Studies on the Immortalisation of Rodent Embryo Fibroblasts by Simian Virus 40 Large Tumour Antigen.

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Abstract.

Introduction of simian virus 40 large tumour antigen (T antigen) into primary rodent fibroblasts in vitro results in them acquiring an infinite proliferative potential, i.e. becoming immortal. To study the mechanism by which T antigen immortalises rodent embryo fibroblasts I assayed mutants of T antigen for their ability to complement the growth defect of a rodent embryo fibroblast cell line that had been conditionally immortalised with a temperature sensitive mutant (tsA58) of T antigen. I also assayed the same T antigen mutants for their ability to immortalise secondary rodent embryo fibroblasts. I identified several functional domains of T antigen which are required for both the immortalisation of secondary rodent embryo fibroblasts and the maintenance of immortalisation in the T antigen-dependent cell line at the non-permissive temperature. Interestingly, an amino-terminal deletion mutant, dl1135, which was negative for immortalisation readily complemented the growth defect of the conditionally immortal cells. I found that the inability of the dl1135 mutant to immortalise could be overcome by cotransfection with either of two carboxy-terminal point mutants (5031 or 5041), both of which themselves are negative for immortalisation.

To clarify the role of these mutants in this trans-complementation I constructed temperature sensitive mutants of dl1135, 5031 and 5041 by introducing the tsA58 point mutation into them. Using combinations of these mutants in immortalisation assays at both the permissive and non-permissive temperature I have demonstrated the presence of a functional domain of T antigen, lacked by dl1135, which is required transiently for the initiation of immortalisation. Once immortal cell lines have been established this functional domain is no longer required and the growth of these cell lines can be maintained by the dl1135 T antigen. I have thereby demonstrated that the initiation and maintenance of immortalisation by T antigen are functionally separable.

I have also attempted to isolate novel immortalising cDNAs from a newt limb blastema cDNA expression library by transfecting the library into rodent embryo fibroblasts and selecting for immortal cell lines. I describe the cloning of a cDNA fragment which, although it is unable to encode a protein, is clearly able to stimulate cellular proliferation.
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STATEMENT REGARDING COLLABORATION

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PUBLICATION

At the time this thesis was finished the work presented in Chapter 3 was being prepared for submission to the Journal of Virology for publication.
**Abbreviations.**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double-distilled water</td>
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<tr>
<td>dATP</td>
<td>Deoxyadenosine-5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine-5'-triphosphate</td>
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<tr>
<td>dGTP</td>
<td>Deoxyguanosine-5'-triphosphate</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine-5'-triphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>kb</td>
<td>Kilobase pair</td>
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<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>polyA</td>
<td>poly-adenylation</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) methylamine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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Chapter 1.

General Introduction

1.1: Introduction.

The work presented in this thesis is predominantly concerned with determining the mechanisms through which SV40 large tumour antigen (T antigen) induces proliferation in primary rat embryo fibroblasts. Such primary cells in culture have a finite mitotic life span, after which they senesce. Expression of T antigen in these cells, before they have senesced, allows them to overcome their finite mitotic life span and results in the establishment of immortal cell lines. The immortal phenotype of these cell lines is dependent on the continuous presence of T antigen, though it has not been determined whether all of the functions of T antigen which were required initially to induce the immortal state are required to maintain it.

In this introduction, I firstly discuss the growth restrictions of primary cells in culture and how studies on the oncogene products of the DNA tumour viruses have allowed the identification of a number of the pathways that are involved in cellular growth control. I particularly discuss the growth regulatory role of two tumour suppressor proteins, p53 and pRB, both of which are targeted by the oncogene products of the DNA tumour viruses. I then go on to describe the functions of SV40 large T antigen that are involved in SV40 replication, cellular transformation and immortalisation.

1.2: Primary cells in culture have a finite mitotic life span.

When mammalian cells isolated from an embryo or an animal are cultured in vitro, they initially proliferate but stop dividing after a finite number of divisions (Hayflick and Moorhead, 1961). At this point the cultures undergo crisis and the cells senesce. For example, rodent embryo fibroblasts undergo approximately 30 population doublings before their capacity for growth is exhausted, while human fibroblasts undergo as many as 60-70 doublings (Bayreuther et al., 1988). Such senescent cells
cannot be induced to enter mitosis even if supplemented with fresh growth medium. However, the cells do not die but remain metabolically active as assayed by their ability to synthesise RNA and protein and remain responsive to mitogens by the expression of some immediate early genes (Tavassoli and Shall, 1988). It has been observed that cells from progressively older animals undergo progressively fewer divisions in culture before undergoing senescence, suggesting that there is a correlation between the age of the animal and the in vitro life span of cells derived from that animal (Hayflick and Moorhead, 1961; Todaro and Green, 1963). Analysis of senescent fibroblasts suggests that the cells arrest in the G1 and possibly G2 phases of the cell cycle rather than Go, the point at which cells arrest if bought into quiescence by either serum deprivation or contact inhibition (Gelfant, 1977; Grove and Cristofalo, 1977; Cristofalo, Phillips and Brooks, 1985).

Since cells from the same animal species undergo a relatively constant number of divisions and the number of population doublings for a given cell type is highly reproducible, it has been suggested that cellular senescence may be a programmed event and that entry into senescence is a manifestation of ageing at the cellular level (Goldstein, 1990). Seven distinct types of pre- and post-mitotic fibroblasts have been identified based on their morphology, proliferative potential and patterns of protein expression, suggesting that fibroblasts in vivo and in vitro progress along a unidirectional seven stage terminal differentiation pathway (Bayreuther et al., 1988).

The molecular basis for programmed entry into senescence is not known although it has been suggested that it may be linked to the random accumulation of cellular damage (Orgel, 1973; Goldstein, 1990). This hypothesis suggests that as cells divide in vitro they accumulate mutations, karyotypic changes and other forms of DNA damage such as loss of DNA methylation and that these lead to changes in the expression of positive and negative regulators of cell growth or to a predisposition to karyotypic instability, resulting in loss of proliferative potential (Cristofalo, Phillips and Brooks, 1985; Sherwood et al., 1988). It has also been proposed that the progressive loss of telomeric DNA and the other essential sequences from the ends of chromosomes determines the finite proliferative potential (Harley, Futcher and Greider, 1990), but as yet it has not been demonstrated whether this relationship between telomere length and the finite proliferative potential regulates the mitotic life span or is merely an effect of cellular ageing.

Others have suggested that senescence is regulated via a genetic program (Orgel, 1973; Bayreuther et al., 1988; Goldstein, 1990). A number of genes that may be involved in regulating senescence have been identified from senescent cells although whether these are involved in directly determining the mitotic life span or are involved in regulating growth downstream of such a factor is not known (Murano et al., 1991; Nuell et al., 1991). Negative growth regulatory genes, such as the retinoblastoma
susceptibility gene \((RB)\) and \(p53\) have also been proposed to be involved in regulating senescence (Shay, Pereira-Smith and Wright, 1991). Senescent cells fail to phosphorylate the pRB protein (Stein, Beeson and Gordon, 1990) and thus contain the underphosphorylated form of pRB which inhibits progression through the cell cycle (see section 1.6.2). Another inhibitor of the cell cycle, p21 (section 1.6.1.4), which is up-regulated during both senescence and differentiation and is able to inhibit DNA synthesis following its transfection or microinjection into young HDF (Noda et al., 1994; Steinman et al., 1994; Halevy et al., 1995), has also been suggested to be involved in the regulation of the mitotic life span. The failure of senescent cells to phosphorylate pRB may be due to increased p21 expression, since p21 is an inhibitor of the kinase complex which phosphorylates pRB (Harper et al., 1993; Xiong et al., 1993).

1.3: Immortalisation.

Although the actual cause of senescence is not known, a sub-group of viral and cellular oncogenes have been shown to readily overcome senescence in primary rodent cells. Cells in culture which have overcome senescence are said to have become immortal, since they have acquired an infinite life span. Immortalisation has been suggested to be the first of two steps required to bring about the complete malignant transformation of rodent cells \textit{in vitro} (Weinberg, 1985). Immortal cells remain dependent on the presence of mitogens (although they have a reduced requirement for them), cannot overgrow a confluent monolayer and cannot form tumours in nude mice, thus they are distinct from fully transformed cells. Such immortalised cells are capable of being transformed into fully malignant cells by either the introduction of a second oncogene (Rassoulzadegan et al., 1982; Land, Parada and Weinberg, 1983; Ruley, 1983; Weinberg, 1985) or, at a low frequency, through the occurrence of spontaneous second events such as chromosomal mutations (Land et al., 1986). This 2-hit oncogene model for transformation suggested that the immortalisation step is carried out by a nuclear oncogene product while the transformation step is carried out by a cytoplasmic oncogene product (Weinberg, 1985). Experiments carried out since this model was proposed, however, have proved that the requirements for immortalisation and transformation are more complicated.

It is not known whether immortalisation has a role to play in tumorigenesis \textit{in vivo}, or whether it is merely required for the \textit{in vitro} establishment of transformed cell lines (Strauss and Griffin, 1990; Stamps, Gusterson and O'Hare, 1992). While it is hard to envisage a situation where it may be possible to derive a tumour without overcoming the finite mitotic life span, it remains to be demonstrated whether this is a critical step in tumorigenesis. Nevertheless, studies on the factors required for cellular immortalisation have revealed a great deal of information on the nature of cell cycle regulation and the
Chapter 1.

nature of negative growth control in senescent cells. Some of the growth regulatory proteins which are implicated during cellular immortalisation have also been implicated in human tumorigenesis.

Examples of viral oncogenes which are capable of immortalising primary rodent cells are adenovirus (Ad) E1A (Ruley, 1983), human papillomavirus (HPV) type 16 E7 (Kanda, Watanabe and Yoshike, 1988; Vousden et al., 1988) and simian virus (SV40) and polyomavirus (Py) large T antigens (Petit, Gardes and Feunteun, 1983; Rassoulzadegan et al., 1983; Jat and Sharp, 1986). Examples of cellular oncogenes (or viral homologues of cellular genes) which display this activity are \( \text{myc} \) (Land, Parada and Weinberg, 1983; Kelekar and Cole, 1987), \( \text{fos} \) (Jenuwein and Muller, 1987), \( \text{jun} \) (Schütte, Minna and Birrer, 1989) and some mutants of \( p53 \) (Jenkins, Rudge and Currie, 1984; Jenkins et al., 1985). The majority of these oncogenes have the common feature of a nuclear subcellular localisation (with the possible exception of HPV E7) and are able to stimulate or suppress transcription of a sub-group of viral and cellular promoters (Weinberg, 1985). The continued expression of the immortalising oncogene is required to maintain the immortal state, however whether all the functions which were initially required to overcome the finite life span of the primary cells, or a subset of these functions, are required to maintain the immortal state has not been determined. In contrast to these dominant immortalising genes there are a number of tumour suppressor genes whose products negatively regulate cell proliferation. The growth suppression activities of wild-type \( p53 \) and the retinoblastoma susceptibility gene (RB) have been most extensively studied.

Serial cultivation of rodent embryo fibroblasts occasionally results in spontaneously immortal cell lines which have escaped senescence (Todaro and Green, 1963; Curatolo, Erba and Morasca, 1984). The cellular lesions which enable these cells to continue proliferating are poorly defined however alterations in the gene for the negative growth regulator \( p53 \) have been observed in some but not all spontaneously immortal cell lines (Harvey and Levine, 1991; Rittling and Denhardt, 1992). Increased transcription of the \( c\text{-}\text{myc} \) cellular proto-oncogene has also been observed in spontaneously immortal mouse cell lines (Tavassoli and Shall, 1988). In contrast, normal human and avian cells have rarely been shown to be capable of spontaneous immortalisation, thus the frequency at which cells escape senescence appears to be species specific (Hayflick, 1965; Smith and Pereira-Smith, 1990).

Somatic cell fusions of normal diploid human fibroblasts (HDF) with several immortal cell lines, including HeLa and simian virus 40-transformed cells, have suggested that senescence is dominant over proliferation. Hybrids resulting from such fusions only proliferate for a limited period of time and then senesce (Bunn and Tarrant, 1980; Pereira-Smith and Smith, 1981; Pereira-Smith et al., 1990). Moreover, microinjection of polyA RNA prepared from senescent human diploid fibroblasts into
cells which were otherwise able to proliferate inhibited DNA synthesis (Lumpkin et al., 1986), thus suggesting that senescent cells contain at least one mRNA species which encodes an inhibitor of DNA synthesis. A cDNA (encoding a protein named prohibitin) that is able to block DNA synthesis in HDF and in cancer (HeLa) cells has since been cloned from these senescent cells (Nuell et al., 1991).

The finding that cellular senescence is dominant in cell hybrids and that the inactivation of specific senescence-promoting genes may be important for cells to escape from senescence is compatible with the idea that activation of specific dominant oncogenes can overcome senescence. There is evidence that both the activation of dominant oncogenes and the repression of senescence-promoting genes may be necessary to enable human cells to overcome senescence. A rare immortal HDF cell line has been isolated following the transfection of a dexamethasone-inducible SV40 large T antigen construct by nurturing the transfected cells through crisis (Wright, Pereira-Smith and Shay, 1989). Proliferation of this cell line required the continued expression of T antigen in the presence of dexamethasone and removal of dexamethasone resulted in growth arrest of the cells in the G1 phase of the cell cycle. For the immortalisation of human cells therefore it is necessary to overcome two controls of senescence. Mortality stage 1 (M1) causes a loss of mitogen responsiveness and arrest at the G1/S boundary while mortality stage 2 (M2) causes a failure of cell replication during crisis. In human cells the M1 stage can be overcome by genes such as T antigen which are capable of stimulating DNA synthesis, however inactivation of M2 is a rare event. Since the probability of inactivating M1 and M2 in a single cell is very low, this explains why very few spontaneously HDF have been isolated. Single or various combinations of oncogenes would also be unlikely to immortalise HDF unless M1 and M2 can both be overridden. In contrast, the ability of Py large T antigen to establish immortal human fibroblast cell lines (albeit at a low efficiency compared to rodent fibroblasts) occurs without the cells exhibiting crisis (Strauss et al., 1990). Thus it seems that despite similarities between the large T antigens of Py and SV40, there are clearly differences in their ability to immortalise human fibroblasts. The two T antigens both appear to carry functions enabling them to overcome the M1 stage, however polyoma appears to carry function(s) which make it more efficient at overcoming the M2 stage. Rodent cells which are readily immortalised by oncogenes, or spontaneously following crisis, may either lack M2 or have a greater capacity to overcome it.

Immortalisation involves not only the ability to overcome the limited proliferation of primary cells but also requires the inhibition of programmed cell death by apoptosis. Apoptosis is associated with growth regulation both during development and following DNA damage. A multitude of factors modulate the induction of apoptosis, including growth factors, intracellular signal transduction mechanisms and nuclear proteins which regulate gene expression, DNA replication and the cell cycle.
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(reviewed by Harrington, Fanidi and Evan, 1994). It is possible that these factors all act through a common apoptosis pathway. Immortalising oncogenes need to be able to overcome the effects of proteins which mediate apoptosis, such as the tumour suppressor protein p53, to ensure the survival of the immortalised cells. It is surprising, therefore, that a number of immortalising oncogenes, including Ad E1A and c-myc, have been shown to trigger apoptosis in certain cell types or following growth factor deprivation (White et al., 1991; Evan et al., 1992). Thus apoptosis may be a cellular defence against deregulated growth in inappropriate conditions. This suggests that in order to successfully immortalise a cell, an oncogene must not only deregulate cell growth but also overcome the apoptotic pathway(s) which may result from this deregulated cell growth.

1.4: The oncogenes of the small DNA tumour viruses.

Studies carried out on the functions required by immortalising or transforming oncogenes to induce cellular proliferation have already yielded a vast amount of information regarding the nature of cell growth control. The oncogenes encoded by the small DNA tumour viruses (papovaviruses, papillomaviruses and adenoviruses) have particularly aided the identification and characterisation of a number of cellular proteins involved in regulating cell proliferation.

The large T antigens of both SV40 (708 amino acids) and polyomavirus (785 amino acids) are related in their amino acid sequences as well as in their known biochemical activities. Both are sufficient to immortalise primary rodent fibroblasts in culture (Petit, Gardes and Feunteun, 1983; Rassoulzadegan et al., 1983; Jat and Sharp, 1986). Interestingly SV40 T antigen also has the capacity to fully transform primary rodent cells at a low frequency without the introduction of a second oncogene and can efficiently transform established cell lines (Kriegler et al., 1984; Brown et al., 1986; Jat et al., 1986; Jat and Sharp, 1986). It has been suggested that the amount of SV40 T antigen expressed is important in determining its ability to transform primary cells and that expression of small t antigen may be important where levels of large T antigen are limiting (Bikel et al., 1987). The efficiency of transformation of primary cells by SV40 large T antigen is greatly increased by the cotransfection of a second, cytoplasmic oncogene, such as activated ras (Michalovitz et al., 1987). Polyomavirus encodes a second oncogene, middle T antigen, which is unable to immortalise or transform primary cells alone, but can transform primary cells when introduced in combination with Py large T and transforms established cell lines alone (Rassoulzadegan et al., 1982).

Two gene products of the human papillomaviruses types 16 and 18, the E7 21kDa protein and E6 15.5kDa protein have been identified as oncogenic proteins. E7
efficiently immortalises primary rodent cells (Kanda, Watanabe and Yoshike, 1988) and cooperates with either ras or with v-fos to induce cellular transformation (Phelps et al., 1988). HPV18 E7 and E6 are able to cooperate to transform established rodent cell lines (Bedell, Jones and Laimins, 1987) and cooperate to immortalise primary human keratinocytes (Hawley-Nelson et al., 1989; Munger et al., 1989). In contrast, either HPV16 E7 or E6 alone have been shown to immortalise human epithelial cells (Band et al., 1991; Halbert, Demers and Galloway, 1991). HPV16 E7 can also partially transform established mouse NIH3T3 cells as determined by its ability to induce anchorage-independent growth (Vousden et al., 1988).

The transforming region of the adenovirus genome is represented by the early region, E1A and E1B, genes. The E1A region encodes a series of related proteins through the alternate splicing of the primary transcript. In adenovirus 5 the two most abundant proteins, expressed soon after infection, are the 289 amino acid product of the 13S RNA and the 243 amino acid product of the 12S RNA (reviewed by Boulanger and Blair, 1991). The E1B gene encodes two proteins, one of 19kDa and the other of 55kDa from overlapping coding regions. E1A alone is able to immortalise primary rodent cells (Houweling, van den Elsen and van der Eb, 1980; Ruley, 1983) and can transform primary cells in cooperation with E1B or activated ras (Ruley, 1983; van den Elsen, Houweling and van der Eb, 1983b). E1B has no detectable transforming activity of its own, in either primary cells or established cell lines (van den Elsen, Houweling and van der Eb, 1983a). Introduction of E1A into an established cell line (NIH3T3) has little effect unless its expression is increased by a strong promoter in which case it has been reported to cause the morphological transformation of these cells (Senear and Lewis, 1986). It has been suggested that the role of E1B in the transformation of primary rodent cells by E1A plus E1B may be to increase the level of expression of E1A (Jochemsen et al., 1987). In contrast, E1A 12S has been demonstrated to immortalise primary epithelial cells more efficiently in the absence of the E1B 55kDa and 19kDa proteins (Quinlan, 1994).

It was originally believed that a functionally important property shared by the immortalising oncogenes was their localisation to the nucleus (Weinberg, 1985). These proteins were believed to exert transcriptional regulatory activities in the nucleus. However HPV E7 may prove to be an exception as it has since been reported to be cytoplasmic (Smotkin and Wettstein, 1987). The product of the second transforming oncogene, which results in decreased responsiveness to external growth conditions, occurs in the cytoplasm where the oncogene product is believed to interfere with signal transduction mechanisms.

Thus immortalising DNA virus oncogenes such as SV40 large T antigen, Py large T antigen, Ad E1A and HPV E7 are capable of cooperating with second oncogenes, such as Py middle T antigen or activated ras, to induce the conversion of
normal embryonic fibroblasts to the transformed state. However, the relationship between the ability to transform in cooperation with activated \textit{ras} or Py middle T antigen and to immortalise is not that simple. Mutant E1A and SV40 large T antigen genes which are unable to immortalise primary cells but are able to cooperate with activated Ha-\textit{ras} to transform have been reported (Michalovitz \textit{et al.}, 1987; Schneider \textit{et al.}, 1987; Kuppuswamy, Subramanian and Chinnadurai, 1988; Quinlan, Whyte and Grodzicker, 1988). This suggests that either some of the functions required for these two biological activities are different or that the activated \textit{ras} oncogene is able to provide some of the functions which are required for the immortalisation step in the transformation of primary cells. The ability of activated \textit{ras} to provide some of the immortalisation functions may correlate with the demonstration that expression of the activated Ha-\textit{ras} oncogene from a strong promoter can fully transform early passage rat embryo fibroblasts without requiring the action of an immortalising oncogene (Spandidos and Wilkie, 1984).

1.5: The oncogenes of the small DNA tumour viruses interact with the same cellular proteins through homologous regions.

The amino-terminus of E1A proteins from different adenovirus serotypes contain two regions of homology which are conserved not only between different adenoviruses, but are also homologous to two regions of the SV40 large T antigen, Py large T antigen and HPV E7 proteins (Stabel, Argos and Philipson, 1985; Figge \textit{et al.}, 1988; Dyson \textit{et al.}, 1989b; Dyson \textit{et al.}, 1990). These regions are designated conserved region 1 (CR1; amino acids 40-80) and conserved region 2 (CR2; amino acids 121-140; reviewed by Moran and Mathews, 1987). The E1A 13S products also have a third conserved region (CR3; amino acids 141-188) which appears to be unique to the adenoviruses and is involved in transactivation of the other adenovirus early genes (Lillie \textit{et al.}, 1987). The positions of these conserved regions and the homologies within the CR1- and CR2-like regions of Ad5 E1A, HPV16 E7, Py large T antigen and SV40 large T antigens are shown in Figure 1.1. The striking homology of the CR1-like and CR2-like regions of E1A with those of HPV E7, SV40 and Py large T antigens suggests that these oncogenes might immortalise via a common pathway. This notion is supported by the finding that the CR2-like region of SV40 large T antigen can efficiently substitute for the CR2 of E1A (Moran, 1988). In addition more recent experiments have indicated that SV40 T antigen and E1A contain other functionally homologous regions, since transformation-defective E1A mutants lacking the amino-terminus can cooperate with SV40 T antigen mutants lacking the CR2-like region in transforming baby rat kidney cells in cooperation with \textit{ras} (Yacuik \textit{et al.}, 1991).

E1A has been shown to form complexes with several cellular proteins including
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Figure 1.1: SV40 large T antigen, Py large T antigen and HPV E7 have homology to Ad E1A within conserved regions 1 and 2.

A schematic representation of the adenovirus 5 E1A (Ad5 E1A), human papillomavirus type-16 E7 (HPV-16 E7), polyomavirus large T antigen and SV40 large T antigen are shown. The shaded boxes indicate the regions of homology with the adenovirus serotypes E1A conserved regions 1 (CR1) and 2 (CR2). The adenovirus E1A-13S specific CR3 region is also shown. The amino acid homologies within the CR1- and CR2-like regions are shown below along with the CR2 LxCxE consensus motif. The numbers represent the position of the amino acids. Adapted from Stabel et al. (1985), Figge et al. (1988), Dyson et al. (1989b), Imai et al. (1991).

cyclin A (Pines and Hunter, 1990), pRB (the product of the retinoblastoma susceptibility gene; Whyte et al., 1988), p107, p130 and p300 (Harlow et al., 1986), all of which are believed to be important regulators of cellular proliferation (see section 1.6.2). The p107 and p130 proteins share several properties with pRB such as interaction with the same families of cellular proteins and a region of homology termed the "pocket" region (Hannon, Demetrick and Beach, 1993; Li et al., 1993). The regions of E1A which
interact with these proteins include an amino-terminal region, the CR1 and the CR2 (Whyte, Williamson and Harlow, 1989), and data from a number of laboratories indicate that these same regions are required for the growth stimulatory effects of E1A (Moran et al., 1986; Lillie et al., 1987; Schneider et al., 1987; Kuppuswamy, Subramanian and Chinnadurai, 1988; Moran and Zerler, 1988). E1A mutants which are deficient in pRB, p107, p130 and p300 binding are unable to transform primary baby rat kidney cells in cooperation with an activated ras oncogene (Whyte, Ruley and Harlow, 1988; Whyte, Williamson and Harlow, 1989). The interaction of E1A with pRB, p107 and p130 predominantly requires the CR2, although the CR1 is also required for its efficient interaction with pRB, while the p300 interaction requires the amino-terminus region and CR1 (Egan et al., 1988; Whyte, Williamson and Harlow, 1989).

SV40 large T antigen, Py large T antigen and HPV E7 are also able to bind pRB through their CR2-like regions (DeCaprio et al., 1988; Dyson et al., 1989b; Ewen et al., 1989; Munger et al., 1989; Dyson et al., 1990). Both SV40 large T antigen and HPV16 E7 have also been shown to bind p107 (Davies et al., 1993; Dyson et al., 1989a) and an association between SV40 large T antigen and p130 has also been reported (Hannon, Demetrick and Beach, 1993). It is possible that HPV E7 and Py large T antigen also bind p130 (and p107 in the case of Py T antigen) given the homology between these proteins, although such interactions have yet to be reported. A consensus amino acid motif sequence within the CR2-like regions important for their association with these "pocket" proteins is L-x-C-x-E (Figure 1.1). A number of the other polyomavirus group T antigens also have the CR1 and CR2-like regions of homology (Pipas, 1992).

Moran and colleagues have demonstrated that the CR1-like region of SV40 large T antigen carries a function capable of complementing a p300-binding-deficient E1A mutant in the transformation of baby rat kidney cells in cooperation with ras (Yacuik et al., 1991). However, as yet no interaction between p300 and either SV40 large T antigen, Py large T antigen or HPV E7 has been reported. Recently the E1A-associated p300 protein has been shown to have the properties of a transcriptional adaptor protein and to be a functional homolog of the transcriptional co-activator, CREB-binding protein (CBP; Arany et al., 1995; Lundblad et al., 1995). HPV E7 and SV40 large T antigen, like Ad E1A are also able to associate with cyclin A and cdk2 (Dyson et al., 1992; Adamczewski, Gannon and Hunt, 1993; Tommasino et al., 1993) though it is not known whether this interaction is direct or occurs through another protein or proteins. The CR2 of E1A is sufficient for its interaction with cyclin A and cdk2 (Dyson et al., 1992) suggesting that the interaction may occur indirectly through one or other of the "pocket" proteins.

SV40 large T antigen, Ad5 E1B-55K and HPV16 & 18 E6 interact with the cellular protein p53 (DeLeo et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow et al., 1982; Werness, Levine and Howley, 1990), which, like pRB, is a
Chapter 1. tumour suppressor gene and negatively regulates cellular proliferation (see section 1.6.1). Interestingly, however, the large T antigen of polyomavirus and the Ad12 E1B-55K protein are unable to associate with p53 (Zantema et al., 1985). In non-transformed tissues p53 has a very short half life of approximately 10-20 minutes, however in cells transformed by either SV40 T antigen or adenovirus its half life is increased to as much as 20 hours (Oren, Maltzman and Levine, 1981). While the interaction of SV40 large T antigen stabilises the p53 protein, the interaction of HPV E6 with p53 appears to target the p53 protein for ubiquitin-dependent degradation which is mediated by a cellular protein, E6-AP (Scheffner et al., 1990; Huibregtse, Scheffner and Howley, 1993). This targeted degradation of p53 involves E6 sequences distinct from those required for binding p53 (Crook, Tidy and Vousden, 1991) and recently it has been demonstrated that E6-mediated inhibition of the activities of p53 does not necessarily require the degradation of p53 (Thomas et al., 1995).

Since the work presented in this thesis is primarily concerned with elucidating the requirements for growth stimulation of one of these immortalising oncogenes, SV40 large T antigen, I shall predominantly discuss the role of this oncogene in the SV40 lytic cycle, cellular immortalisation and transformation. However, wherever appropriate I shall compare the activities of T antigen with the activities of the oncogenes of the other small DNA tumour viruses. As discussed above, these oncogenes have been shown to interact with the same families of cellular proteins and I shall firstly discuss the role of several of these tumour suppressor proteins in growth control.

1.6: Growth regulation by tumour suppressor proteins.

1.6.1: The p53 tumour suppressor protein.
1.6.1.1: p53 is a tumour suppressor gene.

p53 was first identified as a cellular tumour antigen complexed with large T antigen in SV40-transformed cells (DeLeo et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Harlow, Pim and Crawford, 1981). In addition, high levels of the p53 protein were observed in many other cell lines transformed with either chemical carcinogens, UV irradiation or viruses other than SV40 (DeLeo et al., 1979; Linzer and Levine, 1979; Rotter et al., 1981; Sarnow et al., 1982) while much lower levels of p53 were observed in normal, non-transformed cells (Benchimol, Pim and Crawford, 1982). While p53 was originally believed to be an activated oncogene capable of immortalising primary cells (Jenkins, Rudge and Currie, 1984) or transforming them in cooperation with activated ras (Eliyahu et al., 1984), it is now known that these p53 cDNAs encode proteins with missense mutations which confer these oncogenic activities upon them (Jenkins et al., 1985; Hinds, Finlay and Levine, 1989) and that the wild type p53 protein suppresses growth.
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Evidence that wild type p53 is a tumour suppressor gene came with the demonstration that wild type p53 suppressed transformation mediated either by mutant p53 plus ras or by E1A plus ras (Finlay, Hinds and Levine, 1989). Eliyahu et al. (1989) demonstrated that wild type p53 could also inhibit transformation of primary REFs by myc plus ras. Further studies demonstrated that the wild type p53 gene suppressed growth or tumorigenicity in a number of transformed cell lines including SV40-transformed cell lines (Mercer et al., 1990), three p53-negative tumour cell lines (one mouse and three human; Chen et al., 1990; Johnston et al., 1991) and several other human cancer cell lines (Baker et al., 1990; Chen et al., 1991). In contrast, expression of mutant p53 alleles in these cell lines had no antiproliferative effect. Alterations in p53 have also been observed in some, but not all spontaneously immortalised mouse cell lines (Harvey and Levine, 1991; Rittling and Denhardt, 1992). These studies, along with others (reviewed by Lane and Benchimol, 1990), clarified the role of wild type p53 as a tumour suppressor gene, rather than a dominant cellular oncogene. The majority of missense mutants of p53, however, seem not only to loose the growth suppressor activity of wild type p53, but appear to gain new activities and act as dominant oncogenes in tumorigenesis.

Abnormalities of the p53 gene have been observed in a number of human and animal tumours (Hollstein et al., 1991; Levine, Momand and Finlay, 1991). A high proportion of human colorectal carcinomas contain mutations in both p53 alleles, generally through the deletion of one allele and point mutation of the other (Nigro et al., 1989). A number of other human tumours, including breast, brain, bladder, bone, ovarian and lung (reviewed by Levine et al., 1994), show this reduction to homozygosity, as do Friend virus-induced erythroleukaemia cells in mice (Mowat et al., 1985). Germline p53 mutations have been described in Li-Fraumeni cancer syndrome families who have high incidences of cancer, including breast carcinoma, brain tumours, soft tissue sarcomas, osteosarcoma, leukaemia and adrenocortical carcinoma (reviewed by Vogelstein, 1990; Frebourg and Friend, 1992; Michalovitz, Halevy and Oren, 1991). The majority of missense mutations observed in these cancers are clustered in four regions between amino acids 130-290 of the 393 amino acid p53 protein and in particular there are three frequently mutated residues (amino acids 175, 248 and 273; Levine, Momand and Finlay, 1991).

1.6.1.2: p53 regulates transcription.

The precise mechanisms through which wild type p53 exerts its tumour suppression activity are not totally resolved. The wild type p53 protein has been shown to both activate (Zambetti et al., 1992) and repress transcription (Seto et al., 1992). Growth suppression by p53 has been suggested to correlate with its transcriptional activation function (Crook et al., 1994). The region of the p53 protein between residues 120 to 290 (where the majority of missense mutations are clustered) acts as a specific
DNA-binding domain (Kern et al., 1991), while the amino-terminal 75 amino acids promote transcription when fused to a DNA-binding domain (for example Gal4; Fields and Jang, 1990). Both the growth suppression activity of wild type p53 and the ability of p53 mutants to cooperate with ras to transform primary rat embryo fibroblasts (REFs), have been shown to be dependent upon nuclear localisation (Shaulsky et al., 1991). Transcriptional activation by p53 has been shown to occur through a p53 response element both in vitro (Funk et al., 1992) and in vivo (Zambetti et al., 1992) and the affinity of p53 for it's specific DNA targets requires its ability to form tetramers (Shaulian et al., 1995). The carboxy-terminal domain has a site for phosphorylation by a cyclin-dependent kinase (Stürzbecher et al., 1990). The in vitro transactivation activity of purified wild type p53 is activated by the addition of either nuclear extract or a monoclonal antibody (Funk et al., 1992). These results suggest that p53's activity as a transcriptional activator is regulated by post-translational modification and/or interactions with other cellular factors.

Among the genes which are activated by wild type p53 in a sequence specific manner are the WAF1/cip1 gene (El-Deiry et al., 1993; El-Deiry et al., 1994), the GADD45 gene (Kastan et al., 1992), the MDM2 gene (Juven et al., 1993) and the muscle creatine kinase (MCK) gene (Zambetti et al., 1992). Almost all of the missense mutant p53 proteins commonly associated with malignancies (and unable to suppress neoplastic growth) have been shown to be unable to bind efficiently to, and fail to stimulate transcription of a gene with, the p53 responsive element (Raycroft, Wu and Lozano, 1990). Wild type, but not mutant, p53 protein has been shown to bind the TATA-binding protein (TBP) which is one of the basal transcription factors (Seto et al., 1992) and the cooperative DNA binding of p53 with TBP at promoters containing p35-binding sequences is believed to be involved in transcriptional activation by p53 (Chen et al., 1993). Many of the p53 mutants are able to inhibit wild type p53's ability to activate p53 regulated genes upon coexpression (Kern et al., 1992). This transdominant repression of p53 transcriptional activity has been suggested to occur through the ability of mutant p53 proteins to oligomerise with, and inactivate, the wild type protein (Milner and Medcalf, 1991) and to be sufficient for the transformation of REFs in cooperation with ras (Shaulian et al., 1992; Slingerland, Jenkins and Benchimol, 1993). Such complex formation, however, may not be the only explanation for the oncogenicity of p53 mutants. There are two lines of evidence for this: firstly the oncogenic potential of different p53 mutants does not correlate with the efficiency with which they complex wild type p53 (Halevy, Michaelovitz and Oren, 1990) and secondly there are cases in which a mutant p53 could enhance tumorigenicity in the absence of any endogenous wild type p53 (Dittmer et al., 1993; Sun et al., 1993). The observation that in human cancers one of the mutated p53 alleles is often a missense mutation while the other is a larger deletion, rather than both alleles having large deletions, also adds weight to this
hypothesis. Thus it appears that p53 mutants carry oncogenic activities, which positively contribute to neoplastic processes, over and above simply inactivating the endogenous p53 (reviewed by Michalovitz, Halevy and Oren, 1991).

Wild type p53 also represses the transcription of a number of genes which do not have p53 responsive elements including the c-fos, c-jun, retinoblastoma (RB) and proliferating cell nuclear antigen (PCNA) genes as well as the p53 gene itself (Ginsberg et al., 1991; Mercer et al., 1991; Shiio, Yamamoto and Yamaguchi, 1992). The interaction of p53 with TBP appears to be responsible for the p53's transcriptional repression of several minimal promoters (Seto et al., 1992; Mack et al., 1993). Other mechanisms may also contribute to p53-mediated transcriptional repression. p53 also interacts with a number of other transcription factors, including CCAAT binding factor (Agoff et al., 1993), the Wilm's tumour gene product (Maheswaran et al., 1993) and the general transcription factors Sp1 (Borrelli and Glazer, 1993) and ERCC3 (Wang et al., 1994), though the roles of these interactions have yet to be clarified. It seems probable that p53 exerts its tumour suppression activity by promoting the expression of anti-proliferative genes and repressing the expression of growth stimulating genes.

1.6.1.3: p53 is necessary for a G1 checkpoint.

During normal development p53 protein levels decrease as cells differentiate, thus it was originally suggested that p53 plays an important role during embryonic development and differentiation (Rogel et al., 1985). This was also shown during the in vitro differentiation of F9 teratocarcinoma cells (Oren, Reich and Levine, 1982) and erythroleukaemia cells (Richon et al., 1989). Increases in the synthesis and steady-state levels of p53 protein and mRNA are observed prior to DNA synthesis in late G1 upon serum stimulation of quiescent, non-transformed 3T3 mouse fibroblasts (Milner and Milner, 1981; Reich and Levine, 1984). To further investigate the role of p53 in development p53 knockout mice were generated. These mice were found to develop normally, however they did have a predisposition to developing specific types of cancer, including lymphomas, haemangiosarcomas and osteosarcomas, at an early age (Donehower et al., 1992). These results confirmed the role of p53 as a tumour suppressor, however indicated that p53 function is not essential for normal cell division or normal development. Subsequently it has been suggested that p53 has a cell cycle checkpoint function (reviewed by Perry and Levine, 1993). Checkpoints are functions which are not required for the viability of the cell, but monitor progression through the cell cycle and stop the progression through the cell cycle if conditions are not satisfactory (Hartwell and Weinert, 1989; Murray, 1992).

p53 is required for the G1 checkpoint in mammalian cells (Kuerbitz et al., 1992). When the growth of p53 negative cells is inhibited by the introduction of wild type p53, growth arrest occurs within the G1 phase of the cell cycle (Diller et al., 1990; Martinez et al., 1991). Cells whose growth was inhibited by either the microinjection of an anti-
p53 antibody were also arrested in the G1 phase of the cell cycle (Mercer, Avignolo and Baserga, 1984). Injection of an antibody specific for p53 was found to interfere with the ability of quiescent mouse fibroblasts to exit G0 when exposed to serum (Mercer et al., 1982). This antibody has since been found to induce the sequence-specific DNA binding activity of wild type p53 and possibly enables it to activate transcription (Funk et al., 1992; Wolkowicz et al., 1995). p53-deficient mouse embryo fibroblasts are readily established in culture and grow faster than normal embryo fibroblasts due to a faster progression through G1 (Harvey et al., 1993).

p53 levels rapidly increase in cells following DNA damage by either UV- or γ-irradiation (Maltzman and Czyzyk, 1984; Kastan et al., 1991). This occurs mainly through the stabilisation of the normally short-lived p53 protein and appears to be an important component of the G1 arrest that follows DNA damage (Kuerbitz et al., 1992). This growth arrest following DNA damage is believed to be important for the cells to repair their DNA before entering S phase. Cells lacking p53, or expressing only mutant p53, fail to arrest in G1, but can arrest in G2, in response to irradiation and this is thought to result in the cells entering S phase with unrepaired DNA (Kastan et al., 1991). Loss of wild type p53 has been shown to give rise to genetic instability and gene amplification (Livingstone et al., 1992; Harvey et al., 1993). This is believed to be responsible, at least in part, for the elevated incidence of tumorigenesis in p53 knockout mice.

1.6.1.4: **p53 inhibits the cell cycle through key regulators.**

Growth arrest by p53 has been shown to be dependent on its ability to transactivate cell cycle inhibitory genes. Expression of *GADD45* is induced by p53 following DNA-damage implying that the induction of this gene by p53 is involved in growth arrest (Kastan et al., 1992). The product of the *GADD45* gene has been shown to associate with PCNA, stimulates DNA excision repair and inhibits entry of cells into S phase (Smith et al., 1994). The general transcription factor ERCC3, with which p53 associates, is also involved in the regulation of DNA excision repair and has helicase activity (Wang et al., 1994). Recently another protein, p21, whose expression is induced by p53 following DNA-damage has been identified (El-Deiry et al., 1994). This gene was cloned independently by virtue of its induction by p53 (El-Deiry et al., 1993), its association with cyclin/cdk complexes (Harper et al., 1993; Xiong et al., 1993) and its up-regulation in senescent cells (Noda et al., 1994) and as a result has several names, *SDII*, *WAF1*, *CIP1*, *CAP20* and *PICl*. While expression of p21 is regulated independently of p53 following serum stimulation and during differentiation, p53 plays a critical role in p21 induction following DNA damage by γ-irradiation (Macleod et al., 1995).

p21 is one of a family of cyclin-dependent kinase (cdk) inhibitors (reviewed by Hunter and Pines, 1994; Peter and Herskowitz, 1994; Kamb, 1995). These inhibitors
modulate progression through the cell cycle by regulating the activity of a family of cyclin-cdk complexes. Progression of eukaryotic cells through the cell cycle is mediated by the sequential assembly and activation of key cyclin-dependent kinases. Cyclin-dependent kinase activity is regulated through the cell cycle in several ways. Active cdk's are composed of a catalytic subunit, whose activity is regulated by phosphorylation, and a regulatory subunit called a cyclin. Different cyclin subunits are required at different phases in the cell cycle. G1 cyclins are required for the G1/S transition, S phase cyclins for progression through S phase and G2 or mitotic cyclins for entry into mitosis. The levels of each cyclin are regulated through the cell cycle by transcription and by modulation of their rates of degradation (reviewed by Lew and Reed, 1992).

Some of the cdk-inhibitors modulate the activity of specific cdk-cyclin complexes. However p21 binds to, and inhibits, a wide variety of cyclin-cdk complexes, including cyclin D-cdk4 and cyclin E-cdk2 which are normally activated during progression through the G1 phase of the cell cycle and cyclin A-cdk2 which is active during progression through S phase (Harper et al., 1993; Xiong et al., 1993; Dulic et al., 1994; El-Deiry et al., 1994; see Figure 1.2). The cyclin D-cdk4 complex binds directly to, and phosphorylates, the pRB protein (Ewen et al., 1993). Underphosphorylated pRB binds to and negatively regulates the activities of members of the E2F family of transcription factors whose functions are important for regulation of the cell cycle through the G1/S boundary (see section 1.6.2). Phosphorylation of pRB on cdk sites releases E2F from this inhibitory complex, thereby reversing the growth suppressive activities of the pRB-like proteins in the G1 phase of the cell cycle. Thus the inactivation of the cyclin D-cdk4 complex by p21 results in the continued inactivation of the E2F family by the pRB family yielding cell cycle arrest in G1 (Figure 1.2).

The p53 protein itself is regulated through the cell cycle by phosphorylation on serine 315 by either cyclin A-cdc2 and cyclin B-cdc2 (Bischoff et al., 1990). This phosphorylation is believed to regulate the antiproliferative activities of p53 during the progression through G1 and S phase. p53 is also phosphorylated by casein kinase II on serine 389 (Meek et al., 1990) and by a novel casein kinase I-like enzyme on serines 4, 6 and 9 (Milne et al., 1992). Casein kinase II has been implicated in the transduction of mitogen stimulation signals to the nucleus (Carroll and Marshak, 1989) and may itself be regulated by phosphorylation, mediated by cdc2 in a cell cycle-dependent manner (Litchfield et al., 1991). Mutation of the cdc2 and casein kinase II phosphorylated residues however did not inhibit the transcriptional activation function of p53 (Marston, Crook and Vousden, 1994).

p21 also binds PCNA, which is a component of the DNA replication machinery and therefore is required for DNA replication and repair (Waga et al., 1994). p21 can inhibit PCNA-dependent DNA replication but does not interfere with PCNA-dependent
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Figure 1.2: Cell cycle dependent regulation of the E2F transcription factor family.

This simple schematic diagram shows the cell cycle with particular respect to the cellular targets of SV40 large T antigen. The proteins with which T antigen associates are shown in bold. The phosphorylation of pRB and p107 and the release of active members of the E2F family during progression through the G1 phase of the cell cycle are shown. The cyclin-ckd complexes whose activities are associated with each phase of the cell cycle are shown. p53's activation (following DNA damage) of the cdk-inhibitor p21 and the inhibitory effects of p21 and two other cdk-inhibitors p15 and p16 are shown. Adapted from Hunter & Pines (1994).

nucleotide repair (Li et al., 1994). Thus p53, through activation of p21 expression, regulates, and can arrest, progression through the G1 phase of the cell cycle and the ability of a cell to replicate its DNA. However the cells arrested in G1 following DNA-damage retain the ability to repair their DNA.

The possibility that p53 is also involved in a G2/M phase cell cycle block has also been investigated. Cells transformed with a temperature sensitive mutant of p53 and an activated ras gene growth arrest when shifted to the non-permissive temperature due to the mutant protein resuming the growth suppressive activities of wild type p53 at
this temperature. This growth arrest occurs in both the G1 and G2 phases of the cell cycle (Stewart et al., 1995). A G2 arrest is also usually observed in mouse fibroblasts exposed to inhibitors of spindle formation, however this arrest fails to occur in fibroblasts from p53 knockout mice (Cross et al., 1995).

1.6.1.5: p53 mediates apoptosis.

p53 also can mediate cell death by apoptosis following DNA damage. Reintroduction of wild type p53 expression into p53-negative or mutant tumour cells results in apoptosis (Shaw et al., 1992). Moreover thymocytes derived from the p53 knockout mice fail to apoptose following DNA-damage induced by irradiation (Clarke et al., 1993; Lowe et al., 1993). The mechanisms through which p53 mediates apoptosis are poorly defined though they have been suggested to involve its transcriptional repression functions (Sabbatini et al., 1995). Apoptosis can also occur through pathways independent of p53. Thymocytes from the p53 knockout mice remain susceptible to apoptosis induced by several other agents, including glucocorticoids (Clarke et al., 1993; Lowe et al., 1993). Indeed apoptosis is believed to play a role during embryonic development and the p53 knockout mice develop normally. Thus p53 can regulate cellular proliferation either through causing a G1, and possibly a G2, growth arrest or through causing cell death by apoptosis. The loss of both p53 alleles, or the loss of one followed by the mutation of another, is thought to predispose cells to cancer due to the loss of the cell cycle checkpoint arrest following DNA damage. Since such cells will continue to replicate their damaged DNA, the possibility of oncogenic mutations occurring increases during subsequent cell cycles.

1.6.1.6: Viral and cellular oncogenes inactivate p53.

During the progression of an uninfected cell through the cell cycle the growth suppression activities of p53 may be regulated by phosphorylation or by other means such as an association with an inhibitory protein. The discovery that the product of a cellular proto-oncogene, MDM2, which was observed to be amplified in human sarcomas (Oliner et al., 1992), binds to p53 and inhibits p53-mediated transactivation (Momand et al., 1992), suggests that the protein product of this proto-oncogene may negatively regulate p53 in proliferating cells. Indeed over expression of the MDM2 oncogene can overcome growth suppression by wild type p53 in REFs cotransformed with ras (Finlay, 1993). Moreover, when quiescent Balb/c 3T3 cells are stimulated, with serum, to re-enter the cell cycle the levels of the MDM2 gene product and MDM2-p53 complexes are increased in the late G1 phase of the cell cycle (Olson et al., 1993).

The oncogenes of the small DNA tumour viruses (SV40 large T antigen, adenovirus E1B-55K and HPV E6) are believed to target p53 to enable the virally infected cell to progress through G1 to S phase where the viral genome can be replicated. All three of these oncoproteins inactivate the transcriptional transactivation functions of p53 (Mietz et al., 1992; Yew and Berk, 1992). E6 does this through
Chapter 1. promoting its degradation (Scheffner et al., 1990). While it is possible that the ability of T antigen and E1B-55K to bind to p53 inactivates its transactivation functions, the ability of E1B-55K to bind to p53 does not seem to be absolutely required to do so (Yew and Berk, 1992).

The role of p53 interactions in transformation by the adenovirus oncogenes E1A and E1B has yielded several surprising results. Adenovirus E1A, while it has been shown to immortalise primary rodent cells (Houweling, van den Elsen and van der Eb, 1980), has also been shown to reduce the anchorage-independent growth and tumorigenicity of established human tumour cell lines (Frisch, 1991). E1A has been shown to induce the stabilisation of p53 and p53-mediated apoptosis in established or transformed cell lines (Debbas and White, 1993; Lowe and Ruley, 1993) and this may explain the tumour suppression properties of E1A. This induction of apoptosis by E1A appears to be specific to the 13S gene product and requires the transcriptional activation functions (Chinnadurai, 1992; Quinlan, 1993). In cells transformed by adenovirus, the apoptosis induced by E1A can be overcome by the E1B-19K protein as well as by the p53 binding function of the E1B-55K protein (White et al., 1991; Rao et al., 1992; Debbas and White, 1993). The ability of the E1B-19K protein to overcome p53-mediated apoptosis has been suggested to be due to E1B-19K alleviating p53-mediated transcriptional repression (Sabbatini et al., 1995). HPV E7 has also been reported to induce apoptosis, as well as proliferation, in certain cell types (Howes et al., 1994).

While the inactivation of T antigen in cell lines conditionally immortalised by a temperature sensitive mutant of T antigen by Jat et al. (1989) results in these cell lines growth arresting without significant cell death, similar cell lines isolated by other investigators have been reported to undergo cell death by apoptosis when T antigen is inactivated (Yanai and Obinata, 1994; Zheng et al., 1994). This is thought to be due to the sudden release of a large amount of stable p53. As is the case with adenovirus, stabilisation of p53 in SV40-transformed cells can also occur indirectly of a direct T antigen-p53 association (Deppert and Haug, 1986; Deppert, Haug and Steinmayer, 1987; Tiemann and Deppert, 1994). SV40 large T antigen and p53 can also overcome growth suppression by p53 in the absence of direct p53 binding (Michael-Michalovitz et al., 1991). The recent identification of a kinase activity which is activated by T antigen and phosphorylates p53 suggests a role for this T antigen-dependent phosphorylation in the binding-independent stabilisation of p53 by T antigen (Muller, Boldyreff and Scheidtmann, 1993). The fact that the oncoproteins of the small DNA tumour viruses have evolved several means to modulate the activity of the p53 protein suggests that one or more blocks by p53 must be overcome for them to exert their oncogenic activities.
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1.6.2: The retinoblastoma family of growth regulatory proteins.

1.6.2.1: pRB is a tumour suppressor protein.

Retinoblastoma, a malignant tumour of the eye, is one of the most studied examples of a human malignancy where loss of both alleles of a tumour suppressor gene is a critical step in tumorigenesis. It occurs in both sporadic and hereditary forms both of which result from the inactivation of both alleles of the retinoblastoma susceptibility (RB) gene. Inactivation of both alleles of the RB gene is necessary for the development of the disease (Benedict et al., 1983). A child who inherits a defective allele from one parent is predisposed to the disease, since the possibility of their second allele being inactivated is higher than a child with two intact alleles (Knudson, 1971; reviewed by Hansen and Cavanee, 1988). Unlike the p53 tumour suppressor, the mutant RB alleles isolated from human cancers do not encode dominant mutant proteins, thus mutations of the RB gene are usually recessive and have not been reported to exhibit any overt oncogenic effects in their own right. The role of the RB gene product in oncogenesis, therefore, appears to be entirely associated with its loss of function. RB gene abnormalities are also associated with osteosarcomas, small cell lung carcinomas, bladder carcinomas, gastric carcinomas, breast carcinomas, acute myelogenous leukaemia and chronic lymphocytic leukaemia (reviewed by Knudson, 1993).

The RB gene product (pRB) is a 928 amino acid phosphoprotein with an approximate molecular weight of 110kDa and corresponds to the 105kDa protein which had been reported to be complexed with the adenovirus E1A proteins (Harlow, Franza and Schley, 1985; Whyte et al., 1988). pRB also associates with SV40 large T antigen (DeCaprio et al., 1988), Py large T antigen (Dyson et al., 1990) and HPV E7 (Dyson et al., 1989b; Munger et al., 1989). The regions of pRB required for the interactions with these viral oncoproteins correspond to common sites for mutation in human retinoblastomas suggesting that the viral oncoproteins are targeting a domain which carries an important function for negative growth regulation by pRB (Hu, Dyson and Harlow, 1990). Reintroduction of wild type pRB expression into a number of RB-deficient human cancer cell lines suppresses their growth and reduces their tumorigenicity in nude mice (Bookstein et al., 1990; Wang et al., 1993b). Such experiments have allowed the mapping of the growth suppression activities to a domain which includes the minimal region of the protein required for binding to T-antigen and E1A (Hu, Dyson and Harlow, 1990; Kaelin, Ewen and Livingston, 1990; Qin et al., 1992). Thus the majority of loss-of-function RB mutations, T/E1A-binding and growth suppression all map to the same region. This region has been termed the "pocket" domain.

1.6.2.2: pRB is required for normal development.

Unlike p53, the pRB tumour suppressor protein appears to play a critical role in regulating cell growth during development. Mice homozygous for one of several
inactivating mutations in RB are not viable and die in utero between the 14th and 15th
day of gestation (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The embryos
exhibit defects in erythropoiesis and in the central and peripheral nervous systems due to
a large amount of neuronal cell death. The development of other tissues at the time of
embryo death appears to have proceeded normally, indicating that the majority of cell
divisions in the embryo had occurred normally despite the absence of pRB. It is
possible that pRB is only required to halt cell division upon entry into a terminal
differentiation pathway. This is believed to occur at different times in different cell
lineages and earliest in the haematopoietic and neural systems. Analysis of chimeric
mice partially composed of RB-I- cells indicates that RB-I- cells can contribute
substantially to the formation of most tissues, including the blood, brain and spinal
chord, the tissues with defects in the knockout mice (O'Williams et al., 1994). Thus the
deleterious effects of RB deficiency can be overcome by factors produced by RB+/+
cells and acts in a paracrine fashion to sustain cell viability. The lethal phenotype in
homozygous RB knockout mice could be overcome by the transfer of a human RB mini-
transgene into the mice (Lee et al., 1992). Mice heterozygous for the RB mutations did
not develop retinoblastoma but were predisposed to other malignancies, with some mice
developing pituitary tumours (Jacks et al., 1992).

1.6.2.3: pRB regulates transcription during the cell cycle.

pRB appears to exert its growth suppressive effects during the G1 phase of the
cell cycle (reviewed by Hollingsworth, Hensey and Lee, 1993; Weinberg, 1995).
Microinjection of pRB into cells lacking wild type pRB results in a G1 arrest (Goodrich
et al., 1991). This growth arrest in G1 appears to require the action of pRB at a defined
checkpoint, since microinjection of pRB after the cell has progressed more than two
thirds of the way through G1 fails to result in growth arrest and the cells proceed to S
phase. This time point has been suggested to coincide with the cell cycle "restriction (R)
point" (Pardee, 1989), passage through which commits cycling cells to cell division
rather than quiescence. Prior to this restriction point progression through the cell cycle
is dependent on the presence of appropriate growth factors and lack of contact
inhibition.

The growth inhibitory functions of pRB are regulated through the cell cycle by
phosphorylation. Whereas pRB is underphosphorylated early in G1, the majority of the
protein is hyperphosphorylated in mid to late G1 (Buchkovich, Duffy and Harlow,
1989). The time of phosphorylation correlates approximately with the restriction point
(Figure 1.2). Phosphorylation of pRB takes place in multiple stages and at multiple
phosphorylation sites as the cell progresses through the cell cycle (DeCaprio et al.,
1992). It is firstly phosphorylated during G1, then again during S and again at the G2-M
boundary. Phosphorylation mapping has shown that phosphorylation occurs at specific
sites at each stage of the cell cycle. The protein is then dephosphorylated during mitosis
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by phosphoprotein phosphatase type 1 (Durfee et al., 1993). The fact that pRB is differentially phosphorylated at multiple sites in the other stages of the cell cycle as well as in G1 suggests that these phosphorylations may either repress or activate multiple functions of pRB. Phosphorylation of pRB in G1 fails to occur in the presence of inhibitors of cell growth, such as transforming growth factor β1 (Laiho et al., 1990) or α-interferon (Thomas et al., 1991). Irreversibly growth arrested cells, such as terminally differentiated monocytes and granulocytes (Furukawa et al., 1990) or senescent HDF (Stein, Beeson and Gordon, 1990) have lost the ability to phosphorylate pRB in response to mitogenic stimulation.

While Ad E1A binds pRB regardless of its phosphorylation state (i.e. it complexes pRB at all points of the cell cycle), SV40 T antigen and HPV E7 preferentially bind the underphosphorylated form in a cell cycle dependent manner (i.e. during G1 Ludlow et al., 1989; Ludlow et al., 1990; Imai et al., 1991). Since the underphosphorylated form of pRB is specifically targeted by these viral oncogenes, it was suggested that this is the form which is active in inhibiting cell cycle progression through G1. The growth inhibitory function of the underphosphorylated form can be inactivated either by phosphorylation during the cell cycle or by its interaction with T antigen, E1A or E7. The discovery that the G1 cyclin-dependent kinases, cyclin D-cdk4 or cyclin D-cdk6, are possibly responsible for the phosphorylation of pRB during G1 further supported this hypothesis (Kato et al., 1993). The D-type cyclins (D1, D2 and D3) are unique in that they are the only cyclins able to bind pRB directly and this has been suggested to target pRB for phosphorylation by either cdk4 or cdk6 (Figure 1.2; Ewen et al., 1993). Cyclin E-cdk2 complexes are also implicated in the phosphorylation of pRB during late G1 since over expression of cyclin E can lead to pRB hyperphosphorylation in transfected cells (Hinds et al., 1992). As mentioned previously (section 1.6.1) the activity of these cyclin-dependent kinases is regulated through the cell cycle by the level of expression of their specific cyclins, by phosphorylation and by cdk-inhibitors such as p21.

In its underphosphorylated state pRB has been shown to bind to, and negatively regulate the activity of, three members of the E2F family of transcription factors, E2F-1, 2 and 3 (Figure 1.2; Chittenden, Livingston and Kaelin, 1991; Hiebert et al., 1992; Flemington, Speck and Kaelin, 1993). This interaction occurs through the pRB "pocket" region and the SV40 T antigen, Ad E1A and HPV E7 proteins compete with these transcription factors to bind pRB (Huang, Lee and Lee, 1991). Thus phosphorylation of pRB on its cdk sites during the mid- to late-G1 phase, or the disruption of pRB-E2F complexes by T antigen, E1A or E7, releases the E2F proteins from their inhibitory complexes thereby allowing them to activate genes required for DNA replication (Suzuki-Takahashi et al., 1995). The E2F transcription factor family activates transcription through binding to a consensus nucleotide sequence and this sequence is
found in the promoters of a number of genes which are important for growth control including c-myc, N-myc, c-myb, dihydrofolate reductase, thymidine kinase, DNA polymerase α, cdc2, EGF receptor, p107 and the E2F-1 gene itself (Dalton, 1992; Hiebert et al., 1991; Mudryj, Hiebert and Nevins, 1990; Neuman et al., 1994). E2F proteins complexed with pRB are still able to bind E2F consensus sites but not only is E2F consensus site-mediated transactivation inhibited but it appears to be actively repressed by these complexes (Weintraub, Prater and Dean, 1992). It has recently been suggested that this occurs through pRB interacting with, and inactivating, the basal transcription complex (Weintraub et al., 1995). E2F-1, as well as being negatively regulated by pRB during early G1, becomes negatively regulated by phosphorylation by cyclin A-cdk2 activity in the S to G2 phase transition (Krek et al., 1994) and this phosphorylation also influences the affinity of the pRB-E2F-1 association (Peep et al., 1995). Thus E2F-1 activity appears to be tightly regulated to a specific phase of the cell cycle. The importance of pRB's interaction with E2Fs in regulating progression through the cell cycle is demonstrated by the over expression of E2F-1 in quiescent cells resulting in them progressing through G1 and into S phase (Johnson et al., 1993). Over expression of E2F-1 also overrides wild type pRB-induced growth arrest in an RB-defective osteosarcoma cell line (Qin et al., 1994).

Recently, the product of the MDM2 oncogene has also been implicated in the regulation of pRB and E2F transactivation, in addition to its previously characterised interaction with p53 (see section 1.6.1). Xiao et al., (1995) have demonstrated that MDM2 binds pRB and, as with p53, inhibits the growth regulatory functions of pRB. This inhibition of pRB growth suppression correlated with an increase in E2F transactivation function and it is possible that this occurs through the release of E2F proteins from their inhibitory complex with pRB. In another study, MDM2 was found also to be able to interact directly with the transcriptional activation domain of E2F-1 and with DP-1 (Martin et al., 1995). DP-1 is a member of a family of transcription factors with which the E2F family of proteins can heterodimerise in order to stimulate transcription (reviewed by La Thangue, 1994). This interaction was also shown to result in an increase in E2F transactivation and occurs through residues which are conserved between the activation domain of E2F-1 and p53. Thus it seems that the MDM2 protein, as well as inhibiting p53's transcription activation and repression activities, may also bind to pRB allowing the release of active E2F-1 (and E2F-2 and 3) and bind to E2F-1/DP-1 complexes to increase their activity.

1.6.2.4: p107 and p130 also regulate transcription during the cell cycle.

Two pRB-related proteins which have a great deal of homology within the "pocket" domain have been identified through their interactions with E1A and T antigen and their cross-reactivity with pRB antibodies (Dyson et al., 1989a; Ewen et al., 1989; Hu et al., 1991; Mayol et al., 1993). These proteins, p107 and p130, are also
believed to be involved in cell cycle regulation. Over expression of p107 brings about
cell cycle arrest at the G1 to S phase boundary in a number of human tumour cell lines
(Zhu et al., 1993). Both p107 and p130 are able to form complexes with cyclin A-cdk2
and cyclin E-cdk2 suggesting that they may be regulated by phosphorylation similarly to
pRB, though possibly at different times during the cell cycle (Lees et al., 1992; Hannon,
Demetrick and Beach, 1993; Li et al., 1993). The exact role of these two proteins in
cellular growth control is presently unclear, however they are able to form complexes
with two other members of the E2F-family of transcription factors, E2F-4 (p107 and
p130) and E2F-5 (p130 only; Ginsberg et al., 1994; Hijmans et al., 1995; Vairo,
Livingston and Ginsberg, 1995). p107 is found in cyclin A/E2F complexes during the S
phase of the cell cycle (Figure 1.2; Shirodkar et al., 1992), but is able to suppress E2F
function independently of its ability to bind to cyclin A/cdk2 (Smith and Nevins, 1995).
p130 appears to be the predominant E2F-associated protein in the G0/early-G1 phase
following the serum stimulation of quiescent cells, while p107 is major E2F-associated
protein near the G1/S phase border. p130/E2F complexes were also found to be
associated with cyclin E-cdk2 kinase in late G1, suggesting that the p130/E2F
complexes are dissociated by the phosphorylation of p130 (Cobrinik et al., 1993). p130-
E2F complexes are specifically disrupted by SV40 T antigen in the transformation of
NIH3T3 cells (Wolf et al., 1995). It seems, therefore, that these two proteins are able to
negatively regulate the expression of genes, transactivated specifically by the E2F-4 and
5 transcription factors, at specific times in the cell cycle. As yet it is not known whether
each of the E2F family members transactivate different genes, bind different E2F sites
and/or bind different members of the DP family of transcription factors.

1.6.2.5: pRB and p107 interact with transcription regulators other than E2F.

pRB and p107 are also able to directly repress transcription independently of
their interactions with members of the E2F family (Bremner et al., 1995; Dagnino et al.,
1995), and each has been shown to repress transcription from different promoters. In the
case of pRB, this E2F-independent repression of transcription has been shown to be
regulated by phosphorylation induced by cyclin A or cyclin E and maps to the "pocket"
region.

The ability of pRB to bind and regulate, positively or negatively, a number of
other nuclear proteins has been reported, including transcription factors such as Elf-1
(Wang et al., 1993a), MyoD (Gu et al., 1993), Id-2 (Iavarone et al., 1994), ATF-2 (Kim
et al., 1992b), BRG1 (Dunaief et al., 1994) and BRM (Singh, Coe and Hong, 1995) as
well as the c-Abl kinase (Welch and Wang, 1993). The involvement of pRB in the
regulation of the transcriptional activity of some of these transcription factors appears to
have a greater role in the differentiation of specific cell types than in cell cycle
regulation. Elf-1 is a lymphoid specific Ets transcription factor that regulates inducible
gene expression during T-cell activation (Thompson et al., 1992). The MyoD family is
involved in regulating the transition of proliferating myoblasts to terminally differentiated myotubes and thus has both myogenic and cell cycle suppressive activities (reviewed by Weintraub et al., 1991). Id-2 is a member of a family of helix-loop-helix proteins which regulate the DNA binding and transcriptional activation activities of basic helix-loop-helix (bHLH) proteins (Benezra et al., 1990). Over expression of Id-2 enhances cellular proliferation and also reverses pRB-mediated growth inhibition (Iavarone et al., 1994). It is possible that pRB regulates the activity of a number of bHLH transcription factors through its interaction with Id-2. pRB also regulates transforming growth factor β1 and 2 gene expression both of which down-regulate cellular proliferation in many cell types including most normal and transformed epithelial, fibroblast, lymphoid and haematopoietic cells (Kim et al., 1991). In the case of activation of TGF-β2 gene expression this has been shown to occur through the direct interaction of pRB with ATF-2 (Kim et al., 1992b). Both BRG1 and hBRM are homologues of the Saccharomyces cerevisiae SNF2/SWI2 transcriptional activator (Dunaief et al., 1994; Singh, Coe and Hong, 1995). The interaction of pRB with human BRG1 or BRM is involved in the ability of both BRG1 and BRM to induce cell cycle arrest in several human carcinoma cell lines. Both the BRG1 and BRM proteins include a region which has homology to the HPV E7 proteins CR2-like region, including the LxCxE motif critical in T antigen, E1A and E7 for their ability to bind to pRB. The observation that all three of these viral oncoproteins were able to disrupt pRB-BRG1 complexes and that BRG1 bound only the underphosphorylated, growth arresting form of pRB suggested that pRB-BRG1 (and possibly pRB-BRM) complexes play a critical role in cell cycle arrest (Dunaief et al., 1994). The interaction of pRB with BRM is necessary for pRB’s ability to up-regulate glucocorticoid-receptor-mediated transcription of genes through glucocorticoid response elements (Singh, Coe and Hong, 1995). c-Abl kinase is an ubiquitously expressed tyrosine kinase which binds to DNA. Binding of pRB to the ATP-binding region of this kinase inhibits its kinase activity (Welch and Wang, 1993). Phosphorylation of pRB results in the release of the active kinase during the S phase of the cell cycle.

pRB is able to regulate transcription of the c-fos, c-jun, TGF-β1, c-myc and insulin-like growth factor II genes through a motif which was termed the retinoblastoma control element (Kim et al., 1992a; Pietenpol et al., 1991; Robbins, Horowitz and Mulligan, 1990). While pRB up-regulates transcription from the c-jun, TGF-β1 and IGF-II promoters, it represses transcription from the c-fos and c-myc promoters. The transcription factor Sp1 can bind to and stimulates transcription from the retinoblastoma control element and pRB was found to positively regulate Sp1 transcriptional activity via these elements (Kim et al., 1992a). There is evidence that the ability of pRB to stimulate Sp1-mediated transcription occurs through the ability of pRB to bind an inhibitor of Sp1 (Sp1-I) thereby resulting in the release of transcriptionally active Sp1.
Chapter 1. (Chen et al., 1994). The method by which pRB down-regulates transcription from other promoters through the same element is poorly understood, however the ability of pRB to regulate expression of both c-jun and c-fos indicates that pRB indirectly regulates AP-1 transcriptional activity, as well as the activity of other transcription factors. Thus pRB may inhibit cellular proliferation through other transcription factors in addition to the E2F family.

It is possible that the pRB-related proteins p107 and p130 also inhibit other transcription factors in addition to members of the E2F family, although few such interactions have been defined. p107 has been demonstrated to inhibit c-myc related transactivation through a direct binding of p107 to the transactivation domain of the c-myc protein (Beijersbergen et al., 1994). Over expression of c-myc releases p107-induced growth arrest in SAOS-2 cells suggesting that the c-myc protein is a target for p107-mediated growth suppression at the G1/S phase boundary. Thus p107 is able to inhibit the transcription of genes which are transactivated by c-myc. At present the genes which are up-regulated by c-myc are poorly defined, however constitutive expression of human c-myc in several cell lines results in elevated levels of cyclin A and E mRNAs (Jansen Dürre et al., 1993). c-myc has also been shown to negatively autoregulate the transcription of its own gene (Penn et al., 1990) and to repress the transcription of cyclin D1 (Philipp et al., 1994), one of the cyclins believed to regulate the phosphorylation of pRB.

1.6.2.6: The interactions of viral oncogenes with the pRB-family.

Through interacting with pRB and the pRB-related proteins, p107 and p130, the oncoproteins of the small DNA tumor viruses are able to interfere with the regulation of a range of transcription factors. Each of these transcription factors may be involved, to a greater or lesser extent, in regulating the expression of genes involved in cell cycle progression. Thus the ability of these oncoproteins to inactivate the pRB-family may be involved in their ability to deregulate cellular proliferation. Clearly the pRB family exhibit their growth suppressive activities through inhibiting transcription factors required for growth stimulation and cell cycle progression.

Interestingly pRB has been shown to inhibit apoptosis following the irradiation of SAOS-2 cells (Haas-Kogan et al., 1995). p53-dependent apoptosis is also induced by pRB-deficiency during the embryonic development of mouse lens fibre cells in RB knockout mice embryos (Morgenbesser et al., 1994). Since continuous c-fos expression is observed prior to apoptosis (Smeyne et al., 1993), a possible mechanism for the inhibition of apoptosis by pRB may involve its ability to suppress c-fos gene expression. Several genes important for cell cycle regulation, including cyclin D and E2F, as well as the viral oncogenes E1A and E7, are associated not only with the induction of cell proliferation but also with the induction of apoptosis (Rao et al., 1992; Freeman, Estus and Johnson, 1994; Howes et al., 1994; Wu and Levine, 1994). The ability of these
proteins to bind pRB may be involved not only in their cell cycle regulatory roles but also in their ability to induce apoptosis. Constitutive expression of the c-myc proto-oncogene in the absence of serum is also associated with the induction of apoptosis (Askew et al., 1991; Evan et al., 1992). Since there is no evidence for the existence of a c-myc-pRB interaction in vivo, it is possible that p107, which c-myc is able to bind, may also be involved in the induction of apoptosis. Thus, it seems that pRB, and, potentially, other members of the pRB-family, may not only suppress cellular proliferation but also suppress apoptosis. The role of these proteins may therefore be to arrest growth in the absence of apoptosis and these roles may be important in the development of many differentiated cell types.

1.6.3: Other tumour suppressor proteins.

In addition to the RB and p53 genes a number of other tumour suppressor genes have also been identified, although none of these have been studied as extensively as the RB or p53 gene products. Since none of the products of these tumour suppressor genes are directly implicated in the growth stimulatory activities of the small DNA tumour viruses, I shall only briefly mention them here. Several of these tumour suppressor genes were identified on the basis of particular chromosomal deletions and allelic losses that occur in close linkage with hereditary diseases. It is interesting to note that abnormalities in these genes are involved only in tumorigenesis in specific tissues, suggesting that the products of these genes normally negatively regulate cell growth in specific cell types and are not more generally involved in the regulation of the cell cycle.

The WT1 gene locus, mutations in which are associated with Wilms's (kidney) tumour (reviewed by Van Heyningen and Hastie, 1992), encodes a transcription factor which is important for kidney development (Kreidberg et al., 1993). Interestingly, the WT1 gene product (WT1) associates with p53 thereby regulating the transactivation activities of both p53 and WT1 (Maheswaran et al., 1993). Other tumour suppressor genes include the DCC ("deleted in colon carcinoma") gene, the neurofibromatosis type-1 (NF1) and type-2 (NF2) genes, the adenomatous polyposis coli (APC) gene, the von Hippel-Lindau syndrome (VHL) gene and the breast cancer 1 (BRCA1) gene (reviewed by Knudson, 1993; Vogelstein and Kinzler, 1994).

Several members of the cyclin-dependent kinase inhibitor family have also been implicated as tumor suppressor genes. The importance of one of these cdk-inhibitors, the p53-inducible p21, in regulating cell cycle progression by inactivating specific kinases at multiple stages of the cell cycle, was discussed previously (section 1.6.1.4). It has been suggested that the gene encoding another of these cdk-inhibitors, p16 (INK4/MTS1), is homozygously deleted in as many as 90% of the primary tumours and 75% of the tumour-derived cell lines examined (reviewed by Kamb, 1995). The p16 gene is located on human chromosomal region 9p21 and is believed to be involved in
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familial (hereditary) melanoma. The gene for a second cdk-inhibitor, p15 (INK4B/MTS2) is located immediately adjacent to the p16 gene and thus this gene may also be deleted in tumorigenesis. Both p15 and p16 are able to bind to, and inhibit cyclin D-cdk4 or cdk6 complexes but are not known to inhibit any of the other cdk complexes (Figure 1.2; Serrano, Hannon and Beach, 1993; Hannon and Beach, 1994). Two other cdk inhibitors, p21 (section 1.6.1.4) and p27 (KIP1) are more promiscuous inhibitors of cdks, being able to bind to and inhibit cyclin A/E-cdc2 (p21 only), cyclin A/D/E-cdk2, cyclin D-cdk4 or cyclin D-cdk6 (Harper et al., 1993; Xiong et al., 1993; Toyoshima and Hunter, 1994). As yet there is no evidence for deletions in the p21 or p27 genes being involved in tumorigenesis. Another two cdk inhibitors have also been cloned. The first, p18 is a homologue of p15 and p16 which predominantly inhibits the activity of the cdk6 kinase (Guan et al., 1994) and has not yet been implicated in tumorigenesis. The gene for the second, p57 (KIP2), which is more closely related to p21 and p27, is located at 11p15.5, a region which is implicated in both sporadic cancers and with a familial cancer syndrome (Beckwith-Wiedemann syndrome) suggesting p57 may be a tumor suppressor (Matsuoka et al., 1995).

1.7: SV40 and polyomavirus genome organisation.

SV40 (5243bp) and Py (5292bp) are members of the papovavirus group of small icosahedral DNA viruses which contain double-stranded DNA genomes (Tooze, 1981). Sequence comparisons suggest that these viruses have co-evolved with their hosts (SV40 with monkey, Py with mouse and the related BK virus with human) from a common ancestor (Soeda et al., 1980). Lytic infection of cells permissive for viral infection results in full viral gene expression, synthesis of progeny particles and eventually cell death. In contrast, non-permissive cells survive viral infection and progeny particles are not produced however the early viral proteins are expressed. The SV40 lytic infection is discussed in section 1.8.1.

The location of the coding sequences on the viral genome for the SV40 and Py proteins are shown in Figure 1.3. Lytic infection by these viruses can be divided into two phases, early and late, defined by the onset of viral DNA replication. During the early phase of infection the viral genes from the "early" region (Figure 1.3) are expressed (Griffin, 1981). Following viral and cellular DNA replication the "late" region genes are expressed which encode the structural components of the virus particle. Both DNA molecules have a single origin of replication which is located in the region of transcriptional initiation for both the early and late genes and contains multiple large T antigen binding sites.

The Py early region encodes three proteins, designated small t, middle T and large T antigens while SV40 encodes only two early proteins, designated small t and
The viral genomes of SV40 and Py are shown with the origin of viral DNA replication (ORI) at the top. The early and late regions are indicated. Regions of the viral genome encoding the virus specific proteins; the different reading frames from which the proteins are translated are indicated by different shading. The positions of the polyadenylation signal sequences are also shown (pA). Numbers indicate the nucleotide position. N= amino-terminus, C= carboxy-terminus.
large T antigens. In each case these proteins are translated from differentially spliced mRNAs which have common translational start sites resulting in the early proteins having homologous amino-termini. Differential splicing of the early transcript then gives rise to the different proteins through the translation of different reading frames (Griffin, 1981). The late regions of both SV40 and Py encode three proteins VP1, VP2 and VP3 which are the structural components of the virion. VP3 corresponds to the carboxy-terminal portion of VP2 and this coding sequence partially overlaps the VP1 coding sequence (Figure 1.3). The late region mRNAs comprise a short 5'-untranslated leader segment attached to alternative body segments (Ziff, 1980) which contain the two large open reading frames from which the proteins are translated (Griffin, 1981). In contrast to the early region mRNAs, no splicing occurs within the late protein coding regions. The different mRNAs are generated by juxtaposing the 5'-"leader" segment to alternative downstream initiation codons.

1.8: SV40 large tumour antigen.

Simian virus 40 causes a lytic infection of monkey cells and an abortive infection of either rat or mouse cells. The replication of the viral genome (5243bp) in the lytic infection of monkey cells is entirely dependent on cellular chromosomal replication proteins and cellular metabolism, except for the SV40-encoded large T antigen which is required for the initiation of replication (Tegtmeyer, 1972; Tooze, 1981; Li and Kelly, 1984). This dependence on cellular replication proteins means that SV40 DNA replication can only occur in the S phase of the cell cycle. Thus the SV40 early gene products, large T and small t antigen have evolved the ability to stimulate cell growth and cellular DNA synthesis so that the cell can support viral replication. SV40 infection of monkey cells results in the production of thousands of daughter virions per cell upon lysis after no more than a few days (Tooze, 1981). Rodent cells infected with SV40 are not permissive for viral DNA replication and often survive the infection. If T antigen is expressed, following integration of the SV40 DNA into the genome, these cells display altered growth properties and often acquire the ability to induce tumours in experimental animals. This appears to be due to T antigen stimulating cellular growth despite the infection being abortive.

SV40 large T antigen is a multifunctional phosphoprotein of 708 amino acids and is predominantly located in the cell nucleus (Soule and Butel, 1979), although a small fraction is found in the plasma membrane (Santos and Butel, 1982). Several biochemical activities are associated with T antigen's role in viral DNA replication. It localises to the nucleus by virtue of a nuclear localisation signal between amino acids 126 to 132 and this localisation is thought to be regulated by phosphorylation on serine 112 by casein kinase II (Kalderon et al., 1984; Lanford and Butel, 1984; Jans and Jans,
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1994). T antigen has been shown to be an ATPase (Tjian and Robbins, 1979; Clark, Lane and Tjian, 1981), DNA and RNA helicase (Stahl, Droge and Knippers, 1986; Dean et al., 1987a; Scheffner, Knippers and Stahl, 1989), has topoisomerase activity (Marton et al., 1993), binds RNA covalently (Carroll et al., 1988) and is capable of both specific and non-specific binding of DNA (Carroll, Hager and Dulbecco, 1974; Tjian, 1978; McKay, 1981; Prives et al., 1982). While these activities have been shown to be necessary for viral DNA replication, none of them are required for the immortalisation or transformation of rodent cells (Gluzman et al., 1977; Stringer, 1982; Kalderon and Smith, 1984; Manos and Gluzman, 1984; Cole et al., 1986; Tevethia et al., 1988; Peden et al., 1990). T antigen is capable of both activating and repressing transcription from a number of viral and cellular promoters (Reed, Stark and Alwine, 1976; Alwine, Reed and Stark, 1977; Hansen et al., 1981; Brady and Khoury, 1985; Loeken, Kuoury and Brady, 1986; Robbins, Rio and Botchan, 1986). Large T antigen also binds a number of host cellular proteins such as p53 (Lane and Crawford, 1979; Linzer and Levine, 1979), pRB (DeCaprio et al., 1988; Ewen et al., 1989), p107 (Dyson et al., 1989a), p130 (Hannon, Demetrick and Beach, 1993), p185 (Kohrman and Imperiale, 1992), DNA polymerase α (Smale and Tjian, 1986; Gannon and Lane, 1987) and heat shock protein-73 (hsp-73; Sawai and Butel, 1989). The specific regions of T antigen which are required for the interaction with some of these proteins and some of its biochemical activities have been identified and are shown in Figure 1.4.

1.8.1: The role of SV40 T antigen in SV40 DNA replication and lytic infection.

Upon SV40 infection of permissive host cells, transcription initiating at the early promoter leads to synthesis of the early viral genes (large T and small t antigens). T antigen accumulates primarily in the nucleus and interacts with growth regulatory proteins, for example p53 and pRB, thereby altering gene expression and inducing the cells to enter the cell cycle and progress to S phase in preparation for viral DNA replication (reviewed by Fanning and Knippers, 1992). The methods by which T antigen induces the cells to enter the cell cycle give T antigen its oncogenic capacity in cells which are non-permissive for SV40 replication and shall be discussed later (section 1.7.3).

SV40 replication has been studied extensively as a model for mammalian DNA replication and as a result a cell-free replication assay has been developed (Waga, Bauer and Stillman, 1994). SV40 DNA replication involves the ATP-dependent assembly of T antigen double hexamers around specific DNA sequences within the SV40 origin of replication (Dean et al., 1987b; Reynisdottir et al., 1993) which requires the zinc finger region of T antigen (Loeber et al., 1991), followed by the local unwinding of the SV40 genome which involves the ATPase activity of T antigen (Stahl, Droge and Knippers, 1986; Dean et al., 1987a; reviewed by Stillman, 1989; Borowiec et al., 1990).
Figure 1.4: Map of SV40 large T antigen functions.

Some of the functions of T antigen which have been mapped to particular regions are shown. The phosphorylated residues which have been identified in mammalian cells are also shown (reviewed in Prives, 1990; Fanning, 1994). The references for the functions shown are as follows: (1) Donreiter et al. (1990), Smale & Tjian (1986), Gannon & Lane (1987); (2) Sawai, Rassmusen & Butel (1994); (3) Kohrman & Imperiale (1992); (4) Gruda et al. (1993); (5) Figg et al. (1988), Yacuik et al. (1991); (6) Decaprio et al. (1988), Dyson et al. (1989b), Ewen et al. (1989); (7) Kalderon et al. (1984), Lanford, Kanda & Kennedy (1986); (8) Paucha et al. (1986), Arthur, Höss & Fanning (1988); (9) Stahl, Droge & Knippers (1986); (10) Prives et al. (1982); (11) Loeb, Parsons & Tegtmeyer (1989); (12) Kierstead & Tevethia (1993); (13) Clark, Lane & Tjian (1981), Clark et al. (1983), Bradley et al. (1987); (14) Soprano et al. (1983); (15) Pipas (1985), Tornow et al. (1985).

Replication continues with the recruitment of the DNA polymerase α-primase complex through direct association with T antigen and a cellular single-stranded DNA-binding factor, RF-A, in a manner which is dependent on SV40 origin DNA (reviewed by Fanning and Knippers, 1992). T antigen now functions as a DNA helicase to unwind the two strands thereby forming the replication fork and the two T antigen hexamer complexes move away from each other along the DNA. DNA synthesis commences...
with the synthesis of Okazaki fragments by DNA polymerase α-primase, which are extended into leading DNA strands by the activity of the DNA polymerase δ complex (reviewed by Stillman, 1989; Borowiec et al., 1990).

The replication functions of T antigen are known to be regulated both positively and negatively by phosphorylation (reviewed by Prives, 1990; Fanning, 1992; Fanning, 1994) and it is possible that this regulation is important for ensuring that T antigen binds the SV40 origin of replication at specific sites as the cell enters the S phase of the cell cycle. T antigen can be phosphorylated on multiple serine and threonine residues clustered at two sites, one towards the amino-terminus and the other at the carboxy-terminus (Figure 1.4). Analysis of each of these phosphorylated residues demonstrated that T antigen is activated by phosphorylation on threonine 124, possibly in a cell cycle dependent manner by cyclin A-cdk2 at the onset of S phase (Adamczewski, Gannon and Hunt, 1993), but is inhibited by phosphorylation on serines 120 and 123, possibly by casein kinase I (Cegielska and Virshup, 1993). Serines 120 and 123 are dephosphorylated by the catalytic subunit of protein phosphatase 2A (PP2A-C) thereby activating T antigen for DNA replication. It is interesting that SV40 small t antigen associates with PP2A-C (Pallas et al., 1990) thereby inhibiting its ability to dephosphorylate T antigen (Scheidtmann et al., 1991). Small t antigen has been shown to inhibit SV40 DNA replication in cell-free replication assays (Carbone et al., 1992), however in permissive monkey cells small t has been shown to stimulate viral DNA synthesis (Cicala et al., 1994). Virion production, although not dependent upon small t antigen, is more efficient in the presence of small t antigen (Tooze, 1981). It is possible that small t antigen plays a role in regulating the ratio of phosphorylated to unphosphorylated T antigen present during the progression of the cell through the cell cycle and this role is important for efficient virion production.

T antigen then represses the early promoter (thereby autoregulating the amount of T antigen synthesised) and activates the late transcription unit which encodes the three viral capsid proteins (VP1, VP2 and VP3). The carboxy-terminus of T antigen is required for host range specificity and efficient virion assembly (Fanning and Knippers, 1992; Pipas, 1992; Tooze, 1981).

Replication of the other polyomaviruses also appears to depend on their large T antigens, most of which have similar activities to those required for the efficient replication of SV40 by SV40 T antigen. The T antigens of JC virus, BK virus, SA12 virus and SV40 have 75% identity while lymphotrophic polyomavirus, K virus, hamster polyomavirus and murine polyomavirus (Py) have 45-55% homology to SV40 (Pipas, 1992). However these T antigen proteins do have a great deal of homology in the regions required for DNA replication, for example in their nuclear localisation signals and their zinc finger motifs. Like SV40 large T antigen, Py T antigen is a nuclear protein which binds to Py origin of viral DNA replication and has both ATPase and
DNA helicase activities. Both SV40 and Py T antigens, which have been most extensively studied, seem to replicate their respective viral genomes similarly, however they do appear to differ in their ability to bind the cellular protein, p53, since Py T antigen does not bind p53. It is possible that this reflects differences in growth regulation between the cell types that these two viruses normally replicate.

1.8.2: Transcriptional regulation by SV40 large T antigen.

SV40 large T antigen is able to positively or negatively regulate the transcription of a number of cellular genes in addition to the SV40 early and late regions. This transcriptional regulation plays a role in stimulating cell cycle progression following SV40 infection. Many of these activities may be due to the ability of T antigen to bind and inactivate the activities of the tumor suppressor gene products pRB and p53, both of which interact with, and positively or negatively regulate, a number of transcription factors (as discussed in section 1.6.1 and 1.6.2). However T antigen has also been shown to interact with a number of other cellular transcription factors and these interactions may also be important for its ability to stimulate growth. It is most likely, therefore, that a diverse set of mechanisms underlie the changes in cellular gene expression bought about by T antigen following SV40 infection. The ability of T antigen to activate the SV40 late promoter, the Rous sarcoma virus LTR promoter and the adenovirus E2 promoter have been most well studied.

During SV40 lytic infection T antigen represses transcription from the SV40 early promoter but stimulates transcription from the SV40 late promoter (Tooze, 1981). T antigen's ability to repress SV40 early transcription occurs through it binding directly to sequences in the SV40 origin of DNA replication (Rio and Tjian, 1983). T antigen mutants defective for DNA binding are defective for this autoregulation (Reed, Stark and Alwine, 1976; Alwine, Reed and Stark, 1977; Rio and Tjian, 1983). In addition, high affinity binding sites for T antigen have been observed in the cellular genome and are implicated in the repression of transcription from several promoters (Gruss et al., 1988; Wagner and Knippers, 1990).

In contrast, direct binding of T antigen to DNA is not absolutely required for its ability to stimulate the SV40 late promoter, however T antigen mutants which fail to bind to viral DNA have a reduced ability to transactivate (Brady and Khoury, 1985; Zhu et al., 1991b). In the presence of T antigen, cellular factors which bind to the regions of the late promoter required for activation have altered and more stable binding characteristics (Gallo et al., 1990). These observations suggest that T antigen activates the late promoter by activating cellular trans-acting proteins which subsequently interact with sites within the promoter. These T antigen-activated cellular factors may also be responsible for the activating transcription from the promoters of the cellular genes that T antigen up-regulates.
T antigen contains multiple domains which function to activate transcription, each of which may be involved in activating transcription from different promoter elements. Through binding and inactivating the pRB-family of proteins T antigen releases members of the E2F family of transcription factors, which leads to the transcriptional activation of genes with E2F sites (Chittenden, Livingston and Kaelin, 1991; Huang, Lee and Lee, 1991). T antigen may also lead to the repression of transcription from promoters which require members of the pRB family for their stimulation (section 1.6.2). Similarly T antigen interferes with the transcription from promoters which are regulated by p53 (Mietz et al., 1992; section 1.6.1). However, neither the pRB or p53 binding regions of T antigen are implicated in transactivating the SV40 late promoter, the RSV LTR promoter or the Ad E2 promoter (Zhu et al., 1991b). The transactivating function required by T antigen to stimulate transcription from these three promoters is retained, albeit at a decreased level compared to wild type, in a truncation mutant which retains only the amino-terminal 138 amino acids and is disrupted by mutations within the amino-terminal 85 amino acids. Mutations within, or near, the zinc finger region (amino acids 300-350) of T antigen also partially decreased the transactivation function of T antigen (Zhu et al., 1991b).

The SV40 late promoter, RSV LTR promoter and Ad E2A promoter do not have in common any sequence elements which are required for efficient transcription from these promoters, thus the mechanism by which T antigen mediates transcription from these promoters does not occur through a common element. The ability of T antigen to bind the TATA-box-binding protein (TBP), through a region in its amino-terminal 172 residues, is required for T antigen mutants to activate transcription from the SV40 late promoter (Gruda et al., 1993). Rice and Cole (1993) have demonstrated that T antigen can activate transcription from a number of simple modular promoters consisting of an upstream activating sequence (either a CAATT box, an ATF site, an Sp1 site or an AP1 site) and a TATA box (from either the HSP70 promoter, the Ad E2A promoter or the SV40 early promoter). The final level of transcription was found to be more dependent on the nature of the TATA box than the nature of the upstream sequence. T antigen mutants which failed to activate transcription from the SV40 late and RSV LTR promoters also failed to activate transcription from these modular promoters (Rice and Cole, 1993). Although the SV40 late promoter has no TATA box, TBP is also known to be important for transcription from promoters which lack obvious TATA boxes (Wiley, Kraus and Mertz, 1992). It has been suggested therefore that T antigen mediates transcriptional activation by interacting with and stabilising the overall transcription complex (the components of the basal transcription machinery and the factors that recognise upstream sequences).

T antigen's transcription activation of the SV40 late promoter has been shown to require binding sites (SPH/OCT motifs) for transcription-enhancing factor 1 (TEF-1;
Caseaz, Sundseth and Hansen, 1991). This transcription factor is bound by T antigen through different regions than those required for its interaction with TBP (Gruda et al., 1993) possibly through a region between amino acids 131-259 (region X; Dickmanns et al., 1994). Fragments of T antigen were used to show that the binding of both TEF-1 and TBP by T antigen could stimulate wild type levels of transcriptional activation from the SV40 late promoter and a simple modular TEF-1 site/TATA box promoter. This further supports the suggestion that T antigen causes transcriptional activation through direct interactions with multiple factors in the transcription complex. Since it seems unlikely that T antigen is able to bind all of the proteins responsible for interacting with the range of upstream elements in the modular promoters used by Rice and Cole (1993), it is possible that T antigen is also able to stabilise the basal transcription complex through TBP without interacting directly with proteins bound to upstream elements. Thus it seems that the promiscuous nature of T antigen-mediated transcriptional activation is due to the simplicity of the promoter structure. A TATA or initiator element plus an upstream factor-binding site seem to be the only promoter elements required for T antigen to transactivate (Gilinger and Alwine, 1993; Rice and Cole, 1993).

It is interesting to note that adenovirus E1A can also bind TBP (Lee et al., 1991) and is able to activate transcription from the same modular promoters (Taylor and Kingston, 1990). E1A is also able to bind a 300kDa protein, p300, which has been suggested to be functionally homologous to the transcriptional coactivator CREB-binding protein (CBP; Arany et al., 1995; Lundblad et al., 1995). CBP is a co-activator for the cAMP response element-binding protein (CREB) and p300 can substitute for CBP in stimulating CREB-activated gene expression. E1A inhibits cAMP-induced gene expression through the cAMP response element and this has been shown to correlate with its ability to bind either CBP or p300 (Lundblad et al., 1995). p300 is present in TBP complexes (Abraham et al., 1993), thus E1A may interfere with the transcriptional activating functions of p300-TBP complexes. The CR1 domain of E1A, required for its interaction with p300, is conserved in T antigen. As yet an interaction between T antigen and p300 has not been conclusively demonstrated. However E1A mutants defective for p300 binding are able to be complemented in ras cooperative transformation assays by T antigen mutants with intact CR1-like regions (Yacuik et al., 1991). If an interaction between T antigen and p300 (or another CBP-like protein) does exist it may represent another mechanism through which T antigen can regulate transcription.

In addition to these interactions, T antigen has been reported to bind the transcription factor AP-2 and inactivate it in vitro (Mitchell, Wang and Tjian, 1987). Whether this interaction plays a role in the ability of T antigen to regulate transcription in vivo has yet to be demonstrated. T antigen has also been observed in c-jun-containing
complexes and this interaction is believed to be involved in the ability of c-jun and T antigen to down-regulate myelin P0 gene expression in Schwann cells (Bharucha, Peden and Tennekoon, 1994). T antigen transactivation also results in an up-regulation in the expression of a number of other transcription factors, including c-myc (Batsche, Lipp and Cremisi, 1994) and Sp1 (Saffer, Jackson and Thurston, 1990). The ability of T antigen to increase c-myc expression occurs through E2F sites in the c-myc promoter and is mediated through the pRB binding domain of T antigen. Through increasing the expression of other transcription factors, T antigen may indirectly stimulate transcription from a number of other genes which are transactivated by these transcription factors.

1.8.3: Induction of cellular proliferation by SV40 large T antigen.

Introduction of large T antigen into primary rodent cells results in the cells acquiring an infinite proliferative potential but does not necessarily result in them becoming fully transformed (Petit, Gardes and Feunteun, 1983; Jat and Sharp, 1986). Only a small proportion of these immortal cells progress to the transformed state. Inactivation of the T antigen in these immortal cells results in a rapid and irreversible loss of proliferative potential in either the G1 or G2 phases of the cell cycle demonstrating that the T antigen protein is continuously required to maintain the proliferative state (Jat and Sharp, 1989). Introduction of large T antigen into established cell lines, however, can result in these cell lines acquiring a more fully transformed phenotype (Kriegler et al., 1984; Brown et al., 1986; Jat et al., 1986; Jat and Sharp, 1986). A large amount of research has been carried out to identify the functions of T antigen which are required for its ability to stimulate growth. As a result a number of cellular assays have been used to investigate the growth stimulatory effects of SV40 large T antigen mutants. These assays have studied the ability of T antigen to induce DNA synthesis in quiescent cells, to immortalise primary cells from different species, to transform primary cells in cooperation with a second, cytoplasmic oncogene such as activated ras or Py middle T antigen, to allow anchorage independent growth of established cells, to allow growth of established cells in low serum concentrations or at high saturation densities, and to induce tumours in experimental animals. Some of the regions identified by these various assays appear to be similar, however, other regions which have been identified as necessary in one assay are not required in others. It is clear therefore that the functions required by T antigen to stimulate growth are different depending on the cellular growth assay used.

The ability of T antigen to induce viral DNA replication is genetically separable from its ability to transform cells. This was demonstrated by the isolation of a number of T antigen mutants which are defective for viral DNA replication but retain the ability to transform established cell lines (Gluzman and Ahrens, 1982; Stringer, 1982; Manos and Gluzman, 1984; Peden and Pipas, 1985). A number of DNA replication-positive,
transformation-negative T antigen mutants have also been characterised (Cosman and Tevethia, 1981; Rutila, Imperiale and Brockman, 1986; Peden et al., 1990). Similarly, the viral DNA replication functions of Py large T antigen are separable from its ability to immortalise (Larose, St Onge and Bastin, 1990). Analysis of T antigen mutants defective for nuclear localisation have shown that the nuclear localisation signal (NLS) is not required for the transformation of established cells in culture (Kalderon et al., 1984; Lanford and Butel, 1984; Fischer-Fantuzzi and Vesco, 1987). These results suggested that T antigen was acting in the same way as cytoplasmic oncoproteins in its ability to transform or that a proportion of T antigen was able to localise to the nucleus by a mechanism independent of its nuclear localisation signal. Several of these NLS-T antigen mutants have also been reported to immortalise primary cells efficiently (Fischer-Fantuzzi and Vesco, 1987; Vass-Marengo et al., 1986). Immortalisation by such NLS-mutants seems to correlate with the entry of a proportion of the T antigen into the nucleus independently of the NLS (P.S.Jat, Transformation Studies Laboratory, Ludwig Institute for Cancer Research, University College Branch, unpublished data).

A great deal of attention has focused on the involvement of large T antigen's interaction with the tumour suppressor gene products p53 and pRB in immortalisation and transformation. Both p53 and pRB have been shown to interact with proteins involved in regulating the cell cycle, are capable of suppressing tumour formation and mutations in both genes are implicated in human tumorigenesis (discussed in sections 1.6.1 and 1.6.2). The region of large T antigen necessary for binding to and stabilising p53 has been identified between amino acids 347 to 627 (Tevethia et al., 1988; Lin and Simmons, 1991; Zhu et al., 1991a). More recently this region has been divided into two domains, encompassing amino acids 351 to 450 and 532 to 627, which are required either for p53 binding or for the overall stability of the T antigen protein (Kierstead and Tevethia, 1993). As discussed previously (section 1.4) T antigen complexes with the underphosphorylated form of pRB through a domain of the protein (amino acids 102 to 115) that has sequence homology to conserved region 2 (CR2) of the Ad E1A proteins (Stabel, Argos and Philipson, 1985; Ludlow et al., 1989). These regions have been shown to be functionally homologous between E1A and T antigen by domain swapping (Moran, 1988) and by cooperation between various E1A and T antigen mutants (Yacuik et al., 1991). The pRB, p107 and p130 all bind to this domain of T antigen (DeCaprio et al., 1988; Ewen et al., 1989). The p107 and p130 proteins share several properties with pRB such as interaction with the same families of cellular proteins and homology in the pocket region. Similar regions are found in the HPV E7 protein and Py large T antigen both of which are also able to bind to pRB (Phelps et al., 1988; Dyson et al., 1989b; Dyson et al., 1990). Since Ad E1A, HPV16 E7 and Py large T antigen, as well as SV40 large T antigen, all have the ability to establish continuous in vitro proliferation of primary cells (Petit, Gardes and Feunteun, 1983; Rassoulzadegan et al., 1983; Ruley,
T antigen has homology to conserved region 1 (CR1) of the Ad E1A proteins within its amino-terminus, as do HPV E7 and Py large T antigen. This region is required for E1A’s ability to bind to a cellular 300kDa protein (p300) and mutations in this domain of E1A result in loss of transforming activity in cooperation with E1B or activated ras (Lillie et al., 1987; Moran and Zerler, 1988; Whyte, Ruley and Harlow, 1988; Jelsma et al., 1989) and immortalisation of baby rat kidney epithelial cells (Schneider et al., 1987; Kuppuswamy, Subramanian and Chinnadurai, 1988; Quinlan and Douglas, 1992). The ability of E1A to bind to p300 is, therefore, believed to be one of the functions which is important for E1A’s growth stimulatory effects. Moran and colleagues have shown that the CR1-like region of T antigen carries a function capable of complementing an E1A mutant, deficient in p300 binding, in the cooperative transformation of baby rat kidney cells with ras (Yacuik et al., 1991). As yet however no interaction between p300 and T antigen has been documented.

Induction of DNA synthesis in quiescent cells has been mapped to T antigen coding sequences including amino acids 1-272 (Soprano et al., 1983), 1-259 (Dobbelstein et al., 1992) and 115-708 (Tjian, Fet and Graessmann, 1978). These results suggest that the function required for induction of DNA synthesis resides between amino acids 115 to 259 and could possibly correspond to the sequence-specific DNA binding function of T antigen. However a T antigen mutation that abolished the DNA binding activity of the T antigen fragment encompassing amino acids 1-259 did not abolish its ability to stimulate cellular DNA synthesis (Dobbelstein et al., 1992). This region alone is not sufficient to stimulate DNA synthesis but requires another function in either the carboxy-terminus (possibly p53 binding) or the amino-terminus (pRB-family binding). The functions mapping to the 115-259 region and the pRB-binding region were shown to act independently, since two single mutants (one with a point mutation in the pRB-binding region and one with a point mutation which inactivated the 115-259 function) could cooperate in trans to induce DNA synthesis (Dobbelstein et al., 1992). Adenovirus E1A also induces DNA synthesis and this activity required the presence of either the p300- or pRB-binding regions, but not both, suggesting that the inactivation of these two proteins acts in the same pathway or that these two pathways have similar biological effects (Howe et al., 1990).

A number of regions of T antigen have been shown to be required for efficient immortalisation of primary rat or mouse cells, production of dense foci or transformation with ras of established cell lines. The regions required appear to vary depending on the type of growth assay, cell type, and possibly even the species, used when assaying the functions different T antigen mutants. It is generally accepted that the carboxy-terminus of T antigen beyond amino acid 627 can be removed without loss
of immortalisation, transformation or tumorigenic activity (Tevethia et al., 1988). Furthermore, SV40 small t antigen is not required for immortalisation, transformation or tumorigenicity (Thompson et al., 1990), although it has been shown to enhance the transformation activity of low levels of T antigen through the amino-terminal 82 amino acids shared by the two proteins (Montano et al., 1990) and to be necessary for the low frequency transformation of human cells (De Ronde et al., 1989).

Amino-terminal fragments of the T antigen protein have been shown to be functional in a number of growth assays. Amino acids 1-121 of T antigen have been shown to be sufficient to transform C3H10T1/2 (Srinivasan, Peden and Pipas, 1989), amino acids 1-137 or 1-311 to immortalise primary rat embryo fibroblasts (Colby and Shenk, 1982; Asselin and Bastin, 1985), amino acids 1-147 to immortalise secondary rat embryo fibroblasts (Sompayrac and Danna, 1991), to transform F111 cells (Sompayrac and Danna, 1988) and to cooperate with an activated ras oncogene to transform rat embryo fibroblasts (Cavender et al., 1995). In contrast, similar mutants encoding amino acids 1-138 and 1-140 have been shown to be unable to immortalise primary mouse embryo fibroblasts (Tevethia et al., 1988). One of these mutants (dl1137; amino acids 1-121) has also been shown to be capable of inducing choroid plexus tumours, but not lymphomas, in transgenic mice (Chen et al., 1992), whereas wild type T antigen was able to induce both. Thus transformation by this amino-terminal fragment of T antigen appeared to be cell type specific. This mutant is able to bind the pRB-family but not p53 (J.M.Pipas, Department of Biological Sciences, University of Pittsburgh, unpublished data; G.M.Mazars, Transformation Studies Laboratory, Ludwig Institute for Cancer Research, University College Branch, unpublished data). Choroid plexus tumours developed more rapidly when the dl1137 fragment was expressed in p53-null mice (Symonds et al., 1994) suggesting a role for p53 in determining the efficiency of tumorigenesis by T antigen. A similar amino-terminal fragment of Py large T antigen (amino acids 1-259) is also sufficient to immortalise rat embryo fibroblasts (Holman, 1994).

Mutations within the amino-terminal 82 amino acids have been shown to lead to defective transformation ability in both the transformation of an established cell line and transformation in a ras cooperation assay (Montano et al., 1990; Marsilio et al., 1991). Moreover a T antigen mutant lacking amino acids 17-27, which lacks the ability to complement E1A mutants defective in binding p300 in ras cooperative transformation (Yacuik et al., 1991), has been shown to be defective for the transformation of REF52 (Pipas, Peden and Nathans, 1983) and C3H10T1/2 as well as non-established REFs in cooperation with activated ras (Michalovitz et al., 1987). Mutants with deletions within this extreme amino-terminal region still retain the ability to bind to the pRB-family and p53. A fusion protein comprising the region for interacting with pRB from Py large T antigen and the p53 binding domain of SV40 T antigen was also defective for transformation (Manfredi and Prives, 1990). Taken together these results indicate that T
antigen's ability to bind p53 and the pRB-family is not sufficient for transformation. However the T antigen mutant lacking amino acids 17-27 is able to induce the development of T-cell specific thymic lymphomas in transgenic mice but is lacking in T antigen's ability to induce choroid plexus tumours (Symonds et al., 1993). Wild type T antigen can induce lymphomagenesis in both T- and B-cells, thus this region of T antigen appears to be required for the transformation of some, but not all, cell types. T antigen mutants with deletions in their amino-terminus retain the ability to immortalise primary mouse embryo fibroblasts at only a slightly reduced efficiency compared to wild type T antigen (Zhu et al., 1991a), suggesting that, in mouse cells at least, this region has a greater involvement in transformation than in immortalisation (since these mutants are unable to transform mouse C3H10T1/2 cells; Zhu et al., 1992).

T antigen mutants that are defective for interaction with pRB show a substantial reduction in inducing dense foci in Rat-1 (Kalderon and Smith, 1984), Rat-3 (Rutila, Imperiale and Brockman, 1986) and REF52 (Zhu et al., 1992) cells. In contrast similar mutants are able to immortalise non-established mouse and rat embryo fibroblasts (Tevethia et al., 1988; Chen and Paucha, 1990). Thus the amino acids required for complexing with pRB and p107 are important for the transforming functions of T antigen, however, they do not appear to be necessary for immortalisation. In contrast, both the CR1 and CR2 regions of E1A are required for the immortalisation of primary baby rat kidney cells (Schneider et al., 1987; Kuppuswamy, Subramanian and Chinnadurai, 1988; Quinlan and Douglas, 1992). This may reflect either a difference between the requirements of T antigen and E1A for immortalisation or a difference between the requirements to immortalise fibroblast and epithelial cells. The pRB-family binding region is also required for the immortalisation of rat embryo fibroblasts by Py large T antigen (Larose et al., 1991) and HPV16 E7 (Chesters et al., 1990). These results indicate that inactivation of pRB is important for the immortalisation activities of E1A, E7 and Py large T antigen. SV40 T antigen may overcome the growth restriction mediated by pRB/p107/p130 through a mechanism which is independent of direct binding and is unique to SV40 T antigen. It is possible that this mechanism involves p53 binding since recent work indicates that p53 and the pRB-family may both be involved in the inactivation of the E2F family of transcription factors either through p21's inhibition of pRB phosphorylation or through the activities of MDM2 (sections 1.6.1 and 1.6.2).

The region of T antigen that is required for interaction with p53 has been demonstrated to be necessary in a number of growth assays. Mutants defective for complexing p53 were shown to be defective for dense focus formation in REF52 cells but not necessary for transformation of another established rat cell line, C3H10T1/2 (Zhu et al., 1992). Thompson et al. (1990) have shown, using large amino-terminal deletion mutants (mutants lacking amino acids 1-127, 1-175 and 127-250 amongst
Chapter 1.

others) that the T antigen domain located between amino acids 250-708 is sufficient to immortalise C57BL/6 mouse embryo fibroblasts. Indeed immortalisation of C57BL/6 mouse embryo fibroblasts by T antigen mutants cosegregates very well with their ability to bind to p53 (Zhu et al., 1991a; Kierstead and Tevethia, 1993) indicating that the immortalisation and p53 binding domains are colinear and may represent two activities of the same domain. However it is important to remember that amino-terminal T antigen fragments (for example amino acids 1-121 or 1-147), which are unable to bind to p53, are able to function in other growth assays. Recently both the amino-terminus (amino acids 1-147) and carboxy-terminus (amino acids 176-707) of T antigen have been shown to cooperate with activated ras to transform primary rat embryo fibroblasts (Cavender et al., 1995). These results suggested that T antigen contains two independent ras cooperation activities. These two activities may also be able to act independently of each other in other growth assays and this may explain the discrepancy between the results obtained for different assays. The observation that amino-terminus fragments are able to immortalise rat embryo fibroblasts but not mouse embryo fibroblasts suggests that the activity carried by this region may have a greater role to play in the immortalisation of rat cells than in mouse cells.

1.9: Aims of this thesis.

Since the control of cellular proliferation appears to be regulated by a number of mechanisms and these mechanisms may vary in different cell types, I have undertaken to analyse the functions of T antigen required to overcome these growth restrictive mechanisms in two simple immortalisation assays. I firstly attempted to determine the functions of T antigen which are required to maintain the immortal state of a conditionally immortal, clonal rat embryo fibroblast cell line which is dependent on the presence of T antigen for continued proliferation. Secondly, I attempted to determine whether the same functions of T antigen are required to immortalise a heterogeneous population of secondary rat embryo fibroblasts (REFs). These experiments were carried out by electroporation of T antigen mutant DNAs into the cells such that this extremely efficient transfection protocol would allow more sensitive characterisation of the immortalisation capability of these T antigen mutants. I have attempted to reconcile the data obtained from both assays to identify the minimal regions of T antigen which are required for REF immortalisation. The identification of such regions will allow the subsequent analysis of their functions and an insight into the regulation of cell growth. The results of these experiments are described in Chapter 3.

I was particularly intrigued by my observation that the function perturbed by a small deletion within an amino-terminal region of T antigen was required to establish immortal cell lines from rat embryo fibroblasts, but was not required to maintain the
immortal phenotype in the conditionally immortal REF cell line under the non-permissive conditions. These results suggested that one of the functions initially required for immortalisation by T antigen is no longer required to maintain immortalisation once a T antigen-dependent cell line has been established. The demonstration that two immortalisation-deficient T antigen mutant proteins could complement each other in trans to immortalise, allowed me to design experiments to further analyse the significance of this amino-terminal function in immortalisation. These experiments, along with the characterisation of some of the immortal cell lines derived from them, are described in Chapters 4 and 5.

I also undertook to examine the basis of the immortal phenotype displayed by cells isolated and cultured from the blastema of a regenerating newt limb. I carried out a functional screen for newt limb blastema cDNAs which were able to confer an increased proliferative potential upon rat embryo fibroblasts. The results from these experiments are described in Chapter 6.
tas8 and tsa14 are two conditionally immortal cell lines derived from Fischer rat embryo fibroblasts (REF) following infection with a recombinant retrovirus transducing the tsA58 mutant of SV40 large T antigen. tsa14 cells were used for all the experiments described in Chapter 3. The isolation of several other tsA58 conditionally immortal REF cell lines is described in Chapter 4 and these cell lines along with tsa8 and tsa14 were used in the experiments described in Chapter 5.
Chapter 2.

Materials and Methods.

2.1: Cell Culture.

2.1.1: Cells and growth media.

All media and components were obtained from GibcoBRL unless stated otherwise.

2.1.1.1: tsaS, tsal4 and derivatives.

tsaS and tsal4 cells, and all derivatives thereof, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 2mM glutamine, 100 units/ml penicillin and 100μg/ml streptomycin both before and after transfection. Untransfected tsaS and tsal4 cells were maintained at the permissive temperature of 33.0°C while transfected cells were cultured at either 33.0°C or the non-permissive temperature of 39.5°C as indicated.

2.1.1.2: Rat embryo fibroblasts and derivatives.

Rat embryo fibroblasts (REFs) were prepared from 13-day old Sprague-Dawley rat embryos. 13 day pregnant rats were sacrificed by cervical dislocation and the uterus was removed. The embryos were removed from the uterus under sterile conditions and immediately placed into Leibovitz's L-15 medium. Heads and tails were removed and the remaining tissues were disaggregated by fine mincing followed by treatment with trypsin-EDTA to produce a cell suspension. The cells were subsequently sedimented by centrifugation (1000rpm for 5 minutes) and plated. Primary and secondary (second passage) REFs, and all derivatives thereof, were maintained at 37.0°C in DMEM supplemented with 10% (v/v) FCS, 2mM glutamine, 100 units penicillin and 100μg/ml streptomycin. All REF cell lines conditionally immortalised with tsA58 mutants of T antigen were maintained at 33.0°C.

2.1.1.3: NIH3T3 and derivatives.

NIH3T3 cells (Todaro and Green, 1963), and derivatives thereof, were maintained in DMEM supplemented with 10% (v/v) calf serum plus 2mM glutamine, 100 units penicillin and 100μg/ml streptomycin at either 33.0°C, 37.0°C or 39.5°C as
required.

2.1.2: DNA transfection by electroporation and selection for transfectants.

tsa8, tsa14 (p24-30) and all other tsA58 conditionally immortal cell lines, grown at the permissive temperature (33.0°C), or primary REF cells, grown at 37.0°C, were passaged 24 hours before electroporation to ensure logarithmic growth. 10^7 cells per electroporation were trypsinised, washed and resuspended in HEPES buffered saline (137mM NaCl/ 5mM KCl/ 0.7mM Na2HPO4/ 6mM D-Glucose/ 21mM HEPES pH 7.1; Parker and Stark, 1979). The DNA was added and the cells were electroporated at 260V/960μF using a Bio-Rad Laboratories' Gene Puiser according to manufacturer's instructions (Anderson, Spandidos and Coggins, 1991). The conditions used were optimised for the stable transfection of tsa14 cells using a total of 50μg plasmid DNA (Ikram & Jat, unpublished data).

Cotransfections into tsA58 cell lines, including tsa8 and tsa14, were carried out using a 20:1 molar ratio of non-selectable DNA to selectable DNA (pBShygro) to a total of 50μg DNA. All plasmid DNA preparations to be used for transfection were purified twice by caesium chloride:ethidium bromide density gradient ultracentrifugation. DNA was precipitated prior to electroporation with 8ml >98% (v/v) ethanol and 0.2M NaCl (in a total volume of 14ml) and incubated overnight at -20°C. The precipitated DNA was pelleted by centrifugation at 4000rpm for 30 minutes and resuspended in HEPES buffered saline. 48 hours after electroporation the cells were trypsinised, replated (to ensure the transfected cells were relatively sparse) at 33.0°C and selected in 100 μg/ml of hygromycin-B (Calbiochem-Novabiochem Ltd) (Blochlinger and Duggerlmann, 1984). 24 hours later half of the plates were shifted to 39.5°C and all cultures were maintained until distinct colonies were visible (2-3 weeks). During this period the culture medium was changed every 3-4 days.

Cotransfections into secondary rat embryo fibroblasts were carried out using a 20:1 molar ratio of non-selectable DNA to selectable DNA (pSV2neo or pDR2 derivatives). 48 hours after electroporation the cells were trypsinised and replated to ensure the transfected cells were relatively sparse. Stable transfectants were isolated at 37.0°C (or 33.0°C where indicated) by selection in 250μg/ml Geneticin-G418 (GibcoBRL) or 100μg/ml hygromycin-B. The growth medium was changed every 3-4 days for 2-3 weeks (unless indicated otherwise).

Three plasmid cotransfections were carried out using a 10:10:1 molar ratio of non-selectable DNA to selectable DNA. Plasmids pBShygro and pSV2neo encode resistance to the antibiotics hygromycin-B and Geneticin-G418 respectively (Riley et al., 1990; Southern and Berg, 1982). Either plasmid pUC19 (Pharmacia Biotech) or pKS (pBluesript™, Stratagene) were used for control transfections.
2.1.3: Isolation of clonal cell lines.

Representative colonies for expansion into cell lines were isolated by surrounding individual colonies with sterile cloning rings, sealed to the plate with sterile vacuum grease, and trypsinising. The cells were subsequently replated on 3cm tissue culture plates and wherever possible expanded into clonal cell lines.

2.1.4: Methylene blue staining.

Once colonies were isolated the remaining colonies on the plates were visualised by methylene blue staining. The culture media was removed from the plate and 2% (w/v) methylene blue (BDH) in 50% (v/v) ethanol/water was added for 5 minutes. The plates were then washed in water, allowed to dry and the number of colonies counted.

2.1.5: Proliferation assays.
2.1.5.1: Colony assays.

The cell lines were trypsinised from tissue culture plates to yield a single cell suspension. The trypsin was inactivated by the addition of an equal volume of DMEM with 10% (v/v) FCS and the cells were counted using a haemocytometer. 10^3 cells were plated onto either 6cm or 10cm tissue culture plates, in duplicate, at 33.0°C. 24 hours after plating representative plates were transferred to the non-permissive temperature (39.5°C) while the others were maintained at the permissive temperature. The culture medium was changed every 3-4 days for 14 days after which the colonies were visualised by methylene blue staining.

2.1.5.2: Cell number assays.

The cell lines were trypsinised and counted as previously described. 10^4 cells were plated on several duplicate 10cm plates at 33.0°C. Half of the plates were transferred to 39.5°C 24 hours after plating (day 1). The cells were harvested from plates at each temperature on the days indicated and counted using a haemocytometer. The number of cells per plate and the fold increase in cell number compared to day 1 was determined.

2.2. Immunostaining.

10^4 cells were plated onto two 6cm plates, containing several sterile glass coverslips, and incubated at 33.0°C. After 24 hours one of the plates was transferred to 39.5°C. The culture medium was changed 48 hours later and the following day the cells were fixed using methanol (BDH) from -20°C for 10 minutes. If the coverslips were not to be stained immediately they were stored in 1X phosphate buffered saline (PBS, GibcoBRL) with 0.02% (w/v) sodium azide (BDH) at 4°C.

The fixed cells were incubated with 100μl supernatant from hybridoma clone
412, which produces the anti-T antigen monoclonal PAb412 (Harlow et al., 1981; Gurney, Tamowski and Deppert, 1986), at room temperature for 1 hour. The cells were washed six times in HBSS between each antibody incubation. HBSS is a solution of 9.9g/l Hank's buffered salts (HBSS, Imperial Laboratories) containing 5% (v/v) newborn calf serum (Gibco BRL) made up in water and buffered to pH7.4 using 20mM HEPES free base (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]; Sigma) and 20mM of HEPES sodium salt (Sigma). The cells were then incubated with 30μl of a fluorescein isocyanate (FITC) conjugated antibody against mouse IgG2a (which had been diluted 1:100 in HBSS; Southern Biotechnology Associates, Inc.) at room temperature for 1 hour. The coverslips were washed in HBSS, rinsed twice in 1X PBS, mounted in anti-fade mountant (a solution of 22mM 1,4-diazobicyclo-[2,2,2] octane (Sigma) prepared in glycerol) and sealed to microscope slides with nail varnish.

The immunostained cells were viewed using a Zeiss Axiophot microscope and photographed using Fuji chrome ASA400 colour film.

2.3: Protein Analysis.

All chemicals were obtained from BDH-Merck unless stated otherwise.

2.3.1: Preparation of cell lysates.

Cell lysates were prepared from either 10cm or 15cm plates of non-confluent cells which had been incubated at the appropriate temperature for at least 72 hours and had their growth medium changed not more than 24 hours previously.

2.3.1.1: Cell lysates for direct immunoblotting.

The cells were harvested from the plates by scraping into ice cold 1X PBS plus 0.02% (w/v) sodium azide (1ml for 10cm plates and 2.5ml for 15cm plates). The cells were sedimented by centrifugation at 1000rpm for 5 minutes and resuspended in 175μl 1X PBS. 20μl of 10X lysis buffer (3% (w/v) SDS, 10% (v/v) β-mercaptoethanol) was added and the cells were boiled for 5 minutes (Garrels, 1979). The cell lysates were frozen and stored at -20°C until use.

2.3.1.2: Cell lysates for immunoprecipitation.

The cells were lysed directly on the tissue culture plates for 30 minutes in ice cold modified RIPA lysis buffer (10mM Tris pH7.2, 150mM NaCl, 1% (v/v) Triton X-100 (Sigma), 0.1% (v/v) SDS, 1.0% (w/v) sodium deoxycholate, 5mM EDTA). The lysates were scraped from the plates and any insoluble protein was sedimented by centrifugation at 10,000rpm for 10 minutes. The cell lysates were frozen and stored at -20°C until use.
2.3.2: Protein quantification.

The relative protein concentration of samples was determined in triplicate for each cell lysate using a Bio-Rad Laboratories protein assay system, according to manufacturer's instructions (modified from Bradford, 1976). 2-5μl of each lysate was adjusted to a volume of 800μl with 1X PBS and 200μl of Bradford reagent was added. After 5 minutes the absorbance was measured at 595nm in a Beckman DU-50 spectrophotometer. A standard calibration curve was prepared using bovine serum albumin (Boehringer Mannheim) to give a comparative, but not absolute, protein concentration.

2.3.3: Immunoprécipitations.

Proteins were immunoprecipitated from the cell lysates using standard protocols (Harlow and Lane, 1988). Briefly, 100-200μg cell lysate was incubated with 100-300μl of supernatant, from a hybridoma clone producing the required monoclonal antibody, at 4°C for 1 hour. This was followed by addition of 1μl of goat anti-mouse immunoglobulin (Ig) antibody (DAKO) and incubation at 4°C for 30 minutes. Immunocomplexes were collected by adding 150μl 15% (w/v) protein A-sepharose CL-4B (Pharmacia Biotech) and incubating at 4°C on a rotary shaker for 1 hour (Ey, Prowse and Jenkin, 1978). The protein A-sepharose-immunocomplexes were then sedimented by centrifugation (10,000rpm for 10 seconds) and washed three times in 500μl modified RIPA buffer. The sediment was subsequently resuspended in Laemmli sample buffer (80mM Tris-HCl pH6.8, 2% (w/v) SDS, 15% (w/v) glycerol, 100mM dithiothreitol, 2mM EDTA and 0.001% (w/v) bromophenol blue) and boiled for 5 minutes prior to fractionation by SDS-PAGE.

The antibodies used have all been described previously and the references for each antibody are provided in the text. PAb419 and PAb423 supernatants were kindly provided by J.Gannon, PAb108 by J.M.Pipas, PAb106 and PAb114 by E.Gurney. All other hybridoma supernatants were prepared by P.Jat and stored as frozen aliquots with 0.02% (w/v) sodium azide.

2.3.4: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed according to the method of (Laemmli, 1970). Gels were prepared from a 40% (w/v) acrylamide stock solution (containing a ratio of 29.1 acrylamide:0.9 NN'-methylenebisacrylamide; BDH acrylogel 3), in 373mM Tris-HCl pH8.8 and 0.1% (w/v) SDS. Gels were polymerised by addition of ammonium persulphate (0.3% (w/v) final; Bio-Rad Laboratories) and TEMED (N,N,N',N' tetraethylenemethylendiamine, 0.0006% (w/v) final; BDH). For direct immunoblots 25-50μg of each cell lysate was boiled in Laemmli sample buffer (80mM Tris-HCl pH6.8, 2% (w/v) SDS, 15% (w/v) glycerol, 100mM dithiothreitol, 2mM EDTA and 0.001%
(w/v) bromophenol blue) and fractionated by SDS-PAGE on either 10% or 8%-15% gradient polyacrylamide gels as indicated. For immunoprecipitated proteins the samples were also boiled in Laemmli sample buffer for 5 minutes and fractionated on either 6.5% or 10% polyacrylamide gels. Electrophoresis was carried out at a constant current of 25-40mA in electrode buffer containing 3.03g/l Tris base, 14.42g/l glycine and 0.1% (w/v) SDS. Proteins were stacked through approximately 1.0cm of 4% polyacrylamide, 125mM Tris-HCl pH6.8 and 0.1% (w/v) SDS gel. Proteins were fractionated alongside prestained low range SDS-PAGE standards (Bio-Rad Laboratories).

2.3.5: Immunoblotting.

Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell) by electrophoretic transfer (in a wet tank blotting system, Bio-Rad Laboratories' Trans-Blot Cell) in 25mM Tris, 190mM glycine and 20% (v/v) methanol (Towbin, Staehelin and Gordon, 1979). Transfer was carried out overnight at a constant current of 200mA at 4°C. The nitrocellulose filter was then blocked by incubation in 5% (w/v) skimmed milk powder (Marvel, Premier Beverages) and 0.05% (v/v) Tween 20 in 1/2X PBS (PBS/Marvel) at 4°C for 2 hours to reduce non-specific protein binding. The filter was then incubated overnight with the primary monoclonal antibody (as hybridoma supernatant) diluted 1:50 in PBS/Marvel. The filters were washed three times (15 minutes each at 4°C) in PBS/Marvel prior to addition of the secondary antibody for detection. Immunocomplexes were either detected with 125I-labelled second antibody or Horseradish peroxidase-linked second antibody.

2.3.5.1: Detection with 125I-labelled second antibody.

The filters were incubated with 125I-labelled sheep anti-mouse Ig antibody (Amersham Life Sciences, 2.5μCi per blot) in 50ml PBS/Marvel at 4°C for 2 hours. They were then washed twice in PBS/Marvel (4°C for 10 minutes) and twice in 1/2X PBS and 0.05% (v/v) Tween 20 (PBS-T; 4°C for 10 minutes). The filters were wrapped in clink film and exposed to X-ray film (either Fuji-RX or Kodak X-OMAT™-AR) for between 1-14 days.

2.3.5.2: Detection with Horseradish peroxidase-linked second antibody.

The filters were incubated with Horseradish peroxidase-linked sheep anti-mouse Ig antibody (Amersham Life Sciences ECL western blotting analysis system) in 50ml PBS/Marvel at 4°C for 2 hours. They were then quickly rinsed twice in PBS-T and washed three times in PBS-T at 4°C for 20 minutes. The filters were then developed in the HRP detection reagents according to manufacturer's instructions (Amersham Life Sciences) and exposed to X-ray film (either Fuji-RX or Kodak X-OMAT™-AR) for 1-60 minutes.
Chapter 2.

2.4: DNA Manipulation.

All chemicals were obtained from BDH-Merck unless stated otherwise.

2.4.1: Genomic DNA preparation from cultured REF cell lines.

Genomic DNA was prepared from confluent monolayers of cells on 6cm or 10cm tissue culture plates. The cells were lysed on the tissue culture plates in 10mM Tris-HCl pH8.0, 150mM NaCl, 10mM EDTA and 0.2% (w/v) SDS at room temperature for 30 minutes. The lysate was then scraped into a polypropylene tube, proteinase K (Boehringer Mannheim) added to a final concentration of 200μg/ml and incubated at 53°C overnight. The protein was removed by extracting the lysate three times with phenol/chloroform/isoamylalcohol (mixed in a ratio of 24:24:1 respectively). The DNA was then precipitated by the addition of an equal volume of >98% (v/v) ethanol and centrifuged at 2500rpm for 10 minutes. The DNA pellet was washed once in 70% (v/v) ethanol in ddH2O and resuspended in 10:1 TE (10mM Tris-HCl pH8.0 and 1mM EDTA pH8.0). DNA concentrations were calculated from the absorbance of the DNA solutions at 260nm (Sambrook, Fritsch and Maniatis, 1989).

2.4.2: Restriction digestion of DNA.

_BamHI, BglII, HindIII, HpaI, KpnI, PstI, SalI, SfI, XbaI and XhoI_ were all purchased from New England BioLabs while _EcoRI_ and _PvuII_ were purchased from Boehringer Mannheim. All restriction digests were carried out according to manufacturer's recommendations and in the restriction buffers supplied for each particular enzyme.

2.4.3: Polymerase chain reaction (PCR) amplification of DNA fragments.

PCR amplification of genomic DNA was carried out on 100ng template DNA while amplification of plasmid DNA (to screen for inserts after ligation) was carried out on approximately 10ng template DNA. Amplification was performed with 0.5μg of each oligonucleotide primer, 2.5 units _Thermus aquaticus (Taq)_ DNA polymerase (Promega Corporation) in 10mM Tris-HCl pH8.4, 50mM KCl, 0.1% (w/v) gelatin, 0.05% (v/v) Nonidet P-40, 0.05% (v/v) Tween20 (10X PCR buffer provided by Promega), 0.2mM dNTPs (Pharmacia Biotech) with 1-4mM MgCl2 (from Promega; the optimum MgCl2 concentration was titrated for each primer set). Amplification was performed with 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60-70°C (optimised for each pair of primers) for 1 minute and polymerisation at 72°C for 3 minutes. To prevent contamination problems, separate pipettes and tips were used for pre- and post-PCR sample handling. Reaction components were kept as aliquots and used only for PCR. Highly pure water was used (tissue culture grade, Imperial
Laboratories). Control PCR amplifications were performed with either one of the two primers alone and with no template DNA.

2.4.4: DNA electrophoresis.

2.4.4.1: Analytical electrophoresis.

DNA fragments (either PCR fragments or restriction fragments) were loaded in 2.5% Ficoll (400), 0.2% (w/v) bromophenol blue and 0.2% (w/v) SDS and fractionated by electrophoresis on either 1.0% or 1.8% (w/v) agarose (GibcoBRL) gels, prepared in 1X TAE (40mM Tris-acetate and 2mM EDTA) with 1μg/ml ethidium bromide (Sambrook, Fritsch and Maniatis, 1989). Electrophoresis in 1X TAE was carried out in IBI electrophoresis tanks at 75 volts for 2-3 hours. Samples were loaded alongside double stranded DNA molecular weight markers, either λ-phage digested with HindIII or 1kb-ladder (GibcoBRL). Ethidium bromide stained DNA fragments were visualised on either a short or a long wave transilluminator and photographed on Polaroid type 57 high speed film.

2.4.4.2: Preparative electrophoresis.

DNA fragments for subsequent cloning or for use as DNA probes were loaded in 2.5% Ficoll (400), 0.2% (w/v) bromophenol blue and 0.2% (w/v) SDS and separated by electrophoresis on either 1.0% or 1.8% (w/v) low melting point agarose (GibcoBRL) gels, prepared in 1X TAE (40mM Tris-acetate and 2mM EDTA). Electrophoresis was carried out in 1X TAE at 15-35 volts either for 6 hours or overnight. The gels were then stained with 1μg/ml ethidium bromide. The required DNA fragments were visualised on a long wave transilluminator and cut out using a clean scalpel blade.

2.4.5: Purification of DNA fragments.

2.4.5.1: Phenol extraction.

DNA fragments isolated in LMP agarose were heated to 65°C in 400μl 10:1 TE pH8.0 for 5 minutes. An equal volume of phenol (equilibrated in 150mM NaCl, 10mM Tris-HCl pH 8.0 and 1mM EDTA) was added, mixed and separated by microfuging at 13,000rpm for 5 minutes. The aqueous top layer was removed and re-extracted once with phenol and once with chloroform. The DNA was then precipitated by the addition of 2 1/2 volumes of >98% (v/v) ethanol plus 1/10th volume of 3.0M sodium acetate pH5.2 (0.3M final) and incubation at -20°C for at least 2 hours. The precipitated DNA was pelleted by centrifugation at 13,000rpm for 15 minutes, washed with 70% (w/v) ethanol and allowed to dry. The DNA was resuspended in 10-50μl 10:1 TE pH8.0.

2.4.5.2: Purification of DNA using glass milk.

DNA fragments were purified from LMP agarose using a glass milk purification system according to the manufacturer's instructions (Geneclean® II Kit, Bio101). The DNA was eluted in 10-50μl 10:1 TE pH8.0.
2.4.6: Ligation of DNA fragments.

The amount of pure DNA fragment isolated and purified was estimated by fractionation on mini-agarose gels and comparison to a known amount of standard DNA.

2.4.6.1: Ligation of restriction fragments.

DNA fragments with identical protruding termini, created by restriction digest, were ligated into linearised vector in 1X ligation buffer (50mM Tris-HCl pH7.6, 10mM MgCl₂ and 10mM dithiothreitol) with 0.5 Weiss unit of T4 DNA ligase (Pharmacia Biotech) in the presence of 1mM ATP (Sambrook, Fritsch and Maniatis, 1989). The vector DNA was often dephosphorylated by calf intestine phosphatase (Boehringer Mannheim) prior to purification to avoid self-religation. An equal molar ratio of insert to vector DNA was used for the ligations. Ligations were incubated overnight at 16°C.

2.4.6.2: Ligation of PCR fragments into pCRII™.

PCR fragments were ligated by virtue of their 3' A-overhang (created by the terminal transferase activity of Taq DNA polymerase) into a vector with a single 3' T-overhang, pCRII™. PCR fragments were ligated, either straight from the PCR reaction or having been isolated and purified, in accordance with the manufacturer’s instructions (TA Cloning® Kit, Invitrogen Corporation).

2.4.7: Transformation of plasmid DNA or ligations into competent bacteria.

2.4.7.1: Bacterial strains.

The following three E. coli strains were used:

JS4, a recA1 derivative of MC1061:
Genotype: F' araD139, Δ(ara, leu)7697, Δ(lac)X74, galU, galK, hsdR2 (rk⁻ mk⁻), mcrA, mcrBC, rpsL (Str') thi, recA1.

JS5, a derivative of JS4 which allows blue/white selection of recombinants:
Genotype: F' araD139, Δ(ara, leu)7697, Δ(lac)X74, galU, galK, hsdR2 (rk⁻ mk⁻), mcrA, mcrBC, rpsL (Str') thi, recA1/F::Tn10 (tet') proAB, lacIq, lacZΔM15.

E. coli JS4 and JS5 were kindly provided by J. Sedivy, Yale University.

OneShot™, provided with the Invitrogen TA Cloning® Kit to allow blue/white selection of recombinants:
Genotype: F' endA1, recA1, hsdR17 (rk⁻ mk⁺), λ− supE44, thi-1, gyrA96, relA1, φ80ΔlacZΔM15, Δ(lacZYA-argF)U169deoR.

E. coli OneShot™ competent cells were transformed according to the manufacturer's instructions.

2.4.7.2: Preparation of transformation-competent bacteria.

500ml of LB medium (25g/l Luria broth base dissolved in ddH₂O and autoclaved) was inoculated with a 5ml overnight culture of either E. coli JS4 or JS5 and incubated at 37°C with vigorous shaking until the optical density at 600nm reached 1.0-
1.2. The bacteria were harvested by centrifugation at 2500rpm for 20 minutes, resuspended in 50ml ice cold 0.1M CaCl₂ and incubated on ice for 20 minutes. The bacteria were centrifuged again and resuspended in 5ml of ice cold 85:15 solution of 0.1M CaCl₂ and glycerol. The competent bacteria were stored frozen as 50μl aliquots.

2.4.7.3: Transformation and selection for transformants.

One 50μl aliquot of frozen competent bacteria was thawed on ice. 950μl ice cold 0.1M CaCl₂ was added and 100μl was added to each DNA to be transformed. The DNA was incubated with the bacteria on ice for 30 minutes followed by heat shock at 42°C for 90 seconds. The transformations were returned to ice for 2 minutes, 1ml of LB medium was added and the transformations were incubated at 37°C with vigorous shaking for 30 minutes. The cells were concentrated by centrifugation (2500rpm for 10 minutes) and resuspended in 100μl LB medium. The transformations were plated on 10cm LB plates with 100μg/ml ampicillin (Sigma). For blue/white selection with *E.coli* JS5 the plates were spread with 40μl 2% (w/v in dimethylformamide) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Sigma) and 100μl 100mM IPTG (isopropylthio-β-D-galactoside; Sigma) 30 minutes prior to plating the bacteria.

2.4.8: Plasmid DNA preparation.

2.4.8.1: Small scale preparation.

5ml of LB medium with 100μg/ml ampicillin (Sigma) was inoculated with a single bacterial colony and incubated at 37°C with vigorous shaking overnight. 1.5ml of culture was transferred to an Eppendorf tube and microfuged at 13,000rpm for 5 minutes. All but 100μl of the supernatant was removed and the pellet was resuspended. 200μl of freshly prepared 0.2M NaOH/1% (w/v) SDS was then added, the contents of the tube vortexed gently and the tube incubated on ice for 5 minutes. 150μl of ice-cold 3M potassium: 5M acetate (294.4g/l potassium acetate in 11.5% (v/v) glacial acetic acid) was added, the tube vortexed gently and incubated on ice for 10 minutes. An equal volume of phenol/chloroform/isoamylalcohol (24:24:1) was added, vortexed vigorously, microfuged at 13,000rpm for 5 minutes and the supernatant transferred to a fresh tube. 2½ volumes of >98% (v/v) ethanol were added, mixed and microfuged for 15 minutes. The supernatant was removed and the DNA pellet washed in 1ml 70% (v/v) ethanol. The pellet was allowed to dry and was resuspended in 10:1 TE pH8.0 containing 20μg/ml DNase-free RNase (Boehringer Mannheim). Typically 1.5ml of culture yielded 2-3μg of plasmid DNA.

2.4.8.2: Large scale preparation.

700ml of superbroth (32g/l tryptone (Oxoid), 20g/l yeast extract (Oxoid) and 10g/l Na₃-[N-Morpholino]propanesulphonate) containing 100μg/ml ampicillin (Sigma) was inoculated with an overnight culture of bacteria and grown at 37°C with vigorous shaking until the optical density at 600nm reached between 1.5 to 2.0. 150μg/ml
chioramphenicol (Sigma) was the added and the cultures were incubated overnight at 37°C with vigorous shaking. Bacteria were harvested at 2500rpm for 20 minutes, the pellet resuspended in 40ml 50mM glucose, 25mM Tris-HCl pH8.0 and 10mM EDTA with 5mg/ml lysozyme (from hen egg white, Boehringer Mannheim) and incubated at room temperature for 10 minutes. 80ml of freshly prepared 0.2M NaOH/1% (w/v) SDS was then added, the contents of the tube mixed gently and the tube incubated on ice for 5 minutes. 40ml of ice-cold 3M potassium: 5M acetate (section 2.4.8.1) was added, the tube mixed gently and incubated on ice for 10 minutes. The tube was then centrifuged at 2500rpm for 20 minutes and the supernatant filtered through muslin. 100ml propan-2-ol was added and the tube centrifuged at 10,000rpm for 20 minutes. The supernatant was drained and the DNA pellet allowed to dry. The pellet was dissolved in 8ml 50:1 TE (50mM Tris-HCl pH8.0 and 1mM EDTA pH8.0). Exactly 9g caesium chloride was dissolved in 8.63g of the DNA-TE solution followed by 370μl ethidium bromide (10mg/ml). The tube was centrifuged at 2500rpm for 10 minutes and the supernatant decanted into a 12ml QuickSeal tube (Beckman). The tube was filled with caesium chloride solution (1g/ml), sealed and was ultracentrifuged at 55,000rpm for at least 18 hours. The ethidium stained band, corresponding to supercoiled plasmid rather than nicked plasmid, was removed and re-ultracentrifuged in 1g/ml caesium chloride. Again the ethidium stained supercoiled plasmid was removed and an equal volume of isoamylalcohol was added and mixed. The aqueous and organic layers were allowed to separate and the top layer removed. This was repeated until all the ethidium bromide had been removed. The DNA solution was diluted to 8ml with ddH2O, 6ml propan-2-ol was added and the DNA precipitated at -20°C for at least two hours. The precipitated DNA was pelleted at 4000rpm for 30 minutes and was resuspended in 1ml 10:1 TE. The DNA was reprecipitated with 2½ volumes of >98% (v/v) ethanol and 0.3M sodium acetate pH5.2 at -20°C for 2 hours. The precipitated DNA was pelleted at 4000rpm for 30 minutes, washed in 70% (v/v) ethanol and was resuspended in 1ml 10:1 TE pH8.0. DNA concentrations were calculated from the absorbance of the DNA solution at 260nm (Sambrook, Fritsch and Maniatis, 1989). Generally between 2-10mg plasmid DNA was recovered from a 700ml starting culture.

2.4.9: Southern blotting and DNA hybridisation.

DNA fragments were fractionated on 1.0% or 1.8% agarose gels as described previously (section 2.4.5). The fractionated DNA was transferred to nitrocellulose by modification of the method of Southern (1975). The gel was treated with 0.4 NaOH for 20 minutes and transferred to a nitrocellulose (Zetabind®, CUNO) filter in the same solution overnight. The filter was then washed for 10 minutes in 2X SSC (1x SSC: 150mM NaCl and 15mM sodium citrate) and the DNA cross-linked to the filter by exposure to ultraviolet light in a Stratagene Stratalinker.
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Complementary DNA was detected by the hybridisation of a radiolabelled DNA probe. 50ng purified DNA fragment was denatured by boiling for 5 minutes and cooled rapidly on ice. A random primed labelling kit (Boehringer Mannheim) was used to incorporate $^{32}$P-dCTP (74MBq/ml, Amersham Life Sciences) into the DNA by the addition of 0.5nmol each of dATP, dTTP and dGTP, 2 units Klenow DNA polymerase and 10% (v/v) B.M. reaction mixture (which contains a mixture of random hexanucleotide primers) to the denatured DNA. 20-50μCi $^{32}$P-dCTP was then added and the reaction incubated at 37°C for 30 minutes. Labelled DNA was purified from unincorporated $^{32}$P-dCTP by passing the reaction through a Sepharose 4B (Pharmacia Biotech) spin column (equilibrated in 1X TNE: 150mM NaCl, 10mM Tris-HCl pH8.0 and 1mM EDTA pH8.0) in 150μl 1X TNE at 1000rpm for 5 minutes. 1μl of the purified radiolabelled-DNA was counted in a scintillation counter (Beckman Instruments) to check the activity of the probe.

The Southern filter was pre-hybridised in a shaking incubator at 42°C (at least 1 hour) in hybridisation buffer containing 0.5mg/ml calf thymus DNA (Sigma), 50% (v/v) formamide (Fluka), 5X SSCPE (from a 20X stock solution: 3M NaCl, 300mM sodium citrate, 176mM NaH$_2$PO$_4$ and 20mM EDTA), Denhardt's solution (from a 50X stock: 1% (w/v) Ficoll (type400, Sigma), 1% (w/v) polyvinylpyrrolidone and 1% (w/v) bovine serum albumin (Sigma) dissolved in distilled water and filter sterilised), 1% (w/v) SDS and 10% (w/v) dextran sulphate (Sigma). Calf thymus DNA (from a 10mg/ml stock which had been autoclaved to break it down in size) was denatured by boiling for 10 minutes prior to the addition to the hybridsation buffer. The radiolabelled DNA probe was denatured by boiling for 10 minutes, added to hybridisation buffer and hybridised overnight in a shaking incubator at 42°C.

The hybridised filters were washed twice in 2X SSC and 0.1% (w/v) SDS at room temperature with shaking, followed by 0.1X SSC and 0.1% (w/v) SDS at 60-70°C with shaking. The filters were then exposed to X-ray film (either Fuji-RX or Kodak X-Omat™-AR) at -70°C for times ranging from 1 hour to 3 days.

2.4.10: Double stranded DNA sequencing.
2.4.10.1: Dideoxy- sequencing.

2-6μg plasmid DNA was denatured in 0.2M NaOH at room temperature for 10 minutes. The denatured DNA was neutralised by the addition of 0.25M (final) ammonium acetate (pH4.6) and immediately precipitated with 2$^{1/2}$ volumes of >98% (v/v) ethanol. The DNA precipitate was pelleted and washed as described previously and resuspended in 10μl ddH$_2$O. The appropriate sequencing primers are added (to a final concentration of 4-6pmol), annealed and dideoxy-sequencing (Sanger, Nicklen and Coulson, 1977) was carried out using a Pharmacia T7 Sequencing Kit according to the manufacturer's instructions. Dideoxy-terminated DNA molecules were labelled with
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10mCi $\alpha^{35}$-dATP (370MBq/ml; Amersham Life Sciences). Sequencing reactions were heated to 80°C for 5 minutes and fractionated on 6-8% polyacrylamide-8.3M urea gel in 1X TBE (90mM Tris-borate and 1mM EDTA) on an IBI STS45 vertical slab gel sequencing apparatus at 60W for 3-6 hours. The gel was fixed in 5% (v/v) methanol and 5% (v/v) glacial acetic acid in ddH20, dried onto 3MM paper using a gel drier at 80°C for 1 hour and exposed to X-ray film (Kodak X-OMAT™-AR) overnight.

2.4.10.2: ABI automated sequencing.

Plasmid DNA was sequenced with the appropriate sequencing primer using a PCR based method and dye-terminators as supplied in the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit (the Perkin Elmer Corporation). The sequencing reactions were analysed on an Applied Biosystems 373A DNA Sequencer System according to manufacturer's instructions.
Several regions of SV40 large T antigen are required for the immortalisation of rat embryo fibroblasts.

3.1: Introduction.

Since the growth stimulatory effects of T antigen have been assayed in a variety of assays, for example immortalisation of primary or secondary cells, transformation of a variety of different immortal cell lines or cooperation with activated \( \text{ras} \) to transform primary or established cell lines, it is difficult to compare the conflicting data which comes from these assays. I have attempted to simplify this by assaying a range of T antigen mutants in a cell line which is already dependent on T antigen for continued growth. This cell line (tsa14) was established from rat embryo fibroblasts by infection with a recombinant retrovirus that transduces the temperature sensitive large T antigen from the SV40 early region mutant \( tsA58 \) (Tegtmeier, 1975). tsa14 cells grow continuously at the permissive temperature (33.0°C), but rapidly undergo growth arrest upon shift up to the non-permissive temperature (39.5°C; Jat and Sharp, 1989) due to the instability of the \( tsA58 \) T antigen (see Figure 3.1). This growth arrest is irreversible although the cells remain metabolically active and it is believed that upon shift up these cells undergo senescence (Gonos \textit{et al}, in preparation). The inability of this cell line to grow at the non-permissive temperature can be overcome in this cell line by exogenous introduction of either wild type T antigen, Ad5 E1A or HPV16 E7 (Jat and Sharp, 1989; Vousden and Jat, 1989).

The advantage of assaying T antigen's immortalisation functions using this cell line is that it will allow the identification of regions which are required to maintain the proliferative state in a clonal population of genomically stable cells. In contrast, primary cultures represent a mixed population of cells which may be prone to karyotypic instability. Thus the immortalisation of such cultures may involve selection for endogenous mutations as well as the action of the transfected T antigen mutant or may represent the immortalisation of a sub-population of cells. Assaying these mutants in a clonal cell line would hopefully rationalise the results obtained with such mutants. It could be argued however that the T antigen functions identified as necessary to maintain
Figure 3.1: Immunofluorescent staining of *tsa14* cells at 33.0°C and 39.5°C with anti-T monoclonal antibody PAb412.

*tsa14 33.0°C*

*tsa14 39.5°C*

*tsa14* cells were plated onto coverslips, incubated at either 33.0°C or 39.5°C for 72 hours and fixed. The cells were stained with anti-T monoclonal antibody PAb412 and detected with FITC-conjugated goat anti-mouse IgG2a antibody (see Materials and Methods). The stained cells were photographed using phase optics (left) and immunofluorescent conditions (right).
immortalisation in a conditionally immortal cell line are not sufficient to immortalise a primary cell. It is possible that certain T antigen functions are required to immortalise primary cells but once immortal only a subset of these functions, or different functions altogether, are required to maintain the immortal state in established rat embryo fibroblast cell lines. Indeed, complementation of tsa14 growth by Ad5 E1A has been shown to be dependent upon the amino-terminal and CR1 domains but does not require the CR2 (Riley et al., 1990), while immortalisation of primary cells by E1A required the CR2 as well as the amino-terminal region and CR1 (Schneider et al., 1987; Kuppuswamy, Subramanian and Chinnadurai, 1988; Quinlan and Douglas, 1992). To verify the tsa14 immortalisation assays, I assayed the same series of T antigen mutants for their ability to immortalise secondary (p2) rat embryo fibroblasts (REFs). I identified several regions of T antigen which were necessary for both assays, however I was intrigued by the result obtained with one mutant, d/1135, which was defective in its ability to immortalise secondary REFs but retained the ability to maintain immortalisation in the tsa14 cell line. tsa14 cells appear, therefore, to allow the identification of regions which are required for the maintenance of immortalisation and which, alone, are not sufficient for the immortalisation of REFs.

The identification of three mutants which were able to immortalise at a decreased efficiency compared to wild type but were unable to maintain immortalisation in tsa14 cells suggested that these T antigen mutants may either immortalise a sub-population of secondary REFs or require second events for their ability to immortalise. The inability of these three mutants to maintain immortalisation in tsa14 cells was complemented by the cotransfection of another mutant deficient in tsa14 maintenance. I further show that two mutants defective for immortalising REFs can complement each other in trans, thereby indicating that the domains necessary for both tsa14 maintenance and REF immortalisation can function independently. The data presented here suggests that different domains of T antigen are required for the initiation and maintenance of immortalisation and that they can complement each other in trans. My data also suggests that mutations towards the carboxy-terminus of the protein may perturb amino-terminal functions.

3.2: Results.

3.2.1: Complementation of the growth defect of tsa14 cells at the non-permissive temperature.

To determine which regions of T antigen were required to complement the growth of tsa14 cells at the non-permissive temperature, I used the SV40 T antigen mutants shown in Table 3.1. The majority of these mutants have been described previously and have been shown to produce the indicated proteins. Recombinant
<table>
<thead>
<tr>
<th>Mutant</th>
<th>Recombinant Plasmid</th>
<th>Encoded Protein</th>
<th>CR1-like region</th>
<th>pRB-family binding</th>
<th>p53 binding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE (wild-type)</td>
<td>pSE</td>
<td>wild type large T and small t antigens</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Jat &amp; Sharp (1989)</td>
</tr>
<tr>
<td>small t</td>
<td>pSRrtcDNA</td>
<td>wild type small t antigen (sm t)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Sompayrac, personal communication</td>
</tr>
<tr>
<td>T176-708</td>
<td>py2-70K</td>
<td>dl(1-175) aa</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Thompson et al. (1990)</td>
</tr>
<tr>
<td>T128-708</td>
<td>py2xmet128-70K</td>
<td>dl(1-127) aa</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Thompson et al. (1990)</td>
</tr>
<tr>
<td>df1135</td>
<td>df1135</td>
<td>dl(17-27) aa; sm t</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>Pipas, Peden &amp; Nathans (1983)</td>
</tr>
<tr>
<td>5002</td>
<td>5002</td>
<td>L(19) -&gt; F &amp; P(28) -&gt; S; sm t</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>Peden et al. (1990)</td>
</tr>
<tr>
<td>5003</td>
<td>5003</td>
<td>A(23) -&gt; V; sm t</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Pipas &amp; Peden, unpublished data</td>
</tr>
<tr>
<td>3213</td>
<td>3213</td>
<td>E (107 &amp; 108) -&gt; L; sm t</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Christensen &amp; Imperiale (1995)</td>
</tr>
<tr>
<td>K1</td>
<td>K1</td>
<td>E (107)-L; sm t</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Kalderon &amp; Smith (1984)</td>
</tr>
<tr>
<td>df1137</td>
<td>df1137</td>
<td>1-121 aa; sm t</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Pipas, Peden &amp; Nathans (1983)</td>
</tr>
<tr>
<td>T147D</td>
<td>pKPT147Ddl</td>
<td>1-147 aa</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Sompayrac &amp; Danna (1988)</td>
</tr>
<tr>
<td>df1061</td>
<td>df1061</td>
<td>1-590 aa; sm t</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Pipas, Peden &amp; Nathans (1983)</td>
</tr>
<tr>
<td>5031</td>
<td>5031</td>
<td>D(402) -&gt; N &amp; V(404 &amp; 413) -&gt; M; sm t</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Pipas &amp; Peden, unpublished data</td>
</tr>
<tr>
<td>5041</td>
<td>5041</td>
<td>G(426) -&gt; E &amp; S(430) -&gt; N; sm t</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Pipas &amp; Peden, unpublished data</td>
</tr>
<tr>
<td>5080</td>
<td>5080</td>
<td>A(23) -&gt; V &amp; P(584) -&gt; L; sm t</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Peden et al. (1989); Tack et al. (1989)</td>
</tr>
<tr>
<td>5030</td>
<td>5030</td>
<td>A(417) -&gt; S; sm t</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Pipas &amp; Peden, unpublished data</td>
</tr>
<tr>
<td>5061</td>
<td>5061</td>
<td>G(431) -&gt; ALE; sm t</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Pipas &amp; Peden, unpublished data</td>
</tr>
<tr>
<td>ts458</td>
<td>pZip ts458</td>
<td>A(438) -&gt; V; sm t</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Tegtmeyer (1975); Jat &amp; Sharp (1989)</td>
</tr>
</tbody>
</table>

This table shows the SV40 large T antigen mutants used in this study, the recombinant plasmid and the protein(s) encoded. The ability of the mutant proteins to bind pRB or p53 and the presence of an intact CR1-like region is also shown. 
(-) = defective, (+) = functions, dl = deletion, aa = amino acids
plasmids encoding these T antigen mutants were transfected by electroporation into tsal4 cells using a plasmid expressing hygromycin-B resistance as a coselectable marker. The transfected cells were assayed for their ability to produce hygromycin resistant colonies at the non-permissive temperature (39.5°C) compared to the permissive temperature (33.0°C). Previously it had been observed that some recombinants are capable of inducing colonies at the non-permissive temperature but when isolated these colonies do not always expand into cell lines. Mutants were therefore only considered capable of complementing growth if they produced colonies at the non-permissive temperature and if representative colonies could be expanded into cell lines and serially cultured. Representative cell lines stably transfected with each mutant were also isolated at the permissive temperature and assayed for growth upon shift up to the non-permissive temperature.

Due to the large number of mutants assayed and the resulting number of cell lines generated, ten separate experiments were carried out (experiments 2, 3, 4 & 7 were carried out by E.S.Gonos and are included here for completion). The results of these are shown on Table 3.2 (and summarised in Figure 3.10). The numbers of colonies yielded at the non-permissive temperature (39.5°C) and at the permissive temperature (33.0°C) are shown along with the percentage complementation (percentage number of colonies obtained at 39.5°C compared to 33.0°C) for each experiment. The average efficiency of colony formation at the non-permissive temperature (39.5°C) compared to the permissive temperature (33.0°C) is also shown (average % complementation) and the total number of cell lines derived from the total number of colonies isolated from all ten experiments is shown. As had been demonstrated previously (Jat and Sharp, 1989) reintroduction of wild type T antigen (recombinant plasmid SE) readily complemented the growth defect at the non-permissive temperature. The average efficiency of colony formation at the non-permissive temperature relative to the permissive temperature was 64.6% and all the colonies isolated at the non-permissive temperature were readily expanded into cell lines. In contrast, when control DNA (either pUC19 or pKS) was cotransfected with the same amount of hygromycin-B selectable recombinant plasmid, the efficiency of colony formation was 14.3% and only 1 colony, from a total of 21 isolated, expanded to yield a cell line. Since recombinant plasmid SE, as well as other recombinant plasmids used in the transfections, was capable of expressing SV40 small t antigen as well as large T antigen I investigated the effect of small t antigen on tsal4 complementation. Transfection of a recombinant plasmid expressing only small t antigen yielded only 10.8% the number of colonies at the non-permissive temperature compared to the permissive temperature and of 4 colonies isolated none expanded into cell lines.

Function(s) carried towards the amino-terminus of large T antigen were clearly required for complementation. A recombinant which encodes a mutant protein lacking
Table 3.2: Complementation of the growth defect of tsal4 cells by T antigen mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Number of densely staining colonies</th>
<th>39.5°C / 33.0°C (% complementation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>expt. 1</td>
<td>expt. 2</td>
</tr>
<tr>
<td>SE(wild-type)</td>
<td>315/705</td>
<td>713/953</td>
</tr>
<tr>
<td>pUC19/pKS</td>
<td>90/652</td>
<td>18/627</td>
</tr>
<tr>
<td>small t</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T176-708</td>
<td>-</td>
<td>66/441</td>
</tr>
<tr>
<td>T128-708</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>d1135</td>
<td>196/754</td>
<td>(26.0%)</td>
</tr>
<tr>
<td>S002</td>
<td>-</td>
<td>60/362</td>
</tr>
<tr>
<td>S003</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3213</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>d1137</td>
<td>23/690</td>
<td>(3.3%)</td>
</tr>
<tr>
<td>T147D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S031</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S041</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S050</td>
<td>56/747</td>
<td>(7.5%)</td>
</tr>
<tr>
<td>S050</td>
<td>-</td>
<td>14/32</td>
</tr>
<tr>
<td>S050</td>
<td>-</td>
<td>85/165</td>
</tr>
</tbody>
</table>

The indicated recombinants were transfected into tsal4 cells using a recombinant plasmid carrying hygromycin-B resistance as a coselectable marker, as described in Materials and Methods. Transfected tsal4 cells were cultured at either the permissive temperature of 33°C or at the non-permissive temperature of 39.5°C until colonies were clearly visible. They were then stained and counted. The indicated number of 39.5°C colonies were isolated and expanded into cell lines. *Experiment (expt) was only carried out once. Experiments 2, 3, 4 & 7 were carried out by E.S.Gonos.

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The amino-terminal 175 amino acids (T176-708) was found to be negative for complementation. The efficiency of complementation was a little above that obtained with the control transfection (pUC19/pKS) but none of the 37 colonies isolated at 39.5°C were successfully expanded. Interestingly a recombinant encoding a protein with a smaller amino-terminal truncation (T128-708) was capable of partial complementation. The average efficiency of colony formation at the non-permissive temperature was 40.6% of that achieved at the permissive temperature and 8 out of 23
colonies isolated at the non-permissive temperature were successfully expanded into cell lines. Mutant dl1135, which has a smaller deletion in the amino-terminus of the protein (amino acids 17-27), yielded colonies at the non-permissive temperature at approximately the same efficiency (37.8%) but a higher proportion of these were able to expand (4 out of 6). Two mutants with amino acid substitutions within this region were also able to complement growth at a slightly lower efficiency. Mutant 5002 (L[19]-F and P[28]-S) yielded colonies at 29.0% and mutant 5003 (A[23]-V) at 28.2%. Although the efficiency of complementation by these two mutants was lower than that obtained with the larger amino-terminal truncation mutant T128-708, all the colonies isolated at the non-permissive temperature with mutants 5002 and 5003 were successfully expanded into cell lines (6 out of 6 each).

The amino-terminal 175 amino acids of T antigen are known to have several functions, one of which is the sequestration of the retinoblastoma susceptibility gene product (pRB), and other members of the pRB family of pocket proteins, via the conserved region 2 (CR2)-like domain (DeCaprio et al., 1988; Dyson et al., 1989a; Ewen et al., 1989; Hannon, Demetrick and Beach, 1993). To determine whether it was this region which was required for the complementation of the growth defect in tsal4 at the non-permissive temperature, two mutants that are both defective for pRB binding were used (DeCaprio et al., 1988; Christensen and Imperiale, 1995). The first of these mutants (3213; E[107]-L & E[108]-L) gave a higher efficiency of complementation than wild type T antigen (101.1%) while the second (K1; E[107]-L) gave the same efficiency as wild type (64.6%). Both mutants yielded colonies which were readily expanded into cell lines at the non-permissive temperature (4 out of 4 and 6 out of 6 respectively). This data suggests that the complementation of tsal4 cells by T antigen at the non-permissive temperature does not require the sequestration of the pRB family of pocket proteins.

Having established that amino-terminal functions of T antigen were required for complementation I used several mutants encoding amino-terminal fragments of T antigen to determine whether this region alone is sufficient to maintain growth in tsal4 at the non-permissive temperature. Mutants dl1137 and T147D encode stable proteins comprising the amino-terminal 121 and 147 amino acids respectively. Both of these mutants were unable to complement the growth defect; efficiencies of colony formation at the non-permissive temperature relative to the permissive temperature of 5.5% and 16.3% respectively were obtained. Neither mutant yielded colonies which were able to expand at the non-permissive temperature. Another mutant, dl1061, which encodes a larger protein including the amino-terminus (amino acids 1-590), was also unable to complement tsal4 growth at the non-permissive temperature (as indicated by a complementation efficiency of 8.3% and that no colonies could be expanded). Thus the functions carried by the amino-terminus of T antigen, while they are required for
complementation, alone are not sufficient.

To determine whether functions carried by the carboxy-terminus of the T antigen protein were also required for growth complementation I used several mutants encoding proteins with amino acid substitutions in the carboxy-terminal half of the protein. Mutants 5031 (D[402]-N, V[404]-M & V[413]-M), 5041 (G[426]-E & S[430]-N) and 5080 (A[23]-V & P[584]-L) encode proteins which are unable to bind p53. These three mutants all failed to complement the growth defect of tsal4 at 39.5°C, giving average efficiencies lower than that obtained with the pUC19/pKS controls (4.0%, 1.5% and 7.1% respectively) and no colonies were successfully expanded at the non-permissive temperature. The substitution at amino acid 23 in mutant 5080 is the same as that in mutant 5003, which was capable of complementation, thereby suggesting that the amino acid substitution which disrupts activity in mutant 5080 is the one at amino acid 584. This residue lies within a hydrophobic region of T antigen and this mutation is thought to disrupt the tertiary structure of the protein (Peden et al., 1989). Mutants 5030 (A[417]-S) and 5061 (G[431]-ALE) along with mutant 5031 are defective in viral DNA replication in vivo and in vitro (J.M.Pipas,, Department of Biological Sciences, University of Pittsburgh, unpublished data) however 5030 and 5061 are both able to bind to p53 (unlike 5031). Both these mutants were able to complement tsal4 growth at efficiencies close to wild type T antigen (45.0% and 56.7% respectively and every colony isolated at 39.5°C was successfully expanded).

3.2.2: Ability of tsal4-derived cell lines isolated at the permissive temperature to grow at the non-permissive temperature.

The results obtained by directly selecting for growth at the non-permissive temperature were confirmed by a third assay. Representative hygromycin-B resistant colonies were isolated at the permissive temperature (33.0°C) following transfection of each mutant and expanded into clonal cell lines. The growth of these cell lines at the non-permissive temperature (39.5°C) was compared to that at the permissive temperature by plating 1000 cells on duplicate plates and growing them at either temperature. The numbers of colonies obtained at 39.5°C and at 33.0°C after 14 days growth are shown in Table 3.3 along with the percentage efficiency colony formation at 39.5°C compared to 33.0°C. These assays confirmed that wild type T antigen along with mutants 5003, K1, 5030 and 5061 were capable of complementing tsal4 growth at the non-permissive temperature since all the tsal4 cell lines isolated at the permissive temperature after transfection with these mutants were capable of growth at the non-permissive temperature. Although only 4 of the 6 colonies isolated at the non-permissive temperature following transfection of dl1135 yielded cell lines, all six cell lines isolated at the permissive temperature were able to grow at the non-permissive temperature. This indicates that any function(s) perturbed by the deletion of amino acids
Table 3.3: Quantitative cell growth analysis of cell lines derived from 33.0°C after transfection with either one or two T antigen mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Total number of stained colonies obtained on 2 dishes at 39.5°C / 33.0°C (%)</th>
<th>no. of cell lines which grow at 39.5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cell line 1</td>
<td>cell line 2</td>
</tr>
<tr>
<td>SE (wild-type)</td>
<td>112/132 (84.8%)</td>
<td>290/333 (87.1%)</td>
</tr>
<tr>
<td>pUC19/pKS</td>
<td>0/648 (0.0%)</td>
<td>0/175 (0.0%)</td>
</tr>
<tr>
<td>small t</td>
<td>10/160 (6.3%)</td>
<td>16/83 (19.3%)</td>
</tr>
<tr>
<td>T176-708</td>
<td>8/250 (3.2%)</td>
<td>12/210 (5.7%)</td>
</tr>
<tr>
<td>dH137</td>
<td>37/138 (21.3%)</td>
<td>214/257 (84.1%)</td>
</tr>
<tr>
<td>S003</td>
<td>228/240 (95.0%)</td>
<td>-</td>
</tr>
<tr>
<td>K1</td>
<td>107/187 (57.2%)</td>
<td>196/202 (97.0%)</td>
</tr>
<tr>
<td>dH137 + dH1137</td>
<td>4/230 (1.7%)</td>
<td>9/244 (3.7%)</td>
</tr>
<tr>
<td>T176-708 + T147D</td>
<td>31/398 (7.8%)</td>
<td>3/369 (0.8%)</td>
</tr>
<tr>
<td>T176-708 + T147D</td>
<td>10/367 (2.7%)</td>
<td>41/325 (12.6%)</td>
</tr>
</tbody>
</table>

The indicated 33°C derived cell lines were analysed for growth at either 33°C or 39.5°C. 1000 cells were plated per 60mm or 100mm dish and cultured for two weeks at 33°C or 39.5°C. Colonies were then stained and counted.

* those cell lines which gave over 30.0% the number of colonies at 39.5°C compared to 33.0°C were considered positive

**This line gave very dense colonies at 39.5°C indicating that the cotransfected mutants were capable of maintaining growth at 39.5°C.

→ arrows indicate that data for these lines is continued on the lower portion of the table
17-27 are not required for the maintenance of tsal4 growth. Cell lines derived at the permissive temperature, after the transfection of mutants which were incapable of direct complementation at the non-permissive temperature, were also negative in this assay; T176-708 (12 cell lines assayed), dl1137 (12 assayed), T147D (10 assayed), dl1061 (12 assayed), 5031 (5 assayed), 5041 (4 assayed), 5080 (6 assayed) and small t antigen (4 assayed) all yielded cell lines at the permissive temperature which were unable to grow upon shift up to the non-permissive temperature. The majority of colonies isolated at the permissive temperature following transfection of the amino-terminal truncation mutant T128-708 were unable to grow at the non-permissive temperature; however two cell lines did appear to be able to support a decreased amount of growth at the non-permissive temperature. T128-708 cell line 1 gave 39.6% growth while cell line 2 gave 76.6%. However colonies obtained from these cell lines at the non-permissive temperature were not as dense or as large as those obtained at the permissive temperature, indicating that these cell lines did not grow as rapidly at the non-permissive temperature as they did at the permissive temperature. This may represent the ability of mutant T128-708 to partially complement tsal4 growth since I was only able to expand 8 out of the 23 colonies selected directly at the non-permissive temperature into cell lines.

3.2.3: tsal4 complementation by cotransfection of two mutants.

Moran and colleagues have shown that Ad E1A mutants which alone were unable to cooperate with activated ras to transform primary baby rat kidney cells were able to transform when used in combination (Moran and Zerler, 1988). I examined whether T antigen mutants were capable of complementing each other to overcome the growth defect in tsal4 cells. I cotransfected the amino-terminal truncation mutant T176-708 with either mutant dl1137 (1-121 amino acids only), T147D (1-147 amino acids only), dl1061 (1-590 amino acids only), 5031 (D[402]-N, V[404]-M & V[413]-M) and 5041 (G[426]-E & S[430]-N) each of which alone were unable to complement the growth defect of tsal4 at the non-permissive temperature. The results of these cotransfections are shown in Table 3.2, along with the results for the single mutant transfections carried out simultaneously (experiments 7-10). The combination of T176-708 with either dl1137, T147D or dl1061 clearly had some ability to complement tsal4 growth, giving efficiencies of 33.6%, 31.6% and 27.9% respectively. In each case a number of the colonies isolated at the non-permissive temperature were successfully expanded into cell lines. In contrast, however, the cotransfection of T176-708 with either 5031 or 5041 yielded very few colonies at the non-permissive temperature (efficiencies of 9.6% and 8.1% compared to the permissive temperature were obtained) and no colonies were successfully expanded into cell lines at the non-permissive temperature.
I attempted to verify these complementation results by assaying cell lines isolated at the permissive temperature for growth at the non-permissive temperature. The results obtained from representative cell lines are shown in Table 3.3. These cotransfections only yielded one cell line at the permissive temperature which clearly was able to grow at the non-permissive temperature. This line came from the combination of T176-708 with dl1061 (cell line 7). Although this line gave a relatively low efficiency of colony formation (27.6%) at the non-permissive temperature when compared to the permissive temperature these colonies were very dense suggesting that they grew particularly well.

3.2.4: Analysis of protein expression in tsa14 cell lines.

Wherever a mutant T antigen protein failed to complement the growth defect of tsa14 cells, I analysed the stably transfected cell lines obtained at the permissive temperature (33.0°C) for expression of the mutant protein to ensure that lack of expression of the mutant protein was not the reason for the lack of complementation. Analysis of T antigen expression at the permissive temperature is complicated by the presence of the endogenous tsA58 T antigen. For this reason I assayed mutant protein expression in stably transfected cells originally isolated at the permissive temperature but which had been shifted to the non-permissive temperature (39.5°C) 72 hours previously. Under these conditions most of the endogenous tsA58 T antigen will have been degraded; that which remains is denatured and as a result is unable to bind p53. Therefore those T antigen mutants which were able to bind to p53 were co-immunoprecipitated from cell lysates, prepared from cells at the non-permissive temperature, using an antibody against p53. Analysis of protein expression was also carried out on several tsa14 cell lines which had been directly complemented by a mutant at the non-permissive temperature and therefore continuously maintained at the non-permissive temperature. This was carried out to ensure that the mutant protein was not eliciting its growth maintenance effect through either stabilising the endogenous tsA58 protein or recombining to create wild type T antigen expression. Protein extracts were prepared as indicated and either immunoblotted directly or selectively immunoprecipitated and then immunoblotted.

To ensure that mutant dll06l (amino acids 1-590) was able to be detected by monoclonal antibodies which recognise epitopes within the amino-terminus of T antigen (PAb419, PAb108 and PAb416) and not by antibodies which recognise epitopes towards the carboxy-terminus (PAb114, PAb106 and PAb423; Harlow et al., 1981; Gurney, Tamowski and Deppert, 1986; Mole et al., 1987), immunoprecipitations with these antibodies were carried out on a cell lysate prepared from a REF cell line immortalised with this mutant (see section 3.2.5). The immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with the anti-T monoclonal antibody PAb416. The result is
Figure 3.2: Specificity of monoclonal antibodies against T antigen for truncation mutant \textit{dl}1061.

Extracts were prepared from a REF cell line immortalised by T antigen mutant \textit{dl}1061 in modified RIPA (0.15M) buffer. Immunoprecipitations with the indicated monoclonal antibodies against T antigen (and M73 against Ad E1A as a control) were carried out on 200\mu g extract and fractionated on by SDS-10\% PAGE alongside prestained marker (M) whose molecular masses are shown on the right. The fractionated proteins were transferred to nitrocellulose and blotted by a monoclonal antibody against T antigen (PAb416). Immunocomplexes were detected using $^{125}$I-labelled goat anti-mouse Ig antibody (as described in Materials and Methods). The positions of the \textit{dl}1061 T antigen and the immunoglobulin heavy chains are shown.

shown in Figure 3.2. Only monoclonal antibodies which recognise the amino-terminus of T antigen (PAb419, PAb416 and PAb108; lanes 1, 2 & 6 respectively) can immunoprecipitate the \textit{dl}1061 protein despite the fact that this protein represents a relatively large fragment of T antigen. A control antibody, PAbM73 (anti-E1A; Harlow, Franza and Schley, 1985), PAb114, PAb106 and PAb423 (lanes 3, 4, 5 & 7 respectively) all failed to immunoprecipitate the \textit{dl}1061 protein.
3.2.4.1: Detection of T antigen mutants with deleted amino-termini.

T antigen mutant protein T176-708 cannot be directly immunoblotted with antibodies PAb419, PAb108 or PAb416 since the epitopes for these antibodies lie in the amino-terminal region that is deleted in this mutant (Harlow et al., 1981; Gurney, Tamowski and Deppert, 1986; Mole et al., 1987). The T128-708 protein also lacks the epitope for antibodies PAb419 and PAb108. In order to detect these truncated proteins it was necessary to use an antibody towards the carboxy-terminus, but PAb423, which recognises an epitope towards the carboxy-terminus, still detects a high level of the denatured tsA58 T antigen at the non-permissive temperature in direct immunoblots. Since the tsA58 protein does not complex with p53 at the non-permissive temperature and both of these mutants are able to complex p53, I firstly immunoprecipitated the mutant T antigen from the cell extracts using an antibody against p53 (PAb421; Harlow et al., 1981). Co-immunoprecipitated T antigen was then detected by immunoblot using the anti-T antigen monoclonal antibody PAb423. The results of these experiments are shown in Figures 3.3 and 3.4. Figure 3.3 (lane 1) shows the amount of T antigen co-precipitated with p53 in a tsa14 cell line, isolated at the permissive temperature, which was stably transfected with recombinant SE. Recombinant SE expresses high levels of wild type T antigen and this cell line continued to proliferate when shifted to the non-permissive temperature (SE cell line 7 in Table 3.3). In contrast, a cell line isolated at the permissive temperature following transfection with control DNA which failed to grow when shifted to the non-permissive temperature (pKS cell line 7 in Table 3.3), did not show any T antigen co-precipitating with p53 (lane 2). Figure 3.5 shows the amount of T antigen which was detected in these cell lines when the same extracts were directly immunoblotted with antibodies PAb419 & PAb108 (SE cell line 7- lane 1 and pKS cell line 7- lane 2). Some tsA58 T antigen was still detected in the tsa14 pKS cell line even though it was not co-immunoprecipitated with p53 and this cell line failed to grow at the non-permissive temperature. This was also true for another pKS cell line which is shown in Figure 3.6 lane 1 (pKS cell line 8). In contrast, SE cell line 7 (Fig. 3.5, lane 1) showed a much higher level of expression of T antigen as well as a low level of small t antigen.

Analysis of the T176-708 T antigen detected, after co-precipitation with p53, in RIPA lysates from two cell lines (cell lines 6 and 11, Table 3.3) isolated at the permissive temperature following transfection of T176-708, is shown in Fig. 3.4 (lanes 2 & 3). This shows that the growth of these two cell lines was not maintained by the T176-708 T antigen despite its continued expression, and its ability to bind p53, at the non-permissive temperature. The protein detected in lanes 2 & 3 comigrates with the T176-708 protein immunoprecipitated from RIPA lysates prepared from an NIH3T3 cell line which stably expresses this mutant protein. Several other T176-708 cell lines which were isolated at the permissive temperature and failed to grow when shifted to the non-
Cell lysates were prepared in modified RIPA (0.15M) buffer following incubation of the indicated cell lines at 39.5°C for at least 72 hours. The cell lines were originally isolated at 33.0°C or 39.5°C as indicated. Cell lines originally isolated at 33.0°C were shifted to 39.5°C 72 hours prior to lysis. The lysates were immunoprecipitated with anti-p53 monoclonal antibody PAb421 and fractionated by SDS-6.5% PAGE alongside prestained marker (M) proteins whose molecular masses are shown on the left. Co-precipitating T antigen was immunoblotted with anti-T monoclonal antibody PAb423. Immunocomplexes were detected using HRP-linked sheep anti-mouse Ig antibody (Amersham Life Sciences). The positions of wild type (wt), tsA58 and T128-708 T antigens are indicated. CL= cell line, SE= wild type T antigen (T Ag).

permissive temperature, also expressed the T176-708 protein at high levels.

Figure 3.3 shows the level of expression of the mutant T128-708 protein in a tsa14 cell line isolated directly at the non-permissive temperature (lane 6) following transfection of T128-708, compared to three cell lines isolated at the permissive temperature (lanes 3, 4 & 5). The cell line directly isolated at the non-permissive temperature (as well as several other T128-708 cell lines isolated at the non-permissive temperature; data not shown) clearly had a high level of expression of T128-708 and a very low level of full length T antigen which may correspond to a small amount of stabilised tsA58 protein. The three cell lines isolated at the permissive temperature, however, failed to show any expression of T128-708, at least as measured by its ability to complex p53. Two of these cell lines did show some ability to grow at the non-
Figure 3.4: T176-708 T antigen expression in tsa14 T176-708 cell lines.

Cell lysates were prepared in modified RIPA (0.15M) buffer following incubation of the indicated cell lines at 39.5°C for at least 72 hours. Both tsa14 cell lines were originally isolated at 33.0°C and shifted to 39.5°C 72 hours prior to lysis. The lysates were immunoprecipitated with anti-p53 monoclonal antibody PAb421 and fractionated by SDS-6.5% PAGE alongside prestained marker (M) proteins whose molecular masses are shown on the left. Co-precipitating T antigen was immunoblotted with anti-T monoclonal antibody PAb423. Immunocomplexes were detected using HRP-linked sheep anti-mouse Ig antibody (Amersham Life Sciences). The positions of tsA58 and T128-708 T antigens are indicated. CL= cell line.

permissive temperature (cell lines 1 & 2 in Table 3.3) while the third did not (cell line 4 in Table 3.3). It is possible that this reflects a separate function, other than p53 binding, carried by the T128-708 which enables some growth at the non-permissive temperature, the selection of some other cellular mutation or a combination of both of these possibilities.

3.2.4.2: Detection of T antigen mutants with deleted carboxy-termini.

The three mutants which encode amino-terminal fragments of T antigen (dl1137, T147D and dl1061) can all be detected by direct immunoblotting with antibodies PAb419 and PAb108 and are readily distinguished from tsA58 T antigen by virtue of their smaller size. Figure 3.5 shows the level of expression of dl1137 or T147D in four
Cell lysates were prepared in modified RIPA (0.15M) buffer following incubation of the indicated cell lines at 39.5°C for at least 72 hours. The cell lines were originally isolated at 33.0°C or 39.5°C as indicated. Cell lines isolated at 33.0°C were shifted to 39.5°C 72 hours prior to lysis. The lysates were boiled in Laemmli sample buffer, fractionated by SDS-PAGE (8%-15% gradient) alongside prestained marker (M) proteins whose molecular masses are shown and immunoblotted with anti-T monoclonal antibodies PAb419 and PAb108. Immunocomplexes were detected using HRP-linked sheep anti-mouse Ig antibody (Amersham Life Sciences). The positions of wild type (wt), tsA58, dl1137 and T147D large T and small t antigens are indicated.  CL= cell line, SE= wild type T antigen.
Cell lysates were prepared in modified RIPA (0.15M) buffer following incubation of the indicated cell lines at 39.5°C for at least 72 hours. The cell lines were originally isolated at 33.0°C or 39.5°C as indicated. Cell lines isolated at 33.0°C were shifted to 39.5°C 72 hours prior to lysis. The lysates were boiled in Laemmli sample buffer, fractionated by SDS-PAGE (8%-15% gradient) alongside prestained marker (M) proteins whose molecular masses are shown and immunoblotted with anti-T monoclonal antibodies PAb419 and PAb108. Immunocomplexes were detected using HRP-linked sheep anti-mouse Ig antibody (Amersham Life Sciences). The positions of tsA58 and dl1061 large T and small t antigens are indicated. CL= cell line, SE= wild type T antigen.
representative cell lines from a number of the tsa14 cell lines which expressed these truncated proteins. These cell lines were isolated at the permissive temperature and ceased growing upon shift up to the non-permissive temperature (dl1137 cell line 8-lane 3 and T147D cell lines 9, 7 & 8-lanes 4, 5 & 6; growth data in Table 3.3). The position of migration of T147D protein was determined by analysing an extract prepared from a T147D immortalised REF cell line (lane 14; see section 3.2.5). dl1137 cell line 8 clearly expressed both the truncated dl1137 T antigen (amino acids 1-121) and small t antigen, while T147D cell line 8 clearly expressed the truncated T147D T antigen. Both T147D cell lines 7 and 9 also expressed the truncated T antigen, albeit at a lower level (longer exposure shown). Two tsa14 cell lines which expressed the truncation mutant dl1061 are shown in Figure 3.6, lanes 4 and 5. Again these two cell lines were isolated at the permissive temperature, following transfection with mutant dl1061, and they failed to grow upon shift up to the non-permissive temperature (dl1061 cell lines 7 & 8 in Table 3.3). Both of these cell lines clearly expressed the dl1061 T antigen. The position of migration of the dl1061 protein (lane 10) was confirmed by analysing a RIPA lysate prepared from a dl1061 immortalised REF cell line (see section 3.2.5). The lack of growth complementation in cell lines transfected with either dl1137, T147D and dl1061 was clearly not due lack of expression of these three mutant proteins. The dl1137, T147D and dl1061 cell lines showed varying levels of tsA58 T antigen at the non-permissive temperature but not as high a level as the wild type T antigen expression required to maintain growth in SE cell line 7. It is important to note that these lines failed to grow at the non-permissive temperature while expressing this level of tsA58 T antigen along with the truncated proteins and therefore the level of tsA58 detected here is not sufficient for complementation.

3.2.4.3: Detection of T antigen mutants with deletions in the carboxy-terminal half of the protein.

Representative tsa14 cell lines isolated at the permissive temperature following transfection with either mutant 5031 or 5041 also failed to grow upon shift up to the non-permissive temperature. Cell lysates (prepared in denaturing lysis buffer rather than the modified RIPA buffer used for the preparation of the other cell lysates) from two of each of these lines (5031 cell lines 3 & 4 and 5041 cell lines 2 & 4) were immunoblotted directly using monoclonal antibody PAb416. Figure 3.7 shows the level of expression of the mutant T antigen in each of these cell lines (lanes 3, 4, 5 & 6). For comparison, the level of expression of tsA58 T antigen detected at the non-permissive temperature in a tsa14 cell line transfected with control DNA (pKS cell line 5 in Table 3.3) and the level of expression of wild type T antigen in tsa14 SE cell line 5 is also shown (lanes 1 & 2 respectively). Both cell lines isolated at the permissive temperature following transfection of 5031 or 5041 expressed detectable levels of the mutant T antigen whereas no T antigen was detected in the pKS cell line (unfortunately there is some distortion on
Cell lysates were prepared from the indicated cell lines in denaturing lysis buffer. These lines were isolated and maintained at 33.0°C but were incubated at 39.5°C for 72 hours prior to lysis. Lysates were boiled in Laemmli buffer and fractionated by SDS-10% PAGE alongside prestained marker (M) and immunoblotted with anti-T antigen monoclonal antibody PAb416. Immunocomplexes were detected using 125I-labelled sheep anti-mouse Ig antibody (Amersham Life Sciences). The positions of the T antigens (T Ag) are indicated on the right.

CL= cell line, SE= wild type T antigen.

this autoradiograph due to the large amount of T antigen detected in the neighbouring lane, lane 2).

3.2.4.4: Detection of T antigen in tsa14 cotransfected with two mutants.

tsa14 cells transfected with a combination of T antigen mutants (T176-708 with either dl1137, T147D or dl1061) yielded colonies directly at the non-permissive temperature which could be expanded into cell lines. In contrast, however, the majority of cell lines isolated at the permissive temperature from these transfections failed to grow when shifted to the non-permissive temperature. Only one cell line isolated at the permissive temperature following transfection of the T176-708 plus dl1061 combination was capable of some growth at the non-permissive temperature (cell line 7, Table 3.3). Protein analysis was carried out on representative cell lines from these experiments firstly to determine whether cell lines isolated at the permissive temperature expressed both mutant proteins, and secondly, to determine whether cell lines isolated directly at
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the non-permissive temperature retained expression of both mutants or somehow selected for stabilisation of the tsA58 T antigen. In addition, it is possible to generate wild type T antigen by recombination following transfections involving the combination of T176-708 plus dl1061. Therefore, RIPA lysates were prepared from the cell lines either maintained continuously at the non-permissive temperature (where possible), or following transfer to the non-permissive temperature 72 hours previously, to minimise the detection of the tsA58 T antigen. Expression of T176-708 was detected by association with p53 in PAb421 immunoprecipitates and immunoblotted with PAb423 while the carboxy-terminal truncation mutants dl1137, T147D and dl1061 were detected by direct immunoblotting with PAb419 and PAb108. Since the epitopes for antibodies PAb419 and PAb108 lie within the amino-terminal 82 amino acids of T antigen, these antibodies will not immunoblot T176-708 and complicate the detection of the other mutant proteins. This was demonstrated by the failure of PAb419 and Pab108 to detect T176-708 in extracts from an NIH3T3 cell line stably expressing T176-708 (Fig. 3.6, lane 11) or in extracts from tsal4 cell lines expressing mutant T176-708 (cell lines 11 & 6 in Fig.3.6, lanes 2 & 3).

Figure 3.8 shows the expression of T176-708 in two lines from the permissive temperature and five cell lines from the non-permissive temperature from transfections of T176-708 plus either dl1137 or T147D. Neither dl1137+T176-708 cell line 12 (lane 1) or T147D+T176-708 cell line 10 (lane 2) showed any T176-708 T antigen associated with p53. Moreover, neither of these cell lines expressed detectable levels of dl1137 or T147D (Figure 3.5, lanes 7 & 8). In contrast the dl1137+T176-708 and T147D+T176-708 cell lines selected directly at the non-permissive temperature all showed expression of the T176-708 protein (Fig. 3.8, lanes 3-7). Of these lines only dl1137+T176-39.5°C cell line 1 (lane 3) showed a significant amount of full length T antigen co-precipitating with p53. Since recombination between dl1137 and T176-708 cannot recreate a wild type T antigen reading frame, this is likely to be tsA58 T antigen which has become stabilised. dl1137+T176-708-39.5°C cell lines 1 & 2 both expressed detectable levels of dl1137 T antigen (Fig 3.5, lanes 9 & 10) while cell line 3 did not (Fig. 3.5, lane 11). T147D+T176-708-39.5°C cell lines 1 & 2 expressed detectable levels of T147D (Fig. 3.5, lanes 12 & 13). Thus three of the five representative cell lines analysed, dl1137+T176-708-39.5°C cell line 2 and T147D+T176-708-39.5°C cell lines 1 and 2, the growth of which was maintained following direct selection at the non-permissive temperature expressed both transfected mutants and did not have high levels of tsA58 coprecipitating with p53. Therefore, mutants dl1137 and T147D are able to complement mutant T176-708 to maintain the growth of tsal4 cells at the non-permissive temperature in the absence of tsA58 stabilisation or recombination to recreate wild type T antigen. However this maintenance of immortalisation appears to require direct selection at the non-permissive temperature, since cell lines isolated at the permissive
Figure 3.8: T176-708 T antigen expression in tsa14 dl1137 or T147D plus T176-708 cell lines.

Cell lysates were prepared in modified RIPA (0.15M) buffer following incubation of the indicated cell lines at 39.5°C for at least 72 hours. The cell lines were originally isolated at 33.0°C or 39.5°C as indicated. Cell lines originally isolated at 33.0°C were shifted to 39.5°C 72 hours prior to lysis. The lysates were immunoprecipitated with anti-p53 monoclonal antibody PAb421 and fractionated by SDS-6.5% PAGE alongside prestained marker (M) proteins whose molecular masses are shown on the left. Co-precipitating T antigen was immunoblotted with anti-T monoclonal antibody PAb423. Immunocomplexes were detected using HRP-linked sheep anti-mouse Ig antibody (Amersham Life Sciences). The positions of tsA58 and T176-708 T antigen proteins are indicated. CL= cell line.

temperature, and therefore maintained for a number of passages in the presence of tsA58 T antigen, failed to express either of the transfected T antigen mutants when shifted to the non-permissive temperature.

dl1137+T176-708-39.5°C cell line 3 appeared only to express T176-708 but still grew at the non-permissive temperature. However tsa14 cell lines isolated at the permissive temperature following the transfection of T176-708 (T176-708-33.0°C cell lines 11 & 6), which expressed similar levels of T176-708 protein (Fig. 3.4, lanes 2 & 3)
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as detected by co-immunoprecipitation with p53, failed to grow upon shift up to the non-
permissive temperature. Moreover, transfection of T176-708 alone into tsal4, did not
yield any expandable colonies directly at the non-permissive temperature (Table 3.2). Therefore the dl1137+T176-708-39.5°C cell line 3 may have selected some other
cellular lesion which along with T176-708 expression allowed it to grow continuously at
the non-permissive temperature or it expresses the dl1137 protein at a much lower level
than the other cell lines examined.

The results obtained with the cell lines isolated after cotransfection of mutants
T176-708 and dl1061 are shown in Figure 3.6 & 3.9. Cell lines 7 and 8 were both
isolated at the permissive temperature. Cell line 8 did not proliferate when shifted up to
the non-permissive temperature (Table 3.3). The dl1061 protein was readily detected in
this cell line (Fig. 3.6, lane 7) but no T176-708 protein (Fig. 3.9, lane 5) was detected by
its interaction with p53, thus confirming that expression of dl1061 alone is insufficient
to maintain growth. In contrast, cell line 7 was capable of continued growth at the non-
permissive temperature (Table 3.3). This cell line, along with two cell lines isolated
directly at the non-permissive temperature, clearly expressed both dl1061 (Fig. 3.6,
lanes 6, 8 & 9) and T176-708 (Fig. 3.9, lanes 4, 6 & 7) proteins. Thus maintenance of
growth in cell lines isolated directly at the non-permissive temperature and in the one
cell line isolated at the permissive temperature which was able to grow at the non-
permissive temperature required the expression of both mutant proteins. Interestingly,
in contrast to the T176-708 plus dl1137 or T147D combinations, in the three
dl1061+T176-708 cell lines capable of growth at the non-permissive temperature a
relatively high amount of T antigen migrating at the same size as full length T antigen
was observed in both the direct immunoblots and by co-immunoprecipitation with p53.
One explanation for this is the possibility that the endogenous tsA58 T antigen has
become stabilised at the non-permissive temperature. Alternatively, I may have selected
for cell lines expressing a greater amount of the tsA58 protein. Previous studies have
shown that when tsA58 is synthesised at a higher level more protein is detected at the
non-permissive temperature (Jat et al., 1991). The full length protein was only detected
in one of the cell lines isolated at the non-permissive temperature with the T176-708
plus dl1137 or T147D combinations. The fact that three of the dl1137+T176-708 or
T147D+T176-708-39.5°C cell lines are able to maintain growth without stabilisation of
tsA58 T antigen suggests that the full length protein observed in the dl1061+T176-708
cell lines may be due to a second event selected following the effects of the two mutant
proteins. Alternatively, the reading frames encoding the T176-708 and dl1061 proteins
may have recombined to generate wild type T antigen. While there is certainly selection
pressure for this to occur in the cell lines isolated directly at the non-permissive
temperature, there should be no such pressure at the permissive temperature
dl1061+T176-708-33.0°C cell line 7). It is interesting to note that the level of full
Cell lysates were prepared in modified RIPA (0.15M) buffer following incubation of the indicated cell lines at 39.5°C for at least 72 hours. The cell lines were originally isolated at 33.0°C or 39.5°C as indicated. Cell lines originally isolated at 33.0°C were shifted to 39.5°C 72 hours prior to lysis. The lysates were immunoprecipitated with anti-p53 monoclonal antibody PAb421 and fractionated by SDS-6.5% PAGE alongside prestained marker (M) proteins whose molecular masses are shown on the left. Co-precipitating T antigen was immunoblotted with anti-T monoclonal antibody PAb423. Immunocomplexes were detected using HRP-linked sheep anti-mouse Ig antibody (Amersham Life Sciences). The positions of tsA58 T Ag and T176-708 T antigens are indicated. CL= cell line.

Length T antigen is higher in this cell line (Figure 3.6, lane 6) than that observed in SE cell line 7 (Fig. 3.5, lane 1) however this SE cell line grows much better at the non-permissive temperature. T176-708-33.0°C cell line 7 formed colonies at an efficiency of 27.6%, whereas SE cell line 7 formed colonies at an efficiency of 62.0%, at the non-permissive temperature compared to the permissive temperature (Table 3.3). It therefore seems unlikely that the full length T antigen corresponds to the wild type protein. Moreover, it is not possible to generate wild type T antigen by recombination in the
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T176-708 plus dl1137 or T147D combinations, each of which complement tsa14 growth.

3.2.5: Immortalisation of secondary rat embryo fibroblasts.

Having established that several different regions of T antigen were required for complementing the growth defect of tsa14 cells, I examined whether precisely the same regions were required for the immortalisation of secondary REFs or whether additional regions were also required. The T antigen mutants were cotransfected into secondary REFs with pSV2neo, a plasmid which encodes resistance to the drug Geneticin-G418. Mutants were only considered to be positive for immortalisation if they induced G418-resistant colonies, the majority of which could then be expanded into cell lines. The results of nine experiments are shown in Table 3.4 (and summarised in Figure 3.11). The average efficiency of immortalisation, expressed as the percentage number of colonies obtained with the mutant compared with the number obtained with wild type T antigen (plasmid SE), and the number of cell lines successfully established from all nine experiments are shown in the right hand two columns. Wild type T antigen yielded a large number colonies in each experiment and all the colonies isolated were successfully expanded into cell lines. In contrast, control transfections with pUC19/pKS control DNA plus pSV2neo resulted in very few colonies (2.9% compared to wild type T antigen) and none of the 36 colonies isolated could be expanded into cell lines. Small t antigen alone was unable to immortalise, yielding fewer colonies than the control transfection (0.6%).

Mutant proteins lacking the amino-terminal 175 (T176-708) or 127 (T128-708) amino acids yielded very few colonies (2.6% and 5.1%) respectively. Moreover, none of the colonies isolated from transfections of mutant T176-708 could be expanded, while 1 of 25 colonies isolated with mutant T128-708 was successfully expanded. Thus p53 binding by T antigen is not sufficient for immortalisation and one or more amino-terminal domain(s) is necessary for the immortalisation of REFs. The lack of immortalisation by these amino-terminally truncated T antigen proteins may be due to the deletion of several functional domains of T antigen for example the CR1-like and CR2-like regions. I attempted to clarify these results using smaller point and deletion mutants. Mutant dl1135, which lacks amino acids 17-27, also yielded very few colonies (7.6% compared to wild type T antigen) and only 1 of 40 colonies isolated was successfully expanded. Single amino acid substitutions within the amino-terminus only partially decreased the efficiency of colony formation. Mutants 5002 and 5003 yielded colonies at efficiencies of 20.5% and 51.1% respectively. All the colonies isolated after transfection with 5002 were readily expanded into cell lines. These results suggest that a function carried by the CR1-like region, which is disrupted by the dl1135 mutation but not by the 5002 and 5003 mutations, is necessary for immortalisation. Mutant 3213,
Table 3.4: Ability of SV40 large T antigen mutants to immortalise secondary REFs.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Number of densely staining colonies obtained following transfection of mutant into REFs (% of SE)</th>
<th>Immortalisation efficiency (% SE)</th>
<th>cell lines derived/colonies isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>expt. 1</td>
<td>expt. 2</td>
<td>expt. 3</td>
</tr>
<tr>
<td>SE (wild-type)</td>
<td>4,900 (100.0%)</td>
<td>3,840 (100.0%)</td>
<td>2,520 (100.0%)</td>
</tr>
<tr>
<td>pUC19/pKS</td>
<td>32 (0.7%)</td>
<td>-</td>
<td>168 (6.7%)</td>
</tr>
<tr>
<td>small t</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T176-708</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T128-708</td>
<td>56 (1.1%)</td>
<td>24 (0.6%)</td>
<td>732 (29.8%)</td>
</tr>
<tr>
<td>T124-708</td>
<td>941 (19.2%)</td>
<td>1,062 (27.6%)</td>
<td>374 (14.0%)</td>
</tr>
<tr>
<td>3003</td>
<td>2,502 (51.1%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3213</td>
<td>372 (7.6%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K1</td>
<td>-</td>
<td>234 (6.1%)</td>
<td>-</td>
</tr>
<tr>
<td>T147D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T1061</td>
<td>360 (7.3%)</td>
<td>-</td>
<td>409 (16.2%)</td>
</tr>
<tr>
<td>S031</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S041</td>
<td>-</td>
<td>81 (2.1%)</td>
<td>294 (11.7%)</td>
</tr>
<tr>
<td>S080</td>
<td>62 (1.3%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S030</td>
<td>-</td>
<td>723 (18.8%)</td>
<td>-</td>
</tr>
<tr>
<td>S061</td>
<td>-</td>
<td>189 (4.9%)</td>
<td>-</td>
</tr>
<tr>
<td>T176-708+S031</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T176-708+S041</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T135+5031</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T135+5041</td>
<td>-</td>
<td>-</td>
<td>395 (15.7%)</td>
</tr>
</tbody>
</table>

The indicated recombinants were transfected into secondary REFs using pSV2neo as a coselectable marker as described in Materials and Methods. Transfected REFs were cultured for 2-3 weeks with 250μg/ml G418. Representative plates were stained and colonies were counted. The indicated number of cell lines were isolated and the total number of cell lines successfully derived is shown. expt. = experiment; N.D. = not done. aExperiment was only carried out once.
which encodes a protein with a defective CR2-like region and is unable to bind pRB, p107 and probably p130 (Christensen and Imperiale, 1995), also yielded a low number of colonies (7.6% compared to wild type T antigen) but all 6 colonies isolated were readily expanded into cell lines. In contrast, mutant K1 which also cannot bind to pRB (DeCaprio et al., 1988), yielded a much higher number of colonies (85.4%) and all those colonies isolated expanded successfully. Since these two CR2-defective mutants were only assayed once (in separate experiments) it is possible that the differences in the efficiencies of colony formation may be due to variation between the two experiments. However the clear result is that deletion of the CR2-like region does not negate immortalisation by T antigen.

In contrast to the results I obtained with tsa14 complementation, I, like others, observed that mutants which encode only the amino-terminus of T antigen can still immortalise REFs. Even though mutants dl1137 and T147D, which encode T antigen proteins of amino acids 1-121 and 1-147 respectively, yielded colonies at decreased efficiencies compared to wild type T antigen (6.3% and 28.3% respectively), the majority of colonies isolated successfully expanded into cell lines (36 of 50 and 19 of 24 respectively). Moreover, mutant dl1061 which produces a larger amino-terminal fragment of the protein (amino acids 1-590) yielded colonies at a slightly higher efficiency (29.5%) and almost all of the isolated colonies were expanded into cell lines (22 of 24). The colonies obtained following transfection of the dl1061 mutant expanded more rapidly than those isolated from the dl1137 and T147D transfections. These results are interesting when compared to the results obtained with other mutants, such as 5031 and 5041 which contain the whole amino-terminus region intact but are unable to immortalise REFs. The efficiency of colony formation with these mutants was low (7.7% and 5.8% respectively). None of the colonies isolated after transfection with 5041 grew and only 3 colonies out of the 26 isolated grew after transfection with 5031. It is curious that the amino-terminal region of these mutant proteins is intact yet, unlike mutants dl1137, T147D and dl1061, these mutants fail to immortalise.

The amino acid substitution at position 584 in 5080 has been suggested to destabilise the T antigen protein thereby destroying its ability to interact with both p53 and pRB; this mutant was also found to be incapable of immortalising REFs. Mutants 5030 and 5061, which have amino acid substitutions at positions 417 and 431 respectively but retain the ability to bind to p53, were both able to immortalise REFs albeit at decreased efficiency (18.8% and 23.5% compared to wild type T antigen respectively). Nearly all the colonies isolated were able to yield cell lines in both cases.

3.2.6: REF immortalisation by cotransfection of two mutants.

The data obtained by transfection of individual mutants demonstrated that several regions of T antigen are required for the efficient immortalisation of secondary
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REFs. These regions correspond to the amino-terminus including the CR1-like domain or the carboxy-terminus which is involved in the association with p53 and the stability of the protein. To determine whether these domains could complement each other in trans, I cotransfected amino-terminus mutants (dl 1135 and T176-708) with carboxy-terminus mutants (5031 and 5041), each of which alone were unable to immortalise. The results obtained are shown in Table 3.4 (experiments 3-7). The efficiency of colony formation for these cotransfections was low compared to wild type T antigen possibly because the transfections involved three independent plasmids. However approximately half the colonies picked were successfully expanded in each case. These results show that the amino-terminal and carboxy-terminal domains of T antigen can complement each other in trans to immortalise secondary REFs.

3.3: Discussion.

SV40 large T antigen is capable of immortalising primary rodent embryo fibroblasts (Petit, Gardes and Feunteun, 1983; Jat and Sharp, 1986). Such T antigen immortalised cells can proliferate indefinitely but remain absolutely dependent upon T antigen's continued presence for maintenance of growth and its inactivation results in a rapid and irreversible entry into a post mitotic state (Jat and Sharp, 1989). I have assayed the ability of various T antigen mutants to either immortalise secondary rat embryo fibroblasts (REFs) or maintain the potential of a conditionally immortal rat cell line (tsa14) which was derived by immortalising REFs with the thermolabile tsA58 T antigen. This cell line proliferates indefinitely at the permissive temperature but rapidly ceases dividing upon transfer to the non-permissive temperature. The loss of proliferative potential in this cell line can be readily complemented by the exogenous introduction of either wild type T antigen, adenovirus 5 E1A or HPV16 E7 (Jat and Sharp, 1989; Vousden and Jat, 1989). Several conclusions can be drawn from the results which I have obtained:

[1] Several regions of T antigen are required, to a greater or lesser extent, for immortalisation (a region colinear with the p53 binding region, the hydrophobic region of T antigen and one or more regions within the amino-terminal 175 amino acids; summarised in Figures 3.10 and 3.11).

[2] One of these regions (defined by the deletion of amino acids 17-27) is required for the immortalisation of REFs but is not necessary for maintaining growth in the conditional cell line suggesting that the functions initially required to immortalise secondary REFs are not all continuously required once the resulting cell lines have been established. Thus the complementation of growth in the conditionally immortal cell line (tsa14) assays functions which are required for the maintenance of immortalisation.
Figure 3.10: The ability of SV40 T antigen to complement the growth defect of tsal4 requires several regions.

This figure summarises the data presented in Table 3.2. Recombinants expressing the indicated T antigen mutant proteins were cotransfected into tsal4 cells with a recombinant expressing resistance to hygromycin B. The transfected cells were assayed for their ability to grow at 39.5°C, the non-permissive temperature for the tsal4 cells. The graph shows the percentage number of colonies obtained at the non-permissive temperature compared to the permissive temperature (33.0°C) for a number of experiments (average complementation). Colonies isolated at the non-permissive temperature were then assayed for their ability to expand into cell lines. Where the majority of colonies isolated were successfully expanded into cell lines the columns are filled. If a proportion of the columns were successfully expanded the columns are shaded, and if no colonies successfully expanded the columns are not shaded. The position of the mutations and the regions identified as necessary for the maintenance of tsal4 growth are summarised below.
Figure 3.11: The ability of SV40 T antigen to immortalise secondary REFs requires several regions.

This figure summarises the data presented in table 3.4. Recombinants expressing the indicated T antigen mutant proteins were cotransfected into secondary rat embryo fibroblasts with a recombinant expressing resistance to Geneticin (G418). The transfected cells were incubated at 37.0°C for 2-3 weeks after which representative colonies were isolated and wherever possible expanded into cell lines. The graph shows the percentage efficiency of immortalisation as compared to wild-type T antigen (SE). In experiments where the majority of the colonies isolated were able to expand into cell lines the columns are filled. Where a proportion of the colonies yielded cell lines the columns are shaded, while wherever no colonies where successfully expanded the columns are not shaded. The position of the mutations and the regions identified as necessary to immortalise secondary REF are summarised below.
Amino-terminal fragments of T antigen are sufficient to immortalise secondary REFs albeit at a decreased efficiency compared to wild type T antigen. However these truncated proteins are unable to maintain growth in the conditionally immortal cell line suggesting that immortalisation by such amino-terminal fragments may be due to either second events or the immortalisation of a subset of cells within the heterogeneous REF population.

Point mutations within the carboxy-terminus perturb functions in the amino-terminus; T antigen mutants which have intact amino-termini but carry point mutations closer to the carboxy-terminus are unable to immortalise while amino-terminal fragments are able to immortalise at low efficiency.

The functions required for both REF immortalisation and complementation of tsa14 growth can be provided in trans by separate mutant T antigen molecules. This is shown by amino- or carboxy-terminus mutants, which alone are incapable of immortalising REFs or complementing tsa14, being able to cooperate in REF immortalisation (T176-708 or dl1135 plus 5031 or 5041) or tsa14 complementation (T176-708 plus dl1137, T147D or dl1061).

Together, the results show that the functions required for both REF immortalisation and maintenance of growth can be provided in trans by separate T antigen molecules suggesting that immortalisation by T antigen requires the cooperative action of independent functional domains to both initiate and maintain the immortal state.

3.3.1: Requirement for the amino-terminus of T antigen in immortalisation.

The data I have presented here shows that one or more functions carried by the amino-terminal 175 amino acids of T antigen are required both for the maintenance of the immortal state in tsa14 cells and the immortalisation of secondary REFs. This region is defined by mutant T176-708 which is inactive in both assays and can be further subdivided into three distinct domains, the CR1-like domain, the CR2-like domain and a region downstream of the CR2-like domain as far as amino acid 175, each of which are involved to different degrees in the initiation and maintenance of immortalisation. This amino-terminal region also includes the region (amino acids 17-27) that overlaps the CR1-like domain, which is necessary for T antigen's ability to complement the p300-associated transforming function of Ad E1A (Yacuik et al., 1991). The boundaries of these regions are indicated in Figures 3.10 & 3.11 and are defined by mutants T128-708, 3213 (amino acids 107 and 108 substituted) and mutant dl1135 (amino acids 17-27 deleted). Mutant dl1135 is able to maintain growth in tsa14 cells but is unable to immortalise REFs. This raises the intriguing possibility that the functions initially required to immortalise primary rat cells are not all required once the cell lines have been established and that the complementation of growth in the conditional cell line
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assays for the maintenance of proliferation. This mutant retains the p53, pRB and p107-binding activities demonstrating that these activities alone are not sufficient to immortalise secondary REFs. Interestingly dl1135 is unable to transform REF52 (Pipas, Peden and Nathans, 1983) and C3H10T1/2 (J.M.Pipas, unpublished data). Also, in cooperation with activated ras, it is unable to transform primary baby rat kidney cells (Yacuik et al., 1991) and is greatly reduced for non-established rat embryo fibroblasts (Michalovitz et al., 1987). Neither is dl1135 able to overcome the wild type p53-mediated growth arrest at the non-permissive temperature of a REF cell line transformed by activated ras and a temperature sensitive p53 mutant (Quartin et al., 1994). Interestingly a similar T antigen mutant, with amino acids 5 to 35 deleted, is also unable to transform C3H10T1/2 or REF52 cells (Zhu et al., 1992), but has been shown to immortalise primary mouse embryo fibroblasts at only a slightly lower efficiency than wild type T antigen (Zhu et al., 1991a). Moreover, targeting of dl1135 expression specifically to B and T lymphocytes and the choroid plexus epithelium of the brain using the lymphotrophic papovavirus transcription signals in transgenic mice causes thymic lymphomas but not choroid plexus tumours. This T-cell specific lymphomagenesis is in contrast to the lymphomagenesis of both T- and B-cells and the induction of choroid plexus tumours by wild type T antigen (Symonds et al., 1993). This data suggests that the T antigen function eliminated by the deletion of amino acids 17 to 27 may only be necessary for immortalisation or transformation in some species or cell types.

The dl1135 deletion removes one of the conserved amino acids (position 19) in the CR1-like domain of T antigen and this is one of the 2 amino acids substituted in mutant 5002. Mutant 5002 is unable to transform REF52 cells and can only transform C3H10T1/2 cells at 10% the efficiency of wild type T antigen (Srinivasan, Peden and Pipas, 1989; Peden et al., 1990). My data shows that mutant 5002 is able to both complement the tsal4 growth defect and immortalise secondary REFs suggesting that either the 2 amino acid substitutions in the CR1-like domain are not totally eliminating the activity of this region or the deletion of amino acids 17 to 27 in mutant dl1135 is inactivating a different function, specifically necessary for REF immortalisation. It has previously been shown that the CR1 of El A was necessary for ElA's ability to complement the growth defect of tsa14 cells (Riley et al., 1990). This region of ElA has several activities, one of which is association with the cellular protein p300 (Whyte, Williamson and Harlow, 1989). ElA mutants defective for the interaction with p300 have been shown to be defective for transformation of baby rat kidney cells in cooperation with ras (Moran and Zerler, 1988; Whyte, Ruley and Harlow, 1988). CR1 mutants of ElA can be complemented in these assays by T antigen (Yacuik et al., 1991) and this is dependent upon the presence of amino acids 17 to 27 since mutant dl1135 is unable to complement. As yet there is no evidence for a physical association between T antigen and p300. My results suggest that other regions of T antigen may be sufficient
for maintenance of the immortal state in tsa14 when amino acids within the CR1-like domain are mutated (as in mutants d/1135 and 5002). These functions would presumably be lacked by E1A and specific to T antigen, since E1A CR1 mutants failed to maintain immortalisation in tsa14 cells.

The amino terminal truncation mutant T128-708 has partial activity in maintaining the growth of tsa14 cells at the non-permissive temperature, but is unable to immortalise REFs. The efficiency of colony formation at the non-permissive temperature was similar to that obtained with mutant d/1135 but only approximately one third of the colonies isolated at the non-permissive temperature were able to grow into cell lines. This low level of maintenance of tsa14 growth by T128-708 appears to require direct selection for a high level of expression at the non-permissive temperature since cell lines established at the permissive temperature following transfection were unable to grow well when shifted to the non-permissive temperature. This appears to be due to the failure of the cell lines isolated at the permissive temperature to retain expression of the T128-708 protein (Fig. 3.3). In contrast, a cell line isolated at the non-permissive temperature clearly expressed the mutant protein. Mutant T128-708 lacks both the first and second regions I have analysed. The second region, which corresponds to the CR2-like domain of T antigen and is necessary for T antigen's ability to complex pRB, p107 and p130, is not necessary for the maintenance of growth in tsa14 cells at the non-permissive temperature however may be involved in efficient immortalisation. Mutants 3213 and K1 both have substitutions at amino acid 107 but 3213 also has a second substitution at amino acid 108. Neither of these mutants are capable of binding pRB (DeCaprio et al., 1988; Christensen and Imperiale, 1995) however the abilities of these mutants to bind p107 and p130 have not been determined. Both mutants are able to complement tsa14 growth at similar efficiencies to wild type T antigen. Mutant K1 immortalised secondary REFs relatively well compared to wild type, whereas 3213 immortalised at a much lower efficiency. Nevertheless colonies isolated after transfection with either mutant were readily established and grew as well as wild type T antigen cell lines (data not shown). This suggests that binding of the pRB family of proteins by T antigen may be involved in determining the efficiency of immortalisation. This result is in accordance with the work of other researchers who have also demonstrated that pRB binding is not absolutely required for immortalisation by T antigen (Tevethia et al., 1988; Chen and Paucha, 1990). Immortalisation of baby rat kidney cells by Ad E1A is affected by mutation of its CR2 domain (Schneider et al., 1987; Subramanian et al., 1988; Quinlan and Douglas, 1992). Previous work using mutants of adenovirus E1A to maintain growth in tsa14 cells also demonstrated that the CR2 of E1A was not required (Riley et al., 1990). In contrast, similar mutations in the CR2-like region of HPV16 E7 have been shown to decrease both the ability of the E7 protein to complement tsa14 growth at the non-permissive temperature and its ability to
cooperate with ras to transform rat embryo fibroblasts (Chesters et al., 1990).

The third of the amino-terminal regions I have identified, which appears to be involved in both the tsa14 complementation and REF immortalisation activities of T antigen, is the region that is immediately downstream of the pRB binding region. In contrast to T128-708, mutant T176-708 is completely inactive in tsa14 complementation, as well as REF immortalisation. The region spanning amino acids 128 to 175 overlaps part of a region (spanning amino acids 131 to 259) which is required for the initiation of DNA synthesis by T antigen (Dobbelstein et al., 1992). This region, termed region X, overlaps a region which has recently been suggested to interact with several cellular proteins including the TATA-binding protein (TBP) and enhancer-binding transcriptional activator, TEF-1 (Gruda et al., 1993). Cotransfection of dl1137 (amino acids 1-121 only) and T176-708 can also complement the growth of tsa14 cells, however neither of these mutants encode amino acids within this third region (128-175). It seems therefore that this region only becomes necessary for maintenance of growth in the absence of the amino-terminal 121 amino acids of T antigen. The functions provided by the dl1137 (and T147D) protein may be sufficient to override the effect of the absence of amino acids in this region (amino acids 128-175).

In my studies I have found that neither one of the mutants with deletions in their amino-terminus (dl1135, T128-176 and T176-708) were able to immortalise secondary REFs. In contrast, it has been shown that this amino-terminal region of T antigen is neither required (Thompson et al., 1990; Zhu et al., 1991a), nor alone is sufficient (Tevethia et al., 1988), for the immortalisation of C57BL/6 mouse embryo fibroblasts. Indeed, studies in mouse embryo fibroblasts (MEFs) have mapped immortalisation to regions within the carboxy-terminal half of the protein and these regions correlate strongly with the ability to bind to p53 (Zhu et al., 1991a; Kierstead and Tevethia, 1993). The difference between the results obtained with these mutants in the immortalisation of rat and mouse embryo fibroblasts suggest that the inactivation of p53 by T antigen may play a greater role in the immortalisation of MEFs while amino-terminus functions are required for the immortalisation of REFs. These results are in sharp contrast with the results of my own (mutants dl1137 and T147D), and other researcher's (Colby and Shenk, 1982; Asselin and Bastin, 1985; Sompayrac and Danna, 1991), studies which indicate that amino-terminal fragments of T antigen can immortalise primary or secondary rat embryo fibroblasts. Interestingly mutant dl1061, which encodes a larger amino-terminal fragment (amino acids 1 to 590) was relatively efficient at immortalising secondary REFs despite its inability to bind p53, suggesting that another function, between amino acids 147-590, may improve the immortalisation efficiency of T antigen. This function may correspond to the function carried by region X defined by Dobbelstein et al. (1992). Early studies with mutants dl1137 and dl1061 suggested they are unable to transform REF52 cells (Pipas, Peden and Nathans, 1983),
however recently mutant dl1137 as well as mutant T147D have been shown to be capable of transforming REF52 but only if they are over expressed from a strong promoter (Sompayrac and Danna, 1992). All three of these mutants, dl1137, T147D and dl1061 are clearly able to transform mouse C3H10T1/2 cells (Sompayrac and Danna, 1988; Srinivasan, Peden and Pipas, 1989 & J.M.Pipas, unpublished data). Mutational analysis of large T antigen carried out by Cole and colleagues (Zhu et al., 1992) has confirmed that transformation of C3H10T1/2 is abrogated by a deletion in the amino-terminus (amino acids 5 to 35) while all other mutants tested were able to transform this cell line. In contrast, transformation of REF52 cells relied upon the integrity of this region and two other regions (mapped by mutations between amino acids 408 to 519 and 587 to 589). Both T147D and dl1061 are also able to transform non-established REFs in cooperation with activated ras albeit at a decreased efficiency compared to wild type T antigen (Michalovitz et al., 1987; Cavender et al., 1995). In contrast to the immortalisation of REFs, dl1137, T147D and dl1061 are not able to maintain immortalisation in tsa14 cells at the non-permissive temperature. This raises the following question; if these mutants are unable to maintain growth in a T antigen immortalised cell line, how are they able to immortalise REFs? There are several possible explanations for this:

[1] It is possible, since these mutants immortalise with lower efficiencies than wild type, that they only confer the immortal phenotype on a sub-population of cells in the heterogeneous REF population which already carry an appropriate predisposition to immortalisation by these mutants.

[2] It is possible that these cell lines may have acquired cellular mutations which cooperate with the functions carried by the amino-terminus of T antigen for their establishment. Karyotypic changes are certainly observed when mouse cells are passaged in culture (Todaro and Green, 1963) and the transfer of immortalising oncogenes into rat fibroblasts has been suggested to induce both high rates of sister chromatid exchange and the appearance of abnormal karyotypes (Cerni, Mougneau and Cuzin, 1987).

[3] T antigen may inactivate p53 through mechanisms other than direct binding; T antigen has the ability to induce cellular processes which result in the stabilisation of p53 in the absence of direct T antigen-p53 binding, but the location of these functions within the T antigen molecule has not yet been determined (Deppert, Steinmayer and Richter, 1989; Tiemann and Deppert, 1994).

Evidence for the hypothesis that these amino-terminal fragments of T antigen may selectively immortalise a sub-population of cells has been provided by the demonstration that mutant dl1137 is able to induce choroid plexus tumours in transgenic mice but no lymphomas were detected even though expression of this mutant was targeted to both the choroid plexus and B- and T-lymphoid cells (Chen et al., 1992).
Chapter 3. The possibility that these mutants may immortalise in the context of a cellular mutation, such as the mutation of p53, is suggested by the observation that when dll1137 transgenic mice were bred with p53-null mice, the choroid plexus tumours developed more rapidly (Symonds et al., 1994). Therefore, further work has been initiated to determine the status of p53 in these cell lines; evidence so far suggests that the p53 protein is stabilised in some, but not all, of the dll1137 cell lines.

3.3.2: Requirement for the carboxy-terminus of T antigen in immortalisation.

I have identified two regions within the carboxy-terminus which are required both for T antigen's ability to immortalise secondary REFs and to maintain immortalisation in tsa14 cells. The first of these is indicated by the substitution of amino acid 584 in mutant 5080. This residue lies within one of two hydrophobic stretches (amino acids 571 to 589 and 619 to 627) which are required for stability of T antigen's tertiary structure. When these amino acids are altered, in the context of the whole protein, T antigen becomes defective for p53 binding, oligomerisation, phosphorylation, ATPase activity, immortalisation of C57BL/6 MEFs and transformation of REF52 (Cole et al., 1986; Tevethia et al., 1988; Peden et al., 1989; Tack et al., 1989; Zhu et al., 1991a; Zhu et al., 1992). The amino acid substitution at position 584 results in a T antigen defective for binding both p53 and pRB although the amino acid sequences responsible for these interactions remain intact (Peden et al., 1989; Chen et al., 1992).

The second region in the carboxy-terminus which is required both for efficient REF immortalisation and tsa14 complementation appears to be colinear with the p53 binding domain. Both mutants 5031 and 5041, which fail to bind to p53, were unable to immortalise REFs or complement the growth defect of tsa14 at the non-permissive temperature. In contrast mutants 5030 and 5061, which retain the ability to bind to p53, were both able to immortalise REFs and complement tsa14. However, association with p53 cannot be the sole requirement for either REF immortalisation or tsa14 complementation since mutant T176-708 retains the ability to bind p53 but is inactive in both assays. Moreover, Ad E1A and HPV E7 are both capable of complementing tsa14 growth but neither bind p53 (Jat and Sharp, 1989; Vousden et al., 1993) whereas Ad E1B and HPV 16 E6 both interact directly with p53 but are unable to maintain the growth of tsa14 cells. Interestingly, it has been demonstrated that both E1A and E7 are able to overcome p53-induced growth arrest in a REF cell line conditionally transformed with a temperature sensitive p53 mutant plus activated ras, suggesting that they are able to inactivate p53 through mechanisms other than direct binding or through mechanisms which act downstream of p53 (Vousden et al., 1993). dll1137 is also able to overcome the p53-induced growth arrest of a similar cell line (Quartin et al., 1994), suggesting that it is another function carried by E1A and E7, but lacking in dll1137 which is required to
Chapter 3.

maintain tsal4 growth. Alternatively, it is possible that there is another function of T antigen colinear with the p53 binding domain which is involved in the ability of T antigen to stimulate growth. Recently the existence of another function within this region has been suggested by a single amino acid substitution in this region which does not affect T antigen's interaction with p53 but negates its ability to transform REFs in cooperation with activated ras (Cavender et al., 1995).

It is interesting that T antigen mutants dl1137, T147D and dl1061 lacking the carboxy-terminal amino acids are able to immortalise secondary REFs whereas mutants with point mutations in this region (5031 and 5041) are inactive. This is surprising since both of these mutants have the amino-terminal 121 amino acids intact, which are sufficient for the smallest protein (encoded by the dl1137 mutant) to immortalise. It is possible that, in addition to disrupting T antigen's ability to complex p53, the amino acid substitutions carried by mutants 5031 and 5041 may be perturbing activities carried by the amino-terminus of the protein. This has certainly been demonstrated to be the case with mutant 5080, where one amino acid substitution, in a region necessary for the stability of the protein's tertiary structure, affects a large number of the protein's functions. This possibility is further supported by the observation that mutants dl1137, T147D and dl1061 are able to supply the amino-terminal functions which mutant T176-708 lacks to overcome the growth defect of tsal4, whereas mutants 5031 and 5041 lack this ability. Complementation of tsal4 growth by two cooperating mutants appears to rely on selective pressure for the both mutant proteins to be expressed, since all except one (dl1061+T176-708) of the cell lines from these cotransfections, which were isolated and maintained at the permissive temperature, were unable to grow when shifted to the non-permissive temperature. The inability of these tsal4 cell lines to grow at the non-permissive temperature seemed to correlate with loss of expression of one or both of the mutant proteins. In contrast, most of the cell lines examined which were selected directly at the non-permissive temperature following cotransfection of two mutants retained expression of both mutant proteins.

In the immortalisation of the heterogeneous population of secondary REFs however, mutants 5031 and 5041 are capable of trans-complementing both mutants T176-708 and dl1135 (which lacks amino acids 17-27) albeit at a lower efficiency than wild type. This appears to represent an anomaly in the data since, if the amino acid substitutions in mutants 5031 and 5041 are affecting an amino-terminal function, these would not be expected to immortalise in cooperation with a mutant lacking the entire amino-terminus, T176-708. One way to rationalise this data is the possibility that a number of activities are involved. The first would be lacking in T176-708 and dl1135 for immortalisation of REFs but clearly present in 5031 and 5041; the second activity corresponds to the inactivation of p53 which is lacking in dl1137, T147D and dl1061 as well as 5031 and 5041 and is demonstrated by the inability of these proteins to maintain
the proliferation of tsal4 cells; and the third corresponds to functions present in \( d/1137, \) T147D and \( dl/1061 \) but missing in 5031 and 5041 as assayed by their differing abilities to immortalise REFs. Mutants \( dl/1137, \) T147D and \( dl/1061 \) are only deficient in the second activity and thus can be readily complemented in the tsal4 growth assay by the action of T176-708 which can bind p53. It seems that the amino-terminal function required for complementation of the T176-708 mutant, which can be provided by \( dl/1137, \) T147D and \( dl/1061 \) but is lacking in 5031 and 5041, may play a greater role in the maintenance of the tsal4 cell line than in the immortalisation of the heterogeneous REF population. It is possible that in tsal4 cells, this activity may be inactivated in the full length 5031 and 5041 mutant proteins due to the presence of regions lacking in \( dl/1137, \) T147D and \( dl/1061 \). The effect of the intact regions for specific (amino acids 131-259) and non-specific DNA binding (amino acids 277-362) or for binding other proteins (for example DNA polymerase \( \alpha \)) may be to inactivate this amino-terminus function, possibly by sequestering the 5031 or 5041 protein. These effects may be overcome in secondary REF cells, possibly by the selection for a higher level of expression of these T antigen mutants. Alternatively, the coexpression of three types of full, or almost full, length T antigen mutants (tsA58, T176-708 and 5031 or 5041) each of which are capable of the majority of the functions mapping between amino acids 176-708 on T antigen, may result in other interference within the tsal4 cell line. It is clear, however, that some of the functions required for both maintenance of growth in tsal4 cells and immortalisation of REFs can be provided \textit{in trans} by separate T antigen molecules.

3.3.3: The requirements for rat and mouse fibroblast immortalisation by T appear to be different.

In contrast with data obtained from C57BL/6 mouse embryo fibroblast immortalisation assays, I have demonstrated that the carboxy-terminus (amino acids 128-708) of T antigen is insufficient to immortalise rat embryo fibroblasts. In fact, small deletions within the amino-terminus (amino acids 17-27) destroy T antigen's ability to immortalise REFs, whereas amino-terminal fragments of T antigen as small as amino acids 1-121 are sufficient to immortalise REFs, albeit inefficiently. Primary mouse embryo fibroblasts are unable to be immortalised by similar amino-terminal fragments of T antigen (Tevethia \textit{et al.}, 1988) while carboxy-terminal fragments of T antigen (amino acids 128-708) retain the ability to immortalise (Thompson \textit{et al.}, 1990). Mutations within two regions of T antigen (amino acids 351 to 450 and 533 to 626, which are carried within the carboxy-terminal mutant) resulted in loss of ability to immortalise mouse embryo fibroblasts (Kierstead and Tevethia, 1993). Taken together these data suggest that T antigen carries two domains which can act separately to immortalise, one of which has a greater role in the immortalisation of rat embryo fibroblasts, while the other has a greater involvement in mouse embryo fibroblast
immortalisation. This is further supported by the fact that, while mutant dl1135 is unable to immortalise rat embryo fibroblasts, a similar mutant with amino acids 5-35 deleted is able to immortalise mouse embryo fibroblasts (Zhu et al., 1991a). In the context of the tertiary structure of full length T antigen, however, mutations within the carboxy-terminus (mutants 5031, 5041 and 5080) affect its ability to immortalise rat embryo fibroblasts. Recently both of these domains, the amino-terminal fragment (amino acids 1-147) and the carboxy-terminal fragment (amino acids 251-708), have been shown to transform rat embryo fibroblasts in cooperation with ras (Cavender et al., 1995). In the context of full length T antigen, however, mutant dl1135, which encodes a protein with amino acids 17-27 deleted and therefore has an intact carboxy-terminus, has been shown to be unable to cooperate with ras to transform baby rat kidney cells (Yacuik et al., 1991). This lends further support to the hypothesis that the tertiary structure of full length T antigen influences the functions of these individual domains. It remains to be seen whether smaller mutations like those carried by 5031 and 5041 affect the ability of the amino-terminal region to transform REFs in cooperation with ras. Two mutants with larger deletions within this area (deleted amino acids 341 to 449 or 271 to 416) retain the ability to transform in cooperation with ras but only at approximately 5% the efficiency of wild type T antigen (Michalovitz et al., 1987; as compared to the 26-65% obtained with amino acids 1 to 147 alone by Cavender et al., 1995).

3.3.4: Summary.

I have demonstrated that several regions of T antigen are required, to a greater or lesser extent, for immortalisation. The first of these is located within the amino terminal 82 amino acids common to both large T and small t antigens and appears to be required only for the initiation of immortalisation in REFs and not for the subsequent maintenance of immortalisation in the established cell line. This region includes the amino acids necessary for T antigen's ability to complement the p300-associated transforming function of adenovirus E1A (CR1-like region; Yacuik et al., 1991). The second region that I have identified encompasses the amino acids immediately downstream of the CR2-like region as far as amino acid 175. The third region is colinear with the p53 binding region while the fourth region corresponds to the hydrophobic region of T antigen, mutations in which have previously been suggested to perturb the tertiary structure of the protein (Tack et al., 1989).

The functions carried by some of the T antigen regions identified remain to be clearly defined. The multifunctional nature of T antigen has made it difficult to assign particular functions to particular regions since one region may be responsible for more than one activity. In addition to the well defined interactions with p53, pRB, p107 and p130, T antigen also binds DNA polymerase α through amino acids 1 to 82 and 271 to 517 (Smale and Tjian, 1986; Gannon and Lane, 1987; Dornreiter et al., 1990), heat shock protein-73 through amino acids 1 to 97 (Sawai and Butel, 1989; Sawai,
Rasmussen and Butel, 1994), p185 through amino acids 2 to 108 exclusive of amino acids 17 to 27 (Kohrmann and Imperiale, 1992), and AP-2 through an unknown region (Mitchell, Wang and Tjian, 1987). T antigen is also a potent transcriptional activator of both viral and cellular promoters (Loeken, Kourouy and Brady, 1986; Robbins, Rio and Botchan, 1986; Rice and Cole, 1993). Transactivation by T antigen does not require DNA binding but possibly occurs through direct protein-protein interactions (Brady and Khoury, 1985; Gallo et al., 1990; Zhu et al., 1991b). In vitro T antigen has been shown to bind directly two proteins involved in its transcriptional activation functions, transcription-enhancing factor 1 (TEF-1) through amino acids 5 to 383 and TATA-binding protein (TBP) through amino acids 5 to 172 (Gruda et al., 1993). T antigen's ability to bind DNA and ATP, as well as its ATPase and helicase activities, have previously been shown not to be involved in any of its growth stimulatory effects (Kalderon and Smith, 1984; Zhu et al., 1991a; Zhu et al., 1992). Future work will hopefully help to clarify the functions of each region I have identified as necessary for the immortalisation of rat embryo fibroblasts or for the maintenance of growth in the established tsal4 cell line. It will be intriguing to see whether these functions can explain the differences in the requirements for immortalisation between rat and mouse primary cells.
Chapter 4.

Initiation and maintenance of immortalisation by SV40 large T antigen are functionally separable.

4.1: Introduction.

One of the most interesting results to come from my studies on the ability of various T antigen mutants to either maintain growth in tsa14 cells or immortalise secondary rat embryo fibroblasts was the result obtained with mutant dl1135. This T antigen mutant was clearly capable of maintaining immortalisation in the tsa14 cell line however was unable to immortalise secondary rat embryo fibroblasts. The dl1135 protein appears therefore to carry all the functions required to maintain immortalisation in an established cell line (when that cell line is shifted to conditions where it would otherwise cease dividing); however it lacks one or more of the functions which are required for the immortalisation of secondary REFs. These data suggest that there is a function carried by T antigen, which dl1135 lacks, that is required to initiate immortalisation but is not required to maintain it. I have presented data in Chapter 3 which shows that this "initiation" function, which is destroyed by the deletion of amino acids 17-27 in dl1135, can be trans-complemented by cotransfection of two T antigen mutants with amino acids 17-27 intact (mutants 5041 and 5031).

To investigate further the role of these mutants in this complementation I have constructed double mutants by introducing the tsA58 point mutation into each of these mutants (dl1135, 5041 and 5031). I carried out REF immortalisation assays at the permissive and non-permissive temperature for tsA58 following the cotransfection of one non-temperature sensitive (single) mutant and one temperature sensitive double mutant. The requirement for both mutants in this trans-complementation can therefore be demonstrated by a decrease in these mutant's ability to form colonies at the non-permissive temperature compared to the permissive temperature. Colonies isolated at the permissive temperature and successfully expanded into cell lines can then be assayed for their continued dependence on either mutant protein by selectively inactivating one or other of the mutant proteins through shifting the cell lines to the non-permissive temperature for the protein containing the tsA58 point mutation.
Chapter 4.

In this chapter I describe the construction of recombinant plasmids able to express the temperature sensitive double mutants and the results of the immortalisation assays outlined above. I go on to describe my attempts to characterise the integrity of the mutant proteins in the resulting immortal cell lines.

4.2: Results.

4.2.1: Construction of temperature sensitive double mutants of T antigen.

The recombinants encoding the $d_{11}135$, 5031 and 5041 mutant proteins have the SV40 genome (bearing their respective mutations) linearised via its BamHI site (at SV40 nucleotide position 2533) cloned into the BamHI site of pBR322 (Pipas, Peden and Nathans, 1983, and J.M.Pipas, Department of Biological Sciences, University of Pittsburgh, unpublished data). Recombinants encoding the $d_{11}135$, 5031 and 5041 $t_{s}A_{58}$ double mutations were constructed, using carefully selected restriction sites, by ligating fragments containing the $d_{11}135$, 5031 and 5041 mutations into the T antigen open reading frame of a plasmid able to express the $t_{s}A_{58}$ T antigen (pUC19$t_{s}A_{58}$).

pUC19$t_{s}A_{58}$ contains the KpnI (SV40 nucleotide position 294) to BamHI (SV40 nucleotide position 2533) fragment of the SV40 $t_{s}A_{58}$ genome cloned into the KpnI and BamHI sites of pUC19. This fragment of the SV40 genome encompasses the SV40 enhancer, the large T and small t antigen open reading frames and the SV40 polyadenylation signal.

The double mutants were constructed by replacing the SfiI (SV40 nucleotide position 5234) to PvuII (SV40 nucleotide position 3506) fragment of the pUC19$t_{s}A_{58}$ with the same fragment from the three T antigen mutants, which encompasses the mutated nucleotides of all three mutants. A map of the resulting double mutants is shown in Figure 4.1, with the restriction sites used for their construction highlighted in bold type. Due to the presence of a number of PvuII sites within the pUC19$t_{s}A_{58}$ recombinant this plasmid was first restriction digested with SfiI and BamHI and both of the resulting fragments (2978bp and 2701bp) were isolated and purified. The 2701bp SfiI-BamHI fragment which encompasses the T antigen open reading frame was then digested with PvuII and the resulting 973bp fragment was isolated and purified. This 973bp PvuII-BamHI fragment contains the 3' end of the large T antigen reading frame including the $t_{s}A_{58}$ point mutation. The $d_{11}1135$, 5031 and 5041 recombinants were restriction digested with SfiI and PvuII and the 1728bp fragment, which contains the 5' end of the large T antigen reading frame and the point mutations or deletions of the three mutants, was isolated and purified. The recombinants encoding the double mutants were subsequently constructed by a three part ligation of the 1728bp SfiI-PvuII fragment (containing the $d_{11}1135$/5031/5041 mutations), the 973bp PvuII-BamHI fragment (containing the $t_{s}A_{58}$ mutation) and the 2978bp BamHI-SfiI fragment (containing the
Figure 4.1: Construction of temperature sensitive double mutants.

The temperature sensitive double mutants were constructed through the ligation of the three restriction fragments shown in the map above. A more detailed description of the construction is given in the text. The restriction sites used to assemble the double mutants are shown in bold. The sequence surrounding the mutations in the T antigen reading frame is shown in Figure 4.2.
Figure 4.2: Sequence of T antigen mutations.

A: Sequence of T antigen mutants in region of \( d/1135 \) deletion.

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGG CAG CTA ACG GAC CTT CTA GGT CTT GAA AGG AGT GGC TGG GGG AAT ATT</td>
<td>Leu Gin Leu Met Asp Ser Ala Trp Gly Asn Ile Pro Leu Met Arg Gly Tyr</td>
</tr>
<tr>
<td>TGG CAG CTA ACG GAC CTT CTA GGT CTT GAA AGG AGT GGC TGG GGG AAT ATT</td>
<td>Leu Gin Leu Met Asp Ser Ala Trp Gly Asn Ile Pro Leu Met Arg Gly Tyr</td>
</tr>
</tbody>
</table>

DNA sequence and encoded amino acids of the temperature sensitive double mutants (\( ts1135, ts5031 \) and \( ts5041 \)), surrounding the sites of the mutations, aligned with the DNA and amino acid sequence of wild-type T antigen and the \( tsA58 \) mutant. Mutated nucleotides and resulting amino acid substitutions are shown in bold. Only substituted amino acids are shown; --- indicates the amino acid encoded is the same as the wild-type sequence. The broad bars in figure A show the nucleotides and amino acids deleted in mutant \( 1135 \). The position of the \( PvuII \) site used to construct the temperature sensitive double mutants is shown (B). The figures above each line represent the SV40 nucleotide numbers (SV40 numbering system, Tooze, 1981). The figures below each line represent the amino acid number.

B: Sequence of T antigen mutants in region of \( tsA58, 5041 \) and 5031 point mutations and \( PvuII \) site.

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGG CCA AAA ATG GAT TCA GGT TAT GAC TTT TTA AAA TCG ACG GCT GTT TTG CTT GAA TTA TGT GGG GGG</td>
<td>Trp Leu Phe Lys Gly Pro Leu Asp Ser Gly Lys Thr Thr Leu Ala Ala Leu Leu Glu Leu Cys Gly</td>
</tr>
<tr>
<td>TGG CCA AAA ATG GAT TCA GGT TAT GAC TTT TTA AAA TCG ACG GCT GTT TTG CTT GAA TTA TGT GGG GGG</td>
<td>Trp Leu Phe Lys Gly Pro Leu Asp Ser Gly Lys Thr Thr Leu Ala Ala Leu Leu Glu Leu Cys Gly</td>
</tr>
</tbody>
</table>

DNA sequence and encoded amino acids of the temperature sensitive double mutants (\( ts1135, ts5031 \) and \( ts5041 \)), surrounding the sites of the mutations, aligned with the DNA and amino acid sequence of wild-type T antigen and the \( tsA58 \) mutant. Mutated nucleotides and resulting amino acid substitutions are shown in bold. Only substituted amino acids are shown; --- indicates the amino acid encoded is the same as the wild-type sequence. The broad bars in figure A show the nucleotides and amino acids deleted in mutant \( d/1135 \). The position of the \( PvuII \) site used to construct the temperature sensitive double mutants is shown (B). The figures above each line represent the SV40 nucleotide numbers (SV40 numbering system, Tooze, 1981). The figures below each line represent the amino acid number.

The ligations were transformed into \( E.coli \) strain JS4 and the resulting recombinants were verified by restriction mapping and DNA sequencing. The DNA sequence and position of the amino acid substitutions or deletions of the resulting double mutants (named \( ts1135, ts5031 \) and \( ts5041 \)) are shown in Figure 4.2A (around the position of the \( d/1135 \) deletion) and B (around the position of the 5031, 5041 and \( tsA58 \).
point mutations). The position of the *PvuII* site, fortunately just upstream of the *tsA58* point mutation, is also marked.

### 4.2.2: Ability of cotransfected mutants to immortalise secondary REF at the permissive and non-permissive temperature.

Cotransfections of *dl1135* with either 5041 or 5031 were previously shown to cooperate to immortalise REFs (Chapter 3, Table 3.4). To determine the requirement of each mutant in immortalisation by *trans*-complementation, secondary REFs were cotransfected with *dl1135* plus either 5041 or 5031 mutants using a combination of one temperature sensitive double mutant and one non-temperature sensitive mutant. The transfected cells were then assayed for their ability to form colonies at either the permissive temperature (33.0°C) or non-permissive temperature (39.5°C) for the *tsA58* mutation. The data from four such cotransfections is presented in Table 4.1. The number of densely staining colonies formed at the non-permissive temperature and permissive temperature from each transfection is shown along with the percentage efficiency of colony formation at the non-permissive temperature compared to the permissive temperature (percentage efficiency of colony formation 39.5°C/33.0°C). The average percentage efficiency of colony formation at the non-permissive temperature compared to the permissive temperature is also shown, along with the number of cell lines successfully expanded at the permissive temperature. It is important to remember that the number of colonies obtained by cotransfection of two T antigen mutants is generally 10-fold decreased compared to the number of colonies obtained with wild type T antigen and the numbers presented in Table 4.1 show the relative efficiency of colony formation for each combination at the non-permissive compared to the permissive temperature, not the immortalisation efficiency compared to wild type T antigen. Within experiments there was a degree of fluctuation between transfection efficiency and possibly even a difference in the efficiency of Geneticin-G418 to select for G418-resistant transfected cells at the non-permissive temperature compared to the permissive temperature. It is therefore important to compare the average efficiencies of colony formation at 39.5°C compared to 33.0°C for the combinations of mutants with those obtained with wild type T antigen, *tsA58* and the combinations of non-temperature sensitive mutants.

Wild type T antigen yielded an overall average of 77.6% the number of colonies at the non-permissive temperature compared to the permissive temperature for the four experiments. All 12 colonies isolated at the permissive temperature readily yielded cell lines. Control transfections with only pSV2neo plus carrier DNA (pKS) yielded only a small number of colonies at either the permissive or non-permissive temperature. It is interesting to note that consistently more colonies were obtained at the non-permissive temperature than at the permissive temperature with these control transfections. This
Table 4.1: Immortalisation of secondary REFs by trans-complementation between ts and non-ts T antigen mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Number of densely staining colonies 39.5°C/33.0°C</th>
<th>39.5°C/33.0°C average percentage efficiency of colony formation 39.5°C/33.0°C</th>
<th>no. of cell lines derived from no. of colonies isolated 39.5°C/33.0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>expt. 1 (%)</td>
<td>expt. 2 (%)</td>
<td>expt. 3 (%)</td>
</tr>
<tr>
<td>SE (wild-type)</td>
<td>918/768 (119.5%)</td>
<td>230/610 (37.7%)</td>
<td>104/145 (71.7%)</td>
</tr>
<tr>
<td>pKS</td>
<td>4/0 (-)</td>
<td>7/2 (-)</td>
<td>8/0 (-)</td>
</tr>
<tr>
<td>pUC19ts458</td>
<td>283/718 (39.4%)</td>
<td>302/928 (32.5%)</td>
<td>234/603 (38.8%)</td>
</tr>
<tr>
<td>ts1135</td>
<td>6/64 (9.4%)</td>
<td>17/82 (20.7%)</td>
<td>-</td>
</tr>
<tr>
<td>ts5041</td>
<td>6/7 (85.7%)</td>
<td>8/9 (88.9%)</td>
<td>-</td>
</tr>
<tr>
<td>ts5031</td>
<td>0/0 (-)</td>
<td>1/3 (33.3%)</td>
<td>-</td>
</tr>
<tr>
<td>d1135</td>
<td>-</td>
<td>-</td>
<td>15/6 (250.0%)</td>
</tr>
<tr>
<td>5041</td>
<td>-</td>
<td>-</td>
<td>10/0 (-)</td>
</tr>
<tr>
<td>5031</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>d1135 + 5041</td>
<td>-</td>
<td>32/20 (160.0%)</td>
<td>19/20 (95.0%)</td>
</tr>
<tr>
<td>d1135 + 5031</td>
<td>-</td>
<td>41/116 (24.7%)</td>
<td>33/142 (23.2%)</td>
</tr>
<tr>
<td>ts1135 + 5041</td>
<td>3/43 (7.0%)</td>
<td>25/155 (16.1%)</td>
<td>24/116 (20.7%)</td>
</tr>
<tr>
<td>ts1135 + 5031</td>
<td>43/232 (18.5%)</td>
<td>78/254 (30.7%)</td>
<td>7/132 (5.3%)</td>
</tr>
<tr>
<td>d1135 + ts5041</td>
<td>25/153 (16.3%)</td>
<td>16/25 (64.0%)</td>
<td>12/32 (37.5%)</td>
</tr>
<tr>
<td>d1135 + ts5031</td>
<td>0/2 (0.0%)</td>
<td>7/12 (58.3%)</td>
<td>0/3 (0.0%)</td>
</tr>
</tbody>
</table>

Secondary rat embryo fibroblasts were cotransfected with the indicated T antigen mutants along with pSV2neo as a co-selectable marker. 48 hours after transfection, the cells were split onto 4 plates, two of which were incubated at the permissive temperature for tsA5S (33.0°C) and two at the non-permissive temperature (39.5°C) for three to four weeks. Representative colonies were isolated at 33.0°C and expanded into cell lines wherever possible. The plates were then stained with methylene blue and the remaining colonies counted. The results from four independent experiments are shown in the table, along with the average efficiency of colony formation at 33.0°C compared to 39.5°C and the number of cell lines successfully established compared to the number of colonies isolated. N.D. = not done. ⁰Experiment was only carried out once.
may reflect the ability of the early passage fibroblasts to divide faster and undergo more divisions at the non-permissive temperature compared to the permissive temperature (Ikram, Norton and Jat, 1994), thereby giving them a greater capacity to acquire cellular mutations.

The tsA58 recombinant (pUC19tsA58) yielded colonies at a lower efficiency at the non-permissive temperature than at the permissive temperature (34.6% 39.5°C/33.0°C). This result gives a representation of the conditionality of T antigen protein containing the tsA58 mutation. All 30 colonies isolated at the permissive temperature following transfection of tsA58 were successfully expanded into cell lines. Transfection of the ts5041, ts5031, dl1135, 5041 or 5031 mutants alone failed to yield many colonies at either the permissive or the non-permissive temperature (between 0.0-0.6% the number of colonies obtained with wild type T antigen). The one ts5041 colony isolated failed to yield a cell line. Transfection of the ts1135 mutant alone yielded more colonies than the other single mutant transfections (with more at the permissive temperature than the non-permissive temperature, but only approximately 5% the number obtained with wild type T antigen), however only one of the 14 colonies isolated at the permissive temperature yielded a cell line.

Cotransfection of the non-temperature sensitive combination of mutants (dl1135 plus either 5041 or 5031) serves to determine whether the temperature difference has any effect on colony formation by trans-complementation and whether any one of these three original single mutants are temperature sensitive. The combination of dl1135 with 5041 appeared to be as efficient at immortalising REFs at the non-permissive temperature as at the permissive temperature yielding an average of 95.8% the number of colonies at 39.5°C compared to 33.0°C. This relative colony formation efficiency was higher than that obtained with wild type T antigen, thus indicating that neither mutant dl1135 nor mutant 5041 are temperature sensitive in their own right. In contrast, the combination of dl1135 and 5031 yielded fewer colonies at the non-permissive temperature giving an average efficiency of colony formation of only 28.3% compared to the permissive temperature. This relative immortalisation efficiency was lower than that obtained with the tsA58 mutant suggesting that the 5031 mutant protein may be somewhat temperature sensitive.

In transfections where one of the cotransfected mutants carries the tsA58 mutation the mutant protein expressed should have been rapidly denatured and degraded at the non-permissive temperature. All combinations incorporating a temperature sensitive T antigen allele (ts1135 plus 5041 or 5031 and dl1135 plus ts5041 or ts5031) yielded fewer colonies at the non-permissive temperature compared to the permissive temperature. Combinations of ts1135+5041, ts1135+5031 and dl1135+ts5031 yielded colonies at the non-permissive temperature at a lower relative 39.5°C/33.0°C efficiency than the tsA58 mutant alone (14.3%, 16.7% and 22.5% respectively compared to 34.6%
for tsA58). The dl1135+ts5041 combination yielded colonies at the non-permissive temperature at a slightly higher efficiency relative to the permissive temperature than the tsA58 mutant (44.4% compared to 34.6%), but still not as high as either the non-temperature sensitive combination of dl1135+5041 (95.8%) or wild type T antigen (77.6%). A proportion of the colonies isolated at the permissive temperature from each of the combination transfections could be expanded into cell lines.

The observation of a temperature sensitive colony formation phenotype by these cooperating mutants indicates that the introduction of the tsA58 point mutation into these proteins has successfully caused the proteins to become thermolabile. It also confirms that the presence of both mutant proteins was required for the immortalisation of rat embryo fibroblasts by cooperating T antigen mutants. This result is the expected one since transfection of any one of these three mutants alone failed to immortalise REFs (Chapter 3, Table 3.4).

4.2.3. The amino-terminal function is not required for the maintenance of the immortal phenotype once cell lines are established.

Following the isolation of cell lines immortalised by the trans-complementing mutants I determined whether both T antigen mutants were continuously required for the maintenance of immortalisation. In each of the cell lines successfully established at the permissive temperature one or other of the mutant proteins could be selectively inactivated by shifting the cell lines to the non-permissive temperature.

The growth of these cell lines in the presence of both mutants (at the permissive temperature for tsA58) or just one of the mutants (at the non-permissive temperature) was assayed in two ways. The first method involved determining the number of colonies formed at the non-permissive temperature (39.5°C) compared to the permissive temperature (33.0°C) following the seeding of 1000 cells on duplicate plates at the permissive temperature and incubation at either temperature. The results of these colony assays (expressed as percentage number of colonies obtained at 39.5°C compared to 33.0°C) are shown in Figures 4.3 (dl1135+5041 cell lines) and 4.4 (dl1135+5031 cell lines). The results for the tsA58 cell lines are shown in both figures to aid comparison between graphs. The data from the colony assays were confirmed by a second growth assay using representative cell lines isolated from each cotransfection. This method involved plating the same number of cells (10,000) on duplicate dishes and determining the fold increase in cell number, compared to the number of cells counted on the day the cells were shifted to the non-permissive temperature (the day after plating), obtained after 7 days and 14 days at either the permissive or non-permissive temperature. The results of these cell number assays for representative cell lines are shown in Figures 4.5 (dl1135+5041 cell lines) and 4.6 (dl1135+5031 cell lines). Again the results for the tsA58 cell lines are shown in both figures to aid comparison between graphs.
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The colony assays carried out on six cell lines isolated at the permissive temperature following transfection of the recombinant expressing wild type T antigen (SE) are shown in Figure 4.3A. All six of the cell lines assayed grew well when shifted to the higher temperature, the lowest percentage number of colonies obtained at the non-permissive temperature being 77.4% compared to the permissive temperature (cell line 3).

The majority of cell lines isolated at 33.0°C following cotransfection of the non-temperature sensitive combination of mutants, dl1135+5041 (Figure 4.3B) and dl1135+5031 (Figure 4.4A) yielded a similar number of colonies at both temperatures, indicating that the temperature shift did not unduly effect the growth of these cell lines. All except one of the dl1135+5041 cell lines yielded as many, or more, colonies at 39.5°C as at 33.0°C and the one cell line (line 6) which did not grow so well still yielded 63.9% of the number of colonies at 33.0°C at 39.5°C. The results obtained by colony assay for five of these cell lines were confirmed by the cell number assays, the results of which are shown in Figure 4.5B. Although two of the five cell lines selected (cell lines 2 and 5) grew at decreased rates at both temperatures compared to the other three (cell lines 1, 3 and 4), all five cell lines grew better (cell lines 1, 4 and 5), or almost as well (cell lines 2 and 3), at 39.5°C compared to 33.0°C. Twelve of the sixteen dl1135+5031 cell lines grew as well at the non-permissive temperature compared to the permissive temperature (Figure 4.4A) and the results for three of these cell lines were confirmed by the cell number assays shown in Figure 4.6B (cell lines 7, 9 and 11). The fourth cell line (cell line 10) gave approximately a 50% decrease in growth at 39.5°C when assayed by cell number compared to the 103.5% efficiency of colony formation at 39.5°C compared to 33.0°C given by the colony assay. This cell line grew very slowly and this may explain the difference between the results obtained with this line. Four dl1135+5031 cell lines (lines 1, 6, 8 and 13) gave decreased efficiencies of colony formation at 39.5°C. Since, in the original REF immortalisation experiments (Table 3.4) carried out with 5031, 3 of the 26 colonies isolated yielded cell lines, it is possible that these cell lines only express 5031 and that this reflects the temperature sensitivity of the 5031 mutant which was observed in the immortalisation data obtained at the permissive and non-permissive temperatures (Table 4.1). Further analysis is necessary to investigate this possibility.

The results obtained when the colony assays were carried out on the tsA58 cell lines isolated at the permissive temperature are shown in duplicate in Figures 4.3C and 4.4B. The 30 cell lines showed varying degrees of conditionality, with only 12 of the 30 yielding less than 50% the number of colonies obtained at the permissive temperature at the non-permissive temperature and only 2 cell lines yielding less than 20% (cell line 2 yielded 8.4% and cell line 16 yielded 14.1%). Most of the cell lines showed some degree of conditionality with 19 of the 30 giving less than 77.4% conditionality (77.4%
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is the lowest result obtained by the same assay on the wild type T antigen cell lines). The results of cell number assays on seven of these tsA58 cell lines are shown in Figure 4.5A (and duplicated in Figure 4.6A). The growth data obtained by cell number assay shows that six of the seven tsA58 cell lines assayed grew at a greatly reduced rate at the non-permissive temperature. Despite differences in the growth rates of these cell lines at the permissive temperature, the results obtained by this assay confirm the results obtained by the colony assays; all of the cell lines assayed showed varying degrees of conditionality with the exception of cell line 6 which grew almost as well at 39.5°C as at 33.0°C. The contrast between these results and those obtained with the dl1135+5041 (Fig. 4.5B) and dl1135+5031 (Fig. 4.6B) cell lines, where a number of the cell lines assayed actually grew better at 39.5°C than at 33.0°C, should be noted. In general, cells incubated at 39.5°C usually grow better than cells at 33.0°C (Ikram, Norton and Jat, 1994), however the majority of the tsA58 cell lines grew at a reduced rate at 39.5°C.

In cell lines which carry the ts1135 double mutant the majority of cell lines yielded fewer colonies at the non-permissive temperature. This was most strikingly observed with the ts1135+5041 cell lines (Fig. 4.3D). All except one of these cell lines were conditionally immortal, yielding fewer colonies at the non-permissive temperature, with 14 of the 22 yielding fewer than 20% the number of colonies obtained at 33.0°C at 39.5°C. The cell number assays on six of these cell lines confirmed this result (Fig. 4.5C). A similar result was obtained with the ts1135+5031 cell lines where 30 of the 36 cell lines assayed yielded less than 77.4% the number of colonies at the non-permissive temperature compared to the permissive temperature and 13 of these 30 yielded less than 20%. Again the cell number assays carried out on six representative lines (Fig. 4.6C) confirmed these results. These results demonstrate that the ts1135 mutant protein is required to maintain immortalisation in these cell lines.

In contrast to this, all the cell lines isolated at the permissive temperature following transfection of either the dl1135+ts5041 or dl1135+ts5031 combinations were able to continue growing when shifted to the non-permissive temperature. The results obtained with the dl1135+ts5041 cell lines are shown in Figure 4.3E. All 22 cell lines isolated yielded colonies at the non-permissive temperature at over 77.4% the number obtained at the permissive temperature. The cell number assays carried out on six of these cell lines (Fig. 4.5D) showed that these cell lines grew almost as well (cell line 2) or better (cell lines 1, 3, 4, 5 and 6) at 39.5°C than at 33.0°C. Although only a few of the colonies isolated at the permissive temperature following transfection of the dl1135+ts5031 combination were successfully expanded into cell lines, all 6 of these cell lines grew well when shifted to the non-permissive temperature for the mutant bearing the tsA58 point mutation (Fig. 4.4D). One of these cell lines gave less than 77.4% the number of colonies obtained at 33.0°C at 39.5°C (cell line 3, giving 61.2%). Despite some of these cell lines growing less rapidly than others, the four cell lines
The cell lines isolated at the permissive temperature after cotransfection of the trans-complementing T antigen mutants *dl1135*+*5041* were assayed for their ability to grow at the non-permissive temperature for the mutant proteins bearing the *tsA58* point mutation (39.5°C). An equal number of cells (approx. 1000) were plated on four dishes, two of which were shifted to 39.5°C the following day while two were maintained at 33.0°C. Plates were fed every three to four days. After 2 weeks the plates were stained with methylene blue and colonies were counted. The percentage colony formation at non-permissive temperature is expressed as a percentage of the number of colonies obtained at the permissive temperature.
Figure 4.4: Ability of cell lines established at 33.0°C by d/l1135 + 5031 combinations to grow at 39.5°C: Colony assays.

A. d/l1135 + 5031 lines

B. tsA58 lines

C. ts1135 + 5031 lines

D. d/l1135 + ts5031 lines

The cell lines isolated at the permissive temperature after cotransfection of the trans-complementing T antigen mutants d/l1135+5031 were assayed for their ability to grow at the non-permissive temperature for the mutant proteins bearing the tsA58 point mutation (39.5°C). An equal number of cells (approx. 1000) were plated on four dishes, two of which were shifted to 39.5°C the following day while two were maintained at 33.0°C. Plates were fed every three to four days. After 2 weeks the plates were stained with methylene blue and colonies were counted. The percentage colony formation at non-permissive temperature is expressed as a percentage of the number of colonies obtained at the permissive temperature.
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Figure 4.5: Ability of cell lines established at 33.0°C by d/1135 + 5041 combinations to grow at 39.5°C: Cell number assays.

A. tsA58 lines

B. d/1135 + 5041 lines

C. ts1135 + 5041 lines

D. d/1135 + ts5041 lines

The cell lines isolated at the permissive temperature after cotransfection of the trans-complementing T antigen mutants d/1135+5041 were assayed for their ability to grow at the non-permissive temperature for the mutant proteins bearing the tsA58 point mutation (39.5°C). An equal number of cells (approx. 10,000) were plated on 10cm dishes. Half the dishes were shifted to 39.5°C on day 1 while the remainder were maintained at the permissive temperature (two of which were trypsinised and the total number of cells per plate were counted. Plates were fed every three to four days. Two plates from each temperature were trypsinised and counted on day 7 and day 14. The fold increase in cell number over the total number of cells present on day 1 was calculated for each temperature and are shown.
Figure 4.6: Ability of cell lines established at 33.0°C by \( dl1135 + 5031 \) combinations to grow at 39.5°C: Cell number assays.

A. \( tsA58 \) lines

B. \( dl1135 + 5031 \) lines

C. \( ts1135 + 5031 \) lines

D. \( dl1135 + ts5031 \) lines

The cell lines isolated at the permissive temperature after cotransfection of the \( trans \)-complementing T antigen mutants \( dl1135+5031 \) were assayed for their ability to grow at the non-permissive temperature for the mutant proteins bearing the \( tsA58 \) point mutation (39.5°C). An equal number of cells (approx. 10,000) were plated on 10cm dishes. Half the dishes were shifted to 39.5°C on day 1 while the remainder were maintained at the permissive temperature (two of which were trypsinised and the total number of cells per plate were counted). Plates were fed every three to four days. Two plates from each temperature were trypsinised and counted on day 7 and day 14. The fold increase in cell number over the total number of cells present on day 1 was calculated for each temperature and are shown.
assayed by cell number all grew better at the non-permissive temperature than the permissive temperature (Fig. 4.6D).

These results suggest that once these cell lines were established, following the transfection of the combination of mutants, the ts5041 or ts5031 mutant proteins could be inactivated by shifting the cell lines to the non-permissive temperature and the dll135 mutant protein was sufficient to sustain their growth. In contrast, if the 5031 or 5041 mutant proteins were cotransfected with the ts1135 double mutant the resulting cell lines were unable to grow at the non-permissive temperature. These results suggest that while one or more of the functions carried by the 5041 or 5031 proteins are required to initiate immortalisation in cooperation with dll135, these functions can be removed once the cell lines are established and the functions carried exclusively by the dll1135 protein are sufficient to maintain immortalisation.

4.2.4. Analysis of protein expression in the established cell lines at the permissive and the non-permissive temperatures.

To confirm that the dll135 mutant protein alone was sufficient to maintain growth in the established cell lines, it was necessary to show that both mutant proteins were expressed and that the mutant proteins bearing the tsA58 point mutation were temperature sensitive (and that the proteins which did not bear the tsA58 mutation were not). It was also important to show that the coexpressed proteins retained their individual integrity. I had already shown genetically that the dll135 and 5041 proteins were not temperature sensitive, and that the ts1135, ts5041 and ts5031 proteins were temperature sensitive, in the immortalisation assays carried out at the two temperatures (Table 4.1). The observation that immortalisation by the dll1135+5031 combination was also temperature sensitive revealed, unexpectedly, that the 5031 protein without the tsA58 mutation was somewhat temperature sensitive for at least one of the functions necessary for its ability to cooperate with dll135 to immortalise REFs.

Another possibility which has to be addressed is that homologous recombination may have occurred between the two mutant T antigen reading frames thereby creating a reading frame capable of expressing either the wild type protein or the tsA58 protein. Even though this possibility seems unlikely considering the low frequency at which homologous recombination occurs, there is selection pressure for it to occur in these experiments. Recombination between the ts1135 and 5041 or 5031 reading frames would most probably generate the tsA58 reading frame since the intact 5' end of the 5041/5031 reading frame could recombine with the 3' end of the ts1135 reading frame (which bears the tsA58 deletion) to recreate T antigen bearing just the tsA58 point mutation (outlined in Figure 4.7). A temperature sensitive phenotype was observed when these ts1135+5041/5031 cell lines were shifted to the non-permissive temperature. In contrast, recombination between the reading frames expressing dll1135 and 5041 or
Figure 4.7: The possible results of recombination between the mutant reading frames

Recombination between ts1135 and 5041/5031 open reading frames could yield T antigen molecules bearing the only the tsA58 point mutation:

\[ \text{ts1135} \quad \text{tsA58} \quad \text{5041/5031} \quad \text{tsA58} \]

Recombination between 1135 and ts5041/ts5031 open reading frames could yield wild type T antigen molecules:

\[ \text{1135} \quad \text{ts5041/ts5031} \quad \text{tsA58} \quad \text{wild type T antigen} \]

5031 would result in a wild type T antigen reading frame and this would result in cell lines which are non-conditional. Again this was the phenotype observed with these cell lines. Another way either the wild type or tsA58 T antigen reading frames could be recreated could be by trans-splicing between the two mutant's mRNAs. The argument against these events is that, when the mutants are cotransfected, a temperature sensitive colony formation phenotype was always seen initially when one of the mutant proteins carried the tsA58 point mutation. This would suggest that if either of these two events were occurring they were happening after the initial stages of immortalisation. Analysis of the two T antigen mutant proteins expressed in these cell lines was therefore desirable.

4.2.4.1. Identification of T antigen monoclonal antibodies capable of distinguishing between the T antigen mutant proteins.

To analyse the expression of each of the two mutants being coexpressed in the same cell lines it was firstly necessary to find a method of distinguishing between them. A large number of monoclonal antibodies have been raised against T antigen and a number of the epitopes for these antibodies have been mapped. Several monoclonal antibodies against T antigen are known to recognise epitopes in the amino-terminal 82 amino acids of T antigen due to their ability to immunocomplex with both large T and small t through the 82 amino acids they have in common. Two of these, PAb419 and PAb108 recognise epitopes within this amino-terminal 82 amino acids (Harlow et al., 1981; Gurney, Tamowski and Deppert, 1986; Mole et al., 1987) which are destroyed by
the 11 amino acid deletion carried by mutant dl1135 (Clark et al., 1983).

There are also a number of monoclonal antibodies which recognise the region of T antigen between residues 271 to 527 (Gurney, Tamowski and Deppert, 1986), however whether any of these are specific for an epitope which is destroyed by the point mutations present in the 5041 and 5031 mutants was not known. Using supernatants from a panel of hybridomas expressing these antibodies (kindly provided by E. Gurney, Department of Biology, University of Utah) I successfully identified two antibodies (PAb106 and PAb114) which were unable to immunoprecipitate the 5041 and 5031 T antigen mutant proteins under the same conditions as they were able to precipitate wild type T antigen (data not shown). The epitopes for these two antibodies were particularly sensitive to denaturing conditions therefore these antibodies could only be used to immunoprecipitate proteins under relatively mild lysis buffer conditions (modified RIPA buffer) and neither could be used for directly immunoblotting T antigen.

To demonstrate the ability of these antibodies to distinguish between the mutant T antigens at both 33.0°C and 39.5°C it was first necessary to obtain cells which expressed each T antigen mutant individually. NIH3T3 cells were therefore transfected (by electroporation) with the recombinant plasmids expressing each of the three mutants along with a selectable marker (pSV2neo). Transfected cells were selected in Geneticin-G418 at 37.0°C for two weeks. The G418-resistant colonies from each transfection were subsequently pooled and these cells were used for analysis of the mutant T antigen's expression. Cell lysates were made in modified RIPA buffer from the cell lines at either 33.0°C or from cells which had been shifted to 39.5°C 72 hours previously. The cells were fed 24 hours prior to lysis. 150µg of each cell lysate was immunoprecipitated with anti-T antigen antibodies PAb416, PAb419, PAb108 and PAb114. PAb106 was also used for preliminary experiments however it became clear that this antibody was not able to immunoprecipitate T antigen as efficiently as the other antibodies used (data not shown). The epitope for PAb416 lies between amino acids 82 and 106 (Mole et al., 1987; Mole, Iggo and Lane, 1989) so this antibody should recognise all three mutant proteins. A control immunoprecipitation was carried out with a monoclonal antibody against adenovirus E1A (PAbM73; Harlow, Franza and Schley, 1985). For reasons which are discussed later the supernatants from the PAb419 immunoprecipitations were immunoprecipitated again with PAb114. The immunoprecipitated proteins were fractionated in duplicate by SDS-PAGE and immunoblotted with either PAb419 and PAb108 or PAb416. The results are shown in Figure 4.8.

The control antibody, PAbM73, failed to immunoprecipitate any protein which co-migrated with T antigen in lysates from the cell lines expressing the three mutant T antigen proteins, at either temperature, which could be immunoblotted with either PAb416, PAb419 or PAb108 (panels A, B & C, lanes 6 & 12). A protein which
Figure 4.8: Specificity of T antigen monoclonal antibodies for mutant proteins.

**A. *dl1135.***

33.0°C | 39.5°C | 33°C
---|---|---
Immuno-precipitated with PAb:

<table>
<thead>
<tr>
<th>kDa</th>
<th>112</th>
<th>84</th>
<th>112</th>
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<td>108</td>
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<td>419</td>
<td>416</td>
<td>108</td>
<td>114</td>
</tr>
<tr>
<td>M73</td>
<td>114</td>
<td>149/114</td>
<td>M73</td>
<td></td>
</tr>
</tbody>
</table>

Immunoblotted with PAb(s):

416

T Ag

419 & 108

**B. *5041.***

33.0°C | 39.5°C | 33°C
---|---|---
Immuno-precipitated with PAb:

<table>
<thead>
<tr>
<th>kDa</th>
<th>112</th>
<th>84</th>
<th>112</th>
<th>84</th>
</tr>
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<tbody>
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<td>416</td>
<td>416</td>
<td>419</td>
<td>108</td>
<td>114</td>
</tr>
<tr>
<td>419/114</td>
<td>419</td>
<td>416</td>
<td>108</td>
<td>114</td>
</tr>
<tr>
<td>M73</td>
<td>114</td>
<td>149/114</td>
<td>M73</td>
<td></td>
</tr>
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Immunoblotted with PAb(s):

416

T Ag

419 & 108

See overleaf for figure legend.
(A, B & C) Cell lysates were prepared in modified RIPA (0.15M) buffer from NIH3T3 cell lines, at either 33.0°C or 39.5°C, which expressed T antigen mutants dl1135, 5041 or 5031. Immunoprecipitations with the indicated monoclonal antibodies against T antigen (and M73 against Ad E1A as a control) were carried out on 150µg cell lysate (supernatants from PAb419 immunoprecipitates were re-immunoprecipitated with PAb114- 419/114 lanes 5 & 11) and fractionated on duplicate SDS-10% PAGE gels. The fractionated proteins were transferred to nitrocellulose and blotted by the indicated monoclonal antibodies against T antigen. Immunocomplexes were detected using an HRP-labelled goat anti-mouse Ig antibody (as described in Materials and Methods).

(D) Specificities of T antigen monoclonal antibodies for mutants. (+ denotes that this mutant protein will be immunoprecipitated by this antibody at the indicated temperature; - denotes that this mutant protein will not be immunoprecipitated by this antibody at the indicated temperature.)
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migrated slightly higher than T antigen was observed in some experiments (panel B, lane 6, for example). The identity of this protein is not known although its appearance in long exposures of immunoblots with either PAb416 or PAb419 and PAb108 (data not shown) suggest it is a protein immunoprecipitated by PAbM73 and detected by the horseradish peroxidase-linked sheep anti-mouse Ig antibody.

The $dl_{1135}$ protein in lysates prepared from cells at either temperature was immunoprecipitated by both PAb416 (Fig. 4.8, panel A, lanes 1 & 7) and PAb114 (lanes 4 & 10) and detected by immunoblotting with PAb416. As observed previously by others, the $dl_{1135}$ was not immunoprecipitated by either PAb419 (lanes 2 & 8) or PAb108 (lanes 3 & 9). The unprecipitated $dl_{1135}$ protein in the supernatants from the PAb419 immunoprecipitations (33.0°C and 39.5°C) could subsequently be immunoprecipitated with PAb114 (lanes 5 & 11). The fractionated $dl_{1135}$ proteins were not immunoblotted by PAb419 or PAb108 (panel A, lanes 1-12, lower portion), the neighbouring PAb416 immunoprecipitation on the 5031 33.0°C cell lysate lane (lane 13) is included here to show that the immunoblot had worked.

The 5041 protein in lysates prepared from either 33.0°C or 39.5°C was also immunoprecipitated by PAb416 (Fig. 4.8, panel B, lanes 1 & 7) but, in contrast to the $dl_{1135}$ protein, it was also immunoprecipitated by PAb419 (lanes 2 & 8) and PAb108 (lanes 3 & 9). The immunoprecipitated protein was successfully immunoblotted by either PAb416 (upper portion) or PAb419 plus PAb108 (lower portion). A similar result was obtained following immunoprecipitation of the 5031 33.0°C and 39.5°C cell lysates with these three antibodies (Fig. 4.8, panel C, lanes 1, 2, 3, 7, 8 & 9). Immunoprecipitation of the 5041 protein with PAb114 failed to precipitate the protein at either 33.0°C or 39.5°C (panel B, lanes 4 & 10) and, of course, PAb114 failed to immunoprecipitate any protein from the supernatants of the PAb419 immunoprecipitations (lanes 5 & 11). Interestingly while PAb114 was unable to immunoprecipitate any 5031 protein from lysates prepared from cells at 39.5°C (panel C, lane 10) it was able to immunoprecipitate the 5031 protein at 33.0°C (lane 4), albeit at a decreased ability compared to the amount of protein immunoprecipitated by either PAb416, PAb419 or PAb108. The 5031 protein precipitated by PAb114 from the 33.0°C cell lysates was also detected by PAb419+PAb108 immunoblotting but was only visible on longer exposures (not shown). This temperature sensitivity of a particular epitope of the 5031 mutant protein is consistent with the temperature sensitive immortalisation phenotype observed with the $dl_{1135}+5031$ combination (Table 4.1). When the supernatants from PAb419 immunoprecipitations of 5031 33.0°C cell lysates were subsequently immunoprecipitated with PAb114 no 5031 protein was observed (panel C, lane 5) since the 5031 protein had been precipitated by PAb419. Therefore to separate the $dl_{1135}$ and 5031 proteins in lysates from cells immortalised by both mutants it would be necessary to carry out PAb419 immunoprecipitations (to selectively
precipitate the 5031 protein) and then immunoprecipitate the supernatants from these immunoprecipitations with PAb114 (to selectively immunoprecipitate the dl1135). Lysates from cell lines immortalised by the dl1135+5041 combinations can be immunoprecipitated directly with the PAb419 and PAb114 antibodies since these will specifically immunoprecipitate the 5041 and dl1135 proteins respectively at both 33.0°C and 39.5°C. The specificities of these antibodies to the mutant T antigens is summarised in Figure 4.8D.

To ensure that the two combinations of mutant proteins could be successfully separated by selective immunoprecipitation, I mixed equal amounts (150μg) of lysate from each NIH3T3 cell line, dl1135 plus either 5041 or 5031, prepared at 33.0°C or 39.5°C. The dl1135 plus 5041 mixed lysates were immunoprecipitated separately with PAb419 or PAb114 (and PAbM73-33.0°C lysates only), while the dl1135 plus 5031 mixed lysates were immunoprecipitated firstly with PAb419 followed by PAb114 for the reasons mentioned previously. The immunoprecipitated proteins were then fractionated on duplicate SDS-polyacrylamide gels and immunoblotted with either PAb416 (which will blot all three mutant proteins) or PAb419 and PAb108 (which will blot only the 5041 or 5031 proteins). The results are shown in Figure 4.9. The presence of both mutant proteins in each mixed lysate was demonstrated by the PAb416 immunoblot (upper portion of Fig. 4.9), the 5041 or 5031 protein was immunoprecipitated with PAb419 (5041-lanes 2 & 4; 5031-lanes 7 & 9) and the dl1135 protein was immunoprecipitated by PAb114 (lanes 3, 5, 8 & 10). The fact that the mutant T antigen proteins were separated by the selective immunoprecipitation of these mixed cell lysates was demonstrated by the PAb419 and PAb108 immunoblot (Fig. 4.9, lower portion). Only the T antigen immunoprecipitated by PAb419 was successfully immunoblotted by PAb419 and PAb108 (i.e. 5041-lanes 2 & 4 or 5031-lanes 7 & 9). The protein present in the PAb114 immunoprecipitations (lanes 3, 5, 8 & 10) was not immunoblotted by PAb419 or PAb108 indicating this protein is the dl1135 T antigen since the epitopes for these two antibodies are destroyed in this mutant.

These selective immunoprecipitations followed by differential immunoblots yielded the results which would be expected if the coexpressed mutant proteins were retaining their individual integrity within the immortal cell lines. If however recombination or trans-splicing were occurring to recreate either wild type or tsA58 T antigen the immunoprecipitated protein would be successfully immunoblotted by both PAb416 and the PAb419 plus PAb108 combination. The regeneration of wild type or tsA58 T antigen would mean that the epitope for both PAb419 and PAb114 would be present on the same T antigen molecules. Thus using these antibodies in selective immunoprecipitations and immunoblotting I should be able to detect the expression of both mutant proteins and that they are retaining their individual integrity. I should also be able confirm that the double mutants bearing the tsA58 mutation are temperature
Cell lysates were prepared in modified RIPA (0.15M) buffer from NIH3T3 cell lines, at either 33.0°C or 39.5°C, which individually expressed T antigen mutant proteins dl1135, 5041 or 5031. 150µg of dl1135 lysate plus 150µg of either 5041 or 5031 lysate were mixed (for both 33.0°C and 39.5°C lysates) and immunoprecipitations were carried out on the mixed lysates using the indicated monoclonal antibodies (*PAb114 immunoprecipitations were carried out on the supernatants from PAb419 immunoprecipitations in 5031 containing mixes). Immunoprecipitated proteins were separated by SDS-10% PAGE, transferred to nitrocellulose and immunoblotted (in duplicate) by the indicated antibodies (PAb416 or PAb419+PAb108). Immunocomplexes were detected using HRP-labelled goat anti-mouse Ig antibody (as described in Materials and Methods).
sensitive in the immortal cell lines.

4.2.4.2 Analysis of T antigen in \textit{tsA58} cell lines at the permissive and non-permissive temperature.

The monoclonal antibodies to be used in these experiments were firstly analysed for their ability to detect the \textit{tsA58} T antigen protein at 33.0°C and 39.5°C, in three of the conditionally immortal \textit{tsA58} cell lines (cell lines 2, 16 and 17, Fig. 4.3C & 4.5A). Cell lysates prepared from these cell lines, maintained at either 33.0°C or shifted to 39.5°C 72 hours previously, were immunoprecipitated with PAb416, PAb419, PAb108, PAb114 and PAbM73. Immunoprecipitated proteins were fractionated by SDS-PAGE and immunoblotted with either PAb416 or PAb419 plus PAb108. The results are shown in Figure 4.10. Both PAb416 and the PAb419+PAb108 combination successfully immunoblotted the immunoprecipitated T antigen. Under the conditions used for these immunoprecipitations (modified RIPA buffer) antibodies PAb416, PAb419 and PAb108 only detected a slight difference in the level of \textit{tsA58} T antigen at 39.5°C compared to 33.0°C in cell lines 2 (panel A, lanes 1, 2, 3, 6, 7 & 8) and 16 (panel B, lanes 1, 2, 3, 6, 7 & 8). A greater difference was observed with these three antibodies in \textit{tsA5S} cell line 17 (panel C, lanes 1, 2, 3, 6, 7 & 8).

In contrast, PAb114 immunoprecipitations showed the temperature dependent denaturation of the \textit{tsA58} T antigen protein very well, with practically no protein detected at 39.5°C in cell lines 2 (panel A, lane 9) and cell line 17 (panel C, lane 9) and very little observed in cell line 16 (panel B, lane 9) while PAb114 did precipitate the \textit{tsA58} protein well in the 33.0°C cell lysates (panels A, B & C, lane 4). The control antibody PAbM73 failed to precipitate any protein which could be immunoblotted by either PAb416, PAb419 or PAb108, from either temperature (lanes 5 & 10, all panels).

It is important to note from these results that the epitopes on T antigen for antibodies PAb416, PAb419 and PAb108 were not as rapidly denatured as the epitope for PAb114 when two of the three \textit{tsA58} cell lines analysed were shifted to the non-permissive temperature. The \textit{tsA58} T antigen protein is still synthesised at 39.5°C but is rapidly denatured and degraded (Jat and Sharp, 1989). Previous studies have shown that when the \textit{tsA58} protein is synthesised at a high level more protein is detected at the non-permissive temperature (Jat \textit{et al.}, 1991). Thus these two cell lines may be expressing a higher amount of the \textit{tsA58} protein perhaps through the integration of multiple copies of the \textit{tsA58} gene into their genomes or by virtue of the gene's integration site in the genome. The contrast in the results obtained with PAb114 compared to PAb416, PAb419 and PAb108 reflects the sensitivity of the epitope for this antibody to denaturation and reflects this antibody's inability to immunoblot denatured T antigen. The ability of both PAb419 and PAb108 to detect T antigen proteins bearing the \textit{tsA58} point mutation at 39.5°C in some cell lines has made it difficult to determine whether or
Figure 4.10: Stability of tsA58 T antigen at 33.0°C and 39.5°C in three tsA58 cell lines.

### A. tsA58 cell line 2

<table>
<thead>
<tr>
<th>Immuno-precipitated with PAb:</th>
<th>33.0°C</th>
<th>39.5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>112kDa</td>
<td>416</td>
<td>416</td>
</tr>
<tr>
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<td>419</td>
<td>108</td>
</tr>
<tr>
<td>64kDa</td>
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<td>114</td>
</tr>
<tr>
<td>41kDa</td>
<td>M73</td>
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</table>

Immunoblot: tsA58 T Ag

Cell lysates from the indicated cell lines were prepared in modified RIPA (0.15M) buffer at 33.0°C and 39.5°C. 400μg of each lysate was immunoprecipitated with the indicated antibodies. Immunoprecipitates were separated by SDS-10% PAGE, transferred to nitrocellulose and immunoblotted (in duplicate) by the indicated antibodies. Immunocomplexes were detected using HRP-labelled goat anti-mouse Ig antibody (as described in Materials and Methods).
not the 5041, ts5041, 5031 and ts5031 mutant proteins are temperature sensitive in the dl1135+5041/5031 cell lines.

4.2.4.3. Analysis of T antigen mutant proteins in cell lines immortalised by cooperation between the mutants.

The integrity of the mutant T antigen molecules was analysed in the dl1135+5041 or dl1135+5031 combination cell lines by carrying out the selective immunoprecipitations followed by differential immunoblotting described previously. These experiments clearly allowed the mutant T antigen molecules to be distinguished when they were expressed in separate cell lines and the cell lysates mixed prior to immunoprecipitation (Fig. 4.9). The results obtained when these experiments were carried out on five representative cell lines immortalised with various dl1135+5041 combinations are shown in Figure 4.11 while the results with five representative dl1135+5031 combination cell lines are shown in Figure 4.12.

If the mutant proteins were being independently expressed and not recombining or trans-splicing to give expression of either wild type or tsA58 T antigen the protein immunoprecipitated by PAb114 (dl1135) would be detected by PAb416 blotting but not by PAb419 or PAb108. However in cell lysates from the five dl1135+5041 cell lines examined this was not the case. The protein immunoprecipitated by PAb114 was clearly capable of being detected by all three antibodies used for the immunoblots (Fig. 4.11, lanes 3, 5, 8 & 10, all three panels). In the dl1135+5031 cell lines the immunoprecipitations were carried out differently. The cell lysates were firstly immunoprecipitated by PAb419 and the supernatant from these immunoprecipitations were subsequently immunoprecipitated with PAb114. In the PAb114 immunoprecipitations however no T antigen protein was detected by immunoblotting with either PAb416 or PAb419 and PAb108 (Fig. 4.12, lanes 3, 5, 8 & 10, all three panels). This suggests that all the T antigen present was removed from the cell lysate by the PAb419 immunoprecipitation and there was no dl1135 protein remaining (which would have been immunoprecipitated by PAb114 and immunoblotted by PAb416 but not PAb419 or PAb108).

The results obtained with the five cell lines examined from each of the dl1135+5041 and dl1135+5031 combinations can be interpreted in two ways. Either wild type, or tsA58, T antigen molecules were recreated by recombination or trans-splicing between the reading frames of the two mutants or the mutant T antigen molecules were forming complexes within the cells which were not dissociated by the lysis buffer. T antigen molecules are certainly capable of forming oligomeric complexes with each other. Large T antigen hexamers, and smaller oligomers, are formed in an ATP-dependent (down to 10μM) manner (Dean et al., 1987b; Reynisdottir et al., 1993) and this oligomerisation has been shown to require the zinc finger region of T antigen (Loeber et al., 1991). The zinc finger region of T antigen is between amino acids 302 to
Figure 4.11: Analysis of expression of T antigen mutant proteins at 33.0°C and 39.5°C in *dl1135 + 5041* cell lines.

**A. dl1135 + 5041 line**

Cell line 1

Immuno-precipitated with PAb: M73 419 114 419 114 419 114 419 114 419 114

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<th>Immuno-precipitated with PAb</th>
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<th>39.5°C</th>
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</tr>
<tr>
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Immunoblotted with PAb(s): T Ag 416

**B. ts1135 + 5041 lines**

Cell line 6

Immuno-precipitated with PAb: M73 419 114 419 114 419 114 419 114 419 114

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Immunoblotted with PAb(s): T Ag 416

**C. dl1135 + ts5041 lines**

Cell line 1

Immuno-precipitated with PAb: M73 419 114 419 114 419 114 419 114 419 114

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<th>39.5°C</th>
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Immunoblotted with PAb(s): T Ag 416

Cell lysates from the indicated cell lines were prepared in modified RIPA (0.15M) buffer at 33.0°C and 39.5°C. 400μg of each lysate was immunoprecipitated with the indicated antibodies. Immunoprecipitates were separated by SDS-10% PAGE, transferred to nitrocellulose and immunoblotted (in duplicate) by the indicated antibodies. Immunocomplexes were detected using HRP-labelled goat anti-mouse Ig antibody (as described in Materials and Methods).
Figure 4.12: Analysis of expression of T antigen mutant proteins at 33.0°C and 39.5°C in \textit{dl1135 + 5031} cell lines.

**A. \textit{dl1135 + 5031} line**

Cell line 2

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B. \textit{ts1135 + 5031} lines

Cell line 1

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Cell line 7

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C. \textit{dl1135 + ts5031} lines

Cell line 1

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Cell line 4

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<tr>
<td>T Ag 419+ 108</td>
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Cell lysates from the indicated cell lines were prepared in modified RIPA (0.15M) buffer at 33.0°C and 39.5°C. 400µg of each lysates was immunoprecipitated with the indicated antibodies (*PAb114 immunoprecipitations were carried out on the supernatants from PAb419 immunoprecipitations). Immunoprecipitates where separated by SDS-10% PAGE, transferred to nitrocellulose and immunoblotted (in duplicate) by the indicated antibodies. Immunocomplexes were detected using HRP-labelled goat anti-mouse Ig antibody (as described in Materials and Methods).
320, which are intact in all three of these mutants (Loeber, Parsons and Tegtmeyer, 1989). When the same mutant proteins are expressed separately in baculovirus systems and then mixed, along with ATP, in DNA replication assays they are known to form tight complexes which can only be dissociated under strong denaturation conditions (J.M.Pipas, personal communication).

Recombination or trans-splicing between the two mutant reading frames in either *dl1135ts5041/ts5031* cell lines would result in the expression of wild type T antigen. Due to the inability of PAb419 to efficiently detect the temperature sensitivity of proteins bearing the *tsA58* point mutation it is impossible to discern whether the PAb419-immunoprecipitated proteins (*ts5041* or *ts5031*) in most of these cell lines were temperature sensitive. In two cell lines however the immunoprecipitated proteins did appear to be temperature sensitive. The amount of protein immunoprecipitated by PAb419 (as detected by PAb416 immunoblotting) in cell lysates from *dl1135+ts5041* cell line 1 was greater at 33.0°C (Fig. 4.11, panel C, lane 2) than at 39.5°C (lane 4). In contrast, more T antigen was immunoprecipitated by PAb114 from the same 39.5°C cell lysate (lane 5) than the 33.0°C cell lysate (lane 3). This would indicate that at least some of the protein precipitated by PAb419 from these cell lysates was temperature sensitive. The protein immunoprecipitated by PAb419 from *dl1135+ts5031* cell line 1 cell lysates was also temperature sensitive (Fig. 4.12, panel C, lanes 2 & 4). This result would not be expected if wild type T antigen were being expressed alone in these cell lines. However, it could be explained if expression were occurring from all four possible reading frames which could result from recombination or trans-splicing events, i.e. expressing wild type, *dl1135, ts5041/ts5031*, and an undetectable *dl1135+ts5041/ts5031* triple mutant, T antigen molecules.

It is also hard to use the expected temperature sensitivity of the *ts1135* protein in the *ts1135+5041/5031* cell lines to interpret whether recombination or trans-splicing is occurring. The result of such events would be expression of T antigen bearing the *tsA58* mutation. Since the temperature sensitive phenotype of *tsA58* bearing proteins was detected well by PAb114 immunoprecipitations but poorly by PAb419 immunoprecipitations, the results obtained with the *ts1135+5041/5031* cell lines (Fig. 4.11B or Fig. 4.12B) could have either been due to the mutant molecules retaining their integrity or due to the expression of T antigen bearing the *tsA58* mutation alone.

4.2.4.4. T antigen-p53 complexes remained intact in cell lysates prepared from the cell lines immortalised by the combinations of mutants.

To investigate the possibility that T antigen complexes were not being dissociated in the cell lysates prepared from these cell lines I analysed whether T antigen-p53 complexes were still present. If T antigen-p53 complexes were not dissociated in the modified RIPA buffer used to lyse the cells it is possible that T antigen oligomeric complexes were not dissociated either.
Cell lysates were prepared from the indicated cell lines at 33.0°C in modified RIPA (0.15M) buffer and immunoprecipitated using a monoclonal antibody against p53 (PAb421). Immunoprecipitates were fractionated by SDS-10% PAGE and transferred to nitrocellulose. Co-precipitated T antigen was immunoblotted with PAb416. Immunocomplexes were detected using a HRP-labelled goat anti-mouse Ig antibody (as described in Materials and Methods).
Chapter 4.

The cell lysates prepared from a number of the immortal cell lines were immunoprecipitated with a monoclonal against p53 (PAb421 Harlow et al., 1981) and the precipitated proteins fractionated by SDS-PAGE. Any T antigen coprecipitated with p53 was detected using the anti-T antigen monoclonal antibody PAb416. Figure 4.13A (dl1135+5041 cell lines) and 4.13B (dl1135+5031 cell lines) shows that T antigen was co-immunoprecipitated with p53 in every cell line examined. This indicates that T antigen-p53 complexes were not dissociated in the cell lysates prepared from any of these cell lines. It is therefore possible that T antigen oligomeric complexes were also present in these cell lysates, thereby complicating my attempts to distinguish between the mutant proteins coexpressed in these cell lines. While the results obtained when the mutant proteins were expressed in separate cell lines and then the cell lysates were mixed suggest that the complexes could not form in the modified RIPA buffer (Fig. 4.9), it is possible that complexes formed within the cells in an ATP-dependent manner, or during the lysis of the cells, are not separated by this buffer.

The only way to successfully distinguish between the mutant proteins when they are coexpressed in the cell lines will be to find lysis conditions which will dissociate T antigen oligomeric complexes. These cell lysis conditions will have to be strongly denaturing enough to break up the protein complexes but not so strongly denaturing that the denaturation sensitive epitope for PAb114 is destroyed. Experiments are currently underway to identify alternative conditions for these experiments.

4.3. Discussion.

Having established that two immortalisation defective T antigen mutants could cooperate to immortalise secondary REFs (Chapter 3), I investigated the role of each of the two mutant proteins in this trans-complementation. Using the combination of mutant dl1135 with either mutant 5041 or mutant 5031 I have shown that the presence of both mutant proteins was required to successfully establish cell lines. This was done by introducing the tsA58 point mutation into either one of the two cooperating mutant proteins and examining the difference in the ability of the mutants to immortalise at the non-permissive temperature, compared to the permissive temperature, for the protein bearing the tsA58 point mutation (Table 4.1). This data also indicated that the double mutant proteins bearing the tsA58 point mutation (ts1135, ts5041 and ts5031) were indeed temperature sensitive, as was the 5031 mutant protein, while the dl1135 and 5041 proteins were not.

Once cell lines were established at the permissive temperature following the cotransfection of the T antigen mutant combinations, the dependence of these cell lines on the continued presence of each mutant protein was determined. Cell lines immortalised at the permissive temperature with the temperature sensitive ts1135 protein
in combination with the non-temperature sensitive 5041 protein were unable to grow upon shift up to the non-permissive temperature. The ts1135+5031 cell lines were also conditionally immortal. In contrast, cell lines containing the non-temperature sensitive dl1135 protein (dl1135+ts5041/ts5031 cell lines) retained the ability to grow at the non-permissive temperature despite the fact that the ts5041 and ts5031 proteins should be denatured and degraded at this temperature. Despite my inability to show that the ts5041 or ts5031 proteins were temperature sensitive biochemically, the temperature sensitive phenotype of these double mutant proteins was demonstrated genetically in the immortalisation assays shown in Table 4.1. This temperature sensitive immortalisation phenotype also rules out the possibility that small t antigen may provide the amino-terminal function lacked by the dl1135 mutant protein. While the small t antigen encoded by mutant dl1135 would bear the same 10 amino acid deletion (17-27) as the dl1135 large T antigen, both mutants ts5041 and ts5031 are able to express wild type small t antigen which would be stable at 39.5°C. It is therefore possible that small t antigen could complement the function(s) lacked by the dl1135 protein in the dl1135+ts5041/ts5031 cell lines at the non-permissive temperature. This is clearly not the case since immortalisation by the dl1135+ts5041/ts5031 combinations was temperature sensitive (Table 4.1). Furthermore, 35S-methionine-labelled extracts prepared from these cell lines and immunoprecipitated with PAb419 indicated that very little small t antigen was synthesised in these cell lines at either temperature (approximately 10-fold less than the amount of labelled T antigen protein synthesised at the permissive temperature; data not shown).

This data demonstrates that the dl1135 mutant T antigen lacks a function necessary for the immortalisation of secondary REFs and that this function can be provided by either the mutant 5041 or 5031 proteins. Once cell lines are established the T antigen functions carried by the dl1135 protein are sufficient to maintain immortalisation in these cell lines. This hypothesis is confirmed by the data obtained previously, where the dl1135 protein was shown to be able to maintain immortalisation at the non-permissive temperature in the conditionally immortal tsa14 cell line (Chapter 3). This data suggests that there is a transient requirement for a function carried by the 5041 and 5031 proteins which is required during the early stage of immortalisation. This "initiation" function appears to be lacking in the dl1135 protein but this protein does carry all the functions necessary for the subsequent maintenance of immortalisation. In the immortalisation of REFs by the cooperating T antigen mutants the 5041 and 5031 proteins provide this initiation function, however once the cell lines are established, the functions carried by these two mutants are no longer required. The demonstration that neither mutant alone could immortalise REFs or maintain the immortal state in tsa14 cells suggests that the 5041 and 5031 protein lack one or more of the maintenance functions necessary for immortalisation. Since neither of these mutant
proteins are able to complex p53, while the dl1135 protein can, sequestration of p53 may be one of the T antigen functions necessary for the maintenance of immortalisation. The necessity for T antigen to bind to p53 in order to maintain immortalisation may be to overcome p53-dependent apoptosis. Zheng et al. (1994) have isolated conditionally immortal REF cell lines using the tsA58 mutant and these cell lines not only growth arrest at the non-permissive temperature, but undergo apoptosis which correlates with the release of wild type p53 from the T antigen-p53 complexes. The activity of a 121 amino acid amino-terminal fragment of T antigen (dl1137) in inducing choroid plexus tumours in transgenic mice is increased in p53-null mice (Symonds et al., 1994). This result suggests that p53-dependent apoptosis decreases the growth rate of the dl1137 tumours in transgenic mice expressing wild type p53. Recently, Cavender et al have suggested that another function carried by T antigen, which is required for its ability to cooperate with ras to transform rat embryo fibroblasts, may be colinear with the p53 binding domain (Cavender et al., 1995).

The requirement for a transient function of T antigen for the initiation of immortalisation suggests that this function plays an important role during an early stage of immortalisation after which this function is no longer required. It is possible that T antigen interacts transiently with a factor during the initiation of immortalisation and that this interaction is not required for the maintenance of immortalisation. Such an interaction would suggest that the activity of this factor may be regulated in a different way once cell lines have become dependent on T antigen. If this factor is regulated within the cells as they age, it is tempting to speculate that such a factor may be involved in the mechanism which determines the finite life span of primary rodent cells. Alternatively, it is possible that the activity of this factor (or some other factor within the same growth regulatory pathway) is regulated through another functional region of T antigen during the maintenance of immortalisation but for some reason this is not able to occur during the initiation of immortalisation.

The function lacking in the dl1135 protein is not known. The deletion of amino acids 17-27 removes one of the amino acids of the CR1-like domain of T antigen (the leucine at residue 19; see Figure 1.1). This amino acid is conserved in the CR1 domains of Ad E1A, HPV16 E7 and the large T antigens of SV40, polyomavirus, human JC virus, human BK virus, baboon SA12 virus and monkey lymphotrophic papovaviruses (Dyson et al., 1990; Imai et al., 1991). This region has been shown to be required for the ability of Ad E1A to complex the cellular 300kDa protein, p300 (Whyte, Williamson and Harlow, 1989). However an association of T antigen with this protein has yet to been demonstrated. E1A mutants defective for the interaction with p300 are defective for the transformation of baby rat kidney cells in ras cooperation assays. CR1 mutants of E1A can be complemented in these assays by wild type SV40 T antigen, however not by the dl1135 mutant. This suggests either that a T antigen-p300 interaction, although
not yet identified, does occur or that CR1 mutations affecting E1A's ability to associate with p300 are affecting another, as yet unidentified, function which the CR1 of E1A and T antigen have in common. The p300 protein has recently been shown to have homology to, and to be a functional homologue of, the transcriptional co-activator CBP. More recently two proteins involved in transcriptional activation have also been demonstrated to interact with the amino terminus of T antigen in vitro. These are the TATA-binding protein and enhancer-binding transcriptional activator TEF-1 (Gruda et al., 1993). The "initiation" function of T antigen in immortalisation might therefore involve transcription activation. Mutations within the amino terminal 85 amino acids decrease T antigen's ability to transactivate the RSV LTR promoter, the SV40 late promoter and several simple modular promoters (Zhu et al., 1991b; Rice and Cole, 1993). The amino-terminal 121 amino acids of T antigen (dl1137) are sufficient to transactivate the adenovirus E2 promoter (Srinivasan, Peden and Pipas, 1989). DNA polymerase α and heat shock protein-73 also associate with T antigen through its amino-terminus (Smale and Tjian, 1986; Gannon and Lane, 1987; Sawai and Butel, 1989; Sawai, Rasmussen and Butel, 1994; Dornreiter et al., 1990).

Future work will hopefully clarify whether the mutant proteins are expressed separately and maintain their integrity within the cell lines immortalised by the dl1135+5041/5031 combinations. This may be clarified at the level of protein expression using the antibodies I have identified as specific for the mutant proteins. Alternatively, it is possible that the problem could be resolved by isolating mRNA from these cell lines and using a reverse-transcriptase PCR (RT-PCR) technique with oligonucleotide primers specific for the mutant or wild type coding sequences in the region of the mutations. The results from either of these lines of investigation will hopefully clarify whether the functions of T antigen required for the immortalisation of secondary rat embryo fibroblasts can be separated into initiation and maintenance functions. If the results from these experiments indicate that the mutant proteins are functioning independently in these cell lines, these experiments will represent the first demonstration that one or more functions of an immortalising oncogene are required only transiently during the initial stages of immortalisation and that maintenance of immortalisation, once cell lines are established, does not require all the oncoprotein's functions. It is clear however that the region of T antigen destroyed by the dl1135 deletion is required for immortalisation, whether it only be at an early "initiation" stage or not. Future work using this region of T antigen in a yeast two-hybrid screen or in an affinity purification may allow the identification and characterisation of proteins which interact with T antigen through this region.
Chapter 5.

Analysis of ts1135 conditionally immortal cell lines.

5.1: Introduction.

The conditionally immortalised cell lines originally isolated by Parmjit Jat (Jat and Sharp, 1989) following the infection of rat embryo fibroblasts with a retrovirus transducing the temperature sensitive large T antigen mutant tsA58 growth arrested irreversibly after inactivation of the T antigen at the non-permissive temperature for 72 hours or more. Following growth arrest at the non-permissive temperature these cell lines are unable to grow even if the stability of the tsA58 T antigen is restored by shifting the cells back to the permissive temperature. This growth arrest occurs at either the G1 or G2 phase of the cell cycle and it is believed that these cells are recapitulating senescence (E.S.Gonos et al, in preparation). When these cell lines are shifted to the non-permissive temperature they remain metabolically active and do not show any degree of cell death. In contrast, Zheng et al. (1994) have reported that REFs similarly immortalised with the tsA58 T antigen mutant not only growth arrest at the non-permissive temperature but also undergo apoptosis. They have suggested that this is due to the dissociation of T antigen-p53 complexes thereby releasing p53.

The cell lines which I isolated at the permissive temperature following the cotransfection of ts1135 with either 5041 or 5031 were also conditionally immortal. In these cell lines, however, the majority of the functions carried by T antigen are retained upon shift up to the non-permissive temperature, since only one of the cooperating T antigen mutants carries the tsA58 point mutation and is thermolabile. Only the T antigen function(s) being provided exclusively by the ts1135 molecule are inactivated at the non-permissive temperature. The functional domain provided by the ts1135 molecule, which is disrupted in the 5041 and 5031 molecules, is the domain required for T antigen's association with p53. It is possible, however, that this domain may also provide other functions necessary for T antigen's growth stimulatory effects. The existence of another activity in this domain has been suggested by Cavender et al. (1995) who demonstrated that a T antigen molecule with a mutation in this domain,
which was still able to associate with p53, was unable to transform in cooperation with an activated ras oncogene. It is possible that the continued presence of the functions provided by the 5041 or 5031 proteins in my conditionally immortal cell lines when their growth is arrested at the non-permissive temperature, may change the characteristics of the growth arrest compared to that observed in the tsA58 cell lines. It may be at that the functions provided by the 5041 or 5031 proteins prevent irreversible growth arrest in these cells and they may be able to resume growth upon being returned to the permissive temperature.

In this chapter I describe two lines of analysis which I carried out to further characterise the conditionally immortal ts1135+5041/5031 cell lines. Firstly, I carried out two proliferation assays on six of these cell lines to determine whether the growth arrest in these cell lines, upon shift up to the non-permissive temperature, is reversible. Secondly, I have attempted to overcome the growth arrest in these six cell lines by reintroducing expression of either wild type T antigen or the non-temperature sensitive dl1135 mutant at the non-permissive temperature.

5.2: Results.

5.2.1: The growth arrest in the ts1135 plus 5041 or 5031 cell lines is irreversible.

The ability of representative conditionally immortal cell lines to grow at either the permissive temperature (33.0°C), the non-permissive temperature (39.5°C) or the permissive temperature following growth arrest at the non-permissive temperature for 7 days (39.5°C to 33.0°C) was determined by two growth assays. The first involved plating 10,000 cells on multiple plates and counting the resulting number of cells per plate, at several time points, after incubation of the plates under the three conditions. This cell number assay yielded data for the whole population of cells initially plated, however, if a sub-population of these cells retained the ability to grow upon being returned to the permissive temperature, then the results would become distorted. This was overcome in the second assay, where 1000 cells were plated on six dishes, and two of each were incubated under each condition. After at least 14 days at their final temperature the resulting colonies were stained and counted. With this colony assay the viability of each cell plated could be determined by its ability to form an individual colony. From the results of the colony assays it was possible to determine whether the majority of the population of cells for each cell line were irreversibly arresting and only a sub-population attained the ability to overcome the growth arrest, or whether the growth arrest was reversible for the majority of the population.

The results of these growth assays are shown in Figures 5.1-6. In each figure the left graph represents the cell number assays, where the number of cells obtained under
each condition is plotted against the number of days. Representative plates were shifted up to 39.5°C on day 1 (and first counted at 39.5°C on day 4) and returned to 33.0°C on day 7 (39.5°C to 33.0°C cells were first counted on day 10). The graphs on the right side of each figure show the results for the colony assays presented as number of colonies obtained for each of the three growth conditions.

In total six tsA58 conditionally immortal cell lines were assayed for their ability to grow following growth arrest at 39.5°C. Four of these were isolated from my pUC19tsA58 transfections while the other two (tsa8 and tsa14) are two of the cell lines originally isolated by Jat and Sharp (1989) using the pZiptsA58 retrovirus; tsa14 is the cell line I used to assay the maintenance of immortalisation by T antigen mutants (Chapter 3). Figure 5.1A and B show the results obtained with the tsa8 and tsa14 cell lines respectively. The majority of the cells appeared to irreversibly growth arrest when shifted to the non-permissive temperature, however a proportion of the cells in each case appeared to be able to resume growth at the permissive temperature. The proportion of cells able to overcome the growth arrest and resume growth at 33.0°C appeared to be greater in the case of tsa14 (as indicated by the colony assay). tsA58 cell lines 2 and 8 (Fig. 5.1C & 5.2A respectively) gave similar results with the colony assays. However, it seems that neither of these two cell lines had successfully arrested following 7 days at 39.5°C, since the number of cells obtained after 7 days was similar at either temperature. A reduction in cell number at 39.5°C was only observed after 14 days in either cell line. Thus in these assays the cells were returned to the permissive temperature before the majority of cells had ceased dividing. tsA58 cell lines 16 and 17 (Fig. 5.2B & C respectively) both arrested rapidly at 39.5°C. In cell line 17, little growth occurred upon shifting back to 33.0°C from 39.5°C, while in cell line 16 some of the cells were able to overcome the growth arrest to eventually yield a similar number of cells as obtained at 33.0°C. In each case, the fact that only a proportion of the cells overcome the growth arrest when returned to the permissive temperature is indicated by the colony assays.

The results obtained for two cell lines immortalised with each non-temperature sensitive combination are shown in Figure 5.3 (dl1135+5041 cell lines) and Figure 5.4 (dl1135+5031 cell lines). These four cell lines are not conditionally immortal and therefore did not arrest at either temperature. The numbers of colonies obtained were similar on plates incubated under the three growth conditions for all four cell lines. The cell number assays for all four cell lines indicated that non-conditional cell lines generally yielded 5-10 fold more cells at 39.5°C than at 33.0°C after 14 days growth.

Figure 5.5 shows the results obtained with three ts1135+5041 cell lines while Figure 5.6 shows the results for three ts1135+5031 cell lines. The results obtained for each of these cell lines were similar. The majority of the cells irreversibly growth
Figure 5.1: Ability of tsA58 cell lines to grow at the permissive temperature following growth arrest at the non-permissive temperature:

A. tsa8 (tsA58)

B. tsal4 (tsA58)

C. tsA58 cell line 2

The indicated cell lines were assayed for their ability to grow at the permissive temperature after growth arrest resulting from incubation at the non-permissive temperature for 7 days. The line graphs indicate the number of cells obtained on day 1, day 4, day 7, day 10, day 14, day 17 and day 21 following the incubation of the cells (10,000 seeded per 10cm plate) at either the permissive temperature (33.0°C; ), the non-permissive temperature (39.5°C; ) or cells which were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature ( ). The bar graphs represent the number of colonies obtained following the incubation of the same cell lines (1000 cells seeded per 6cm plate) after incubation at either the permissive temperature (33°C), the non-permissive temperature (39°C) or that were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature (39°C to 33°C). Colonies were stained after two weeks (33.0°C plates) or three weeks (39.5°C and 39.5°C to 33.0°C plates) with methylene blue and counted.
Figure 5.2: Ability of *tsA58* cell lines to grow at the permissive temperature following growth arrest at the non-permissive temperature: 2.

A. *tsA58* cell line 8

The indicated cell lines were assayed for their ability to grow at the permissive temperature after growth arrest resulting from incubation at the non-permissive temperature for 7 days. The line graphs indicate the number of cells obtained on day 1, day 4, day 7, day 10, day 14, day 17 and day 21 following the incubation of the cells (10,000 seeded per 10cm plate) at either the permissive temperature (33.0°C; ), the non-permissive temperature (39.5°C; ) or cells which were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature ( ). The bar graphs represent the number of colonies obtained following the incubation of the same cell lines (1000 cells seeded per 6cm plate) after incubation at either the permissive temperature (33°C), the non-permissive temperature (39°C) or that were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature (39°C to 33°C). Colonies were stained after two weeks (33.0°C plates) or three weeks (39.5°C and 39.5°C to 33.0°C plates) with methylene blue and counted.
Figure 5.3: Ability of ts \(1135+5041 \) cell lines to grow at the permissive temperature after shift up to the non-permissive temperature.

A. \(ts1135+5041 \) cell line 1

B. \(ts1135+5041 \) cell line 5

The indicated cell lines were assayed for their ability to grow at the permissive temperature following incubation at the non-permissive temperature for 7 days. The line graphs indicate the number of cells obtained on day 1, day 4, day 7, day 10 and day 14 following the incubation of the cells (10,000 seeded per 10cm plate) at either the permissive temperature (33.0°C; ---), the non-permissive temperature (39.5°C; ------) or cells which were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature (---o---). The bar graphs represent the number of colonies obtained following the incubation of the same cell lines (1000 cells seeded per 6cm plate) after incubation at either the permissive temperature (33°C), the non-permissive temperature (39°C) or that were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature (39°C to 33°C). Colonies were stained after two weeks with methylene blue and counted.

arrested in each case, however a varying proportion of the cells, in each cell line, appeared to be able to overcome this growth arrest upon shift back to the permissive temperature. The number of cells able to overcome the growth arrest at 39.5°C or when returned to 33.0°C was least in ts1135+5041 cell line 10 (Fig. 5.5C) and ts1135+5031
Figure 5.4: Ability of dl1135 + 5031 cell lines to grow at the permissive temperature after shift up to the non-permissive temperature.

A. *dl1135 + 5031* cell line 7

B. *dl1135 + 5031* cell line 11

The indicated cell lines were assayed for their ability to grow at the permissive temperature following incubation at the non-permissive temperature for 7 days. The line graphs indicate the number of cells obtained on day 1, day 4, day 7, day 10 and day 14 following the incubation of the cells (10,000 seeded per 10cm plate) at either the permissive temperature (33.0°C; —□—), the non-permissive temperature (39.5°C; ---o---) or cells which were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature (----o--). The bar graphs represent the number of colonies obtained following the incubation of the same cell lines (1000 cells seeded per 6cm plate) after incubation at either the permissive temperature (33°C), the non-permissive temperature (39°C) or that were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature (39°C to 33°C). Colonies were stained after two weeks with methylene blue and counted.

cell line 1 (Fig.5.6A). The cell number assays indicated that in each case the growth rate of this proportion of cells was similar either at 39.5°C or when returned to 33.0°C. Therefore, this may represent the sub-population of cells which failed to growth arrest at 39.5°C and not cells which had growth arrested at 39.5°C and subsequently started to
Figure 5.5: Ability of ts1135 + 5041 cell lines to grow at the permissive temperature following growth arrest at the non-permissive temperature.

A. *ts1135 + 5041* cell line 6

B. *ts1135 + 5041* cell line 7

C. *ts1135 + 5041* cell line 10

The indicated cell lines were assayed for their ability to grow at the permissive temperature after growth arrest resulting from incubation at the non-permissive temperature for 7 days. The line graphs indicate the number of cells obtained on day 1, day 4, day 7, day 10, day 14, day 17 and day 21 following the incubation of the cells (10,000 seeded per 10cm plate) at either the permissive temperature (33.0°C; — o— ), the non-permissive temperature (39.5°C; — — ) or cells which were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature ( — — o — — o — — ). The bar graphs represent the number of colonies obtained following the incubation of the same cell lines (1000 cells seeded per 6cm plate) after incubation at either the permissive temperature (33°C), the non-permissive temperature (39°C) or that were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature (39°C to 33°C). Colonies were stained after two weeks (33.0°C plates) or three weeks (39.5°C and 39.5°C to 33.0°C plates) with methylene blue and counted.
Figure 5.6: Ability of ts1135 + 5031 cell lines to grow at the permissive temperature following growth arrest at the non-permissive temperature.

A. ts1135 + 5031 cell line 1

B. ts1135 + 5031 cell line 7

C. ts1135 + 5031 cell line 17

The indicated cell lines were assayed for their ability to grow at the permissive temperature after growth arrest resulting from incubation at the non-permissive temperature for 7 days. The line graphs indicate the number of cells obtained on day 1, day 4, day 7, day 10, day 14, day 17 and day 21 following the incubation of the cells (10,000 seeded per 10cm plate) at either the permissive temperature (33.0°C; — o— ), the non-permissive temperature (39.5°C; --- o--- ) or cells which were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature (--- o--- ). The bar graphs represent the number of colonies obtained following the incubation of the same cell lines (1000 cells seeded per 6cm plate) after incubation at either the permissive temperature (33°C), the non-permissive temperature (39°C) or that were shifted (the day after plating) to the non-permissive temperature for 7 days then shifted back to the permissive temperature (39°C to 33°C). Colonies were stained after two weeks (33.0°C plates) or three weeks (39.5°C and 39.5°C to 33.0°C plates) with methylene blue and counted.
proliferate when returned to 33.0°C. These cells appeared to have grown irrespective of the presence of the ts1135 T antigen. This also appeared to be the case with tsA58 cell line 17 (Fig. 5.2C) and tsa14 (Fig. 5.1B). Both these cell lines grew similarly following 7 days at 39.5°C whether they were held at 39.5°C or returned to 33.0°C. Only with tsA58 cell line 16 (Fig. 5.2B) and tsa8 (Fig. 5.1A) did the proportion of cells which failed to arrest following 7 days at 39.5°C grow more rapidly when they were returned to 33.0°C, suggesting that the tsA58 T antigen resumed a role in the growth of this sub-population of cells.

It is clear from this data that the presence of the functions carried by the 5041 or 5031 T antigen molecule at the non-permissive temperature was insufficient to prevent the majority of cells from becoming irreversibly growth arrested in the ts1135+5041/5031 cell lines. The variable proportion of cells which were somehow able to overcome this growth arrest did not appear to be influenced by the reintroduction of the functions carried by the ts1135 molecule when the cells were returned to the permissive temperature. Alternatively the ts1135 protein may not have been sufficiently inactivated in this sub-population of cells when they were initially shifted to the non-permissive temperature.

5.2.2: Complementation of the growth defect of the conditional cell lines by the introduction of wild type or dl1135 T antigen.

I made several attempts to complement the growth defect of the conditionally immortal cell lines (both tsA58 and ts1135+5041/5031) which I had isolated. The conditional cell lines were transfected with recombinants expressing either wild type (plasmid SE), dl1135 (dl1135) mutant or no (pKS) T antigen along with a recombinant plasmid expressing hygromycin-B resistance. Transfected cells were selected in hygromycin-B at either the permissive (33.0°C) or non-permissive (39.5°C) temperature. After 2-3 weeks representative colonies were isolated at the permissive temperature, the plates stained and the colonies counted. The results from three experiments are shown in Table 5.1. The number of colonies obtained at 39.5°C and 33.0°C is given along with the percentage number of colonies obtained at 39.5°C compared to 33.0°C (percentage complementation). Where the experiment was carried out more than once the average percentage complementation is shown in the right hand column. I attempted to complement the growth defect in the same cell lines used to determine the irreversibility of the growth arrest (six tsA58 cell lines including tsa8 and tsa14, three ts1135+5041 cell lines and three ts1135+5031 cell lines).

Of these cell lines only the tsa14 cell line was directly complemented by wild type and dl1135 T antigen (yielding complementation at 71.2% and 52.6% respectively). These results confirmed those seen previously (Chapter 3). None of the other cell lines
Table 5.1: Complementation of the growth defect of conditionally immortal cell lines by wild type T antigen or mutant d/1135.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Recombinant encoding:</th>
<th>Number of densely staining colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>39.5°C / 33°C (% complementation)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>tsa8 SE</td>
<td>-</td>
<td>9/325 (2.8%)</td>
</tr>
<tr>
<td>tsa8 pKS</td>
<td>-</td>
<td>28/1013 (2.8%)</td>
</tr>
<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>0/129 (0.0%)</td>
</tr>
<tr>
<td>tsa14 SE</td>
<td>-</td>
<td>1088/1528 (71.2%)</td>
</tr>
<tr>
<td>tsa14 pKS</td>
<td>-</td>
<td>33/530 (6.2%)</td>
</tr>
<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>745/1417 (52.6%)</td>
</tr>
<tr>
<td>tsA58 line 2 SE</td>
<td>-</td>
<td>58/892 (6.5%)</td>
</tr>
<tr>
<td>tsA58 line 2 pKS</td>
<td>-</td>
<td>1/699 (0.1%)</td>
</tr>
<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>1/411 (0.2%)</td>
</tr>
<tr>
<td>tsA58 line 8 SE</td>
<td>-</td>
<td>109/832 (13.1%)</td>
</tr>
<tr>
<td>tsA58 line 8 pKS</td>
<td>-</td>
<td>20/919 (2.2%)</td>
</tr>
<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>81/678 (11.9%)</td>
</tr>
<tr>
<td>tsA58 line 16 SE</td>
<td>-</td>
<td>23/905 (2.5%)</td>
</tr>
<tr>
<td>tsA58 line 16 pKS</td>
<td>-</td>
<td>0/1459 (0.0%)</td>
</tr>
<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>0/312 (0.0%)</td>
</tr>
<tr>
<td>tsA58 line 17 SE</td>
<td>-</td>
<td>15/884 (1.7%)</td>
</tr>
<tr>
<td>tsA58 line 17 pKS</td>
<td>-</td>
<td>0/1264 (0.0%)</td>
</tr>
<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>0/291 (0.0%)</td>
</tr>
<tr>
<td>ts1135+5041 line 6 SE</td>
<td>-</td>
<td>125/781 (16.0%)</td>
</tr>
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<td>ts1135+5041 line 6 pKS</td>
<td>0/108 (0.0%)</td>
<td>1/941 (0.1%)</td>
</tr>
<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>0/145 (0.0%)</td>
</tr>
<tr>
<td>ts1135+5041 line 7 SE</td>
<td>-</td>
<td>126/345 (36.5%)</td>
</tr>
<tr>
<td>ts1135+5041 line 7 pKS</td>
<td>-</td>
<td>2/722 (0.3%)</td>
</tr>
<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>2/219 (0.9%)</td>
</tr>
<tr>
<td>ts1135+5041 line 10 SE</td>
<td>-</td>
<td>25/737 (3.4%)</td>
</tr>
<tr>
<td>ts1135+5041 line 10 pKS</td>
<td>0/124 (0.0%)</td>
<td>0/958 (0.0%)</td>
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<tr>
<td>d/1135 SE</td>
<td>-</td>
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</tr>
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<td>-</td>
<td>30/966 (3.1%)</td>
</tr>
<tr>
<td>ts1135+5031 line 1 pKS</td>
<td>-</td>
<td>1/1545 (0.1%)</td>
</tr>
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<td>d/1135 SE</td>
<td>-</td>
<td>0/584 (0.0%)</td>
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<td>5/634 (0.8%)</td>
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<td>ts1135+5031 line 7 pKS</td>
<td>0/556 (0.0%)</td>
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<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>0/309 (0.0%)</td>
</tr>
<tr>
<td>ts1135+5031 line 17 SE</td>
<td>-</td>
<td>6/559 (1.1%)</td>
</tr>
<tr>
<td>ts1135+5031 line 17 pKS</td>
<td>0/122 (0.0%)</td>
<td>0/391 (0.0%)</td>
</tr>
<tr>
<td>d/1135 SE</td>
<td>-</td>
<td>0/13 (0.0%)</td>
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The indicated recombinants were transfected into the indicated cell line using a recombinant plasmid carrying hygromycin-B resistance as a coselectable marker (as described in Materials and Methods). Transfected cells were cultured at either the permissive temperature of 33°C or at the non-permissive temperature of 39.5°C until colonies were clearly visible. Representative colonies were picked at the permissive temperature. The plates were then stained with methylene blue and counted. ³Experiment was only carried out once.
Chapter 5.

were efficiently complemented by either wild type or dl1135 T antigen, including the tsa8 cell line. Wild type T antigen did show a slight amount of complementation in most of the cell lines. The percentage complementation obtained with wild type T antigen was consistently a little higher than that obtained with the control (pKS) transfections. The average percentage complementation obtained with wild type T antigen ranged from 36.5% (ts1135+5041 cell line 7) to 0.8% (ts1135+5031 cell line 7). The percentage complementation obtained following transfection of dl1135 was generally similar to that obtained with the control transfection. Clearly these cell lines are unable to be complemented directly, following transfection of either wild type or dl1135 T antigen, as efficiently as the tsal4 cell line. This may reflect the fact that the majority of these cell lines are more conditional than the tsal4 cell line. It is possible that because these cell lines growth arrest irreversibly upon incubation at the non-permissive temperature more rapidly than the tsal4 cell line, there is not enough time for the transfected recombinant to be integrated and expressed. It certainly seems that wild type T antigen complemented growth more efficiently in the cell lines which were slightly less conditional (tsASS cell lines 2 & 8 and ts1135+5041 cell line 7; see colony assays, Figs.5.1C, 5.2A, 5.6B respectively).

To determine whether this were the case, cell lines isolated at the permissive temperature following each transfection were assessed for their ability to grow upon shift up to the non-permissive temperature (by colony assay). The percentage number of colonies formed at 39.5°C compared to 33.0°C (percentage complementation) for cell lines derived from each of the twelve original cell lines following transfection of recombinants SE, pKS or dl1135, are shown in Figures 5.7, 5.8 (tsA58 cell lines), 5.9 (ts1135+5041 cell lines) and 5.10 (ts1135+5031) cell lines. The majority of cell lines isolated following transfection of either wild type or dl1135 T antigen into both tsa8 and tsa14 (Fig. 5.7A & B) were successfully maintained by the transfected T antigen. This confirmed the previous results with tsa14 (Chapter 3) and demonstrated that maintenance of growth by dl1135 is not limited to one particular cell line, since it can also maintain the immortal state in the tsa8 cell line. dl1135 also maintained immortalisation in 2 out of 8 cell lines derived from tsa58 cell line 2 and 4 out of 8 cell lines derived from tsa58 cell line 8 (Figs. 5.7C & 5.8A respectively) while wild type T antigen (SE) maintained growth in all four tsa58 cell line 8 derived lines but only maintained growth in two of the tsa58 cell line 2 derived lines at 50% efficiency. The more conditional tsa58 cell lines 16 and 17 yielded cell lines with wild type and dl1135 T antigen (Fig. 5.8B & C) in which proliferation was not as efficiently maintained at the non-permissive temperature. Wild type T antigen was able to maintain some of these cell lines to some extent (mostly below 50% efficiency) while only one of the dl1135 cell lines was even poorly maintained in each case.
Figure 5.7: Ability of tsA58 cell lines isolated at 33.0°C following transfection of either wild type T antigen, mutant d/1135 or pKS to grow at 39.5°C: 1.

A. tsA8 (tsA58) cell lines

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Recombinant: SE  pKS  d/1135

B. tsA14 (tsA58) cell lines

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Recombinant: SE  pKS  d/1135

C. tsA58 cell line 2

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Recombinant: SE  pKS  d/1135

Cell lines isolated at the permissive temperature (33.0°C) following transfection of the indicated recombinant expressing either wild-type T antigen (SE), mutant d/1135 T antigen or no T antigen (pKS) were assayed for their ability to grow at the non-permissive temperature (39.5°C). An equal number of cells (approx. 1000) were plated on four dishes, two of which were shifted to 39.5°C while two were maintained at 33.0°C. After two weeks the plates were stained with methylene blue and colonies were counted. The percentage complementation is expressed as the percentage number of colonies obtained at the non-permissive temperature compared to the permissive temperature (%39.5°C/33.0°C).
Figure 5.8: Ability of tsA58 cell lines isolated at 33.0°C following transfection of either wild type T antigen, mutant d/1135 or pKS to grow at 39.3°C.

A. tsA58 cell line 8

B. tsA58 cell line 16

C. tsA58 cell line 17

Cell lines isolated at the permissive temperature (33.0°C) following transfection of the indicated recombinant expressing either wild-type T antigen (SE), mutant d/1135 T antigen or no T antigen (pKS) were assayed for their ability to grow at the non-permissive temperature (39.5°C). An equal number of cells (approx. 1000) were plated on four dishes, two of which were shifted to 39.5°C while two were maintained at 33.0°C. After two weeks the plates were stained with methylene blue and colonies were counted. The percentage complementation is expressed as the percentage number of colonies obtained at the non-permissive temperature compared to the permissive temperature (%39.5°C/33.0°C).
Figure 5.9: Ability of ts1135+5041 cell lines isolated 33.0°C following transfection of either wild type T antigen, mutant d/1135 or pKS to grow at 39.5°C.

A. ts1135+5041 cell line 6

B. ts1135+5041 cell line 7

C. ts1135+5041 cell line 10

Cell lines isolated at the permissive temperature (33.0°C) following transfection of the indicated recombinant expressing either wild-type T antigen (SE), mutant d/1135 T antigen or no T antigen (pKS) were assayed for their ability to grow at the non-permissive temperature (39.5°C). An equal number of cells (approx. 1000) were plated on four dishes, two of which were shifted to 39.5°C while two were maintained at 33.0°C. After two weeks the plates were stained with methylene blue and colonies were counted. The percentage complementation is expressed as the percentage number of colonies obtained at the non-permissive temperature compared to the permissive temperature (%39.5°C/33.0°C).
Figure 5.10: Ability of \(ts^{1135+5031}\) cell lines isolated at 33.0°C following transfection of either wild type T antigen, mutant \(d^{1135}\) or pKS to grow at 39.5°C.

A. \(ts^{1135+5031}\) cell line 1

Cell lines isolated at the permissive temperature (33.0°C) following transfection of the indicated recombinant expressing either wild-type T antigen (SE), mutant \(d^{1135}\) T antigen or no T antigen (pKS) were assayed for their ability to grow at the non-permissive temperature (39.5°C). An equal number of cells (approx. 1000) were plated on four dishes, two of which were shifted to 39.5°C while two were maintained at 33.0°C. After two weeks the plates were stained with methylene blue and colonies were counted. The percentage complementation is expressed as the percentage number of colonies obtained at the non-permissive temperature compared to the permissive temperature (%39.5°C/33.0°C).
Most of the cell lines derived from the transfection of wild type T antigen into either the ts1135+5041 (Fig. 5.9) or ts1135+5031 (Fig. 5.10) cell lines were maintained to a greater or lesser extent. Fewer of the dl1135 derived cell lines were maintained, however, with only 2 cell lines giving more than 50% the number of colonies at 39.5°C compared to 33.0°C (ts1135+5041 cell line 10#12 and ts1135+5031 cell line 17#14, Figs. 5.9C & 5.10C respectively). None of the cell lines derived from the transfection of the control DNA (pKS) into any of these cell lines (either tsA58, ts1135+5041 or ts1135+5031) were efficiently maintained at the non-permissive temperature.

The results show that growth at the non-permissive temperature in the conditionally immortal ts1135+5041/5031 cell lines could only be inefficiently maintained by wild type T antigen and was barely maintained by reintroduction of the dl1135 mutant protein. Two of the tsA58 cell lines (2 & 8) which I isolated could be maintained by either wild type or dl1135 T antigen while two other, slightly more conditional, tsA58 cell lines (16 & 17) were inefficiently maintained by wild type T antigen and were barely maintained by dl1135 T antigen.

5.3: Discussion.

In the previous chapter I demonstrated that cell lines isolated at the permissive temperature following the transfection of either tsA58, ts1135 plus 5041 or ts1135 plus 5031 into secondary rat embryo fibroblasts were conditionally immortal. Here I have demonstrated that the growth arrest of these cell lines at the non-permissive temperature is irreversible, despite the continued presence of the 5041 or 5031 protein in the ts1135+5041 or ts1135+5031 cell lines. When these conditionally immortal cell lines were returned to the permissive temperature following their incubation at the non-permissive temperature for 7 days, the majority of cells failed to resume growth. However a sub-population of the cells from each cell line were able to continue growing and the size of this population varied in each cell line. In the majority of cell lines this sub-population of cells appears to represent cells which have escaped growth arrest at the non-permissive temperature, rather than cells which have growth arrested and then resumed growth upon returning to the permissive temperature. This is indicated by the appearance of the same sub-population of cells, growing with the same kinetics, at both the permissive temperature and the non-permissive temperature, in most cell lines. Reactivation of the temperature sensitive mutant (ts1135 or tsA58) into this sub-population, by returning these cell lines to the permissive temperature, appears to have little effect on their growth when compared to the cells kept at the non-permissive temperature (i.e. in the absence of this protein). The manner by which these cells revert to the non-conditional phenotype is unknown. It is possible that this reversion occurs as
a result of a second site mutation, in which case it would be interesting to know whether these mutations affect a common pathway in the majority of cell lines and, if so, in which growth regulatory pathway these occur. Since one of the functions of T antigen removed when the ts1135+5041/5031 cell lines are shifted to the non-permissive temperature is the association with p53, it may be that the reversion event occurs in the p53 pathway. This could occur in several ways: the ts1135 T antigen protein may lose its temperature sensitivity and retain the ability to associate with p53; the ts1135 protein may become stabilised by another protein; or a cellular mutation may occur in p53 itself or in the gene for another protein involved in the p53 growth suppression pathway.

It is interesting that, in general, the cell lines conditionally immortalised with the ts1135+5041/5031 combination were more conditional than the tsA58 conditional cell lines (compare the tsA58 cell lines in Figures 5.1 and 5.2 to the ts1135+5041/5031 cell lines in Figures 5.5 and 5.6 and also Figure 4.3C with 4.3D). Most of the ts1135+5041/5031 cell lines yield far less growth at the non-permissive temperature (relative to the permissive temperature) than the tsA58 cell lines. I was surprised by this observation since these cell lines retain the stability of one of the cooperating T antigen mutants at the non-permissive temperature, meaning that at least some of the T antigen functions required for immortalisation are still present. I would have expected these cell lines to be more prone to reversion for this reason. It is possible that the continued presence of the 5041/5031 immortalisation functions in these cell lines drives the cells to divide, however the lack of T antigen's p53 binding activity causes the cells to undergo apoptosis. It has been suggested that T antigen is able to stabilise p53 through mechanisms other than simply binding it, and that this function is carried by a different region of T antigen than the region responsible for p53 binding (Deppert, Steinmayer and Richter, 1989; Tiemann and Deppert, 1994). It is possible that the 5041/5031 proteins bring about the stabilisation of p53, and when ts1135 is denatured at the non-permissive temperature this stabilised p53 is no longer sequestered by the ts1135 protein. The release of a large amount of stabilised p53 in the cells may bring about apoptosis and this would account for their greater degree of conditionality. Some cell death appeared to occur when the ts1135+5041/5031 cell lines were shifted to the non-permissive temperature, although I have not quantified or characterised this phenomenon. A quantifiable amount of cell death certainly occurred when ts1135+5031 cell line 1 is shifted to the non-permissive temperature (Fig. 5.6A) as indicated by the number of cells on the plates falling from almost 10,000 to 1000 in 10 days. It will be interesting to see whether cell death is occurring to a quantifiable degree in the other ts1135+5041/5031 conditional cell lines and whether it is occurring by apoptosis. Interestingly, cell death was not observed in the tsA58 cell lines isolated by myself or previously by Jat and Sharp (1989). Neither is apoptosis observed when conditionally
immortal cell lines isolated from the H.2kbtsA58 transgenic mouse are shifted to the non-permissive temperature (Jat et al., 1991). Zheng et al. (1994), however, did observe apoptosis in rat embryo fibroblasts conditionally immortalised with tsA58 T antigen when they were shifted to the non-permissive temperature. It is possible that the tsA58 protein in their cell lines is not being denatured as efficiently as in our cell lines, and the functions which are provided by the 5041/5031 proteins in my ts1135+5041/5031 cell lines at the non-permissive temperature, are still provided by the tsA58 protein in their cell lines at the non-permissive temperature. The difference in conditionality observed between the ts1135+5041/5031 and tsA58 cell lines suggests that there are differences in the T antigen expressed in these cells when they are shifted to the non-permissive temperature. These differences suggest that the two mutant proteins in the ts1135+5041/5031 cell lines are being expressed independently and not recombining or trans-splicing to express the tsA58 protein. However, this does not rule out the possibility that these differences may be due to four species of T antigen being expressed in these ts1135+5041/5031 cell lines (the ts1135 protein, the 5041 or 5031 protein, as well as the tsA58 protein and the ts1135+5041/5031 triple mutant protein which would result from recombination or trans-splicing).

The inability of the ts1135+5041/5031 cell lines to be maintained efficiently at the non-permissive temperature by either wild type or dl1135 T antigen is hard to explain. The lack of direct complementation may be due to the inability of the transfected cells to express the protein before the cells irreversibly growth arrest or even apoptose upon shift up to the non-permissive temperature. I attempted to overcome this problem by isolating and expanding cell lines at the permissive temperature following transfection and then shifting these cell lines to the non-permissive temperature. The ability of wild type T antigen to maintain growth in a small number of the cell lines, isolated at the permissive temperature following the transfection of recombinant SE, suggests that the level of protein expression may be important. Recombinant SE expresses a large amount of T antigen, which is more than that expressed by the dl1135 recombinant. It is possible that the amount of dl1135 protein expressed in the majority of these cell lines is insufficient to maintain growth upon the denaturation of the endogenous ts1135 protein at 39.5°C. Indeed it is possible that, for some reason, the dl1135 protein is not being expressed at all in the ts1135+5041/5031 cell lines when they are shifted to the non-permissive temperature. It may be that there is some kind of interference due to the amount of T antigen which is already being expressed in these cell lines. Since I have been unsuccessful in my attempts to distinguish between the mutant proteins when they are coexpressed, I have been unable to analyse these cell lines for expression of the transfected dl1135 mutant. However, it is interesting that more of the tsA58 derived conditionally immortal cell lines were rescued by dl1135
expression than those derived from the ts1135+5041/5031 cell lines. The observation that immortalisation could be maintained by dl1135 in most of the tsA58 cell lines while most of the ts1135+5041/5031 cell lines were not maintained by dl1135 provides further evidence that these cell lines are not conditionally immortal simply as a result of the recreation of tsA58 T antigen expression. If tsA58 T antigen expression, as opposed to the independent expression of ts1135 and 5041 or 5031, were the reason for the conditional immortality of these cells, a similar number of these ts1135+5041/5031 cell lines would be expected to be rescued by dl1135.

My inability to find a ts1135+5041/5031 cell line in which immortalisation could be maintained upon the reintroduction of the recombinant expressing the non-temperature sensitive dl1135 protein is disappointing. If such a cell line were identified it would be interesting see whether other immortalising oncogenes could also maintain its growth. That such a cell line retains, at the non-permissive temperature, a mutant T antigen which provides some, but not all, of the functions necessary for immortalisation, could aid the analysis of the activities of other oncogenes in immortalisation. It will be interesting to determine why the growth of these cell lines was not able to be complemented at the non-permissive temperature by reintroduction of non-temperature sensitive dl1135 mutant. Analysis of the phase(s) of the cell cycle in which these ts1135+5041/5031 cell lines growth arrest and comparison to the growth arrest of tsA58 cell lines may also yield interesting results.
Chapter 6.

Expression screening of a newt limb blastema cDNA library for cDNAs capable of immortalising rodent embryo fibroblasts.

6.1: Introduction.

Adult urodele amphibians such as the newt *Notophthalmus viridescens* are able to regenerate their limbs and tail after amputation. This limb regeneration is a position-dependent process; a hand is regenerated following amputation at the wrist whereas a complete arm is regenerated following amputation at the shoulder (reviewed by Stocum, 1984; Brockes, 1994). This regeneration memory for position can be respecified, in a graded and dose-dependent manner, by retinoic acid (Maden, 1982; Maden, 1985; Stocum and Crawford, 1987). Immediately after amputation the wound is covered by rapidly migrating epithelial cells, which form the wound epidermis. Regeneration proceeds with the mesenchymal tissues underlying the wound epidermis giving rise to blastemal cells, the progenitor cells of the regenerating limb (Ferretti and Brockes, 1991; Stocum, 1991). Dissociated mesenchymal blastemal cells (isolated from the limb blastema) can be serially passaged for hundreds of generations without detectable crisis or senescence (Ferretti and Brockes, 1988). These cells have been a valuable resource for studies on the nature of limb regeneration in the newt since they can be manipulated *in vitro* and subsequently reimplanted underneath the wound epidermis of an early regenerate and become incorporated into the host blastema (Brockes, 1992; Lo, Allen and Brockes, 1993; Pecorino, Lo and Brockes, 1994).

The nature of the immortal phenotype of these blastemal cells in culture is unknown. I have attempted to clone any immortalising factors expressed in the limb blastemal cells using the immortalisation of rat embryo fibroblast as a screen. A eukaryotic cDNA expression library was constructed using mRNA isolated from regenerating newt limb blastemas (by Clontech Laboratories, Inc.). I have screened this library for cDNAs able to establish immortal cell lines following transfection of the library into rat embryo fibroblasts. Any cDNAs isolated from these cell lines (by PCR) were characterised and were used to isolate the original cDNA clone from the library.
These library clones were subsequently retransfected into secondary REFs and again assayed for their ability to immortalise.

Several cDNAs were isolated by PCR from cell lines established following transfection of the library. In this chapter I describe how this expression screening technique has allowed the isolation of a cDNA fragment which is able to induce proliferation.

6.2: Results.

6.2.1: Description of the newt limb blastema cDNA library construction and conversion to recombinant plasmids.

mRNA was isolated from newt limb blastemal cells from regenerating newt limbs rather than from cultured cell lines established from the limb blastema. This was carried out by Philip Gates (Developmental Biology Laboratory, Ludwig Institute for Cancer Research, University College Branch). The cDNA library was constructed from this mRNA by Clontech Laboratories, Inc., in an expression vector, λDR2, which allows expression of the cloned cDNA in mammalian cells. The basic structure of λDR2 consists of an Epstein-Barr virus-derived plasmid vector embedded within vector arms derived from lambda phage (Swirski et al., 1992). Thus the high cloning efficiencies obtained with λgt10 and λgt11 can also be obtained with λDR2. The λDR2-newt limb blastema cDNA library could thus be stored, maintained and screened by DNA hybridisation similarly to any other lambda phage library. The cDNA was cloned directionally into the unique BamHI-XbaI sites of the phage vector (see Figure 6.1).

The λDR2-cDNA phages could be converted to pDR2-cDNA plasmids for transfection into mammalian cells by virtue of two bacteriophage P1-derived loxP recombination sites. These 34bp loxP (locus of crossing-over) sites consist of an 8bp asymmetric core region between two 13bp inverted repeats, which are binding sites for the Cre (causes recombination) recombinase, a member of the Int family of recombinases. Cre-mediated recombination occurs between two directly oriented loxP sites and results in the excision of the DNA between the loxP sites as a circular molecule with a single loxP site (reviewed by Sauer, 1993). The activities of the Cre recombinase involved in its ability to mediate recombination at loxP sites are its topoisomerase activity and its ability to generate a 6bp staggered cut within the 8bp core region with a 5' overhang. This Cre-loxP site-specific recombination can be achieved in vivo, by either transfecting eukaryotic cells or infecting prokaryotic cells expressing the Cre recombinase, or in vitro, by incubating the DNA carrying two loxP sites with purified Cre recombinase in the appropriate buffer conditions. In the case of the preparation of pDR2-cDNA plasmids, this was achieved by infecting a restriction minus bacterial strain, expressing Cre recombinase (E.coli AM1), with the λDR2-cDNA library. The
recombination and circularisation events produced pDR2 plasmids carrying the cDNA inserts (Figure 6.1). Large scale conversions of the λDR2 newt limb blastema cDNA library to recombinant pDR2 plasmids (pDR2-library) were carried out by Diana Wylie (Developmental Biology Laboratory, Ludwig Institute for Cancer Research, University
The pDR2 plasmid carries the hygromycin resistance gene allowing the selection of transfected cells in hygromycin-B. Stable cDNA expression in mammalian cells was driven from the Rous Sarcoma virus (RSV LTR) promoter (Gorman et al., 1982) and transcripts were polyadenylated by virtue of the SV40 polyadenylation signals.

### 6.2.2: Transfection of the newt limb blastema expression library into secondary rat embryo fibroblasts.

For this expression screening experiment it was necessary to maximise the number of transfection events, thereby ensuring that the maximum number of cDNA clones possible are taken up and expressed in the secondary REFs. If the number of independent cDNA clones present in the whole library were, for example, $10^6$ it would be necessary to ensure that at least $10^6$ independent clones were taken up by the transfected cells. It was also necessary to ensure that transfection of a large amount of plasmid DNA expressing resistance to hygromycin-B did not lead to a confluent monolayer of hygromycin-B resistant cells. It had previously been observed that if transfected REFs were plated at too high a density the cells often appeared to survive the hygromycin-B selection more efficiently, even though these cells did not grow when they were isolated. If the transfected cells were plated more sparsely, however, the selection appeared to be much more efficient. It was therefore necessary to obtain the maximum transfection efficiency and to ensure that the transfected cells were plated at a sufficiently low density to allow efficient selection for colony formation.

Electroporation had previously been shown to be the most efficient method of transfecting REFs, yielding 10-100 fold more colonies than transfection by either calcium phosphate or lipofection (Z.Ikram & P.S.Jat, Transformation Studies Laboratory, Ludwig Institute for Cancer Research, University College Branch, unpublished data). Secondary REFs were therefore transfected by electroporation, with $10^7$ cells being used for each electroporation. Table 6.1 shows the results for four separate experiments. The total number of colonies obtained, following incubation of the transfected cells for 4-5 weeks, are shown. The transfected cells were incubated at 33.0°C since the stability of mRNAs expressed from newt-derived cDNAs was not known. It was felt that incubation of the cells at a temperature closer to the temperature at which newt blastemal cells are normally maintained may increase the stability of the newt-derived mRNAs in the rat cells. The number of cell lines successfully expanded from the number of colonies isolated is also shown in Table 6.1. Plasmid DNA prepared from a single phage plaque, selected at random from the cDNA library, was used as a negative control in each experiment (pDR2-S).

In the first two experiments $10^7$ REFs were electroporated with a total of 40μg of the library DNA. The transfected cells from each of these electroporations were
Table 6.1: Results of electroporations of newt limb blastema eukaryotic cDNA expression library into secondary rat embryo fibroblasts.

<table>
<thead>
<tr>
<th>Electroporation</th>
<th>Amount of pDR2 DNA transfected</th>
<th>Total number of colonies obtained</th>
<th>Number of cell lines derived from number of colonies isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE + pSV2neo</td>
<td>-</td>
<td>2717</td>
<td>N.D.</td>
</tr>
<tr>
<td>SE + pDR2-S</td>
<td>40.0μg</td>
<td>11</td>
<td>N.D.</td>
</tr>
<tr>
<td>pDR2-S</td>
<td>40.0μg</td>
<td>30</td>
<td>0/16</td>
</tr>
<tr>
<td>pDR2-library</td>
<td>40.0μg</td>
<td>56</td>
<td>0/46</td>
</tr>
</tbody>
</table>

| Expt. 2         |                                |                                 |                                                             |
|-----------------|                                |                                 |                                                             |
| pDR2-S          | 40.0μg                         | 52                              | 0/19                                                        |
| pDR2-library    | 40.0μg                         | 148                             | 0/114                                                       |

| Expt. 3         |                                |                                 |                                                             |
|-----------------|                                |                                 |                                                             |
| SE + pSV2neo    | 2.5μg                          | 679                             | N.D.                                                        |
| pDR2-library    |                               |                                 |                                                             |
| (a)             | 2.5μg                          | 3                               | 2/2                                                         |
| (b)             | 2.5μg                          | 1                               | 3/3                                                         |
| (c)             | 2.5μg                          | 1                               | 2/2                                                         |
| (d)             | 2.5μg                          |                                 |                                                             |
| (e)             | 2.5μg                          |                                 |                                                             |
| (f)             | 2.5μg                          |                                 |                                                             |
| (g)             | 2.5μg                          |                                 |                                                             |
| (h)             | 2.5μg                          |                                 |                                                             |
| (i)             | 2.5μg                          |                                 |                                                             |
| (j)             | 2.5μg                          |                                 |                                                             |

| Expt. 4         |                                |                                 |                                                             |
|-----------------|                                |                                 |                                                             |
| pDR2-S          | 1.5μg                          |                                 | 0/20                                                        |
| pDR2-library    |                               |                                 |                                                             |
| (a)             | 1.5μg                          |                                 | 2/14                                                        |
| (b)             | 1.5μg                          |                                 | 1/23                                                        |
| (c)             | 1.5μg                          |                                 | 3/25                                                        |
| (d)             | 1.5μg                          |                                 | 5/24                                                        |
| (e)             | 1.5μg                          |                                 | 6/20                                                        |
| (f)             | 1.5μg                          |                                 | 6/27                                                        |
| (g)             | 1.5μg                          |                                 | 1/29                                                        |
| (h)             | 1.5μg                          |                                 | 2/21                                                        |
| (i)             | 1.5μg                          |                                 | 1/30                                                        |
| (j)             | 1.5μg                          |                                 | 4/18                                                        |

Secondary rat embryo fibroblasts were transfected (10^7 cells per electroporation) with the indicated amount of the pDR2 cDNA library (pDR2-lib) using pKS (Bluescript) as carrier DNA (50μg DNA per electroporation). Control transfections were carried out using a single clone picked at random from the library (pDR2-S). Electroporated cells were plated on to either ten 15cm plates (experiments 1 & 2) or four 15cm plates (experiments 3 & 4), selected in 100μg/ml hygromycin-B and incubated at 33.0°C. Cells transfected with SE+pSV2neo were selected in 250μg/ml Geneticin-G418. The indicated number of colonies were picked after 4-5 weeks and expanded wherever possible. The remaining cells on the plates were then either stained and the remaining colonies counted or genomic DNA was prepared from them. expt.= experiment; N.D.= not done.
plated onto ten large (15cm) tissue culture plates. The number of colonies derived from the electroporation of recombinant SE (which expresses wild type SV40 large T antigen) plus 40µg pDR2-S (experiment 1) was much lower than the number obtained from the transfection of SE plus 1.5µg pSV2neo (followed by selection in G418) which was used as a control for the electroporation. The high number of colonies obtained with the SE+pSV2neo transfection demonstrated that the electroporations were working efficiently, however the fact that only 11 colonies were obtained from the transfection of SE+pDR2-S indicated that the hygromycin selection was not working efficiently. It is possible that the transfection of such a large amount of a plasmid (40µg) which expressed resistance to hygromycin-B may have been toxic to a large number of the transfected cells.

A number of colonies were obtained following the transfection of both pDR2-S and the pDR2-library, and in both experiments the pDR2-library yielded more colonies than pDR2-S thereby indicating that transfection of the pDR2-library may allow the expression of a number of cDNAs with growth stimulatory effects. However, when representative colonies were isolated none of them successfully yielded cell lines.

For the third and fourth experiments multiple electroporations (ten per experiment) were carried out using a smaller amount of the pDR2-library (1.5 or 2.5µg) for each electroporation. Thus a total of 25µg of pDR2-library DNA was transfected into 10^8 cells for experiment 3 and 15µg into 10^8 cells for experiment 4. A similar amount of pDR2-S was used for one control electroporation in each experiment. The transfected cells from each electroporation were plated onto four large plates and each electroporation was kept separate. Experiment 4 yielded approximately 5-fold more colonies than experiment 3 and, after the healthiest looking colonies were picked from each experiment, genomic DNA was prepared from the remainder of the cells on the plates. This was carried out in case it was ever felt necessary to amplify, by PCR, any cDNAs present in these remaining cells. While the majority of the colonies isolated from the pDR2-library transfections failed to yield cell lines, a total of 43 cell lines were successfully expanded (from a total of 263 colonies isolated). 12 cell lines were derived from experiment 3 and 31 from experiment 4. Most of these cell lines grew slowly compared to the rate of growth observed in cell lines immortalised by SV40 large T antigen, however they did grow. It is unlikely that the growth stimulated in rat cells by a newt limb blastema cDNA would very rapid. It is possible that these cell lines may have acquired a second event, which in cooperation with the newt limb blastema cDNA, allowed the cells to become immortal. A proportion of these cell lines may have become established by simply coming through crisis (Todaro and Green, 1963) and these spontaneously immortal cell lines may not have required the expression of a newt limb blastema cDNA at all. However, no spontaneously immortal cell lines were obtained from the control transfections of pDR2-S.
Figure 6.2: Position of primers designed to amplify integrated pDR2-library cDNA sequences by PCR.

The DNA sequence surrounding the cDNA cloning site (between BamHI and Xbal) of pDR2 (Swirski et al, 1992) is shown, along with the position of the Rous Sarcoma virus long terminal repeat promoter (RSV LTR) and the polyadenylation signal sequences from SV40 (SV40 pA). Two pairs of primers were designed to enable amplification of the integrated cDNA following transfection of the library (designated external upstream (E_u) and downstream (E_d) and internal upstream (I_u) and downstream (I_d)). The position of two primers (23mer and 21mer) used for sequencing either the cloned PCR fragments or cDNAs are also shown.

6.2.3: Amplification of pDR2 cDNA inserts from established cell lines.

Once the colonies were isolated following transfection of the library and while they were being expanded, wherever possible, into cell lines, they were grown in the absence of hygromycin-B. This ensured that the only selective pressure on the cells to retain the integrated pDR2-cDNA was the cell's requirement for the cDNA for continued growth. If the pDR2-cDNA was not required for continued growth it could be shed.
from the genome of the rat cells. Genomic DNA was prepared from the cell lines which were successfully established at the earliest passage possible (p3) and a later passage (p6). Any pDR2-cDNA insert which was retained by the cell lines was amplified from the genomic DNA by PCR using primers (designed by myself) specific for sites either side of the cDNA cloning site in pDR2. The position of these primers, designated external upstream (Eu) and downstream (Ed) and internal upstream (Iu) and downstream (Id), are shown in Figure 6.2, along with the restriction sites surrounding the cDNA insert, the position of the RSV LTR promoter and the SV40 polyadenylation signals. Initially PCR amplification of the genomic DNAs from all the cell lines isolated was carried out using the external (Eu/d) primers and 1μl of this PCR was subsequently amplified with the internal (Iu/d) primers. There were 2 reasons for this: Firstly, I was initially worried about the suitability of these primers for PCR and I wished to ensure that any fragments obtained were specific; and secondly, I wanted to insure that I could amplify a cDNA insert, present at only one copy per cell, even if the genomic DNA preparation was not very good. Subsequent PCR amplifications, and all those shown here, were amplified once with the internal (Iu/d) primers. Of the 43 cell lines successfully established only 16 yielded PCR fragments. Those cell lines which failed to yield PCR fragments may have been either spontaneously immortal cell lines which shed the pDR2 DNA once the hygromycin selection was removed or cell lines which retained a newt limb blastema cDNA, which was still required for growth, but had lost or mutated the site for one, or both, of the two PCR primers.

The results obtained following the PCR amplification (with Iu/d) of genomic DNA prepared from eight cell lines, at passages 3 and 6, are shown in Figure 6.3. Genomic DNA prepared from the REF-derived tsa14 cell line at passage 24 was used as a control to demonstrate the specificity of the PCR amplification (lane 12). All eight of these cell lines yielded PCR fragments at the earlier passage (p3). The size of the DNA fragments ranged from below 300bp (cell lines b1 and d2, lanes 6 & 8) to approximately 2000bp (cell line h31- not shown here). Of the 16 cell lines which yielded PCR fragments at passage 3 only four of them retained these cDNAs at the later passage (p6). The PCR amplification of genomic DNA from all four of these cell lines is shown here (cell lines h4, lanes 2 & 3; d21, lanes 4 & 5; c41, lanes 10 & 11 and a21, lanes 17 & 18). Genomic DNA prepared from the other 12 cell lines at the later passage no longer yielded PCR fragments, indicating that the pDR2-cDNA had been shed or that the site for one of the primers had been lost or mutated.

6.2.4: One pDR2-cDNA fragment was isolated independently from two cell lines.

To ascertain whether any of the cDNA fragments amplified by PCR contained the same sequences, I cross-hybridised Southern blots of agarose gels containing these
Figure 6.3: Fragments obtained following PCR with pDR2 internal primers on genomic DNA prepared from REF+pDR2-library cell lines at early and later passages.

The colonies obtained following transfection of the pDR2 newt limb blastema cDNA expression library into secondary REFs were isolated and those which expanded successfully into clonal cell lines were passaged. Genomic DNA was prepared from the indicated cell lines at an early passage (p3) and at a later passage (p6) and amplified by PCR using pDR2 internal (Lu and Ld) primers (as described in Materials and Methods). The resulting fragments were separated on 1.8% agarose gels (with 1.0μg/ml EtBr) in 1X TAE alongside approximately 1.0μg 1kb DNA ladder (GibcoBRL). Approximate sizes are shown on the left side. The results for several representative cell lines are shown here. Genomic DNA prepared from tsa14 cells (p24) was similarly amplified to ensure the PCR conditions were specific.
Genomic DNA was prepared from the cell lines which were successfully established following transfection of the pDR2 newt limb blastema cDNA expression library into secondary REFs. The genomic DNA (from p3) was amplified using the pDR2 internal (I₂ and I₃) primers (as described in Materials and Methods). The resulting fragments were fractionated on 1.8% agarose gels (with 1.0μg/ml EtBr) in 1X TAE alongside approximately 1.0μg/ml 1kb DNA ladder (GibcoBRL). The fractionated DNA was then transferred to Zetabind® membrane (CUNO, Inc.). The filter was probed with the h4 PCR fragment which was isolated and random-prime labelled with α³²P-dCTP.

PCR fragments. This allowed me to determine whether I had isolated duplicate cDNA sequences in the expression screening. If I had been successful in isolating the same cDNA, from two independent transfections, this cDNA could be an ideal candidate to pursue as a factor capable of stimulating proliferation.

Firstly, I probed my Southern blots with any PCR fragments which seemed to be a similar size to others. The PCR fragments obtained from cell lines h4 and d21 (lanes 2, 3, 4 & 5) and from cell lines a21 and f43 (lanes 17, 18 & 19) seemed to be of similar sizes (approximately 1.0kb and 1.5kb respectively). The fragments to be used as DNA probes were isolated on 1.8% low melting point agarose gels, purified and random prime labelled with α³²P-dCTP (as described in Materials and Methods). Hybridisations were carried out in formamide hybridisation buffer and washed up to as high as 70°C in 0.1%
(w/v) SDS/0.1X SSC to minimise cross-reaction. The a21 PCR fragment failed to hybridise to the f43 fragment or another similarly sized fragment from cell line e11 (not shown here). However the PCR fragment obtained from cell line h4 did cross hybridise to the d21 fragment (Fig. 6.4, lanes 5 & 10). The difference in intensity of the hybridisation merely reflected the difference in the amount of DNA loaded on the agarose gel. None of the other PCR fragments cross-hybridised each other.

6.2.5: Characterisation of the h4 and d21 PCR fragments.

The h4 and d21 cell lines were clearly the result of immortalisation by the same pDR2 newt limb blastema library cDNA in two independent experiments (experiment 3, electroporation (h) and experiment 4, electroporation (d); Table 6.1). Furthermore, both cell lines retained the 1.0kb cDNA insert upon passaging (Figure 6.3, lanes 2, 3, 4 & 5). These results clearly suggested that this cDNA, derived from the newt limb blastema, was able to stimulate growth in rat embryo fibroblasts. I therefore concentrate here upon the characterisation of the PCR fragments from these two cell lines.

Both the h4 and d21 cDNA fragments (PCR amplified by the lu & Id primers) were isolated on low melting point agarose (1.8%), purified and ligated, by virtue of the single 3' A-overhang, added by Taq DNA polymerase, into a TA cloning vector, pCRII™ (Invitrogen Corporation). The ligations were transformed into E.coli OneShot™ and selected on ampicillin, X-gal and IPTG. White colonies were verified for the presence of a cloned insert by PCR with the pDR2 lu and Id primers. Two of the resulting clones, pCRIF™h4/28 and pCRII™d21/4, were sequenced using the 23mer and Id primers (Fig. 6.2) by automated sequencing (ABI).

The automated sequencing yielded approximately 450bp of sequence from either end of the PCR fragments, however I was unsure of the accuracy of this sequence. I therefore restriction mapped three other clones, pCRII™ clones h4/26, d21/3 and d21/5, in the hope of identifying shorter restriction fragments to clone and sequence manually. Figure 6.5 shows the basic restriction maps of these three clones and the fragments which were subsequently cloned and sequenced. Clones h4/26 and d21/5 (Fig. 6.5A) were both cloned into pCRII™ in the same direction, and both contained an internal BamHI site approximately 300bp from one end of the cDNA. This BamHI site allowed me to clone two smaller fragments, a 300bp BamHI-BamHI fragment and a 660bp BamHI-SalI fragment, into pBluescript™ (Stratagene) at the appropriate restriction sites within its multiple cloning site. These fragments were subsequently sequenced manually by dideoxy-sequencing using the T3 and T7 primers either side of the multiple cloning site. Interestingly clone d21/3 (Fig 6.5B), did not have a BamHI restriction site within its cloned PCR fragment. However, it did have an EcoRI site approximately 380bp from the pDR2 BamHI site, which was not observed in either clones h4/26 and d21/5. The anomaly between the restriction maps of the three clones could explained by
Figure 6.5: Restriction maps of cloned h4/d21 PCR fragments in pCRII™ showing fragments subsequently cloned into pBluescript™.

A: Map of pCRII™ clones h4/26 and d21/5

Restriction maps of cloned h4/d21 l_u and l_d PCR fragments showing the restriction sites within the pDR2 cDNA cloning site (*), the HindIII sites either side of the pDR2 cDNA cloning site and some of the restriction sites within the pCRII™ cloning site. Sequences derived from pDR2 are shaded. The internal BamHI (pCRII™ clones h4/26 & d21/5) and EcoRI (pCRII™ clone d21/3) restriction sites are also shown. These sites were subsequently used to sub-clone smaller fragments into pBluescript™ for sequencing. The explanation for the difference in restriction map between the d21 PCR fragments was revealed upon sequencing these and other clones (the sequence is shown in figure 6.6) and is explained in the text. The orientation of the cloned PCR fragments with respect to the RSV-LTR of pDR2 is indicated by the arrow. The position of the pDR2 23mer, l_u and l_d primers is shown.
Chapter 6.

Figure 6.6: Alignment of h4/d21 consensus nucleotide sequence with the sequence of the cDNA library clone L3 in the overlapping region.

<table>
<thead>
<tr>
<th>h4/d21</th>
<th>L3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>BamHI</td>
</tr>
<tr>
<td>h4/d21</td>
<td>L3</td>
</tr>
<tr>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td>121</td>
<td>181</td>
</tr>
<tr>
<td>241</td>
<td>481</td>
</tr>
<tr>
<td>301</td>
<td>541</td>
</tr>
<tr>
<td>601</td>
<td>661</td>
</tr>
<tr>
<td>721</td>
<td>781</td>
</tr>
<tr>
<td>841</td>
<td>901</td>
</tr>
<tr>
<td>961</td>
<td></td>
</tr>
</tbody>
</table>

The PCR fragments obtained by amplifying genomic DNA from REF cell lines h4 and d21 using pDR2 Lu and L4 primers were cloned into pCRRII™. The h4/d21 consensus sequence was assembled from an automated sequence of one h4 clone and one d21 clone (using pDR2 23mer & L4 primers for sequencing) and manual sequencing (using pKS- T3 and T7 primers) of the sub-cloned fragments from 3 of these clones (one h4 & two d21- mapped in figure 6.5). Clone L3 was isolated by Philip Gates through screening the A.DR-2 newt limb blastema cDNA library with the h4 PCR fragment. The restriction sites surrounding the pDR2 cDNA cloning site are shown as well as the internal BamHI site. The differences between the DNA sequences are shown in boxes, including the 22 base pair deletion. The nucleotides marked with an asterisk (*) are mutated in one of the d21 PCR clones (pCRRII™ d21/3) giving rise to an EcoRI site (G->A at position 407) and loss of the internal BamHI site (G->T at position 304).
base pair substitutions having been introduced by the *Taq* DNA polymerase during the PCR amplification. pCRII™ clone d21/3 also had the PCR fragment cloned in the opposite orientation compared to clones h4/26 and d21/5. The indicated restriction fragments, a 640bp *EcoRI-EcoRI* fragment and a 380bp *EcoRI-BamHI* fragment, were cloned into pBluescript™ and sequenced by dideoxy-sequencing using the T3, T7 and Id primers.

A consensus sequence was assembled from the automated sequence obtained from pCRII™ clones h4/28 and d21/4 and the manual sequence obtained from the h4/26, d21/3 and d21/5 restriction fragments subcloned into pBluescript™. This consensus sequence is shown in Figure 6.6 (upper sequence). The two base pair substitutions which destroyed the *BamHI* site and gave an *EcoRI* site in pCRII™ clone d21/3 were revealed when the consensus sequence was assembled and are marked with asterisks in Figure 6.6. The position of the restriction sites within the pDR2 cDNA cloning site are shown at each end of the sequence. The cDNA sequence obtained had no polyA tail and as a result no indication could be obtained as to whether the cDNA had been cloned directionally when the library was constructed. Unfortunately, no open reading frame was found on either strand of the cDNA and the sequence had no homology to any known genes when it was used to search the GenEMBL database.

A number of the other PCR fragments were also cloned and sequenced. These including the 1.5kb fragment from a21 and the 0.7kb fragment from c41, both of which were retained during passaging. The sequence obtained from the a21 fragment revealed that it originated from *E.coli*, which probably was a result of a cloning artifact during the construction of the cDNA library. The c41 fragment sequence showed no homology to any previously cloned cDNA and had no open reading frame.

### 6.2.6: Isolation and sequence of cDNA library clones for h4/d21.

Despite the h4/d21 PCR fragment sequence showing no evidence of an open reading frame, I had isolated it from two independent experiments. Moreover, it was retained when the cell lines were passaged and thus warranted further investigation. The fact that it was isolated from two separate experiments possibly could be explained by it having a relatively high abundance in the newt limb blastema cDNA library. Philip Gates used the h4 Id/Id PCR fragment to probe a northern blot containing mRNA from the newt limb blastema. The probe hybridised to an mRNA of approximately 6.0kb which was present at a relatively low abundance. He then used the h4 PCR fragment to screen the λDR2 newt limb blastema cDNA library. From the screening of approximately 1.5x10⁶ plaques only four positively hybridising plaques were identified. Three of these plaques, clones L2, L3 and L4 were subsequently isolated and sequenced by Philip.

The features of these three clones are shown in Figure 6.7 and their relationship
The \(\lambda\)DR2 newt limb blastema library was screened using the h4 I\(_0\)/I\(_d\) PCR fragment. Three cDNA clones were isolated and sequenced. Two of these (clones L2 & L3) were of similar length (approximately 1.3kb) while the other (clone L4) was longer (approximately 1.9kb). L2 and L3 were found to have been cloned into \(\lambda\)DR2 in different orientations and both had an extra 300bp (approximately) as well as the 1.0kb encompassed by the h4/d21 PCR fragments. At the end of this extra 300bp both the clones had polyA sequences. The orientation of these polyA sequences in relation to the pDR2 RSV promoter (RSV LTR) indicates that both the immortalising clones h4 & d21 and clone L3 would express the anti-sense mRNA from the promoter, while clone L2 would express the sense mRNA. The figure shows the relationship of these clones to each other and their orientation relative to the pDR2 RSV promoter (shown as an arrow) and the SV40 polyadenylation signal (SV40 pA). The restriction sites used to clone the cDNAs into the \(\lambda\)DR2 arms are also shown (\(B=\)BamHI and \(X=XbaI\)). The third library clone as well as having the extra 300bp common to L2 & L3 also contained a further 600bp at the other end of its cDNA (i.e. 5' to the cDNA's polyA sequences). Both clones L3 and L4 contained an extra 22bp within their cDNAs which were present in neither the clone L2 cDNA or the original h4/d21 PCR fragments. Base pair numbers given are to the closest 100bp. The shaded area represents the 1.0kb common to the library clones and the original PCR fragments (which is interrupted by the 22bp insert in L3 and L4). Both the library screening and the sequencing of L2, L3 and L4 were carried out by Philip Gates (Developmental Biology Laboratory, Ludwig Institute for Cancer Research, University College Branch).

Figure 6.7 shows the cDNA inserts in the orientation
originally isolated from the cell lines. Interestingly both the original h4/d21 cDNAs and the L2 cDNA contained a 22bp deletion as compared to the sequence of L3 or L4. This, and the other differences between the sequences of h4/d21 and clone L3, is shown in the alignment in Figure 6.6. The position of the BamHI site common to the pCRII™ clones h4/26 and d21/5 and clones L2/3/4, but absent in pCRII™d21/3, is indicated. The L2, L3 and L4 clones all lacked the EcoRI site which was observed in pCRII™d21/3, confirming that these changes in restriction sites were due to errors made by Taq DNA polymerase during the PCR amplification. The sequence of clone L4, which extended 600bp upstream in respect to the orientation of the polyA tail, still failed to show the presence of an open reading frame.

6.2.7: Retransfection of the pDR2-library clones into rat embryo fibroblasts.

The three cDNA clones, L2, L3 and L4, isolated from the newt limb blastema library were assayed for their ability to immortalise secondary rat embryo fibroblasts. Since the h4/d21 cDNAs were isolated as cDNAs which were able to immortalise REFs it is important to see whether this activity was observed with any of the three library cDNA clones. Indeed since a larger amount of each pDR2 clone can be transfected immortalisation should occur in a larger number of cells and the immortalisation efficiency should be enhanced. Since a library clone corresponding to the original 960bp cDNA fragment was not isolated I was unable to retransfect a recombinant pDR2 plasmid expressing precisely the cDNA which was originally isolated. These experiments should also indicate whether immortalisation by this cDNA sequence is dependent on the orientation of the cDNA with respect to the RSV LTR promoter, thereby indirectly demonstrating a necessity for the cDNA to be expressed from this promoter.

The three library cDNA clones were retransfected into secondary REFs alongside the pDR2-S control. As before the transfected cells were incubated at 33.0°C. The results of two experiments are shown in Table 6.2. The number of colonies formed following transfection of these cDNA clones, along with the average percentage efficiency of colony formation for the two experiments, in relation to recombinant SE (which expresses wild type T antigen) is shown. The number of cell lines successfully established from the number isolated is also indicated. Both pDR2-L2 and pDR2-L3 yielded a reasonable number of colonies (39.6% and 28.2% respectively, compared to T antigen) while the pDR2-S only yielded one colony from either experiment (0.5% SE). The longer cDNA clone, pDR2-L4, yielded very few colonies (2.8% SE). The colonies isolated following transfection of either pDR2-L2 or pDR2-L3 yielded a few cell lines, with pDR2-L2 yielding a more than pDR2-L3 (7 out of 22 and 3 out of 17 respectively). As was the case with the original h4 and d21 cell lines, these cell lines grew very slowly compared to cell lines immortalised with T antigen. This suggests the possibility that
Table 6.2: Ability of pDR2 clones L2, L3 and L4 to immortalise secondary REFs.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Number of colonies obtained following transfection of clone into REFs (% of SE)</th>
<th>Average efficiency of colony formation (% SE)</th>
<th>Number of cell lines derived/colonies isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>expt. 1</td>
<td>expt. 2</td>
<td></td>
</tr>
<tr>
<td>SE (T Ag)+pDR2-S</td>
<td>142 (100.0%)</td>
<td>104 (100.0%)</td>
<td>0.5</td>
</tr>
<tr>
<td>pKS+pDR2-S</td>
<td>0 (0.0%)</td>
<td>1 (1.0%)</td>
<td></td>
</tr>
<tr>
<td>pKS+pDR2-L2</td>
<td>82 (57.7%)</td>
<td>21 (21.4%)</td>
<td>39.6</td>
</tr>
<tr>
<td>pKS+pDR2-L3</td>
<td>76 (53.5%)</td>
<td>3 (2.9%)</td>
<td>28.2</td>
</tr>
<tr>
<td>pKS+pDR2-L4</td>
<td>8 (5.6%)</td>
<td>0 (0.0%)</td>
<td>2.8</td>
</tr>
</tbody>
</table>

The indicated clones (1.5 μg) were transfected into secondary REFs using pKS (48.5 μg) as carrier DNA (as described in Materials and Methods). Transfected REFs were cultured at 33.0°C in the presence of 100 μg/ml hygromycin-B for four weeks. Representative plates were stained with methylene blue and colonies were counted. The indicated number of colonies were isolated and the total number of cell lines successfully derived is shown. expt. = experiment; N.D. = not done.

The newt limb blastema cDNAs were only partially able to immortalise REFs and that second events may be required for the cell lines to grow efficiently. It is also interesting to note that both orientations of the cDNA, in relation to the RSV LTR promoter, were able to yield cell lines in these experiments.

The fact that the pDR2-L3 clone, which has the cDNA in the same orientation as it was in the original h4/d21 cDNAs, did not work very efficiently suggests several things about the immortalisation by these clones. Either the smaller cDNA fragment (960bp) was able to immortalise more efficiently than the longer L3 clone; the 22bp deletion in the h4/d21 clones was important for its immortalisation or that in the original expression screening electroporations I managed to isolate the h4/d21 cDNA through the additional effects of second events in these cell lines. If this last possibility is correct then I was fortunate that these second events occurred in two separate cell lines during my screening experiments.

6.2.8: The pDR2-L2 and L3 cDNAs were retained in most of the cell lines derived following retransfection.

Genomic DNA was prepared from the cell lines, at passage 4, isolated following
Chapter 6.

Figure 6.8: Fragments obtained following PCR with pDR2 internal primers on genomic DNA prepared from REF+pDR2-L2 or L3 cell lines.

The colonies obtained following transfection of the pDR2 library clones L2 or L3 into secondary REFs were isolated and genomic DNA was prepared from those which expanded successfully into clonal cell lines. Genomic DNA was amplified by PCR using pDR2 internal (Iu and Iq) primers (as described in Materials and Methods). The resulting fragments were separated on 1.8% agarose gels (with 1.0µg/ml EtBr) in 1X TAE alongside approximately 1.0µg 1kb DNA ladder (GibcoBRL). Genomic DNA prepared from tsa14 cells (p24), or from the cells remaining on whole plates (WP) four weeks after transfection of pDR2-S (the single clone transfection control), was similarly amplified to ensure the PCR conditions were specific. CL= cell line

The transfection of the pDR2-L2 and L3 clones. The continued presence of the cDNAs in these cell lines was determined by PCR amplification using the pDR2 Iu and Iq primers. The fragments resulting from these PCR amplifications are shown in Figure 6.8. Clearly the cDNA insert was retained in the majority of the cell lines isolated. Six of the seven pDR2-L2 cell lines (lanes 2, 3, 4, 8, 11 & 12) and two of the three pDR2-L3 cell lines (lanes 5 & 6) retained the cDNA insert. The control genomic DNAs, prepared from the small number of cells remaining on two plates from the pDR2-S transfection (lanes 9 & 10) and from tsa14 cells, failed to yield PCR fragments. The one pDR2-L2 cell line (CL-4, lane 7) and one pDR2-L3 cell line (CL-3, lane 3) which failed to yield PCR fragments either may have lost or mutated one of the primer sites upstream or downstream of the cDNA or may have shed the cDNA.
6.2.9: Retransfection of the pDR2-L2 and L3 cDNAs along with SV40 T antigen mutants.

Since neither orientation of the newt limb blastema cDNA was efficient at establishing secondary REF cell lines although both orientations yielded colonies relatively efficiently, I attempted to determine whether any of the T antigen mutants, which were deficient in immortalisation (Chapter 3), could cooperate with the newt limb blastema cDNA to immortalise. I chose three T antigen mutants for these experiments, d/l1135, T128-708 and 5041 (see Table 3.1 for position of mutations). The d/l1135 mutant was able to maintain immortalisation in a number of cell lines once they were established (Chapter 4) but was unable to initiate immortalisation in secondary REFs. The T128-708 mutant was also able to maintain immortalisation in a T antigen-dependent established cell line, but at a decreased efficiency compared to d/l135, while being unable to immortalise outright. The 5041 mutant was neither able to immortalise secondary REFs nor able to maintain immortalisation in a T antigen-dependent established cell lines, however it was able to provide the initiation of immortalisation function lacking in the d/l135 mutant (Chapter 4).

The results of two experiments are shown in Table 6.3. As in previous immortalisation experiments the number of colonies formed following the cotransfection of the indicated recombinants are shown and are also expressed as a percentage efficiency colony formation relative to wild type T antigen (%SE). The average efficiency of colony formation compared to wild type T antigen for the two experiments is also shown, along with the number of cell lines derived from the number of colonies isolated. In contrast to the previous experiments described in this chapter these transfections were all carried out at 37.0°C and the resulting colonies were isolated and the plates stained after no more than 2 weeks. Transfection of the control DNA, pDR2-S, only yielded 11.5% the number of colonies obtained with wild type T antigen and none of the 13 colonies isolated successfully expanded into cell lines. Transfection of the three T antigen mutants with pDR2-S yielded colonies at a similarly low efficiency compared to wild type T antigen and only one of the 13 colonies obtained with the d/l135 mutant was successfully expanded. Electroporation of pDR2-L2 alone yielded fewer colonies after 2 weeks than were previously observed after 4-5 weeks (section 6.2.7) while pDR2-L3 yielded no colonies. Both orientations of the newt limb blastema cDNA yielded more colonies relative to wild type T antigen in the previous experiments. This difference was probably due to the shorter amount of time allowed for the colonies to form in the later experiments. No colonies were isolated from either of these electroporations.

Cotransfection of either pDR2-L2 or pDR2-L3 with the d/l135 mutant yielded colonies more efficiently than either recombinant alone. Indeed the average efficiency of colony formation with the d/l135+pDR2-L3 cotransfection was close to that obtained
Table 6.3: Ability of pDR2 library clones L2 and L3 to immortalise secondary REFs in cooperation with SV40 T antigen mutants.

<table>
<thead>
<tr>
<th>T antigen mutant plus library clone</th>
<th>Number of colonies obtained following transfection of T antigen mutant plus library clone into REFs (% of SE)</th>
<th>Efficiency of colony formation (% SE)</th>
<th>cell lines derived/colonies isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>expt. 1</td>
<td>expt. 2</td>
<td></td>
</tr>
<tr>
<td>SE (T Ag wt)+pDR2-S</td>
<td>120 (100.0%)</td>
<td>247 (100.0%)</td>
<td>100.0 N.D.</td>
</tr>
<tr>
<td>pKS+pDR2-S</td>
<td>16 (13.3%)</td>
<td>24 (9.7%)</td>
<td>11.5 0/13</td>
</tr>
<tr>
<td>d/1135+pDR2-S</td>
<td>4 (3.3%)</td>
<td>51 (20.6%)</td>
<td>12.0 1/13</td>
</tr>
<tr>
<td>T128-708+pDR2-S</td>
<td>13 (10.8%)</td>
<td>-</td>
<td>10.8^a N.D.</td>
</tr>
<tr>
<td>5041+pDR2-S</td>
<td>6 (5.0%)</td>
<td>5 (2.0%)</td>
<td>3.5 N.D.</td>
</tr>
<tr>
<td>pKS+pDR2-L2</td>
<td>12 (10.0%)</td>
<td>28 (11.3%)</td>
<td>10.7 N.D.</td>
</tr>
<tr>
<td>d/1135+pDR2-L2</td>
<td>42 (35.0%)</td>
<td>95 (38.5%)</td>
<td>36.8 9/24</td>
</tr>
<tr>
<td>T128-708+pDR2-L2</td>
<td>3 (2.5%)</td>
<td>-</td>
<td>2.5^a N.D.</td>
</tr>
<tr>
<td>5041+pDR2-L2</td>
<td>9 (7.5%)</td>
<td>14 (5.7%)</td>
<td>6.6 N.D.</td>
</tr>
<tr>
<td>pKS+pDR2-L3</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0.0 N.D.</td>
</tr>
<tr>
<td>d/1135+pDR2-L3</td>
<td>68 (56.6%)</td>
<td>308 (124.7%)</td>
<td>90.7 21/34</td>
</tr>
<tr>
<td>T128-708+pDR2-L3</td>
<td>53 (44.2%)</td>
<td>-</td>
<td>44.2^a 4/11</td>
</tr>
<tr>
<td>5041+pDR2-L3</td>
<td>1 (0.8%)</td>
<td>13 (5.3%)</td>
<td>3.1 N.D.</td>
</tr>
</tbody>
</table>

The indicated clones and T antigen mutants were co-transfected into secondary REFs described in Materials and Methods. Transfected REFs were cultured at 37.0°C in the presence of 100-125μg/ml hygromycin-B for two weeks. The indicated number of colonies were isolated and the total number of cell lines successfully derived is shown. Plates were then stained and colonies were counted. expt. = experiment; N.D. = not done.

Upon transfection of wild type T antigen (90.7%). The d/1135+pDR2-L2 cotransfection gave colonies at an average efficiency of 36.8% compared to wild type T antigen. A large number of the colonies isolated following the cotransfection of d/1135+pDR2-L3 were readily expanded into cell lines (22/34), while a smaller proportion of the d/1135+pDR2-L2 colonies expanded successfully (9/24). The T128-708+pDR2-L3 also yielded colonies at a higher efficiency than either recombinant alone (44.2% compared to wild type T antigen). This colony formation efficiency occurred at a lower frequency than d/1135+pDR2-L3 though, and only 4 of the 11 colonies isolated expanded into cell lines. This reflects the results obtained with these two mutants when they were used to maintain the immortal state in tsal4 cells, the d/1135 mutant yields cell lines at the non-permissive temperature more efficiently than T128-708 (Table 3.2). Importantly all the cell lines established from these experiments grew much more rapidly than either the original h4/d21, pDR2-L2 or pDR2-L3 cell lines isolated previously. The cotransfection
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of the T128-708+pDR2-L2 combination failed to immortalise the secondary REFs as did the combinations of either pDR2-L2 or pDR2-L3 with 5041. All three of these cotransfections yielded colonies at a lower efficiency, compared to wild type T antigen, than the pDR2-S control (2.5%, 6.6% and 3.1%, respectively, compared to 11.5%).

Genomic DNA prepared from the majority of the cell lines immortalised following the cotransfection of dll135+pDR2-L2 or L3 yield PCR fragments corresponding to the L2 or L3 cDNA upon amplification with the pDR2 lu and ld primers. These PCR amplification demonstrated that the L2 or L3 fragments were retained during subsequent passages of nearly all these cell lines (carried out by Parmjit Jat; data not shown).

6.3: Discussion.

6.3.1: General discussion.

In this chapter I have described the functional cloning of a cDNA corresponding to a fragment of an mRNA which is expressed in the blastemal cells of the regenerating newt limb. This cDNA fragment was isolated, independently, from two cell lines which were immortalised following the transfection of a eukaryotic cDNA expression library, prepared from newt limb blastema mRNA, into secondary REFs. Characterisation of this cDNA fragment revealed that it probably originated from the 3' untranslated region of an mRNA of approximately 6.0kb. Furthermore, the orientation of the cDNA fragment, with respect to the RSV LTR promoter of the library vector, indicated that, if this cDNA was expressed in these cell lines, it would have expressed the antisense mRNA. However, this cDNA fragment was clearly demonstrated to stimulate growth in secondary rat embryo fibroblasts. The roles of 3' untranslated regions (3'UTRs) in the regulation of protein expression and the possible effects an antisense 3'UTR might have on this regulation are discussed in the following section (section 6.3.2).

cDNA clones which contained this 960bp cDNA sequence were isolated from the original limb blastema library. When these clones were transfected into secondary REFs they were found to be able to yield immortal cell lines but only at a low efficiency. However, transfection of both the L2 and L3 clones resulted in an increase in the numbers of colonies initially formed, in comparison to the pDR2-S control. These results suggested that these cDNA fragments were able to stimulate growth to some degree but that second events may have been required to yield established cell lines. This would imply that the two cell lines from which the h4/d21 cDNA fragments were originally isolated may also have arisen due to the effects of second events along with the blastemal cDNA fragments. Both the h4 and d21 cell lines grew very slowly, as did the three cell lines successfully immortalised following transfection of pDR2-L3. Alternatively, these cDNA fragments may only be capable of immortalising a sub-
population of the secondary REFs. Experiments carried out with mutants of T antigen (dl1137 and dl1135) have previously demonstrated that the functions of T antigen required for stimulating growth differ depending on the specific cell type (Chen et al., 1992; Symonds et al., 1993; discussed in Chapter 3). Since the secondary REF population is heterogeneous, it is possible that a sub-population of these cells have a disposition to the immortalisation mediated by this cDNA fragment.

The activity of these clones in stimulating growth was conclusively demonstrated when they were shown to complement a T antigen mutant (dl1135) which had previously shown to be deficient in the initiation of immortalisation (Chapter 4). The ability of these cDNA fragments to immortalise in cooperation with dl1135, which is able to maintain immortalisation in established T antigen dependent cell lines, suggests that the blastema cDNA fragment is able to initiate immortalisation but requires second events to occur for the immortal state to be maintained. The ability of this cDNA fragment to initially stimulate growth may explain the increase in colony formation observed when either L2 or L3 are transfected alone into REFs while only a small proportion of these colonies go on to yield cell lines. This cooperative immortalisation with dl1135 was most efficient when the cDNA was transfected in the same orientation (pDR2-L3), with respect to the promoter, as it was originally isolated, although some cell lines were successfully isolated following the cotransfection of the opposite orientation (pDR2-L2). Both the dl1135+pDR2-L3 cell lines and the dl1135+pDR2-L2 cell lines grew more rapidly than the few cell lines immortalised by either pDR2-L3 or L2 alone.

The demonstration that transfection of the opposite orientation of the cDNA (pDR2-L2) can yield immortal colonies is concerning. pDR2-L2 alone establishes cell lines at a similar efficiency as the original orientation isolated (pDR2-L3) but at a much lower efficiency in cooperation with the dl1135 T antigen. The difference in the orientation with respect to the RSV LTR promoter in these recombinants suggests that expression of the cDNA was not required. As yet expression of the antisense mRNA in these cell lines has not been detected. Philip Gates has attempted to use primers specific for the h4/d21 DNA sequence to RT-PCR amplify RNA from some of the pDR2-L2 and L3 cell lines, however the results have not convincingly demonstrated mRNA expression from this cDNA. It would be hard to explain the activity obtained with a transfected cDNA expression plasmid which is not expressing an mRNA. If, however, the function of the cDNA is required only transiently for the initiation of immortalisation, as is indicated by its ability to cooperate with dl1135, it is possible that the cDNA may be expressed at higher levels during the early stages of immortalisation. Once the cell lines are established they may no longer require the expression of the cDNA and expression decreases. The action of second events, in the case of the h4, d21 and pDR2-L3 cell lines, or the functions carried by the dl1135 T antigen molecule, in
Chapter 6. the case of \(dl1135+pDR2-L3\) cell lines, may then be sufficient to maintain immortalisation. This hypothesis would explain the difficulty in demonstrating expression of the mRNA in these cell lines. It may be necessary to prepare RNA from cells at an earlier stage following the transfection of the cDNA expression plasmid to demonstrate expression. It would also be informative to cotransfect \(dl1135\) with a recombinant carrying the L3 cDNA without a promoter to determine whether this combination could immortalise. If expression of the mRNA is required, then the cotransfection of such a promoter-less recombinant would fail to complement \(dl1135\) in immortalisation.

Since a 960bp cDNA corresponding to the original h4/d21 sequence isolated from the expression screening was not isolated from the \(\lambda DR2\) library when it was screened with the h4/d21 PCR fragment, it is impossible to say whether a cDNA corresponding to this 960bp is present within the original library. It is possible that this 960bp fragment was created from the cDNA clones corresponding to L2 or L3, through deletion of the extra 300bp, when the library was transfected into the cells. This seems unlikely, however, since similar 960bp fragments were cloned from two independent cell lines. It seems more probable that the cDNA corresponding to the 960bp fragment is present in the library but at a lower abundance than the L2, L3 and L4 clones. This would suggest that the enrichment involved in isolating this clone has been very efficient. Alternatively, the abundance of a cDNA clone corresponding to the 960bp h4/d21 fragment may have been enriched during the Cre-\(loxP\) site-specific recombination-mediated rescue and subsequent growth of the library as pDR2 plasmids. It is possible that the 960bp cDNA fragment originally isolated from the h4 and d21 cell lines is able to establish cell lines more efficiently than the longer L3 fragment or that the 22bp deletion in the h4/d21 fragment may be significant for its growth stimulatory activity. It may be informative to construct a pDR2 clone expressing the 960bp cDNA fragment, with and without the 22bp deletion, to determine the relative efficiency of this cDNA fragment to immortalise, both alone and in cooperation with \(dl1135\), in comparison to pDR2-L3. Further mutational analysis of this fragment may yield interesting data on the minimal region within this cDNA fragment which is required for its activity. The determination of such a region may yield clues as to how this cDNA fragment elicits its effects in stimulating growth.

6.3.2: The role of 3' untranslated regions in regulating protein expression.

The 3' untranslated regions (3'UTRs) of many mRNAs have been implicated in the regulation of gene expression (Jackson, 1993). Once a gene has been transcribed, the level of protein expression from the mRNA relies on transit of the mRNA to the cytoplasm, its translational initiation and its stability. Translational initiation is generally regulated through sequences in the 5' untranslated regions of the mRNA
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(reviewed by Curtis, Lehmann and Zamore, 1995; Hentze, 1995). However, control of the translation of a number of mRNAs has also been shown to involve cis-acting regulator sequences located in their 3'UTRs. These sequences appear to be involved in the polyadenylation and deadenylation states of these mRNAs. This method of regulating gene expression has particularly been implicated in translationally activating dormant mRNAs in oogenesis and during the development of early embryos (reviewed by Curtis, Lehmann and Zamore, 1995; Vassalli and Stutz, 1995).

In many cases 3'UTRs appear to contain specific negative regulatory elements which interact with trans-acting proteins to repress translation. These negative elements probably function in one of two ways. Since polyA tails can function to stimulate translation and are important for mRNA stability, these elements might repress translation by stimulating the loss of the polyA tail. The translational repression of several mRNAs, including the repression of the Caenorhabditis elegans fem3 mRNA (Ahringer and Kimble, 1991) and the Xenopus c-mos mRNA (Sheets, Wu and Wickens, 1995) during oogenesis, correlates with the shortening of their polyA tail. Alternatively, these 3' regulatory elements may act independently of the polyA tail to inhibit translation, as demonstrated by the ability of 3'UTR elements in the mammalian interferon β and lipoxygenase mRNAs to repress the translation of mRNAs without polyA tails (Kruys et al., 1987; Ostareck-Lederer et al., 1994). 3'UTRs also contain sequence-specific endonucleolytic cleavage sites which regulate mRNA stability. The decay rate of several mRNAs can be controlled through the activity of protective factors which bind to the 3'UTRs of these mRNAs and mask endonucleolytic cleavage sites (Brown, Zipkin and Harland, 1993).

3' UTR elements can also act positively to stimulate translation. One method through which this is believed to occur is through alleviating the repression imposed on mRNA translation by a negative element. In a number of cases, including the translational regulation of c-mos, cdk2 and cyclin A1, B1 and B2 mRNAs in Xenopus oogenesis and tissue plasminogen activator in mouse oogenesis, however, translational activation involves the cytoplasmic adenylation of polyA-deficient mRNAs (Vassalli et al., 1989; Sheets et al., 1994; Stebbins-Boaz and Richter, 1994). This cytoplasmic adenylation of mRNAs requires the same polyadenylation sequence as nuclear adenylation plus an additional cytoplasmic polyadenylation element (Fox, Sheets and Wickens, 1989; McGrew et al., 1989). 3'UTR elements also appear to be involved in the specific localisation of mRNAs to particular regions of the cytoplasm (Wilhelm and Vale, 1993).

There is evidence that the 3'UTR regulatory elements are bound by proteins in a manner which correlates with translation from the mRNA. An excellent example of this is the demonstration of a protein which binds to the 3'UTR of the luciferin-binding protein mRNA when the mRNA is not translated but does not bind when the mRNA is
translated (Mittag, Lee and Hasting, 1994). The proteins which bind elements within 3'UTRs and thereby regulate mRNA stability, localisation and translation are poorly defined. Two human RNA-binding proteins (Hel-N1 and Hel-N2) have been characterised which bind in vitro to a number of mRNA 3'UTRs, including those of proteins involved in cell growth regulation such as c-myc, c-fos, granulocyte/macrophage colony stimulating factor and the transcriptional repressor Id1 (Gao et al., 1994). The fact that a number of mRNAs for growth regulatory proteins are bound by proteins through sequences in their 3'UTRs suggests that these regions play a role in growth regulation.

Recently, it has been suggested that 3'UTR sequences from specific mRNAs can act in trans to affect the translation of either the same or different mRNA. The 3'UTRs of several muscle structural genes have been shown to be able to induce differentiation in a differentiation-defective mutant myogenic cell line (NMU2), suppress tumour formation by the NMU2 cell line (which is usually able to give rise to rhabdomyosarcomas in mice) and suppress growth in immortal mouse fibroblasts (C3H10T1/2; Rastinejad and Blau, 1993; Rastinejad et al., 1993). This data indicates that 3'UTRs could act in trans as regulators of growth and differentiation in the absence of coding sequences. It is possible that over expression of 3'UTRs might also act to titrate regulatory factors. This has been demonstrated in C.elegans where over expression of the fem3 3'UTR, which contains a binding site for a negative regulatory factor, results in an increase in fem3 expression (Ahringer and Kimble, 1991). If the same protein binds to similar sites thereby regulating translation from a number of mRNAs, it is possible that over expression of this 3'UTR may result in increased expression from all of these mRNAs. It is possible that the newt cDNA fragment which I have isolated acts through a similar mechanism to immortalise rat embryo fibroblasts.

Polyadenylation of c-mos mRNA is important for the regulation of Xenopus meiotic maturation and it has recently been shown that the expression of antisense c-mos 3'UTR sequences prevents maturation by causing the loss of the c-mos mRNA's polyA tail (Sheets, Wu and Wickens, 1995). The expression of antisense mRNA directed against the 3'UTR of tissue plasminogen activator has also been shown to prevent the activation of dormant mRNA during mouse oogenesis (Strickland et al., 1988). Antisense mRNAs also appear to be involved in the normal regulation of gene expression. An endogenously expressed antisense mRNA has been shown to regulate p53 mRNA translation during the differentiation of mouse erythroleukaemia cells (Khochbin and Lawrence, 1989). Thus, it is clear that the expression of the antisense 3'UTR of an mRNA may interfere with the normal translation regulation of that mRNA. This may come about by the deadenylation of the endogenous mRNA. If the 3'UTR of a particular mRNA is involved in suppressing growth, the antisense 3'UTR of this mRNA may interfere with this negative growth control. The newt blastema cDNA fragment
which I have isolated as able to stimulate growth in rat embryo fibroblasts may do so by encoding an antisense mRNA homologous to the 3'UTR of one or more mRNA involved in negative growth regulation.

Whether the isolated cDNA fragment has a role to play in the regeneration of the newt limb has not been demonstrated. There is no evidence that an antisense transcript of this mRNA is found in the regenerating newt limb blastema. It is possible that identification of the upstream coding sequence of this 6.0kb mRNA may yield some clues as to its possible function in the newt. It may be, however, that the cDNA is acting in a different fashion for the immortalisation of rat embryo fibroblasts. If it is interfering with the normal expression of one or more genes in REFs through its 3'UTR these may not necessarily be rat homologues of the newt gene from which the cDNA originated. If a minimal region which carries the growth stimulatory activity of this cDNA can be identified, it might be interesting to attempt to screen a rat cDNA library for homologous sequences. Subsequent characterisation of any homologous sequences identified will reveal whether the homology lies within a 3'UTR and may yield information about the proteins being encoded by such cDNAs. It may also be informative to do band shift experiments with the RNA to determine whether this 3' untranslated sequence is bound by any proteins. It is clear that a lot of further work will have to be carried out before the mechanism through which this cDNA elicits its growth stimulatory activity is rationalised.
Studies on the oncogenic proteins of a number of DNA tumour viruses, including SV40 large T antigen, have already yielded multiple insights into the mechanisms regulating cellular proliferation and the identification of many of the proteins that may be involved. Although several of T antigen's interactions with cellular proteins have been defined, the role these interactions play in T antigen's ability to immortalise primary cells remain poorly understood. To determine the functions of SV40 large T antigen which are required for its ability to stimulate growth, a large number of mutants have been constructed and analysed in a variety of cellular growth assays. While some of these assays determine the regions of T antigen required to immortalise primary cells from different species, other assays determine the regions of T antigen required to convey a more transformed phenotype on a range of cell lines which are already immortal, such as growth in semi-solid media or tumour induction in nude mice. Such transformation assays may require different functions of T antigen depending on the origin of the cell line used; some of the pathways which would usually be targeted by T antigen may already be activated or inactivated in these cell lines. As a result these studies have yielded a large amount of data which are not entirely consistent. It is clear that the functions of T antigen required to stimulate growth depend on the cellular growth assay used.

In an attempt to identify the functions of T antigen required to immortalise rat embryo fibroblasts I have used two cellular assays. The first assay determined the ability of T antigen mutants to maintain the immortal state in a clonal cell line which is dependent upon T antigen for its continued growth. This cell line, tsal4, was established from rat embryo fibroblasts by infection with a recombinant retrovirus that transduces the thermolabile large T antigen from early region mutant tsA58 (Jat and Sharp, 1989). tsal4 cells grow continuously at the permissive temperature for the tsA58 protein but rapidly undergo growth arrest upon shift up to the non-permissive temperature. The second assay analyses the ability of the same mutants to immortalise
secondary rat embryo fibroblasts. Primary (or secondary) cultures comprise a mixed population of cells that may be susceptible to karyotypic instability. Thus assaying the immortalising activity of T antigen mutants in such cells may involve the selection of endogenous mutations in addition to the action of the transfected T antigen mutant or may represent the immortalisation of a sub-population of cells. Assaying T antigen mutants in the clonal, T antigen dependent tsa14 cell line should not be complicated by these possibilities, however, it is possible that maintenance of the immortal phenotype in tsa14 cells may only require some of the functions necessary for the immortalisation of primary cells. Comparison of the results obtained with a number of T antigen mutants in these assays has allowed me to address these possibilities.

The results obtained show that several regions of T antigen are required, to a greater or lesser extent, for immortalisation. One of these regions corresponds to the hydrophobic region of T antigen which is required for the stability of the tertiary structure of the protein, while another is colinear with the p53 binding region thereby suggesting that sequestration of p53 is required for efficient immortalisation. In contrast to the functions required for the immortalisation of primary mouse embryo fibroblasts by T antigen (Thompson et al., 1990; Zhu et al., 1991a), one or more functions carried by the amino-terminal 175 amino acids were required for the immortalisation of rat embryo fibroblasts. One of these functions maps close to the CR1-like region of T antigen and is perturbed by the deletion of amino acids 17-27, while the other function, which seemed to have a lesser role, maps to amino acids 128-175 and may correspond to a function mapped by Dobbelstein et al. (1992). The CR2-like region responsible for T antigen's interaction with the pRB family of proteins was not required either for maintaining immortalisation in tsa14 cells or for immortalising secondary REFs.

Amino-terminal fragments of T antigen were found to be sufficient to immortalise secondary REFs, albeit at a decreased efficiency, however they were not able to maintain immortalisation in the clonal tsa14 cell line. These observations suggest that immortalisation by these amino-terminal fragments may be due to either second events, such as endogenous mutations, or the immortalisation of a subset of cells within the heterogeneous REF population. Further characterisation of these cell lines may determine whether this inefficient immortalisation is due to interference with the p53 growth regulatory pathway. My results also suggest that point mutations within the carboxy-terminal p53 binding region inactivate functions carried within the amino-terminus of T antigen. Experiments involving the cotransfection of two immortalisation-deficient T antigen mutants, one with an intact amino-terminus and the other with an intact carboxy-terminus, suggest that the functions required for both REF immortalisation and maintenance of tsa14 growth can be provided in trans by separate T antigen molecules. The observation that in a number of tsa14-derived cell lines these mutant proteins remained functionally independent, that is their reading frames were not
recombining to yield expression of a wild type protein, suggests that both the initiation
and maintenance of immortalisation by T antigen requires the cooperative action of
independent functional domains.

One of the regions (defined by the deletion of amino acids 17-27 in mutant
dl1135) required by T antigen for REF immortalisation was found not to be necessary
for maintaining growth in the conditionally immortal cell line. This observation raised
the possibility that the functions initially required by T antigen to immortalise secondary
REFs are not all continuously required once the resulting cell lines are established.
Alternatively this result may have been due to a peculiarity of the tsal4 cell line. In the
immortalisation of secondary REFs, I had demonstrated that mutant dl1135 could be
complemented by two other immortalisation-deficient mutants (5041 and 5031) which
had point mutations within their p53 binding domains. I took advantage of this fact to
further examine the role that the function lacking in dl1135 plays in immortalisation and
to determine whether the lack of a requirement for this function for the maintenance of
immortalisation was applicable to other T antigen immortalised REF cell lines. Using
temperature sensitive double mutants of T antigen (ts1135, ts5041 and ts5031) I
demonstrated that while the function lacking in mutant dl1135 is required to initiate REF
immortalisation, only the functions carried by mutant dl1135 are required to maintain
the immortal state in a number of cell lines. This result is further supported by the
observation that T antigen mutant dl1135 could also maintain the immortal state in a
number of other conditionally immortal tsA5S cell lines.

While the genetic analysis carried out using these temperature sensitive double
mutants in cooperative immortalisation assays suggests that the two cotransfected
recombinants encode the mutant T antigen proteins independently, I have not been able
to separate the two mutant proteins biochemically. It is possible that the cooperative
immortalisation by the two T antigen mutants is due to recombination or trans-splicing
occurring to result in the expression of either a wild type or tsA5S T antigen. If this were
occurring in every cell line, it would suggest that the regions of T antigen required for
immortalisation are not able to function independently. The observation that cell lines
conditionally immortalised with ts1135 plus either 5041 or 5031 behave somewhat
differently to conditionally immortal tsA5S cell lines suggests that expression of
regenerated tsA5S protein in these cell lines is not the reason for their conditionally
immortal phenotype. ts1135+5041/5031 cell lines appear to undergo some apoptosis
when shifted to the permissive temperature while tsA5S cell lines isolated from the same
experiments do not. Furthermore, while several of the tsA5S cell lines were able to be
maintained by the non-temperature sensitive dl1135 mutant, few of the
ts1135+5041/5031 cell lines were able to have their growth maintained at the non-
permissive temperature by the reintroduction of dl1135 expression. Future experiments
are required to conclusively determine whether these mutants are being expressed
independently. It may be possible to separate the two mutant proteins by preparing the cell extracts in more strongly denaturing buffer conditions. Alternatively, it may be possible to carry out RT-PCR, on RNA prepared from these cell lines, using primers specific for each of the mutants to determine whether a wild type or tsA58 reading frame is being created.

I have also described the successful cloning of a cDNA fragment, which originated from a newt limb blastema eukaryotic cDNA expression library, which is able to stimulate growth in secondary REFs. This cDNA was able to express an antisense RNA corresponding to the 3' untranslated region of a 6.0kb newt limb blastema mRNA. Although alone it was only able to immortalise at a relatively low efficiency, suggesting it was either immortalising a sub-population of cells or that endogenous cellular mutations were also required, this cDNA fragment was able to efficiently complement the initiation-deficient, maintenance-efficient T antigen mutant, dll1135, in REF immortalisation. This observation demonstrates that the function that is required for the initiation of immortalisation by T antigen, which is lacked by dll1135, can be provided by a factor other than another T antigen mutant. Since there is no possibility that this newt cDNA mutant can recombine or trans-splice with the dll1135 reading frame to result in expression of wild type T antigen, it suggests that the functions required for immortalisation are able to function independently. Thus the two cooperating T antigen mutants may also be functioning independently in the immortalisation of REFs. While it is unclear how this newt cDNA fragment elicits its growth stimulatory effect, this effect seems to play a similar role as T antigen mutants 5041 and 5031 in the initiation of immortalisation.

Further analysis of both the newt cDNA fragment and the function lacked by dll1135 will yield information on the nature of the initiation step of immortalisation. Identification of a protein binding to T antigen in a manner which requires amino acids 17-27 may be possible using the amino-terminus of T antigen in a yeast two-hybrid screen or in an affinity purification procedure. The structure of this region of T antigen appears to be conserved in a 35kDa cellular protein of unknown identity. The epitope for antibody PAb419 is destroyed in the mutant T antigen (dll1135) which lacks this functional region and this antibody immunoprecipitates this 35kDa cellular protein from mammalian cells (Harlow et al., 1981). I have successfully immunoprecipitated this cellular protein from a number of immortal or transformed mouse, rat and human cell lines (data not shown). Since I have shown that the stability of the epitope for this antibody appears to correlate with a growth stimulatory function of T antigen, it may be informative to immunoaffinity purify this cellular protein and microsequence it. Further characterisation of the activities of this protein, or of proteins which bind the amino-terminal region of T antigen, may explain why this amino-terminal function of T antigen is required only transiently for the initiation of immortalisation.
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