Studies on the Expression and Functional Activities of Bacteriophage T7 RNA Polymerase and Vaccinia Virus Guanylyltransferase in Mammalian Cells.

Colin Andrew Hodgkinson

Department of Biology

Medawar Building

University College London

Gower Street

London WC1E 6BT.

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ABSTRACT

The ability to express exogenously introduced genes in mammalian cells under control of strong viral promoters has been valuable in many areas of cell and molecular biology. Transcription from these promoters is driven by the host cell's RNA polymerases, together with an array of host-derived transcription factors, which influence the level and cell-type specificity of transcription in as yet poorly understood ways. Transcription of exogenous genes by a bacteriophage RNA polymerase in mammalian cells could offer several potential advantages: lack of inherent cell-type specificity, high transcription rates, exquisite promoter specificity, and the possibility of utilising well understood and characterised procaryotic gene control mechanisms. Potential problems exist, arising from the different requirements for RNA processing in procaryotic and eucaryotic cells. This thesis describes investigations into the feasibilty of using T7 RNA polymerase to generate functional mRNAs in mammalian cells.

Introduction of the bacterial choloramphenicol acetyltransferase (CAT) gene under the transcriptional control of a late T7 promoter into mammalian cells together with a eucaryotic expression vector encoding T7 RNA polymerase, by transfection or microinjection did not result in expression of CAT, suggesting a block to transcription and/or post-transcriptional RNA processing. This block was overcome by superinfection of transfected cells with vaccinia virus, which carries its own RNA processing enzymes, including a guanylyltransferase which modifies free 5'-ends by addition of a guanylyl residue in a 5'-5' linkage. Such capping of eucaryotic mRNAs is ubiquitous and essential for mRNA stability and translation initiation. I cloned the vaccinia virus guanylyltransferase (GT) gene into a eucaryotic expression vector to investigate whether expression of cloned GT could cooperate and complement T7 RNA polymerase to generate stable translatable mRNAs in mammalian cells. Expression of the cloned GT was unable to complement T7 RNA polymerase activity, and antiserum generated against a bacterial fusion protein containing the C-terminal third of GT revealed the expressed GT to be located at discrete sites within the nucleus. My results suggest that either further RNA processing events are required to convert T7 transcripts to functional mRNAs, or that the GT protein and T7 transcripts are sequestered into different regions of the nucleus and do not encounter each other.

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Abbreviations

AdoHcy	adenosyl homocysteine
ß-gal	ß-galactosidase
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CIP	calf intestinal phosphatase
cT7pol	native T7 RNA polymerase
dH_2O	de-ionised water
DNase	deoxyribonuclease
EMCV	encephalomyocarditis virus
FLV	feline leukemia virus
FMDV	foot and mouth virus
FITC	fluorescein iso-thiocyante
HIV-1	human immunodeficiency virus, type I
hnRNA	heterologous nuclear RNA
HRP	horseradish peroxidase
HSV-1	herpes simplex virus type 1
Ig	immunoglobulin
IPTG	isopropyl ß-D-thiogalactopyranoside
kb	kilobase-pairs
kDa	kilodalton
Klenow	large fragment of *E.coli* DNA polymerase I
LTR	long terminal repeat
Mab	monoclonal antibody
MMTV	mouse mammery tumor virus
mRNA	messenger RNA
NLS	nuclear location signal
nt	nucleotide(s)

nT7pol	nuclear targetted T7 RNA polymerase
ORF	open reading frame
ϕ10	bacteriophage T7 late promoter (type-III)
p.f.u.	plaque forming units
poly(A)-minus	non polyadenylated
poly(A)-plus	polyadenylated
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	human placental RNase inhibitor
RT	room temperature
S-AdoMet	S-adenosyl methionine
snRNP	small nuclear ribonucleoprotein particle
SNTV	satellite tobacco necrosis virus
TEMED	N,N,N',N'-Tetramethylethylenediamine
TK	thymidine kinase
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol
T7pol	T7 RNA polymerase
Tϕ	T7 bacteriophage late region transcription terminator sequence
UPE	upstream promoter element
UTR	untranslated region
VSV	vesiculostomatitis virus

1.1 Introduction.

High-level expression mediated by the bacteriophage T7 RNA polymerase in mammalian cells may provide a means to utilise dominant mechanisms such as antisense RNA for interfering with gene expression in mammalian systems. In this Introduction I shall outline problems encountered in the study of mammalian development, the mechanisms available to manipulate the mammalian genome, and the factors which currently limit the use of dominant mutations in transgenic animals. Following this, the potential applications of a mammalian expression system based on the procaryotic bacteriophage T7 RNA polymerase enzyme (T7pol) are discussed. Possible problems of obtaining gene expression mediated by procaryotic enzymes in eucaryotic cells are discussed by looking at the differences and similarities between the procaryotic and eucaryotic mechanisms for gene expression from transcription initiation through to translation, with special emphasis on eucaryotic processing of RNAs. Finally I shall discuss the potential use of cloned vaccinia virus elements, capable of processing T7 transcripts in vaccinia-infected cells, to complement T7pol activity and produce expression at the protein level in mammalian cells.

When fertilised, a mouse egg undergoes a remarkable transformation from single cell to mature adult composed of a bewildering range of different cell types assembled into a variety of specialised tissues and organs. During development cells divide rapidly and then differentiate to produce the many different cell-types of the adult. This process involves the coordinated expression of genes and complex cell-cell interactions. The challenge to biologists investigating mammalian development is to understand the molecular events and control mechanisms which underlie this apparently miraculous transition from single cell to small furry rodent.

Much information about the molecular basis of development has come from studies in *Drosophila melanogaster*, where mutations resulting in aberrant development have been

mapped from the mutant phenotype to the genes responsible and from these to the gene product. The success of this "top-down" approach is well illustrated by our recent explosion of knowledge about morphogenesis, resulting from studies of the segmentation and homeotic mutants of *Drosophila* (reviewed by Gehring, 1987). The top-down approach is facilitated by the extensive genetic and physical maps of the *Drosophila* genome and because of the relatively small size of the genome ($1.4x10^8$bp - haploid genome). The top-down approach is inappropriate in mice which possess a larger genome ($3x10^9$bp - haploid genome size) than *Drosophila*, and for which there are less well defined genetic maps. In mice the "bottom-up" approach is more often adopted, progressing from gene product to the gene and finally to the phenotypic effect of interfering with the expression of that gene. In mice it is difficult to take the step from gene to phenotype because until recently it was not possible to target mutations to specific genes in mammals, due to the low frequency of homologous recombination (reviewed by Capecchi, 1989). At best, fragments of foreign DNA could be integrated randomly into the genome of transgenic mice (Overbeek *et al.*, 1985; Mahon *et al.*, 1988).

1.2 Transgenic Animals and the Manipulation of the Eucaryotic Genome.

It has become possible to manipulate the germ-line of mice by directly injecting DNA into the male pro-nucleus of a fertilised oocyte, or introducing DNA into embryonic stem cells (ES cells) by infection with recombinant retrovirus, transfection or electroporation, and reintroducing these ES cells into a developing mouse blastula (reviewed in Palmiter & Brinster, 1985; Gordon & Ruddle, 1985; Jaenisch, 1988). This allows the introduction of foreign genes, *in vitro* engineered genes, and other DNA sequences into the germ-line of mice. A large number of genes have been successfully linked to tissue-specific promoters, and have been expressed in the appropriate tissue, including diptheria toxin A-chain linked to the elastase *I* promoter (pancreas α-cell, Palmiter *et al.*, 1987), and chloramphenicol acetyltransferase (CAT) linked to the α-crystallin promoter (Westphal *et al.*, 1986) and to the glucagon promoter element (Philippe *et al.*, 1988). This approach has allowed identification

Upstream elements	Expression compared to endogenous promoter	Reference
murine ß-globin	2-3%	Magram *et al.* (1985)
human ß-globin	~10% 1-2%	Behringer *et al.* (1987) Chada *et al.* (1985)
murine whey acidic protein promoter	>10%	Schoenenberger *et al.* (1988)
murine protamine *1* promoter	~10%	Peschon *et al.* (1987)
rat α_{2U} globulin	~2%	da Costa Soares (1987)
chicken transferrin	>10%	McKnight *et al.* (1983)
rat myosin light chain	5-10%	Shani (1985)
murine myelin basic protein	≥25%	Readhead *et al.* (1987) Popko *et al.* (1987)
human fetal globin gene	2-8%	Chada *et al.* (1986)
human globins $^{A}\alpha$-, ß-	~10%	Kollias *et al.* (1986)
murine Kappa light chain	~4% >100%*	Brinster *et al.* (1983) Adams *et al.* (1985)
human α-*1*-antitrypsin	~100%*	Rüther *et al.* (1987)
human ß-globin "minilocus"	100%	Grosveld *et al.* (1987)
rat elastase *1*	100% 100%	Swift *et al.* (1984) Ornitz *et al.* (1985)
sheep ß-lactoglobulin	100%	Simons *et al.* (1987)
human insulin	100%	Selden *et al.* (1986)

* gene copy number not determined.

Table 1. Table showing the levels of expression obtained using heterlogous upstream promoter elements inserted into the germ-line of mice.

of *cis*-acting DNA elements that are responsible for correct temporal- and tissue-specific patterns of gene transcription (see Jaenisch, 1988 for review). A common problem is that expressed levels of the transgene are often lower, usually by one order of magnitude, than those from the equivalent endogenous promoter (see Table 1.) and expression of the gene is position dependent and independent of integrated copy number (Jaenisch *et al.*, 1981; Lacy *et al.*, 1983). For ß-globin, these problems were overcome by including sequences 50kb and 20kb remote from the gene in the transgene construct; this allowed tissue-specific expression at levels comparable to the endogenous gene, in a strictly copy number dependent manner in transgenic animals (Grosveld *et al.*, 1987).

Manipulation of the genome by homologous recombination in ES cells, (Thomas & Capecchi, 1986, 1987; reviewed by Capecchi, 1989), allows targetting of foreign DNA to specific sequences within the genome and their reintroduction into animals. This permits the introduction of specific mutations into structural genes or promoter sequences and has been used to disrupt the expression of the murine *hox 1.3*, *hox 1.2*, *int*-2, and *hprt* genes (Thomas & Capecchi, 1986; Mansour *et al.*, 1988). This method of germ-line manipulation necessarily affects the function of a gene product in all cells that normally express that gene. If we wish to be more selective, and only disrupt gene function in a subset of cell-types which express the gene we must adopt a different approach. The use of tissue-specific promoter elements, in conjunction with dominant strategies for disrupting gene function - such as antisense RNA, antibodies to gene products, dominant-negative mutations, or ribozymes - is the most promising way of approaching this goal

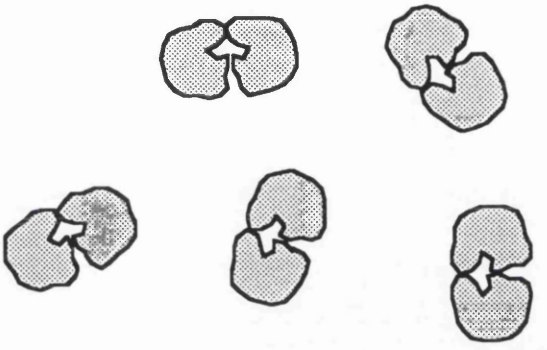
1.3 Dominant Strategies for Disrupting Gene Function

1.3a Dominant Negative Mutation.

One strategy for disrupting the function of a particular protein is to alter the coding sequence of the corresponding gene by site directed mutagenesis in such a way that when re-introduced into a transgenic animal or cell line the mutated protein is capable of inhibiting

Figure 1. Diagram showing the mechanism for inhibition of gene expression by dominant-negative mutation. The activity of a protein, active as a dimer in wild-type cells(A), can be inhibited by over expression of an inactive mutant subunit which results in the formation of inactive mutant-wild-type and mutant-mutant dimers (B). The high level of expression of the mutant subunit prevents formation of any wild-type dimers, the cell becoming a phenotypic mutant for the expression of the target protein.

Wild-type gene expression

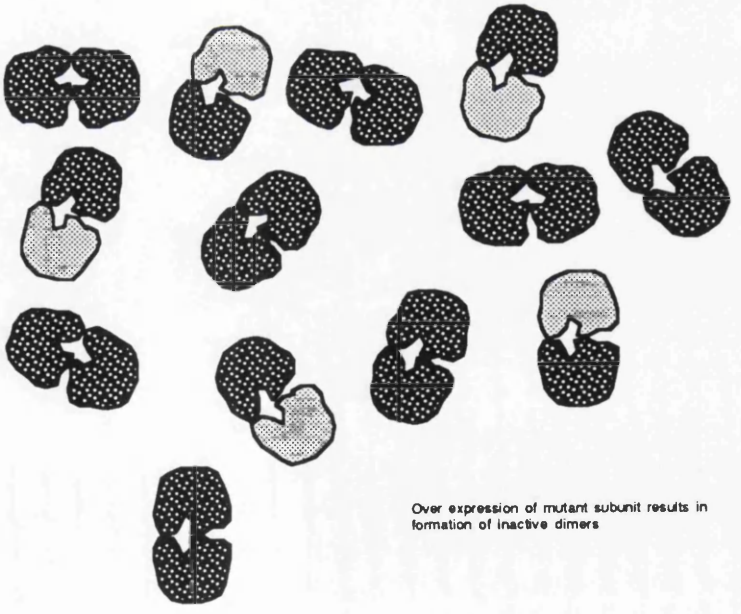


Wild-type dimers formed

Wild-type subunit

Inactive mutant subunit

Dominant-negative mutation



Over expression of mutant subunit results in formation of inactive dimers

the function of the wild-type protein. Such mutations are refered to as "dominant-negative mutations" or "antimorphs" (Herskowitz, 1987). This approach is particularly appropriate for multimeric proteins, as it may be possible to design mutations that allow the assembly of nonfunctional multimers (see Figure 1.) (Novick & Botstein, 1985). A strategy envisaged for the inactivation of monomeric proteins involves production of catalytically inactive mutants that bind a substrate or co-factor. Such mutant proteins would therefore act as competitive inhibitors of the wild-type protein by competing for the substrate/co-factor. This strategy of expressing inactive variant proteins has been successfully utilised to generate transgenic mice with a phenotype that apparently mimics the human disease osteogenesis imperfecta II, a disease caused by a single amino acid change (gly^{391}→arg or gly^{798}→cys or gly^{988}→cys ; Bateman *et al.*, 1987) in proα1(I) collagen, resulting in a perinatal lethal disorder. This occurred when as little as 10% of the proα1(I) collagen RNA was derived from the mutant gene (Stacey *et al.*, 1988).

In the absence of detailed biochemical information concerning the domains, or amino-acids within a protein that are functionally important, an alternative strategy needs to be sought. If the protein under investigation is active as a dimer or multimer a solution might be to express a mutant monomer carrying a signal sequence which causes the active multimers formed to be sequestered in an inapproriate subcellular compartment for function.

1.3b "Ribozymes".

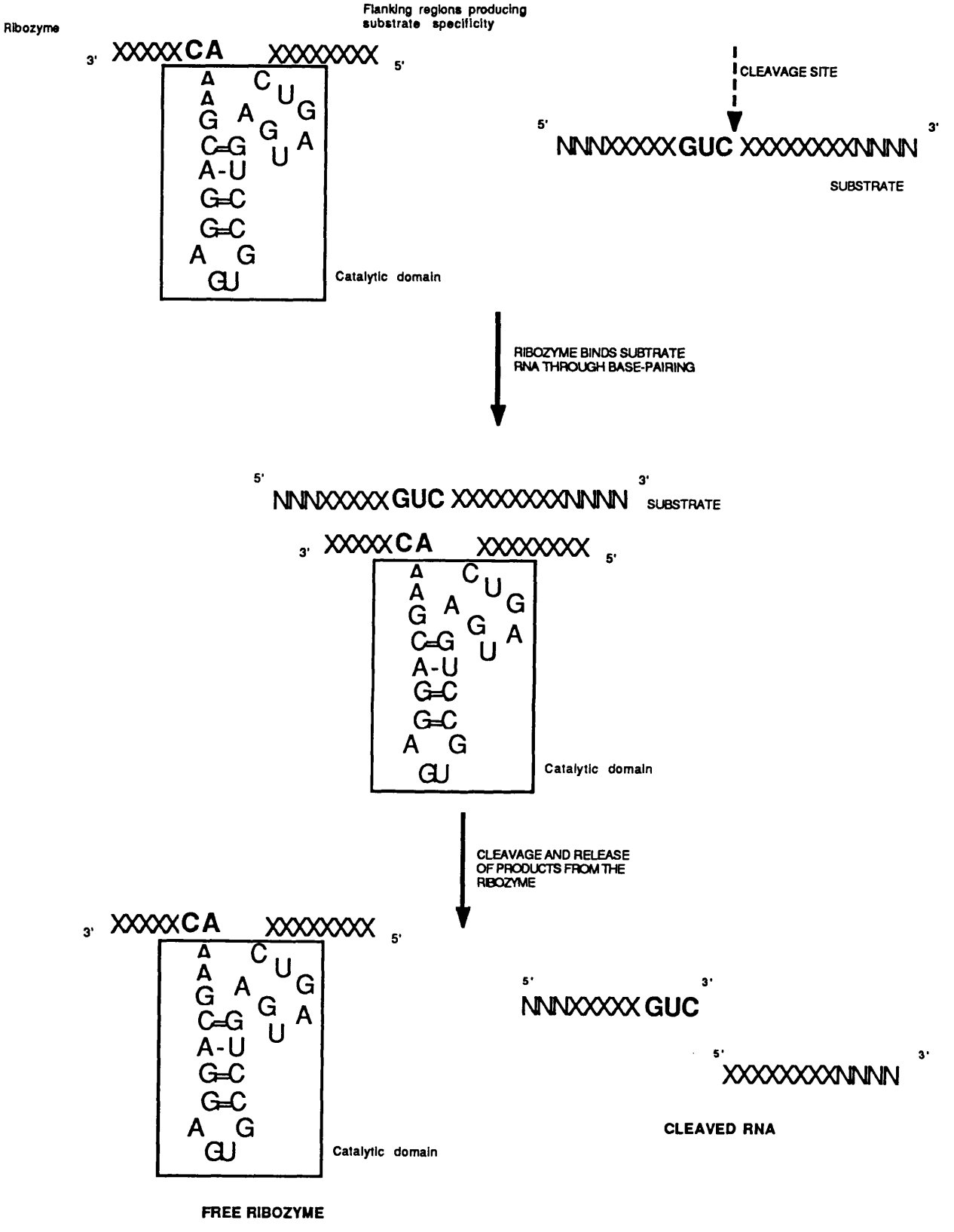
A recent development to emerge in RNA modification has been the discovery of RNA enzymes, dubbed "ribozymes", which possess highly specific endoribonuclease activities (Haseloff & Gerlach, 1988). The involvment of RNA molecules in catalytic processes has been apparent for several years, since RNAs are present in both ribosomes and the small nuclear ribonucleoprotein particles (snRNPs) responsible for intron excision and polyadenylation. Previously these RNA species were thought to have a purely structural role, ensuring correct alignment of associated proteins responsible for the observed enzymatic activities. This simple

view was confounded by the discovery of introns within rRNA transcripts from *Tetrahymena* which are capable of self excision (Cech, 1983). Self splicing has been observed in small circular RNAs that replicate in plants either alone (viroid RNAs) or with the aid of a helper virus (satellite RNAs), this splicing being essential for the replication of the RNA (Forster & Symons, 1987). Self-catalysed cleavage of RNA transcripts from the tandemly repeated satellite-2 DNA sequences of newt has also been observed *in vitro* (Epstein & Gall, 1987).

Studies on the avocado sunblotch viroid (ASBV), the satellite RNAs of tobacco ringspot virus (sTobRV) and lucerne transient streak virus (sLTSV) has shown that the RNA domains associated with these intra-molecular self-cleavage reactions possess conserved sequence and secondary structure motifs. These consist of three stem-loop structures flanking the susceptible phosphodiester bond, and two single stranded regions of highly conserved sequence. The catalytic activities are capable of cleaving a phospodiester bond immediately downstream of the sequences GUC, GUA, GUU (but not GUG). The sites of cleavage are specific and the reactions for all three RNAs share a requirement for divalent metal ions and a neutral, or higher pH. The structure of a minimal ribozyme is shown in Figure 2. The catalytic domain has been defined as a region of conserved sequence and secondary structure containing a stem-loop and two single stranded regions. The susceptible phosphodiester bond is brought into close proximity of the catalytic domain by base pairing between the sequence surrounding the cleavage point and stretches of RNA flanking the catalytic domain. These flanking sequences define the specifity of the cleavage reaction, and altering these sequences provides the means to target cleavage to RNAs other than the natural substrates. Recombinant ribozymes have been produced, targetted to three sites within mRNAs specifying the bacterial protein chloramphenicol acetyltransferase (CAT), and have been shown to catalyse the cleavage of CAT mRNA *in vitro*. These recombinant ribozymes showed activity in conditions similar to those for natural cleavage reactions (ie. high divalent cation concentration, pH$\geq$ 7), but maximal activity was obtained at elevated pH, temperature (50°C) and divalent cation concentrations (pH8.0, 50°C, 20mM $MgCl_2$)

Expression of recombinant ribozymes in mammalian cells could be used to specifically cleave and inactivate a single RNA species during development. Because of the enzymatic

Figure 2. Diagram showing inter-molecular cleavage of RNA by a ribozyme. Base-pairing between the sequences surrounding the catalytic domain of the ribozyme and the cleavage site on the RNA substrate provides specificity for the cleavage reaction. The base-pairing brings the ribozyme catalytic domain and the essential GUC sequence (on the substrate) into close proximity allowing cleavage of the target RNA.


Ribozyme
Flanking regions producing substrate specificity
3'
XXXXCA
XXXXXXX
5'
Catalytic domain
CLEAVAGE SITE
5'
NNNXXXXGUC XXXXXXXXNNNN
3'
SUBSTRATE
RIBOZYME BINDS SUBTRATE RNA THROUGH BASE-PAIRING
5'
NNNXXXXGUC XXXXXXXXNNNN
3'
SUBSTRATE
3'
XXXXCA
XXXXXXX
5'
Catalytic domain
CLEAVAGE AND RELEASE OF PRODUCTS FROM THE RIBOZYME
3'
XXXXCA
XXXXXXX
5'
Catalytic domain
FREE RIBOZYME
5'
NNNXXXXGUC
3'
5'
XXXXXXXXNNNN
3'
CLEAVED RNA

nature of the ribozyme it is possible that relatively low levels of ribozyme expression might be capable of inhibiting expression of a highly expressed gene.

1.3c Antisense RNA.

Antisense RNA is an RNA species that is complementary to, and capable of hybridising to another RNA species, and can be produced either by trancription of the complementary strand of DNA encoding a gene product, or by transcription at an alternative locus. In both naturally occurring and artificial systems usually one of the RNA species involved is an mRNA. Antisense RNAs have been found to occur naturally in procaryotic cells, where they are involved in the replication control and incompatability of plasmid families, the regulation of Tn10 transposition, regulation of gene expression and in the regulation of phage replication (reviewed in Green *et al.*, 1986). It is not yet certain whether antisense RNA has a regulatory function in eucaryotic cells. Antisense transcripts of the locus encoding the protein p53 have been identified in murine erythroleukemia (MEL) cells. p53 is a protein implicated in the control of cell proliferation and is known to be post-transcriptionally regulated and it is possible that the antisense transcripts identified play a role in the regulation of p53 expression (Khochbin & Lawrence, 1989). MEL cells do however show atypical dependence on p53 for proliferation and so it is uncertain whether this putative regulation of p53 expression by antisense RNA reflects the normal cellular mechanism. Antisense transcripts from the c-*myc* locus have also been identified with possible impications for regulation of expression for the c-*myc* gene (Nepveu & Marcu, 1986).

The use of antisense RNA as a means to specifically inactivate genes in eucaryotic cells has been reviewed by Weintraub *et al.*, 1985, and Green *et al.*, 1986. Specific inhibition of gene expression in eucaryotic cells was shown by expression of sense and antisense genes for thymidine kinase from Herpes Simplex virus (HSV-TK) from plasmids micro-injected into mouse L cells (Izant & Weintraub, 1984). The specificity of inhibition was demonstrated by the ability of the HSV-TK antisense constructs to inhibit the expression of the viral gene

product, but not the related, but non-homologous chicken TK gene. This inhibition was dependent upon the ratio of injected antisense:sense constructs being >200:1. Subsequently it was shown that antisense RNA could also inhibit the expression of an integrated HSV-TK gene (Izant and Weintraub, 1985) in an inducible manner, and also inhibit the expression of the endogenous ß-actin gene. This specificity of action allows for the possibility of inhibiting specific 3'-polyadenylation additions or alternative splicing pathways, as a means to determine the role of these alternative pathways *in vivo*.

Antisense genes have been used to study the role of gene products in development and normal cellular metabolism by the inactivation of endogenous genes. Discoidin I-minus mutants in the lower eucaryote *Dictyolstelium discoideum* were obtained by transformation of cells with an antisense discoidin I gene (Crowley *et al*, 1985), and the developmental requirement of the cells for myosin expression was shown by the inhibition of myosin heavy chain expression (Knecht & Loomis, 1987). The normal cellular roles of proto-oncogenes was demonstrated by the inducible expression of antisense *fos* message in Swiss 3T3 cells (Holt *et al*, 1986), which resulted in reduced cell proliferation, suggesting a role of the c-*fos* proto-oncogene product in normal cell growth. The universality of this mechanism for gene inactivation is illustrated by the expansion of its use from bacteria to mammalian and plant cells (Ecker & Davis, 1986).

Direct injection of antisense RNA species synthesised *in vitro*, from SP6 and T7 promoters, into Xenopus oocytes and developing embryos has been used to block expression of ß-globin (Melton, 1984), HSV-TK and CAT (Harland & Weintraub, 1985), and ribosomal protein L1 (Wormington,1986). *Krüppel* (*Kr*) phenocopy mutants have been generated by the injection of SP6 antisense *Kr* transcripts into wild-type *Drosophila* embryos at the syncytial blastoderm stage (Rosenberg *et al*, 1985). These approaches, apart from showing the possibility of using SP6-generated transcripts as a source of antisense species, have highlighted a mechanism for the regulation of genes at the post-transcriptional level which may prove to be important in *Xenopus* development. *Xenopus* embryos have been shown to possess an RNA duplex unwinding activity, or "melting" activity, which is absent in the

unfertilised oocyte, but which precludes the use of antisense inhibition in the early embryo (Rebagliati & Melton, 1987: Bass & Weintraub, 1987). This "melting" activity was not found at the one cell stage in mice (Bevilacqua *et al*, 1988) and it is not yet clear whether it will be a significant barrier to the use of antisense RNA in mammalian embryos.

The use of antisense inhibition has several major advantages for use in the study of gene function. The inhibition relies on cross-hybridisation and so can be used to inhibit one member of a closely related gene family, or potentially inhibit a tissue-specific alternative splice or polyadenylation event. Also no detailed biochemical knowledge of the gene product is necessary for inhibition to be achieved. Expression of an antisense myelin basic protein resulted in the conversion from wild-type to the *shiverer* phenotype in transgenic mice (Katsuki *et al.*, 1988). Regulation of an endogenous gene and protection from virus infection by insertion of an antisense gene into transgenic plants has also been demonstrated (Rodermel *et al.*, 1988; Powell *et al.*, 1989).

1.3d Intra-cellular Expression of Antibody Directed Against a Gene Product.

This strategy, described by Carlson (1988), has been used to inactivate alcohol dehydrogenase I (ADH-I) in yeast. This procedure involved raising monoclonal antibodies against ADH-I, cloning the genes encoding both light and heavy chains of the antibody chosen, removing the signal sequences, and expressing these genes in *Saccharomyces cerevisiae*. The recombinant heavy and light chains associated and were found to bind antigen, resulting in lowered levels of ADH-I activity being assayed in cell-lines expressing the two antibody genes. This may become a generally more useful approach in the future because it has recently been shown that recombinant light or heavy chain variable regions alone sometimes retain binding specificity for antigens (Riechmann *et al.*, 1988).

1.3e Inappropriate, Ectopic or Over Expression of Wild-Type Gene Products.

A mutant phenotype, in which loss of chromosomes V and VII was promoted, was created in yeast by the over-expression of the histone H2A & H2B genes, altering the relative ratios of the histone proteins within the cell (Meeks-Wagner & Hartwell, 1986). Creation of subunit imbalance within other multi-protein structures, such as the cytoskeleton, might also be utilised as a mechanism for the disruption of proper function, although the phenotypic consequences of such mutations would be uncertain, and would, therefore, have to be empirically determined.

Ectopic overexpression of the granulocyte-macrophage colony stimulating factor (GC-CSF) in transgenic mice resulted in macrophage infiltration and accumulation in retinal tissue causing the eyes to become opaque and resulting in blindness, the peritineal and pleural cavities, and in skeletal muscle (Lang *et al.*, 1987). Overexpression of other growth factors may produce similar developmental abnormalities. Tissue-specific expression of rearranged T-cell receptor genes (Uematsu *et al.*, 1988) and immunoglobulin genes (Iglesias *et al.*, 1987) in transgenic mice has been shown to suppress rearrangement and expression of the endogenous genes, and has been used to introduce changes into the B-cell population of an individual, because expression of the rearranged transgene has severe immunoregulatory effects (Herzenberg *et al.*, 1987)

Studies using the v-Ha-*ras* and c-*myc* genes under the control of the Mouse Mammary Tumour Virus promoter (MMTV promoter) (Sinn *et al.*, 1987), and co-expression of the v-*erb*A & B genes in chick embryo fibroblasts (Gandrillon *et al.*, 1987), have suggested synergistic action of these oncogenes *in vivo*. An inducible high level tissue-specific expression system may present the opportunity to regulate the expression of two oncogenes allowing studies into the regulation of transformation by activation and subsequent inactivation of one of the oncogenes, within a specific tissue. Certain genes show tissue-specific patterns of alternative splicing and\or polyadenylation site selection, the reasons for these alternatives being unclear at present.

1.4 Using Procaryotic Transcriptional Controls to Regulate High Level Expression in Mammalian Cells.

Dominant mechanisms for interfering with gene function (see earlier) in general depend for success on high-level expression of the inserted gene for effect. Often, the expression levels obtained from promoters in the context of artificial transgenes are much lower than expected, and as a result they cannot be used successfully for driving transcription of dominant transgenes. Inducible mammalian promoters such as the MMTV or murine metallothionein promoters are relatively leaky under non-induced conditions and show poor levels of induction (2-3 fold induction by dexamethosone; 3-10 fold induction with cadmium) (Mayo *et al.*, 1982, Brinster *et al.*, 1982). These promoters too are unlikely to be capable of producing high enough levels of expression for dominant interference to occur and in any case induction of the trans-promoter may have pleiotropic effects caused by induction of similarly responsive endogenous promoters. Use of viral UPEs can overcome the problem of low level of transgene transcription but the viral UPEs known to function in mammalian cells are active in more than one tissue and so pleiotropic effects may result when used in transgenic animals.

Another approach is to use bacterial or bacteriophage control elements and proteins in mammalian cells to regulate high level gene expression. DNA-binding proteins that regulate transcription initiation in *E.coli* have been used successfully in conjunction with their DNA recognition sequences to tightly regulate transcription by pol*II* in mammalian cells. Both negative - using *lacI* and the *lac* operator sequence (Brown *et al.*, 1987; Hu & Davidson, 1987; Figge *et al.*, 1988) - and positive regulation - using a *Lex*A/GAL4 hybrid molecule (Brent & Ptashne, 1985) have been achieved. Use of a procaryotic or bacteriophage RNA polymerase in mammalian cells would potentially allow the development of even more tightly regulated control systems, because of the better characterisation of elements regulating procaryotic transcription initiation. Several laboratories have been exploring the possibility of using bacteriophage RNA polymerases in this context; the work I describe in this Thesis is directed towards using T7pol to drive high level expression from a procaryotic promoter

in mammalian cells.

1.4a Bacteriophage T7 RNA Polymerase.

When bacteriophage T7 infects its host, transcription of the phage genome is tightly regulated. The phage early (class I) genes are transcribed by the host RNA polymerase, whilst a newly synthesised T7 RNA polymerase (gene *1*) transcribes the late (class II & III) genes (for review see Studier & Dunn, 1983). About the time the T7pol appears, all host cell transcription ceases, and transcription becomes limited to T7 genes, due in part to the stringent promoter specificity of the T7pol. A burst of T7 transcription occurs in two overlapping stages (McAllister & Wu, 1978): the class II promoters regulate transcription of genes involved in DNA metabolism, and are the first genes to be utilised, followed by the class III promoters which control genes involved in phage structure and assembly.

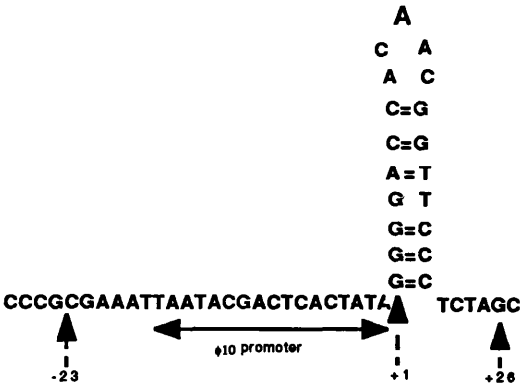
T7 DNA-dependent RNA polymerase (T7pol) [EC 2.7.7.6] was first isolated from T7 infected *E.coli* (Chamberlin *et al.*, 1970). The polymerase enzyme exists as an transcriptionally active monomeric protein, of 107kd molecular weight. The polymerase is distinct from *E.coli* RNA polymerase B, in that it retains 100% activity in 20μg/ml rifampicin, conditions in which the bacterial enzyme is totally inactive. The enzyme is also insensitive to Streptolydigin ($2x10^{-4}$M) and to inhibition by antibodies prepared against *E.coli* RNA polymerase. This enzyme possesses several characteristics which make it especially attractive for use in a heterologous eucaryotic expression system. The active polymerase enzyme exists as a monomer, removing possible problems of complex assembly and subunit association, which might be observed with multi-subunit enzymes, when the polymerase is expressed in mammalian cells. The monomeric nature of the polymerase means that only one gene needs to be introduced into the mammalian germ-line, and also, because the enzyme is small compared to the polymerases of eucaryotic and *E.coli* cells, manipulation of the gene is relatively easy. Importantly the enzyme exhibits almost absolute specificity for transcription initiation at T7 late promoters both *in vivo* and *in vitro* (Golomb & Chamberlin, 1974a: Niles

& Condit, 1975), even genes of the closely related bacteriophage T3 being poorly transcribed (Golomb & Chamberlin, 1974b). The promoter specificity of the T7pol appears to be related the highly conserved nature of the promoter sequence. Seventeen T7 promoters have been identified from the T7 DNA molecule (Carter *et al.*, 1981, Studier & Rosenberg, 1981), and their sequences determined (Panayotatos & Wells, 1979; Rosa, 1979; Dunn & Studier, 1983). None of these sequences are found in any known eucaryotic or procaryotic promoter. The five class III promoters contain the same sequence over 23bp:

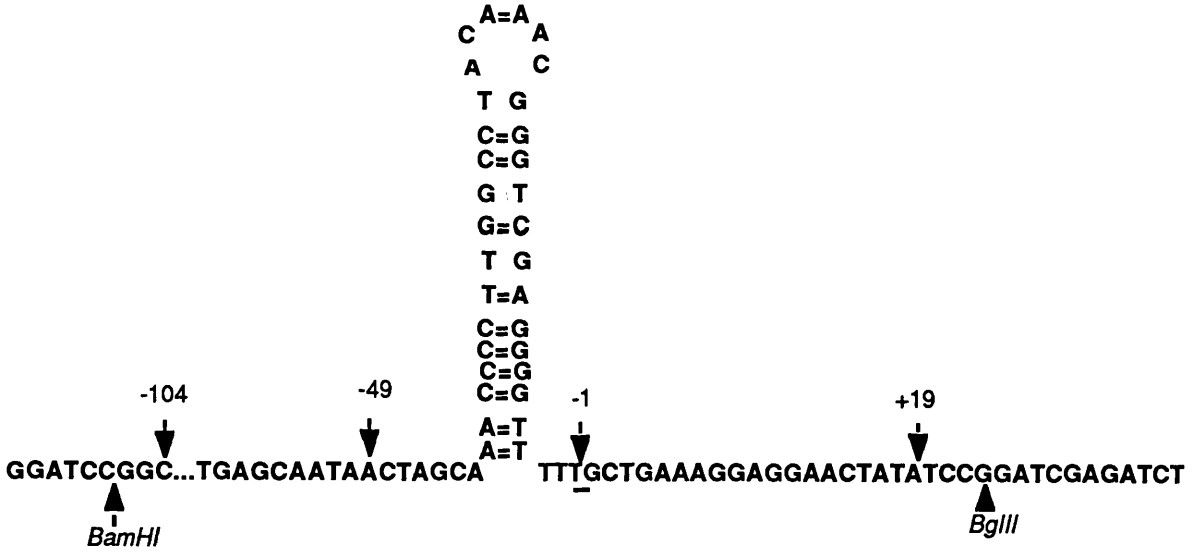
TAATACGACTCACTATAGGGAGA = ϕ10

which includes the transcription start site (underlined), a sequence partially conserved in ten of the class II promoters identified, differing at between two to ten positions. The promoter acts simultaneously as a recognition motif and as a transcriptional start site (see Figure 3). This promoter specificity removes any potential problem of promoter competition, transcription of cellular genes by the phage polymerase, or of recognition of the phage promoter by cellular elements. T7pol requires no other co-factors for the recognition of the promoter, or for the initiation of transcription. This means that the T7 promoter and polymerase will not be competing against endogenous promoters and the cellular polymerase for rate limiting *trans* activating factors, which could cause unexpected pleiotropic effects, by affecting the overall level of expression of an endogenous gene as a result of lower transcription initiation rates at its promoter.

T7pol has been shown to be capable of transcribing long stretches of DNA without terminating, and can transcribe several times around pBR322 DNA both *in vitro* and *in vivo* (McAllister *et al.*,1981; Studier & Moffat, 1986). Transcription *in vitro* can be terminated simply by utilising linear templates, an option not available to *in vivo* systems. Downstream of gene10 in T7 phage is a termination sequence, Tϕ, which has been cloned (Rosenberg *et al.*, 1987) and been shown to be capable of efficient termination of T7 transcription in both linear and supercoiled DNA (see Figure 3). T7 RNA polymerase activity in infected *E.coli* cells is modulated by the expression of a phage-encoded protein, with transcription from T7 late



Nucleotide sequence of the bacteriophage T7 late promoter ϕ 10 showing the potential stem-loop structure formed at the 5'-terminus of the RNA transcript. Positions are numbered realtive to the transcription initiation site.



Nucleotide sequence of the T7 late terminator sequence Tϕ showing the potential stem-loop structure. Nucleotides are numbered relative to the last nucleotide of the terminated transcript.

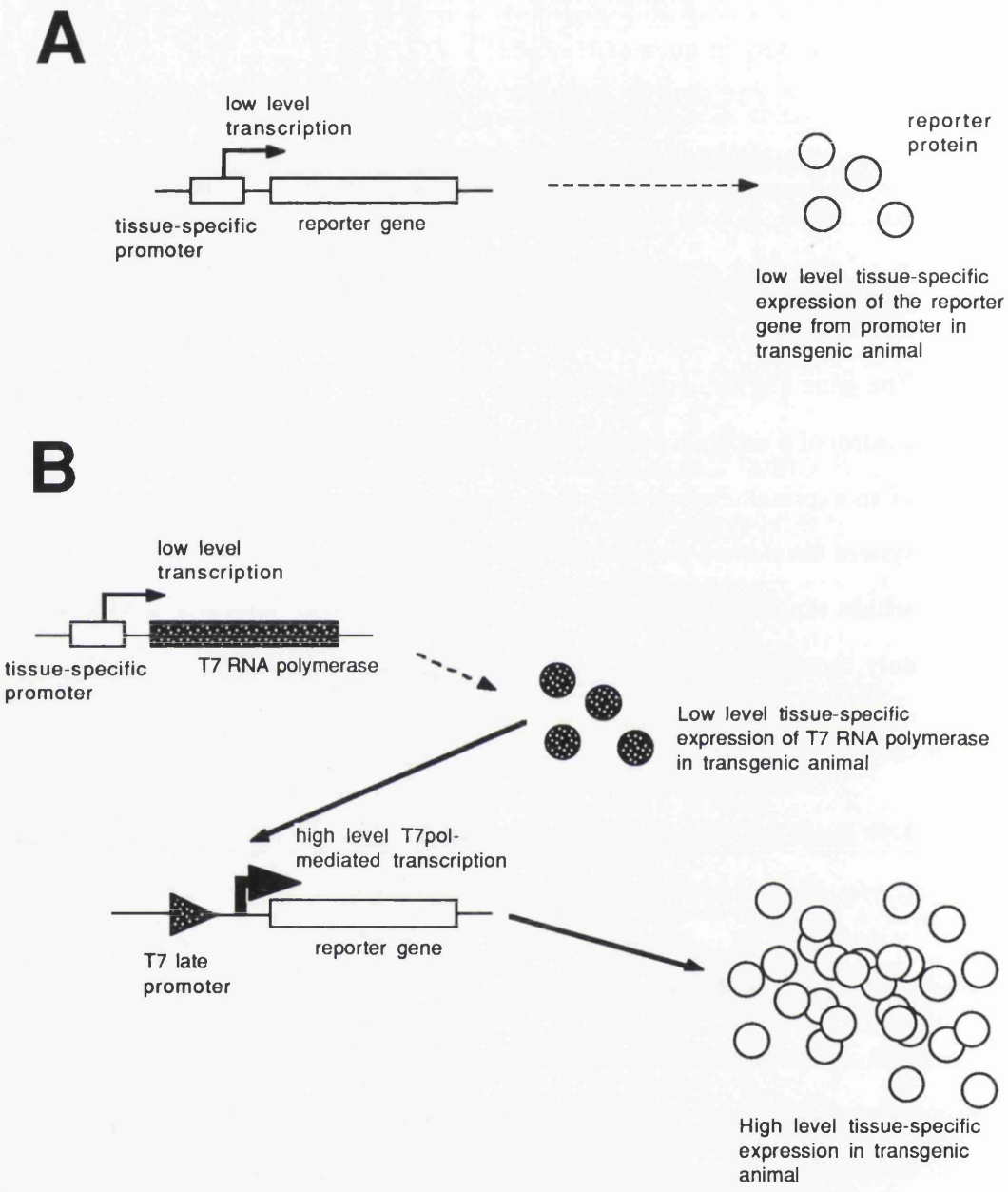
Figure 3. Diagrams showing the potential stem-loop structures formed within RNA transcripts initiated from the bacteriophage T7 late promoter ϕ10 (A) and terminated at the T7 late terminator sequence Tϕ (B).

promoters being shut off at the time of T7 DNA replication (Summers, 1969). This inhibitory activity has been identified as T7 lysozyme (Moffat & Studier, 1987), the enzyme binding to the RNA polymerase and thus inhibiting transcription. The gene encoding T7 lysozyme has been cloned into a mammalian expression vector, and could conceivably be used to modulate T7pol activity in mammalian cells.

The gene encoding T7pol has been cloned (Davanloo *et al.*, 1984) and has formed the basis for high-level expression systems in bacterial cells (Tabor & Richardson, 1985; Studier & Moffat,1986, Rosenberg *et al.*, 1987). During cloning the first ten amino-acids at the N-terminus were deleted, but this affects neither the catalytic activity nor promoter specificity. The gene has also been cloned into the genome of vaccinia virus, under the transcriptional control of a vaccinia promoter (Fuerst *et al.*, 1986) and has been successfully used as the basis of an expression system in eucaryotic cells (Fuerst *et al.*, 1987; Elroy-Stein *et al.*, 1989). This system has demonstrated that the polymerase is active and capable of high level expression within the cytoplasm of CV-1 monkey kidney cells. However the vaccinia/T7pol system is only capable of transient expression since the viral infection ultimately kills the cells.

1.4b Potential Uses of T7pol-Mediated Transcription in Mammalian Cells.

Used in a virus-free system the high specificity of T7pol for T7 late promoters provides a possible basis for a high level expression system for use in cultured mammalian cells and in transgenic animals. The specificity of the polymerase for the T7 late promoters ensures that transcription of the target gene under the control of the T7 late promoter will be unaffected by promoter competition, resulting in high level transcription of any gene placed under the transcriptional control of a late T7 promoter. Therefore, transcription from a T7 late promoter could be used to drive stable high level expression for protein production in culture, a production route that would ensure correct physiological post-transcriptional modification (glycosylation, phosphorylation etc.) of the protein product. This system could be extended to transgenic animals to overcome the problem described earlier


A
low level
transcription
tissue-specific
promoter
reporter gene
reporter
protein
low level tissue-specific
expression of the reporter
gene from promoter in
transgenic animal
B
low level
transcription
tissue-specific
promoter
T7 RNA polymerase
Low level tissue-specific
expression of T7 RNA polymerase
in transgenic animal
high level T7pol-
mediated transcription
T7 late
promoter
reporter gene
High level tissue-specific
expression in transgenic
animal

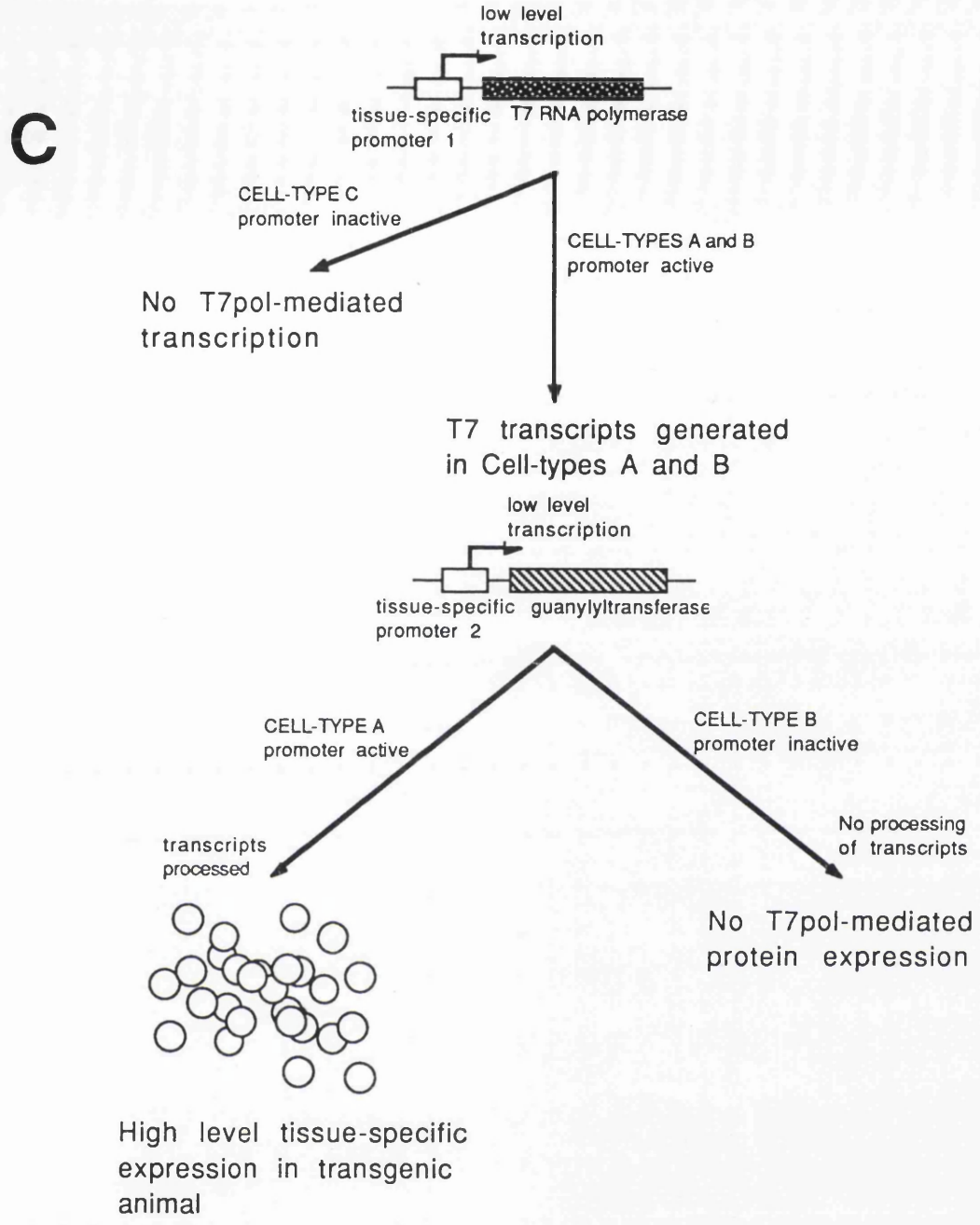


Figure 4. Diagrams showing the potential use of bacteriophage T7 RNA polymerase to drive high level expression in mammalian cells. Low levels of expression of transgenes (A) may be amplified by expression of T7pol which in turn drives high level transcription of the desired gene inserted into the germ-line under the control of the ϕ10 promoter (B). If two or more elements are required for expression from the ϕ10 promoter, cell-type expression may be achieved in the absence of a cell-type specific UPE by use of two or more promoter elements with differing but overlapping patterns of cell-type expression, resulting in the appearence of all elements of the T7 expression system appearing in a single cell-type.

whereby many tissue-specific promoters when reintroduced into transgenic animals show levels of expression lower than the corresponding endogenous promoter (see Figure 4A and Table 1). If T7pol is expressed from such a promoter in a transgenic animal containing a gene under the transcriptional control of a T7 late promoter, the catalytic nature of T7pol may result in an amplification of the level of expression of the desired gene (Figure 4B), in a tissue-specific manner, when compared to levels obtained if the transgene were under the control of the tissue-specific promoter alone (Figure 4A). This amplification of a tissue-specific signal might permit the use of antisense RNA or a dominant negative mutation to functionally inhibit expression of an endogenous gene product within a single tissue or cell-type.

If, as seems probable (see later), that T7pol will require the co-expression of other elements in order for translation to be possible, this may allow the generation of cell-type specificity of T7pol-mediated expression through a combinatorial mechanism in the absence of a single cell-type specific promoter (see Figure 4C). By this mechanism single elements of the expression system may be expressed in a variety of different cell-types, but by linking the elements to different promoters, co-expression of all the elements required for the expression system will only occur in a single cell-type which will therefore be the only site where the target transgene exerts its effect.

Transcription mediated by T7pol in mammalian cells would provide a large pool of pre-mRNA precursors within a cell, which could be useful in the studies of RNA stability, processing and transport, studies which are at present limited because of the absence within nuclei of high levels of a single RNA species. T7pol-mediated expression has already been used in this way to investigate the mechanisms utilised by two DNA-binding proteins in promotion of transcription initiation in yeast (Chen *et al.*, 1987).

Potential problems exist, however, in the use of a procaryotic RNA polymerase in eucaryotic cells. Eucaryotic pre-mRNAs are subject to 5' and 3' modification prior to transport from the nucleus. Both the 5'-capping and polyadenylation of eucaryotic mRNAs

have been implicated in mRNA stability and translation (See section 1.5d), but these are modifications absent from procaryotic messages, and may not be performed on T7 transcripts generated *in vivo*. Chen *et al.* (1987) have reported accurate transcription initiation from a T7 promoter integrated into a yeast chromosome, but also reported that the transcripts were not translated. This probably reflects the inability of the cellular guanylyltransferase to cap the T7 transcripts, rendering them unrecognisable to the yeast ribosome. If a eucaryotic expression system based upon T7pol is to be capable of high level expression at the protein level, this block to translation must be overcome.

Also it has been shown that T7pol, when introduced into mammalian cells, is excluded from the nuclei (Dunn *et al.*, 1988; Lieber *et al.*, 1989; this Thesis). Nuclear proteins in general possess one or more nuclear location signal (NLS) that are recognised by components of the cellular transport apparatus and specify transport into nuclei through nuclear pores. For example SV40 large-T antigen possesses a single NLS (pro-lys-lys^{128}-lys-arg-lys-val) and addition of this signal to cytoplasmic proteins is sufficient to cause them to accumulate in nuclei (Kalderon *et al.*, 1984). Addition of the SV40 large-T NLS to T7 RNA polymerase results in rapid accumulation of the polymerase in cell nuclei (Dunn *et al.*, 1988; Lieber *et al.*, 1989; this Thesis). It is unclear whether this targetting is necessary for expression of genes from an integrated T7 promoter, since in yeast it has been possible to obtain transcription from an integrated T7 promoter using the native form of the polymerase (Chen *et al.*, 1987). From this it can be seen that accessibilty of the T7 promoter to the polymerase through the localisation of the chromosomal DNA within the nucleus, and the packaging of the DNA (see below) may also affect the ability of T7pol to mediate expression within eucaryotic cells.

1.5 Control of Gene Expression in Eucaryotic Cells.

Procaryotic and eucaryotic gene expression mechanisms and machinery share some common features, both at the transcriptional and translational levels. In both systems genes are transcribed from a DNA template to produce an mRNA species, which is then translated

at a ribosome. In both cases the expression of a gene can be controlled in response to external factors usually by modulation of transcription initiation. Appearance of a gene product can also be modulated at the post-transcriptional level, by modulation of mRNA stability, translatability, and also by the turnover of the protein product itself. Little is known about the factors that control regulation at these points, however, and transcription initiation appears to be the dominant factor in regulation of gene expression in the majority of cases. The code used to specify the protein is common to both systems; hence bacterial proteins can be expressed in mammalian cells (Mulligan & Berg, 1980), and procaryotic mRNA can be translated in eucaryotic cell-free systems (Paterson & Rosenberg, 1979). Ribosomes from eubacteria and the eucaryotic 80S ribosomes have the same basic structure, as seen by electron microscopy, although the eucaryotic ribosomes are slightly larger (Boublik & Hellman, 1978). The processes of aminoacyl-transfer RNA (tRNA) binding, peptide bond formation, and ribosomal translocation are virtually identical, and all ribosomes display the same division of labour between the small and large subunits.

Never the less there are features of translation, transcription and RNA structural requirements that differ between procaryotic and eucaryotic systems and may affect expression mediated by T7pol within mammalian cells. Regulation of eucaryotic gene expression will be discussed below. Any differences from the eucaryotic model observed in procaryotic expression, and the possible implications of these differences, will also be covered.

Expression of genes within mammalian cells, and other eucaryotic cells, can be regulated in a variety of different ways. Packaging of the genome into chromatin and chemical modification of the DNA itself may affect the accessibility of the DNA to the transcription machinery and thus influence transcription of specific sequences. Expression can be affected by the regulation of transcription initiation, and since messenger RNA is modified prior to nuclear export, further modulation of gene expression by alternative splicing and polyadenylation can occur (see below). Transcription and translation occur in different subcellular compartments and eucaryotic cells may also be able to regulate the

appearance of a gene product by modulation of the rate of mRNA transport between the compartments (see below).

1.5a Active Eucaryotic Genes.

In eucaryotic cells the chromosomal DNA is highly packaged along with protein, to form chromatin and within the nucleus the majority of the DNA is too highly condensed to permit transcription. It follows, therefore, that the actively transcribed sequences must be packaged in a different manner to the bulk of the DNA (reviewed by Weisbrod, 1982, and Thomas, 1984).

In the interphase nucleus DNA is organised into two predominant forms, the 10nm and 30nm fibres. The 10nm fibre, also known as nucleosome filament, is a linear array of nucleosome particles appearing as a "beads-on-a-string" structure in electron microscopy. Each nucleosome contains between 166-241bp of DNA (depending upon the source) associated with an octomeric protein core comprising pairs of each of four types of histone - H2A, H2B, H3 and H4. The DNA is organised in two turns of about 166bp sealed by the binding of a fifth histone protein, H1 (forming the chromatosome). The remainder of the nucleosome DNA forms a linker between chromatosome cores. The 10nm fibre undergoes "higher order" folding forming the 30nm fibre, a left-handed superhelix with approximately six nucleosomes per turn, possibly induced by histone H1 acting as a cross-linker. This 30nm fibre probably represents the bulk of interphase DNA, and transition between the 30nm and 10nm forms is thought to represent a coarse mechanism for regulation of gene expression by determining accessibility for transcription.

Within chromatin there exist regions known as *nuclease hypersensitive sites* (reviewed by Gross & Garrard, 1988), which are believed to represent the open windows that allow access of transcriptional control proteins to *cis* acting DNA elements that regulate transcription initiation. Comprising only 1% of the total DNA present, these regions are

defined by their increased sensitivity to nuclease activity, or to chemical modification which is two orders of magnitude greater than that of bulk DNA. These regions are histone depleted but are associated with proteins including pol*II*, topoisomerases *I* and *II*, a variety of *trans* activating transcription factors and members of the high mobility group (HMG) proteins. These regions represent the control sequences of genes either undergoing transcription or poised to be transcribed. Constitutive hypersensitive sites are often present in the promoter regions of "housekeeping" genes and their positions within the DNA can vary among different cell-types, these sites being termed tissue-specific. Certain hypersensitive sites can be induced prior to transcription of a gene, but are not dependent upon transcription because they can persist after the removal of the inducing agent. Tissue-specific sites that appear transiently during embryogenesis are termed developmental hypersensitive sites. Evidence is also emerging for the presence of so called "superhypersensitive" sites, so-called because of their increased sensitivity to nuclease digestion, which are tissue specific sites located over 10kb from a gene which affect expression of a *cis* located gene (Kioussis *et al.*, 1983; Groudine *et al.*, 1983; Tuan *et al.*, 1985; Forrester *et al.*, 1986).

Another feature associated with active chromatin is the undermethylation at cytosine residues within CpG dinucleotides, where generally the cytosine residue is methylated (Felsenfeld & McGhee, 1982, and reviewed by Bird, 1986). Experiments involving transfection of genes methylated *in vitro* have shown that highly methylated genes are not transcribed when introduced into mammalian cells, whereas undermethylated genes are actively expressed (Stein *et al.*, 1982), but the *in vivo* implications of DNA methylation (absent in insects) are uncertain since undermethylation does not necessarily result in gene expression as seen in the case of the chicken α(2)I collagen gene which in five cell types - chick embryo fibroblast (CEF), RSV transformed CEF, brain, erythrocyte and spermatozoa - has an invariant *HpaII* methylation pattern, with some sites totally unmethylated, irrespective of whether or not the gene is expressed (McKeon *et al.*, 1982).

1.5b Regulation of Transcription Initiation.

The cloning of individual genes has allowed *cis*-acting DNA sequences that control the temporal and tissue-specific expression of genes to be identified. In higher eucaryotes two elements which bind protein factors - promoters and enhancers - have been found to be necessary for the regulation of genes encoding mRNAs (Reviewed by Dynan & Tjian, 1985; Serfling *et al.*, 1985; Maniatis *et al.*, 1987). Promoters, typically about 100bp in length (Dynan & Tjian, 1985), are located immediately upstream from the transcription start site, and are required for efficient and accurate transcription initiation. Usually this element contains an element known as the "TATA box" approximately 30bp upstream from the transcription initiation site, which ensures correct initiation of the mRNA. A typical promoter also contains at least one further 8-12bp element upstream of the TATA box. These additional elements are called upstream promoter elements (UPEs). Enhancers as their name suggests enhance the level of transcription and are characterised by their ability to act upon *cis* located promoters. Enhancers can be located at large distances (over 3kb) both 5' and 3' to the gene, and act in an orientation-independent manner. Enhancers, like promoters, are composed of multiple short motifs, some of which have been found to appear both in promoters and enhancers, for example a component of the immunoglobulin enhancer, the "octamer" element, also appears in a number of promoters including the immunoglobulin heavy chain and U2 snRNA promoters (Bohmann *et al.*, 1987). Along with this structural similarity promoters and enhancers appear to possess functional similarities. The SV40 promoter when placed adjacent to the TATA box of a deleted ß-globin promoter is able to restore high level transcription of the gene (Treisman & Maniatis, 1985). Any operational difference between the two classes of upstream element (ie. action at a distance), therefore, appears to stem from differences in the arrangement and number of UPEs and not from any fundamental difference in the mechanisms by which these elements act.

Proteins recognising UPEs have been purified to homogeneity, and are providing insights into the mechanisms by which the DNA sequences contained within promoters and enhancers exert influence upon transcription (Ptashne, 1986; Wang & Giaever, 1988; Ptashne,

1988). Much of the information concerning DNA sequences governing tissue-specificity, and the role of specific UPEs, has come from re-introduction of mutated sequences and truncated control regions into cultured cells or animals and observing changes in patterns of expression (Hammer *et al.*, 1987).

1.5c Transcription termination.

The process of transcription termination is poorly defined in both procaryotic and eucaryotic systems. In procaryotes termination of transcription takes place at strictly defined positions, corresponding to the end of the translated mRNA. *rho*-dependent termination depends upon the activity of the termination factor *rho*, an ATP dependent DNA-RNA helicase (Brennan *et al.*, 1987) which may operate by unwinding the nascent RNA chain from the DNA template up to the RNA polymerase stalled at a specific sequence, and in this way promote transcription termination. *rho*-independent termination can also occur in bacteria, the presence of an inverted repeat in the terminator sequence promotes the formation of a hairpin loop in the nascent transcript. This is thought to cause the polymerase to pause and allow its immediate release. Such stem-loop structures are seen in bacteriopage T7 Tϕ terminator sequences (see Figure 3).

In eucaryotes RNA polymerase *II* initiates transcription at the promoter and reads through all intron and exon sequences finally passing into the 3' flanking region of the gene. Termination of transcription (reviewed by Proudfoot, 1989) often occurs between 600-4,000bp beyond the normal 3' end of the mature mRNA (which is defined by an endonucleolytic cleavage; see below) and can occur heterogeneously over a kilobase or more of sequence as in the case of the mouse α-amylase (Hagenbüchle *et al.*, 1984) and ß-globin genes (Citron *et al.*, 1984) rather than at a defined position. snRNA and histone genes, however, differ from other genes transcribed by pol*II* in that transcription termination occurs at clearly defined sequence positions relative to the 3' end of the mature RNA.

The exact mechanism for transcription termination for the majority of pol*II* transcripts remains unclear, partially due to the short half-lives of the primary transcript, which is rapidly processed, and of the 3' excised sequences (less than 10 minutes) (Citron *et al.*, 1984). There is evidence, however, that links transcription termination to the 3' processing of the primary transcript. In the mouse ß-globin gene, termination occurs over a 1kb region, but transfer of these sequences to the adenovirus E1A gene does not promote authentic transcription termination unless the poly(A) signal (see below) is included (Falck-Pederson *et al.*, 1985), and further to this, point mutations in this poly(A) signal abolishes these termination events. Also a single point mutation in the human α2-globin gene in the poly(A) signal (from AATAAA to AATAAG) prevents normal 3' processing and promotes transcription beyond the flanking region in which termination usually occurs (Whitelaw & Proudfoot, 1986).

Two models have been proposed for the mechanism of transcription termination in eucaryotes. The first proposes a specific elongation factor associated with the transcription complex, which dissociates from the complex when a poly(A) signal is encountered. Without this elongation complex the RNA polymerase becomes prone to random termination beyond the end of the gene (Logan *et al.*, 1987). The second implicates the 3' processing mechanism more directly, proposing that 3' processing of the nascent RNA chain occurs whilst the RNA polymerase is transcribing sequences further downstream. The newly formed 5' end of the nascent chain is uncapped and, therefore, suseptible to 5'→3' exonuclease digestion (see below) and it is proposed that an RNA-DNA helicase (similar to the procaryotic transcription terminator *rho*) is coupled to this exonuclease activity, unwinding the nascent RNA chain from the DNA template and promoting termination in an analogous manner to that thought to operate in procaryotic *rho* dependent transcription termination. RNA secondary structure in the flanking region is also implicated in termination, in an analogous manner to *rho*-independent termination, promoting polymerase stalling. A potential stem-loop structure has been proposed in the 3' flanking region of the chicken $ß^H$-globin gene and has been implicated in the transcription termination at this gene (Pribyl & Martinson, 1988). This second model is supported by evidence from termination of transcription of the polyoma

virus late gene (which possesses a weak poly(A) signal) on episomal DNA, where termination only occurs after multiple transcription rounds of the circular DNA template. When a stronger poly(A) signal is substituted, accurately processed, monocistronic transcripts are produced, as would be predicted by this model (Lanoix & Acheson, 1988).

snRNA genes possess a consensus sequence immediately downstream of the 3' terminal stem-loop structure present at the end of all snRNAs. Deletion of this consensus sequence disrupts 3' end formation and results in snRNAs with extended 3'-termini (Kunkel & Pederson, 1985). It is likly that snRNAs generate their 3'-termini by accurate termination of transcription, possibly promoted by the presence of specific transcription factors associated with transcription from promoters of snRNA genes. Interestingly the consensus sequence does not function on transcripts from a heterologous promoter, instead the polymerase reading through and a downstream poly(A) signal utilised (Hernandez & Weiner, 1986).

Transcriptional termination of the sea urchin histone H2A gene has been characterised. Unlike other genes, the 3' processing signals can be removed without affecting transcription termination, and the termination sequences can function efficiently in a heterologous α-globin gene (Johnson *et al.*, 1986). Three sequence elements, all of which are required for termination, have been identified, one contained within the coding region of the histone gene and the other two in the 3' flanking region. Termination appears to occur within the first of the required sequences found in the 3' flanking region.

Tissue-specific patterns of polyadenylation have been determined for over 50 genes (reviewed by Leff *et al.*, 1986; Andreadis *et al.*, 1987), these processes being important in determining iso-enzymetric structure and possibly important in message stability and translational efficiency.

1.5d Modification of cellular mRNAs.

In eucaryotic cells pre-mRNAs are subject to three major post-transcriptional modifications that characterise the transformation from primary transcript to mature message. These modifications are polyadenylation (for review see Birnstiel, 1985); intron excision (Cech, 1983), and the addition of a methylated 5'-guanosine cap structure (reviewed by Banerjee, 1980). The fully processed transcript is then exported from the nucleus into the cytoplasm. The requirement for each of these modifications for further RNA processing and transport into the cytoplasm is unclear. Introduction of RNA, synthesised *in vitro* from an SP6 promoter, has shown eucaryotic cells to be capable of accurate intron excision (Green *et al.*, 1983) and polyadenylation (Moore & Sharp, 1985) of exogenous RNAs. In general these modifications do not occur in procaryotic cells, translation of the RNA transcript occurring prior to, or immediately following transcription termination. Procaryotic genes are colinear with the proteins encoded by them, no introns having been discovered in procaryotic genomes, with the exception of certain members of the Archaebacteria, so there is no requirement for intron excision in these systems. Some procaryotic RNAs have been shown to possess a short poly(A) tail (approximately 24 residues) of unknown function (Kozak, 1983) but the vast majority do not. Furthermore no 5'-cap structure is associated with procaryotic transcripts, the message terminating as the 5'-triphosphate or diphosphate.

i) Polyadenylation.

With the exception of some histone mRNAs, eucaryotic pre-mRNAs undergo a modification at the 3' end. This process involves a two step reaction with a site-specific cleavage followed by the addition of approximately 200 adenosine residues (reviewed by Proudfoot, 1989). Two *cis* acting sequences are required for polyadenylation, the invarient hexamer, AAUAAA, which is highly conserved in nature (Proudfoot & Brownlee, 1976), and which is found approximately 18-30nt upstream of the polyadenylation site, and a second region, a GT-rich sequence located downstream of the cleavage site. The cleavage and polyadenylation reactions appear to be linked because cleaved non-polyadenylated mRNA is not detected *in vivo* or *in vitro* except in exceptional circumstances. This coupling is, however,

not obligatory because cleaved RNAs are detected *in vitro*, when polyadenylation is inhibited by the presence an inhibitor such as EDTA (Moore & Sharp, 1985), and pre-mRNAs can be polyadenylated at their 3' ends *in vitro* in the absence of cleavage. These substrates whether terminated at the accurate *in vivo* polyadenylation site or at a position 3' to this site, all required the presence of an intact AAUAAA signal sequence, which suggests that both pathways involve the same mechanism and that cleavage and polyadenylation both require the AAUAAA signal. Three *trans*-acting factors are required for accurate polyadenylation and these include a cleavage activity, a polyadenylase activty (Takagaki *et al.*, 1988) and possibly a snRNP, designated U11 (Christofori & Keller, 1988). Histone mRNAs containing no AAUAAA signal can also be polyadenylated in amphibian oocytes, and erroneous 3' ends have been observed to be polyadenylated, but these reactions occur with poor efficiency and yield short poly(A) tails and so this may reflect an alternative mechanism (Manley,1983). Pre-existing polyadenylated mRNA can be further polyadenylated in the cytoplasm of cells (Brawerman, 1981), the importance of this modification being poorly understood.

3'-end formation of non-polyadenylated histone mRNAs also involves processing of the message. The snRNP U7 mediates an endonucleolytic cleavage by base pairing with the $^{G}/_{A}$AAAGA consensus cleavage signal found downstream of all histone mRNA 3' termini. The cleavage site is also flanked by a palindromic sequence capable of forming a stem-loop structure close to the 3' end of the mature mRNA (Birchmeier *et al.*, 1983) and mutations within this palindrome which destroy the ability to form a stem-loop structure also prevent 3' processing (Birchmeier *et al.*, 1984). This cleavage event has been implicated in cell-cycle regulation of histone mRNA accumulation (Lüscher & Schümperli, 1987).

The function of the poly(A) tail remains uncertain. Injection of RNA into Xenopus oocytes has shown that the presence of the poly(A) tail increases the stability of the injected message and also was required for translation of the message (Drummond *et al.*, 1985). The poly(A) tail has also been implicated in message transport (see later) by the discovery of a nuclear membrane bound ATPase stimulated by poly(A) (Bernd *et al.*, 1982). This ATPase activity is thought to provide the energy required for mRNA nucleo-cytoplasmic

translocation.

ii) Intron excision.

The coding regions of many eucaryotic genes (including rRNA and tRNA genes) contain intervening non-coding DNA sequences, introns. These sequences are transcribed along with the rest of the gene and must be excised from the RNA to form functional RNAs. Three classes of RNA splicing reactions are defined, based on how the splice sites are specified (reviewed by Cech, 1983). It is apparent that although there are similarities and conservation of mechanisms between lower and higher eucaryotes, precise details of snRNP and protein factor binding varies between systems studied.

Pre-tRNAs possess no consensus sequences to define their splice sites, neither sequences surrounding or within the intron being conserved. A feature common to all tRNA introns is, however, that they all interupt the tRNA sequence one base 3' to the anticodon, and do not alter the highly conserved secondary and tertiary structure of the species. It is the conserved structure of the mature tRNA that appears to function as the major recognition element for splicing.

Pre-rRNAs of *Tetrahymena* are able to excise introns by an autocatalytic reaction, which *in vitro* requires no protein intervention. The excised intron undergoes a second reaction to form a covalent circular structure. The discovery of *cis*-dominant splice defective mutants has established that the integrity of the intron is essential for splicing.

The final class of splicing reactions involves pre-mRNAs, where introns are excised as a circular species (lariat RNAs) in a reaction mediated by the major snRNPs, U1, U2, U4, U5 and U6 (reviewed by Padgett *et al.*, 1985; Maniatis & Reed, 1987 and Green, 1989). The reaction involves cleavage of the RNA at the 5' intron/exon splice junction and formation of the lariat RNA by formation of a 5'-2' linkage between the newly formed free-5' end of the intron and a nucleoside positioned close (18-30bp) to the 3' end of the intron sequences, designated the branch site. U1 snRNP binds the 5' splice site and this is followed by the binding (in an ATP dependent manner) of the U2 snRNP to the branch site. Binding of U2

snRNP requires the presence of U1 snRNP and a protein factor -U2 snRNP auxilary factor (U2AF)- which binds to the 3' splice site and which facilitates binding of U2 snRNP to the branch site. Following this, a pre-formed snRNP complex (U4/U6/U5) is incorporated into the splicing complex, principly in association with bound U1 and U2 snRNPs. Finally U4 snRNP dissociates from the complex, leaving an active spliceosome complex in association with the RNA.

Consensus sequences important for intron excision have been identified at the 5' and 3' extremities of intervening sequences, and at the branch sites, but understanding of splice site selection remains poorly understood. This is because these consensus sequences occur more frequently within an RNA than authentic splice sites and so cannot be the sole determinants of a splice junction. Mutation of splice sites or branch points can abolish authentic splicing events, but frequently these lead to alternative splicing events at cryptic splice or branch sites not usually utilised.

Splicing is not essential for nucleo-cytoplasmic transport of mRNA (see below) as had been previously thought. *Cis* and *trans*-acting mutants in yeast have been identified that cause transport of pre-mRNA (ie. unspliced) into the cytoplasm (Legrain & Rosbach, 1989). Deletion of either the 5' splice site or the branch site resulted in transport of unspliced message. Similarly two temperature sensitive mutants (*rna* mutants *rna6* and *rna9*) have been identified which transport pre-RNA when grown at the non-permissive temperature, although the *trans*-acting factors encoded by these genes have not yet been identified. These results have lent weight to the suggestion that splicing occurs either during transcription or soon after transcription termination, and that the factors responsible for splicing compete with the mRNA export machinery for newly synthesised RNAs.

Alternative splicing is an important process (reviewed by Breitbart *et al.*, 1987) that allows the generation of protein diversity in eucaryotes. Differential incorporation of exons often occurs in a tissue-specific or developmental manner - for example certain Troponin T exons are incorporated in differentiated myotubes but not myoblasts (Breitbart & Nadal-

Ginard, 1987). The mechanisms that allow alternative splicing are again poorly understood, although in the example given two developmentally regulated, muscle-specific *trans*-acting factors have been identified that are responsible for this alternative splicing event.

iii) 5'-Capping of mRNAs.

A 7-methyl-guanosine cap structure located at the 5'-terminal, with a unique 5'-5' linkage (Reviewed by Shatkin, 1976) is a feature of all eucaryotic cellular mRNAs and of mRNAs from both DNA and RNA viruses (Reviewed by Banerjee, 1980) with the exception of the picornaviruses (Hewlet *et al.*, 1976, Lee *et al.*, 1977), and certain RNA plant viruses including satellite tobacco necrosis virus (STNV) (Leung *et al.*, 1979), its helper virus (Lesnaw & Reichman, 1970), and cowpea mosaic virus (CMV) (Stanley *et al.*, 1978). The snRNAs U1, U2, U4, U5 (Maniatis & Reed, 1987) and U6 (Reddy *et al.*, 1987) have also been found to contain related structures. Isolated virions of poliovirus, a picornavirus, have been shown to possess a 12kDa protein covalently linked to the 5'-terminus of the viral genome, although the mRNA produced terminates at the 5'-end as pUp. Encephalomyocarditis virus (EMCV) and foot-and-mouth-disease virus (FMDV) possess a similar structure to that of poliovirus in both the virion RNA and the mRNA. Feline leukemia virus (FLV) was also reported to possess an uncapped mRNA, but this appears to be due to an initial lack of distinction between the virion RNA, which does not possess a cap structure, and the cytoplasmic mRNA which is now thought to contain a 5'-terminal cap structure (Thomason *et al.*, 1976; Thomason *et al.*, 1978). STNV contains a di- or tri-phoshate (p)ppA at the 5'-end, similar to bacterial and bacteriophage mRNAs.

It has been shown that 5'-terminal capping of mRNAs is an early event in message processing and has been implicated in the initiation of transcription, since the presence of S-adenosylhomocysteine, an inhibitor of the guanylylytransferase-associated methyltransferase activities, has been shown to inhibit transcription initiation by pol*II* (Jove & Manley, 1982; Ernst *et al.*, 1983; Spencer & Garcìa, 1984).

a) Structure of the 5'-Cap.

The cap structure is formed by a unique 5'-5' linkage (for review see Shatkin, 1983) containing three phosphate groups between a guanosine residue and the penultimate 5' residue (see Figure 5). The capping base is always ^{7}methyl guanosine, a feature constant throughout all cellular and viral caps, except for Sindbis virus where infected cells have been shown to contain a low percentage of viral message containing a 2,2,7-trimethyl guanosine residue (Dubin *et al.*, 1979) The cap structure isolated from eucaryotic mRNAs has been characterised and possesses the general structure ^{7}mG(5')ppp(5')$N_1{}^{m}pN_2{}^{m}pN_3$ where N is a purine or pyrimidine. The methylation status of the two initial bases following the cap varies generating the following structures: ^{7}mGpppN$_1$pN$_2$ (Cap-0), ^{7}mGppp^{m}N$_1$pN$_2$ (Cap-1), and ^{7}mGppp^{m}N$_1$p^{m}N$_2$ (Cap-2). Increased methylation of the cap generally occurs with increasing complexity of the organism from which the RNA has been isolated. Yeast shows a high proportion of Cap-0 bearing mRNA whereas human cells seem to possess mRNA bearing only only Cap-1 or Cap-2 structures. Studies on the cap structure ^{7}mGppp^{m}ApAp using NMR techniques revealed that the three bases form a closely stacked array, which results in increased thermodynamic stability (Kim & Sharma, 1977) and that due to conformational constraints the ^{7}mGp residue is able to intercalate between the two adenosine bases (Hickey *et al.*, 1977). This feature may play a role in stability of the message and also affect interaction with proteins of the cap-binding complex.

b) Biological Function.

The cap structure has been shown to possess two distinct functions, relating to RNA stability and translational efficiency. The relationship of mRNA capping to RNA stability has been shown, by injection of *in vitro* generated SP6 transcripts into *Xenopus* oocytes, where it was possible to show that message stability is increased by the presence of a 5'-cap (Drummond *et al.*, 1985). Experiments involving injection of capped and uncapped reovirus RNA into the cytoplasm of *Xenopus* oocytes has suggested that the stability conferred to an mRNA species by the presence of a cap structure results from increased resistance to 5'→3' exonuclease activity (Furuichi, 1977), and data from studies upon c-*myb* mRNA, which shows tissue-specific cap site selection, has suggested that the abundance of this mRNA

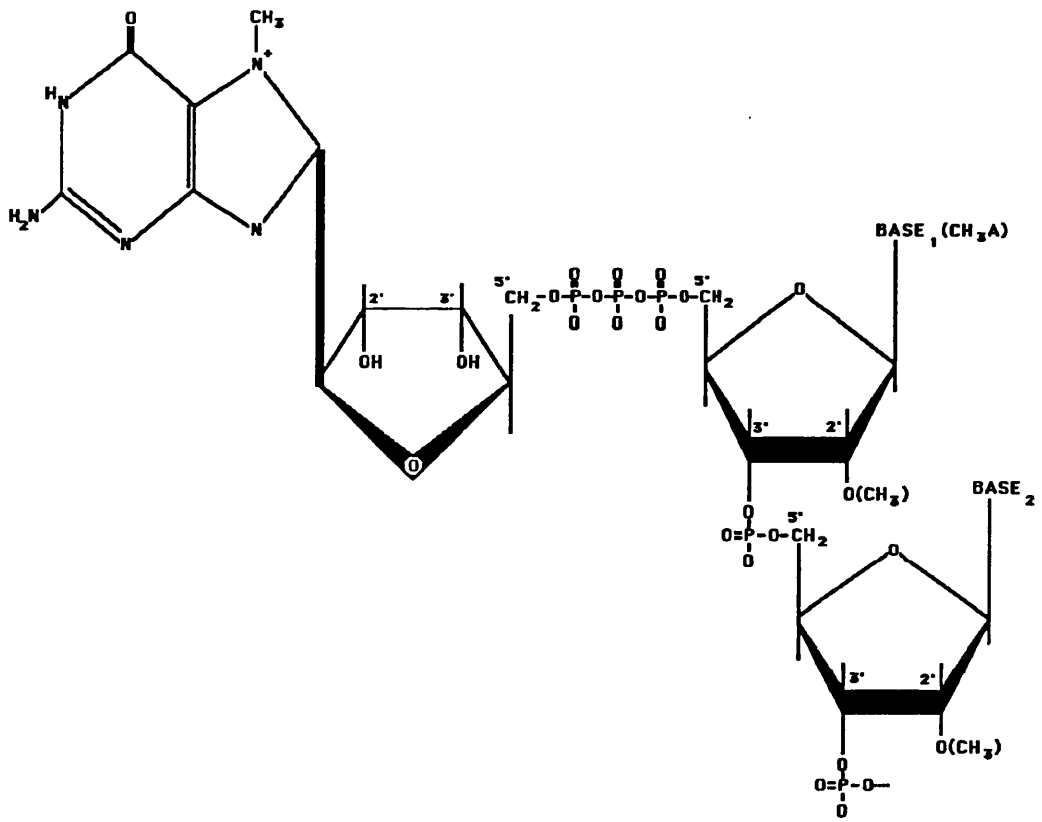


Figure 5. Diagram of the methyl-guanosine cap structure located at the 5'-termini of eucaryotic mRNAs. When $base_1$ is adenosine, it is methylated at the N-6 position.

species is regulated in part by the choice of cap site utilised (Watson *et al.*, 1987). The importance of the cap for RNA stability within the nucleus was shown by Green *et al.* (1983) using SP6 transcribed ß-globin transcripts generated *in vitro*. Injection of transcripts into the nuclei of *Xenopus* oocytes showed that capped mRNA remained intact for up to 24 hours, but uncapped material was totally degraded within 5 hours.

The requirement of a cap structure for efficient translation of mRNA was first demonstrated in wheat germ cell-free translation systems using message from vesicular stomatitis virus (VSV) and reovirus (Both *et al.*, 1975). These experiments showed that capped-and-methylated VSV and reovirus mRNAs were translated, whereas the corresponding unmethylated-capped and uncapped messages were untranslatable. Removal of cap structures from eucaryotic mRNAs by periodate treatment (Kemper, 1976), potato nucleoside pyrophosphatase (Zan-Kowlczewska *et al.*, 1977) and tobacco pyrophosphatase (Shimotohno *et al.*, 1977) in cell-free extracts from wheat-germ, reticulocytes, *Artemia salina* (brine shrimp) and ascites also showed the cap dependence of translation. Requirement for the cap was shown, however, to depend on the cell-free extract used (Rose & Lodish, 1976; Held *et al.*, 1977) and also on the K^+ concentration, cap dependence increasing with increasing K^+ concentration (Wodnar-Filipowicz, 1978). It was further shown that the methylation status of the cap is also important for translation and ribosome binding (Muthukrishnan *et al.*, 1975). Lack of the 7-methyl group from the cap can reduce ribosome binding and translation by as much as 90% (Lodish & Rose, 1977; Muthukrishnan *et al.*, 1978). In the absence of the 7-methyl group the presence of the 2-O-methyl group on the penultimate nucleoside increases *in vitro* ribosome binding by 5%. Both reticulocyte lysates and wheat germ cell-free extracts depend on the presence of a 7-methyl group for the formation of stable translation initiation complexes (Muthukrishnan *et al.*, 1976). These cell-free extracts have been shown to be capable of adding of the 7-methyl moiety to the cap structure, but not the 2-O-methyl group (Muthukrishnan *et al.*, 1975a & b).

Using the synthetic polynucleotides, $^7mGpppG^mC(U)_n$ and $^7mGpppG^mc(A,C)_n$ it was shown that the capped species was able to bind more efficiently to the 40S ribosomal subunits

than the corresponding uncapped species (Both *et al.*, 1976). Addition of a methyl-cap structure to uncapped mRNA increased the rate and extent of binding of vaccinia virus mRNA to wheat germ ribosomes (Muthukrishnan *et al.*, 1976 & 1978), and when capped and uncapped mRNAs were mixed, the capped mRNA selectively and rapidly associated with the ribosomes. Injection of uncapped RNA into the nuclei of cells and its subsequent instability and lack of translation has suggested that the cellular guanylyltransferase is not available to cap exogenous mRNAs. Paterson and Rosenberg (1979) have shown by obtaining *in vitro* translation of λ *cro* mRNA following capping of the RNA (using vaccinia guanylyltransferase to produce a Cap-1 structure), that procaryotic mRNAs possess all the signals necessary for translation initiation except for the cap structure.

1.6 Regulation of mRNA translation: Ribosome recognition.

The limited ability of procaryotic mRNAs to be translated by eucaryotic ribosomes (and of eucaryotic mRNA to be translated at procaryotic ribosomes) suggests dissimilarities in the initiation processes of the two systems. One similarity is that both systems apparently require the ribosome binding site to be relatively free of secondary structure. In procaryotic translation initiation the evidence supports the role of mRNA/rRNA base pairing in the selection of initiation sites (reviewed by Kozak, 1983). This conclusion was drawn from the original observation that complementary sequences capable of base-pairing are present just upstream from the initiator codon in mRNA and at the 3'-end of the *E.coli* 16S rRNA (Shine & Dalgarno, 1974), and largely confirmed by the sequencing of the ribosomal binding sites of over 150 bacterial and phage mRNAs (reviewed by Gold *et al.*, 1981), which has revealed the presence of the Shine-Dalgarno sequence preceding each cistron on the mRNA. Some mRNAs are known to lack this recognition sequence; in these examples the initiation codon is too close to the 5'-terminus of the mRNA to permit the presence of the Shine-Dalgarno sequence. The spacing between the Shine-Dalgarno sequence and the initiator codon is critical, on average seven bases between the end of the run of purines and the A of the AUG initiation codon (Stormo *et al.*, 1982). The importance of the spacing is demonstrated by the

fact that the functional initiation codon is not always the fist AUG or GUG following the Shine-Dalgarno sequence, for example in the *E.coli trpC* initiation site, GAGGGUAAAUGAUG, (Christie & Platt, 1980), or the *E.coli lacI* initiation site GGUGGUGAAUGUG (Steege, 1977). In each of these examples the Shine-Dalgarno bases are shown in the larger type, the non-functional AUG and GUG initiation codons are underlined, and the correct initiation site is in bold.

In eucaryotic systems no sequence analgous to the Shine-Dalgarno box is known to promote recognition of mRNA by the ribosome. Recognition of mRNA and translation initiation appears to be mediated by protein factors, designated initiation factors (eIF : e denoting eucaryotic) interacting with the 5'-terminal cap structure (Kozak & Shatkin, 1976; reviewed by Rhoads, 1988) followed by "scanning" of the mRNA sequence downstream of the cap to find the first suitable AUG initiation codon (Kozak, 1983).

Four protein factors - eIF-4A, eIF-4B, eIF-4E and p220 acting as the eIF-4 complex - promote mRNA binding to the 43S translation initiation complex [comprised of the 40S ribosomal subunit associated with methionine tRNA ($tRNA^{met}$) and GTP bound eIF2] to form the 48S initiation complex. The mRNA is bound by the eIF-4 complex through the 5'-cap structure, the exact form of the cap-binding complex being uncertain since the individual factors can be purified individually, or as intermediate, possibly transient, complexes of two or more proteins ("cap-binding protein complex" (CBPC) composed of eIF-4E and p220; eIF-4F composed of CBPC + eIF-4A). The first factor to interact with an mRNA is eIF-4E, also known as Cap Binding Protein (CBP), the only known factor to possess affinity for mRNA in the absence of ATP hydrolysis (Sonenberg, 1981; Hellman *et al.*, 1982). This binding is able to occur irrespective of mRNA secondary structure (Pelletier & Sonenberg, 1985; Lawson *et al.*, 1986). p220 becomes associated with mRNA-bound eIF-4E, permitting binding of eIF-4A and eIF-4B. It has been shown that eIF-4A and -4B are not cross-linked to mRNA in the absence of eIF-4E and p220 (Grifo *et al.*, 1983) indicating that they do not directly interact with the cap structure. The second factor to contact the mRNA is eIF-4A, in an ATP dependent interaction, resulting in cross-linking of eIF-4A to the mRNA cap (Edery *et al.*,

1983). eIF-4A unwinds mRNA secondary structure, an activity enhanced by eIF-4B, important because secondary structure appears to reduce the efficiency of translation (Pelletier & Sonenberg, 1985; Kozak, 1986a). Following these interactions, the 60S ribosomal subunit binds to the 48S initiation complex, forming the 80S initiation complex and displacing eIF-4 complex in readiness for peptide bond formation. These complex RNA/protein interactions involved in ribosome recognition demonstrate the importance of the 5'-cap structure to the process of message translation in eucaryotic cells.

1.7 Nucleo-cytoplasmic transport of RNA.

The transport of newly synthesised RNA from the nucleus into the cytoplasm is also a poorly understood phenomenon. Not all RNAs are exported from the nucleus suggesting some specificity within the system. The major exported species are the tRNAs, rRNAs and mature mRNAs. Export of 5S rRNA and tRNA is inhibited by a monoclonal antibody directed against the nuclear pore, which also inhibits protein import (Featherstone *et al.*, 1988). This along with electron microscopy studies showing RNA-bound gold particles associating with the pore (Dworetzky & Feldherr, 1988) demonstrates that these RNAs are transported through the nuclear pore complexes. A saturable transport system for tRNAs has been identified (Zasloff, 1983), and point mutations within the tRNA that abolish transport suggest that the tRNAs, at least, harbour specific transport signals (Zasloff *et al.*, 1982; Zasloff, 1983; Tobian *et al.*, 1985). The *rev* protein of the human immunodeficiency virus type 1 (HIV-1) promotes transport into the cytoplasm of mRNA containing a *rev*-responsive element within the sequence of the message (Felber *et al.*, 1989).

As described in previous sections, the function of nuclear modification of mRNA with respect to translocation are uncertain. Splicing of RNA *per se* is not necessary for transport (Greenspan & Weissman, 1985; Legrain & Rosbach, 1989), although these experiments utilised a pseudo-intron, and it is conceivable that genuine intron sequences may contain nuclear retention signals that must be spliced from the mRNA before transport can

occur. This latter explanation appears unlikely, however, because of the low level of sequence conservation within eucaryotic introns. Also HIV-1 *rev*-mediated mRNA transport functions independently of splicing (Felber *et al.*, 1989). Certain mRNAs do, however, require the presence of an excisable intron for efficient cytoplasmic accumulation - for example SV40 late mRNAs show an absolute requirement for the presence of an excisable sequence within the primary transcript for transport to occur (Ryu & Mertz, 1989). Polyadenylation may play a role in message transport by interaction of the poly(A) tail with a nuclear membrane bound ATPase (see Section 1.5d), but this has yet to be established.

1.8 Vaccinia Virus and Vaccinia\T7pol Hybrid Expression Systems.

Vaccinia virus is a member of the poxvirus family, possessing a 185kb double-stranded DNA genome. Vaccinia virus replicates in the cytoplasm of infected-cells (for review see Moss, 1985), and the virus particles are capable of synthesising 5'-capped (Cap-1 structure: see Section 1.5c), methylated, poly(A)-plus messenger RNA. This is made possible because the virus encodes, along with a virally encrypted DNA polymerase, it's own polyadenylase, methyltransferase, and guanylyltransferase enzymes. Infection of cells by vaccinia virus results in a rapid shut-off of host-cell gene expression mediated by a viral intervention in cellular gene expression at several levels. Transcription of nuclear genes falls to zero over the first six hours of infection, but transport of mRNAs out of the nucleus rapidly becomes blocked slowing host gene expression before inhibition of transcription is complete. Translation of host mRNAs starts to be inhibited 20 minutes post-infection, becoming complete after 4 hours. This latter inhibition appears to result from preferential degradation and increased instability of host mRNAs, vaccinia late mRNAs requiring a virally encoded gene product to prevent degradation (Pacha & Condit, 1985). This ability to produce post-transcriptionally modified mRNAs has led to the use of vaccinia virus in hybrid expression systems coupled to RNA polymerase enzymes from bacteriophage T7 (Fuerst *et al.*, 1986,1987; Elroy-Stein *et al.*, 1989) and T3 (Deuschle *et al.*, 1989).

The vaccinia virus guanylyltransferase is an early gene, levels of the enzyme rising to a maximum (~10-fold above input levels) after 5 hours, this rise being unaffected by inhibitors of viral DNA replication (Boone *et al.*, 1977). The guanylyltransferase complex has been purified to homogeneity from viral core particles (Martin *et al.*, 1975; Monroy *et al.*, 1978), each of which contains an estimated 60-80 molecules of the enzyme (Shuman & Hurwitz, 1981). This enzyme was shown to be capable of the addition of a 7-methyl cap structure to uncapped vaccinia RNA and synthetic polyribonucleotides (Martin & Moss, 1975). The holoenzyme (127kDa), is composed of two subunits (93kDa and 23kDa) and possesses three catalytic activities; mRNA guanylyltransferase, mRNA(guanine-7-) methyltransferase and mRNA(nucleoside-2'-) methyltransferase, which can be summarised by the following reaction schemes:

(1) pppNpN → ppNpN + Pi

(2) pppG + ppNpN → GpppNpN + PPi

(3) G(5')pppN- + S-AdoMet → ^{7}mG(5')pppNpN + AdoHcy

(4) ^{7}mG(5')pppNpN + S-AdoMet → ^{7}mG(5')pppN^{m}pN + AdoHcy

The guanylyltransferase activity along with the 7-methyltransferase activity are provided by the 93kDa subunit, which has been shown to catalyse these reactions in the absence of the small subunit *in vitro* (Monroy *et al.*, 1978). The fact that the large subunit alone is capable of adding a Cap-0 structure - the minimum structure required for ribosome recognition - makes the vaccinia enzyme a candidate for use in heterologous expression systems based on T7 RNA polymerase. The gene encoding the large subunit has been localised to the *HinDIII*D fragment of the genome, and the 5'- and 3'- limits of the gene identified (Morgan *et al.*, 1984). The sequence of the entire 16kbp *HinDIII*D fragment has been determined (Niles et al., 1986), facilitating the cloning of the gene.

Previous expression systems based on vaccina/T7pol hybrid expression have suffered from a major drawback -the lethality of vaccinia infection to cells. This means that expression of any cloned gene in this system can only be transient. A stable high level

mammalian expression is desirable both for protein production and for possible use in transgenic animals. This thesis examines the possibility of utilising T7 RNA polymerase to drive high level protein expression from a late T7 promoter in uninfected cultured cells by coupling T7pol-mediated transcription to the RNA modifying activity of a cloned vaccinia virus guanylyltransferase.

Chapter 2. Materials and Methods.

2.1 Bacteriological Methods.

i) Bacterial Strains.

For general cloning and sub-cloning of recombinant plasmids *E.coli* strains LE392 [F^-, *hsdR*514 (r_k^-, m_k^+), *sup*E44, *sup*F58, *lac*IY1 or Δ(*lac*IZY)6, *gal*K2, *gal*T22, *met*B1, *trp*R55, λ^-] (Murray, 1977) and DH5 [F^-, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, (r_k^-, m_k^-), *sup*E44, *rel*A1?, λ^-] (Hanahan, 1985) were used. The *E.coli* strain H4.4, which expresses high levels of *lac*I, was used with pUR-type vectors (Rüther & Müller-Hill, 1983) for the production of fusion proteins.

ii) Growth Media and Agar Plates. (Maniatis, 1982)

All specialised media components were obtained from Difco Laboratories Ltd, Michigan and solutions were sterilised by autoclaving and stored at room temperature.

Solutions.

LB medium
per litre:

10g	bacto-tryptone
5g	yeast extract
10g	NaCl

1x agar
per litre:

15g	bacto-agar

Ampicillin was used at 40μg/ml in both liquid media and agar plates. The ampicillin was prepared as a 1000x concentrated stock from the sodium salt by dissolution in dH_2O, filter sterilised through a 0.22μm filter (Millipore Corp.) and stored frozen at -20°C.

All strains of *E.coli* used were grown in LB at 37°C, with constant shaking of liquid cultures. Agar plates were prepared by heating sterile 2x agar solution until the agar had melted, and combining this with an equal volume of 2x LB medium. Under sterile conditions this solution was poured into 10cm diameter petri dishes (Falcon) and allowed to solidify at room temperature. When plates were supplemented with 40μg/ml ampicillin, the LB agar solution was cooled to below 55°C before the ampicillin was added. Plates were stored at 4°C

and were air dried at 37°C for 1 hour prior to use, to remove surface liquid.

iii) Preparation of Competent Bacteria.

a) Calcium chloride method.

200ml of LB medium was inoculated with 0.5ml from an overnight bacterial culture and incubated with shaking at 37°C until the culture reached an A_{550} between 0.2-0.4. The cells were pelleted (3000rpm, Heraeus Minifuge T, 2250 rotor, 4°C, 7min), resuspended in 20ml ice cold 100mM CaCl, and incubated on ice for 20 minutes. The cells were pelleted as before, resuspended in 1ml ice-cold 100mM CaCl, and placed on ice for 3-18 hours before transformation.

b) Rubidium chloride method.

This protocol, adapted from Hanahan (1985), was used to produce frozen stocks of competent bacteria.

Solutions.

TfbI	30mM	potassium acetate
	100mM	rubidium chloride
	10mM	calcium chloride
	50mM	manganese chloride
	15%	glycerol (v/v)

Solution adjusted to pH5.8 with 0.2M acetic acid

TfbII	10mM	MOPS (3-(N-Morpholino)propane sulphonic acid)
	75mM	calcium chloride
	10mM	rubidium chloride
	15%	glycerol (v/v)

Solution adjusted to pH6.5 with KOH

Both solutions were filter sterilised and stored at -20°C.

200ml of LB medium were inoculated with 50μl of an overnight culture of DH5 cells, and the culture shaken at 37°C until the Absorbance at 550nm (A_{550nm}) was between 0.2-0.4. The cells were chilled on ice for 5 minutes and pelleted at 6,000rpm at 4°C for 5 minutes

(Heraeus Minifuge T, 3360 rotor). The bacteria were resuspended in 40ml of ice-cold TfbI by gentle vortexing and placed on ice for 5 minutes. The bacteria were then repelleted, resuspended in 5ml TfbII and placed on ice for 15 minutes. Aliquots (usually 200μl) were placed into round bottomed screw-cap cryotubes (NUNC) on ice, and then snap-frozen in liquid nitrogen and stored at -70°C.

iv) Transformation of bacteria with plasmid DNA

DNA was added to competent cells in 0.1 volumes, at less than 100ng DNA per 100μl cell suspension, and incubated on ice for 10 minutes, followed by a 5 minute heat-shock at 37°C. The cells were transferred to 3ml LB (no antibiotics), incubated at 37°C for 15 minutes, pelleted and resuspended in 100μl LB. The cells were then spread onto LB-agar plates (supplemented with antibiotics if appropriate) and incubated at 37°C overnight. Colonies were picked into 3ml liquid cultures with sterile Gilson tips.

v) Long term storage bacteria strains.

Long term stocks of bacterial strains and bacteria hosting recombinant plasmids were produced by adding glycerol to 15% to overnight bacterial cultures and storing these at -20°C (Maniatis *et al* 1982). Bacteria were recovered by inoculating 3ml cultures with 10μl of the glycerol stock and growing the culture overnight at 37°C with constant agitation.

2.2 Mammalian Cell Culture Methods.

i) Media and culture materials.

All media, balanced salt solutions, trypsin (0.05% in DMEM) and Versene (0.02% in DMEM) solutions were obtained from the Imperial Cancer Research Fund, Clare Hall. Fetal calf serum (FCS) was obtained from from Gibco-BRL. Disposable plastic culture-ware was obtained from Falcon Ltd. Cells were incubated in a 5% CO_2/95% air atmosphere, with 100% humidity, at 37°C.

ii) Culture of adherent cell lines.

Vero and HeLa cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum. These adherent cells were passaged when confluent by removing the growth medium, adding equal volumes of trypsin and versene solutions and incubating at room temperature until the cells became detached. The cells were spun down, (3000rpm, MSE benchtop centrifuge, 30 sec) the trypsin/versene removed and resuspended in fresh growth medium. Fresh culture flasks were seeded to a density 5-fold lower than confluence.

iii) Storage of cell lines.

Frozen stocks of the cell lines used were stored under liquid nitrogen. For storage, cells were resuspended in FCS + 10% dimethylsulphoxide (DMSO) at an approximate density of 10^6/ml and placed in round bottomed cryotubes (NUNC) in 1ml aliquots. These were frozen by storage at -70°C for 24 hours before transferal to liquid nitrogen storage tanks. Cell lines were revived from storage by transferring the contents of a thawed vial into 10ml of growth medium, pelleting the cells and resuspending them in 10ml of fresh growth medium and the cell suspension was then transferred to a sterile culture flask.

iv) Transfection of mammalian cells for transient expression.

Solutions.

1) 10X Hepes-buffered saline (HBS).

8.18% NaCl
5.94% Hepes
0.2% Na_2HPO_4

The pH of this solution was adjusted to 7.12 using 1M NaOH

2) 2M $CaCl_2$

All the solutions used were sterilised by filtration through 0.22μm nitrocellulose filters (Millipore Corp.).

Transfections were performed on Vero cells in monolayer culture according to Gorman (1985) using the calcium phosphate precipitation method. Cells were passaged the day before transfection and replated in 35mm diameter dishes at a density of $\approx 10^4/cm^2$. Next day, three hours before the addition of the DNA precipitate the medium was replaced with 3ml DMEM + 10% FCS. The precipitate was prepared by mixing the DNA solution with dH_2O to a volume of 140μl, adding 20μl 2M $CaCl_2$, mixing, adding 160μl 2X HBS and vortexing the solution immediately. The precipitate was left to stand at room temperature for 30 minutes with occasional vortexing before addition to the monolayer. The precipitate was added to the cells and left in contact for 18 hours. The cells were then washed three times with DMEM (minus Ca^{2+}), fed with fresh DMEM + 10% FCS and incubated for a further 24-36 hours at 37°C before harvesting. In experiments involving the viral infection of transfected cells, the virus was added immediately following the removal of the precipitate from the cell monolayer.

v) Assay for chloramphenicol acetyltransferase.

The assay was performed according to Gorman (1985). Transfected mammalian cells were washed three times with PBS and incubated in 1ml Tris-EDTA-NaCl (40mM Tris-HCl, pH7.4, 1mM EDTA, 150mM NaCl) for 5 minutes at room temperature. The cells were harvested by scraping from the dish and pelleted in a microfuge,(10,000g, 4°C, 2 minutes). The cell pellet was resuspended in 50μl 0.25M Tris pH7.8 and the cells disrupted by three rounds of freeze-thawing (ethanol/dry-ice bath for 5 minutes followed by incubation in a 37°C water-bath for five minutes).The cell debris was spun down, 10,000g, 4°C, 5 minutes, and the supernatant taken. This was either stored at -20°C or used immediately. Before introduction into the enzyme assay the supernatant was heated to 60°C to reduce the background due to endogenous CAT activity (Mercola *et al.*, 1983).

The assay reaction was set up as follows:

70μl	1M Tris-HCl pH7.8	
5μl	dH_2O	
50μl	cell extract	
5μl	^{14}C-chloramphenicol (Amersham)	
*20μl	4mM acetyl CoA	* Made fresh every assay

The reaction was incubated at 37°C for 60 minutes and then extracted with 1ml ethyl acetate by vortexing for 20 seconds. The mixture was centrifuged, 10,000g, 4°C, 30 seconds, and the upper organic phase taken. The samples were dried down in a Savant "Speed Vac" and the chloramphenicol taken up in 10μl fresh ethyl acetate. These samples were applied to a silica gel TLC plate (Camlab). The TLC was developed in an ascending chromatography tank containing chloroform:methanol (95:5), dried and autoradiographed overnight on preflashed X-omat XAR-5 film (Kodak Ltd.).

vi) Vaccinia Virus.

All preparation of virus stocks and infection of cell monolayers was performed according to Mackett, Smith and Moss (1985). Vaccinia virus, strain WR, was a gift from Dr. M. Mackett.

a) Preparation of a small virus stock.

A vaccina virus stock ($\approx 4x10^4$ p.f.u.) was used to infect a HeLa cell monolayer in a $75cm^2$ flask. The cells were incubated at 37°C until the majority of the cells displayed viral cytopathic effects, when they were trypsinised from the flask and pelleted (4000rpm, 4°C, 2 min). The cells were resuspended in 10mM tris-HCl (pH 9.0), 1mM EDTA and the virus released by three rounds of freeze-thawing. The cell debris was removed by centrifugation (6000rpm, Heraeus Minifuge T, 3360 rotor, 4°C, 5min), the virus remaining in the supernatant. This stock was titred (usually around $2.9x10^7$ pfu/ml), snap frozen, and stored at -70°C. These stocks were used to generate larger viral stocks as detailed below.

b) Preparation of a large viral stock.

Monolayer culture flasks were inoculated with $\simeq 4x10^8$ Hela spinner cells and the cells allowed to settle overnight. The medium was removed and the cells infected using half the product from a small virus stock preparation diluted in 16ml PBS. After one hour the virus inoculum was removed and fresh medium added. Following incubation at 37°C for 48 hours the cells were scraped into the medium and pelleted (4000rpm, Heraeus Minifuge T, 3360 rotor, 4°C, 2min). The virus was released from the cells, as before, by three rounds of

freeze-thawing, and was separated from the cell debris as before. This stock was stored in aliquots at -70°C.

c) Infection of a confluent monolayer.

Solutions.

10x Phosphate buffered saline (PBS).

80g	NaCl	
20g	KCl	
15g	Na_2HPO_4	
20g	NaH_2PO_4	per litre

Following removal of the medium, a newly confluent monolayer of cells was washed once with sterile PBS. The virus stock was added to the monolayer in a volume approximately 1/10 the original volume of culture medium, at a multiplicity of 30 p.f.u./cell and the cells incubated at 37°C for one hour. The cells were then fed with fresh medium and incubation continued at 37°C.

d) Plaque assay.

10-fold serial dilutions of the virus stock were made in Hanks balanced salt solution + 1% BSA at 25°C. The medium was removed from a newly confluent monolayer of Vero cells, and the cells washed once in PBS. The virus dilution was added to the monolayer and left in contact for 1 hour. The monolayer was then washed with PBS, fed with medium and incubated at 37°C for a further 48 hours. The cells were fixed (see 2.4ii.Immunofluorescence) and the plaques counted. The titre was calculated from the dilution of virus which produced approximately 50 plaques per dish and assayed as plaque forming units per millilitre (p.f.u./ml).

$$\text{Titre} = \frac{\text{No. of plaques x dilution factor x 1000}}{\text{volume of diluted virus added } (\mu l)}$$

vii) Herpes infection of a monolayer.

Herpes simplex virus, type 1, (HSV-1) stock of $1x10^{10}$ p.f.u./ml was a gift from Drs. L. Kemp and D. Latchman. Infection was performed as for vaccinia virus. Routinely 30 p.f.u./cell were used.

viii) Microinjection of cultured cells.

This was performed according to Graessmann & Graessmann (1983). Borosilicate glass capillaries (Type GC150T-15) for needles were obtained from Clarke Electromedical Ltd, Pangbourne, Reading. Needles were formed by hand pulling a constriction into the capillary over a bunsen flame and then by heating the capillary at the constriction, under tension, on a custom made needle puller.

Vero cells were allowed to settle overnight onto uncoated coverslips and were subsequently injected and stained attached to the coverslip. Plasmid DNA was routinely injected at a concentration of 0.1mg/ml. Proteins were injected at concentrations between 0.5-1.0mg/ml. Prior to injection all samples were centrifuged in a microfuge (10,000g, 4°C, 5 minutes) to remove particulate matter and prevent blockage of the needles.

2.3 Molecular Biological Methods.

i) Phenol and chloroform extraction.

"UNC" phenol

500g	phenol ("Analar" grade)
111ml	2M Tris pH7.5
144ml	dH_2O
28ml	m-cresol
1.1ml	2-ß-mercaptoethanol
555mg	8-hydroxyquinoline

Chloroform/IAA

96%	chloroform
4%	iso-amyl alcohol

The phenol was heated to 65°C in a water-bath until melted, and the other ingredients were added. The solution was stored in a dark bottle at room temperature.

Solutions containing nucleic acids were phenol extracted by first adding NaCl to 0.5M, then adding an equal volume of UNC phenol/chloroform/IAA (50:50 v/v), resulting in a doubling of the original volume, shaking in a polypropylene tube and centrifugation, (10,000g, 20°C, 1 minute). The upper aqueous phase was withdrawn, extracted with an equal volume of chloroform/IAA, centrifuged as before, and the upper phase taken.

ii) Ethanol precipitation of nucleic acid.

0.1 volumes of 5M NaCl was added to the sample, and 2.5 volumes of 95% ethanol (-20°C) added. The sample was incubated on dry-ice for 15 minutes, centrifuged (10,000g, 4°C, 10 minutes), washed once with 70% ethanol and allowed to air dry. The nucleic acid was then taken up into the appropriate buffer. Where small quantities of the DNA/RNA were present, 5μg yeast tRNA was added prior to the addition of the ethanol to act as a carrier.

iii) Small Scale Plasmid Preparation.

Solutions.

Solution 1.	25mM	Tris pH8.0
	10mM	EDTA
	50mM	glucose
Solution 2.	0.2M	NaOH
	1%	SDS
Solution 3.	3M	potassium acetate
	2M	acetic acid
TE-8	10mM	Tris (pH8.0)
	1mM	EDTA

3ml bacterial cultures were grown overnight, 1.5ml taken and the cells pelleted in eppendorf tubes. The cells were resuspended in 100μl solution 1, incubated at room temperature for 5 minutes. 200μl solution 2 was added, the tubes incubated on ice for 5 minutes, 150μl solution 3 added, followed by a further 10 minutes on ice. The solution was spun (10 minutes, 10,000g, 4°C), the supernatant taken, extracted once with

phenol/chloroform/IAA, once with chloroform/IAA, and the nucleic acid precipitated by the addition of 1ml 95% ethanol (at -20°C) followed by incubation at -70°C for at least 15 minutes. The nucleic acid was pelleted (10 minutes, 10,000g, 4°C), the supernatant discarded, the pellet air dried, resuspended in TE-8 and stored at -20°C

iv) Large Scale Plasmid Preparation.

Solutions.

Solution B.	50mM	Tris pH8.0
	25%	sucrose
	1mg/ml	lysozyme*

* lysozyme is unstable in solution even when stored frozen at -20°C and so solid lysozyme was added immediately prior to use of solution B.

Lysis Buffer	150mM	Tris pH8.0
	180mM	EDTA
	3%	Triton X100 (v/v)
Solution C.	10mM	Tris pH8.0
	1M	NaCl
	1mM	EDTA
	20%	polyethylene glycol (PEG)

200ml cultures were inoculated with 0.5ml from a 3ml overnight bacterial culture and incubated overnight shaking at 37°C. The cells were pelleted (Beckman J6, 4000rpm, 4°C, 10 minutes), resuspended in 4ml solution B and incubated on ice for 20 minutes when 0.1 volume 500mM EDTA (pH8.0) was added followed by a further 20 minutes on ice. 1/3 volume of lysis buffer was then added and the mixture incubated on ice for 1 hour following which the mixture was centrifuged (35,000rpm, Centricon TFT65.13 rotor, 4°C, 1 hour). The supernatant was taken, made to 0.5M NaCl, phenol/chloroform/IAA extracted, chloroform/IAA extracted, and made 10% w/v PEG 6000 by the addition of solid polyethylene glycol 6000. This solution was incubated on ice for 1 hour, centrifuged, (6,000rpm, Heraeus Minifuge T, 3360 rotor, 4°C, 10 minutes), the pellet resuspended in 500μl TE-8. 10μl DNase-free RNase (10mg/ml) was added , the solution incubated at 37°C for 1 hour, and an equal volume of solution C added. Following incubation on ice for 1 hour the mixture was centrifuged, the pellet taken, resuspended in TE-8, extracted with phenol and

chloroform and ethanol precipitated. The precipitate was resuspended in TE-8. DNA concentration was estimated by absorbance at 260nm and at 280nm (A_{260}, A_{280}).

$$\text{DNA concentration (mg/ml)} = \frac{A_{260} \text{ x extinction co-efficient x dilution factor}}{1{,}000}$$

At 20°C the extinction co-efficient for absorbance at 260nm is 45ml μg^{-1} cm^{-1} for double-stranded DNA and 25ml μl^{-1} cm^{-1} for single stranded RNA (Maniatis *et al*, 1982).

v) Electrophoresis of Nucleic acids.

a) Agarose gels.

Running buffers:	10x TAE	400mM	Tris base
		200mM	sodium acetate
		100mM	EDTA
		pH adjusted to 8.3 with acetic acid	
	10x TBE	890mM	Tris base
		890mM	boric acid
		250mM	EDTA
		pH adjusted to 8.3 with acetic acid	

Loading buffers: 10x loading buffer

0.1%	solid orange G
20%	Ficoll
10mM	EDTA
9.5x	TAE

Nucleic acid samples were electrophoresed through 0.8-2.0% agarose gels in horizontal submarine gel systems supplied by Pharmacia. Agarose was melted in 1x running buffer and samples were heated to 65°C before the addition of loading buffer and the loading of the gel. Gels were stained after electrophoresis by the application of ethidium bromide (5μg/ml in 1x TAE) for 5 minutes, and the nucleic acid visualised on a U.V. transilluminator (U.V. Products Ltd.). Gels were photographed using Polaroid type 665 or type 667 film.

b) Bisacrylylcystamine (BAC) gels.

This gel system is described by Hansen (1981) and was routinely used for the separation and purification of DNA fragments in the range of 75-1000bp.

47.7g of acrylamide (BDH Electran) and 2.25g bis-acrylylcystamine was dissolved in 500ml of water. The solution was filtered through a Whatman qualitative filter and stored in the dark at 4°C.

Vertical gel plates were set up with 1.5mm spacers (see SDS-PAGE, section 2.4). 20ml of solution 1 was added to 4ml 10xTBE and the solution made up to 40ml with dH_2O. The solution was warmed to 37°C and then 400μl 10% ammonium persulphate (freshly prepared) and 100μl TEMED were added. After mixing, the gel was poured immediately, the comb put into position and clamped, and the gel allowed to set. As soon as the gel had set the comb was removed and the gel set up in a tank containing 1xTBE. The gels were run at 200V for approximately 1 hour and the gel stained with ethidium bromide or autoradiographed as appropriate.

vi) Purification of DNA fragments.

a) From agarose gels: Glass milk method.

This protocol, communicated by Dr.Robb Krumlauf, is based upon the method of Vogelstein and Gillespie (1979) and was used to purify DNA fragments, below 6,000bp., from normal agarose (ie. not low melting point agarose) gels run in TAE buffer, for ligation. Powdered flint glass 325 mesh was obtained from Eagle Ceramics Inc., Rockville, MD. (a gift from Dr.R.Krumlauf). The glass milk was prepared as follows: 250ml of glass was suspended in 500 ml of water, stirred well and allowed to sit for 1 hour. The mixture was centrifuged (6,000rpm, Heraeus Minifuge T, rotor 3360, 4°C, 10 minutes) and the supernatant discarded. The glass was resuspended in 200ml of water, 200ml of concentrated nitric acid added and boiled for 5 minutes. The glass was spun out as before, resuspended in water to wash away the acid, this washing process continuing until the pH was neutral. The glass milk was stored in 1ml aliquots at 4°C.

Solutions.

1. sodium iodide solution: 90.8g NaI
0.5g Na_2SO_3

These reagents were dissolved in a final volume of 100ml and filtered. Several crystals of sodium sulphate were then added to the solution which was stored at 4°C in a light-proof container.

2. ethanol wash solution: 50% ethanol
Stored at -20°C. 0.1M NaCl
10mM Tris pH7.5
1mM EDTA

3. elution buffer: 10mM Tris pH7.5
1mM EDTA

Method.

2 volumes of sodium iodide solution were added to the agarose containing DNA, and the mixture heated to 37°C for 15 minutes, with occasional vortexing, until the agarose had dissolved. The glass milk was vortexed to reform the slurry and 1μl of glass powder added to the dissolved gel for every 2μg DNA present. The mixture was incubated on ice for 1 hour, with occasional vortexing. The glass powder was spun out, 6,000rpm, RT, 10 seconds, and the supernatant discarded. The glass powder was resuspended in wash solution, at half the original gel volume, spun out, as before, and this wash process repeated. The wash solution was removed and the glass powder resuspended in a volume of elution buffer to produce a final DNA concentration of 10-100ng/μl. The mixture was incubated at 37°C for 15 minutes and the glass powder spun out, 10,000g, RT, 5 minutes. The supernatant was removed and retained.

Acid phenol method.

This protocol was used to purify DNA fragments of a size greater then 6,000bp, from low melting point agarose gels. The agarose containing DNA was added to 100μl TE-8 and heated to 65°C until the agarose melted. 5μg yeast tRNA was added and the mixture then extracted twice with redistilled phenol (equilibrated with 0.3M sodium acetate pH4.8), or until no material was observed at the interface. The solution was chloroform extracted and

ethanol precipitated before being resuspended in an appropriate buffer.

b) From BAC gels.

elution buffer:	500mM	NH_4Acetate
	10mM	$Mg(Acetate)_2$
	1mM	EDTA
	0.1%	SDS
	30μg/ml	yeast tRNA

The gel fragment was incubated in 300μl of elution buffer overnight on a vertically rotating platform. The eluate was taken, phenol extracted and ethanol precipitated (no salt added). The DNA pellet was washed once with 70% ethanol, air-dried and resuspended in an appropriate buffer.

vii) Restriction digestion of plasmid DNA.

Restriction enymes were obtained from Gibco-BRL, Pharmacia, and New England Biolabs. All enzymes were used according to the supplier's recommendations and when supplied the manufacturer's digestion buffer was used.

10x DNA digestion Buffer:	100mM	Ttris-HCl pH7.5
	100mM	$MgCl_2$
	10mM	EDTA
	10mM	DTT

NaCl was added to this basic digestion buffer to produce a range of buffers which contained zero, 50mM, 100mM, and 150mM NaCl at 1x dilution.

viii) Ligation of DNA.

10x ligase buffer	500mM	Tris pH7.4
	100mM	$MgCl_2$
	100mM	DTT
	10mM	spermidine
	10mM	ATP
	1mg/ml	BSA

Ligations were performed at 14°C in a volume between 20-50μl, for between 3-24 hours. Approximately 50ng of vector DNA was present per reaction. Fragments were added in a molar ratio of 4:1, insert:vector, for ligations involving cohesive ends. In reactions involving the ligation of blunt ended fragments the ratio of insert:vector was increased and the reaction volume was limited to 20μl. T4 DNA ligase was obtained from Pharmacia and 1 unit of T4 ligase was added per reaction.

ix) End labelling of DNA fragments.

10x Nick translation buffer	500mM	Tris-HCl pH7.2
	100mM	$MgSO_4$
	1mM	DTT
	500μg/ml	BSA

This buffer was stored at -20°C in aliquots.

DNA was phenol extracted and ethanol precipitated prior to labelling. Approximately 500ng of DNA was added to a solution containing 1x NT buffer, 0.1mM dNTP (dATP, dGTP, dCTP), 0.1 units Klenow (large subunit) and 10μCi [α-^{32}P]dTTP to produce a final reaction volume of 30μl. The reaction was incubated at 37°C for 15 minutes, 5μg carrier tRNA added, and then phenol extracted and ethanol precipitated.

x) Dephosphorylation of DNA terminal nucleotides.

10x CIP dilution buffer.	500mM	Tris pH9.0
	10mM	$MgCl_2$
	1mM	$ZnCl_2$
	10mM	spermidine

Calf-intestinal alkaline phosphatase (CIP) was obtained from Pharmacia. Typically 2μg of DNA was incubated with 0.2 units of CAP for 1 hour at 37°C. The sample was the heated to 65°C for 15 minutes to inactivate the CIP.

xi) *In vitro* transcription.

In vitro transcription was performed using a modification of the protocols of Melton *et.al.* (1984), and Tabor and Richardson (1985). SP6 RNA polymerase and T7 RNA

polymerase were obtained from Gibco-BRL. T7 RNA polymerase was also obtained from Dr.John Dunn and Dr.Bill Studier, Brookhaven National Laboratories, New York. RNasin (placental RNase inhibitor) was obtained from Promega Biotech Ltd. All solutions used were treated with diethylpyrocarbonate (DEPC) where possible (see below) and autoclaved, or were prepared using DEPC-treated dH_2O.

a) DEPC-treatment of solutions.

100μl of a 10% DEPC stock solution (BDH) was added to each 100ml of solution. The solution was shaken, incubated at room temperature for 30 minutes and then autoclaved.

b) Storage buffer for T7 RNA polymerase.

20mM	$NaPO_4$ pH7.7
200mM	sodium acetate
0.1mM	EDTA
1mM	DTT
50%	glycerol

c) *In vitro* transcription reaction.

Solutions.

5x T7 transcription buffer.

200mM	Tris-HCl pH8.0
40mM	$MgCl_2$
10mM	spermidine-$(HCl)_3$
125mM	NaCl

Template for the transcription reaction was produced by linearising the appropriate plasmid. This was then phenol/chloroform and chloroform extracted and finally ethanol precipitated, before being resuspended in TE-8.

The transcription reaction was set up as follows:

44.5μl	dH_2O
20μl	5x transcription buffer
10μl	100mM DTT
2.5μl	RNasin (40units/μl)
20μl	2.5mM each of ATP, GTP, CTP & UTP
2μl	linearised plasmid (2-5μg)
1μl	T7 RNA polymerase (BRL- 50units/μl)

This reaction mixture was incubated at 37°C for 1 hour and the DNA template removed by the addition of 1μl RNase-free DNase (BRL- 10 units/μl) and incubation of the reaction mixture at 37°C for a further 15 minutes. This protocol was used to generate uncapped RNA species. The yield of RNA, typically 2μg, was estimated by ethidium bromide staining of a sample, following TAE buffered gel electrophoresis.

d) Generation of capped transcripts.

Capped transcripts were generated by the inclusion of the cap analogue GpppG (Pharmacia) in the transcription reaction at a concentration of 0.5mM which resulted in the production of capped but not methylated transcripts.

xii) Oligonucleotides.

Oligonucleotides were obtained from the ICRF oligonucleotide synthesis service and were supplied with -OH groups at both the 5' and 3'ends. Oligonucleotides were also obtained from Dr. Colin Goding, Marie Curie Institute, Oxted, and were supplied bound to the column matrix.

a) Elution of oligonucleotides from the column matrix.

1ml syringes were attached to either end of the column, and 0.5ml 35% (w/v) ammonia solution (BDH Analar) solution was passed through the column, between the two syringes, six times. The ammonia solution was then allowed to remain in contact with the column matrix for 30 minutes before being collected. The elution process was repeated twice more, each time using fresh ammonia solution. The three column eluates were pooled and then heated to 65°C for 8 hours. The solution was then freeze-dried and the oligonucleotide resuspended in 300μl TE-8, and this solution phenol/chloroform extracted, chloroform extracted and ethanol precipitated. After pelleting, the oligonocleotide was resuspended in 0.5ml TE-8, the concentration of DNA estimated by absorbance at 260nm and the concentration adjusted to 1mg/ml with TE-8.

b) Annealing of oligonucleotides.

10x annealing buffer:	1M	NaCl
	0.1M	Tris pH7.8
	10mM	EDTA

The two complimentary single-stranded oligonucleotides were mixed in equimolar proportions (approximately 1μg of each oligonucleotide). The reaction mixture (100μl) was heated to 65°C for 5 minutes, cooled to 57°C and maintained at this temperature for 20 minutes and then cooled to 30°C and maintained at this temperature for up to 2 hours.

c) 5'-Phosphorylation of oligonucleotides.

10x kinase buffer:	400mM	Tris pH9.5
	100mM	$MgCl_2$
	50mM	DTT
	10mM	ATP

1μg DNA, either annealed oligonucleotide or synthetic linker fragments, was added to a reaction volume of 50μl containing 1 unit T4 polynucleotide kinase (Pharmacia). The reaction was incubated at 37°C for 1 hour, fresh T4 polynucleotide kinase was added, and incubation continued at 37°C for a further hour and then the reaction mixture was phenol/chloroform extracted and ethanol precipitated.

xiii) Polymerase chain reaction (PCR).

Taq DNA polymerase was obtained from Anglian Biotec Ltd. and was used to amplify and to mutate plasmid sequences according to Saiki *et al.* (1988).

5x amplification buffer:	250mM	Tris-HCl pH9.0
	75mM	ammonium sulphate
	35mM	magnesium chloride
	250mM	KCl
	0.85mg/ml	BSA

The plasmid containing the sequence to be amplified was linearised by restriction

enzyme digestion, phenol extracted, and ethanol precipitated prior to the PCR reaction.

The reaction was set up as follows:

10μl	dimethyl sulphoxide (DMSO)
20μl	5x amplification buffer
8μl	dNTPs (2.5mM each)
1μl	oligonucleotide 1 (1mg/ml)
1μl	oligonucleotide 2 (1mg/ml)
10-25ng	linearised plasmid DNA

The reaction mixture was made up to 100μl with dH_2O and overlaid with 60μl of paraffin oil. The mixture was heated to 95°C for 15 minutes and cooled to 55°C. 1μl *Taq* polymerase (1 unit/μl) was added to the mixture and the following cycle initiated. Using a Cambio "intelligent heating block" the reaction was incubated at 55°C for 30 seconds (annealing), 72°C for 1 minute (extension), and 92°C for 30 seconds (denaturing). This cycle was repeated 30 times, the final extension period at 72°C being increased to 10 minutes. The reaction mixture was phenol extracted, chloroform extracted and ethanol precipitated. Samples were run on agarose or bis-acrylylcystamine gels and ethidium bromide stained to determine yield and to ensure that the amplified fragment was the correct size.

xiv) Plasmids.

The following plasmids were gifts from Drs.J.J.Dunn and F.W.Studier, sent as stab cultures in *E.coli* host strains HMS174, BL21, or BL21(DE3): pAR3132, pAR3126, pAR2529, pAR3157, pAR2529. The construction of these plasmids is described in Rosenberg *et.al.* (1987), Davanloo *et al.* (1984) and Dunn *et al.* (1988). The *HinDIII*D fragment of the vaccinia genome cloned into pUC was obtained from Dr.B.Moss.

pSV2cat (5001bp) (Gorman *et al*, 1982b) contains the bacterial gene chloramphenicol acetyl transferase [CAT] (bases 4218-5001) under the transcriptional control of the SV40 early region promoter (bases 1-323). Downstream of the CAT gene are the SV40 small-t intron (bases 4217-3606) and SV40 early region polyadenylation signal (bases 3606-2618) (see Chapter 3. Figure 15). The plasmid also carries the ampicillin resistance gene.

pRSVcat (5027bp) (Gorman *et al.*, 1982a) : Derived from pSV2cat by deletion of the 503bp *HinDII/AccI* fragment containing the SV40 early promoter and the insertion of the RSV LTR as a 524bp *PvuII-HinDIII* fragment (see Chapter 4. Figure 22).

pSV0cat (Gorman *et al.*, 1982b) : Derived from pSV2cat by insertion of *HinDIII* linkers at the *AccI* site (position 503), digestion with *HinDIII* and recircularisation resulting in deletion of the SV40 early promoter (see Chapter 4. Figure 22).

pT7cat : Constructed by insertion of the 1633bp *HinDIII/BamHI* fragment (bases 3369-5001) from pSV2cat containing the CAT gene, SV40 small-t intron and SV40 early polyadenylation signal into the *HinDIII/BamHI* sites of pGEM2 (Promega Biotech), placing the CAT gene under the transcriptional control of the T7 late promoter ϕ10 (see Chapter 3. Figure 15).

pCA1.3 and pCA3.1 : pT7cat was digested with *BamHI* and phosphatase treated to prevent recircularisation. The 138bp *BamHI/BglII* fragment from pAR2529 (Rosenberg *et al.*, 1987) containing the T7 transcription terminator Tϕ was inserted at the *BamHI* site of pT7cat. Clones were screened for the insertion of the Tϕ fragment in the positive orientation (pCA1.3) by digestion with *BamHI* and *EcoRI* which yields a fragment of 148bp and for insertion in the negative orientation (pCA3.1) by digestion with *BamHI* and *HpaI* which produces a 271bp fragment (see Chapter 3. Figure 15).

pTAC4 : This was constructed by insertion of the 1633bp *HinDIII-BamHI* fragment from pSV2cat into the *BamHI-HindIII* sites of pGEM1 (Promega Biotech.), placing the CAT gene under the transcriptional control of a bacteriophage SP6 promoter (see Chapter 4. Figure 22).

pGEM0cat : This plasmid is a derivative from pT7cat where the 46bp *PvuII\HinDIII* fragment (bases 55-101) containing the ϕ10 promoter has been deleted. Due to the presence of a *PvuII* site within the CAT gene it was necessary to alter the *PvuII* (position 101) site of

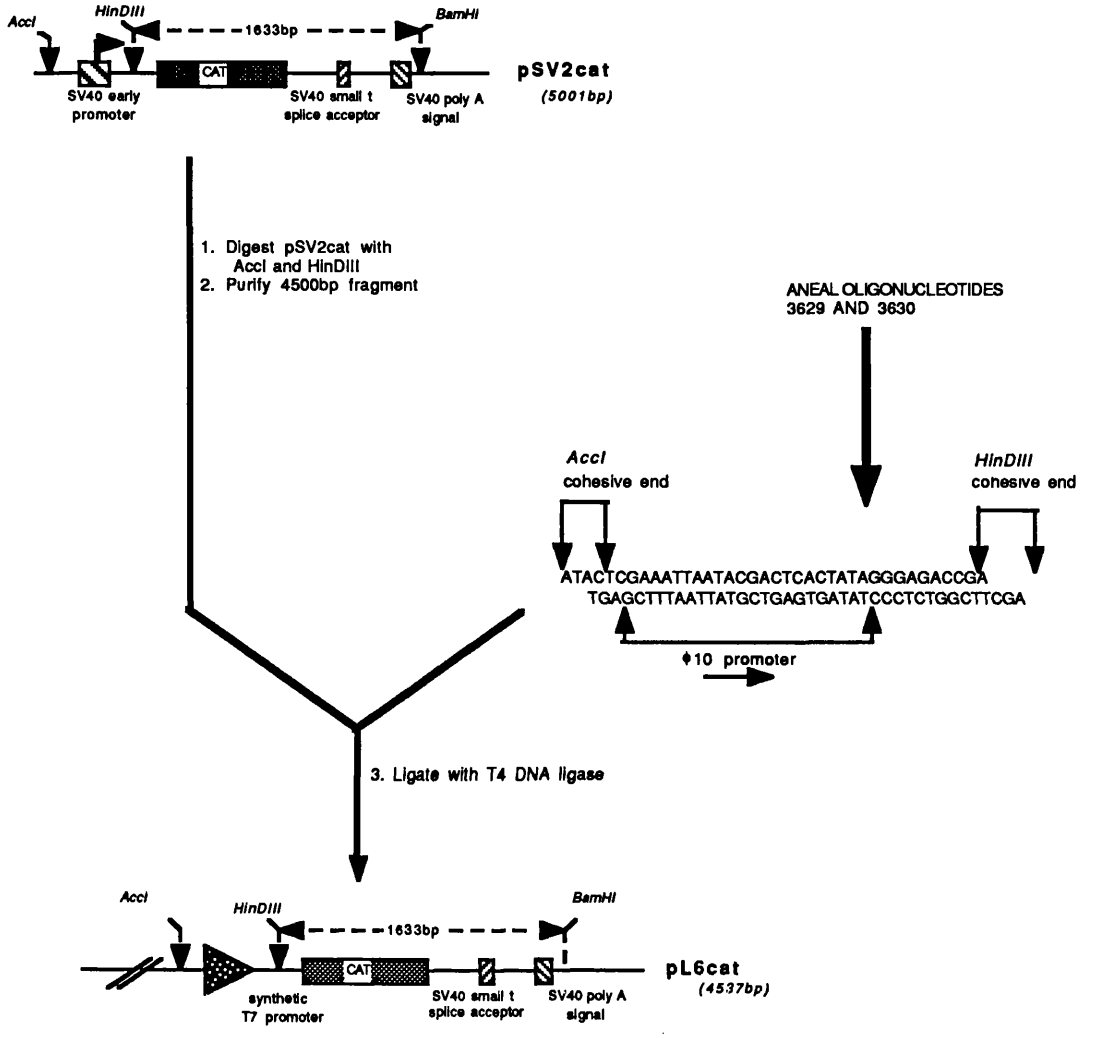


Figure 6. Diagram showing the construction of pL6cat. Annealing of two olignucleotides (3629 and 3630) produces a double stranded DNA fragment, containing the ϕ10 promoter sequence, with *AccI* (5') and *HinDIII* (3') cohesive ends. Insertion of this synthetic linker at the *AccI* (position 501) and *HinDIII* (position 0/5001) sites of pSV2cat, replacing the SV40 early region promoter, produces a plasmid with the CAT gene under the control of the ϕ10 promoter in the pSV2 plasmid background.

pGEM2 by insertion of a *HinDIII* linker (forming the plasmid pGEM-H2). This allowed simultaneous deletion of the ϕ10 sequences by digestion of pGEM-H2 with *HinDIII* and *BamHI* followed by the the insertion of the 1633bp *HinDIII/BamHI* fragment (bases 3369-5001) containing the CAT gene, SV40 small-t intron and SV40 early region polyadenylation signal from pSV2cat (see Chapter 4. Figure 22).

pL6cat : This was formed by digestion of pSV2cat with *AccI* and *HinDIII* followed by the insertion of two oligonucleotides designed so that when anealed the ϕ10 promoter is formed with *AccI* (5') and *HinDIII* (3') cohesive ends (see Figure 6).

[3629 (T7 sense) 38mer]

$^{5'}$ATACTCGAAATTAATACGACTCACTATAGGGAGACCGA$^{3'}$

[3630 (T7 antisense) 40mer]

$^{5'}$AGCTTCGGTCTCCCTATAGTGAGTCGTATTAATTTCGAGT$^{3'}$

This results in the deletion of the SV40 early promoter and places the CAT gene under the transcriptional control of the bacteriophage ϕ10 promoter in a plasmid background based on pSV2cat rather than pGEM (see Chapter 4. Figure 22). This plasmid was screened for by digestion with *EcoRI* and *AccI*

pXm30PK-B (Kalderon *et al.*, 1984) : Contains a chicken pyruvate kinase cDNA under transcriptional control of the SV40 early promoter followed by the SV40 early region polyadenylation signal.

pAR3126 (Dunn *et al.*, 1988) : Derivative of pXm30PK-B having the *EcoRI* to *BglII* region containing the pyruvate kinase cDNA replaced with an *EcoRI* to *BamHI* fragment containing the coding region for amino-acids 10-883 of T7pol, fusing the gene in-frame to the N-terminus of SV40 large-T antigen (see Chapter 3 Figure 13).

pAR3132 (Dunn *et al.*, 1988) : Derived from pAR3126 by insertion of a synthetic 36mer containing the SV40 large-T NLS at the *EcoRI* site of pAR3126 placing the NLS at the N-terminus of the T7pol coding region (see Chapter 3 Figure 13).

pSV2catB2 : pSV2cat derivative produced by insertion of *BglII* linker at the *BalI* site (position 4486), which allows the removal of the bulk of the CAT gene including the translation initiation codon as a *HinDIII/BglII* fragment.

pH3D : pUC derivative containing the vaccinia virus *HinDIIID* fragment inserted at the *HinDIII* site. The cloned fragment includes the coding region for the 93kDa sub-unit of the vaccinia guanylyltransferase complex.

pH3Dc1 : Derived from pH3D by digestion of the parental plasmid with *ClaI* followed by recircularisation of the plasmid deleting 10642bp of the *HinDIIID* fragment (bases 3019 to 14,860) leaving two *HinDIII-ClaI* fragments of 3018bp (containing the guanylyltransferase coding region) and 1.2kb within the *HinDIII* site of the pUC vector and a unique *ClaI* site at position 3418.

pH3Db2 : Derived from pH3Dc1 by insertion of a *BglII* linker (8mer) at the *ClaI* site (position 3418).

pFUB5 : THis plasmid contains the coding region specifying the C-terminal third of vaccinia guanylyltransferase (amino-acids 618-843) appended to the C-terminus of the ß-gal coding region in pUR278 (Rüther & Müller-Hill, 1983), under the control of an IPTG induicble promoter. The plasmid was constructed as follows : pH3Db2 was digested with *BamHI* and *HinDIII* generating fragments of 4486bp (vector), 2269bp, 1358bp, 514bp and 87bp. The 2269bp fragment corresponding to 3'-terminal third of the guanylyltransferase coding region was gel purified and inserted in-frame into the ß-gal coding sequence between the *BglII* and *HinDIII* sites of pUR278 (see Figure 7).

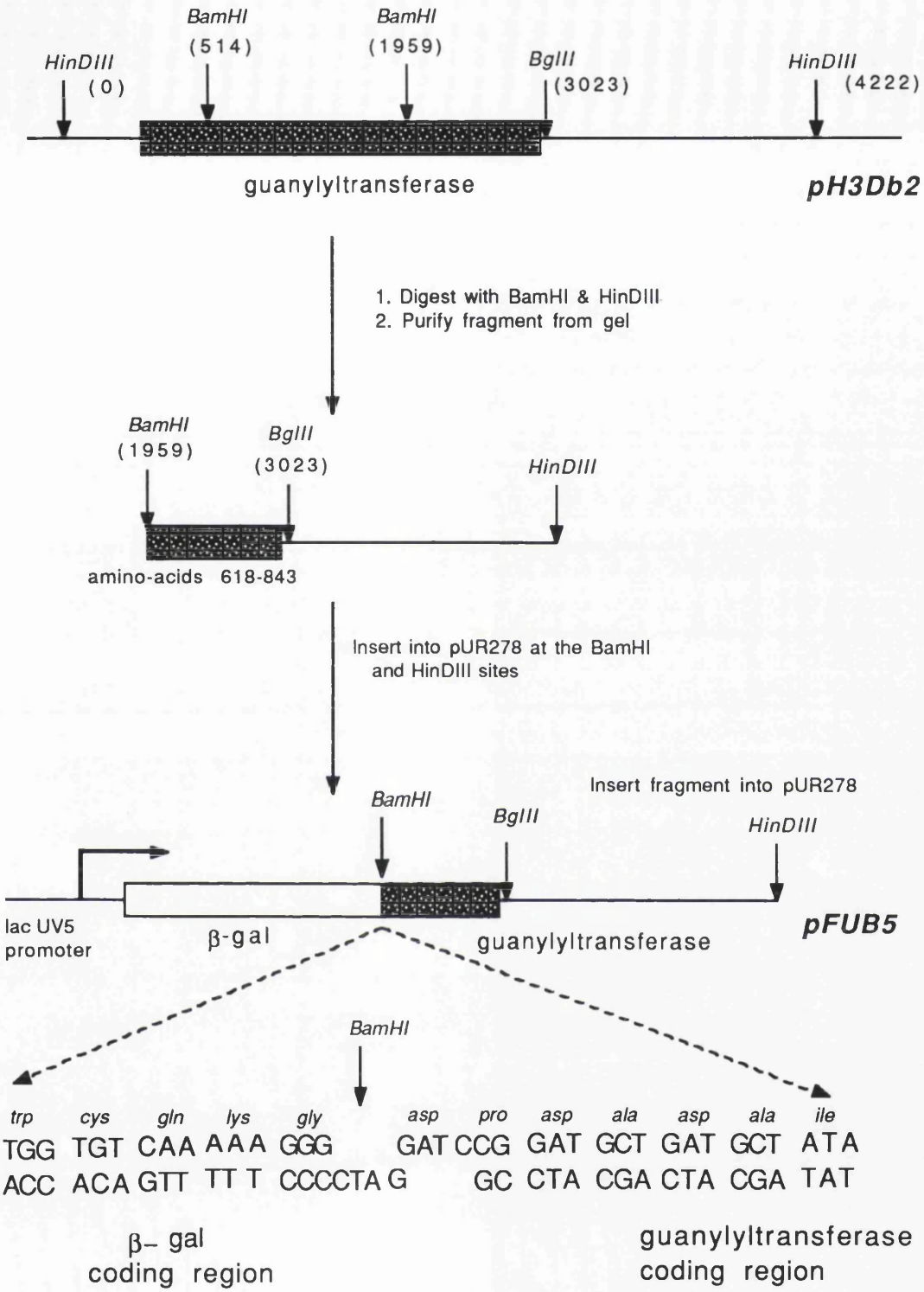


Figure 7. Diagram showing the construction of the plasmid pFUB5 which contains a gene encoding a ß-gal/guanylyltransferase fusion protein. Insertion of a 1900bp fragment from pSV2gt into the *HinDIII/BamHI* sites of pUR278 (B) places the C-terminal third of the vaccinia guanylyltransferase gene in-frame at the C-terminus of ß-gal (A).

pSV2gt : This plasmid was constructed by the insertion of the 3021bp *HinDIII-BglII* fragment containing the entire vaccinia guanylyltransferase coding region, generated by the digestion of pH3Db2 with *HinDIII/BglII/BglI*, between the *HinDIII* and *BglII* sites of pSV2catB2, replacing the bulk of the CAT coding region, and placing the guanylyltransferase under the transcriptional control of the SV40 early promoter and upstream of the SV40 small-t intron and early region polyadenylation signal (see Figure 8).

pXm30gt : This plasmid was constucted by insertion of the 3021bp *HinDIII/BglII* fragment from pH3Db2 containing the entire vaccinia guanylyltransferase coding region between the *HinDIII* and *BglII* sites of pXm30PK-B, replacing the pyruvate kinase cDNA sequences. This plasmid differs from pSV2gt by the absence of the SV40 small-t intron sequences (see Figure 8).

pSV2gt-AN : Derived from pSV2gt by the insertion of an oligonucleotide carrying the the SV40 large-T NLS into the guanylyltransferase coding region close to the C-terminus. The plasmid was constructed as follows: pSV2gt was digested with *AsuII* and the ends filled in with Klenow and in two separate reactions *XhoI* [GCTCGAGC - 8mer] and *SstII* [GCCGCGGC - 8mer] linkers were inserted at the unique *AsuII* site ($TT^{\downarrow}CGAA$) [position 2545]. This created two plasmids (designated pSV2AX1 and pSV2AS2 respectively) containing unique and different restriction sites at the same position within the coding region of the guanylyltransferase. Two oligonucleotides were designed (SVGT1 & SVGT2 : See below) such that when annealed they form a d/s DNA sequence containing the SV40 large-T NLS possessing *XhoI* (5') and *SstII* (3') cohesive ends [See Figure 9A], and which, when inserted at the newly formed *XhoI* and *SstII* sites, places the NLS in-frame into the guanylyltransferase coding region.

[5156 (SVGT-2) 32mer]

$^{5'}$GGATTCGACCTTTCTCTTCTTCTTAGGAGGGC$^{3'}$

[5157 (SVGT-1) 38mer]

$^{5'}$TCGAGCCCTCCTAAGAAGAAGAGAAAGGTCGAATCCGC$^{3'}$

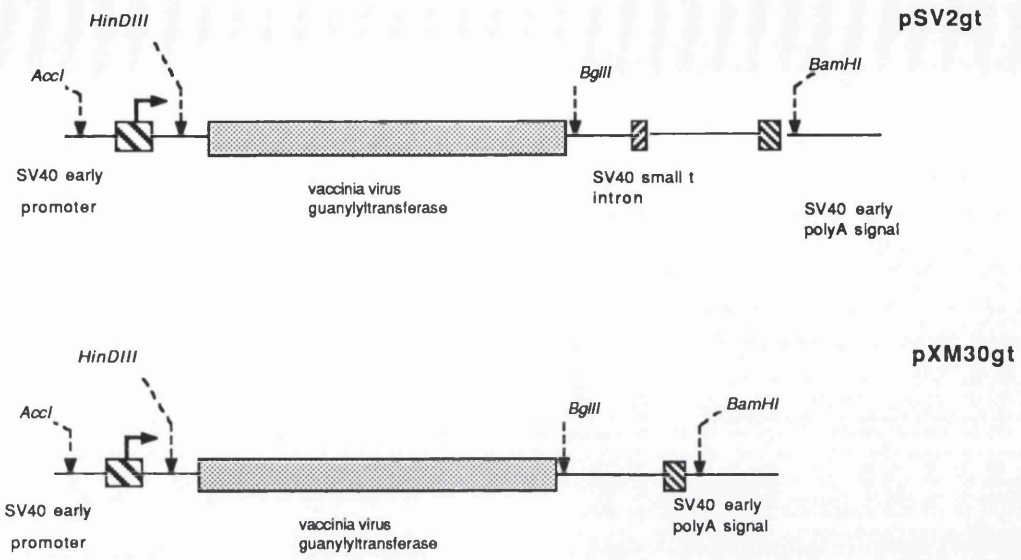


Figure 8. Diagrams of the eucaryotic expression vectors pSV2gt and pXm30gt carrying the gene encoding vaccinia virus guanylyltransferase.

A

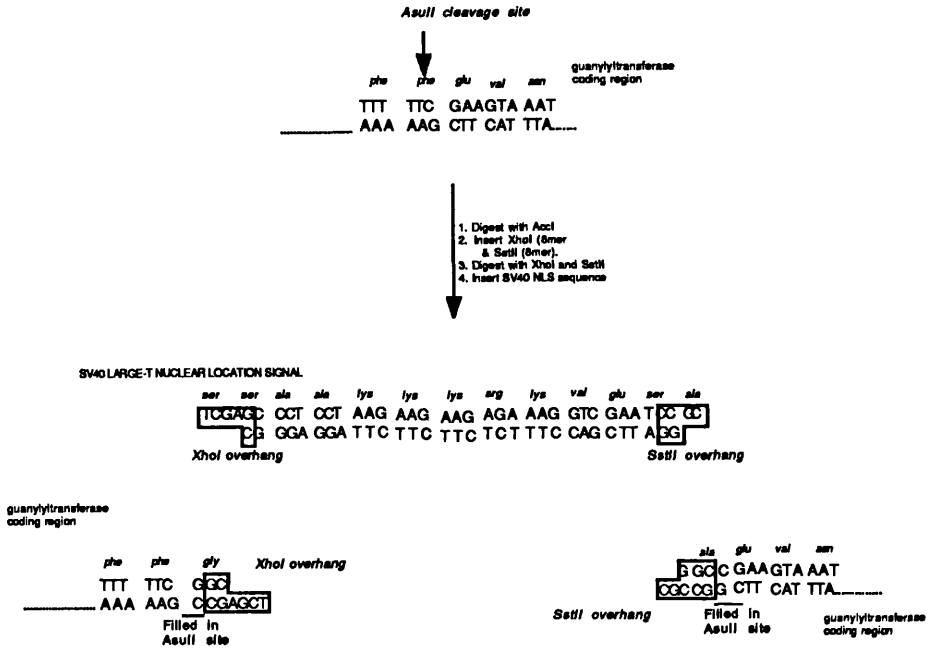
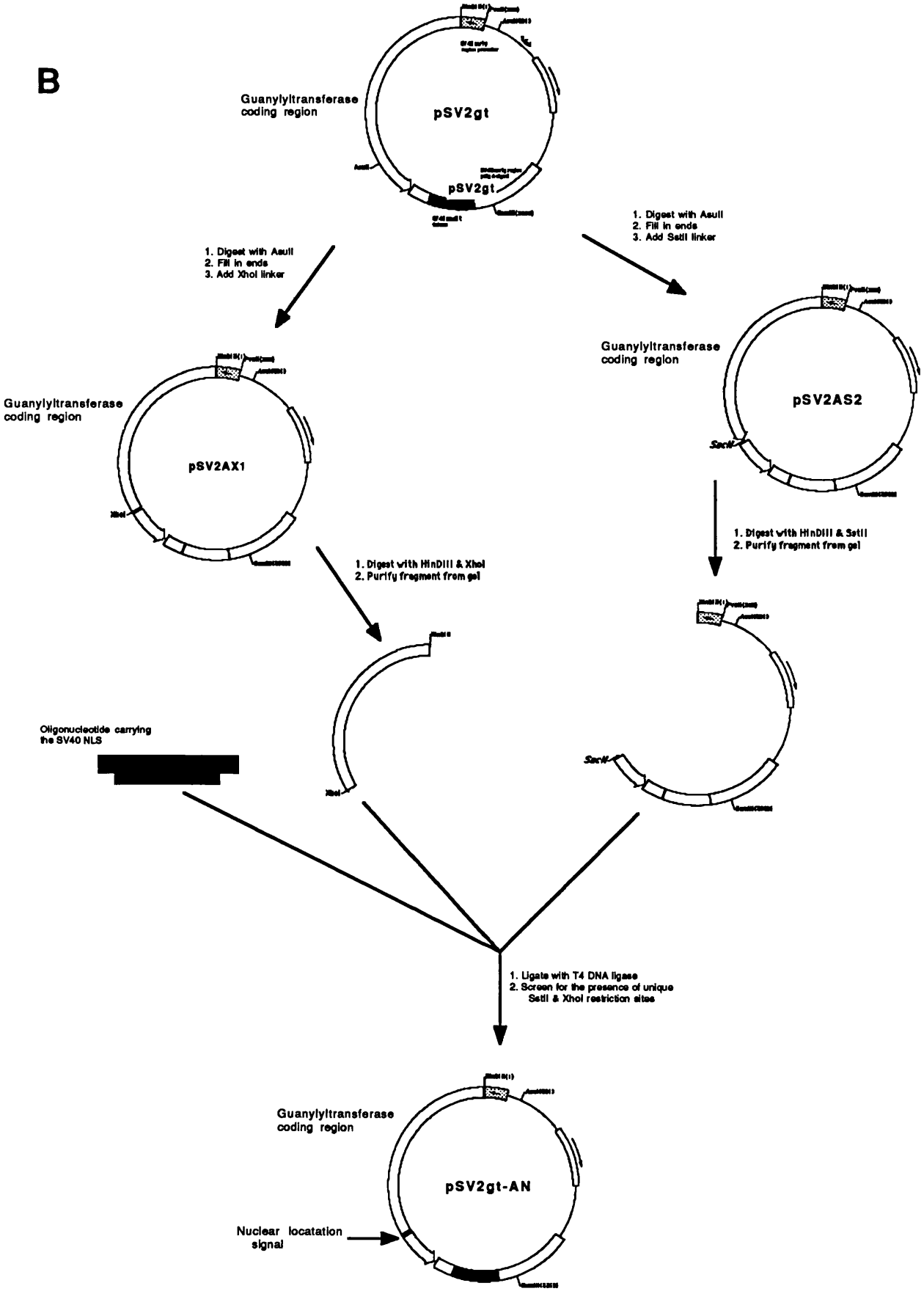


Figure 9. Construction of the plasmid pSV2gt-AN. Insertion of the oligonucleotide containing the sequence for the SV40 large-T NLS into the engineered *XhoI* and *SstII* sites (A) places the NLS in-frame within the vaccinia virus guanylyltransferase coding region (B).


B
Guanylyltransferase coding region
pSV2gt
pSV2gt
1. Digest with AsuII
2. Fill in ends
3. Add XhoI linker
1. Digest with AsuII
2. Fill in ends
3. Add SstII linker
Guanylyltransferase coding region
pSV2AX1
Guanylyltransferase coding region
pSV2AS2
1. Digest with HinDIII & XhoI
2. Purify fragment from gel
1. Digest with HinDIII & SstII
2. Purify fragment from gel
Oligonucleotide carrying the SV40 NLS
1. Ligate with T4 DNA ligase
2. Screen for the presence of unique SstII & XhoI restriction sites
Guanylyltransferase coding region
pSV2gt-AN
Nuclear locatation signal

Plasmid pSV2AX1 was digested with *HinDIII* and *XhoI* to produce two fragments, 4967bp and 2549bp, the 2549bp fragment containing the majority of the guanylyltransferase coding region. pSV2AS2 was digested with *HinDIII* and *SstII* producing fragments of 4966bp and 2548bp. The 2549bp fragment from the pSV2AX1 digestion, the 4966bp fragment of the pSV2AS2 digestion, which contains the remainder of the guanylyltransferase gene and all the signals necessary for gene expression and plasmid replication and selection, along with annealed SVGT1\SVGT2 were combined together in a ligation mixture and *E.coli* cells transfected with the result. Recombinants were screened for the presence of both *XhoI* and *SstII* sites, indicating that the plasmid obtained contained the synthetic sequence, along with all the information required for transcription of the engineered gene (see Figure 9B). Only a plasmid derived from both pSV2AX1 and pSV2AS2 would contain both restriction sites. The clone selected was designated pSV2gt-AN.

pSV2gt-DV : Derived from pSV2gt by PCR deletion of a region present in the 5'-UTR of guanylyltransferase transcripts. See Chapter 5 for details of construction

pSV2gt-AD : as pSV2gt-DV (see above).

xv) Sequence Analysis.

Analysis of DNA sequences was performed on an IBM PC-AT using the Microgenie: Sequence Software program (Beckman).

2.4 Biochemical and Immunological Methods.

i) SDS-PAGE.

Elecrophoresis of proteins was performed according to Laemmli (1970).

a) Preparation of polyacrylamide gels.

Solutions.

1. 30% acrylamide (BDH Electran).

2. 1% bis-acrylamide (BDH Electran).

These solutions were filtered through a Whatman qualitative filter and stored in dark glass bottles at 4°C.

3. Resolving gel buffer. 3M Tris-base pH8.8

4. Stacking gel buffer. 0.5M Tris base pH6.8

The pH of these solutions was adjusted with concentrated HCl.

5. SDS running buffer. 25mM Tris-base
192mM glycine
0.1% SDS

6. Destain. 40% methanol
10% acetic acid

7. Staining solution. Destain + 0.125% coomassie brilliant blue

8. 10% (w/v) ammonium persulphate.

9. N,N,N',N',-Tetramethylethylenediamine (TEMED).

10. Water-saturated iso-butanol.

Electrophoresis was performed using the Gibco-BRL vertical gel system. Plates were washed with detergent, rinsed three times with distilled water and dried with acetone prior to assembly. Spacers of 0.8mm and 1.5mm thickness were used, in conjunction with 15ml and 30ml of resolving gel solution respectively. The percentage of acrylamide within the gel [See Table 2.] was adjusted depending upon the molecular weight of the proteins of interest.

The resolving gel was poured, overlaid with water-saturated iso-butanol and allowed to set. The butanol was washed out with dH_2O, the stacking gel poured and the comb placed into the stacking gel solution. The gel was allowed to set and the gel positioned in the gel tank after the removal of the bottom spacer. The reservoirs were filled with 1x running buffer, the gel comb removed and the wells washed out with running buffer. Air bubbles below the gel were removed and the gel was then loaded with the protein samples.

When gradient gels were poured, because of the slow rate at which these gels were poured, the 10% ammonium persulphate solution was replaced with 0.1% ammonium persulphate. This was to slow polymerisation and prevent polymerisation of the gel matrix before pouring of the gel was complete.

	7%	10%	20%	stack
30% acrylamide	2.34	3.34	6.67	0.84
1% bis-acrylamide	0.91	1.3	0.67	0.65
resolving buffer	2.5	2.5	2.5	-
stacking buffer	-	-	-	1.25
water	4.0	2.73	-	2.2
10% SDS	0.1	0.1	0.1	0.05
10% ammonium persulphate	0.1	0.1	0.1	0.05
TEMED	8μl	8μl	8μl	5μl

Volumes shown in millilitres.

TABLE 2. Composition of Polyacrylamide gels for SDS-PAGE.

Gels were run at constant voltage, 50V while the material was in the stacking gel, and 100V through the resolving gel. Gels were run until the bromophenol blue was in the lower third of the gel. Gels were stained by incubation in staining solution on a rotary shaker for at least 30 minutes. The gels were then destained by incubation in "Destain" until the background staining was reduced and bands could be distinguished.

b) Preparation of protein samples.

2xSDS sample buffer (reducing)	125mM	Tris base (pH6.8)
	4%	SDS
	20%	glycerol
	10%	ß-mercaptoethanol (v/v)
	0.005%	bromophenol blue (w/v)

Protein samples were mixed 1:1 with 2xSDS sample buffer and boiled for 5 minutes in a water bath. The samples were centrifuged, 10,000g, RT, 30 seconds to remove any insoluble material. Samples were either used immediately or stored frozen at -20°C.

ii) Immunofluoresence.

FITC conjugated goat anti-rabbit Ig. antiserum was obtained from Sigma. Biotinylated sheep anti-mouse Ig and biotinylated donkey anti-rabbit Ig antisera, FITC-streptavidin, and Texas-Red streptavidin complex were all obtained from Amersham International.

Solutions.

DABCO mountant.	90%	glycerol
	10%	PBS
	2.5%	1,4-diazobicyclo[2,2,2]octane (triethylenediamine)

Virus infected or micro-injected Vero cells cultured on coverslips were washed three times with PBS and fixed by incubation in 4% formaldehyde (in PBS) for 10 minutes. The cells were washed three times in PBS, permeabilised by incubation in 0.5% NP40 (in PBS) for 10 minutes, then washed a further three times with PBS. Staining for all antigens was by indirect immunofluoresence.

Cells were stained using the protein specific antibodies/antisera, as appropriate for the antigen (see below), and excess antibody removed from the cells by three washes with PBS. The commercially obtained second layer antibodies were diluted to 1/200 using sterile PBS and were applied to cells, usually 50μl/coverslip, incubated for 1 hour at RT, and washed three times with PBS. When biotinylated second layer antibodies were used, excess second layer antibody was washed off and the cells incubated with either streptavidin-FITC or streptavidin-Texas Red for 1 hour, followed by three washes with PBS. The coverslips were mounted onto microscope slides using DABCO mountant. Stained coverslips were observed using an Olympus fluorescence microscope, and photographed onto Ektachrome 400 film (Kodak) rated at 800ASA.

a) Staining for T7 RNA polymerase.

Rabbit anti-T7 RNA polymerase anti-serum, a gift from J.J.Dunn and W.Studier, was used diluted 1:200, and left in contact with the cells for 1 hour.

b) Staining for chloramphenicol acetyltranferase (CAT).

Two monoclonal antibodies raised in mice were obtained as ascites fluid from Dr.D.Lane (NIMR). The two antibodies were used simultaneously, at a dilution of 1:1,000, and were left in contact with the cells for two hours.

c) Staining for guanylyltransferase.

Antibodies raised in rabbits against the vaccinia virus guanylyltransferase (large subunit) ß-galactosidase fusion protein were used at a dilution of 1:200, and were left in contact with the cells for 1 hour.

iii) Raising of polyclonal antisera.

Polyclonal antisera were raised in rabbits. Prior to the first immunisation the rabbits were bled to provide a pre-immune serum for control purposes. Rabbits were injected subcutaneously at five sites once a week for three weeks. A test bleed was then made by venu-puncture and the antiserum obtained tested for immunoreactivity. Immunisation was

continued with two further injections in successive weeks, followed in the third week by the removal of blood from the animal until sufficient anti-serum had been obtained.

Once taken, anti-sera were stored at 4°C overnight and the cells were then removed by centrifugation (6,000rpm, Heraeus Minifuge T, Rotor 3360, 4°C, 15 minutes). Sera were stored at -20°C, or at 4°C after the addition of sodium azide to 0.02% final concentration.

a) Production of anti-sera against fusion proteins: Induction of bacterial expression.

3ml cultures of LB containing 10μM IPTG were inoculated with H4.4 bacteria containing the pUR vectors (Rüther and Müller-Hill, 1983). These cultures were grown overnight at 37°C with constant agitation and the bacteria pelleted. The bacteria were resuspended in 163μl 100mM Tris (pH7.0), 10mM EDTA, 15% glucose and an equal volume of 2x SDS loading buffer added. The samples were boiled for 10 minutes and stored frozen under liquid nitrogen. The samples were subjected to SDS-PAGE as described above, 750μl of fusion protein extract being applied to each gel for the production of immunogen. The gels were then Western blotted onto nitrocellulose.

b) Electroblotting of proteins.

Solutions.

10x blot buffer:	24.2g	Tris pH8.3
	112.5g	glycine

When diluted the 1x buffer was made 20% (v/v) with respect to methanol.

After electrophoresis, the polyacrylamide gel was overlaid with three sheets of Whatmann 3mm paper soaked in 1x blotting buffer, the gel lifted and all air bubbles expelled. The gel was overlaid with a sheet of nitrocellulose, (Type BA85, Schleicher & Scheull) soaked in blotting buffer, all air bubbles excluded and the nitrocellulose overlaid with three sheets of 3mm paper soaked in blotting buffer. The whole was then placed in the electroblotter (BioRad) and blotted overnight at 250mA.

c) Immuno-peroxidase development of the blot.

The nitrocellulose filter was blocked with PBS + 3% BSA (blocking buffer) at 37°C for 1 hour, washed twice with PBS + NP40 (0.5%) and twice with PBS. Anti-ß-galactosidase antibodies (Rabbit polyclonal serum kindly supplied by Dr.Bernado Villareal-Ramos) were diluted 1:1000 in blocking buffer and incubated with the filter for 1 hour at 37°C. The filter was washed as before and porcine anti-rabbit antibodies conjugated with HRP (DAKO) diluted 1:1000 in blocking buffer were added to the filter and incubated at 37°C for 1 hour. The filter was then washed twice in PBS-NP40 followed by three washes in PBS.

The substrate solution was prepared by dissolving 0.06g 4-chloro-napthol in ice-cold methanol and adding this and 100μl 30% H_2O_2 to 90ml PBS. This solution was added to the filter and incubated until bands developed. The reaction was stopped by washing the blot in PBS.

Antibodies against the guanylyltransferase/ß-galactosidase fusion protein were raised using an immunogen produced as described by Abou-Zeid *et.al.* (1987). Using the plasmid pFUB5 (see Figure 7) fusion protein was induced in bacteria (as described in section 2.4iiia) subjected to SDS-PAGE and Western blotted onto nitrocellulose. The band corresponding to the fusion protein was determined from test-strips immuno-enzymatically stained using the anti-ß-gal polyclonal antiserum (see above), cut out, dried, and solubilised in 4ml DMSO. The protein was precipitated by the addition of 4ml carbonate buffer (50mM, pH9.0) in 35μl aliquots every 10 seconds with constant vortexing. The precipitate was centifuged (6000rpm, Heraeus Minifuge T, rotor 3360, 4°C, 15 minutes) and washed twice by resuspension in 20 ml RPMI followed by repelleting as before. The precipitate was finally resuspended in 1ml RPMI, this final suspension being stored at -20°C or used immediately. 0.1ml of the suspension was injected at each site. Half-lop rabbits were inoculated with the nitrocellulose/immunogen suspension. Approximately 33μg fusion protein was injected at each site, as estimated from SDS-PAGE gels stained with Coomassie blue.

Chapter 3. T7 RNA polymerase in mammalian cells.

3.1 Introduction.

In eucaryotic cells transcription takes place within a specialised membrane-bound organelle, the nucleus. Entry into this subcellular compartment is regulated, large proteins (≥40kDa) being excluded unless they are associated with a nuclear location signal (NLS). In procaryotic cells access to the genetic material is not restricted in this way. T7pol (~100kDa) being of procaryotic origin is unlikely to possess a nuclear location signal and would therefore be expected to be excluded from the nucleus when expressed in mammalian cells. This might prove to be a problem when attempting to obtain transcription with T7pol from T7 promoters integrated into chromosomal DNA. Therefore, I have investigated the subcellular distributions of the native form of T7pol (cT7pol) and an engineered form of T7pol (nT7pol) that contains the NLS from SV40 large-T antigen (Dunn *et al.*, 1988) in order to determine whether an NLS is required to target T7pol to the cell nucleus and whether nuclear accumulation is necessary for T7pol-mediated gene expression in Vero cells.

Within *E.coli*. T7pol requires the presence of a terminator sequence (Tϕ) for efficient transcription termination. I have investigated the requirement, if any, of this sequence for T7pol-mediated transcription within mammalian cells. The reporter gene I used for these studies was the bacterial chloramphenicol acetyltransferase (CAT) gene, which has no mammalian analogue, and can be assayed both enzymatically and by antibody staining.

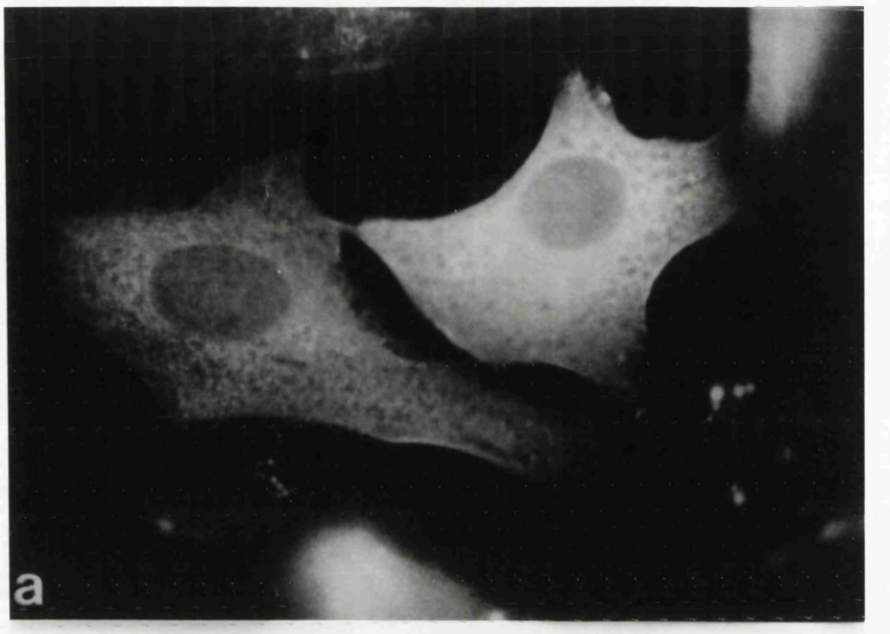
I have used two complementary approaches, microinjection and transfection to introduce genes or gene products into mammalian cells. Microinjection permits DNA, RNA, or protein to be introduced into the nucleus or cytoplasm of cells, but analysis is restricted to the single cell level. Calcium phosphate transfection allows DNA to be introduced into large numbers of cells, so that they can be subjected to biochemical analysis.

3.2a. Microinjected T7 RNA polymerase protein is excluded from the nucleus unless it possesses a nuclear location signal.

Recombinant T7pol purified from *E.coli* (obtained from Dr.J.J.Dunn), was injected at a concentration of 0.5mg/ml into the cytoplasm of Vero cells growing on glass coverslips. Following injection the cells were incubated at 37°C for one hour before fixation and permeabilisation. The cells were then stained for T7pol using a polyclonal rabbit anti-T7pol antiserum followed by FITC-conjugated sheep anti-rabbit antibodies. Cells injected with the native form of T7pol (cT7pol) showed bright fluorescent cytoplasmic staining and unstained nuclei [Figure 10a.], indicating that cT7pol is excluded from the nuclei of injected cells as expected. In contrast, cells injected with a modified T7pol containing the SV40 NLS (nT7pol) displayed a dual distribution of fluorescence staining. The nuclei of injected cells were stained as expected [Figure 10b.], but in addition there was punctate staining over the whole cell. This punctate staining was at the cell surface, because it was still observed when injected cells were stained in the absence of permeabilising detergents [Figure 11a], even though this treatment abolished the nuclear staining by preventing access of antibodies to the cell interior [compare with Figure 11b].

This cell-surface staining is of concern because display of a foreign antigen, such as T7 RNA polymerase, on the surface of a cell might render that cell vulnerable to attack by cellular and humoral components of the immune system, if the nT7pol were expressed in a transgenic animal. The absence of surface staining in cells injected with cT7pol suggests either that degradation of the nT7pol preparation had occurred during storage and\or transport, or that this type of staining was an inherent property of nT7pol. Also, although both protein species were injected at the same nominal concentration, the levels of staining in cells injected with nT7pol were lower than those of cells injected with cT7pol [Figure 10]. This further suggested that the level of intact protein in the nT7pol sample was lower than estimated and that the punctate surface staining might have resulted from the presence of degraded forms of the protein.

Figure 10. Immunofluorescence staining against T7pol of Vero cells injected into the cytoplasm with native T7pol (cT7pol) or with a nuclear targetted form (nT7pol). Vero cells were injected into the cytoplasm with cT7pol (A) or nT7pol (B) at a nominal concentration of 0.5mg/ml. The cells were formaldehyde fixed and permeabilised with NP-40 before staining for T7pol using a rabbit anti-T7pol polyclonal antiserum and FITC-cojugated sheep anti-rabbit Ig second layer antibodies (see Materials and Methods).


a

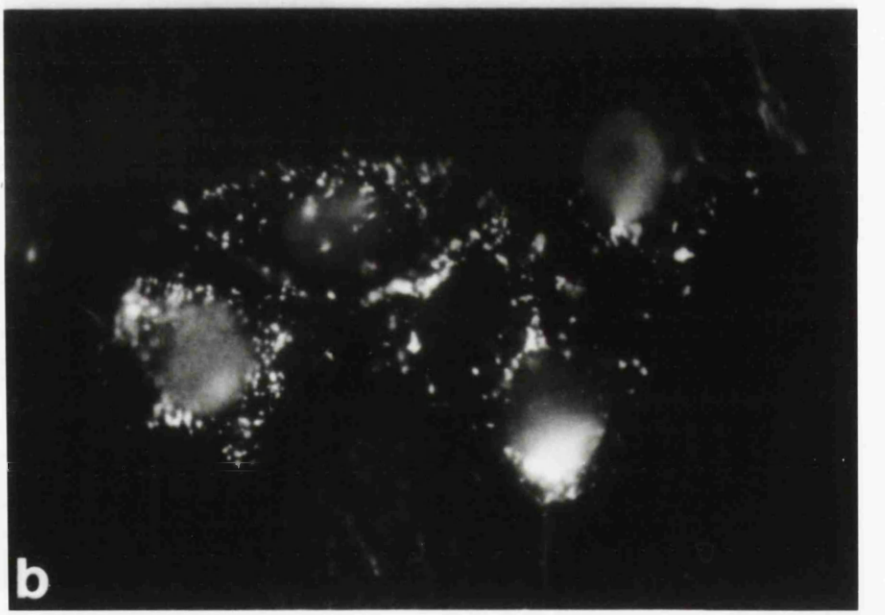
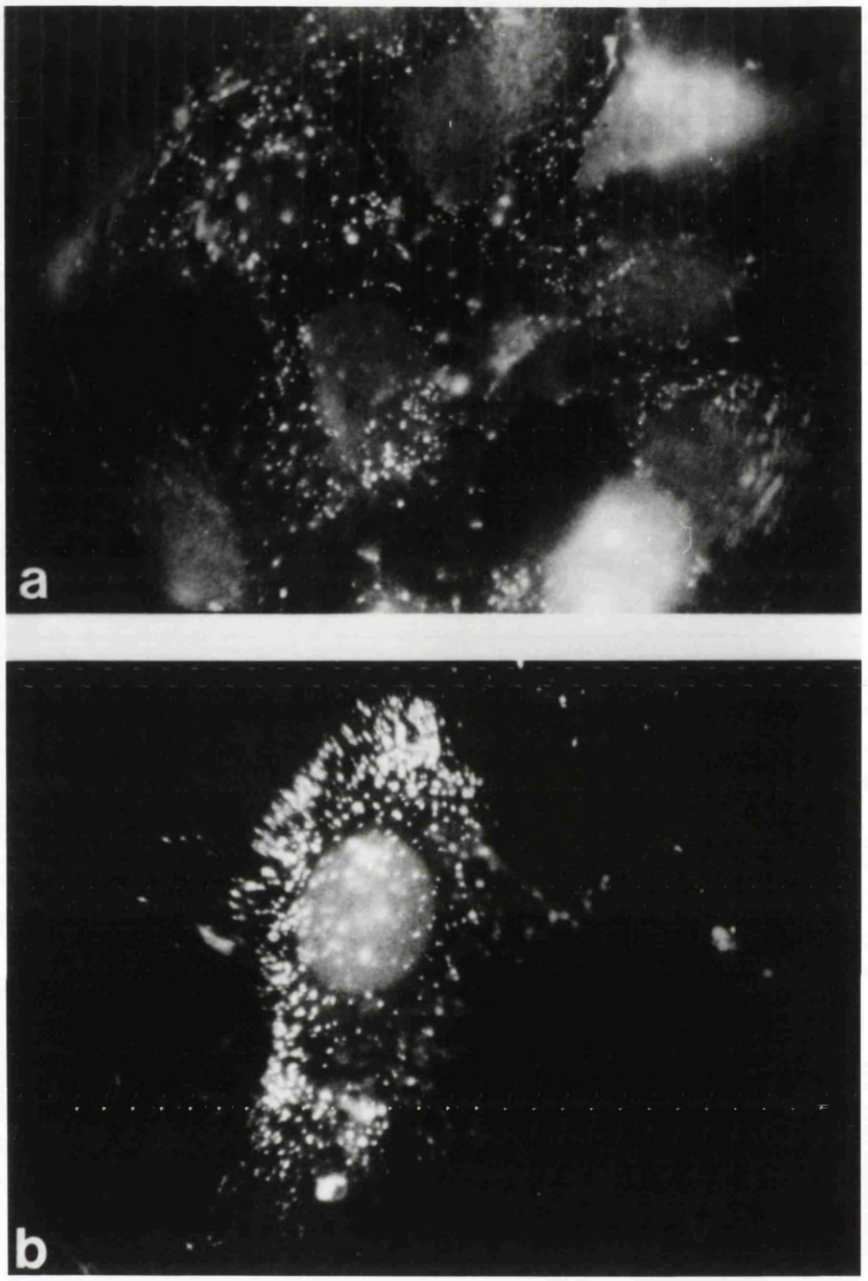

b

Figure 11. Immunofluorescence staining against T7pol of Vero cells injected into the cytoplasm with nT7pol and stained in the presence (b) or absence (a) of permeabilising agents. nT7pol (at a nominal concentration of 0.5mg/ml) was injected into the cytoplasm of Vero cells. 1 hour after injection cells were formaldehyde fixed and permeabilised with NP-40 (b) or formaldehyde fixed alone (a), and then stained for T7pol using FITC-conjugated anti-rabbit Ig second layer antibodies (see Materials and Methods). Staining cells in the absence of permeabilisation abolishes nuclear staining (b) but retains the punctate staining (a), indicating that the nT7pol is displayed at the cell surface.


a
b

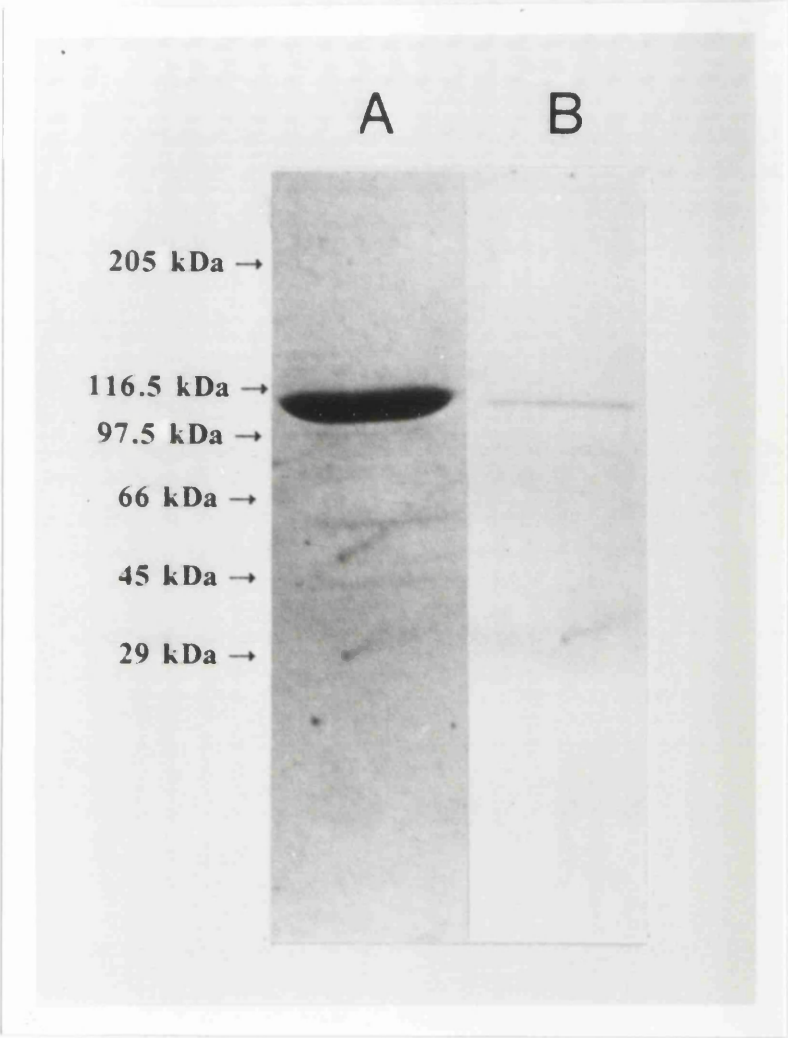


Figure 12. Coomassie-blue stained SDS-PAGE gel of the T7pol samples used to show the integrity of the T7pol samples use in microinjection. Samples of cT7pol (lane A) and nT7pol (lane B) [both samples nominally 1μg] were electrophoresed through a 10-20% gradient SDS-PAGE gel and stained with coomassie-blue.

In order to test the integrity of the T7pol samples used for micro-injection, samples of the cT7pol and nT7pol were subjected to SDS-PAGE through a 10-20% gradient polyacrylamide gel, and stained with coomassie-brilliant blue [Figure 12]. The gel indicates that the majority of both the cT7pol (lane A) and the nT7pol (lane B) were intact at the time of injection, with an apparent molecular weight of 98 kDa. Figure 12 shows, however, that the nT7pol sample (lane B) was at a lower concentration than originally estimated. This probably explains the lower average levels of fluorescence observed in cells injected with nT7pol compared to those injected with cT7pol.

3.2b The nuclear-targetted form of T7 RNA polymerase does not appear on the cell surface when expressed from a microinjected plasmid vector.

The plasmid pAR3126 contains the gene encoding cT7pol under transcriptional control of the SV40 early region promoter and polyadenylation signals. The plasmid pAR3132 is similar to pAR3126 except for the inclusion of sequences encoding the nuclear location signal from SV40 large-T antigen at the 5'-end of the T7pol coding sequences [Dunn *et al.*, 1988; See Figure 13]. These plasmids were injected separately into the nuclei of Vero cells at a concentration of 0.1mg/ml. The injected cells were incubated at 37°C overnight, fixed and permeabilised, and stained for T7pol as previously described. Cells injected with pAR3126 displayed a brightly fluorescing cytoplasm with a dark nucleus [Figure 14a.]. This result together with the protein injections in the previous section shows that the cT7pol behaves in the same manner whether synthesised within the cell, or supplied exogenously. Cells injected with pAR3132 showed a brightly staining nucleus, with unstained nucleoli, and no detectable cytoplasmic or surface staining [Figure 14b.], indicating that the nT7pol had been sequestered into the nucleus. The subcellular distributions of the two different polymerase molecules (native and nuclear-targetted) are consistent with the notion that large proteins accumulate within the nucleus as a result of a selective transport mechanism rather than by passive diffusion and binding (reviewed by Dingwall & Laskey, 1986). Clearly insertion of a nuclear location signal within the primary sequence of the T7pol molecule is sufficient to allow

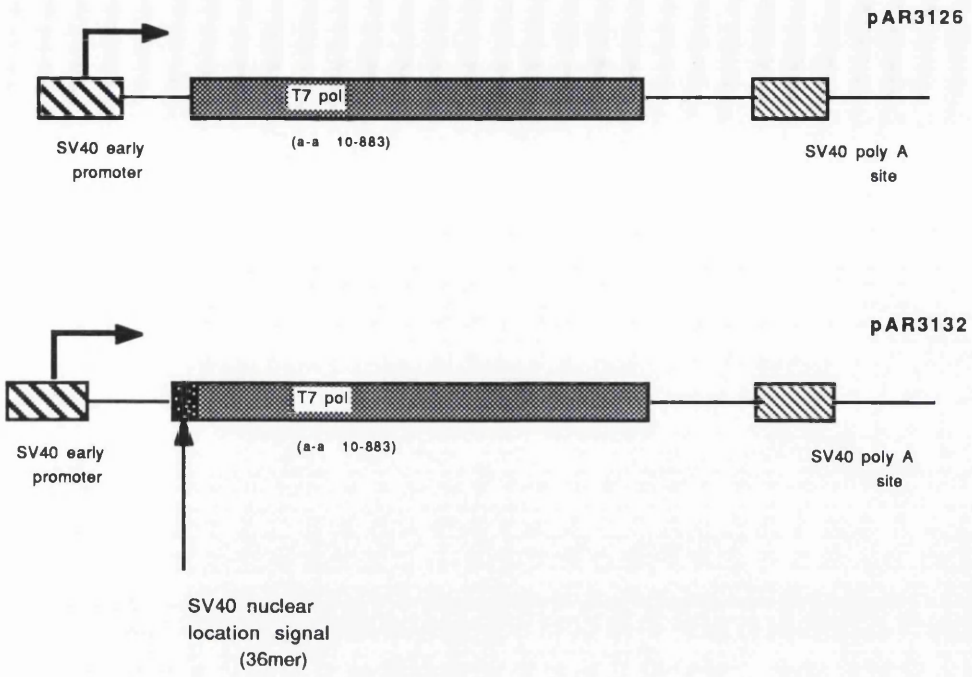


Figure 13. Diagram showing the T7pol expression vectors pAR3126 and pAR3132 (Dunn *et al.*, 1988).

Figure 14. Immunofluorescence staining against T7pol in Vero cells following nuclear injection of a T7pol expression vector. The T7pol expression vectors pAR3126 (cT7pol0) or par3132 (nT7pol), at a concentration of 0.1mg/ml, were injected individually into the nuclei of Vero cells. The cells were incubated overnight, fixed and permeabilised and stained for T7pol using FITC-conjugated second layer antibodies (see Materials and Methods).

accumulation within the nucleus.

The reason for the display of some of the nT7pol at the cell surface when purified protein was injected remains unclear. When expressed from a plasmid within the cell, no nT7pol was transported to the cell surface. Injection of protein resulted in two distinct distributions of the protein, some accumulating in the nucleus and some at the cell surface. This may reflect the presence of two competing processes, one resulting in nuclear accumulation and the other resulting in display of the "foreign" protein at the cell surface, perhaps following proteolytic processing such as occurs during antigen presentation in T-cells. Nuclear accumulation of proteins is a rapid process (Richardson *et al.*, 1988, Newmeyer & Forbes, 1988), but injection of protein into the cytoplasm may transiently saturate the transport mechanism, allowing some of the protein to be processed and end up on the cell surface. Translation of the nuclear-targetted form within the cytoplasm of the cell may avoid surface presentation by allowing the nascent polypeptide chain to associate with the nuclear transport machinery early in translation, with rapid nuclear translocation following the completion of translation. Alternatively, it is possible that the nT7pol sample contained multiple degradation products not detected by SDS-PAGE [Figure 12] and it is these "aberrant" polypetides, some of which would no longer be linked to an NLS, that are scavenged from the cytoplasm and displayed at the cell surface.

3.3 Construction of vectors containing the CAT gene under the control of the T7 late promoter ϕ10.

Three plasmids (pT7cat, pCA1.3 and pCA3.1) were constructed to investigate the DNA sequence elements required for T7pol mediated gene expression in mammalian cells. All three contained the CAT gene under the control of a T7 late promoter, followed by the SV40 small-t splice donor and acceptor sites and the SV40 early region polyadenylation signal. pCA1.3 also contained the bacteriophage T7 transcription terminator sequence (Tϕ) in correct orientation downstream of all the other elements, and pCA3.1 contained the same terminator

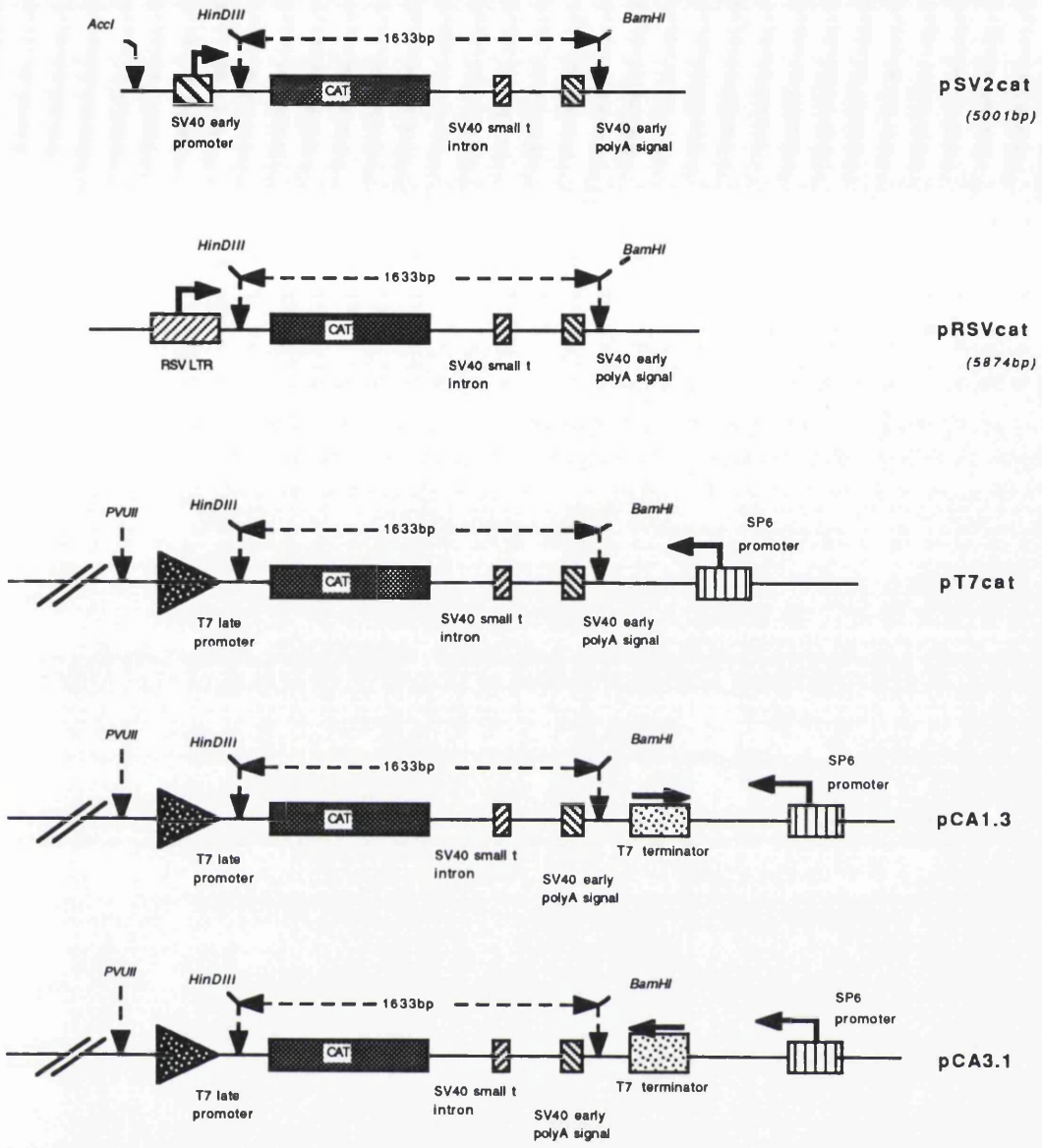


Figure 15. Diagrams of the pGEM-based ϕ10cat plasmid constructs pT7cat, pCA1.3 and pCA3.1 along with the eucaryotic CAT expression vectors pSV2cat and pRSVcat.

sequence in reverse orientation. Collectively these plasmids are termed "ϕ10-CAT" constructs (see Figure 15).

3.4 Co-injection of T7pol expression vectors and CAT reporter constructs is insufficient to produce expression of CAT protein.

The plasmids pT7cat, pCA1.3 and pCA3.1 were mixed individually with one or other of the two plasmids pAR3132 or pAR3126 (encoding nT7pol and cT7pol respectively) at a concentration of 0.1mg/ml/plasmid. These mixtures were then injected into the nuclei of Vero cells growing on glass coverslips. Injected cells were incubated overnight, fixed and permeabilised, and stained for both CAT and T7pol proteins, as described in Materials and Methods. Staining for T7pol served as a control for the success of the microinjection and the viability of the injected cells. As controls for the staining procedures, cells on separate coverslips were injected with a mixture of either pAR3132 or pAR3126 along with pRSVcat, and stained using the same dilutions of antibody and at the same time as the test cells. The results obtained from these injections are shown in Figure 16 and Table 3. They show that no combination of cT7pol or nT7pol with any ϕ10-cat construct is able to produce expression of CAT at the protein level in microinjected cells.

Co-injection of pRSVcat with either pAR3126 or pAR3132 shows that it is possible to co-stain for both CAT and T7pol proteins within the same cell (see Figure 16b & f; 16d & h.). CAT protein is distributed within both the cytoplasm and nucleus of injected Vero cells (Figure 16f & h), presumably due to the low molecular weight of the protein (23kDa), which permits diffusion of the protein through the nuclear pores.

From this data it is also possible to conclude that unlike the situation in bacterial cells (Tabor & Richardson, 1985; Studier & Moffat, 1986), the presence of both a T7 polymerase and a T7 promoter within a eucaryotic cell does not appear to alter the short-term viability of the injected cells, since the percentages of cells surviving injection (shown in Table 4) are

Figure 16. Immunofluorescent staining for T7pol and CAT proteins in Vero cells co-injected into the nucleus with a T7pol expression vector and a ϕ10cat construct. Vero cells were injected with the T7pol expression vector pAR3132 (nT7pol) along with pT7cat (a & e) or pRSVcat (b & f), or with pAR3126 (cT7pol) and pT7cat (c & g) or pRSVcat (d & h) at a concentration of 0.1mg/ml/plasmid. The cells were incubated overnight, fixed, permeabilised and stained for T7pol using a rabbit polyclonal antiserum and FITC-conjugated anti-rabbit Ig (a to d) and for CAT using murine monoclonal antibodies followed by biotinylated anti-mouse Ig and streptavidin-Texas-Red (both Amersham) (e to h) [see Materials and Methods].


a
b
c
d
e
f
g
h

	T7pol expression vector injected			
	pAR3126 (cT7pol)		pAR3132 (nT7pol)	
	stained for:		stained for:	
Plasmid co-injected	T7pol	CAT	T7pol	CAT
pT7cat	40/60	0/60	22/38	0/38
pCA3.1	73/91	0/91	67/81	0/81
pCA1.3	32/75	0/75	48/77	0/77
pRSVcat	34/60	39/60	43/80	35/80

Table 3. Table showing representative results of cell numbers staining for T7pol and CAT when co-injected with a T7pol expression vector and a ϕ10-CAT construct.

	T7pol expression vector injected	
	pAR3126 (cT7pol)	pAR3132 (nT7pol)
Plasmid co-injected	% surviving injection	% surviving injection
pT7cat	35%	56%
pCA3.1	65%	44%
pCA1.3	64%	52%
pRSVcat	66%	51%

Table 4. Table showing the percentage of cells surviving co-injection of a T7pol expression vector along with a ϕ10-cat construct or pRSVcat. The results shown are averages from two separate experiments, survival being scored for T7pol staining.

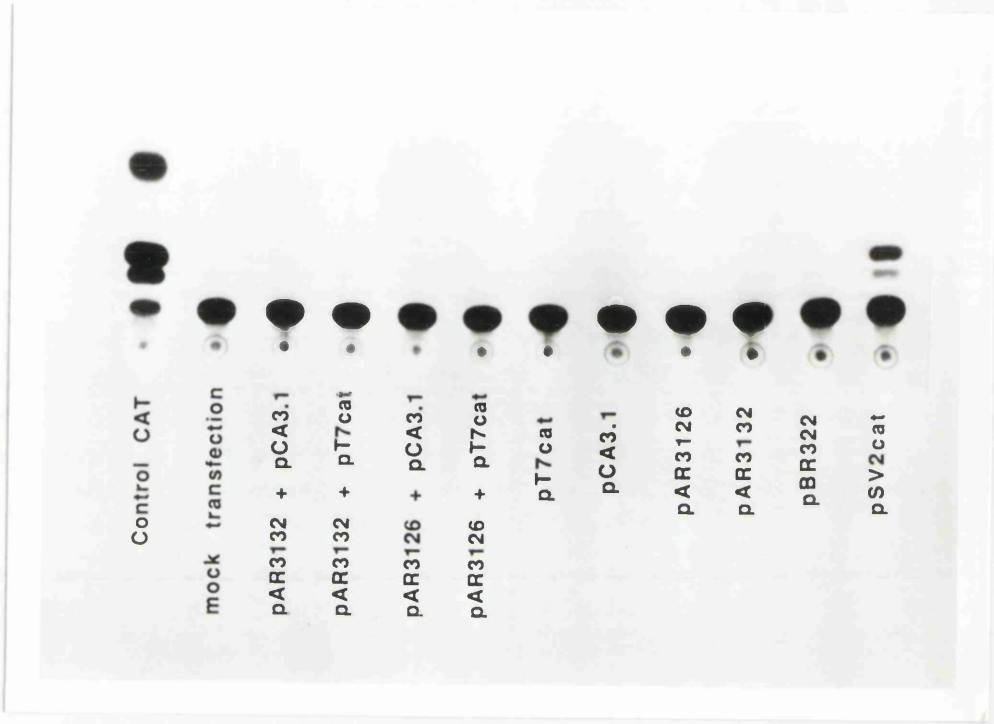


Figure 17. CAT assay of lysates from Vero cells transfected with a T7pol expression vectors along with a ϕ10cat construct. Vero cells were transfected in 35mm diameter culture dishes with 2μg of pT7cat or pCA3.1, either individually or along with 2μg of a T7pol expression vector (pAR3126 or pAR3132). Cell lysates were prepared after 48 hours and assayed for CAT activity (see Materials and Methods). Control cells were transfected with the positive control pSV2cat (2μg) or the negative control pBR322 (2μg). Also shown is the result from the inclusion of 3 units of pure CAT protein (BCL) in the reaction mixture.

similar throughout the experiment, irrespective of the constructs injected.

3.5 Co-transfection of T7pol expression vectors together with plasmids bearing target promoters is also insufficient for expression of CAT protein.

35mm diameter dishes of Vero cells were transfected, as described, with the plasmids pAR3132 or pAR3126 along with one of pT7cat, pCA3.1 or pSV2cat. A total of 4μg DNA was added per dish (2μg of each plasmid). In control transfections each of these plasmids was transfected together with 2μg of pBR322 DNA. The cells were harvested 36 hours after the initial addition of the DNA/$CaPO_4$ precipitate, and assayed for CAT activity. The results of these transfections are shown in Figure 17. These results show that when the ϕ10cat constructs (pT7cat and pCA3.1) were transfected individually, or with either pAR3126 or pAR3132, no CAT activity was observed. CAT activity was easily detected when pSV2cat was transfected as a positive control. These results are consistent with the microinjection data shown previously and indicate that there is some block to production of stable T7pol transcripts, or to the translation of these transcripts in mammalian cells.

3.6 Injection of T7 CAT *in vitro* transcripts demonstrates a requirement for a 5'-cap structure for translation *in vivo*.

There are many potential reasons for the failure to obtain expression of CAT in the experiments described above. Some possible explanations are:

1) T7pol may not be capable of transcription in mammalian cells.
2) transcripts may not be accurately processed.
3) transcripts may be unstable.
4) transcripts may not be transported from the nucleus.
5) T7 transcripts may not be capable of translation at mammalian ribosomes.

To test if T7 transcripts generated from the ϕ10-cat template are capable of being translated in mammalian cells, Uncapped and 5'-capped T7 run-off transcripts were generated *in vitro* and injected into Vero cells. The T7 CAT message was generated using purified T7 RNA polymerase (see Materials and Methods), treated with DNaseI to remove the DNA template (see Figure 18), phenol extracted and ethanol precipitated, and injected into the nuclei of Vero cells at a concentration of ~0.1mg/ml. The transcripts possessed the SV40 polyadenylation signal but a poly(A) tail was not generated in the *in vitro* transcription reaction. Injected cells were incubated for 4 hours post-injection before fixation and permeabilisation, and stained for CAT protein using two mouse anti-CAT monoclonal antibodies and the Amersham biotinylated second antibody and Texas-Red system.

Cells injected into the cell nuclei with capped message stained strongly for CAT protein [Table 5 and Figure 19d], indicating that T7 transcripts can be both stable and translatable in Vero cells. In contrast, injection of capped message into the cytoplasm [Figure 19b], or injection of uncapped message into either the nuclei [Figure 19c] or cytoplasm [Figure 19a] of cells did not result in the appearance of CAT protein. This indicates that the 5'-cap structure is necessary but not sufficient for *in vivo* translation of the T7 *in vitro* transcript, and suggests that the cell modifies the microinjected capped transcript within the nucleus, probably by polyadenylating 3'-end of the RNA and\or splicing the intron from the message. Removal of the DNA template by DNase treatment of the sample prior to injection [Figure 18 lane B], removes the possibility, albeit an unlikely one, that expression of CAT protein resulted from recombination events that brought the CAT coding sequences under the control of an active endogenous promoter.

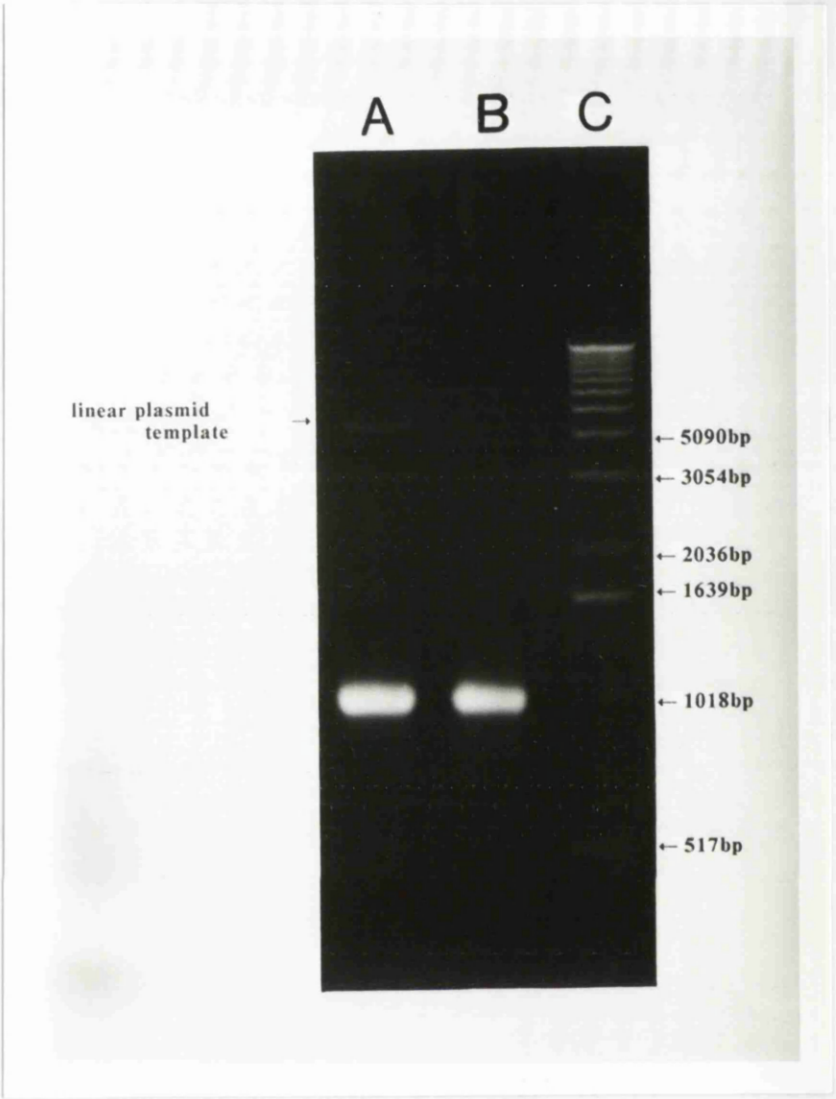


Figure 18. Bis-acrylylcystamine (BAC) gel of *in vitro* transcription reaction products stained with ethidium bromide. Run-off CAT transcripts from a *BamHI* linearised pT7cat template were generated *in vitro* using T7 RNA polymerase. Samples representing 5% of the total yield were applied to the BAC gel prior to (A) and following DNase*I* treatment to remove the DNA template (B) from the sample. Molecular weight markers (1kb ladder -BRL) were run in lane C. Following electrophoresis the gel was stained with ethidium bromide (5μg/ml) and the gel visualised on a UV transilluminator (UV Products Inc.).

Figure 19. Immunofluorescence staining for CAT protein in Vero cells injected with capped and uncapped T7pol *in vitro* transcripts. Poly (A)-minus run-off T7pol CAT transcripts, both capped (b & d) and uncapped (a & c), were generated *in vitro* from a *BamHI* linearised pT7cat template. The transcription reaction was DNase*I* treated to remove the template, phenol extracted and ethanol precipitated, and the RNA resuspended at ~0.05mg/ml. These RNA preparations were injected into the cytoplasm (a & b) or the nuclei (c & d) of Vero cells. The cells were then incubated for 4 hours and stained for CAT protein using the Amersham biotin-Texas-Red system (see Materials & Methods).

RNA Injections:

5'-capped	injected into	CAT expression
No	cytoplasm	0/85
No	nucleus	0/56
Yes	cytoplasm	0/53
Yes	nucleus	21/90

Table 5. Vero cells injected with *in vitro* transcribed RNA encoding CAT were stained for CAT protein 4 hours post-injection. The transcripts generated *in vitro* were produced lacking a 5'-terminal cap structure or possessing a cap by the inclusion of the cap analogue GpppG in the transcription reaction. Results shown are representative of three separate injection experiments.

3.7 Discussion.

The micro-injection and transfection experiments have both shown that the presence of a reporter gene under the control of a T7 promoter together with T7pol within a mammalian cell, is insufficient to produce expression of that target gene at the protein level. These results are similar those obtained in RK13 (rabbit kidney) cells using an analogous system based on bacteriophage T3 RNA polymerase and a T3 promoter (Deuschle *et al.*, 1989). Addition of a nuclear location signal to the T7pol species failed to overcome the block to expression. The presence of the transcription terminator Tϕ in either orientation failed to have any impact in overcoming the block that apparently exists to expression mediated by T7pol from the ϕ10 promoter *in vivo*.

3.7a Is T7pol Transcriptionally Active in Mammalian Cells?

The non-appearance of CAT protein in the previous transfection and micro-injection experiments could be explained by a failure of the T7pol to transcribe within mammalian cells, for example because the T7 promoter is masked by proteins binding at or near it, or because the T7pol itself is inactivated by post-transcriptional modification. T7pol has previously been used to drive transient expression from a T7 late promoter in a vaccinia virus/T7pol hybrid expression vector system (Fuerst *et al.*, 1986, 1987). In this system CAT, ß-gal, HIV-1 *env*, and hepatitis B antigen have been expressed at the protein level, at levels 400-600 times greater levels than from conventional $CaPO_4$ transfection systems. Analysis of the T7 transcripts indicated that in the vaccinia vector T7pol initiated correctly at the T7 promoter. Extracts of mammalian cells that stably express T7 RNA polymerase, including nuclear extracts from the PMN cell line (Lieber *et al.*, 1989) which expresses a nuclear targetted form of T7pol, have shown T7pol activity in *in vitro* transcription assays. These results show that the T7pol synthesised within mammalian cells is competent to transcribe.

In yeast cell lines containing both T7pol and the ϕ10 promoter, T7pol has been shown to be capable of initiating correctly at the T7 promoter integrated into the yeast chromosome, and also of elongating the chain (Chen *et al.*, 1987). In that study the yeast strain used was *his3*$^-$ and the T7 promoter inserted upstream of the *his3* gene. Although transcripts were correctly initiated, the cells remained *his*$^-$, indicating that no functional protein had been produced. This suggests that the RNA produced in this system was either untranslatable or unstable, and also indicates that promoter inaccessability is unlikely to be the reason for the failure to obtain expression at the protein level in my own experiments, described above.

It might be argued that expression is blocked due to a failure of T7pol to terminate transcription, resulting in the production of large unstable transcripts that are rapidly degraded. However, in the experiments of Chen *et al.* (1986) in yeast, and Lieber *et al.* (1989) in mouse cells, no transcription terminator was included downstream of the reporter gene, and yet accumulation of T7 transcripts was observed in the total RNA fraction in both systems. Fuerst and Moss (1989) have shown that inclusion of Tϕ downstream of a reporter gene results in efficient transcription termination in mammalian cells. Deletion of the Tϕ sequence, although it resulted in random downstream termination, actually caused an increase in the level of expressed protein. In any case, the presence of the SV40 polyadenylation signal downstream of the CAT gene in my ϕ10-CAT constructs means that any read-through of the terminator sequence, or production of large transcripts in the absence of a terminator, will be unlikely to affect final transcript length since the immature transcripts should be cleaved at the SV40 polyA site.

Uncapped T7 *in vitro* transcripts were not translated following microinjection into Vero cells, whether injected into the nucleus or cytoplasm (section 3.6). *In vitro* capped transcripts were translated, but only if they were injected into the nuclei of Vero cells. This indicates that the presence of a 5'-cap structure is necessary but not sufficient for translation of T7pol transcripts in mammalian cells. These results suggest that capped transcripts undergo additional essential modification(s) within the nucleus, possibly by the addition of a poly(A) tail, intron excision, and/or methylation of the 5'-cap. Exogenous hnRNA has been shown

to be accurately spliced and polyadenylated when adenovirus late pre-mRNA is injected into the nuclei of mouse L cells (Moore & Sharp, 1985) and by injection of ß-globin pre-mRNA (synthesised *in vitro* using bacteriophage SP6 RNA polymerase) into the nuclei of *Xenopus* oocytes (Green *et al.*, 1983). Correct splicing of introns from yeast pre-tRNA species synthesised by T7pol *in vitro* has also been reported in yeast cell-free systems (Reyes & Abelson, 1986). The inability of uncapped RNA to be translated following injection into cell nuclei could result from a failure of the transcript to be capped by the cellular guanylyltransferase, which may have knock-on effects further along the pre-mRNA processing pathway at the levels of polyadenylation, intron excision or mRNA transport.

The evidence so far obtained from the yeast (Chen *et al.*, 1986) and vaccinia/T7pol hybrid systems have suggested that T7pol synthesised in mammalian cells is both transcriptionally active and capable of recognising and correctly initiating transcription at the ϕ10 promoter *in vivo*. Also these systems have shown that transcription can be terminated by the presence of Tϕ, but that this is not a prerequisite for the accumulation of stable mRNAs. Taking together my RNA injection results and evidence from injection of RNAs into *Xenopus* oocytes (Furuichi *et al.*, 1977; Lockard & Lane, 1978; Drummond *et al.*, 1985), this leads to the conclusion that the block to expression of T7pol transcripts at the protein level is likely to be at the level of RNA processing, most probably 5'-cap synthesis, which affects the translatability of mRNA *in vivo*.

Chapter 4. *In Vivo* Protein Expression Mediated By T7pol Can Be Modulated by Infection With Vaccinia Virus.

4.1 Introduction.

In the previous chapter I described experiments that show there is a block to expression that prevents the use of T7pol for expression of proteins in mammalian cells. Injection of appropriately processed *in vitro* transcripts into cell nuclei leads to translation of the message, which tends to rule out either a defect in transport of mRNA from the nucleus to the cytoplasm, or a defect in translation of the mRNA as the primary block to expression. The conclusion I draw is that T7pol-mediated expression is blocked at either the transcriptional level or at the level of post-transcriptional RNA modification.

I have not formally shown that T7pol can transcribe in mammalian cells (see Discussion 3.7). However, it has been shown that T7pol is capable of transcription from a ϕ10 promoter integrated into the yeast chromosome, although the resulting transcripts were not translated (Chen *et al.*, 1987). T7pol encoded in a recombinant vaccinia virus vector is also capable of transcribing from the ϕ10 promoter carried on a transfected plasmid, the transcripts generated by this system also being translated into protein (Fuerst *et al.*, 1986,1987; Elroy-Stein *et al.*, 1989). Deuschle *et al.* (1989) using an analogous system (with T3 RNA polymerase rather than T7pol) found that although the presence of both the T3 promoter and T3 RNA polymerase in mammalian cells was insufficient to produce expression at the protein level, infection of cells containing these two components with vaccinia virus could override the block to expression. Translation of T7 transcripts in the vaccinia/T7pol hybrid system is likely to result from the presence within the viral core particles of all the virus coded enzymes required for mRNA maturation, including a guanylyltransferase and a polyadenylase. This further suggests that it is a failure of the cellular pre-mRNA modifying enzymes to act on T7 transcripts that prevents T7pol-mediated expression in uninfected mammalian cells.

This chapter will describe experiments that I performed to test the effect of vaccinia virus super-infection on the outcome of experiments similar to those described in the last chapter. As before, ϕ10-cat reporter plasmids were microinjected or transfected into Vero cells along with pure T7pol, or T7pol expression vectors. These cells were subsequently superinfected with vaccinia virus, and CAT activities in infected cells compared to those of uninfected cells. The results allow some tentative conclusions to be drawn about the nature of the block to T7pol-mediated expression in mammalian cells.

4.2 Vaccinia virus superinfection overcomes the block to T7 RNA polymerase-mediated gene expression.

Cells in 35mm dishes were transfected with 4μg of plasmid DNA, infected with vaccinia virus ($\approx$30 p.f.u./cell) or mock-infected 16 hours later, harvested after a further 24-36 hours and the CAT activities in cell lysates assayed as described in Materials and Methods (also see Chapter 3. Section 3.5). The results are shown in Figure 20. Again no CAT expression is observed in uninfected cells transfected with any combination of T7pol expression vector (pAR3126 or pAR3132) and ϕ10-cat template (pT7cat or pCA3.1), whether transfected individually or together. When cells transfected with pT7cat or pCA3.1 alone were infected with vaccinia virus a high level of CAT activity was observed. When each of these plasmids was co-transfected with either pAR3126 (cT7pol) or pAR3132 (nT7pol) and superinfected with vaccinia virus, the level of CAT activity was further increased. Whatever the reason for the high level of CAT activity in the absence of T7pol, the fact that T7pol can enhance CAT activity suggests that T7pol can generate functional CAT mRNA in vaccinia-infected cells. Under the conditions described above, the presence of a nuclear location signal on the T7pol species does not appear to affect the level of CAT expression achieved (see Chapter 6.).

Why does vaccinia virus infection stimulate expression of CAT activity from the ϕ10-cat plasmids in the absence of T7pol? This observation was surprising because Fuerst *et al.*

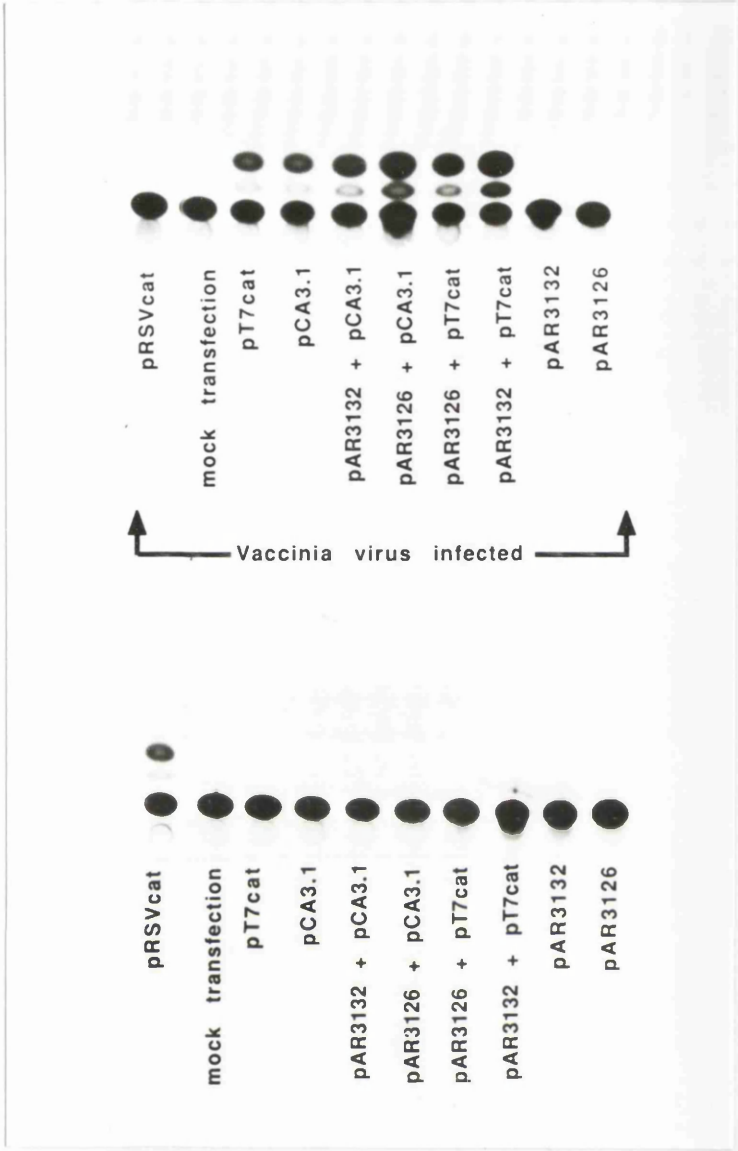


Figure 20. CAT assay of cell lysates from vaccinia virus-infected Vero cells transfected with T7pol expression vectors and ϕ10cat constructs. Vero cells were transfected with 2μg of pT7cat and pCA3.1 individually, or together with 2μg of a T7pol expression vector (pAR3126/3132) by the calcium phosphate method. The precipitate was removed after 18 hours, the cells washed and subsequently infected with vaccinia virus (strain WR) at a multiplicity of 30 p.f.u./ml, or mock-infected. Following overnight incubation the cells were harvested and cell lysates prepared and assayed for CAT activity (see Materials and Methods).

(1986) in experiments analogous to my own reported no CAT expression from a ϕ10-cat type plasmid transfected into vaccinia-infected cells. Their results appeared to conflict with my own and this deserved further investigation.

Several possible reasons for the high level of vaccinia-induced CAT expression in my experiments come to mind:

1) specific or non-specific transcription initiation by cellular enzymes in response to virus-induced shock.

2) non-specific transcription initiation by vaccinia RNA polymerase on double-stranded plasmid DNA.

3) specific transcription initiation by the vaccinia RNA polymerase starting at a cryptic vaccinia promoter present in the plasmid, or from the ϕ10 promoter itself.

4.3 Superinfecting vaccinia virus, but not HSV-1, can generate active CAT mRNA from a transfected CAT-containing plasmid.

To discover whether the high level of vaccinia-induced CAT expression was the result of some general viral cytopathic effect, or to a specific property of vaccinia virus, cells were transfected as before and infected with either vaccinia virus or herpes simplex virus type-1 (HSV-1). Following infection the cells were harvested after 8 hours (to take account of the faster lytic cycle of HSV) and cell extracts were assayed for CAT activity. The results [Figure 21] show that HSV-1 infection of cells transfected with pCA3.1 either alone, or together with a T7pol expression vector, does not result in the appearance of detectable levels of CAT activity. In contrast, vaccinia virus infection results in the appearance of CAT activity in cells transfected with pCA3.1; co-transfection with the T7pol expression vector results in enhanced

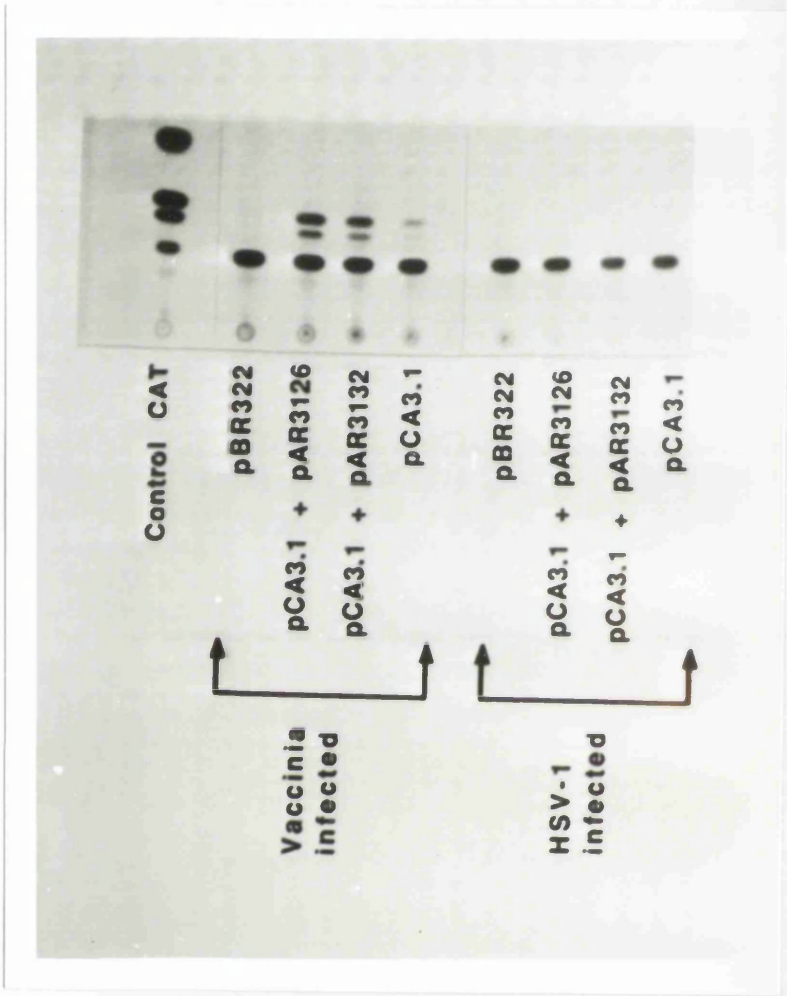


Figure 21. CAT assays of Vero cell lysates from cells transfected with a ϕ10cat construct and subsequently infected with vaccinia virus or HSV-1. Vero cells were transfected by the calcium phosphate method with pCA3.1 alone or along with one of the T7pol expression vectors; pAR3126 or pAR3132. Following removal of the DNA precipitates cells were infected with virus, vaccinia or HSV-1, at a multiplicity of 30 p.f.u./ml, or mock-infected. Cells were harvested 6 hours later and lysates assayed for CAT activity.

levels of CAT activity as before. The overall levels of CAT expression are reduced when compared to cells harvested at 24 hours post-infection [See Figure 20], but this is likely to reflect the shorter time allowed post-infection for the production of CAT. From this experiment it can be concluded that the appearance CAT activity in cells transfected with a ϕ10-cat construct alone and subsequently infected with vaccinia virus is not due to a general virus-induced cytopathic effect, but to some special effect of vaccinia virus.

4.4 The level of vaccinia-induced CAT activity depends on the CAT vector background.

Other possible reasons for the appearance of CAT activity in vaccinia infected cells transfected with a ϕ10-cat construct alone are that the virus-coded RNA polymerase is initiating transcription either non-specifically on double-stranded plasmid DNA, or specifically at a cryptic vaccinia promoter occurring by chance in the plasmid, or at the ϕ10 promoter itself. Therefore, additional plasmids were constructed to assess the contribution of the plasmid background to vaccinia-induced CAT expression. These plasmids are described briefly below (for details of their construction and plasmid maps see Materials and Methods and Figure 22).

pTAC4 : contains the CAT gene under the control of the SP6 promoter followed by the SV40 small-t intron and SV40 early polyadenylation signal inserted into pGEM1.

pGEM0cat : a promoterless CAT vector derived from pT7cat by deletion of the *PvuII-HinDIII* fragment containing the ϕ10 promoter (see Figure 6, Materials and Methods).

pL6cat : based on pSV2cat (Gorman *et al.*, 1982b), the SV40 promoter has been replaced by the ϕ10 promoter placing the CAT gene under the control of the ϕ10 promoter in a vector background based on pSV2cat rather than pGEM.

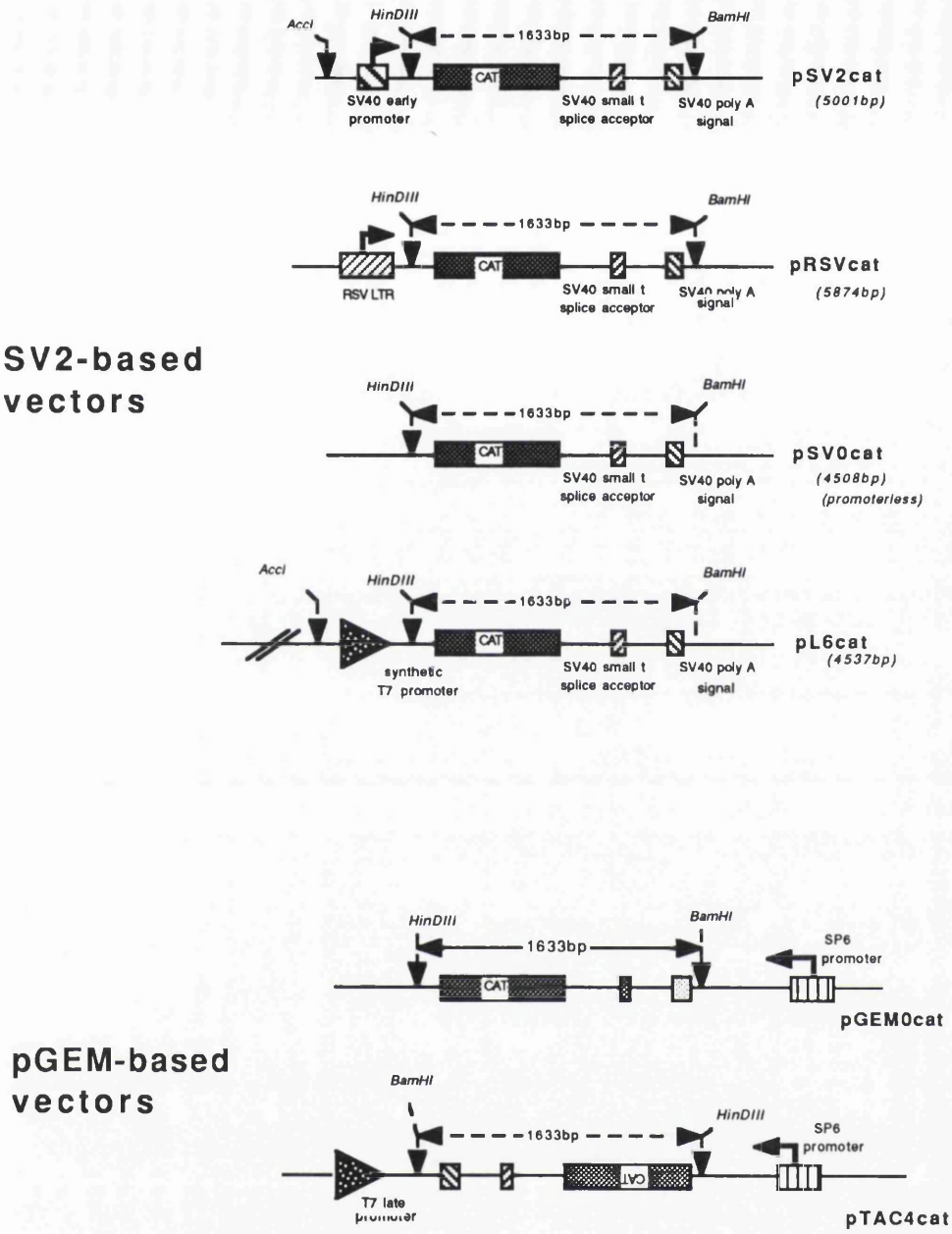


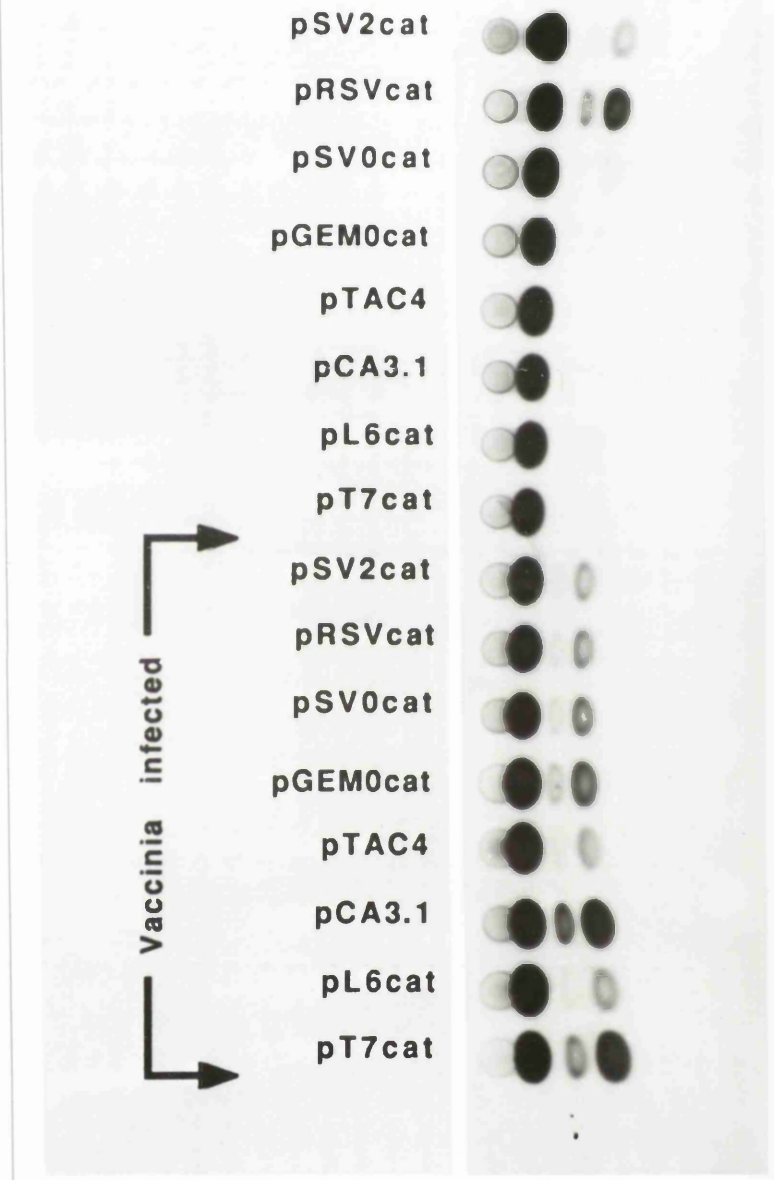
Figure 22. Diagrams of plasmids constructed containing the CAT gene in the pSV2 and pGEM plasmid backgrounds (see Materials and Methods).

pSV0cat : a promoterless pSV2cat-type construct.

Vero cells in 35mm diameter culture dishes were transfected by the calcium phosphate method with one of the plasmids described above, or with one of two control plasmids, SV2cat or pRSVcat. The cells were mock-infected or infected with vaccinia virus after 18 hours, and harvested 24 hours later. Cell extracts were made and assayed for CAT activity. The results of these assays are shown in Figure 23.

In the absence of vaccinia virus infection, CAT expression was not observed in transfected cells except in control cells transfected with pSV2cat or pRSVcat, where the CAT gene is under the control of a eucaryotic promoter. Following vaccinia virus infection of the transfected cells CAT expression was observed in cells transfected with pT7cat and pCA3.1 as before. Lower, but significant levels of CAT were also observed in cells transfected with pTAC4, pL6cat, pSV0cat and pGEM0cat, cells containing pTAC4 and pGEM0cat generally showing higher levels than those containing pSV2-type vectors. The levels of CAT expressed in cells containing pT7cat and pCA3.1 were higher than in cells where no T7 promoter was present, however, transfer of the ϕ10 promoter into the SV2-type plasmid background did not increase the level of CAT expression observed which indicates that that the presence of the T7 promoter *per se* is not the cause for the high levels of virally-mediated transcription initiation. No change, or a reduction in CAT expression was observed following infection of cells transfected with pSV2cat or pRSVcat. A reduction in CAT activity might be expected in these cases due to the general shut-down of cellular transcription in vaccinia infected cells. In general slightly higher levels of CAT expression were observed in cells containg a pGEM-based vector when compared to a pSV2-type plasmid. From these results it is possible to conclude that there is general transcription iniation from plasmid DNA in vaccinia-infected cells, this being worse in pGEM vectors than pSV2-type vectors, and that this effect is compounded by the presence of a T7 promoter in pGEM-based plasmids. Therefore, the discrepancy between my results and those of Fuerst *et al.* (1986) may result from different CAT reporter vectors used in our respective studies.

Figure 23. CAT assay showing the effect of promoter and plasmid background on the level of CAT expression stimulated in transfected cells subsequently infected with vaccinia virus. Vero cells were transfected with plasmids bearing the CAT gene in two different plasmid backgrounds, pSV2-type [pSV2cat, pRSVcat, pL6cat and pSV0cat - see Figure 21] and pGEM-type [pT7cat, pCA3.1, pGEM0cat and pTAC4 - see Figures 14 & 21]. Within these plasmids the CAT gene is under the transcriptional control of different promoters; the SV40 early promoter [pSV2cat]; the RSV LTR [pRSVcat]; the T7 late promoter ϕ10 [pT7cat, pCA3.1 and pL6cat]; the SP6 promoter [pTAC4], and in promoterless contructs [pSV0cat and pGEM0cat]. Cells were washed free of precipitate and infected with vaccinia virus at a multipilicity of 30 p.f.u./ml, or mock-infected and harvested 24 hours later when cell lysates were prepared and assayed for CAT activity.


pSV2cat
pRSVcat
pSV0cat
pGEM0cat
pTAC4
pCA3.1
pL6cat
pT7cat
Vaccinia infected
pSV2cat
pRSVcat
pSV0cat
pGEM0cat
pTAC4
pCA3.1
pL6cat
pT7cat

Taken together, these results strongly suggest that the high levels of CAT expression observed with the GEMINI-based vectors in vaccinia infected cells arise as a result of non-specific, bi-directional initiation of the viral RNA polymerase within the plasmid sequences, and not through any specific initiation event at either the T7 or SP6 bacteriophage promoter. To recap, this conclusion arises from the observations that reversing the orientation of the CAT gene within the GEMINI vectors does not alter the high level of virus-induced CAT expression, and that insertion of the ϕ10 sequences into the low background environment of the pSV2-type vectors does not result in an increase in the levels of CAT following viral infection. Also deletion of the ϕ10 sequence from pT7cat (resulting in pGEM0cat) does not reduce the level of virus-induced level of CAT activity when compared to the parental plasmid. The region of the GEMINI vectors that is responsible for the high levels of virus-induced CAT expression presumably lies outside the *HinDIII-BamHI* fragment containing the CAT sequences since this is common to all of the CAT containing plasmids tested.

4.5 The enhancement of vaccinia-induced CAT activity by T7 RNA polymerase depends on the presence of a T7 promoter.

The experiments described in Section 4.2 show that when a T7pol expression vector is co-transfected into vaccinia-infected Vero cells, together with a CAT gene under the transcriptional control of a T7 promoter there is an increase in the amount of CAT activity over the amount observed in vaccinia-infected cells containing the CAT vector alone. This suggests that T7pol may initiate transcription from the ϕ10 promoter to generate active mRNA. However, other explanations are possible. For instance, T7pol may interact with the vaccinia transcription machinery and enhance the rate of non-specific transcription. In the latter scenario, the levels of enhancement would be expected not to depend on the presence of a ϕ10 promoter; conversely, a requirement for the ϕ10 promoter would be indicative of specific transcription initiation. The experiments described below were conducted to distinguish between these possibilities.

Cells were transfected with pAR3126 (cT7pol) together with pT7cat containing the CAT gene under the transcriptional control of the ϕ10 promoter or with the analogous plasmid lacking any promoter (pGEM0cat). The cells were then infected with vaccinia virus, or mock infected, and cell lysates assayed for CAT activity. The results are shown in Figure 24. These results again show that in the absence of vaccinia virus infection, no CAT expression was observed when both the T7pol gene and the target gene are present. When cells were transfected with the plasmid pT7cat or pGEM0cat individually and infected with vaccinia virus, the expected background levels of CAT expression were observed. No CAT expression was observed in vaccinia infected or uninfected cells which had been transfected with pAR3126 (cT7pol) alone (not shown). In vaccinia-infected cells co-transfected with pAR3126 and pT7cat, higher levels of CAT expression are observed than when pT7cat was transfected alone. The enhancement of CAT expression is abolished in infected cells co-transfected with pAR3126 (cT7pol) and pGEM0cat, the level of CAT expression remaining at that observed for vaccinia-infected cells transfected with the CAT vector alone. This experiment demonstrates that both a T7 promoter and the T7 RNA polymerase are required for the enhancement of CAT expression, suggesting that T7pol can accurately initiate transcription from the ϕ10 promoter in vaccinia-infected cells. Similar results were also observed in experiments where the pSV2cat-based pL6cat construct was used in place of pT7cat (not shown).

Although the previous experiment indirectly suggests that T7pol is both active and capable of recognising the ϕ10 promoter in vaccinia-infected cells, it says nothing about the transcriptional activity of T7pol in uninfected cells. Transcriptional activity in vaccinia-infected cells could conceivably result from virus-induced activation of the T7pol, possibly by incorporation of the T7pol into a hybrid transcription complex with vaccinia-coded enzymes, the T7pol component providing specificity for the ϕ10 promoter. However, the experiments of Chen *et al.* (1987) (see Discussion) have shown that T7pol can initiate transcription from an integrated ϕ10 promoter in yeast cells, so vaccinia is clearly not required for T7pol-mediated transcription in yeast cells.

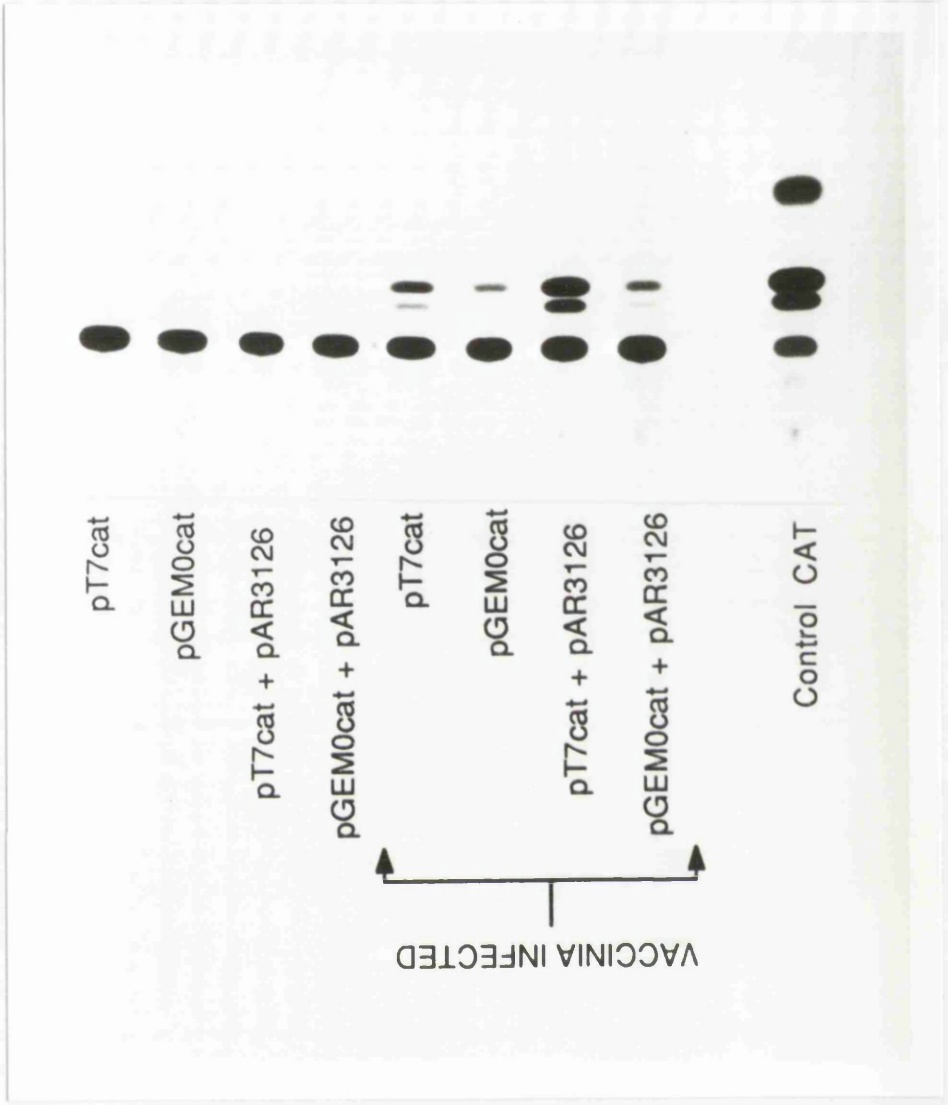


Figure 24. CAT assay showing that enhancement of CAT expression in vaccinia virus-infected cells requires the presence of both a T7 promoter and T7pol. Vero cells were transfected with pT7cat or pGEM0cat, alone or along with the T7pol expression vector pAR3126 and infected 18 hours later with vaccinia virus, or mock-infected. Cells were harvested 24 hours following infection and lysates prepared and assayed for CAT activity.

4.6 Investigating the Subcellular Site of Vaccinia Virus Influence on T7pol Mediated Expression.

The results presented so far show that vaccinia virus can induce a certain level of CAT expression from a transfected ϕ10-CAT reporter plasmid in the absence of T7pol, but that this vaccinia-induced CAT expression is further augmented when T7pol is also present in the infected cell (sections 4.2 and 4.5). The T7pol-mediated enhancement of CAT expression depends on the presence of a ϕ10 promoter and presumably results from transcription initiating from within ϕ10 itself (section 4.5). The extent of T7pol-mediated enhancement of CAT expression seems to be independent of whether the T7pol is targetted to the nucleus or not, because both cT7pol and nT7pol stimulate a similar level of enhancement (section 4.2). Vaccinia virus is a "cytoplasmic" virus; most of its life cycle including transcription, RNA processing, DNA replication and packaging takes place in the cytoplasm of infected cells (see Moss, 1985 for review). It is conceivable, therefore, that transcription from the transfected ϕ10-CAT vectors and all the subsequent events leading to synthesis of CAT enzymatic activity also take place wholly or predominantly in the cell cytoplasm. Indeed, this is not unlikely, because in transfection experiments most input DNA does not reach the cell nucleus (Bradshaw *et al.*, 1981).

It is important to discover where the transfected ϕ10-CAT plasmids are transcribed and the transcripts processed in vaccinia infected cells, because this has a bearing on the interpretation of the transfection experiments, and may influence how we approach our ultimate goal, which is to establish a virus-free expression system based on T7pol. The microinjection experiments described below were intended to provide clues to the subcellular site of interaction between vaccinia virus, T7pol and ϕ10-CAT in the transfection experiments.

I performed two experiments designed to indicate whether vaccinia virus-induced transcription of a ϕ10-CAT reporter gene occurs in the nucleus or cytoplasm of cells. Firstly Vero cells were injected into the cytoplasm with a mixture of cT7pol (0.05mg/ml) along with

	CAT Staining	
	mock infected	vaccinia infected
Cytoplasmic injection:		
T7pol + pCA3.1		
0hr	0/76	0/89
8hr	0/75	21/110
24hr	0/83	69/102
BSA + pCA3.1		
0hr	0/82	0/86
8hr	N .D.	N.D.
24hr	0/89	40/67

Table 6. Results of CAT staining on Vero cells injected cytoplasmically with T7pol and the ϕ10cat construct pCA3.1, or with BSA and pCA3.1 followed by infection with vaccinia virus. Vero cells were injected with T7pol (0.05mg/ml) and pCA3.1 (0.5mg/ml) or with BSA (0.05mg/ml) along with pCA3.1, the cells incubated for 1 hour and then infected with vaccinia virus (~30 p.f.u./cell), or mock-infected. The cells were incubated overnight before staining for T7pol/BSA and CAT. The results shown are representative of two repetitions of the injections.

pCA3.1 (0.1mg/ml), or with BSA (0.05mg/ml) together with pCA3.1 (0.1mg/ml). One hour after injection, cells were mock-infected or infected with vaccinia virus (~30p.f.u./cell), and incubated overnight before fixation and staining for CAT (see Materials and Methods). The results of these injections are shown in Table 6. These show that introduction of a CAT-containing plasmid into the cytoplasm only results in the appearance of CAT protein when the cells are superinfected with vaccinia virus. These results also show that the presence of T7pol is not essential for the production of the CAT protein. The results indicate that vaccinia virus is able to exert an effect on a CAT template in the cytoplasm of cells but do not address the question of whether the viral RNA modifying enzymes can act on T7 transcripts produced in the nucleus.

In order to address this latter question the following experiment was performed. The plasmids encoding cT7pol (pAR3126) or nT7pol (pAR3132) were injected together with the ϕ10-CAT vector pCA3.1 or the positive control pRSVcat into the nuclei of Vero cells. After allowing 4 hours at 37°C for recovery, the cells were infected with vaccinia virus (30 p.f.u./cell) or were mock-infected, and incubated overnight before fixation and staining for T7pol and CAT. Control cells were injected into the nucleus with pXm30PK-B (Kalderon *et al.*, 1984) which contains a chicken muscle pyruvate kinase cDNA under the transcriptional control of the SV40 early promoter, together with pCA3.1 or pRSVcat, and subjected to the infection procedure before fixing and staining for pyruvate kinase and CAT. These cells provided a control for the efficiency of the injection procedure, and the viability of injected cells.

If the vaccinia virus transcriptional apparatus is restricted to the cell cytoplasm, one would predict that microinjecting the ϕ10-CAT vector directly into the nucleus would fail to allow vaccinia-induced CAT expression. The results shown in Table 7 provide support for this view. The majority of cells that were injected with pRSVcat together with either pAR3126 or pAR3132 co-stained for both T7pol and CAT, whether or not the cells were superinfected with vaccinia virus. Similarly, the majority of cells injected with pXm30PK-B and pRSVcat expressed both pyruvate kinase and CAT. However, even after vaccinia

DNA injected into nucleus:	mock infected: Stained for: T7pol	CAT	PK	vaccinia infected: Stained for: T7pol	CAT	PK
pAR3132 + pCA3.1	30/87	0/87	-	22/62*	5/62*	-
pAR3132 + pRSVcat	31/41	28/41	-	-	-	-
pAR3126 + pCA3.1	28/56	0/56	-	23/62*	12/62*	-
pAR3126 + pRSVcat	56/87	58/87	-	-	-	-
pXm30PK + pCA3.1	0/55	0/55	22/55	0/54	0/54	25/54
pXm30PK + pRSVcat	0/71	42/71	41/71	-	-	-

* No examples of double-positives.

Table 7. Results showing the effect of infection with vaccinia virus on CAT expression in Vero cells injected into the nucleus with a T7pol expression vector along with a ϕ10cat construct. Cells were injected into the nucleus with a T7pol expression vector (pAR3126 or pAR3132) along with either pCA3.1 or pRSVcat. Cells were incubated for four hours before overnight infection with vaccinia virus (~30 p.f.u./ml). Control cells were injected as before and mock-infected. Cells were then stained for T7pol and CAT as described. Cells were also co-injected with pXm30PK-B along with pCA3.1 or pRSVcat and infected and mock-infected as above, stained for T7pol and CAT, counted and subsequently stained for pyruvate kinase using a rabbit polyclonal antiserum followed by FITC-conjugated sheep anti-rabbit Ig. The results shown are representative of two repetitions of the injections.

infection, relatively few cells injected in the nucleus with the ϕ10-CAT construct pCA3.1 together with pAR3126 or pAR3132 expressed CAT protein. This contrasts with previous experiments (see Table 6) where cytoplasmic injection of the same ϕ10-CAT vector into cells that were subsequently superinfected with vaccinia virus resulted in a high level of CAT expression in the majority of cells. A simple and plausible interpretation of this data is that the vaccinia virus helper effect for expression from pCA3.1 is restricted to the cytoplasm of infected cells.

Although some vaccinia-infected cells that were nominally injected in the nucleus with pCA3.1 along with pAR3126 or pAR3132 did stain positively for CAT protein, none of these cells co-expressed T7pol. A possible explanation for this anomaly is that the CAT-positive cells were not successfully injected in the nucleus, but instead the plasmid DNA was accidently discharged into the cytoplasm. Since the injection needle must traverse the cytoplasm in order to reach the nucleus it is to be expected that this will sometimes happen.If pCA3.1 is released into the cytoplasm of vaccinia infected cells, non-specific transcription of the plasmid by the viral RNA polymerase will lead to CAT expression (see section 4.6). It might be expected that in those cells injected in the nucleus with pCA3.1 and pAR3132, and superinfected with vaccinia virus, the nT7pol encoded by pAR3132 would transcribe the CAT gene on pCA3.1 and these transcripts might be correctly processed by the vaccinia enzymes to create stable translatable mRNAs. That this does not appear to happen, judging by the lack of detectable CAT protein in these experiments, suggests that one or more of the vaccinia RNA processing enzymes may not gain access to the nuclear interior.

4.7 Site of RNA Modification in Vaccinia Infected Vero Cells.

The experiments described in previous sections have indirectly suggested that the vaccinia RNA modifying enzymes are capable of using T7-generated transcripts as substrates, but that these enzymes are restricted to the cytoplasm of infected cells and are unable to act on T7 transcripts generated in the nucleus. The following experiment was performed,

involving the injection of *in vitro* generated uncapped T7-CAT transcripts into vaccinia-infected and mock-infected Vero cells in order to determine more directly whether the vaccinia encoded RNA modifying activities are excluded from the nucleus.

Uncapped, non-polyadenylated T7-CAT RNA transcripts were generated *in vitro* from *BamHI*-linearised pT7cat template (see Materials and Methods). The reaction mixture was DNase*I* treated to remove the DNA template, phenol/chloroform extracted, ethanol precipitated and resuspended at a concentration of approximately 0.05mg/ml. This RNA was injected into the cytoplasm of vaccinia-infected (overnight infection) or uninfected Vero cells, which were then incubated for 4 hours and fixed and then stained for CAT (see Materials and Methods). Samples of the RNA used in the injections were run on agarose gels to ensure that the RNA had not degraded at any point prior to injection (gel not shown). The results from these experiments are shown in Figure 25 and the results of all the RNA injections (including those described in section 3.6) are summarised in Table 8. They show that when injected into the cytoplasm of uninfected cells, uncapped and non-polyadenylated RNA was not translated (Figure 25a). Injection of uncapped, poly(A)-minus RNA into the cytoplasm of vaccinia-infected cells resulted in translation of the message and the accumulation of CAT protein (Figure 25b) suggesting that vaccinia virus is capable of contributing all the RNA modifying functions required to produce a translatable message within the cytoplasm of infected cells. Injection of uncapped message into the nucleus of vaccinia-infected cells would show whether the vaccinia RNA processing enzymes are able to access the nucleus, but nuclear injection of virus-infected cells is technically difficult so it was not possibleto perform these injections. Data shown in Chapter 5, however, indicates that the vaccinia virus RNA modifying enzymes are retained within the cytoplasm of infected cells.

Figure 25. Immunofluorescence staining for CAT protein in vaccinia infected and mock-infected Vero cells injected cytoplasmically with uncapped poly(A)-minus T7 transcripts. Vero cells were infected with vaccinia virus (30p.f.u./cell) (**a**) or mock-infected (**b**) and incubated overnight before injection of uncapped poly(A)-minus T7-CAT RNA transcripts (~0.5mg/ml) generated *in vitro* from a *BamHI* linearised pT7cat template into the cytoplasm. The cells were incubated for 4 hours, fixed, permeabilised and stained for CAT using mouse monoclonal antibodies and the Amersham biotinylated Texas-Red system (see Materials and Methods)

RNA Injections:

5'-capped	vaccinia virus	injected into	CAT expression
No	No	cytoplasm	No
No	No	nucleus	No
Yes	No	cytoplasm	No
Yes	No	nucleus	Yes
No	Yes	cytoplasm	Yes
No	Yes	nucleus	N.D.

Table 8. Table summarising the results of experiments involving the injection of *in vitro* generated T7cat RNA transcripts into the cytoplasm or nuclei of Vero cells.

4.8 Discussion.

The results from these experiments have provided circumstantial evidence that in this system vaccinia virus exerts its effects on gene expression solely within the cytoplasm of cells. Firstly uncapped poly(A)-minus RNA injected into the cytoplasm of a vaccinia-infected cell is translated efficiently, showing that the vaccinia RNA modifying enzymes are present and available within the cytoplasm to the T7 transcript. Secondly, injection of the ϕ10cat template into the nucleus of Vero cells and subsequent infection with vaccinia virus showed that when the template for T7pol transcription is within the nucleus the vaccinia virus RNA modifying enzymes are unable to operate on the T7 transcripts. This result also tends to exclude the possibility that vaccinia infection leads to a breakdown of the nuclear envelope and intermixing of nuclear and cytoplasmic contents which might otherwise allow processing of RNA in the cytoplasm by cellular enzymes normally located in the nucleus.

The results from experiments involving microinjection of *in vitro* synthesised transcripts into vaccinia-infected or uninfected cells are summarised in Table 8. Along with results from nuclear injection of the ϕ10cat template and subsequent vaccinia infection, the most parsimonious explanation for these findings is that cellular enzymes can polyadenylate and splice exogenous RNA transcripts in the nucleus, but not in the cytoplasm, but cannot 5'-cap exogenous RNA in either compartment, while vaccinia-encoded enzymes can 5'-cap and polyadenylate exogenous RNA in the cytoplasm. Inherent in this explanation is the assumption that translation of exogenous transcripts requires that they be both 5'-capped and 3'-polyadenylated.

T7pol-generated transcripts do not appear to be an *in vivo* substrate for the cellular guanylyltransferase enzyme. This might be explained by a stringent substrate specificity not shared by the vaccinia enzyme, for which the T7 transcripts do represent an *in vivo* substrate, or by physical segregation of the cellular enzyme from the T7 transcripts.

Guanylyltransferase activity has been purified from a variety of sources; from vaccinia virions (Martin *et al.*, 1975; Monroy *et al.*,1978), from the yeast *Saccharomyces cerevisiae* (Itoh *et al.*, 1987) and from HeLa cells (Ensinger & Moss, 1976, Wei & Moss, 1977). The guanylyltransferase enzyme purified from vaccinia virus is routinely used for *in vitro* capping of T7 and SP6 transcripts (See Materials and Methods) and has, therefore, been shown to be capable of addition of 7-methyl guanosine to both pyrimidine or purine residues *in vitro*, a finding confirmed *in vivo* in vaccinia infected cells (Fuerst & Moss, 1989). The enzyme from HeLa cells was purified from the soluble protein component and has been shown to form ^{7}mGpppA and ^{7}mGpppG with RNA and synthetic polyribonucleotides. The enzyme isolated from HeLa cells differs from the vaccinia-encoded activity. In the vaccinia enzyme, the guanylyltransferase and (guanine-7-)methyltransferase are situated on the same protein (93kDa) and the mRNA(nucleoside-2'-)methyltransferase is located on a 23kDa associated protein (Martin *et al.*, 1975; Monroy *et al.*, 1978). These three activities are all found on quite distinct proteins in HeLa cells. The HeLa cell guanylyltransferase is a protein of 56kDa, considerably smaller than the viral protein. Using a transcription product from phage λ c17 (Rosenberg & Paterson, 1979) it has been shown that the cellular enzyme is capable of *in vitro* addition to ppC at the 5'-end (Venkatesan *et al.*, 1980). Venkatesan *et al.* (1980) have also shown that the enzyme is capable of catalysing the transfer of the guanosine residue to ppC terminated RNA *in vitro*, so although not certain it is possible that this reaction would be catalysed *in vivo* because in cellular mRNAs both N_1 and N_2 can be any of the four bases. T7 transcripts generated from the pGEM2 vector initiate with pppC and so should constitute a substrate for the cellular guanylyltransferase. A physical explanation rather than a mechanistic one seems to be more likely. The nascent RNA chain is capped early in transcription (See Banerjee, 1980), not entirely surprising since the cap blocks 5'→3' exo-ribonuclease activity. The cellular enzyme is thought to associate with the pol*II* transcription complex allowing early 5'-processing before transcription is complete and this sequestration of the guanylyltransferase to its site of activity may make it unavailable to exogenous RNAs or those transcribed by an exogenous procaryotic polymerase. Fuerst and Moss (1989) in their vaccinia/T7pol hybrid expression system also report an apparent close coupling of vaccinia RNA polymerase and guanylyltransferase activities in vaccinia

transcription. Within this system although some capping of the T7 transcripts occurs, the majority remain uncapped with resulting 5'→3' degredation.

Chapter 5. Cloning and Expression of Vaccinia Virus Guanylyltransferase.

5.1 Introduction.

The data presented in the previous two chapters strongly suggest that the main block to expression of T7-generated transcripts in mammalian cells is rooted in the inability of cellular 5'-end RNA-processing enzymes to use the T7pol transcripts as a substrate. In contrast, the evidence suggests that the vaccinia virus encoded guanylyltransferase can, and does, cap T7pol transcripts in vaccinia-infected cells. Therefore, it seemed reasonable to investigate whether co-expressing the vaccinia capping enzyme in the absence of other viral gene products would be sufficient to complement T7pol mediated transcription *in vivo* and provide the basis for a virus-free mammalian expression system. Before this could be tested, the vaccinia virus guanylyltransferase coding sequences had first to be isolated and cloned into a suitable expression vector. To confirm that my attempts to express the guanylyltransferase were successful, I had also to raise an antibody against the enzyme. This Chapter describes how I achieved these aims and my subsequent attempts to use the cloned guanylyltransferase to complement T7pol mediated expression in mammalian cells.

The vaccinia virus capping enzyme is a heterodimer composed of a large subunit (93kDa) and a small subunit (23kDa), possessing three catalyltic activities; guanylyltransferase, 7-methyl and 2'-O methyl transferase activities. The capping enzyme has been purified to homogeneity (Martin & Moss, 1975; Monroy *et al.*, 1978) and it has been shown that both the guanylyltransferase and 7-methyltransferase activities are associated with the large subunit, and a 2'-O-methyltransferase activity associated with the small-subunit. The large subunit is capable on its own of adding a Cap-0 structure the minimum requirement for ribosome recognition and may, therefore, be sufficient for modifying T7 transcripts to translatable form in the absence of the small subunit.

5.2 Cloning of Vaccinia Virus Guanylyltransferase.

The coding region for the large subunit of the vaccinia virus capping-complex has been localised to one end of the *HinDIII*D fragment of the vaccinia genomic DNA (Morgan *et al.*, 1984). The *HinDIII*D fragment (~16kbp), subcloned into the *HinDIII* site of a pUC type vector (BRL) was obtained from Dr.B Moss. Inspection of the sequence of the *HinDIII*D fragment (Niles *et al.*, 1986) showed that the region encoding the guanylyltransferase could be isolated as a 3018bp *HinDIII-ClaI* fragment. The downstream *ClaI* site was altered to a *BglII* site by insertion of linkers and the guanylyltransferase coding region was isolated as a *HinDIII/BglII* fragment. This was then inserted between the *HinDIII/BglII* sites of pSV2catB2 (see Materials and Methods), and pXm30PK-B. These plasmids were designated pSV2gt and pXm30gt respectively (see Figure 26 and Figure 8, Materials and Methods).

The relatively large size of the enzyme and the cytoplasmic life cycle of vaccinia virus suggested that the guanylyltransferase would be restricted to the cytoplasm of cells. I therefore took steps to target the guanylyltransferase to the nucleus by engineering an SV40 NLS into its primary structure. The preferred site of insertion was close to either the N- or C-terminus, because although the effect upon enzyme activity of introducing these targetting sequences at these sites was unknown, in general addition of extra amino-acids at either terminus of a protein is likely have less effect on overall structure, and thus enzyme activity, than addition at an internal site. A synthetic double-stranded oligonucleotide encoding the SV40 large-T NLS was obtained and inserted into the *AsuII* site of pSV2gt within the guanylyltransferase gene, close to the C-terminus (see Figure 9, Materials & Methods and Figure 26) forming the plasmid pSV2gt-AN.

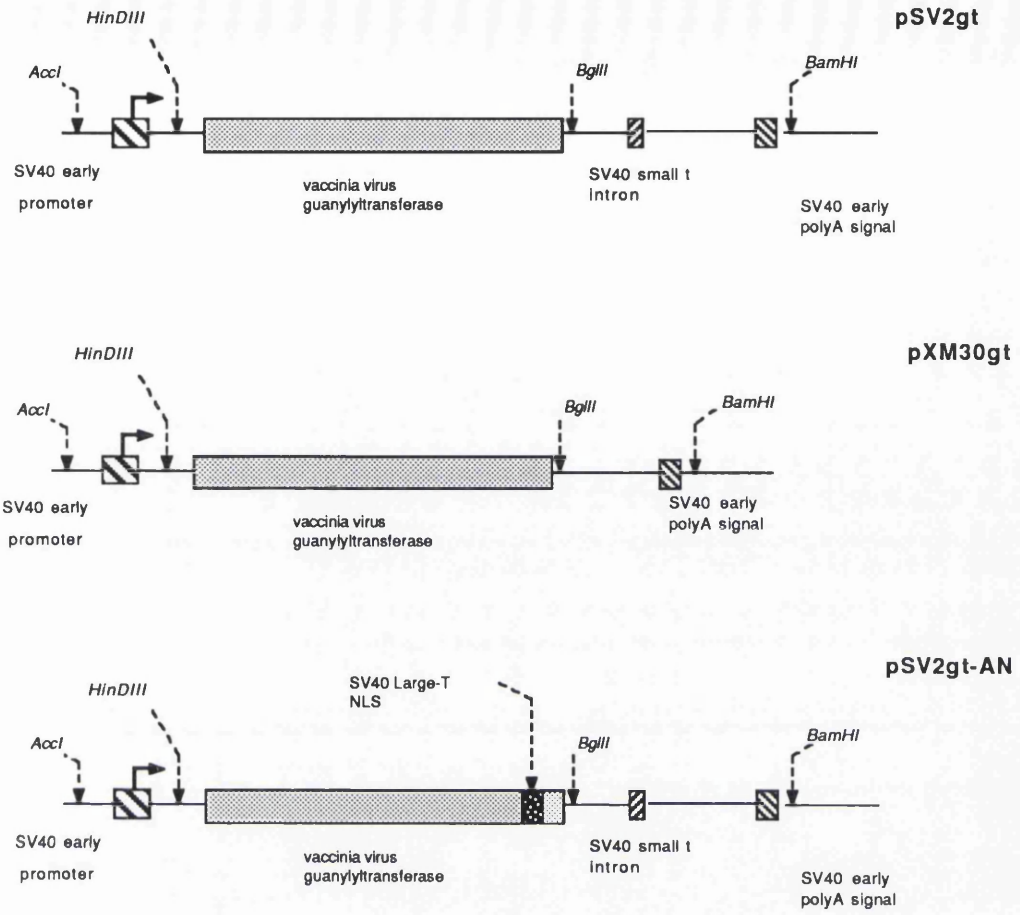


Figure 26. Diagrams of the eucaryotic expression vectors $pSV2_{GT}$ and pXm30-GT which carry the gene encoding the vaccinia virus guanylyltransferase, and the plasmid pSV2gt-AN which carries the gene encoding a nuclear targetted form of the protein.

5.3 Production of Antibodies Against Vaccinia Guanylyltransferase.

No antibodies against the 95kDa guanylyltransferase protein were available. The enzyme has been purified (Martin *et al.*, 1975; Monroy *et al.*, 1978), but these protocols required a complex purification of the enzyme from large quantities of viral cores. An alternative method for obtaining immunogenic material for antibody production is to genetically engineer fusion proteins containing sequences of the protein under investigation linked to a bacterial protein. The fusion protein is expressed in bacterial cells and can be then purified and used as an inoculum. I adopted this approach, and generated a fusion protein containing the C-terminal region of the guanylyltransferase (amino acids 614-843) appended to the C-terminus of the bacterial protein ß-galactosidase (ß-gal) [see Figure 7, Materials and Methods]. A *BamHI/HinDIII* fragment from pH3Db2, corresponding to the C-terminal third of guanylyltransferase gene, was inserted into the *HinDIII/BamHI* sites of pUR278 (Rüther & Müller-Hill, 1983), such that the coding region was inserted in-frame at the C-terminus of the ß-gal coding region, creating the plasmid pFUB5.

E.coli cells (strain H4.4) were transfected with this plasmid and ampicillin resistant clones screened by *HinDIII\BglII* digestion of isolated plasmid DNA. Positive clones were then grown up in the presence or absence of IPTG and whole cell lysates subjected to SDS-PAGE and Western blotting. The blot was stained by immunoperoxidase using antibodies against ß-gal as a first layer and horseradish peroxidase-conjugated anti-rabbit Ig as a second layer, and developed using 4-chloro-napthol (results shown in Figure 27). Uninduced cells containing the parental pUR278 plasmid (lane A) show very little staining with anti-ß-gal antibodies, indicating that the *lac* promoter is effectively repressed under non-inducing conditions. IPTG induction of these cells results in the production of native ß-gal (lane B). Cells containing pFUB5 under non-inducing conditions (lane C) also show little anti ß-gal staining. When induced (lane D), clones containing pFUB5 synthesise a protein with an approximate molecular weight of 140kDa, larger than native ß-gal (lane E) and corresponding to the expected size of the fusion protein, as well as multiple bands of lower molecular weight that probably correspond to proteolytic degradation products.

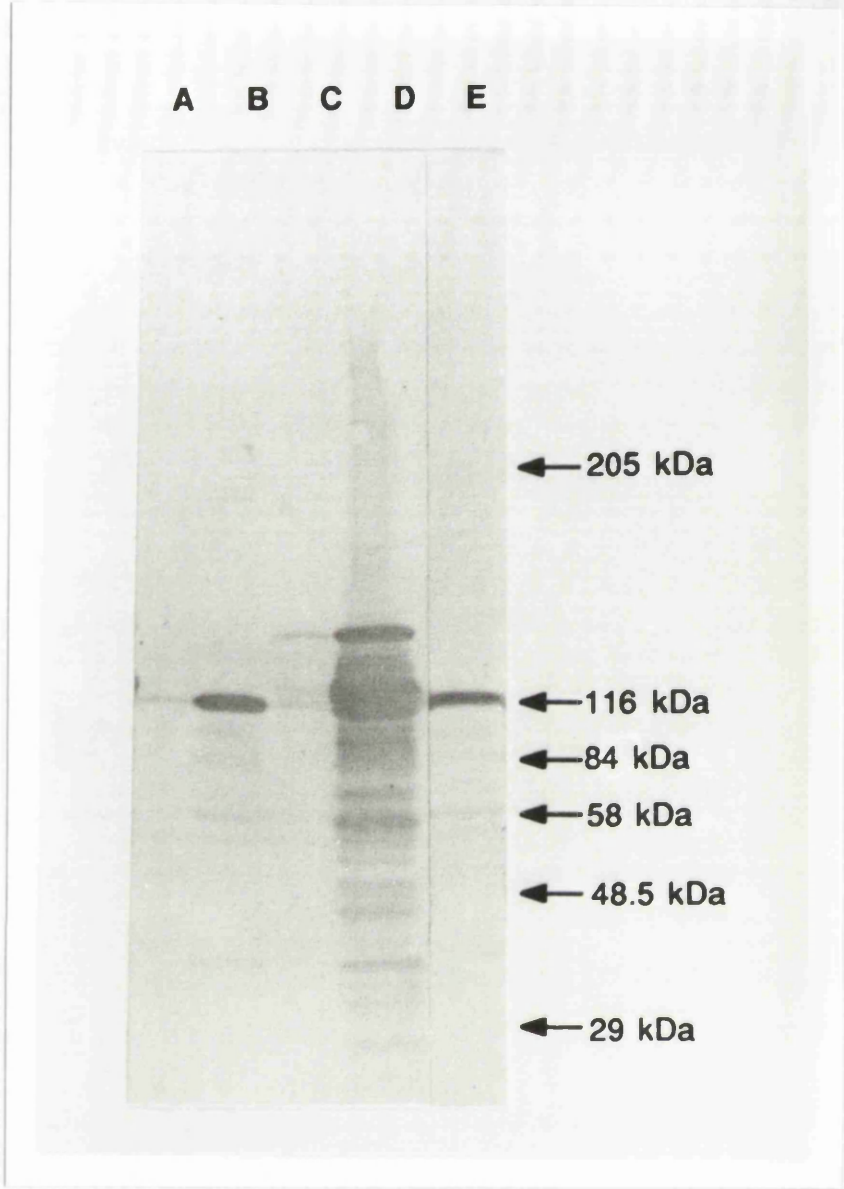


Figure 27. Immuno-peroxidase staining against ß-gal on a Western blot of lysates from *E.coli* cells containing plasmid encoding the ß-gal/guanylyltransferase fusion protein. Cultures of bacteria containing pUR278 (lanes a and b) and pFUB5 (lanes c and d) were grown in the absence (lanes a and c) or the presence (lanes b and d) of the inducing agent IPTG (10μM). After overnight incubation cell lysates were prepared and Western blotted onto nitrocellulose. The blot was stained with a rabbit anti-ß-gal polyclonal antiserum followed by goat-anti-rabbit Ig conjugated HRP (DAKO) and developed using 4-chloronapthol as the enzyme substrate. Pure ß-gal was also run (lane e) as a control for the staining and as an additional molecular weight standard.

Preparative gels of this induced fusion protein were Western blotted onto nitrocellulose and the region of the nitrocellulose containing the full-length fusion protein was dissolved in dimethyl sulphoxide, precipitated as described in Materials and Methods, and the antigen bearing particles injected subcutaneously into half-lop rabbits (see Materials & Methods). Antisera were collected and their specificity tested as described below.

Vero cells were infected with vaccinia virus at a multiplicity of ~30p.f.u./cell. The cells were incubated overnight before fixation and were then stained using the rabbit polyclonal anti-fusion protein antiserum (anti-GTase), or with pre-immune serum from the same animal. Both the pre-immune and immune sera were used at a dilution of 1:400, followed by an FITC-anti-rabbit Ig second layer (Sigma), diluted 1:200. Control cells were mock-infected and stained in the same way.

No staining was observed using the pre-immune serum in either vaccinia infected [Figure 28b], or mock-infected [Figure 28a] cells. Anti-GTase staining of mock-infected cells was also negative [Figure 28c]. Vaccinia-infected cells stained with the anti-GTase serum showed strong staining of cytoplasmic bodies [Figure 28d and at higher magnification in Figure 29]. This pattern of staining closely resembles that observed when cells infected with the closely related rabbit poxvirus are stained for the viral RNA polymerase (Morrison & Moyer, 1985). This result might have been expected in view of the results obtained by Broyles and Moss (1987), which suggested a close association of vaccinia RNA polymerase and guanylyltransferase activities. This result is also consistent with results from Chapter 4 which suggested that the vaccinia-mediated effect on T7 transcripts in infected cells was a cytoplasmic event.

Figure 28. Indirect immunofluorescence staining for vaccinia virus guanylyltransferase in vaccinia-infected cells using a polyclonal antiserum raised against the ß-gal/guanylyltransferase fusion protein (anti-GTase). Vero cells were infected overnight with vaccinia virus (~30p.f.u./cell) (b and d), or mock-infected (a and c) before fixation and permeabilisation. Cells were then stained with pre-immune serum (a and b) or anti-GTase (c and d), both diluted to 1:400 in PBS, followed by FITC-conjugated anti-rabbit Ig (Sigma) (see Materials and Methods).

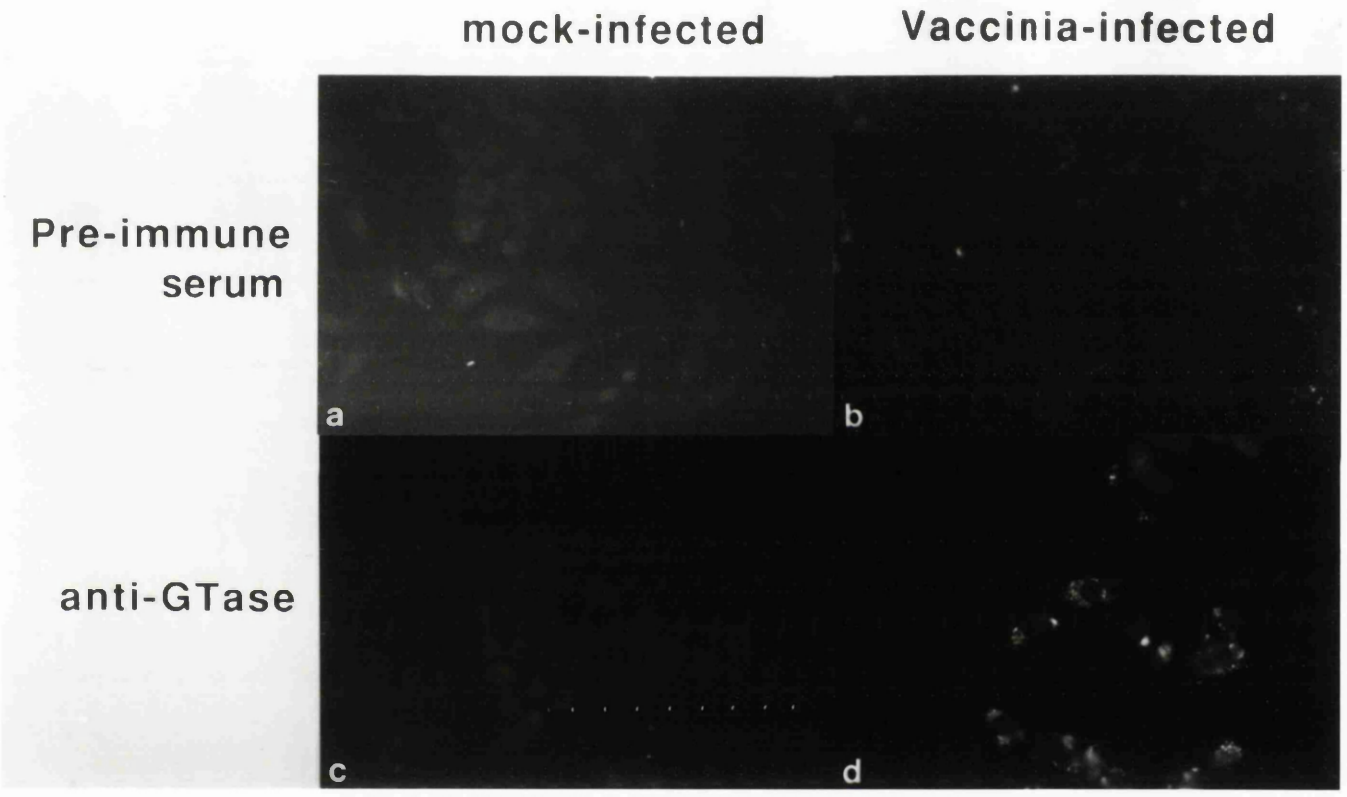

mock-infected
Vaccinia-infected
Pre-immune serum
anti-GTase
a
b
c
d

Figure 29. Immunofluorescence staining against vaccinia virus guanylyltransferase in vaccinia infected Vero cells stained with anti-GTase antiserum showing cells at higher magnification. (For details see Figure 26)

5.4 Virus-Free Eucaryotic Expression of Vaccinia Guanylyltransferase.

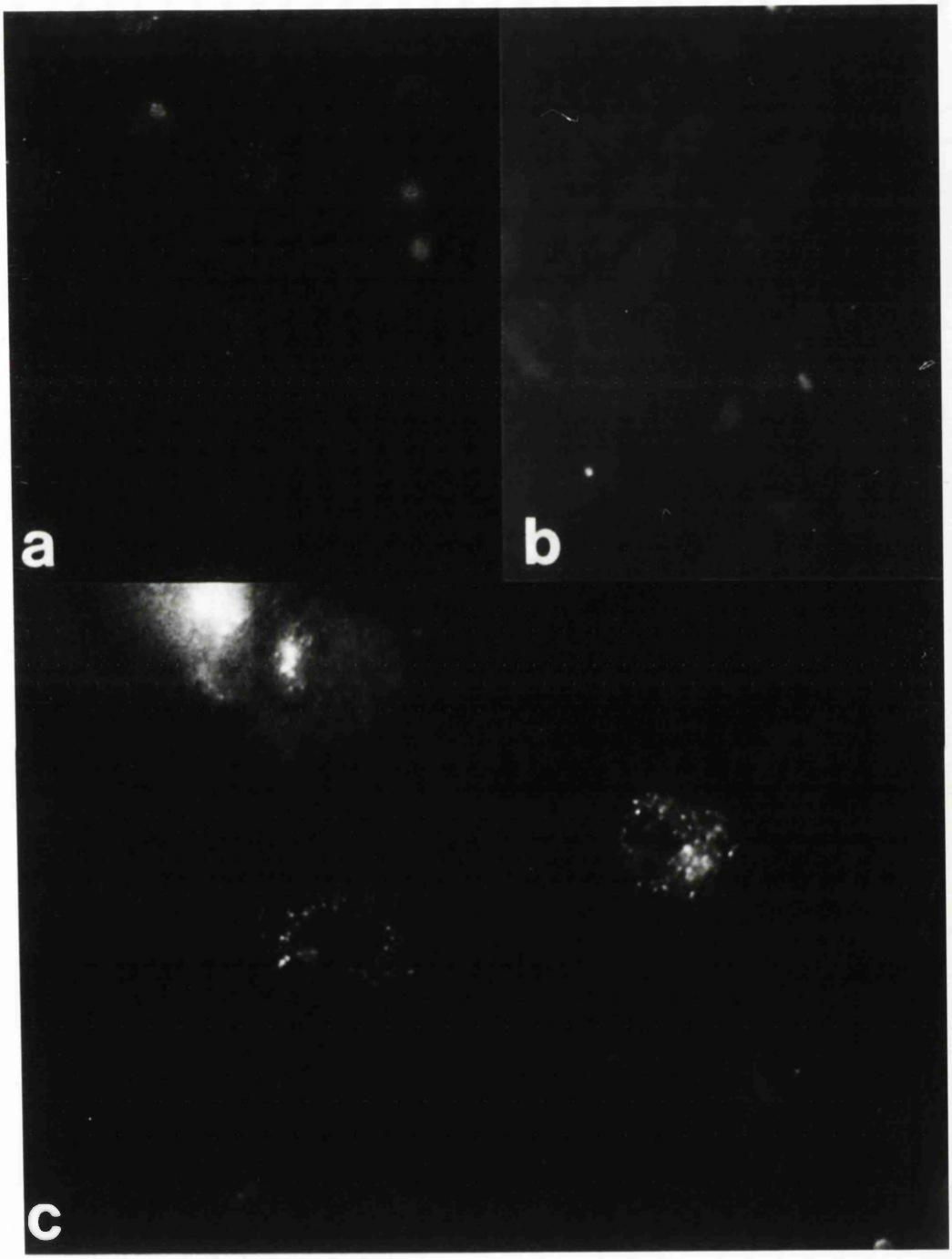
To examine the subcellular distribution of guanylyltransferase when expressed in the absence of other viral gene products, Vero cells were injected with pSV2gt (0.1mg/ml), incubated overnight, fixed and permeabilised, and stained with the anti-GTase antiserum. These injected cells show a punctate pattern of nuclear staining [Figure 30a and c] which was absent from uninjected cells. No staining was observed in cells injected with an irrelevant plasmid (pXm30PK) and stained using the same reagents [Figure 30b]. These results show that the cloned guanylyltransferase gene is expressed in injected cells, although the nuclear location is surprising in view of the large size of the protein and the pattern of cytoplasmic staining observed in vaccinia infected cells. The lack of staining in cells injected with pXm30PK demonstrates that the staining observed in pSV2gt injected cells does not result simply from injection of the cell. This conclusion requires confirmation by Western blotting or immuno-precipitation to show the size of the protein recognised in vaccinia infected cells by the antiserum.

The nuclear location of the anti-guanylyltransferase staining was unexpected and possible explanations will be discussed later in this Chapter. Judging by the intensity of staining, the level of protein expressed is low in comparison to the levels of other proteins expressed from similar plasmids with the same promoter (eg. nT7pol in plasmid pAR3132). Attempts were therefore made to increase the level of guanylyltransferase expression (see later).

5.5 Effect of Cloned Guanylyltransferase on T7pol Mediated Expression.

Vero cells were injected with mixtures of pAR3132 or pAR3126, pSV2gt or pXm30gt, and pCA3.1 or pRSVcat. The cells were incubated overnight and stained for T7pol (as a positive control for the efficiency of injection) and CAT. The results are shown in Table 9, and show that cells injected with either one of the guanylyltransferase expression vectors

Figure 30. Anti-GTase staining of Vero cells injected into the nucleus with one of the the guanylyltransferase expression vectors $pSV2_{GT}$ and pXm30-GT. Vero cells were injected with $pSV2_{GT}$ (a) or pXm30PK-B (b), incubated overnight before fixation. Cells were stained with anti-GTase serum (1:400 in PBS) and FITC-conjugated sheep anti-rabbit serum (1:200 in PBS - Sigma). The nuclei of two anti-GTase stained cells expressing the cloned guanylyltransferase at higher magnification (c).


a
b
c

DNA injected in nucleus	Stained for: T7pol	CAT
pAR3132 + pSV2gt + pCA3.1	54/96	0/96
pAR3132 + pXm30gt + pCA3.1	27/60	0/60
pAR3132 + pRSVcat + pCA3.1	32/90	34/90
pAR3126 + pSV2gt + pCA3.1	33/81	0/81
pAR3126 + pXm30gt + pCA3.1	28/95	0/95
pAR3126 + pRSVcat + pCA3.1	31/60	33/60

Table 9. Results showing cell staining for CAT and T7pol proteins in Vero cells co-injected into the nucleus with a T7pol expression vector (pAR3126 or pAR3132), along with the ϕ10cat construct pCA3.1 and a vaccinia guanylyltransferase expression vector (pSV2gt or pXm30gt).

Vero cells were injected into the nucleus with the plasmid mixtures (0.1mg/ml), incubated overnight and stained for CAT and T7pol proteins (see Materials and Methods). The results shown are representative of two repetitions of the injections.

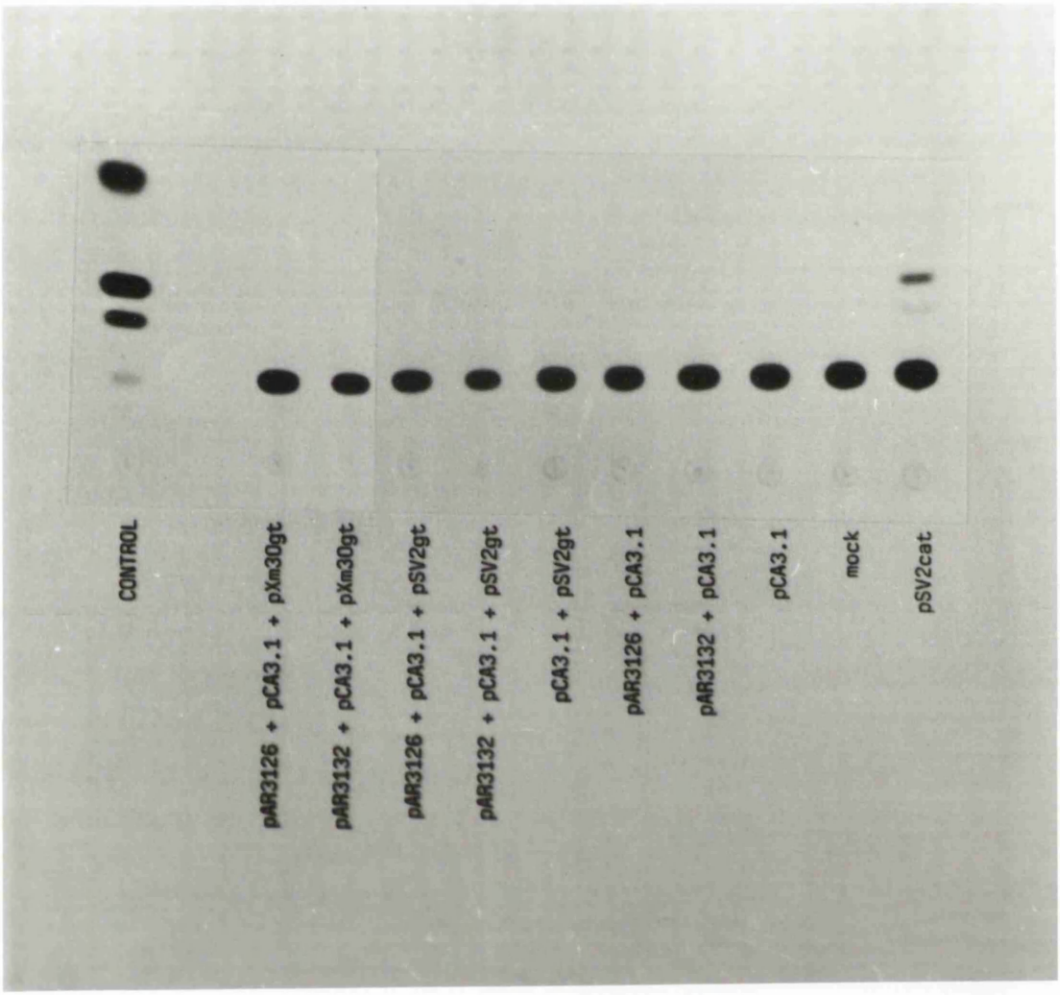


Figure 31. CAT assay of lysates from Vero cells co-transfected with the cloned guanylyltransferase gene along with a ϕ10cat construct and T7pol expression vector. Vero cells were transfected by the calcium phosphate precipitate method with the guanylyltransferase expression vectors alone or along with T7pol expression vectors and ϕ10cat constructs (6μg DNA/dish : 2μg/plasmid). Cells were harvested 48 hours after transfection and cell lysates assayed for CAT activity.

along with the T7pol and target genes is insufficient for the expression of CAT from the T7 promoter within these cells. The pRSVcat controls were positive as expected.

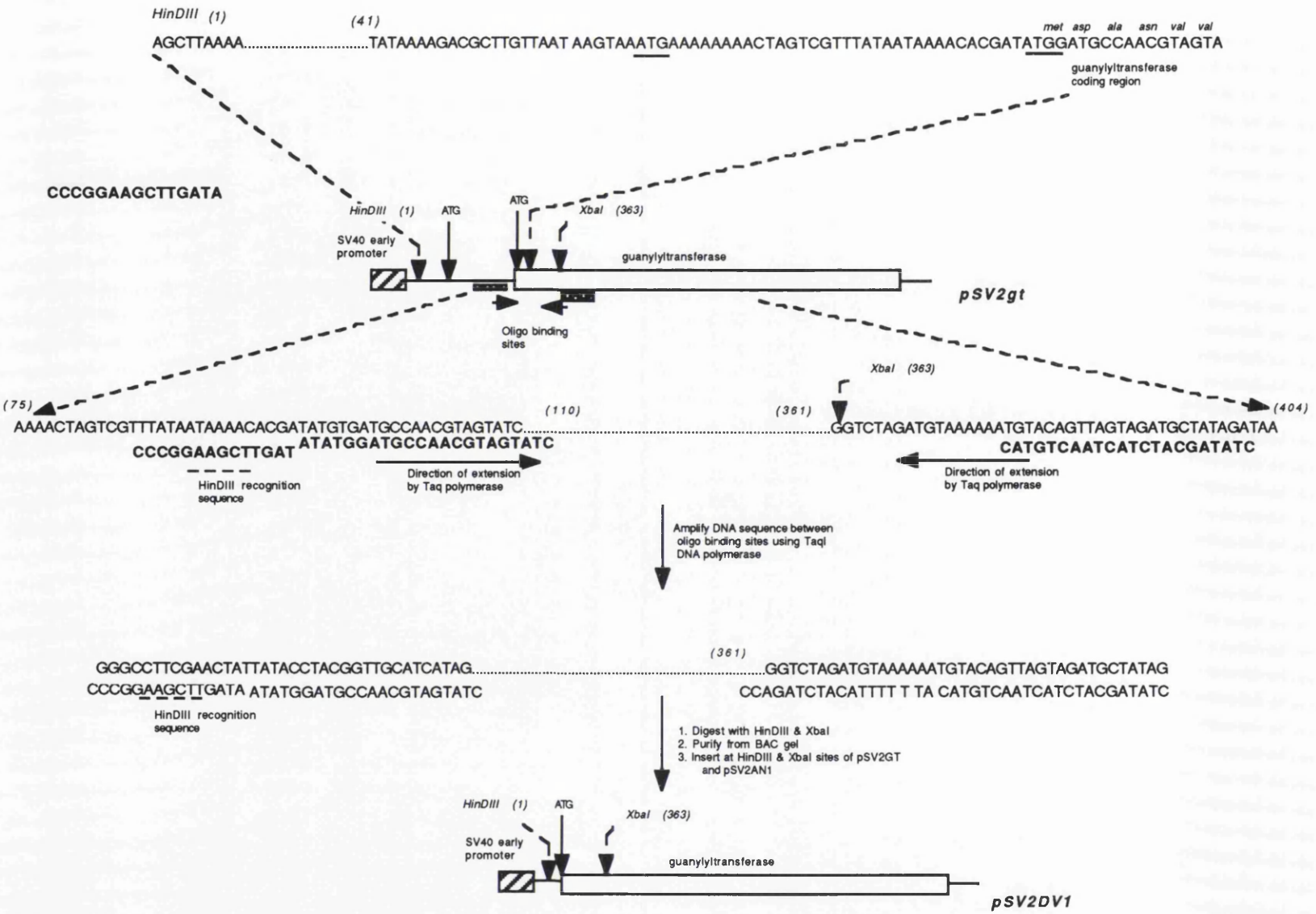
To confirm these results, and to see whether CAT was being produced at levels undetectable by immunofluoresence, Vero cells were co-transfected with pAR3126/3123, pCA3.1 and either pSV2gt or pXm30gt as previously described. The cell extracts were assayed for CAT activity and the results are shown in Figure 31. These show no expression of CAT in the cells transfected with the target gene along with the genes for T7pol and guanylyltransferase. The control transfections show that both the transfection procedure and the CAT assay were functioning correctly.

5.6 Enhancement of Guanylyltransferase Expression.

The level of expression of guanylyltransferase observed in cells injected with pSV2gt was low considering it was driven by the SV40 early promoter. This becomes more evident when the levels of expressed guanylyltransferase are compared to the levels of CAT and T7pol obtained when expressed from the same promoter (pSV2cat and pAR3132/26 - see Figure 16, Chapter 3). Low levels of expression similar to those obtained using pSV2gt were observed for cells injected with the plasmid pXm30gt (data not shown). The low levels of expression of the cloned guanylyltransferase made it seem unlikely that accumulation in the nucleus is due the presence of an NLS within the primary sequence (pSV2gt-AN) rather than by association with cellular components would affect the overall ability of the guanylyltransferase to complement the activity of T7pol, and so efforts initially concentrated on increasing the levels of expressed guanylytransferase within the mammalian cells.

Inspection of the sequence of the *HinDIII-BglII* fragment cloned from pH3Db2 revealed the presence of a translation intiation site (ATG), followed by a three amino acid open reading frame (ORF), upstream of the ORF encoding the guanylyltransferase. It seemed possible that this could have reduced the efficiency of translation from the authentic

Figure 32. Diagram showing the deletion of the upstream ORF from the 5'-UTR of Guanylyltransferase gene by PCR. The 102bp region of DNA between the *HinDIII* site and the correct translation initiation site contains no unique restriction sites and so this region was deleted by PCR (polymerase chain reaction). Two oligonucleotide primers were obtained, one of which corresponded to a 25bp sequence spanning the authentic translation initiation codon, and the other a 22bp sequence complementary to a region just downstream of the unique *XbaI* site located within the coding region. The upstream oligonucleotide contained at its 5'-terminus the sequence necessary for formation of a new *HinDIII* site during amplification. The sequence between the two oligonucleotides was amplified by 30 cycles of anealing, chain extension by *Taq* DNA polymerase, and DNA melting. The PCR product was digested which *HinIII/XbaI*, purified from a BAC gel and ligated between the *HinDIII/XbaI* sites of pSV2gt and pSV2gt-AN.


HinDIII (1)
(41)
met asp ala asn val val
AGCTTAAAA.....TATAAAAGACGCTTGTTAAT AAGTAAATGAAAAAAAACTAGTCGTTTATAATAAAACACGATATGGATGCCAACGTAGTA
guanylyltransferase coding region
CCCGGAAGCTTGATA
HinDIII (1)
ATG
ATG
XbaI (363)
SV40 early promoter
guanylyltransferase
pSV2gt
Oligo binding sites
XbaI (363)
(75)
(110)
(361)
(404)
AAAACTAGTCGTTTATAATAAAACACGATATGTGATGCCAACGTAGTATC.....GGTCTAGATGTAAAAAATGTACAGTTAGTAGATGCTATAGATAA
CCCGGAAGCTTGAT ATATGGATGCCAACGTAGTATC
CATGTCAATCATCTACGATATC
HinDIII recognition sequence
Direction of extension by Taq polymerase
Direction of extension by Taq polymerase
Amplify DNA sequence between oligo binding sites using TaqI DNA polymerase
(361)
GGGCCTTCGAACTATTATACCTACGGTTGCATCATAG.....GGTCTAGATGTAAAAAATGTACAGTTAGTAGATGCTATAG
CCCGGAAGCTTGATA ATATGGATGCCAACGTAGTATC
CCAGATCTACATTTT T TA CATGTCAATCATCTACGATATC
HinDIII recognition sequence
1. Digest with HinDIII & XbaI
2. Purify from BAC gel
3. Insert at HinDIII & XbaI sites of pSV2GT and pSV2AN1
HinDIII (1)
ATG
XbaI (363)
SV40 early promoter
guanylyltransferase
pSV2DV1

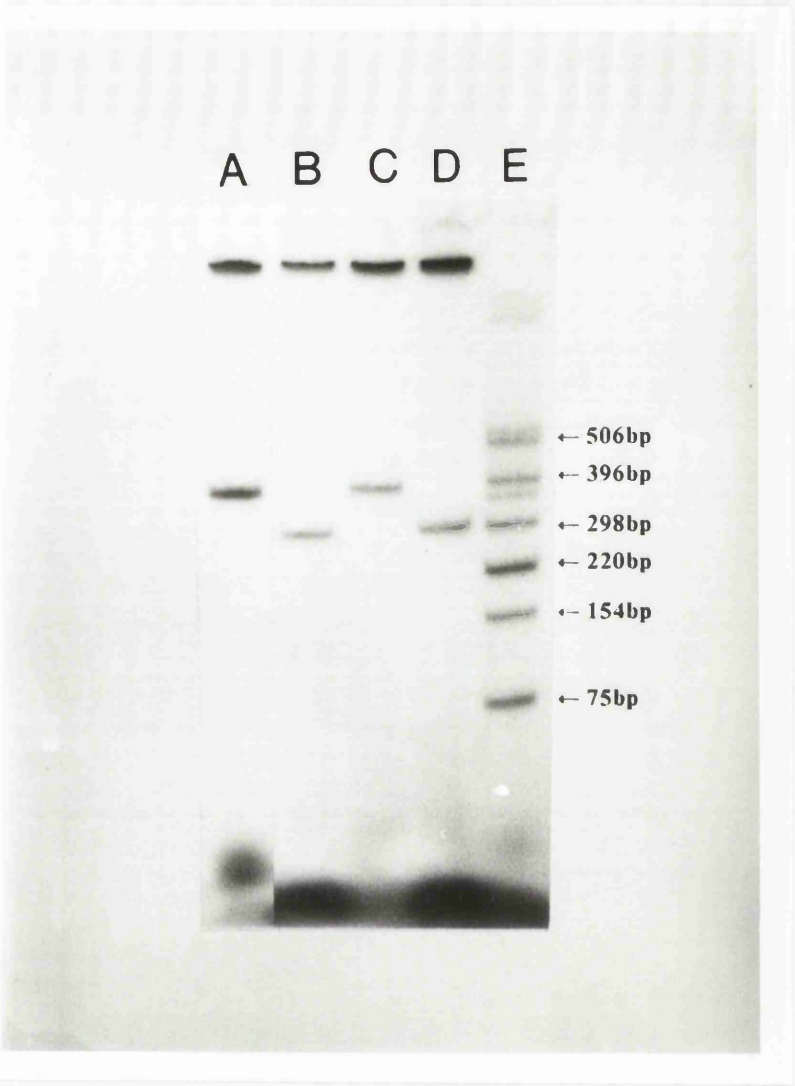


Figure 33. Autoradiograph of ^{32}P-labelled *HindIII/XbaI* fragments from plasmids containing the deletion within the 5'-UTR of the guanylyltransferase gene. The plasmids containing the deletion within the 5'-UTR of the guanylyltransferase, $pSV2_{DV1}$ (lane B) and $pSV2_{AD1}$ (lane D) along with the parental plasmids $pSV2_{GT}$ (lane A) and $pNUC_{AS1}$ (lane C) were digested with *HinDIII* and *XbaI*, the fragments labelled with ^{32}P-labelled dTTP by Klenow addition, and run on a BAC gel. ^{32}P-labelled molecular weight markers (*HinFI* digested pBR322) were present in lane E.

initiation site, so in an attempt to increase the level of guanylyltransferase expressed I decided to delete the ORF immediately upstream of the guanylyltransferase gene.

The 102bp region of DNA between the *HinDIII* site and the correct translation initiation site contains no unique restriction sites and so this region was deleted by PCR (polymerase chain reaction). Two oligonucleotide primers were obtained, one of which corresponded to a 25bp sequence spanning the authentic translation initiation codon, and the other a 22bp sequence complementary to a region just downstream of the unique *XbaI* site located within the coding region. The upstream oligonucleotide contained at its 5'-terminus the sequence necessary for formation of a new *HinDIII* site during amplification [see Figure 32]. The 290bp region between the two oligonucleotides was amplified and the PCR product digested with *HinDIII* and *XbaI*. The fragment was gel purified on a BAC gel, eluted and inserted between the *HinDIII\XbaI* sites of pSV2gt and pSV2gt-AN. The resulting recombinants were screened by digestion with *HinDIII* and *XbaI*, end-labelled and run on a BAC gel. Autoradiography revealed the expected reduction in the size of the small *HinDIII/XbaI* fragment from 363bp seen in pSV2gt (lane A) and pSV2gt-AN (lane C) to 280bp [See Figure 33] indicating that the upstream region of the guanyltransferase gene including the short open reading frame had been successfully deleted. The plasmids were designated pSV2gt-DV (lane B), for the native form of the enzyme, and pSV2gt-AD (lane D) for the nuclear targetted form.

Vero cells were injected with pSV2gt-DV (0.1mg/ml) and incubated overnight before staining with anti-GTase antiserum. Cells injected with this deleted form of the gene [Figure 34 c & d] appeared to express higher levels of guanylyltransferase than those injected with pSV2gt [Figure 34a]; the nuclei of cells injected with pSV2gt-DV showing the punctate nuclear staining observed in cells injected with pSV2gt and additionally diffuse nuclear staining. The punctate staining of cells injected with pSV2gt suggested that the expressed protein was associated with a nuclear structure, and the diffuse pattern of staining achieved by injection with pSV2gt-DV suggests that the protein is expressed at sufficient levels to saturate all these sites. Similar levels of guanylyltransferase expression and the same staining

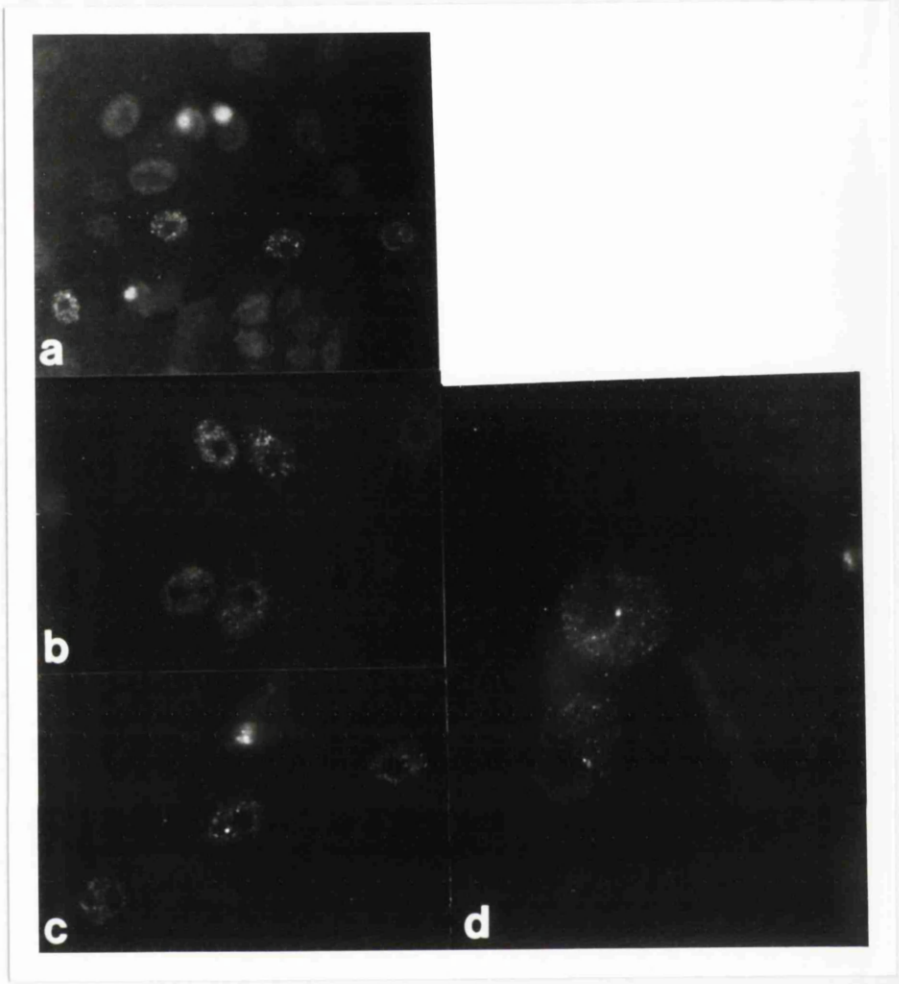


Figure 34. Anti-GTase staining of Vero cells injected with guanylyltransferase expression vectors lacking the ORF upstream of the guanylyltransferase translation initiation site. Vero cells were injected with $pSV2_{GT}$ (a), $pSV2_{AD1}$ (b) or $pSV2_{DV1}$ (c and d) at a concentration of 0.1mg/ml. The cells were stained following fixation and permeabilisation, using the anti-GTase antiserum and FITC-conjugated anti rabbit Ig antibodies (see Materials and Methods).

pattern was observed in Vero cells injected into the nucleus with pSV2gt-AD (Figure 34b) at a concentration of 0.1mg/ml. Despite this increase in levels of expression, the overall level of guanylyltransferase protein expressed was still lower than observed for other proteins expressed from comparableexpression vectors.

Cells were co-injected with the plasmids pAR3132 or pAR3126, pSV2gt-DV or pSV2gt-AD, and pCA1.3. Following injection, the cells were incubated overnight prior to staining for T7pol and CAT proteins. The results of these injections (Shown in Table 10) revealed no expression of CAT protein except in control injections containing pRSVcat. Therefore, although the guanylyltransferase was expressed at enhanced levels, this still was insufficient to overcome the block to T7pol-mediated expression of the CAT gene at the protein level.

DNA injected in nucleus	Stained for: T7pol	CAT
pAR3132 + pSV2gt-DV + pCA1.3	34/87	0/87
pAR3132 + pSV2gt-DV + pCA3.1	65/112	0/112
pAR3132 + pSV2gt-AD + pCA1.3	44/80	0/80
pAR3132 + pSV2gt-AD + pCA3.1	73/138	0/138
pAR3132 + pRSVcat	34/76	34/76

Table 10 Cell numbers staining for T7pol and CAT proteins following co-injection of a T7pol expression vector along with a ϕ10cat construct and the guanylyltransferase expression vector pSV2gt-DV or pSV2gt-AD (nuclear targetted). Cells were injected with the plasmids (0.1mg/ml/plasmid) incubated overnight and stained for T7pol and CAT proteins by indirect-immunofluorescence (as described in Materials and Methods). The results shown are representative of two separate repetitions of these injections.

5.8 Discussion.

In this Chapter I described the production of an antiserum against the vaccinia virus guanylyltransferase, and its use to monitor expression of a cloned guanylyltransferase gene in mammalian cells.

5.8a How Does the Cloned Guanylyltransferase Access the Nucleus When Expressed in Uninfected Cells ?

In vaccinia infected cells, the guanylyltransferase is localised to the cytoplasm. When expressed, in the absence of viral infection, the protein accumulates in the nucleus despite the large size of the polypeptide and the lack of a known nuclear location signal. How, therefore, does the protein gain access to the nucleus?

Two DNA-dependent RNA polymerase enzymes have been purified from rabbit poxvirus (RPV) virions, a virus closely related to vaccinia virus. One (137kDa) is encoded in the RPV genome, the second has a molecular weight of 170kDa (Morrison & Moyer, 1986). The 170kDa protein has been shown by Western blotting using monoclonal antibodies raised against either purified RPV polymerase, or against the largest subunit (~215kDa) of *Drosophila* pol*II*, to be a component of the RK (rabbit kidney) cell pol*II* complex (equivalent to the ~220kDa subunit of pol*II* in HeLa cells). Antibodies directed against the usually nuclear 170kDa protein show that during infection with RPV the protein becomes associated with the "viriosomes", the cytoplasmic sites of viral replication, protein synthesis and packaging.

Vaccinia virus is closely related to RVP and a similar situation, in which the cellular pol*II* is "hi-jacked" by the infecting virus, may exist in vaccinia infected cells. Staining of vaccinia infected Vero cells with antiserum directed against the guanylyltransferase/ß-gal fusion protein showed staining consistent with localisation of vaccinia guanylyltransferase at

the viriosomes (Figures 28 & 29; Morrison & Moyer, 1986). Association of the vaccinia guanylyltransferase enzyme with the viral RNA polymerase and been suggested (Broyles & Moss, 1987) because the two enzymes co-purify. If in vaccinia virus-infected cells, as in RPV infected cells, the cellular pol*II* is sequestered in the viriosomes, the requirement for close coupling of transcription initiation and 5'-capping would suggest that the vaccinia guanylyltransferase will be associated, either physically or geographically, with the sequestered cellular polymerase subunits as well as those of viral origin. Use of monoclonal antibodies raised against the purified RPV polymerases and the largest subunit of *Drosophila* pol*II* has shown conservation of epitopes between the RNA polymerase species, and analysis of coding regions for a variety of eucaryotic pol*II* species also suggests that polymerase molecules from different sources may be structurally related, further suggesting that the vaccinia guanylyltransferase and cellular pol*II* subunit could interact directly. In uninfected cells expressing the cloned vaccinia guanylyltransferase, association between the expressed guanylyltransferase and the pol*II* subunit may also be possible, but unlike the situation in vaccinia-infected cells where the pol*II* may be prevented from entering the nucleus by sequestration into the viriosome structures, in uninfected cells the pol*II* subunits are free to enter the nucleus unhindered. Association of the expressed guanylyltransferase and the pol*II* subunit in the cytoplasm of uninfected cells would result in entry of the guanylyltransferase into the nucleus "on the back" of newly synthesised pol*II* molecules. This phenomenon of nuclear entry by association has been observed for antibodies directed against steroid receptors, which become nuclear located along with the receptor molecules when steroid hormone binds to the receptors.

The possibility that the guanylyltransferase may be associated with the polymerase, thus restricting the enzyme to the site of action of pol*II* is appealing since capping activity is essential to mRNA stability and this is a better strategy to achieve capping of all mRNAs rather than relying on random encounters. Association between the vaccinia guanylyltransferase and the large subunit of pol*II* from Vero (and other mammalian cells) may be possible given the extensive homology at the DNA and primary protein sequence levels between RNA polymerase species from eucaryotic sources, and from the close

immunological relationships between the protein species (Weeks *et al.*, 1982; Allison *et al.*, 1985; Ollis *et al.*, 1985). The apparent lack of coupling between guanylyltransferase and T7 RNA polymerase, despite the sequence similarity that exists between T7pol and eucaryotic RNA and DNA polymerase species, is probably due to the fact that the similarity is confined to regions thought to be responsible for DNA binding (Allison *et al.*, 1985). The hypothesis of guanylyltransferase/pol*II* association could be tested using antibodies directed against cellular pol*II* to precipitate protein from cells expressing the vaccinia guanylyltransferase and investigating by Western blot techniques whether guanylyltransferase is co-precipitated. Efforts to obtain monoclonal antibodies capable of cross-reacting with cellular and viral RNA polymerase species were unsuccessful, the cross-reacting antibodies Mab137-170 and Mab170 (Morrison & Moyer, 1986) no longer being available (Richard Moyer, personal communication).

5.8b Why is Guanylyltransferase Expressed at Low Levels in Uninfected Vero Cells ?

In experiments where pSV2gt was injected into the nuclei of Vero cells the overall level of guanylyltransferase expression was low when compared to the levels of T7pol and pyruvate kinase expressed from the same expression vectors. The low levels of expression from pSV2gt and pXm30gt resulted in part from the presence within the mRNA of the short ORF 5'- to the guanylyltransferase coding region as shown by the increase of expression when the ORF was deleted. According to the ribosome scanning model for eucaryotic translation initiation (Reviewed in Kozak, 1983), mRNA binds to the 43S translation initiation complex (comprised of the 40S ribosomal subunit, $tRNA^{met}$ and GTP-eIF2) the ribosome scans along the RNA until an initiation codon is encountered, when translation commences. In this model reinitiation at internal sites occurs with low efficiency, this event being dependent upon the intercistronic length (Kozak, 1987). In systems such as pSV2gt, where a mini-cistron exists upstream of the correct ATG, reinitiation is strongest when the termination codon associated with the mini-cistron is $\geq$ 79nt upstream of the second ATG codon. This is not the case for pSV2gt where termination codon and the second initiation

codons are separated by only 23nt. This suggests that the guanylyltransferase expression observed from pSV2gt and pXm30gt results from "leaky" initiation (read through of the first initiation codon encountered) and not from reinitiation by the ribosome (re-binding at an internal site). This may account for the low level of guanylyltransferase expression observed. This theory is further strengthened when the sequence environments surrounding the two initiation codons are examined. The sequence observed to result in optimal translation initiation has been determined to be ACCATGG, with the possibility that cytosine bases at positions -4 and -5 (relative to the A of the ATG initiation codon) also increase initiation efficiency (Kozak, 1986b). Mutations occurring within this sequence have been found to be capable of modulating the expression of pre-proinsulin over a 20-fold range. The base at position -3 has a dominant effect, translation becoming more sensitive to mutations at positions -1, -2, and +4 when the purine at -3 is substituted for a pyrimidine. It has been shown that single base substitutions around an upstream out of frame ATG codon affect the efficiency with which it acts as a barrier to initiation at downstream sites. In the case of pSV2gt the initiation site of the upstream mini-ORF has the environment, TAAATGA, a weak initiation site that is likely to be passed over by a 43S complex, the guanylyltransferase cistron possessing a stronger initiation site, GATATGG.

Such "leaky" scanning has been implicated in the regulation of translation of cellular mRNAs, where the 5'-proximal ATG codon lies in an unfavourable context and in the same reading-frame as the second ATG codon and ribosome initiates at both sites. This results in the appearance of "long" and "short" forms of the encoded protein, and since the N-terminal amino-acids may determine the cellular distribution and\or activity of the protein, this could be perceived as a regulatory mechanism (Kozak, 1986c). Certainly patterns of gene expression predicted by this model have been observed for a variety of genes including some onco-genes (Rosson & Reddy, 1986), hormone receptors (Merlino *et al.*, 1985) and growth factors (Derynck *et al.*, 1985). Ribosomal binding and initiation at internal ATG codons has been reported from *in vitro* studies on mRNA from VSV (Herman, 1986), poliovirus (Pelletier & Sonenberg, 19888, 1989), and EMCV (Jang *et al.*, 1988, 1989), rendering the mRNA functionally bi- or poly-cistronic.

When the upstream ORF was deleted, producing the plasmid pSV2gt-DV the level of expression of the guanylyltransferase increased as expected, the first initiation codon encountered by a scanning 43S translation initiation complex being that of the guanylyltransferase ORF. The levels of expression achieved, as observed by immunofluorescence, were still low compared to similar expression vectors utilising the same promoter, but encoding different proteins (pSV2cat, pAR3126, etc.). Alternative explanations must, therefore, be sought to explain this low level of guanylyltransferase expression. The level of expressed protein can be affected by the turnover rates of mRNA and protein, as well as the rates of transcription and translation initiation. The cellular enviroment encountered by the vaccinia guanylyltransferase in uninfected cells will be different to that in virally infected cells but it is unlikely that this will destabilise the protein. Increased turnover of message could conceivably account for the low level of expression at the protein level. Currently the factors regulating message stability, apart from the presence of a 5'-terminal cap-structure, are poorly defined. *Cis*-acting elements within the primary sequence of mRNAs are known to affect stability (reviewed by Cleveland, 1989), and this is an area of contemporary research activity.

The data presented in this thesis has shown that the introduction of a reporter gene under the transcriptional control of the T7 late promoter ϕ10 along with T7pol is insufficient to result in expression of the reporter gene at the protein level. My results along with those of other groups have suggested that the failure to produce translatable T7 transcripts lies in the failure of the T7 transcript to be 5'-capped by the cellular machinery. Although vaccinia virus infection of cells containing the T7pol and reporter gene overcomes this block, expression of the vaccinia guanylyltransferase in a virus-free system was unable to complement the T7pol activity and produce a translatable message.

6.1 Can T7pol-Mediated Protein Expression Be Supported In Mammalian Cells ?

I have shown in this thesis that microinjection of a ϕ10cat construct, together with a T7pol expression vector, into the nuclei of Vero cells is insufficient to result in the expression of CAT at the protein level, as determined by immunofluorescence staining. Similar injections involving the co-injection of a T7pol gene along with pRSVcat results in levels of CAT that are easily visualised by the same staining procedure (see Chapter 3. Figure 16). Similarly, transfection of Vero cells with the same plasmid constructs results in the expression of CAT only from the eucaryotic expression vector. These findings are in agreement with those of Deuschle *et al.* (1989) who used an analogous T3 RNA polymerase expression system.

In contrast, using mouse L cells stably transfected with both a gene encoding a nuclear targetted T7pol and a ϕ10cat construct, Lieber *et al.* (1989) reported T7pol-mediated expression at the protein level in the absence of any exogenous RNA modifying enzymes. Similar cell lines expressing a non-nuclear targetted form of T7pol did not show this expression, suggesting that nuclear targetting of the T7pol species was necessary for

expression from the integrated ϕ10 promoter (see Section 6.3). Their levels of expression, determined by assay for CAT enzymatic activity, were approximately 6-fold higher than the levels obtained from cell lines containing stably integrated copies of pRSVcat. The absence of an exogenous source of RNA modifying enzymes suggests that in the experiments of Lieber *et al.* (1989) either 5'-modification of the transcript was unnecessary and the T7 transcripts were being translated in a cap-independent manner, or that the T7 transcripts are being correctly modified by the cellular machinery. Both of these explanations are difficult to reconcile with the results presented in this Thesis and with those from other researchers. Injection of T7- and SP6- generated *in vitro* transcripts into the nuclei of mammalian cells and *Xenopus* oocytes has suggested that the 5'-cap structure is necessary for stability and translation of the injected message (Furuichi *et al.*, 1977; Lockard & Lane, 1978; Drummond *et al.*, 1985; this Thesis) and that the cellular capping machinery cannot use exogenous RNA as a substrate, presumably because of the normal close coupling of capping with transcription by pol*II*. Further to this, in *his*$^-$ yeast expressing T7pol and possessing an integrated copy of the *his*3 gene under the transcriptional control of ϕ10, transcription of *his*3 by T7pol occurs and the transcripts accumulate to high levels within the cell, however the cells remain phenotypically *his*$^-$ indicating that the transcripts are not translatable due to the presumed absence of a 5'-cap structure. Thus cap-independent translation of T7 transcripts appears to be unlikely to occur in mammalian cells. This conclusion is supported by the results of Fuerst *et al.* (1986,1987,1989) who have shown that although T7 transcripts generated by their recombinant vaccinia virus/T7pol expression system can constitute up to 30% of total cytoplasmic RNA (See section 6.3), the transcripts are translated with low efficiency, and this is thought to be related to the relatively low efficiency of the capping reaction in their system resulting from the presence of a stem-loop structure (see Figure 3) at the 5'-terminus of the T7 transcripts. Cap-independent translation of a cellular mRNA has been reported, but this occurs in poliovirus infected cells and does not appear to be a general phenomenon (Sarnow, 1989).

What then, can be the basis for the discrepancy between the results of Lieber *et al.* (1989) and those of others including myself? The ϕ10cat construct (pG2.2) transfected by

Lieber and his colleagues into the murine cell line was a pGEM2 based plasmid, similar to pT7cat except that it lacks the SV40 small-t intron sequences. Since this is a pGEMINI based vector the 5'-conformation of transcripts will be similar to those from pT7cat. Transcripts generated from both these plasmids will lack a 5'-terminal stem-loop structure (see Figure 3, Chapter 1), also absent from the *his*3 transcripts in the yeast system of Chen *et al.* (1987), and these transcripts should, therefore, be equally likely to be capped by the cellular guanylyltransferase given the similiarity of structure at the 5'-terminus. Therefore the difference in patterns of expression between the three systems is unlikely to arise from differential 5'-processing of the transcripts.

Another possibly significant difference in the structure of the CAT reporter plasmids used by Lieber *et al.* (1989) and myself was that my constructs but not those of Lieber *et al.*, possess the SV40 small-t intron. The absence of a splice site may be an important factor in the appearance of CAT at the protein level, the presence of different sequences in regions distal to the cap site of the transcript possibly affecting 5'-processing. The presence of an intervening sequence within a T7pol generated transcript is likely to result in association of that message with snRNAs for splicing. Such an association may sequester the mRNA into a sub-nuclear compartment, either physical or functional, which prevents the cellular guanylyltransferase from capping the RNA. The absence of any intervening sequence within the transcript may result in the RNA entering an alternative pool of nuclear RNA that is available to the cellular guanylyltransferase for capping to occur. Such segregation of RNAs within the nucleus might explain how protein expression was obtained using a CAT construct lacking an intervening sequence. This explanation however seems unlikely for several reasons - in the yeast system of Chen *et al.* (1987) the *his*3 transcripts also lacked introns and although accumulation of the RNA occurred, no translation of the message resulted; the majority of hnRNA remains uncapped; and the apparent close coupling of 5'-capping to transcription suggests that capping is restricted to the nascent pol*II* transcripts.

The bulk of the evidence from my own work and from other researchers shows that expression mediated by a bacteriophage RNA polymerase cannot be sustained in mammalian

cells without viral intervention. The findings of Lieber and his colleagues directly contradict this view; this is both surprising and difficult to account for. This is the only claim so far made for protein expression in a eucaryotic cell mediated by a procaryotic RNA polymerase in the absence of viral cooperation, and currently I am unable to provide a satisfactory explanation for this discrepancy between the two sets of findings. It will be interesting to see if the results of Lieber *et al.* can be upheld by other laboratories.

6.2 Is Nuclear Targetting of the T7pol Molecule Necessary ?

Inclusion of an NLS in the primary sequence allows accumulation of T7pol in the nucleus of mammalian cells, wheras the native protein is largely excluded from the nucleus (Dunn *et al.*, 1988, Lieber *et al.*, 1989, this Thesis). It remains uncertain whether it is desirable, or indeed necessary for function of the T7pol species, to target the T7pol protein in this way. cT7pol has been shown to be capable of transcribing an integrated gene in yeast (Chen *et al.*, 1987). This suggests that the inclusion of an NLS within the primary sequence of the T7pol protein might prove to be unnecessary for transcription from T7 promoters integrated into the chromosomes of mammalian cells, although Lieber and his co-workers have reported that in their system an NLS is necessary for transcription by T7pol.

The bacterial protein *lacI* has been shown to be functionally active, in the absence of an NLS, in regulating expression from SV40 early promoters in plasmids and integrated into the genome of CV-1 cells (Brown *et al.* 1987; Figge *et al.*, 1988). This is most likely because of the relatively small size of the *lacI* protein (38.6kDa), which like the 23kDa CAT protein (see Figure 16, Chapter 3), may be small enough to diffuse slowly through the nuclear pores.

The desirability of targetting the polymerase molecule should be considered with respect to several factors. In bacterial cells the over expression of T7pol in the presence of a late T7 promoter can be lethal (Tabor & Richardson, 1985; Studier & Moffat, 1986), although this currently does not appear to be a problem in mammalian cells (see Table 4.

Chapter 3; Lieber *et al.*, 1989). Targetting of the protein may, however, overcome several problems encountered in the use of T7pol in yeast cells. A slowing in the growth rate of yeast cells was observed when cT7pol levels reached 4% of the total cellular protein (Chen et al. 1987), although it is uncertain whether this reflects an inherent toxicity of the T7pol to the cells, or merely results from the the diversion of a high proportion of metabolic resources to the production of this one protein. High levels of T7pol expression are required in the yeast cells since the entry of the T7pol is dependent upon leakage through the nuclear membrane. Inclusion of a nuclear location signal in the polymerase effectively confines the protein to its site of action. As a result of this it should not be necesary to express as high levels of nT7pol as cT7pol in order to achieve a useful concentration of the enzyme within the nucleus. This would reduce both the metabolic load on the cell and the liklihood of toxicity problems.

6.3 Potential Significance of Secondary Strucrure At The 5'-End Of T7pol Transcripts.

The low expression levels of the cloned guanylyltransferase and the apparent association of the guanylyltransferase with a cellular structure which results in nuclear accumulation of the protein may explain its apparent failure to overcome cap T7 transcripts. There is a third factor which may play a role in the failure of the vaccinia enzyme to overcome the block to translation of the T7 transcripts. Fuerst and Moss (1989) report that in the T7/vaccinia virus expression system, correctly initiated T7 transcripts were generated and the levels of these transcripts reached 30% of total cytoplasmic RNA late in infection; However, the amount of ß-gal translation product derived from these transcripts was much less than expected for such a high accumulation of mRNA. This disparity between levels of RNA and expressed protein was thought to be due to inefficient capping of T7 transcripts by vaccinia virus guanylyltransferase, probably due to the formation of a stem-loop structure at the 5'-terminus of the T7 transcripts (Rosenberg *et al.*, 1987; see Figure 3, Chapter 2). Fuerst and Moss (1989) created constructs that lacked the capacity to form the stem-loop structure; absence of 5' secondary structure resulted in increased capping of the RNA, but

despite this, expression of ß-gal was even lower than before. Fuerst and Moss (1989) suggested that this indicated that the RNA was rendered more susceptible to exonuclease degradation from the 5'-end; in the absence of close coupling btween transcription initiation and capping, this probably results in capping of partially degraded transcripts. Investigation of the 5'-sequence of CAT transcripts generated from my ϕ10cat constructs shows that the 5' stem-loop structure is not formed because the second group of bases in the split palindrome have been deleted (Figure 3, Chapter 2). This shows that the apparent failure to obtain 5'-capping of the T7 transcripts *in vivo* by the cloned vaccinia guanylyltransferase enzyme is not a result of the 5'-conformation of the T7-generated transcripts inhibiting addition of the cap. However, the apparent increase in transcript stability conferred by the presence of the 5' stem-loop (Fuerst and Moss, 1989) suggests that the presence of this feature within the RNA transcript may be worth further investigation for the stabilisation of transcripts in order to allow capping to occur on full length message, or for the stabilisation of uncapped antisense message (see section 6.6).

6.4 5-'Cap-independent Translation In Mammalian Cells.

Thus far it has been demonstrated that the cellular components involved in mRNA maturation are unable to modify T7 transcripts such that these species can be translated at a eucaryotic ribosome *in vivo.* Also it has been shown that expression of the vaccinia guanylyltransferase protein in Vero cells is unable to overcome this block in the translational process. A question that needs to be addressed is whether the presence of a 5'-cap structure is an absolute requirement for translation because although most eucaryotic mRNAs are capped it has been possible to obtain accurate translation of uncapped procaryotic mRNAs in *in vitro* translation systems (Anderson *et al.*, 1976) and the mRNAs generated by some viruses do not possess a conventional 5'-cap structure. The best characterised of these viruses are the picornaviruses (*Picornaviridae*) which includes poliovirus and encephalomyocarditis virus (EMCV). Viruses of this family are single stranded RNA viruses, of plus strand polarity. The genome size is on average 7,500nt long and encodes a single polyprotein, which

is proteolytically processed to produce the mature capsid and non-capsid proteins. The genomic RNA is not conventionally capped dispite acting as an mRNA and as template for virus replication; instead the 5'-terminus is covalently linked to a virally encoded oligopeptide (VP_g). The mRNAs produced are polyadenylated and characterised by the absence of a cap structure or VP_g cap protein, dispite being translationally avtive, and an unusually long 5'-UTR, of between 650-1,300nt. Using the mRNA of Satellite Tobacco Mosaic Virus (STMV) it has been shown that the translational efficiency of the species in *in vitro* translation systems is unaffected by the addition of a ^{7}mGpppG cap (Smith & Clark, 1979). The clue to the unusual translation initiation mechanism employed by these viruses, where ribosome binding occurs at an internal site within the mRNA (Bienkowska-Szewczyk & Ehrenfeld, 1988; Pelletier and Sonenberg, 1989; Jang *et al.*, 1989), comes fron two sources. Firstly it has been shown that hybridisation of oligonucleotides to the 5'-end of the 5'-UTR did not inhibit translation of EMCV mRNA, as would be predicted by the scanning model of translation initiation (Shih *et al.*, 1987). Also unlike cellular mRNAs where translation initiates at the ATG codon most proximal to the 5'-terminus, in the case of EMCV there are 10 AUG codons in the 888nt 5'-UTR preceeding the AUG codon at which translation initiates, and 8 AUGs prior to the translation initiation site in the 743nt 5'-UTR of poliovirus. These mRNA species appear to be stabilised by the presence of a stem-loop structure close to the 5'-terminus (Larsen *et al.*, 1981; Currey *et al.*, 1986), the importance of the loop being shown by the deletion of a single base at the base of the hairpin, which resulted in a temperature-sensitive mutant (Racaniello & Meriam, 1986).

The feasibility of using these 5'-UTR sequences to stabilise T7 transcripts and increase the translational efficiency of this mRNA in mammalian cells, has been demonstrated in the vaccinia\T7pol hybrid expression system (Elroy-Stein *et al.*, 1989). Inclusion of the EMCV 5'-UTR upstream of the CAT gene resulted in increased production of CAT protein such that it represented >10% of total cellular protein at the end of infection, compared to <2% of total protein when no EMCV UTR was present in the mRNA. Although useful in the vaccinia based expression system it is uncertain whether the same effects will be observed in virus-free systems. All viruses so far characterised that generate uncapped mRNAs have a

cytoplasmic life-cycle and so although facilitating translation, the presence of the EMCV 5'-UTR or a similar sequence may not stabilise mRNA synthesised within the nucleus.

Aside from their unconventional mechanism for translation initiation, the picornaviruses also utilise a diverse range of mechanisms which allow virally encoded mRNAs to compete effectively against cellular message for ribosome binding and thus to be translated preferentially. Infection of a cell with poliovirus results in the shutdown of translation of cellular mRNAs due the virally induced proteolytic cleavage of p220 from the cap-binding complex (see Chapter 1, 1.6). This effectively prevents cap-dependent translation while allowing the uncapped poliovirus mRNA to be translated (Schneider & Shenk, 1987). mRNAs from EMCV and mengovirus (Abreu & Lucas-Leonard, 1976; Hackett *et al.*, 1978; Jen *et al.*, 1978; Svitkin *et al.*, 1978) have been shown to initiate translation more efficiently than cellular messages, mengovirus mRNA initiating 35 times more efficiently than ß-globin mRNA. Infection of cells by virus results in an alteration of plasma membrane permeability which alters the Na^+/K^+ ratios within the cell. Translation of cellular mRNA is inhibited by high sodium concentrations, in contrast to several viral RNA species where translation is enhanced. Although EMCV 5'-leader sequences were shown to be effective at increasing translation of T7 transcripts (Elroy-Stein *et al.*, 1989), this took place in a cellular environment altered by viral infection. The effect of the EMCV 5'-leader sequence on mRNA translational competitivness in the absence of viral infection needs to be determined in order to ascertain whether this approach would be useful in a virus-free expression system.

A 200nt 5'-UTR (the tripartite leader sequence) found on adenovirus late mRNAs possesses extensive secondary structure (predicted by Zucker & Stiegler, 1981) and has been shown to eliminate the requirement for an active cap-binding complex for translation of these RNAs during viral infection (Dolph *et al.*, 1988). As a result, translation of adenovirus mRNAs is resistant to poliovirus super-infection of the cells (Castrillo and Carrasco, 1987). Use of this leader sequence might overcome the block to translation of T7 transcripts, but adenovirus mRNAs also possess a 5'-cap structure, and so the presence of the tripartite leader alone may be insufficient to stabilise the message in mammalian cells.

6.5 Is A 5'-Cap Required For Antisense Inhibition Of Gene Expression ?

This Thesis has concentrated on obtaining T7pol-mediated expression at the protein level but it is uncertain whether, for antisense RNA inhibition of gene expression, the antisense RNA species requires a 5'-cap. If neither cap nor 5'-leader sequence are required for antisense inhibition, inhibition of gene expression mediated by T7pol transcripts might still be possible. Some suggestion that this might be possible comes from the yeast system of Chen and his colleagues where T7 transcripts accumulated even though they were not translated. The sequence requirements and mechanism of antisense inhibition are as yet uncertain. The mode of delivery of the antisense message, either endogenously expressed within the cell nucleus or injected into the cytoplasm, appears to be important for the mechanism of inhibition. Expression of an antisense TK RNA expressed from a plasmid in TK^+ cells altered the distribution of sense mRNA (Kim and Wold, 1985). In the absence of the expressed antisense message the majority of TK mRNA was observed in the cytoplasm but when antisense RNA was co-expressed most of the sense mRNA was found within the the nucleus. This altered distribution of message appears to be specific to the mRNA; with only 40% of the antisense RNA was retained within the nucleus while the remaining 60% was present in the cytoplasm. Thus the presence of the antisense message appears to inhibit the transport of the complementary mRNA from the nucleus by an as yet undetermined mechanism. Direct injection of antisense message into the cytoplasm of *Xenopus* oocytes results in duplex formation between sense and antisense RNAs (Rebagliati & Melton, 1987, Bass & Weintraub, 1987) and it is likely that this prevents translation and promotes degradation of the mRNA. The fact that at least two distinct mechanisms have been identified for antisense inhibition, suggests that structural design considerations for the antisense transcript will depend upon the proposed site of action. Since *in vivo* the primary site of action is likely to be the nucleus, stability within the nucleus will probably be a major consideration.

The optimal length of an antisense species for efficient inhibition is unknown. Inhibition of HSV-TK expression in mouse L cells was achieved using 1,364nt and 423nt

species (Izant and Weintraub, 1984), and expression of the chicken TK gene has been inhibited using 52nt from the 5'-UTR (Izant and Weintraub, 1985). Using antisense *env* message, generated from a transfected plasmid source, Chang and Stoltzfus (1987) have shown that 3'-terminal non-coding sequences and 5'-end regions are equally efficient at inhibiting RSV replication, although inhibition did show length dependence when sequences closer to the 3'-end are used - longer RNA species being required for optimal expression inhibition. This contrasts with results from injection studies in *Xenopus* oocytes (Melton, 1984) and from studies in *E.coli* (Hirashima *et al*, 1986) which showed short RNA species complementary to the 5'-end of the mRNA to be most efficient at inhibiting gene expression. This apparent anomaly probably reflects the involvment of more than one inhibitory mechanism in the action of antisense RNA.

6.6 Alternative Approaches for Obtaining High Level Expression in Mammalian Cells.

The basis of the T7pol expression system relies on the successful introduction of foreign transcription machinery into mammalian cells. As shown, these cells appear to be extremely resistant to such measures, permitting transcription, but not allowing appearance of the gene product at the protein level.

The cloned vaccinia guanylyltransferase enzyme appears to associate with a cellular component, possibly the largest subunit of pol*II*, which results in nuclear accumulation of the protein (see Chapter 5). If this is the case, given the similarities shown by the largest subunits of eucaryotic pol*II* species at the primary protein sequence (Allison *et al.*, 1985) it is likely that any attempted use of a guanylyltransferase species, cloned or endogenous, would result in association of the guanylyltransferase with the cellular polymerase in preference to association with T7pol, which would effectively make the guanylyltransferase unavailable for capping of T7 transcripts. This conclusion is further supported by the close immunological relationship that exists between the largest subunits of the pol*II* molecules of higher eucaryotes (Weeks *et al.*, 1982) which shows that certain epitopes and possibly contact

surfaces are conserved. In the absence of detailed biochemical data to determine the domains responsible for enzymatic activity and pol*II* association, there is at present little prospect of either preventing association of the guanylyltransferase with the cellular RNA polymerase or promoting association of the guanylyltransferase with T7pol.

An alternative might be to combine the T7pol and guanylyltransferase activities at the gene level. This would produce a large protein possessing both RNA polymerase and guanylyltransferase activities. In this way it might be possible to couple capping activity to the T7 transcription. It is however, unknown what effect this coupling would have on the three enzymatic activities (RNA polymerase, guanylyltransferase and 7-methyltransfrase).

Unless 5'-capping can be achieved for T7 transcripts *in vivo* it seems unlikely that a T7pol-based expression system will be useful for directing expression at the protein level. Accumulation of untranslatable T7 transcripts in the yeast system of Chen and his co-workers suggests that T7pol-mediated transcription may however be useful for antisense inhibition of gene expression.

An alternative approach that might result in expression at the protein level involves the use of virally encoded *trans*-activating factors and viral UPEs. In this system weak tissue-specific promoters could be used to drive the expression of a viral *trans* activating factor, which would in turn promote transcription of a gene under the control of responsive viral UPEs in cooperation with the cellular transcription machinery. For this system to function, the viral promoter would be required to be transcriptionally inactive in the absence of the specific *trans* activating factor. A candidate for this may be the *tat* gene from HIV-1, used in conjuction with the viral LTR. Micro-injection of a CAT reporter gene under the transcriptional control of the HIV-1 LTR into Vero cells results in no expression of the gene (Frankel & Pabo, 1988; Green & Lowenstein, 1988; C.Hodgkinson, unpublished data). If the gene for the *tat* protein is co-injected with the LTR-CAT construct, high levels of expression are observed in the injected cells. Such an approach has been successfully used to target expression of a reporter gene in the eyes of transgenic mice (Khillan *et al.*, 1988). Expression

of other *trans* activating factors such as the adenovirus E1a gene product in transgenic mice has resulted in transformation of some cell-types and this may place a limitation on the use of some viral factors (Phelphs *et al.*, 1988; Koike *et al.*, 1989).

6.7 Conclusions.

The use of T7 RNA polymerase to drive high level expression at the protein level, of a target gene within mammalian cells is blocked at some level, very likely at the level of 5'-end processing of the primary transcript. Attempts to overcome this block by the expression of the cloned vaccinia virus guanylyltransferase have so far proven unsuccessful. Antibodies raised against a guanylyltransferase/ß-gal fusion protein showed that the guanylyltransferase is excluded from the nucleus during viral infection but accumulates in the nuclear compartment, presumably in association with a cellular component, when the cloned gene is expressed in the absence of vaccinia infection. The guanylyltransferase may gain access to the nucleus by associating with the cellular RNA pol *II* and traversing the nuclear envelope "piggy-back" fashion. An association with RNA pol*II* or another cellular protein could also prevent the vaccinia guanylyltransferase from capping T7 transcripts, by sequestering the enzyme away from nascent transcripts.

The failure to achieve *in vivo* capping of the T7 transcripts so far prevents the use of this T7pol-based expression system for protein expression in mammalian cells. T7pol-based expression may however still be prove useful in generation of high levels of antisense transcripts *in vivo* for hybrid-arrest of gene expression.

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Appendix.

In this Appendix I shall be concerned with the main areas of critisism directed at the data contained in this Thesis and outline the experiments required to remove these critisisms.

Transcription By T7pol In Mammalian Cells.

Although there is evidence from other researchers that T7pol is transcriptionally active from a ϕ10 promoter in eucaryotic cells (see Section 3.7a), and I have shown indirectly that T7pol is active in vaccinia virus-infected Vero cells (see Chapter 4), I have not demonstrated that T7pol is capable of transcription from a ϕ10 promoter in Vero cells in the absence of vaccinia virus. This might be shown by extraction of RNA from cells co-transfected with a T7pol expression vector along with a ϕ10-CAT construct and subjecting this RNA to analysis by Northern blotting, S1 nuclease analysis or primer extension. The simplest method of analysis would be to probe Northern blots of RNA from transfected cells with the 1633bp *HinDIII\BamHI* fragment from pSV2cat radiolabelled to high specific activity.

Quantitation Of CAT Assays.

Throughout this Thesis results of CAT assays from lysates of transfected cells have been shown qualitatively. It is, however, desirable for the levels of CAT expression to be quantified so that the level of induction arising from the action of vaccinia virus on the differing plasmid backgrounds can be more directly compared and the level of any contribution arising from T7pol transcription determined.

Quantitation of the assays can be performed in two ways. Autoradiographs can be scanned by a densitometer to determine the percentage of chloramphenicol converted into

mono- and di-acetylated forms, or following autoradiography the regions of the TLC plate corresponding to spots on the autoradiograph can be scraped from the plate into scintillation fluid and counted directly.

Survival Following Microinjection.

Table 4 (Chapt 3) shows the percentage of cells co-injected with a T7po expression vector and a ϕ10cat construct that remained competent to express T7pol and not necessarily, as is claimed, the percentage of cells surviving microinjection. "Survival" was scored as the number of cells expressing T7pol but there is no way to determine whether non-expressing cells are killed by the injection procedure, or simply are not capable of express the T7pol protein. Also, expression of T7pol might compromise the ability of the cells to divide, so that although the cells express the injected gene, the cells are unable to replicate. This possibility might be tested by staining the cells 48 hours after injection to see if the number of cells expressing T7pol is greater than the number injected. The results might be difficult to interpret, however; because of systematic errors in the injection procedure, the number of productive injections cannot be determined, and it is uncertain whether sufficient levels of the episomal DNA would be retained in the daughter cells following cell division to allow for assay by immunofluorescence.

Expression Of Vaccinia Virus Guanylyltransferase.

Although there is some evidence to suggest that the antiserum raised against the ß-gal\guanylyltransferase fusion protein recognises the vaccinia guanylyltransferase (Chapter 5) two experiments need to be performed to confirm this. First, immunoprecipitation of labelled protein from vaccinia virus-infected Vero cells would show whether the antiserum is capable of recognising a protein of the correct molecular weight. Second, probing Western blots of the fusion protein with the whole antiserum and with antiserum from which the anti-

ß-gal component has been removed would show whether the antiserum is capable of recognising the whole fusion protein and also the guanylyltransferase component of the fusion protein.

The low level of expressed protein from the guanylyltransferase expression vectors remained low following the deletion of the upstream ORF from the message and may result from the instability of the guanylyltransferase mRNA (Chapter 5). This can be studied by Northern blotting of cells transfected with one of the expression vectors and probing for guanylyltransferase mRNA. If the mRNA is unstable, lower levels of message would be observed compared to those of other mRNAs produced from the same promoter.