Identification and Analysis of Alternative and Aberrant 
Forms of the Transcription Factor ATF1

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ABSTRACT

The CREB/ATF transcription factor family form dimers which regulate a diverse array of genes through the recognition of related promoter elements. The factors CREB, CREM and ATF1 are particularly homologous and define a sub-family within which heterodimerisation and alternative splicing generates further complexity and therefore increased transcriptional versatility.

Transcriptional responses involving CREB are mediated via direct interactions with additional transcription factors to confer cell or promoter-specific patterns of regulation. To clone CREB binding proteins, a cDNA expression library was screened with a radio-labelled CREB protein probe. This 'Far-Western' screen proved remarkably powerful and identified a novel alternatively spliced ATF1 isoform termed vATF1.

A chromosomal translocation associated with the tumour malignant melanoma of soft parts (MMSP) links the N-terminus of the Ewing's sarcoma oncogene (EWS) to the C-terminus (including the DNA binding domain) of ATF1. The resulting chimeric protein EWS-ATF1 is predicted to de-regulate genes containing ATF binding sites resulting in tumorigenesis. Consistent with this, exogenous expression of EWS-ATF1 in JEG3 cells, influences the activity of test promoters containing ATF binding sites. Specific effects range from strong activation to repression. Furthermore an identical pattern of test promoter activity is observed in two MMSP-derived cell lines, DTC1 and SU-CCS-1, which express endogenous EWS-ATF1.

The identification of promoters that are selectively active in MMSP cell lines enabled a drug targeting strategy to be tested in vitro. Sequence encoding Herpes Simplex Virus thymidine kinase, which converts ganciclovir into a cytotoxic agent, was linked to an EWS-ATF1 activatable promoter. As expected DTC1 cells incorporating this 'suicide gene' are rendered sensitive to ganciclovir. It is anticipated that this approach which exploits the transcriptional properties of EWS-ATF1 could form the basis for a selective gene therapy strategy for MMSP.
IF-

IF you can keep your head when all about you
Are losing theirs and blaming it on you;
If you can trust yourself when all men doubt you,
But make allowance for their doubting too;
If you can wait and not be tired by waiting,
Or being lied about, don't deal in lies,
Or being hated don't give way to hating,
And yet don't look too good, nor talk too wise.

If you can dream-and not make dreams your master;
If you can think-and not make thoughts your aim;
If you can meet with Triumph and Disaster
And treat those two imposters just the same;
If you can bear to hear the truth you've spoken
Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to broken,
And stoop and build 'em up with worn-out tools.

If you can make one heap of all your winnings
And risk it all on one turn of pitch-and-toss,
And lose, and start again at your beginnings
And never breath a word about your loss;
If you can force your heart and nerve and sinew
To serve your turn long after they are gone,
And so hold on when there is nothing in you
Except the Will which says to them : "Hold on !"

If you can talk with crowds and keep your virtue
Or walk with kings-nor lose the common touch,
If neither foes nor loving friends can hurt you,
If all men count with you, but none too much;
If you can fill the unforgiving minute
With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And-which is more-you'll be a Man, my son !

Rudyard Kipling
To Mum and Dad
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### COMMON ABBREVIATIONS

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATF1</td>
<td>activating transcription factor 1</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic leucine zipper domain</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3’-5’monophosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response-element-binding-protein</td>
</tr>
<tr>
<td>CREM</td>
<td>cAMP-response-element-modulator-protein</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>diaminotetra-acetic acid</td>
</tr>
<tr>
<td>EWS-ATF1</td>
<td>Ewing’s sarcoma-activating transcription factor 1</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethane sulphonic acid</td>
</tr>
<tr>
<td>KID</td>
<td>kinase inducible domain</td>
</tr>
<tr>
<td>MMSP</td>
<td>malignant melanoma of soft parts</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIC</td>
<td>pre-initiation complex</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP dependent protein kinase A</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>Tris</td>
<td>hydroxymethyl methyl amine</td>
</tr>
<tr>
<td>vATF1</td>
<td>variant activating transcription factor 1</td>
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Chapter One

GENERAL INTRODUCTION

1.1 Gene Regulation

The complexities of cellular growth, differentiation and cell survival in a changing environment depend upon the regulated production of appropriate functional proteins encoded from the repertoire of genes that constitute the cellular genome. In eukaryotes several stages intervene between the initial transcription event which generates the primary RNA transcript through to the production of messenger RNA (mRNA) and finally translation of this template into protein (for review see Latchman, 1990). For the vast majority of genes however expression is regulated primarily at the level of transcription. Consequently the transcription process has been and indeed remains the subject of intense research from which detailed insights regarding regulatory mechanisms are emerging. The key components that make up the eukaryotic transcriptional machinery and which function to make transcription accurate and rate regulated are described next.

1.2 RNA polymerases

Eukaryotic cells contain three distinct RNA polymerases referred to as I, II and III which each transcribe a different set of genes (for review see Latchman, 1991). Synthesis of the primary mRNA transcript from protein-coding genes is performed by RNA polymerase II and this process is the focus for the remainder of this introduction.

RNA polymerase II consists of 12 subunits which together comprise over 500 kDa. Some of the subunits are shared with RNA polymerases I and III (Woychik et al., 1990). A unique feature of the largest subunit of RNA polymerase II however is the presence of a C-terminal domain (CTD) which contains, depending upon the species, between 26 and 52 copies of a heptapeptide repeat bearing the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-
Ser. Intact RNA polymerase II isolated from cells exists in two forms: IIo, in which the CTD is highly phosphorylated and IIa, an unphosphorylated form. Various lines of evidence suggest that phosphorylation of the CTD stimulates the release of RNA polymerase II from the pre-initiation complex (PIC) (see section 1.4a) facilitating the transition to the RNA elongating form of the polymerase (Herschbach and Johnson, 1993 and references therein). The remaining subunits of RNA polymerase II (Young, 1991; Corden, 1993 and references therein) along with a host of additional proteins termed transcription factors, function in the assembly and regulation of the RNA polymerase II transcriptional machine. The inherent complexity of transcriptional regulation is underlined by a recent estimate suggesting that up to 10% of human genes encode transcription factors (Kingston and Green, 1994).

1.3 Promoter elements

Many transcription factors regulate specific genes through the recognition of DNA sequence elements present in the region upstream of the transcription start site termed the promoter. DNA sequence elements have been identified by DNA footprinting and mutagenesis studies and are described on the basis of function as either core or upstream elements.

Core promoter elements of which two types are recognised, bind general transcription factors (see section 1.4a) and in the presence of these proteins are capable of directing an accurate although very low and unregulated (basal) level of transcription in vitro (Ham et al., 1992; Weinmann, 1992 and references therein). First to be identified was the TATA box, an AT-rich sequence of consensus TATA$^A$/$T$ A$^A$/$T$, located approximately 30 nucleotides upstream of the transcription start site (+1). Although the promoters of many protein encoding genes contain the TATA box element a significant proportion, particularly those of housekeeping genes do not (for review see Dynan, 1983). Studies of these TATA-less promoters identified the Initiator element (Inr)
which encompasses the transcription start site and has the consensus sequence YAYTCYYY, where Y corresponds to a pyrimidine. Like the TATA box the Inr can direct the specific initiation of transcription and can be found as an alternative or in addition to the TATA box (for review see Weis and Reinberg, 1992). The way in which these core promoter elements interact with general transcription factors to direct basal transcription is discussed later (see section 1.4a).

In addition to core elements, protein coding genes frequently possess multiple upstream promoter elements generally of between 5-20 bp long. These elements are critical for regulating the transcriptional activity of the gene since they are targeted by specific DNA binding transcription factors termed activators or repressors depending on whether they function to increase or decrease transcriptional activity. Some of these elements are located relatively close to the transcriptional start site ('promoter-proximal' elements). Others termed enhancers or silencers (depending on whether they regulate the rate of transcription in a positive or negative manner) are located substantially further upstream (for reviews see Serfling et al., 1985 and Maniatis et al., 1987). Some upstream elements are found in the promoters of a very wide variety of genes and often contribute to constitutive expression. A classic example is the Sp1 box which has the consensus sequence GGGCGG and binds the transcription factor Sp1 which is present in all cell types (for review see Latchman, 1991). Another is the CCAAT box which can bind a number of different transcription factors including CCAAT box transcription factor/Nuclear factor 1 (CTF/NF1) and CCAAT box enhancer binding protein (C/EBP) which are expressed in a tissue and developmental specific manner (for review see Johnson and Mcknight, 1989). Numerous, less common upstream promoter elements however confer specialised types of signal-dependent transcriptional regulation for example in response to heat shock, hormones and growth factors (for reviews see Maniatis et al., 1987 and Latchman, 1991).
1.4 Transcription factors

Transcription factors are classified into at least three groups as follows.

a) General transcription factors

Eukaryotic RNA polymerase II alone is unable to accurately initiate transcription. For an accurate, low (basal) level of transcription a number of additional proteins termed general transcription factors (GTFs) are required. The GTFs were originally defined by purification schemes as fractions required to reconstitute transcription from core promoter elements \textit{in vitro} (for review see Weinmann, 1992) and include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and TFIJJ. Experiments to reconstitute basal transcription using purified and later recombinant factors has led to a detailed model of how GTFs interact to orchestrate the assembly of a stable PIC as well as insights into specific functions of individual GTF's (for reviews see Conaway and Conaway 1993; Drapkin et al., 1993; Zawel and Reinberg, 1993 and Buratowski, 1994).

Formation of the PIC involves the ordered step wise assembly of the GTFs together with RNA polymerase II as follows (also see Diagram 1). Firstly TFIID binds to the TATA element. TFIID is a large complex composed of the TATA-binding protein (TBP) and TBP associated factors (TAFs) and is the only GTF that contains a sequence specific DNA binding activity. For basal transcription TBP alone is sufficient for TATA element recognition and subsequent assembly of other GTFs. TBP is one of the most highly conserved proteins in eukaryotic evolution and structurally resembles a saddle made up of two halves. The inner surface of TBP interacts with the minor groove of the TATA element causing distortions in the DNA, whilst the outer surfaces interact with other transcription factors (for reviews see Hernandez, 1993; Klug, 1993 and Travers and Schwabe, 1993). For transcriptional regulation by upstream factors however the TFIID complex (TBP + TAFs) is essential (see section 1.4c). Binding of TFIID is facilitated by TFIIA which may counteract the
Diagram 1  Schematic representation of the assembly of a stable pre-initiation complex (PIC) on a TATA box containing promoter. For GTF details refer to text. Although steps are indicated with unidirectional arrows, as they are generally depicted, assembly occurs in the absence of ATP and each step is reversible. Adapted from Zawel and Reinberg, (1992).
effects of inhibitors that displace TFIID from the promoter (Auble and Hahn, 1993 and references therein). Next TFIIB interacts with the TBP-DNA complex and recruits an RNA polymerase II-TFIIF complex. Polymerase recruitment activity is carried out by the TFIIF subunit possibly via an interaction with TFIIB (Ha et al., 1993 and references therein). The factors recruited at this stage are sufficient, at least under under some conditions, to initiate transcription (Parvin and Sharpe, 1993). Formation of the complete PIC however involves recruitment of TFIIE and TFIIH. TFIIE facilitates the recruitment of TFIIH which converts the PIC into an elongation complex (an event known as promoter clearance) capable of efficient transcriptional elongation. Stimulation of promoter clearance by TFIIH depends on its inherent helicase and kinase activities which unwind DNA and phosphorylate the CTD of RNA polymerase II (see section 1.2) respectively (Serizawa et al., 1993; Guzder et al., 1994 and Drapkin and Reinberg, 1994 and references therein). Subsequent events involved in RNA elongation are less well defined but probably involve TFIIJ which is the last factor to enter into the PIC in addition to several other activities which have been found to influence elongation (for review see Krumm et al., 1993).

Promoters utilising an Inr (see section 1.3) rather than a TATA box may use alternative pathways for PIC assembly involving one of several initiator binding proteins that have been identified (for review see Weis and Reinberg, 1992).

b) Promoter specific transcription factors

These factors bind in a sequence specific manner to the regulatory upstream promoter elements of genes (see section 1.3) and are classified as either activators or repressors. In fact the majority function as activators.

Transcriptional activators are typically composed of discrete, functionally independent modules/domains which mediate DNA binding,
transcriptional activation and in some cases multimerisation and regulation. In addition to providing a convenient means of classification (see below), the modular structure has critical implications for gene regulation and is of particular relevance to this thesis. Firstly the modular structure of some factors is exploited via alternative splicing and/or alternative use of translation initiation codons to generate multiple, functionally disparate factors, from the same gene (see section 1.6, 1.9, 1.15 and Chapter 3). Secondly it allows certain tumour specific chromosomal translocations to generate chimeric transcription factors which behave aberrantly and subvert normal growth control mechanisms. Frequently the hybrid transforming protein retains the DNA-binding domain of the particular transcription factor but the regulatory activities are disrupted by the fusion partner (see section 1.17 and Chapters 4 and 5).

The modular structure of transcription factors has been revealed by 'domain-swapping' experiments which demonstrate that these discrete domains can be exchanged between proteins without loss of function. The initial demonstration involved the yeast transcriptional activator GAL4 (Brent and Ptashne, 1985). The DNA binding domain of GAL4 was replaced by that of LexA, a bacterial repressor. The hybrid protein remained an activator but was dependent on the LexA binding site. LexA itself was unable to activate transcription. Following the identification and sequence analysis of activation and DNA binding domains it became clear that they could be grouped into distinct families with common features as follows.

i Classes of activation domain

Activation domains are grouped primarily according to similarities in amino acid content (for review see Johnson et al., 1993).

Acidic activation domains ('acid blobs'), contain a high proportion of acidic residues and have been identified in a large number of transcriptional
activators. Amongst the most intensively studied are those present in the yeast transcription factors GAL4 (Ma and Ptashne, 1987) and GCN4 (Hope and Struhl, 1986) and in the Herpes Simplex Virus transactivator protein, VP16 ( Triezenberg et al., 1988).

Glutamine rich domains represent a second class of activator element, first identified in the mammalian transcription factor Sp1 (Courey and Tjian, 1988). Other glutamine rich activation sequences have subsequently been identified for example in the NF-YA subunit of the ubiquitous CCAAT box binding protein NF-Y (Li et al., 1992).

A third class of activating sequence characterised by a high frequency of proline residues, was discovered in CTF/NF1 (Mermod et al., 1989) and is also present in the lymphoid cell-specific activator Oct-2 (Gerster et al., 1990).

A less common activation domain, characterised as serine/threonine rich, has also been identified in Oct-2 (Tanaka and Herr, 1990) and within the immunoglobulin enhancer binding proteins ITF-1, ITF-2 (Henthorn et al., 1990) and TFE3 (Beckman et al., 1990).

Although the primary function of activation domains is to interact specifically with other components of the transcriptional apparatus thereby stimulating transcription (see section 1.5) the way in which such specificity is attained is not clear. Significantly with the exception of some potential α-helical regions, most activation domains have not been predicted to form specific secondary structures. Furthermore detailed mutagenesis studies suggest that the most important amino acid residues for activation are not necessarily the most predominant. In the case of acidic activation domains for instance whilst the net negative charge has been demonstrated to be a necessary feature for activation it is not the sole determinant of activation function. Thus for GAL4 (Gill and Ptashne, 1987) and VP16 (Cress and Triezenberg, 1991) not all missense mutations that decrease transcriptional activation correspond to acidic residues. Indeed for VP16 the single most critical residue for
transcriptional activation was identified as Phe\(^{442}\) (Cress and Triezenberg, 1991). This residue was proposed to make a vital hydrophobic contact either in tertiary folding interactions or through interactions with GTFs that mediate activation (see section 1.5). In agreement with this the mutation has been shown to be detrimental to the interaction between VP16 and TFIIB (Lin et al., 1991) and also TBP (Ingles et al., 1991) (see section 1.5). In a number of other cases the most important elements for activation are similarly found to correspond to bulky hydrophobic residues that are interspersed with the most preponderant amino acids within the domain (Gill et al., 1994 and references therein). The current view is that hydrophobic interactions drive the initial interaction of the activation domain with its target with specificity achieved by the periodicity of the cohesive elements within the activation domain. Upon interaction with its target the activation domain attains a specific three-dimensional structure associated with increased stability (for review see Tjian and Maniatis, 1994).

ii Classes of DNA binding domain

Several distinct groups of DNA binding domains have been identified on the basis of primary sequence relationships and three dimensional structures and allow classification of transcription factors into families (for reviews see Harrison et al., 1991; Krajewska, 1992; Pabo and Sauer, 1992; Wolberger, 1993). Most of the well characterised families use \(\alpha\)-helicities to contact DNA within the major groove with the majority of known transcription factors falling into four classes referred to as helix-turn-helix (HTH), zinc fingers, basic-region helix-loop-helix (bHLH) and basic-region leucine zipper (bZIP).

The HTH was the first DNA recognition motif identified and amongst eukaryotic transcription factors it most notably occurs in those containing a homeodomain (Scott et al., 1989). The zinc finger motif first identified within the GTF TFIIIA is present in a very large number of eukaryotic transcription
factors with most examples containing multiple zinc finger motifs (Struhl, 1989 and references therein).

The DNA binding domains of the remaining two groups are bipartite consisting of a motif required for dimerisation adjacent to a basic motif which directly contacts the DNA. Dimer formation mediated through the dimerisation motif is necessary for efficient DNA binding by the adjacent basic motif. Furthermore dimerisation can provide an important aspect to the regulation of these factors since in some cases homodimers and specific heterodimeric complexes can form and different complexes may possess distinct DNA binding and transcriptional properties (for discussion see Jones, 1990).

Most relevant to this thesis is the bZIP motif first proposed for the sequence-specific DNA binding dimeric protein C/EBP (Landschulz et al., 1988). As mentioned above the bZIP DNA binding domain contains two distinct subdomains: the leucine zipper which mediates dimerisation and a basic region which contacts the DNA. Leucine zippers are characterised by a heptad repeat of leucines over a region of 30-40 residues with a conserved repeat of hydrophobic residues occurring three residues to the N-terminal side of the leucines (Landschulz et al., 1988). This forms an α-helix where one side of the helix is composed predominantly of hydrophobic amino acids. Two interacting α-helices then dimerise in parallel with each α-helix over-twisting slightly, going from 3.6 to 3.5 amino acids/turn to form a coiled coil structure. This is stabilised through interactions between residues within the hydrophobic interface which pack in a regular 'knobs and holes' pattern (O’Shea et al., 1989; Oas et al., 1990). At specific positions flanking the dimerisation interface there are a large number of charged amino acids and it is thought that electrostatic interactions between these residues in opposing helices are critical determinants of dimerisation specificity (Krylov et al., 1994 and references therein). Although leucine zipper mediated dimerisation is required for efficient DNA binding, the basic domain is responsible for contacting DNA.
The basic region typically contains 30 residues and is arginine and lysine rich. Studies on GCN4 suggest that the basic region is relatively unstructured but adopts an α-helical conformation when bound to DNA with the two positively charged α-helices lying in the major groove (Weiss et al., 1990; Saudek et al., 1991a,b). Two closely related models for the complex have been proposed (Vinson et al., 1989; O'Neil et al., 1990). In the 'scissors grip' model, the α-helix from the basic region is kinked so that it follows the curve of the major groove (Vinson et al., 1989). In the 'induced helical fork' model, the α-helices are not kinked and basic regions form a single helix continuous with the leucine zipper helix and therefore extend away from the DNA (O'Neil et al., 1990).

Similarly to the bZIP motif, bHLH proteins have a basic region which contacts DNA and a neighbouring region that mediates dimer formation (Voronova and Baltimore, 1990). The dimerisation region is proposed to form an α-helix, a loop and a second α-helix and was first identified in MyoD and the immunoglobulin enhancer binding proteins E12 and E47 (Murre et al., 1989).

c) Coactivators

Coactivator proteins (also referred to as adaptors or mediators) are envisioned to bridge or stabilise interactions between sequence specific transcriptional activators and the GTFs. As such coactivators are operationally defined as factors required for activator function but that do not affect activator-independent basal transcriptional activity (see section 1.5).

The first coactivators identified were those in the TFIID complex. Initial studies revealed that whilst purified TBP was sufficient for basal transcription of core promoters in vitro, activation was not stimulated by sequence specific transcriptional activators, whereas partially purified TBP was. Subsequently the TFIID complex was found to be composed of TBP in association with at least eight TAFs (250, 150, 110, 80, 60, 40, 30α, 30β) held together by multiple
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TAF-TAF and TAF-TBP interactions (Chen et al., 1994 and references therein) and capable of reconstituting activation *in vitro* (Dynlacht et al., 1991; Tanese et al., 1991). With the exception of the tightly associated TAFs, most coactivators are defined primarily by function although some have been cloned including the yeast ADA2 gene (Berger et al., 1992).

1.5 Mechanisms of transcriptional activation

Eukaryotic activators stimulate transcription by increasing the formation rate of the PIC. As mentioned earlier (see Diagram 1) each step in this process is reversible and activators act at multiple rate limiting steps in this assembly pathway by increasing the rate of recruitment of a given GTF and/or enhancing the stability of the formed complex. This may occur directly through physical contacts and/or indirectly for example as a consequence of induced conformational changes in GTFs which facilitate binding of downstream factors.

Factors which serve as targets for interactions with activators have been identified by studies using affinity columns containing immobilised activator molecules (Zawel and Reinberg, 1993; Drapkin et al., 1993; Tjian and Maniatis 1994 and references therein). Amongst the first interactions to be identified were between the acidic activation domain of VP16 and TFIIB (Lin et al., 1991) and mutational analysis has demonstrated the importance of this interaction for transcriptional activation (Lin and Green, 1991; Roberts et al., 1993). All activators tested to date appear to require the TFIID complex (composed of TBP and the TAFs - see section 1.4a) for full activation *in vitro*. It was therefore
proposed that TAFs are coactivators, serving as functional links between transcriptional activation domains and the PIC. Consistent with this several activator-TAF interactions have been reported and shown to be important for transcriptional activation. These include interactions between the glutamine rich activation domains of Sp1 with TAFII110 (Hoey et al., 1993; Gill et al., 1994), the C-terminal activation domain of VP16 with TAFII40 (Goodrich et al., 1993) and the activation domain of NTF1 with both TAFII150 and TAFII60 (Chen et al., 1994). Direct evidence for the role of individual TAFs in the process of activation by specific transcriptional activators has come from experiments using partial TBP-TAF complexes (Chen et al., 1994 and references therein). Additional evidence suggests that TAFs may also mediate interactions between TFIID and other components of the basal complex (Choy and Green, 1993). In support of this TAFII40 has been shown to interact selectively with TFIIB (Goodrich, 1993). Significantly since TAFII40 and TFIIB interact independently with distinct regions of VP16 a three way complex involving these factors is possible (Goodrich, 1993). The ability of activators to contact more than one target which in turn mediate additional interactions within the PIC helps to explain the phenomenon of synergy (also termed cooperativity), whereby two activators stimulate transcription multiplicatively as opposed to additively (for review see Carey, 1991).

The role of activators in accelerating PIC assembly by recruiting GTFs and stabilising interactions within this complex is referred to as true activation. Additionally activators are thought to override the repressive effects of chromatin components, a process referred to as antirepression (Croston et al., 1991). These effects were originally overlooked because purified 'naked' DNA was used in experimental systems. This produced high levels of basal transcription and only relatively modest increases in response to activators compared to those observed in vivo. In contrast, with reconstituted chromatin templates basal transcription was repressed but was relieved by activators (for
reviews see Felsenfeld, 1992; Croston and Kadonaga, 1993). It is envisaged that the PIC competes for promoter occupancy with chromatin components, nucleosomes in particular. Activators influence this competition by promoting the assembly and stabilisation of the PIC and also by antagonising repressive chromatin components (see Diagram 2).

1.6 Transcriptional repressors

Although the majority of known transcription factors are activators, some function to inhibit transcription. Whilst many repressors act by neutralising the action of activators, some have an inherent negative effect and interfere directly with the general transcriptional machinery (for reviews see Goodbourn, 1990; Clarke and Docherty, 1993; Herschbach and Johnson, 1993 and Latchman, 1993). In the former case the repressor may exert its effect in a number of different ways as outlined below.

Some interfere with activator nuclear localization as exemplified by the IκB family of transcriptional inhibitors. These block the nuclear import of members of the Rel family of activators by masking the Rel nuclear localisation signal (Beg et al., 1992 and references therein).

Other repressors compete with activators for a common DNA binding site or bind an adjacent or overlapping sequence to that recognised by the activator preventing binding by steric hindrance. DNA binding sites for the Drosophila repressor protein Kruppel for example, often overlap binding sites for transcriptional activators (Zuo et al., 1991).

Alternatively a repressor may interact with a DNA bound activator and mask the activation domain, a phenomenon known as quenching or masking (Latchman, 1993). Both DNA binding and non-DNA binding factors may negatively regulate transcription in this manner. The c-myc gene for instance is repressed by myc-PRF which masks the activation domain of the transcription factor CF1 that occupies an adjacent promoter element (Kakkis et al., 1989). The
Diagram 2  Schematic representation of mechanisms of transcriptional activation. (A) True activation. Upstream activators recruit GTFs and stabilise interactions within the preinitiation complex (PIC). (B) Antirepression. Upstream activators antagonise repressive chromatin components, particularly nucleosomes, promoting assembly of the PIC. The net effect is the increased rate of PIC formation at the transcriptional start site (+1). Adapted from Kingston and Green, (1994).
yeast repressor GAL80 meanwhile does not itself bind DNA but interacts with the C-terminal region of the GAL4 activator masking the nearby acidic activation domain (Salmeron et al., 1990 and references therein).

As discussed earlier, many transcription factors bind DNA and activate transcription as dimers, the interaction being mediated through specialised dimerisation interfaces (see 1.4b ii). Some repressors work by competing for association with one of the activator subunits with two types of negative regulatory interaction observed. Firstly a negatively acting factor may form a dimeric complex which fails to bind DNA. The HLH antagonists Id (Benezra et al., 1990) and emc (Ellis et al., 1990; Garrell and Modolell, 1990) for example, are able to dimerise with other HLH factors but lack DNA binding basic regions. Secondly, the repressor may form a dimeric complex which is able to bind DNA, but lacks domains required for transcriptional activation. In this case the negative factor may compete for occupation of regulatory promoter elements and/or function by sequestering activators in transcriptionally inert heterodimeric complexes. Examples of such behaviour include members of the CREB/ATF family such as CREM (Foulkes et al., 1991a,b); the C/EBP-related factor LIP (Descombes and Schibler, 1991) and two members of the AP1 family, JunB (Chiu et al., 1989; Schutte et al., 1989) and FosB-S (Mumberg et al., 1991; Nakabeppu and Nathans, 1991; Yen et al., 1991).

Interestingly, many of these repressors are the products of genes which also encode transcriptional activators and are generated by exploiting the modular nature of transcription factors through alternative splicing or alternative use of translation initiation codons as discussed previously (see section 1.4b), (for reviews see Frankel and Kim; 1991; Foulkes and Sassone-Corsi, 1992). The generation of activators and repressors from the same transcript may be subject to developmental, tissue specific or signal-responsive regulation providing an important level of gene control.
1.7 Regulation of transcription factor activity

Due to the critical role of transcription factors in the control of gene expression it is imperative that the activities of these factors are modulated appropriately. Inappropriate gene expression is for example central to the process of tumourigenesis where mutation or over-expression of specific cellular genes (proto-oncogenes), results in their conversion into 'cancer causing' oncogenes (for review see Lewin, 1991). Regulation is achieved by controlling transcription factor synthesis so that it is made only when necessary or alternatively by regulating the activity of the pre-existing factor so that it is present in an active form only when required (for review see Latchman, 1993).

Regulation of transcription factor synthesis is most evident for factors which regulate expression of cell type-specific or developmentally regulated genes and represents a metabolically inexpensive control mechanism (for review see Latchman, 1991). MyoD for example is expressed only in muscle cells where it plays a critical role in regulating muscle-specific gene expression (Edmondson and Olson, 1993 and references therein). The regulated synthesis of a specific transcription factor may be achieved by any of the methods which are normally used to regulate the production of individual proteins such as RNA splicing, mRNA translation or indeed at the level of transcription (for review see Latchman, 1990).

Although metabolically more expensive, mechanisms which regulate the activity of a pre-existing transcription factor enable gene expression to be rapidly induced. Several mechanisms, although not necessarily mutually exclusive, are known which serve to regulate activator activity. These involve altering the subcellular localisation of the activator (for example from cytoplasm to nucleus) or else affecting the factors DNA binding or activation properties.

The simplest method is for an inducing ligand to bind the transcription factor resulting in a conformational change which activates the factor. For
example after binding copper the yeast transcription factor ACE1 undergoes a conformational change which allows it to bind regulatory sites in the metallothionein gene and activate transcription (Furst et al., 1988).

Activation of transcription factors is frequently achieved by protein modification and most commonly involves phosphorylation mediated by specific kinases within signal transduction pathways (for reviews see Hunter and Karin, 1992; Jackson 1992; Karin and Hunter, 1995). For example, during the cell cycle the yeast factor SWI5 is active only during G1. At other times it is phosphorylated at three sites near or within the basic nuclear localisation signal causing it to reside in the cytoplasm where it is inactive (Moll et al., 1991 and references therein). Meanwhile the DNA binding activity of c-Myb is decreased by casein kinase II (CKII) mediated phosphorylation (Luscher et al., 1991). There are a large number of other examples where phosphorylation regulates the activity of transcription factors, although in many of these cases the precise way in which this is effected remains unclear. One of the best characterised examples of regulation by phosphorylation involves the mammalian transcription factor CREB which mediates transcription in response to activation of the cAMP dependent protein kinase (PKA) signal transduction pathway. This is described in detail later (see section 1.10b, 1.11 and 1.12).

As described earlier, (see section 1.6) the activity of many activators is neutralised by the action of specific transcriptional repressor proteins. In many cases protein modification targets the repressor and disrupts the inhibitory protein-protein interaction. For example the Rel family of activators are sequestered in the cytoplasm due to interaction with the IκB family of repressors (see section 1.6). Phosphorylation of the repressor however disrupts this interaction allowing the activator to enter the nucleus (Ghosh and Baltimore, 1990).

1.8 Transcription factor families
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As discussed earlier transcriptional activators and repressors have been classified into families based on the structural motif used to mediate dimerisation and/or DNA binding (see section 1.4b ii). Within these families, transcription factors are further classified according to their recognition of similar DNA sequences.

Within the bZIP group of transcription factors present in mammalian cells several major families have been identified. These include the FOS/JUN family which respond to serum growth factors (Curran and Franza, 1988; Woodgett, 1990), redox potential (Abate et al., 1990) and photic stimuli (Kornhauser et al., 1992), the C/EBP family that are involved in liver specific transcription and IL6 signal transduction (Akira et al., 1990; Landschulz et al., 1989; Williams et al., 1991) and the TEF/DBP family which function in thyrotrophic cells of the embryonic pituitary (Drolet et al., 1991). Of central importance to this work is the CREB/ATF family which mediate transcriptional responses to a variety of cellular signalling pathways and viral transactivating proteins.

1.9 The CREB/ATF family

The CREB/ATF family of transcription factors specifically bind the core motif CGTCA, originally identified as the cAMP responsive element (CRE) present within the promoters of many cAMP inducible genes (Comb et al., 1986; Montminy et al., 1986; Andrisani et al., 1987; Jameson et al., 1987; Tsukada et al., 1987; Deutsch et al., 1988; Roesler et al., 1988; Knepel et al., 1990; Philippe and Missotten, 1990). Subsequently identical, or very closely related elements were identified as mediating transcriptional responses to other signals including Ca²⁺ (Sheng et al., 1990; Dash et al., 1991; Sheng et al., 1991), DNA damage (Kedar et al., 1991) and cytokine induction (Hooft van Huijsduijnen et al., 1992; Kaszubska et al., 1993). The motif was also identified in elements mediating transcriptional responses through viral transactivating proteins.
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These include several adenovirus early gene promoters transcriptionally activated by the viral E1A protein (Sassone-Corsi, 1988; Lee et al., 1989; Liu and Green, 1990) and elements in the human T-cell leukemia virus type 1 (HTLV-1) LTR which respond to the HTLV p40\textsuperscript{tax} transactivator (Fujisawa et al., 1989; Yoshimura et al., 1990).

Significantly in the context of different promoters this element may not be functionally equivalent. The adenovirus E4 promoter for example, contains five such elements and is strongly activated by E1A but only weakly inducible by cAMP. For the cellular VIP promoter however which contains two such elements, the reverse is true (Lee et al., 1989). Consequently these promoter elements are referred to more generally as ATF sites as opposed to CREs which are defined on the basis of function (see section 1.14).

Members of the CREB/ATF family were originally identified as DNA binding activities that interact specifically with ATF promoter elements and many were purified by DNA affinity chromatography (Hurst and Jones, 1987; Montminy and Bilezsikjian, 1987; Hai et al., 1988). To date eleven different mammalian genes are known to encode distinct members of this family. Whilst members share the same dimerisation and DNA binding motif (the bZIP domain) and are highly homologous in this region (see Diagram 3), they diverge in other regions.

Cloned members include CREB (cAMP Response Element Binding protein) (Hoeffler et al., 1988), CREM (cAMP Response Element Modulator (Foulkes et al., 1991a), ATF1 (Activating Transcription Factor 1), ATF2, ATF3, ATF4, ATF6 (Hai et al., 1989), TREB5 (Yoshimura et al., 1990), ATFa (Gaire et al., 1990), C/ATF (Vallejo et al., 1993) and B-ATF (Dorsey et al., 1995). In several cases the same cDNAs were isolated by different laboratories resulting in some confusion over nomenclature. Thus ATF1 is identical to TREB 36 (Yoshimura et al., 1990), as is ATF2 to CRE-BP1 (Maekawa et al., 1989), HB16 (Kara et al., 1990) to TREB 7 (Yoshimura et al., 1990) ATF3 to LRF1 (Hsu et al., 1991), ATF4 to
### Basic Domain

<table>
<thead>
<tr>
<th>CREB</th>
<th>AEEARKREVRLMKNEAARECRRKKEYVKCLLENQKTLIFELKALKDLYCHKSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATF1</td>
<td>TDDPQLKREI RLKMKEAARECRRKKEYVKCLLENQKTLIEELKTLKDLYSNKSV</td>
</tr>
<tr>
<td>CREM</td>
<td>AEEATRKRERLPLMKNEAARECRRKKEYVKCLLENQKTLIEELKALKDLYCHKAE</td>
</tr>
<tr>
<td>ATF2</td>
<td>NEDPDEKRKVRERNRAASRCQKWKVW QSIEEKKAEDLSSLNQLQSEVTURNEVAQLEKQLLAL</td>
</tr>
<tr>
<td>ATF3</td>
<td>APEEEDKRKMRERRKKKIAA KCRMNKKKKEKTEQ LKSESKNLKAQIEELKN EKLHNYLMLNLH</td>
</tr>
<tr>
<td>ATF4</td>
<td>KGEKLDKKKMEQNKRAATR YQKKAEQATEDGECKLEKKEALKKAEAI QYLKDLLEEV</td>
</tr>
<tr>
<td>TREB5</td>
<td>HLSPEKALRRLKNRVAADQTDARQK ARMSELEQDQVDELQK KLENQKIREKTHH VQVENQHF</td>
</tr>
<tr>
<td>ATF6</td>
<td>SDAVLRRQR M1KNEEASAQS RKKKEYMIGEARLKA ALEN SEQKKEKQR LQLEREYS VSLENRS</td>
</tr>
<tr>
<td>ATF-a</td>
<td>DEDPDERRQR FLENRRAASRCQKRKLWVS SLEKKAEE LTSQNIQLSNEVTURGNEVAQKQLLAL</td>
</tr>
<tr>
<td>C/ATF</td>
<td>KTEKLDKKKMEQNKTAATRYQKKRAEQEALTEDGECCKELEKKEALK KADAKEI QYLKDLLEEV</td>
</tr>
<tr>
<td>B-ATF</td>
<td>DSDDVRRVQRREKNI AAQKSRQRQTQKADYLHLESERLEKNAALKEIKQITEELKYFTS</td>
</tr>
</tbody>
</table>

#### Diagram 3 Amino acid sequence of bZIP domains of CREB/ATF proteins.

Within the basic domain basic residues are indicated in bold type and leucine residues constituting the leucine zipper are highlighted by boxes.
CREB-2 (Karpinski et al., 1992) and TREP 5 to hXBP1 (Liou et al., 1990). Additional CREB/ATF factors have been purified (Andrisani and Dixon, 1990) or at least identified as DNA binding activities (Merino et al., 1989; Tassios and La Thangue, 1990; Kwast-Welfield et al., 1993) so the size of the family is anticipated to be larger still. Thus the diversity of the promoters regulated by ATF sites is paralleled by the diversity of the CREB/ATF family itself.

Although all CREB/ATF factors are highly similar within the bZIP domain some share additional regions of similarity enabling further classification into subfamilies. Thus CREB, CREM and ATF1 share regions of high homology outside of the bZIP domain and constitute the immediate CREB family. Similarly ATF2 and ATFa are grouped because they are highly homologous in the first 100 N-terminal residues and a 13 amino acid stretch at the C-terminus is conserved.

Within the CREB/ATF family additional diversity is generated by alternative RNA splicing which effectively exploits the multiexonic, modular gene structure encoding these factors allowing the regulated expression of an even wider range of proteins. Alternative RNA splicing has been shown for ATF2 (Ivashkiv et al., 1990), ATFa (Gaire et al., 1990), ATF3 (Chen et al., 1994) and is particularly evident for CREB and CREM (see section 1.14c and 1.15).

DNA binding assays and immunoprecipitation studies indicate that intrafamily heterodimers may form, further increasing the repertoire of factors that might participate in specific transcriptional responses. Functional domain swap experiments reveal that the specific 'dimerisation code' is determined by the leucine zipper (Sellers and Struhl, 1989; Ransone et al., 1990; Kouzarides and Ziff, 1989 and see earlier section, b ii). Thus whilst ATF2 and ATF3 form heterodimers, ATF1 will not dimerise with either of these factors (Hai et al., 1989). ATF1 however along with CREM is able to form heterodimeric complexes with CREB (Foulkes et al., 1991a; Hurst et al., 1990; Hurst et al., 1991). In addition certain specific interfamily heterodimeric complexes can
form and this 'cross-talk' allows different intracellular signal transduction pathways to be integrated at the level of transcriptional control. ATF2, ATF3, ATF4 and B-ATF for example can heterodimerise with members of the Fos/Jun family (Hai and Curran, 1991; Dorsey et al., 1995) whilst C/ATF forms heterodimers with C/EBP-like proteins (Vallejo et al., 1993). Evidence indicates that such interactions can have significant consequences for DNA binding stability and/or specificity. For instance the DNA binding stability of CREB/ATF1 heterodimers is lower than that associated with homodimeric CREB (Hurst et al., 1991). Meanwhile whilst a C/ATF-C/EBPβ heterodimer can interact with both palindromic and asymmetric ATF sites, homodimeric C/ATF can bind the palindromic subclass only (Vallejo et al., 1993). Similarly whilst Fos/Jun heterodimers normally bind to a 12-O-tetradecanoylphorbol-13-acetate response element (TRE), heterodimers between Jun and ATF2, ATF3 or ATF4 preferentially bind ATF sites (Hai and Curran, 1991).

Most studies of the CREB/ATF family have focused on the ATF2/ATFa and CREB/ATF1/CREM subfamilies. Interest in the role of ATF2 relates to the ability of this factor to bind several viral transactivating proteins. These include the adenovirus oncoprotein ElA with which it functions as an ElA-inducible activator of the adenovirus E4 promoter (Liu and Green, 1990). ATF2 also interacts with the hepatitis B virus X protein (Maguire et al., 1991) and the HTLV-1 transactivator p40tax (Fujisawa et al., 1989; Yoshimura et al., 1990). Additionally ATF2 interacts with the tumour suppressor gene product Rb which results in activation of the human TGF-β 2 gene (Kim et al., 1992) and with the high mobility group protein HMG1 (Y) stimulating binding of ATF2 to the interferon-β gene promoter (Du and Maniatis, 1994). ATF2 also interacts with NF-kB (Du et al., 1993) and c-Jun (Benbrook and Jones, 1990). More recently ATF2 and ATFa have been demonstrated to be activated as a result of phosphorylation by stress activated protein kinases (SAPKs) (Gupta et al., 1995; Livingstone et al., 1995 and van Dam et al., 1995) including the c-Jun NH2-
terminal protein kinase (JNK) which is itself activated in response to pro-inflammatory cytokines (tumour necrosis factor and II-1) or ultraviolet radiation.

The immediate CREB family (CREB, CREM and ATF1) have been the focus for most research to date with particular emphasis placed initially on CREB and later also on CREM. This was largely because CREB was the first member of the family to be identified and cloned. CREB was originally purified from PC12 cells as a 43kDa nuclear phosphoprotein which bound with high affinity to the CRE within the promoter of the rat somatostatin gene (Montminy and Bilezikjian, 1987). Subsequently CREB was cloned from human placental and rat PC12 cell libraries (Hoeffler et al., 1988; Gonzalez et al., 1989). Although targeted by multiple signal transduction pathways (see section 1.11), the best characterised role for CREB is as a nuclear target of the cAMP signal transduction pathway. It is now clear that the transcriptional regulation of numerous cAMP inducible genes involves interplay between multiple transcription factors of which CREB, CREM and ATF1 are central players. This is outlined next (for reviews see Lee, 1991; Borrelli et al., 1992; Lee and Masson, 1993; Lalli and Sassone-Corsi, 1994).

1.10 Transcriptional regulation by cAMP

The effects of many extracellular messenger molecules including peptide hormones, growth factors and neurotransmitters are conveyed via the intracellular cAMP signal transduction pathway. The components involved are discussed below and for convenience the membrane and cytoplasmic effectors of the pathway are discussed separately from the nuclear factors.

a) Membrane and cytoplasmic effectors

Initiation of the signal transduction cascade involves binding of appropriate ligands to specific G protein (GTP-binding protein) coupled
membrane receptors through which the signal is transmitted to the effector - the membrane associated enzyme, adenyl cyclase (see Cooper et al., 1995 and references therein). Stimulation of adenyl cyclase by the αs subunit of the G protein results in the synthesis of cAMP, which in turn controls the activity of protein kinase A (PKA). Inactive PKA exists as a tetramer composed of two catalytic (C) subunits bound to a dimer of regulatory (R) subunits. The cooperative binding of two cAMP molecules to each R subunit causes dissociation of the holoenzyme releasing the two active monomeric C-subunits. These specifically phosphorylate the serine residue in the context X-Arg-Arg-X-Ser-X which is present in many cytoplasmic and nuclear proteins. For detailed reviews of these components of the pathway see Taylor et al., (1990); Borrelli et al., (1992); Doskeland et al., (1993) and Cooper et al., (1995).

b) Nuclear effectors

The promoters of numerous and functionally diverse cellular genes have been identified as cAMP responsive and include genes encoding early response proteins, cell specific transcriptional activators, neuropeptides and gluconeogenic enzymes (for review see Borrelli et al., 1992). In the majority of cases the critical promoter element(s) that mediate the transcriptional response to elevated cAMP levels corresponds to the CRE, containing the core motif CGTCA (see section 1.9 and 1.14). Generally only one CRE is present per promoter although exceptions exist. The promoter of the α-chorionic gonadotrophin gene for instance, contains two identical CRES (Delegeane et al., 1987) whilst that of the pituitary specific transcription factor GHF-1/Pit-1 contains two different CRES (McCormick et al., 1990).

The identification and cloning of CREB (see section 1.9) which specifically bound the CRE and could be phosphorylated by protein kinase A (PKA) in vitro (Montminy and Bilezikjian, 1987), significantly advanced the understanding of cAMP signalling in the nucleus. Subsequently CREB was
shown to be a substrate for PKA in vivo with the PKA phosphoacceptor site of CREB required for transcriptional activation (Gonzalez and Montminy, 1989; Lee et al., 1990). Cell staining and in vivo footprinting studies have shown that CREB is in the nucleus prior to phosphorylation by PKA (Gonzalez and Montminy, 1989; Nichols et al., 1992). Fluorescence imaging techniques and microinjection experiments revealed that the catalytic subunit of PKA migrates to the nucleus to phosphorylate CREB (Riabowol et al., 1988; Meinkoth et al., 1990; Adams et al., 1991; Hagiwara et al., 1993). A schematic representation of the basic components involved in the cAMP signalling pathway through CREB is outlined in Diagram 4.

1.11 CREB structure and transcriptional activity

In addition to the bZIP domain, other functionally important regions of CREB have been identified as a result of both in vitro (Hai et al., 1988; Horikoshi et al., 1988; Yamamoto et al., 1988; Andriasani et al., 1989; Gonzalez et al., 1991; Wada et al., 1991) and in vivo studies of CREB transcriptional activity. For in vivo assays the effects of endogenous CREB have been overcome in two ways. In one strategy CREB is fused to the DNA binding domain of the yeast activator GAL4 and the activity of the fusion protein determined by testing its ability to activate reporters possessing GAL4 binding sites (Berkowitz and Gilman, 1990; Lee et al., 1990; Hurst et al., 1991; Sheng et al., 1991; Fiol et al., 1994). Alternatively, exogenous CREB is expressed in F9 embryonal carcinoma cells. F9 cells do not respond to cAMP and allow monitoring of exogenous CREB activity (Gonzalez and Montminy, 1989; Yamamoto et al., 1990; Masson et al., 1992). Together these assays reveal that transcriptional activation by CREB depends upon three distinct regions. These are discussed below and illustrated in Diagram 5.

The P-box (Lee et al., 1990) or kinase inducible domain (KID) (Gonzalez et al., 1991), defines a serine rich region of ~ 50 amino acids which includes the
Diagram 4  The cAMP signalling pathway through CREB

At the plasma membrane, the ligand (L) binding to the specific receptor (R) activates the α subunit of the G protein (α, β, γ) which in turn stimulates adenyl cyclase (AC) to generate cAMP. This secondary messenger molecule binds the regulatory subunits (r) of the inactive PKA tetramer resulting in the release of active catalytic subunits (C*) which are translocated to the nucleus where they phosphorylate CREB. CREB dimers activated in this way stimulate transcription of genes from the CRE.
PKA phosphoacceptor site at Ser 133 (Gonzales and Montminy, 1989; Yamamoto et al., 1990). Two additional elements comprise the remainder of the KID as defined by deletion analysis and lie on either side of the PKA recognition site. The C-terminal element termed PDE2 (Lee et al., 1990) or β (Gonzalez et al., 1991), contains the amino acid sequence DLSSD. This is critical for CREB transcriptional activity and appears to interact closely with the PKA site since function is dependent on their close proximity (Gonzalez et al., 1991). The N-terminal element, referred to as PDE1 (Lee et al., 1990) or the α2 region plus the protein kinase C (PKC) consensus phosphorylation site (Gonzalez et al., 1991), is required for full transcriptional activity. The region contains phosphorylation sites for several kinases and appears to be highly phosphorylated in vivo (Lee et al., 1990). In addition to PKC, phosphorylation sites exist for glycogen-synthase-kinase 3 (GSK3), calmodulin-dependent kinases I and II (CamKI and II) and casein kinase II (CK-II) (Gonzalez et al., 1989; Gonzalez and Montminy, 1989; Lee et al., 1990; Sheng et al., 1990; Gonzalez et al., 1991; Sheng et al., 1991; Fiol et al., 1994) as indicated in Diagram 5.

A second region of 14 amino acids termed the α-peptide is encoded by an alternatively spliced exon and functions directly in transcriptional activation (Berkowitz and Gilman, 1990; Yamamoto et al., 1990). Since only certain forms of CREB contain the α-peptide it is believed to serve a specialised role (Ruppert et al., 1992 and see section 1.14c). Stringent positioning is not essential as the α-peptide can be placed in different regions of CREB without significant loss of activity (Yamamoto et al., 1990).

The third region is composed of two parts that flank the P-box termed Q1 and Q2. Both are glutamine rich similar to the activation regions within other transcription factors such as Sp1 and Oct1 (see section 1.4b i) and together are critical for CREB transcriptional activity (Gonzalez et al., 1991; Brindle et al., 1993). The sequence within the Q2 domain is particularly similar to the
DIAGRAM 5  Functional Domains of CREB

Elements contributing to transcriptional activity include Q1 and Q2 corresponding to glutamine rich domains and the α-peptide (α). The basic (B) and leucine zipper (ZIP) regions comprise the carboxy-terminal bZIP domain that participates in dimerisation and DNA binding. The kinase inducible domain (KID) is required for transcriptional activity and comprises several elements denoted α2, PKC, PKA and β in accordance with the nomenclature of Gonzalez et al., 1991. Phosphorylation sites within the KID for various kinases are indicated by arrows. See text for details.
hydrophobic and glutamine rich activation domain B of Sp1 and there is evidence that both of these domains may interact with the same component of the TFIID complex, dTAFII110 (Ferreri et al., 1994).

1.12 Mechanism of PKA-induced CREB mediated transcriptional activation

CREB phosphorylation within the P-box has no effect on either CREB intracellular distribution or DNA binding affinity (at least in the case of strong CREs - see section 1.14a). Current evidence indicates that the KID can play a direct role in transcriptional activation. First the KID domain alone has a limited ability to mediate transcriptional activation when linked to a heterologous DNA binding domain (Lee et al., 1990; Brindle et al., 1993; Quinn, 1993). Second, the KID interacts specifically with a coactivator termed CBP (CREB-binding protein) (Chrivia et al., 1993). This interaction depends on CREB phosphorylation at Ser 133 (Arias et al., 1994; Kwok et al., 1994) and involves direct contacts between phospho-Ser-133 and residues within an α-helical domain of CBP termed KIX (Parker et al., 1996). CBP in turn associates specifically with RNA polymerase II allowing this factor to be recruited to CREB in a phospho-(Ser-133)-dependent manner (Kee et al., 1996). Interestingly recent data identify CBP as a coactivator of c-Jun (Bannister et al., 1995) and c-Myb (Dai et al., 1996) suggesting that CBP functions as a coactivator for a range of transcriptional activators.

In addition to a direct effect, KID phosphorylation by PKA is suggested to induce a conformational change that unmasks the α-peptide and/or glutamine rich activation domains (Q1 and Q2) of CREB facilitating functional interactions with the general transcriptional machinery (Gonzalez et al., 1991; Krajewski and Lee, 1994). A complete resolution of this issue however awaits crystal structure determination of unphosphorylated and phosphorylated CREB.
Notably the full cAMP-inducible transactivation function of CREB may depend upon further phosphorylation events within the KID that are dependent on prior phosphorylation by PKA. Recent evidence for example indicates that a hierarchical phosphorylation of Ser 129 by GSK-3 is necessary for CREB mediated transcriptional response to cAMP (Fiol et al., 1994).

1.13 Role of de-phosphorylation in the regulation of CREB activity

Several studies indicate that de-phosphorylation of CREB represents a key mechanism in the negative regulation of CREB transcriptional activity. Both protein phosphatase 1 (PP1) (Hagiwara et al., 1992; Alberts et al., 1993) and 2A (PP2A) (Wadzinski et al., 1993), have been implicated in this process.

1.14 Factors influencing CRE activity

As described earlier a vast and diverse range of genes are regulated by cAMP and in most cases the critical promoter element has been identified as a CRE containing the core motif CGTCA (see section 1.9). This represents a half site for CREB binding (Lee et al., 1987; Lin and Green, 1988; Yamamoto et al., 1988; Nichols et al., 1992) and in many cases CREs contain the palindrome TGACGTCA, that binds a CREB dimer symmetrically.

The diversity of genes regulated through CREs is mirrored by the variety of biological functions in which CREB (or a CREB relative) is thought to be involved. These include gluconeogenesis (Boshart et al., 1990), pituitary proliferation (Struthers et al., 1991), opiate tolerance (Guitart et al., 1992), neuronal signalling (Comb et al., 1987), spermatogenesis (Foulkes et al., 1992; Foulkes et al., 1993; Delmas et al., 1993), setting of circadian rhythms (Ginty et al., 1993) and the establishment of long term memory (Bourtchuladze et al., 1994; Frank and Greenberg, 1994; Yin et al., 1994). Accordingly these diverse genes are not coordinately expressed in response to cAMP rather they are finely regulated in a cell and promoter specific manner. Indeed elevation of
intracellular cAMP may result in either stimulation or repression of specific genes (Lalli and Sassone-corsi, 1994 and references therein).

Detailed studies of the cAMP signal transduction pathway indicates that this integral non-coordinate regulation is generated at all levels from the cell surface down to the transcriptional machinery itself (for review see Borelli et al., 1992). The regulatory role of nuclear factors in specifying responses subsequent to activation of PKA is considered next.

a) Structure of CREB binding sites

In addition to the core motif CGTCA, flanking bases can significantly influence DNA binding affinity providing a means by which CRE activity can be varied. Firstly depending on the nature of the CREB binding site (CBS), CREB DNA binding activity can be increased by phosphorylation. While symmetric sites such as the somatostatin CRE (TGACGTCA) have high affinity for CREB and DNA-binding is not strongly stimulated by PKA, asymmetric sites such as that in the tyrosine amino transferase gene (TGACGCAG), have low affinity for unphosphorylated CREB and high affinity for phosphorylated CREB (Nichols et al., 1992). These findings are consistent with the observation that basal transcription (ie, when CREB is unphosphorylated) directed by symmetric sites can be higher than for asymmetric sites but that the induced level (ie, when CREB is phosphorylated) is similar for both types (Muchardt et al., 1990). Secondly a particular CBS may bind alternative members of the CREB family with different stability. Depending upon the particular CBS the binding of ATF1 for example is highly unstable compared to CREB (Hurst et al., 1990).

b) Contributions from additional promoter elements

The relative responsiveness of different CREs to cAMP-induced transcriptional activation has been shown to vary. For example whilst the somatostatin and gonadotrophin α-subunit CREs were found to be highly
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responsive to cAMP when transfected into JEG3 human choriocarcinoma cells, the glucagon and parathyroid CREs were much less active (Deutsch et al., 1988). These four CREs however share the palindrome TGACGTCA which lead to the suggestion that flanking bases account for the differences in transcriptional activity. This has subsequently been confirmed with two distinct mechanisms accounting for the effects. Firstly, flanking nucleotides affect the DNA binding stability of CRE binding proteins (Ryseck and Bravo, 1991) similar to the effect of nucleotides within the CRE (see section 1.14a). Secondly, flanking nucleotides may represent binding sites for additional transcription factors that modulate the function of CREB (for reviews see Masson and Lee, 1993; Schmid et al., 1993). For example tyrosine aminotransferase is expressed exclusively in liver parenchymal cells and can be induced by cAMP. Induction by cAMP is mediated by an enhancer region (at -3.6 kb) consisting of two essential elements. One is a CRE that binds CREB, the other interacts with the liver enriched transcription factor HNF4. Both elements are essential for cell specific and cAMP dependent expression (Schmid et al., 1993 and references therein). Meanwhile the ability of CREB to activate the rat glucagon gene in response to cAMP is decreased by additional proteins which bind adjacent to the CRE (Miller et al., 1993). Whilst in most cases the precise mechanisms by which these additional transcription factors modulate CREB transcriptional activity are not clear, direct protein-protein interactions may be involved. Such interactions might influence DNA binding stability or else modulate the interaction of CREB transactivation domains (see section 1.11) with protein kinases or phosphatases or with components of the basal transcriptional apparatus. Alternatively binding of additional transcription factors to the promoter may alter DNA conformation in a manner that affects the function of the CREB binding site. Growing evidence suggests that transcription factors can impart significant DNA conformational changes upon binding (Gustafson et al., 1989; Schrech et al., 1990; Kerppola and Curran, 1991).
c) Contributions from other ATF family members

As mentioned earlier CBSs may be targeted by different member(s) of the CREB/ATF family with different regulatory and/or functional properties. Such considerations are made all the more important given that within the family alternative splicing and heterodimerisation create the potential for even greater diversity (see section 1.9).

Multiple isoforms of CREB were in fact amongst the first to be identified (see Diagram 6). Activator forms termed CREB α and CREB Δ (the latter differing from CREB α by the exclusion of the 14 amino acid α-peptide (see section 1.11)), appear to be ubiquitously expressed in somatic cells with CREB Δ 3-fold higher than CREB α (Berkowitz and Gilman 1990; Ruppert et al., 1992). Other CREB isoforms (CREB αγ, CREB γ, CREB Ω, CREBW and CREB αW) are highly expressed only in adult testis however their function is not obvious since they lack the DNA binding domain and nuclear translocation signal (Yamamoto et al., 1990; Waeber et al., 1991; Ruppert et al., 1992; Waeber and Habener, 1992).

Of particular relevance are the factors ATF1 and CREM (and various isoforms -see later) which comprise the immediate CREB family (see section 1.9). These share extensive homology with CREB, especially in the bZIP and KID regions (see Diagrams 3 and 7), form selective heterodimers with CREB (see section 1.9) and can be regulated directly by PKA (see sections 1.15 and 1.16). Transgenic experiments using dominant negative CREB mutants and homozygous deletions of the CREB gene indicate that some CREB functions (Struthers et al., 1991; Hummler et al., 1994), although not all (Barton et al., 1996), can be effectively performed by the various isoforms of CREM and/or ATF1 (see sections 1.15 and 1.16) suggesting that a degree of functional redundancy exists.

Structural similarities in the organisation of the CREB (Ruppert et al., 1992) and CREM genes (Laoide et al., 1993) suggest a common ancestral origin.
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Significantly however each has acquired unique features and results in the generation of proteins with some distinct properties which are believed to reflect specialised roles. In line with this many also exhibit complex patterns of gene expression. Both CREM and ATF1 are discussed in turn below.

1.15 CREM

The isolation of several CREM isoforms from a mouse pituitary cDNA library with oligonucleotides corresponding to the bZIP region of CREB, represented another significant advance in the understanding of the transcriptional response to cAMP (Foulkes et al., 1991a). Whilst sharing extensive homology with CREB especially in the bZIP and KID regions, these first forms of CREM identified lacked the glutamine rich activation domains present in CREB and were found to antagonise transcriptional activation obtained by the joint action of CREB and cPKA (Foulkes et al., 1991a).

Subsequently it became apparent that the organisation of the CREM gene enabled a large number of additional isoforms to be encoded, including transcriptional activators (Laoide et al., 1993) as illustrated in Diagram 6. Moreover these distinct forms were expressed in a strict cell and tissue specific manner indicative of specialised roles in cAMP signalling.

An activator form of CREM (CREMT) contains two glutamine rich domains (Q1 and Q2) and is highly homologous to CREB (Foulkes et al., 1992). Distinct activators are generated by alternative splicing which removes either Q2 (CREMT1) or Q1 (CREMT2) (Laoide et al., 1993). Notably, activator forms of CREM are particularly abundant in male germ cells where their expression is developmentally regulated by the pituitary follicle-stimulating hormone (FSH) (Foulkes et al., 1993). Intriguingly, this switch to CREMT coincides with the switch in CREB expression from CREB α and CREB Δ to the CREB isoforms which lack the DNA binding domain and nuclear translocation signal (see section 1.14c).
Diagram 6  Schematic representation of CREB and CREM isoforms in relation to CREB/CREM gene structure. Exons are represented by boxes and introns by a line. The CREB gene contains one 5’ non-coding exon and four exons that contain in-frame stop codons that give rise to the Ψ, γ, W, Wα, αγ and Ω isoforms. The glutamine rich N-terminal transactivation domain (Q), kinase inducible domain (KID) and the bZIP domain are each encoded by two exons. For the bZIP domain the N-terminal exon (B) contains part of the basic domain with the remainder within the C-terminal exon (BZIP). The α-peptide and an additional Q domain are encoded by single exons. Homologous exons in CREM are represented in the same manner as CREB. Differential splicing of CREM, can fuse the N-terminal exon of the basic region (B) to either of two splice sites within the C-terminal BZIP exon generating alternative DNA-binding domains referred to as BZIP1 or BZIP2. ICER isoforms are derived from an internal start site of transcription (P2) with alternative splicing of the γ and BZIP domains.
CREM repressor isoforms can be generated by various mechanisms as follows. First removal of the glutamine rich activation domains by alternative splicing generates CREM α, β and γ isoforms (Foulkes et al., 1991a,b; Laoide et al., 1993). CREM α differs from CREM β only in the bZIP domain and involves the use of two alternative bZIP domains encoded by the CREM gene (Laoide et al., 1993). The two alternative DNA binding domains show distinct binding efficiencies (Laoide et al., 1993) and expression patterns vary in a cell and tissue specific manner (Foulkes et al., 1991a,b). CREM γ is equivalent to CREM β but with a 12 amino acid deletion (Foulkes et al., 1991a). Secondly an N-terminally truncated form of CREM (S-CREM) is generated specifically in adult brain by alternate translation initiation from an internal AUG codon within the CREM transcript (Delmas et al., 1992). In contrast to the CREM α/β/γ repressors which contain the KID region but have no glutamine rich domains, S-CREM contains a single glutamine rich domain but lacks the KID (see Diagram 6). Thirdly a family of repressors termed ICER-I, ICER-Iγ, ICER-II and ICER-IIγ and consisting mainly of the bZIP region are generated as a result of initiation from an alternative, intronic promoter (P2) (Molina et al., 1993). Expression of the ICERs is regulated with circadian rhythm in the pineal gland and may be involved in the rhythmic synthesis of the pineal hormone melatonin (Stehle et al., 1993).

As previously mentioned the regulatory KID region in CREM is highly homologous to that of CREB (see Diagram 7) and contains phosphorylation sites for PKC, CamK, p34cdc2, GSK3, CK I, CK II and p70 S6 (de Groot et al., 1993; de Groot et al., 1994). CREM, like CREB therefore is a nuclear effector in multiple signalling pathways. Significantly CREM τ is also phosphorylated at Ser 117 (equivalent to Ser 133 in CREB α) by PKA upon activation of the cAMP signalling pathway. As with CREB this event leads to activation of transcription which is enhanced by additional cooperative phosphorylation events (de Groot et al., 1993). The same serine residue in the repressor isoforms CREM α/β/γ is
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also phosphorylated by PKA in vivo causing a decrease in their repressor activity (Laoide et al., 1993).

1.16 ATF1

ATF1 was originally cloned from a HeLa cell cDNA expression library by virtue of its ability to bind a labelled oligonucleotide probe containing three tandem ATF binding sites (Hai et al., 1989). The cDNA encodes a 271 amino acid protein which shares extensive homology to CREB (70% overall) (see Diagrams 7). Regions of particularly high homology include the bZIP domain (see Diagram 3) and the C-terminal region of the KID domain which contains a PKA phosphoacceptor site at Ser 63 (equivalent to Ser 133 in CREB α). Similarly to CREB, ATF1 is induced by cAMP (Hurst et al., 1991; Rehfuss et al., 1991; Liu et al., 1993; Ribeiro et al., 1994) and can also mediate Ca²⁺ inducible transcriptional activation (Liu et al., 1993).

Other features of ATF1 however suggest some novel functional and regulatory properties. Firstly although details regarding the cell/tissue specific expression of ATF1 are unknown, expression is specifically down-regulated during the differentiation of F9 embryonal carcinoma cells (Masson et al., 1993a). This is in marked contrast to CREB levels which remain constant and results in a significant change in the composition of the CREB and ATF1 dimer population (Masson et al., 1992; Masson et al., 1993a). Secondly ATF1 exists in multiple cell-type specific phospho-variants which are anticipated to possess specialised functions. The variants are generated by differential phosphorylation of specific serine residues within the KID although the specific kinases involved have yet to be identified (Masson et al., 1993b). A strict requirement for the generation of these phospho-variant isoforms is a region of ~27 amino acids at the N-terminus of ATF1 (Hurst et al., 1990; Hurst et al., 1991; Masson et al., 1993b). Significantly this N-terminal region (NTR) of ATF1
Diagram 7  Functional Regions of ATF1 compared to CREB

Known functional regions of CREB and homologous regions of ATF1 are shown. The basic (B) and leucine zipper (ZIP) regions comprise the carboxy-terminal bZIP domain. The kinase inducible domain (KID) is comprised of several distinct elements denoted \( \alpha_2 \), PKC, PKA and \( \beta \) and correspond to the boxed elements shown for sequence homologies (% homology (including conservative changes) within the KID for CREB, ATF1 and CREM is indicated). ATF1 lacks the \( \alpha \)-peptide (\( \alpha \)) and N-terminal glutamine-rich domain present in CREB but instead has a small region at the amino terminus termed the NTR the sequence of which is not obviously related to CREB. The NTR influences DNA binding stability and affects ATF1 conformation as discussed in the text.
diverges considerably from CREB and corresponds to a major structural difference. Thirdly, dependent on the particular CBS, DNA binding by ATF1 is markedly unstable relative to CREB (Hurst et al., 1990; Hurst et al., 1991). This instability is dependent upon specific phosphorylations within the KID and on the presence of the NTR and is thought to result from long range interactions between these regions and the bZIP domain (Hurst et al., 1991; Masson et al., 1993b). Fourthly, when fused to the GAL4 DNA binding domain, transcriptional activation by GAL4/CREB is not affected by the presence of the leucine zipper while activation by GAL4/ATF1 is greatly increased (Hurst et al., 1991). This indicates that ATF1 must be dimerised either to itself or to another bZIP protein to efficiently activate transcription and may relate to the effect of dimerisation on the phosphorylation of ATF1 as discussed below. Finally whilst the C-terminal region of the KID (including the PKA site and β region) is highly conserved within ATF1, CREB (and CREM) the N-terminal region is much less so (see Diagram 7). Given the key regulatory role of the KID the differences in this region might allow discriminatory controls to operate, conferring unique properties on ATF1. Several observations are consistent with this and suggest that the KID regions in CREB and ATF1 are different. Firstly, phosphorylations within the N-terminal region of the CREB KID domain require initial phosphorylation at the PKA site (Lee et al., 1990), while phosphorylation of ATF1 in the same region does not (Masson et al., 1993b). Secondly in contrast to CREB there is evidence that homodimerisation is a requirement for ATF1 phosphorylation in vitro. This suggests that dimerisation can alter the accessibility of the phosphorylation sites and raises the possibility that the phosphorylation of ATF1 may be influenced by other dimerisation partners (Lee et al., 1990; Masson et al., 1993b). Thirdly amino acid residue 36 in ATF1 is a serine which is not conserved in the CREB KID (see Diagram 7) but plays an important role in modulating the DNA-binding stability of ATF1 (Masson et al., 1993b). Fourthly whilst CREB can be activated by GSK-3 (Fiol et
1.17 Malignant Melanoma of Soft Parts and EWS-ATF1

Malignant melanoma of soft parts (MMSP) is a rare and aggressive tumour, probably of neuroectodermal origin, that mainly develops in tendons and aponeuroses of patients between 15 and 35 years of age (Chung and Enzinger, 1983; Epstein et al., 1984). The tumour exhibits a recurrent, balanced chromosomal translocation, specifically t(12; 22) (q13; q12) (Bridge et al., 1990; Speleman et al., 1992) which generates hybrids between the Ewing's sarcoma (EWS) gene on chromosome 22 and the ATF1 gene on chromosome 12 (Zucman et al., 1993a).

The EWS gene was first identified following analysis of the t(11; 22) (q24; q12) translocation associated with Ewing's sarcoma (see below) and encodes a ubiquitously expressed protein of 656 amino acids (Delattre et al., 1992). The function of EWS is unknown however certain sequence homologies with regions characteristic of RNA-binding proteins (reviewed in Burd and Dreyfuss, 1994) have been described suggesting that it may participate in RNA synthesis or processing (Delattre et al., 1992). These regions of homology include three glycine rich regions homologous to those present within glycine rich single stranded nucleic acid binding proteins and an 85 amino acid sequence which forms a putative RNA binding domain. The latter activity has been demonstrated in vitro (Ohno et al., 1994).

The chimeric gene generated on the der (22) chromosome associated with MMSP results in a fusion in which the N-terminal 325 amino acids of EWS replace the N-terminal 65 amino acids of ATF1 (see Diagram 8). The predicted fusion protein (EWS-ATF1) therefore contains most of ATF1 including the functionally important bZIP domain and also the glutamine rich transcriptional activation domain. However the NTR together with the PKA site and
Diagram 8  Structure and functional regions of EWS-ATF1

Functional regions of the 271 amino acid ATF1 are largely inferred from highly homologous regions characterised for CREB (refer to Diagram 7). EWS-ATF1 contains the N-terminus of EWS (aa 1-325) and most of the C-terminus of ATF1 (aa 66-271) including the bZIP domain, Q2 and β region of the KID. The RNA binding domain of EWS is not present in the fusion protein and the function of the region transferred to ATF1 is unknown but is notably rich in glutamine, proline, serine, threonine and glycine residues.
remaining N-terminal elements of the KID region from ATF1 are not retained. Whilst the function of the N-terminal region of EWS which is transferred to ATF1 is unknown (see above) the sequence is particularly rich in glutamine, proline, serine, tyrosine and glycine residues. The reciprocal ATF1-EWS fusion on der (12) is out of frame and generates a stop codon almost immediately C-terminal to the ATF1 sequence. Consequently the deduced translation product (ATF1-EWS) is almost entirely composed of the first 65 amino acids of ATF1 with only three residues contributed by the EWS sequence. In contrast to EWS-ATF1 this product is not thought to be essential for tumour proliferation (Zucman et al., 1993a).

Chromosomal translocations that generate chimeric transcription factors are associated with a variety of cancers (for reviews see Rabbitts, 1994; Ladanyi, 1995). The generation of EWS-ATF1 appears to follow a common theme whereby the chimeric transforming protein retains the DNA-binding domain of a particular transcription factor but the regulatory activities of this factor are disrupted by the other fusion partner (Delattre et al., 1992; Hunger et al., 1992; Crozat et al., 1993; Galili et al., 1993; Grignani et al., 1994; Lu et al., 1994; Prasad et al., 1994; Sanchez-Garcia and Rabbitts, 1994; Sorenson et al., 1994).

Intriguingly the EWS gene has been implicated in the genesis of other tumours following translocation events that generate oncogenic transcription factors of this class. Indeed as mentioned above EWS was first identified following the determination of the genes involved in the t(11; 22) (q24; q12) translocation associated with Ewing's sarcoma (Delattre et al., 1992). Here the N-terminal region of EWS is fused to the DNA binding domain of the Ets family transcription factor FLI-1 (Delattre et al., 1992; May et al., 1993a; Zucman et al., 1993b) or alternatively (though less commonly) to other members of this family namely ERG-1 (Sorenson et al., 1994), ETV-1 (Jeon et al., 1995) and E1AF (Kaneko et al., 1996). Meanwhile in desmoplastic small round cell tumour (DSRCT) the t(11; 22) (p13; q12) chromosomal translocation fuses EWS to the
DNA binding domain of the Wilms tumour oncogene (Ladanyi and Gerald, 1994; Gerald et al., 1995). Most recently a specific chromosomal translocation associated with myxoid chondrosarcoma has been shown to fuse EWS to the entire sequence of CHN, a member of the steroid/thyroid receptor gene superfamily (Clarke et al., 1996).

In all translocations involving EWS, the sequence specific DNA-binding domain encoded by a transcription factor gene is juxtaposed to a very similar region of EWS consisting principally of the N-terminal glutamine, proline, serine, tyrosine and glycine rich region. Interestingly an analogous situation has also been reported for the TLS/FUS gene, an RNA binding protein with extensive sequence similarity (55.6% identity) to EWS. Fusion of TLS/FUS to CHOP, a dominant negative member of the C/EBP family of transcription factors occurs in the translocation t(12; 16) (q13; p11) associated with malignant liposarcoma, while its fusion to ERG is found in myeloid leukemia with the translocation t(16; 21) (p11; q22) (Crozet et al., 1993; Rabbitts et al., 1993; Ichikawa et al., 1994).

Detailed studies of these chimeric transcription factors involving EWS are currently largely confined to EWS-Fli-1. These have confirmed the putative transforming ability of EWS-Fli-1 and demonstrated the essential requirement for both the EWS domain and DNA-binding domain of Fli-1 in this process (May et al., 1993a,b). Additionally EWS-Fli-1 displays identical DNA-binding specificity to Fli-1 (Mao et al., 1994) and significantly acts as an efficient, sequence-specific activator of target promoters containing ETS binding sites (May et al., 1993b; Ohno et al., 1993; Bailly et al., 1994). These properties suggest that EWS-Fli-1 behaves as an aberrant transcription factor which transforms cells via the inappropriate activation of genes that are normally regulated by Fli-1 or, possibly by other members of the ETS family given that additional members of the ETS family linked to EWS can generate the same disease phenotype.
By analogy, a plausible mechanism for transformation by EWS-ATF1 would involve the inappropriate activation of genes which are normally regulated through an ATF binding site by ATF1 or perhaps by a different member of the CREB/ATF family (see section 1.9). A principle aim of this thesis was to characterise the transcriptional properties of EWS-ATF1 in order to address this possibility (see Chapter 4 and 5).

1.18 **Role of the CREB/ATF family in growth control**

The suggested model for EWS-ATF1 mediated tumourigenesis (see section 1.17) seems likely given the apparent involvement of the CREB/ATF family in growth control.

A specific role for members of the immediate CREB/ATF family is anticipated considering the mitogenic effect of cAMP in a range of cells and tissues (for reviews see Boynton and Whitfield, 1983; Dumont et al., 1989). In the thyroid for example, cAMP mediates the growth promoting effects of thyroid stimulating hormone (TSH) (Jin et al., 1986; Tramontano et al., 1988) whilst in the pituitary, cAMP is the second messenger for growth hormone releasing hormone (GHRH) which stimulates proliferation of pituitary somatotrophs (Billestrup et al., 1986; Burton et al., 1991). In the latter case, transgenic experiments have confirmed the role of CREB in transducing the proliferative effects of GHRH acting via cAMP (Struthers et al., 1991) and involves CREB mediated activation of the pituitary-specific transcription factor, growth hormone factor (GHF)-1 (McCormic et al., 1990), which effects pituitary cell proliferation (Castrillo et al., 1991). Significantly subsets of both thyroid (O'Sullivan et al., 1991; Suarez et al., 1991; Parma et al., 1993; Duprez et al., 1994; Michiels et al., 1994) and pituitary tumours (Landis et al., 1989) are associated with situations where specific mutations up-regulate the adenyl cyclase-cAMP pathway.
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The mitogenic effects of cAMP are reflected in the key growth control genes which can be regulated by CREB, ATF1, or CREM. These include the prototypical immediate early genes c-fos (Sassone-Corsi et al., 1988; Berkowitz et al., 1989; Fisch et al., 1989) and c-jun (Lamph et al., 1990) which form part of the Activator Protein-1 (AP-1) complex (for review see Angel and Karin, 1991).

The immediate CREB/ATF family are also implicated in growth control in response to signals other than cAMP (see section 1.11, 1.15, 1.16). Activation of CREB for example, following phosphorylation by a PKA independent pathway has recently been demonstrated to be required for T cell proliferation (Barton et al., 1996). Meanwhile ATF1 participates in the transcriptional regulation of the proliferating cell nuclear antigen (PCNA) promoter through an ATF binding site (Labrie et al., 1993; Labrie et al., 1995). PCNA is a critical component of the DNA replication machinery and elevated expression of this factor has been implicated in transformation (Zerlar et al., 1987; Morris and Mathews, 1990; Waga et al., 1994).

A role in the control of cellular proliferation is not confined to the immediate members of the CREB/ATF family. Heterodimers consisting of ATF2 and c-Jun, which preferentially bind ATF sites rather than TRE elements (Hai and Curran, 1991), have been implicated in cellular proliferation and oncogenic transformation (Hagmeyer et al., 1993; van Dam et al., 1993; Morooka et al., 1995). Additionally the characteristics of ATF3 expression are indicative of an important function in coordinating gene activity in proliferating and mitogen stimulated cells. Specifically ATF3 gene expression in BALB/c 3T3 cells is induced by mitogens in a protein synthesis-independent manner (Chen et al., 1994) and mRNA levels are dramatically increased during the first 2 h of liver regeneration following hepatectomy (Hsu et al., 1991; Hsu et al., 1992). In view of the mitogenic effects of cAMP (see above) it may be significant that ATF3 homodimers or heterodimers with JunD can stimulate cAMP induced gene regulation (Chu et al., 1994). Since neither ATF-3 or JunD
In each of the tumour associated chromosomal translocations summarised in Table 1, the fusion genes generate chimeric transcription factors which contain a similar region of EWS (or TLS). Significantly the chromosomal translocation is often the only identifiable genetic alteration suggesting that the chimeric transcription factor has a fundamental role in tumorigenesis. Moreover the oncogenic potential of EWS-FLI1 (May et al., 1993a, b) and TLS-CHOP (Zinsner et al., 1994) has already been demonstrated via the ability to transform murine fibroblasts. Oncogenicity is anticipated to depend on the ability of the chimeras to function as aberrant transcription factors which deregulate the expression of key growth control genes and disrupt the normal cellular capacity to differentiate appropriately. The key target genes may normally be regulated by the native transcription factor fusion partner. Indeed functional studies of EWS-FLI1 (May et al., 1993b; Ohno et al., 1994; Bailly et al., 1994; Mao et al., 1994), EWS-ATF1 (Brown et al., 1995; Fujimura et al., 1996) and TLS-CHOP (Sanchez-Garcia and Rabbitts, 1994; Zinsner et al., 1994) are consistent with this model.

Interestingly tumours associated with these specific chromosomal translocations (with the exception of liposarcoma) occur predominantly in patients of young age. One possible explanation is that the cell type(s) from which these tumours originate are more abundant in younger individuals.
possess consensus phosphorylation sites for PKA they are assumed to serve as targets of kinase cascades which are themselves activated by cAMP. Interestingly the cyclin D1 promoter may be induced by an ATF-Jun complex through an ATF binding site (Herber et al., 1994). Cyclin D1 is thought to be a key player in driving the cell cycle through the G1 restriction point, integrating extracellular signals with the cell cycle clock (for review see Sherr, 1993).

Finally it is significant that de-regulation of signal transduction involving the CREB/ATF family has previously been directly implicated in malignancy. Type I human T cell leukemia virus (HTLV-1) is the etiologic agent of the adult T cell leukemia/lymphoma (ATLL) and encodes the Tax transactivator. Tax is thought to play a central role in cellular transformation and activates viral and cellular genes via interaction with cellular transcription factors including members of the CREB/ATF family (Alexandre and Verrier, 1991; Alexandre et al., 1991; Zhao and Giam, 1992; Armstrong et al., 1993; Suzuki et al., 1993; Wagner and Green, 1993; Low et al., 1994). Using specific Tax mutants, Smith and Green (1991), correlated the transforming function of Tax with its ability to activate transcription through the ATF pathway.
MATERIALS AND METHODS

To aid reference, the materials and methods are divided into four sections based on whether they are common to more than one results chapter 'General Materials and Methods' (2.1) or else specific to the results described in Chapter 3 (2.2), Chapter 4 (2.3) or Chapter 5 (2.4)

2.1 GENERAL MATERIALS AND METHODS

a) Electrophoretic analysis of nucleic acids

i Agarose gel electrophoresis

Horizontal gels were prepared by melting agarose (Bio-Rad, molecular biology grade) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH8.0] containing 0.5 μg ml⁻¹ ethidium bromide (EtBr). Typically 1% agarose gels were used to resolve DNA fragments > 500 bp long with smaller fragments analysed in 2% agarose gels. The melted gel was cooled to < 65°C and then cast in perspex trays with sample combs (Bio-Rad) and allowed to set at room temperature. DNA samples were mixed with 0.5 volumes of loading buffer (50% v/v glycerol, 0.25% w/v Orange G dye) and run at 100 V in TAE buffer containing EtBr as above using horizontal agarose gel electrophoresis cells (Bio-Rad). DNA bands were visualised on a u.v. transilluminator and photographed using polaroid '667' film.

ii Denaturing-polyacrylamide gel electrophoresis (denaturing-PAGE)

Denaturing polyacrylamide gels were prepared from a solution containing 10% acrylamide (Bio-Rad), 0.5% bisacrylamide (Bio-Rad), 50% urea, in TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH8.0]) which was polymerised by the addition of 1/100 volume of 10% ammonium persulphate
(Bio-Rad) and 1/1000 volume of TEMED (Bio-Rad) and set in a vertical gel assembly apparatus (Bio-Rad). DNA samples were mixed with an equal volume of loading buffer (90% de-ionised formamide, 1 x TBE, 0.25% bromophenol blue, 0.25% xylene cyanol), denatured by incubating for 3 min at 95°C and then loaded on the gel which was run at 25 mA. Gels were wrapped in cling film or else, in the case of ³⁵S-labelled DNA samples, dried down using a Bio-Rad vacuum dryer and visualised by autoradiography.

b) Preparation and transformation of competent bacterial cells

Competent Eschericia coli strain MC1061 were prepared based on the standard CaCl₂ procedure (Maniatis et al., 1982). A fresh bacterial colony was inoculated into 2 ml Luria-Bertani Broth (LB) (1% w/v bacto-tryptone (DIFCO), 0.5% w/v yeast extract (DIFCO), 1% w/v NaCl) and grown for 3 h at 37°C in a rotary incubator, after which the culture was diluted into 100 ml LB and further incubated until an A₅₅₀ nm = 0.2 was attained. The culture was then cooled on ice for 15 min, the cells transferred into two 50 ml polypropylene tubes (Falcon) and centrifuged at 3000 rpm for 15 min in a Beckmann J6 centrifuge. Cells were resuspended in 50 ml ice-cold 0.1 M CaCl₂ [pH 6.0], incubated on ice for 30 min and harvested by centrifugation. Pelleted cells were resuspended in 5 ml of fresh 14% glycerol/0.1 M CaCl₂, and 50 µl aliquots were snap-frozen with liquid nitrogen and stored at -70°C.

For a transformation an aliquot of frozen competent cells was thawed slowly on ice. Next ~10 ng of appropriate plasmid DNA was added and gently mixed. After incubation on ice for 30 min, cells were heat shocked by incubation for 4 min at 37°C, diluted to 500 µl with LB and incubated for 30 min at 37°C to allow cells to recover and express the selectable gene product (usually the antibiotic resistance enzyme β-lactamase) encoded by the transforming plasmid. A fraction of the transformation culture was plated onto
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LB agar plates (LB + 1.5% w/v agar) containing 50 µg ml⁻¹ ampicillin (for β-lactamase encoding plasmids) and incubated o/n at 37°C.

c) Isolation of plasmid DNA from bacteria

i) ‘Mini-prep’ DNA

Refers to small preparations of DNA suitable for restriction enzyme analysis, sequencing and PCR which were quickly prepared by an adapted alkaline lysis protocol (Maniatis et al., 1982). A single bacterial colony harbouring the relevant plasmid, was inoculated into 1.5 ml of LB containing 50 µg ml⁻¹ ampicillin in a 15 ml polypropylene centrifuge tube (Falcon) and incubated for at least 4 h at 37°C in a rotary incubator. The culture was then transferred to a microcentrifuge tube and the cells pelleted by centrifugation at 14000 rpm for 1 min in an Eppendorf microcentrifuge after which the cells were resuspended in 100 µl of solution 1 (50 mM glucose; 25 mM Tris.HCl [pH8.0], 10 mM EDTA [pH8.0]) and incubated at room temperature for 5 min. Next 200 µl of freshly prepared solution 2 (0.2 M NaOH, 0.1% SDS) was added and after incubation on ice for 10 min the solution was mixed with 150 µl of solution 3 (3 M potassium acetate [pH 5.0]) and incubated for a further 5 min on ice. Cell debris was pelleted by centrifugation at 14000 rpm for 5 min in an Eppendorf microcentrifuge and the supernatant transferred to a fresh tube. Proteins were then removed by extraction with an equal volume of phenol/chloroform and nucleic acids obtained by ethanol precipitation. The nucleic acid pellet was washed with 1 ml 70% ethanol, air dried and resuspended in 10 µl TE buffer (10 mM Tris.HCl [pH8.0], 1 mM EDTA pH8.0). At this stage the DNA was sufficiently pure for all manipulations described above with the exception of sequencing. Prior to sequencing using the Sequenase™ Version 1.0 kit (USB), the DNA was first incubated with RNase A (USB) at 50 µg ml⁻¹ for 15 min at room temperature.
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ii 'Maxi-prep' DNA

Refers to the procedure to obtain larger and purer preparations of plasmid DNA suitable for transfection and in vitro transcription reactions. A single bacterial colony harbouring the relevant plasmid, was inoculated into 400 ml of LB containing 50 μg ml⁻¹ ampicillin in a 1 L conical flask and incubated o/n at 37°C with shaking. Cells were harvested by centrifugation at 3000 rpm for 15 min in a Beckmann J6 centrifuge and resuspended in 50 ml of solution 1. After 5 min 100 ml of solution 2 was added followed after a 10 min incubation on ice by 50 ml of solution 3 (for details of solutions 1, 2 and 3 see section 2.1 c i). After 10 min incubation on ice, cell debris was pelleted by centrifugation as before and the supernatant decanted and strained through sterile gauze into a fresh centifugation bottle. Nucleic acid was precipitated by the addition of 120 ml of isopropanol, incubation at room temperature for 30 min and centrifugation as before. Next the nucleic acid pellet was resuspended in 7.5 ml of TE buffer and 2.5 ml of 10 M ammonium acetate in a 15 ml polypropylene centrifuge tube. Following incubation on ice for 20 min the precipitate was pelleted by centrifugation as before and the supernatant transferred to a fresh tube. Nucleic acid was ethanol precipitated and resuspended in 500 μl TE buffer. RNA was digested by the addition of RNAse A (USB) to 20 μg ml⁻¹ and incubation for 15 min at 37°C. The solution was then adjusted to 1.5 M NaCl and DNA precipitated by the addition of 0.25 volumes of 30% PEG in 1.5 M NaCl. After incubation on ice for 30 min the plasmid DNA was recovered by centrifugation for 5 min at 14000 rpm in an Eppendorf microcentrifuge. The pellet was then resuspended in 400 μl of proteinase K buffer (10 mM Tris. HCl [pH8.0], 5 mM EDTA [pH8.0], 0.5% SDS) and treated with 0.5 mg ml⁻¹ proteinase K for 30 min at 37°C. Following phenol extraction and ethanol precipitation plasmid DNA was resuspended in TE buffer and the DNA concentration determined by measuring absorbance at A₂₆₀ nm (based on an A₂₆₀ nm of 1.0 = 50 μg ml⁻¹).
d) Enzymatic manipulation of DNA

All manipulations of DNA including restriction enzyme digests (restriction enzymes supplied by New England Biolabs), the generation of blunt ends by removal of 3' overhangs with bacteriophage T4 DNA polymerase (Boehringer Manheim) or by filling in recessed 3' termini with Klenow (Boehringer Manheim) and ligations with bacteriophage T4 DNA ligase (New England Biolabs) were performed using standard protocols (Maniatis et al., 1982) and with buffers provided by the enzyme suppliers.

e) Construction of recombinant plasmids

Details regarding the construction of specific constructs are described later. Here the general steps involved in construction are outlined.

Vector was typically prepared for ligation by digestion of 5 µg plasmid DNA with the appropriate restriction enzyme(s). All DNA fragments for cloning, with the exception of DNA generated from complementary oligonucleotides, were purified by electroelution from agarose gels (see section 2.1 a i and Maniatis et al., 1982) prior to their inclusion in ligation reactions. Ligation reactions were performed according to standard protocols (Maniatis et al., 1982) typically containing 100 ng of vector and 100 - 300 ng of each insert although with small fragments (< 50 bp) (generally annealed oligonucleotides) as little as 10 ng might be used. One way and two way 'sticky' ligation reactions were incubated for 2 h at room temperature whilst more complicated ligations were incubated for at least 16 h at 11°C. Products of ligation reactions were transformed into competent bacteria which were subsequently plated onto L-agar containing the appropriate antibiotic for selection. Depending upon the degree of stimulation, a number of colonies were isolated and mini-prep DNA prepared. This was analysed either by PCR or restriction enzyme digestion as appropriate to identify positive clones. In situations where stimulation of
transformation was particularly low pools of potential positive clones were screened by PCR in the first instance to speed up identification. Once a positive clone was identified maxi-prep DNA was prepared. Constructs incorporating PCR generated fragments were routinely sequenced as a precaution against polymerase synthesis errors (see section 2.1g).

f) Polymerase Chain Reaction (PCR)

PCR was routinely used to generate specific fragments of DNA for the purpose of constructing recombinant plasmids, identification of positive recombinant clones and amplification of target sequences from cDNA. DNA synthesis was typically performed in a 50 μl reaction containing 10 ng of template DNA, 100 ng of each oligonucleotide primer, 0.2 mM dNTPs and 2.5 U polymerase in 10 mM Tris.HCl [pH 8.3], 50 mM KCl and 1.5 mM MgCl₂ and overlayed with 35 μl mineral oil. Pfu DNA polymerase (Stratagene) was used in reactions to synthesise DNA for cloning because of its 12 fold higher fidelity over Taq DNA polymerase (Perkin-Elmer Cetus) which was used in all other circumstances. Reactions were typically incubated for between 10 and 40 cycles using the following temperature profile: 94°C, 1 min (melting step); 50-60°C (depending on primers), 2 min (annealing step); 72°C, 2 min (elongation step). After the last cycle reactions were incubated at 72°C for 5 min to ensure complete synthesis of all PCR fragments.

g) DNA sequence analysis

DNA was sequenced by the dideoxy method using the Sequenase™ Version 1.0 kit (USB) according to the manufacturers instructions.

h) Tissue culture

All cell lines were maintained as monolayers in 90 mm tissue culture plates (Falcon) in Dulbecco’s modification of Eagles medium (E4)
supplemented with 10% foetal calf serum (FCS) (GIBCO) at 37°C and 7% CO₂. Confluent plates were split four fold with adherent cells removed by treatment with a 3:1 mix of versene: 0.25% trypsin. With the exception of the JEG3 line, cells were washed with fresh media at 37°C before re-plating to remove residual trypsin which inhibited adherence.

i) Transfection of plasmid DNA into mammalian tissue culture cells

Plasmid DNA was transfected by the calcium phosphate precipitation method (Ausubel et al., 1987). Test and carrier DNA totalling 20 μg in 500 μl of a 240 mM CaCl₂ solution was precipitated by drop-wise addition to 500 μl of 2 x HBSP (40 mM HEPES, 275 mM NaCl, 1.4 mM Na₂HPO₄, 1.4 mM NaH₂PO₄, [pH7.1]) with continuous gentle mixing in a 15 ml centrifuge tube (Falcon). After standing at room temperature for 30 min, the precipitate was resuspended by a brief vortex and 500 μl added dropwise onto cells.

j) Assay of chloramphenicol acetyl transferase (CAT) activity

Mammalian cells transfected with plasmid DNA expressing activators and/or plasmids containing specified test promoters linked to the CAT reporter gene were typically assayed for CAT activity 40 h post transfection based on Gorman et al., (1982). Cells were washed with 5 ml PBS and removed with a rubber policeman in 1 ml PBS into a 1.5 ml centrifuge tube. After brief centrifugation the cell pellet was resuspended in 60 μl lysis buffer (250 mM Tris.HCl [pH7.8], 1 mM EDTA) and crude cell extracts prepared by three cycles of freeze-thaw. Cell debris was removed by centrifugation for 1 min at 14000 rpm in an Eppendorf microcentrifuge and the supernatant transferred to a fresh tube. After standardisation of extracts by measurement of A₂₈₀ nm up to 27 μl was incubated in a 79 μl reaction containing 215 mM Tris.HCl [pH7.5], 1.25 mM acetyl CoA (SIGMA) and 0.125 μCi ¹⁴C-chloramphenicol (Amersham).
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The products of the reaction were extracted by vortexing with 200 µl ethyl acetate and after brief centrifugation to separate the aqueous and organic phases 170 µl of ethyl acetate, which contains all forms of chloramphenicol, was transferred to a fresh tube and evaporated under vacuum. The products of the reaction were re-dissolved in 15 µl ethyl acetate, spotted onto a silica gel TLC plate (Schleicher and Schuell), and resolved by ascending chromatography for 45 min in 95% chloroform/5% methanol. After drying, the plate was exposed to X-ray film at room temperature and examined by autoradiography. For quantitation of results, % conversion of unacetylated to acetylated 14C-chloramphenicol (under linear assay conditions) was determined by excision of spots from the TLC plate and quantification of radioactivity using a liquid scintillation counter.

k) Small-scale nuclear extracts

Monolayer cells were washed twice with PBS, harvested and resuspended in 300 µl ice-cold lysis buffer (20 mM HEPES [pH8.0], 20 mM NaCl, 0.5% NP40, 1 mM DTT, protease inhibitors [0.5 mM PMSF, 2 µg ml⁻¹ leupeptin, 2 µg ml⁻¹ trasylol]) per 10⁷ cells. After 5 min on ice tubes were centrifuged for 1 min at 14000 rpm in an Eppendorf microcentrifuge at room temperature. The crude nuclear pellet was resuspended in 60 µl of high salt extraction buffer (20 mM HEPES [pH7.9], 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, protease inhibitors [0.5 mM PMSF, 2 µg ml⁻¹ leupeptin, 2 µg ml⁻¹ trasylol]), left on ice for 15 min, resuspended once more and left on ice for a further 15 min. Nuclear debris was removed by centrifugation as before and the nuclear extract transferred to a fresh centrifuge tube. For Western blots nuclear extract was prepared for loading by the addition of 0.5 volume of 3x SDS sample buffer (see section 2.1m). For immunoprecipitations nuclear extracts were diluted to low salt concentration
by the addition of 2 volumes of dilution buffer (20 mM HEPES [pH7.4]) and heat denatured by incubation for 5 min at 60°C.

l) In vitro transcription and translation

\(^{35}\)S-labelled proteins were synthesised in vitro by transcription of the corresponding cDNA and subsequent translation in rabbit reticulocyte lysate (Amersham). In vitro transcription occurred in an 80 µl reaction volume containing 40 mM Tris.HCl [pH7.5], 6 mM MgCl\(_2\), 2 mM spermidine, 100 µg ml\(^{-1}\) BSA (RNAse free, Boehringer Mannheim), 100 µM GTP, 500 µM each of ATP, CTP and UTP, 500 µM CAP analogue (m\(^7\)GpppG, Boehringer Mannheim) and 10 U RNA polymerase (SP6, T7 or T3, as stated for specific reactions, (Boehringer Mannheim). Linearised plasmid (4 µg) containing the appropriate cDNA was added last to reduce spermidine mediated precipitation. Reactions were incubated for 1 h at 40°C, after which plasmid DNA was digested by the addition of 40 U of DNase I (RNAse free, Boehringer Mannheim) and incubation for 10 min at 37°C. 15 µg tRNA and 1.5 volumes of 0.7 M ammonium acetate were added and after phenol extraction the RNA was recovered by ethanol precipitation. The RNA pellet was resuspended in 40 µl DEPC H\(_2\)O and protein was translated from this RNA in vitro as follows. 1 µl of the RNA preparation was incubated with 24 µl rabbit reticulocyte lysate, 3 µl \(^{35}\)S-methionine (10 µCi µl\(^{-1}\), Amersham) and 2 µl DEPC H\(_2\)O for 1 h at 30°C. Aliquots of the translation reaction were snap-frozen in liquid nitrogen and stored at -70°C.

m) SDS - Polyacrylamide gel electrophoresis (SDS - PAGE)

Protein samples were resolved by SDS - PAGE gels using the Bio-Rad Mini Protean II apparatus. SDS-PAGE gels were prepared with a resolving gel of 10% acrylamide (Bio-Rad) (with a ratio of 37.5 : 1 acrylamide : bisacrylamide) in a buffer containing 375 mM Tris.HCl [pH 8.8], 0.1% SDS and with a stacking
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gel of 5% acrylamide (with a ratio of 37.5 : 1 acrylamide : bisacrylamide) in a buffer containing 125 mM Tris.HCl [pH 6.8], 0.1% SDS). Acrylamide gels were polymerised by the addition of 1/100 volume of 10% ammonium persulphate (Bio-Rad) and 1/1000 volume of TEMED (Bio-Rad). Protein samples were mixed with 0.5 volumes of 3x SDS sample buffer (125 mM Tris.HCl [pH 6.8], 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.005% bromophenol blue, 0.25% xylene cyanol) and denatured by boiling for 2 min. Samples (with Bio-Rad pre-stained protein size markers in the outer lanes) were then loaded and the gel run at 25 mA in SDS running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) for an appropriate time period (typically 1.5 - 2 h).

Resolved protein samples in SDS-PAGE gels were detected by Coomassie staining, autoradiography or Western blotting as described below.

n) Coomassie staining

Proteins were visualised by soaking the SDS-PAGE gel in staining solution (0.2% Coomassie brilliant blue R250 (Bio-Rad), 50% methanol, 7.5% glacial acetic acid) for 10 min followed by transfer to de-staining solution (10% methanol, 7.5% glacial acetic acid) until protein bands could be clearly distinguished from background staining.

o) Autoradiography

35S-labelled proteins generated by in vitro translation with rabbit reticulocyte lysate were resolved by SDS-PAGE, the gels fixed in 50% methanol, 7% acetic acid for 45 min then 10% methanol, 7.5% acetic acid for 30 min. Gels were then dried using a Bio-Rad vacuum dryer and proteins detected by autoradiography.

p) Western blotting
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Protein samples resolved by SDS-PAGE were transferred to nitrocellulose filters (Schleicher and Schuell) for 1 h at 120 mA using the Bio-Rad Mini Trans-Blot apparatus with transfer buffer (25 mM Tris, 190 mM glycine). Filters were incubated for 1 h in blocking solution (PBS containing 1% w/v BSA and 0.05% v/v NP40 (Sigma)). Filters were then incubated o/n with primary antibody diluted appropriately in hybridisation solution (PBS containing 1% w/v BSA and 10% v/v FCS (GIBCO)) at room temperature with gentle rocking. Filters were then washed with 3 x 10 ml changes of PBS over a period of 5 min and incubated with the secondary antibody (alkaline phosphatase-conjugated rabbit anti-mouse or swine anti-rabbit antibody (DAKO) as specified) diluted 1/200 in hybridisation solution for 1 h at room temperature with gentle rocking. Following washing as before, detected proteins were visualised using the nitro blue tetrazolium /bromochloroindoyl phosphate (NBT/BCIP) stain system (Harlow and Lane, 1988).

2.2 Chapter Three

a) Plasmids and constructions

pGEX-CR1α (a gift from Helen Hurst, Hammersmith Hospital, London) contains cDNA encoding full length αCREB cloned into pGEX-KG as previously described (Masson et al., 1993a).

pBV-ATF1 contains the cDNA sequence of variant ATF1 (vATF1) within the EcoRI/Xho1 sites of pBluescript sk (Stratagene) and was obtained from a λ ZAP II clone (identified in Chapter 3) using the Stratagene in vivo excision protocol.

pGV-ATF1 contains the entire sequence of vATF1, with the exception of the most 3' 362 bp of 3' untranslated sequence and was obtained by ligating an Xba1/Kpn1 fragment from pBV-ATF1 with a Xba1/Kpn1 digested pGem3 vector (Promega).
pGV-ATF1-ATG1 and pGV-ATF1-ATG2 were derived from pGV-ATF1 as follows. A SalI/ScaI restriction enzyme fragment from pGV-ATF1 containing the first 343 bp of vATF1 was replaced with a SalI/ScaI PCR product which lacked the first 193 bp of vATF1 and in which the potential ATG translation initiation codons generated by either nucleotides 203-205 or 326-328 in pGV-ATF1 were changed to ATC by site directed mutagenesis in pGV-ATF1-ATG1 and pGV-ATF1-ATG2 respectively.

pZ-VA3 and pZ-VA12 were obtained by replacing a SalI/ScaI restriction enzyme fragment from pZA3 or pZA12 (Ribeiro et al., 1994), encoding the first 141 amino acids of human ATF1 with a SalI/ScaI restriction enzyme fragment from pGV-ATF1 encompassing the 113 bp of alternative ATF1 sequence and 3' 230 bp of normal ATF1 sequence encoding the first 6 amino acids of vATF1 (Chapter 3, Figure 5).

pGem3-VATF1 contains the first 279 bp of vATF1 sequence comprised of 113 bp of alternative sequence followed by 166 bp of normal ATF1 sequence and was obtained by ligating a Hind III/XbaI ended PCR product generated from pBV-ATF1 with a HindIII/XbaI digested pGem3 vector.

b) Mouse UF9 cDNA library

The directional cDNA library was constructed by Siegfried Ruppert, GCRC, Heidelberg essentially according to Ruppert et al., (1988), but with the following modifications. Single stranded cDNA primed with the oligonucleotide 5'-AATTCCTCGAG(T)₁₅ -3' containing a XhoI restriction site, was prepared using AMV reverse transcriptase and 5-methylcytosine. Double-stranded cDNAs, methylated at internal EcoR1 sites were ligated to 12-mer EcoR1 linkers (CCGGAATTCCGG). After re-cutting with EcoR1 and XhoI and size fractionation on a Biogel A50 column the cDNA inserts were ligated to EcoR1/XhoI digested λ ZAP II vector DNA (Stratagene). The average insert size was 2.1 kb (0.7-7.6 kb based on 34 phage minipreps). The original library
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consisted of $5 \times 10^8$ recombinants of which $5 \times 10^6$ phages were plated and amplified once.

c) Preparation of $^{32}$P-labelled CREB probe for Far Western screen

$pGEX-CR1\alpha$ was used to express glutathione S-transferase-CREB (GST-CREB) fusion protein in *E. coli* (strain BL21(DE3)). The pGEX plasmids (Pharmacia) transcribe the target gene (here encoding CREB) from the strong *tac* promoter. Expression is induced by isopropyl β-D-thiogalactopyranoside (IPTG) since they also incorporate the *lacI* gene which encodes the Lac repressor.

For over-expression of GST-CREB, an 800 ml culture of BL21(DE3) transformed with pGEX-CR1α was incubated at 37°C until the logarithmically growing culture reached an A$_{550}$ nm of 0.6 at which point it was adjusted to 1 mM IPTG (SIGMA) and incubated for a further 1 h. Cells were then obtained by centrifugation at 3000 rpm for 15 min in a Beckmann J6 centrifuge and bacterial lysate prepared by sonication in 5 mls lysis buffer (PBSA, 0.5% TX100, 1 mM EDTA, 1 mM DTT and protease inhibitors [0.5 mM PMSF, 2 µg ml$^{-1}$ leupeptin and 2 µg ml$^{-1}$ trasylol]. The lysate was then mixed with 500 µl glutathione agarose beads end over end for 40 min at room temperature, unbound protein removed and the beads washed 3x with 10 ml lysis buffer. Bound protein was eluted by mixing for 20 min end over end with 1 ml of 25 mM glutathione (SIGMA) (dissolved in 50 mM Tris.HCl [pH8.0]), the eluate adjusted to 10% glycerol, quick frozen and stored at -70°C. Total yield was approximately 20 µg as judged by Coomassie staining (Figure 1A). Prior to labelling, full length CREB was produced by treatment with thrombin as follows. Purified GST-CREB 300 µl (6 µg) was adjusted to 150 mM NaCl and
Figure 1  Purification and labelling of CREB. (A) A Coomassie stained SDS PAGE gel shows purification of GST-CREB. P indicates the whole cell pellet + and - induction with IPTG. E represents 10 µl of eluate from GST-beads following incubation with glutathione, the band corresponding to purified GST-CREB is indicated by an arrow. BIO-RAD low range SDS markers (sm) and BSA concentration markers are as indicated. (B) 10 ng of purified GST-CREB before (lane 1) and after thrombin treatment (lane 2) was resolved by SDS PAGE and detected by Western blotting using an anti CREB Ab. Arrows indicate GST-CREB and CREB. (C) Detection of ^32P-labelled CREB. ~ 3 ng of labelled CREB before (lane 1) and after passage through a G50 spin column (lane 2) was resolved by SDS PAGE and detected by autoradiography after 1 min exposure of film to gel.
2.5 mM CaCl₂ and incubated with 680 ng thrombin (Sigma) for 90 min at 25°C yielding full length CREB as verified by Western blotting with an anti CREB antibody (Figure 1B).

Full length CREB was ³²P-labelled using the catalytic subunit of PKA (Sigma) in a 200 μl reaction containing 30 μl CREB (600 ng), 20 μl 10 x kinase buffer (200 mM Tris.HCl [pH 7.5], 1 M NaCl and 120 mM MgCl₂), 20 μl PKA catalytic subunit (17U/μl in 0.3 M DTT), 45 μl ³²P-γ-ATP (10 mCi ml⁻¹, 5000 Ci mmol⁻¹, Amersham) and 85 μl H₂O by incubation for 1 h at 37°C. BSA (enzyme grade) was added to 1 mg ml⁻¹ and the sample passed through a G50 spin column (Pharmacia) equilibrated in 1 x kinase buffer plus 1 mg ml⁻¹ BSA (enzyme grade) in order to remove free label (Figure 3C). Specific activity of ³²P-labelled CREB probe was typically between 10⁷ and 10⁸ cpm per μg.

d) Far Western Library Screen

Host cells for the λ ZAP II UF9 cDNA library were the E. coli XL1 Blue strain (Stratagene) and were prepared for infection as follows. A single tetracycline resistant colony was inoculated into 50 ml LB broth supplemented with 0.2% maltose and 10 mM MgSO₄ and incubated o/n at 37°C with shaking. The culture was diluted with fresh media and grown to A₆₀₀ nm = 0.5, the cells pelleted by centrifugation at 3000 rpm for 5 min in a Beckmann J6 centrifuge and resuspended in 10 mM MgSO₄ at A₆₀₀ nm = 2.0. For infection with phage 15 ml polypropylene tubes (Falcon) containing 170 μl A₆₀₀ nm = 2.0 XL1 Blue cells were inoculated with 10 μl SM buffer (50 mM Tris.HCl [pH7.5], 10 mM NaCl, 2 mM MgSO₄, 0.01% gelatin) containing 5 x 10⁴ pfu of the λ ZAP II UF9 cDNA library, incubated for 15 min at 37°C, mixed with 9 ml H Top agar at 47°C and then poured onto 14 cm LB agarose plates (Falcon). Plates were incubated at 37°C until plaques were just visible (~4 h) at which point a dry nitrocellulose filter (previously soaked in 10 mM IPTG and possessing 2 triangular cuts to aid orientation) was placed on top. The plates were incubated
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for a further 2 h at 37°C and then placed for 3 h at 6°C before the nitrocellulose filters were lifted and soaked in HYB100 (20 mM HEPES [pH 7.7], 100 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.5% NP40) for 4 h at 4°C. Filters were blocked separately in HYB100 + 5% milk powder for 30 min, HYB100 + 1% milk powder for 15 min and then incubated with ³²P-labelled CREB probe (3 ng ml⁻¹) in HYB100 + 1% milk powder o/n at room temperature. After removal of probe, filters were washed 3x with 300 ml HYB100 + 1% milk powder for 5 min, dried and exposed to X-ray film. The Far Western screening assay is outlined in Diagram 1. Plaques corresponding to positive signals were isolated, placed in 0.5 ml SM buffer and the phage eluted by incubation for 2 h at 37°C. Phage supernatant was stored at 4°C, titred and re-screened at a plaque density of ~1000 per 9 cm plate. A positive plaque was isolated and the process repeated but at a density of ~ 20 plaques per 9 cm plate for cloning. cDNA clones within pBluescript plasmid were then obtained from λ ZAP II phage using the Stratagene in vivo excision protocol using f1 R408 helper phage thus avoiding the need for sub-cloning.

e) Peptides

Synthetic peptides corresponding to the leucine zipper (LZ) regions of CREB and JUN were as follows. CREB LZ contains the 36 amino acids (EYVKCLENRVAVLENQNKTLIEELKALKDLYCHKSD). JUN LZ contains the 34 amino acids (ARLEEKVKTLKAQNSELASTANMLREQVAQLKQK). Peptides were prepared by the ICRF peptide synthesis laboratory.

f) Oligonucleotide primers

A description of all oligonucleotide primers used for work relating to the identification and analysis of ATF1 and vATF1 is presented in Diagram 2.
Diagram 1  **Far Western screening strategy.** Bacterially expressed CREB was labelled *in vitro* using cPKA and $^{32P}$-ATP and $\sim 3 \text{ ng/ml}$ was incubated with nitrocellulose filters lifted from plates containing $\sim 5 \times 10^6$ plaques derived from *E. coli* XL1 Blue cells infected with a $\lambda$ ZAP II UF9 cDNA library. After washing and autoradiography, positive plaques were identified by precise orientation of the film with the plate.
Diagram 2 Oligonucleotide primers used for identification and analysis of ATF1 and vATF1

- a – ATF NP
  CTCAGCCTGGTCAACCGTTGCGGG
- b – ATF IDP
  GAAGATACACGGGGCAGAAAAG
- c – ZIP P
  AGTTTTATTTTGATTTTCCAG
- d – ATF COMP1
  CACAGGAGAAGTCATCACCAC
- e – ATF COMP2
  AATGCTGGGATTTTCTCCTT
- f – VP1
  AGCAAAACCCCAAGCCGAGA
- g – ATF VP
  GCCGCAGAAGTCTGTCAGTTA
- h – VP2
  TTCTGTCCACTGTGATTTCAATCA
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g) Cell lines

Undifferentiated F9 (UF9) cells were supplied as monolayers by ICRF central cell services.

h) Isolation of mouse tissues and embryos

Eye and thymus tissue were obtained from 3 week old male Balb C mice. Mouse embryos were supplied by the ICRF animal unit.

i) RNA Preparations

All solutions involved in the preparation/analysis of RNA unless otherwise stated were made with diethylpyrocarbonate (DEPC) treated H$_2$O. This was prepared by addition of 0.2% v/v DEPC (Sigma) to ddH$_2$O with incubation for 8h at 37°C followed by autoclaving.

i) Cytoplasmic RNA from tissue culture cells

Cell monolayers (90mm plates) were rinsed 3 x with ice cold PBS and cells were scraped into 1 ml ice cold PBS and collected by centrifugation for 5 min in an Eppendorf microcentrifuge. Cells were resuspended in 375 µl ice cold lysis buffer (50 mM Tris.HCl [pH8], 100 mM NaCl, 5 mM MgCl$_2$, 0.5% v/v NP40). After 5 min incubation on ice cells were centrifuged for 2 min in an Eppendorf microcentrifuge and the supernatant transferred to a clean tube containing 4 µl 20% SDS and mixed by vortexing. Next 2.5 µl of freshly prepared proteinase K at 20 mg ml$^{-1}$ was added and following incubation for 15 min at 37°C the preparation was extracted 3 x with 400 µl phenol, adjusted to 0.3 M sodium acetate [pH 5.2] and mixed with two volumes of 100% ethanol. Following o/n incubation at -20°C, RNA was recovered by centrifugation for 10 min at 4°C, rinsed with 1 ml of 75% ethanol/25% 0.1 M sodium acetate [pH5.2], dried and resuspended in 100 µl H$_2$O.
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ii Total RNA from mouse tissues / embryos

Approximately 200 mg of tissue was removed from storage in liquid nitrogen and placed into the corner of a polythene bag containing powdered dry ice. Tissue was shattered with a hammer and transferred into a 15 ml polypropylene tube (Falcon) containing 5.1 ml guanidium thiocyanate denaturing solution (Pharmacia). After thorough mixing the viscous contents were centrifuged at 3000 rpm for 15 min in a Beckmann J6 centrifuge. The homogenate was transferred to a fresh 15 ml tube on ice and passed 3 x through a 19g needle and then 4 x through a 23g needle using a syringe to shear DNA. The homogenate was then layered onto 4.9 ml of CsTFA solution (Pharmacia) in a Beckmann 14 x 89 mm polyallomer centrifuge tube and centrifuged at 27000 rpm for 16 h at 15^\circ C using an SW41 rotor in a Beckmann ultracentrifuge. The liquid was removed by aspiration and the RNA pellet re-dissolved in 200 µl TE buffer (Pharmacia), adjusted to 0.2 M potassium acetate and mixed with 2 volumes 100% ethanol. RNA was recovered as previously described.

j) Sources of prepared RNA

Poly(A)+ RNA from brain, heart, kidney, liver, lung, pancreas skeletal muscle, smooth muscle, spleen and testis was purchased from Clontech. Total RNA from differentiated F9 (DF9) cells was supplied by Tim Cole, GCRC, Heidelberg.

k) RNA Analysis

i Quality checks on RNA preparations

The purity of RNA was assessed by determination of the A_{260}/A_{280} nm ratio with highly pure preparations having a ratio of 1.8 - 2.0. RNA integrity was assessed by denaturing RNA gel-electrophoresis. A 1% agarose denaturing
RNA gel was prepared by dissolving an appropriate quantity of agarose (BioRad) in H₂O. After cooling to ~ 60°C the solution was adjusted to give a final concentration of 20 mM MOPS [pH7], 5 mM sodium acetate and 1 mM EDTA by the addition of 10 x gel buffer stock. Formaldehyde was then added to 2.2 M final concentration and the gel set in a perspex gel casting tray (BioRad) and then soaked for 1 h in running buffer (= 1x gel buffer containing 0.5 μg ml⁻¹ EtBr). Samples composed of 1 μg RNA dissolved in 4.5 μl H₂O were made upto 20 μl by the addition of 10 μl de-ionised formamide, 3.5 μl formaldehyde and 2 μl 10 x gel buffer. Before loading, samples were incubated for 15 min at 55°C and then mixed with 2 μl sample buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol. Gels were run at 100V for 3 h before inspection on a u.v. transilluminator. RNA integrity was confirmed by the presence of bands corresponding to ribosomal RNA.

ii Reverse transcriptase PCR (rt-PCR)

cDNA was synthesised from 100 ng poly(A)+ or from 5 μg total RNA which was dissolved in 9.5 μl DEPC H₂O, incubated for 5 min at 65°C and then placed on ice. For cDNA synthesis the reaction volume was made up to 20 μl containing 50 mM Tris.HCl [pH8], 150 mM KCl, 10 mM MgCl₂, 15 mM DTT, each 0.5 mM dNTP, 15 ng μl⁻¹ ATF COMP1 oligonucleotide primer (see Diagram 2), 30 U RNAsin (Boehringer Mannheim) and 12 U reverse transcriptase (Boehringer Mannheim). After incubation for 1.5 h at 42°C the reaction was stopped by incubation for 10 min at 65°C and 1 μl from the reaction was used for PCR analysis. Typical 50 μl PCR reactions were performed as previously described using ATF COMP2 and either ATF NP or ATF VP (see Diagram 2) each at 2 ng μl⁻¹ (Chapter 3, Figure 7). The following temperature profile was used : 1 min at 90°C, 2 min at 55°C, 2 min at 72°C; the last cycle was performed for 10 min at 72°C. RNA derived from cell lines was
analysed by 30 cycles of PCR whilst RNA derived from adult mouse tissues or whole embryos was analysed by 35 or 40 cycles respectively.

iii RNAse protection

Radiolabelled antisense RNA probe for RNAse protection was generated by *in vitro* translation of 1 |μg linearised pGem3-VATFl in a 25 |μl reaction containing 40 mM Tris.HCl [pH8], 6 mM MgCl2, 2 mM spermidine, 400 |μM ATP, GTP and CTP, 5 |μM 32P-αUTP (800 Ci mM, Amersham), 10 mM DTT, 20 U RNAsin and 10U T7 polymerase. Template DNA was added last of all to reduce spermidine mediated precipitation. Reactions were incubated for 30 min at 37°C followed by the addition of 75 |μl H2O and subsequent extraction with 1 volume of phenol. After adjusting to 2 M ammonium acetate, 20 |μg tRNA was added followed by 2.5 volumes of 100% ethanol. The solution was allowed to freeze in dry ice before the RNA probe was recovered by centrifugation for 5 min in an Eppendorf microcentrifuge. The RNA pellet was dried, resuspended in 5 |μl H2O, mixed with 5 |μl TBE loading buffer and after incubation for 2 min at 80°C was loaded onto a 5% denaturing polyacrylamide gel. For gel purification the probe was located by autoradiography, excised and then transferred to a microcentrifuge tube containing 300 |μl elution buffer (0.1 M Tris.HCl [pH7.5], 0.15 M NaCl, 12.5 mM EDTA and 1% SDS) and 15 |μg tRNA. Following o/n incubation at 37°C the eluate was spun through a 0.45 |μm filter unit (Millipore) and the RNA probe recovered by ethanol precipitation as described above. The RNA pellet was resuspended in 25 |μl H2O and stored at -70°C.

For RNAse protection analysis, RNA samples (10 |μg total or 200 ng poly(A)+) dissolved in 30 |μl of hybridisation buffer (80% de-ionised formamide, 40 mM PIPES [pH6.4], 400 mM NaCl, 1 mM EDTA) and containing 0.5 |μl of antisense RNA probe were incubated for 10 min at 85°C and then for 12 h at 45°C. Samples were then digested by addition of 350 |μl digestion buffer (10 mM Tris.HCl [pH7.5], 300 mM NaCl, 5 mM EDTA) containing 20 |μg ml⁻¹
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RNAse A (USB), 1 μg ml⁻¹ RNAse T1 (USB) and additionally for poly(A)+ samples 6 μg tRNA (to prevent over-digestion) and incubated for 30 min at 30°C. After adjusting to 0.25% SDS and adding 5 μl of freshly made 10 mg ml⁻¹ proteinase K, samples were incubated for 10 min at 37°C followed by phenol extraction and ethanol precipitation with 15 μg carrier tRNA. RNA samples were then dissolved in 2 μl H₂O and 4.5 μl TBE loading buffer, incubated for 10 min at 85°C and resolved in a 7.5% denaturing polyacrylamide gel. Protected fragments were visualised by autoradiography.

2.3 CHAPTER FOUR

a) Plasmids and constructions

pSVEWS-ATF1 expresses EWS-ATF1 under the control of the SV40 early promoter and contains the entire EWS-ATF1 coding sequence and an extended C-terminus containing a seven amino acid T-antigen epitope for the KT3 monoclonal antibody (MacArthur and Walter, 1984). pSVEWS-ATF1 was constructed in stages starting from the plasmid pATF1-T (Masson et al., 1993b) which expresses full length ATF1 (except that the bZIP domain is replaced with the bZIP domain of CREB) under the control of the SV40 early promoter. Firstly a Sal1 partial BsmA1 digest of pATF1-T removed the N-terminal 79 amino acids of ATF1 and was replaced with a Sal1/BsmA1 PCR product encoding amino acids 245-340 of EWS-ATF1. The template for the PCR was clone 712, containing EWS cDNA (a gift from Chris Denny, UCLA Medical Centre, California). A Sma1 site was built into the PCR product to aid the second stage of construction as follows. The intermediate construct was digested with Sma1 and BamH1 and ligated with a Sma1/BamH1 restriction fragment obtained from clone 597 containing EWS-Fli1 cDNA (a gift from Chris Denny) encoding the first 244 amino acids of EWS-ATF1 and also includes 123 bp of 5' untranslated sequence from EWS. Finally the 5' untranslated sequence was
removal to aid construction of the deletion mutants described below. This was achieved by removal of a Sal I/Pst I fragment which includes the 123 bp 5' untranslated region in addition to sequence encoding the first 13 amino acids of EWS-ATF1 and replacement with an oligonucleotide encoding the first 13 amino acids of EWS-ATF1 and the 6 bp of untranslated sequence immediately 5' to the ATG translation initiation codon (GAGAAA). A representation of this construct is shown below.

pSVEWS-ATF1 provided the basis for the construction of a series of deletion mutants.

pΔ77 lacks the N-terminal 77 amino acids of EWS-ATF1 and was obtained by replacing EWS-ATF1 sequence upstream of the BglI site (amino acid 84) with an oligonucleotide encoding amino acids 78 - 83.

pΔ166 lacks the N-terminal 166 amino acids of EWS-ATF1 and was obtained by replacing sequence upstream of the BanI site (amino acid 175) with an oligonucleotide encoding amino acids 167 - 174.
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pΔ209 lacks the N-terminal 209 amino acids of EWS-ATF1 and was obtained by replacing sequence upstream of the BamH1 site (amino acid 245) with a PCR product encoding amino acids 210-244.

pΔ325 expresses the ATF1 portion of EWS-ATF1 (ATF1 amino acids 66-271) and was obtained by replacing sequence upstream of the Xcm1 site (amino acid 426) with a PCR product encoding amino acids 326-425.

All of the N-terminal deletion mutants have the sequence GAGAAAATGGCGTCC incorporated at the N-terminus to provide an efficient translation initiation site and result in the addition of two amino acids at the N-terminus that correspond to the normal N-terminus of EWS-ATF1.

pΔQ2 is lacking amino acids 402-441 of EWS-ATF1 and was obtained by replacing a ScaI/XhoI fragment from EWS-ATF1 encoding amino acids 402-487 with a ScaI/XhoI PCR product encoding amino acids 442-487.

pN1 lacks amino acids 87-325 of EWS-ATF1 and was constructed by replacing a BglI/BsmA1 fragment encoding residues 83-340 with an oligonucleotide encoding amino acids 83-86 and 326-340 of EWS-ATF1. The oligonucleotide incorporated a conservative change which generated a BglII site at amino acid residue 326.

pI1 lacks amino acids 87-166 of EWS-ATF1 and was constructed from pN1 by replacing a BglI/BglII fragment encoding amino acids 83-86 and 326-327 of EWS-ATF1 with a BglI/BglII PCR product encoding amino acids 83-86 and 167-327 of EWS-ATF1.

pΔ(-71)somCAT contains the somatostatin promoter to position -71, fused to CAT coding sequence (Montminy and Bilezikjian, 1987).

pΔ(-42)somCAT was obtained by digestion of pΔ(-71)somCAT with AatII, blunting with T4 DNA polymerase and re-ligation.

pVIP25CAT contains the VIP CRE fused to the RSV promoter from positions -50 to +39 and to CAT (Tsukada, et al., 1987).
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pIE4WT contains the two ATF/E4F binding sites from the adenovirus E4 promoter linked to CAT and is identical to pIE4PM1 and pIE4PM2 except that these harbour point mutations in both ATF/E4F binding sites (Jones and Lee, 1991).

pE4CAT contains all adenovirus E4 promoter sequences between -240 and +32 fused to CAT (Lee et al., 1989).

phGH500CAT contains the human growth hormone promoter to position -500 fused to CAT (a gift from Bernard Peers, University of Liege, Belgium).

pSP72MycCAT contains the c-myc P1 and P2 promoters and ~1500 bp of upstream sequence.

pF711CAT contains the human c-fos promoter linked to CAT (Treisman, 1985).

pSV-EA3 and pSV-EA12 were constructed as follows. pSVEWS-ATF1 was digested with NdeI and XbaI and an NdeI/XbaI fragment derived from pZIP3 or pZIP12, containing a hybrid bZIP domain composed of ZTA amino acids 150-196 and CREB amino acids 294-327 including defined mutations (Ribeiro et al., 1994) was inserted accordingly.

Z7CAT contains 7 binding sites for the Epstein-Barr virus activator ZTA fused to CAT (Carey et al., 1992).

2.4 Chapter Five

a) Plasmids and constructions

pRSVCAT contains the RSV LTR linked to CAT (Gorman et al., 1982).

pCAT TM-Control contains the SV40 enhancer and early promoter linked to CAT (Promega).

pΔERSVCAT is identical to pVIP25CAT (see section 2.3a) except that it does not contain any VIP gene sequence (Tsukada et al., 1987).
pGem3EWS-ATF1 was obtained by ligation of a HindIII/Xba1 fragment from pSVEWS-ATF1 (containing the entire EWS-ATF1 coding sequence) to HindIII/Xba1 digested pGem3.

p somTK contains the somatostatin promoter to position -71 linked to the sequence encoding Herpes Simplex Virus thymidine kinase (HSVtk), cloned into Polyneo which has a selectable neomycin marker. p somTK was constructed in a three way ligation in which a Sal1/HindIII fragment isolated from pΔ(-71)somCAT (containing the somatostatin promoter to position -71) and a HindIII/SpeI fragment from pCRIITK (containing the entire coding sequence of HSVtk) were cloned into a Xho1/Xba1 digested Polyneo vector. Both pCRIITK and Polyneo were gifts from Johnathen Harris, Hammersmith Hospital, London).

b) In vitro transcription and translation

35S-labelled EWS-ATF1 protein was synthesised by in vitro transcription of pGem3EWS-ATF1 (linearised with Xba1) with SP6 RNA polymerase (Boehringer) and subsequent translation in rabbit reticulocyte lysate (Amersham).

c) In vitro labelling and immunoprecipitates

Proteins in heat treated nuclear extracts were labelled as follows. Extracts were adjusted to kinase labelling conditions by addition of 10X kinase buffer (1 M NaCl, 200 mM Tris.HCl [pH 7.5], 120 mM MgCl₂, 25 μM ATP), 1 μCi μl⁻¹ 32P-γ-ATP (5000 Ci mmol⁻¹) and 1 U μl⁻¹ PKA catalytic subunit (Sigma). Labelling reactions were performed at 30°C for 30-60 min. Immunoprecipitations were performed using anti-CREB (Hurst et al., 1990) or anti-ATF1 antibody (Hurst et al., 1991) as follows. Antiserum (1-3 μl/100 μl) was added directly to the labelling reactions and left on ice for 1 h. Immunoprecipitates were collected on protein A-Sepharose beads (Pharmacia)
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(10 μl of packed beads/μl of antiserum added) by mixing end over end at room temperature for 20 min. Following removal of supernatant, protein A-Sepharose beads were washed with five changes (700 μl each change) of wash buffer (25 mM HEPES [pH7.0], 125 mM NaCl and 0.1% NP-40 and finally in wash buffer in the absence of NP-40. Washed beads were resuspended in SDS-PAGE sample buffer, boiled and electrophoresed. Gels were fixed in 50% methanol and dried for autoradiography.

d) Affinity purification and Western blotting

For affinity purification nuclear extracts were incubated batch wise with DNA affinity resin by resuspending the resin several times over a period of 30 min, in a 1.5 ml microcentrifuge tube at room temperature as follows. 200 μl of nuclear extract (derived from ~10^7 cells) was incubated with 20 μl of affinity resin. Incubations were performed in the presence of specific and non-specific competitors (5 μM oligonucleotides containing wild type (WT) or mutated (M) ATF-binding sites and 4 μg ml^{-1} poly [dI/dC]) which were added to the DNA affinity resin before mixing with nuclear extract. After binding, the resin was washed with five changes of affinity column wash buffer (20 mM HEPES [pH7.4]; 100 mM KCl) and SDS-PAGE sample buffer was added directly to the washed resin. Competitor oligonucleotides (the ATF binding site TGACGTCA or corresponding mutated sequence is underlined) were as follows: GGATCCATGACGTCA (WT); GGATCCATGAATTCATGGATC (M).

Western blot analysis was performed with anti ATF1 (Hurst et al., 1991) and anti EWS (a gift from Chris Denny, UCLA Medical Centre, California) antibodies.

e) Selection of stable cell lines incorporating p somTK
Confluent monolayers of DTC1 cells (~5 x 10^6 cells) were transfected with 1 µg of p.somatTK or pGem3 (control cells) by calcium phosphate co-precipitation. After 24 h cells were washed and re-plated in the same volume. After a further 48 h cells were divided four fold into selective media containing 250 µg ml\(^{-1}\) G418 (GIBCO; stored as 100 x stock in sterile filtered 100 mM HEPES [pH7.2]). Selection media was changed daily for the first five days and on alternate days thereafter. Selection was maintained for four weeks after which time 37 macroscopic colonies were evident for cells transfected with p.somatTK whilst all control cells (transfected with pGem3 only) were dead. For preliminary drug sensitivity experiments a mixed population of stably transfected cells was analysed. Genomic integration of p.somatTK was confirmed by PCR using primers designed to amplify a 337 bp band composed of somatostatin promoter and HSVtk sequence.

f) Drug sensitivity assays

A mixed population of cells stably transfected with p.somatTK were assayed \textit{in vitro} for sensitivity to ganciclovir (Syntex Pharmaceuticals Ltd; stored at 5 mg ml\(^{-1}\) in sterile H\(_2\)O). Stably transfected and non-transfected (control) DTC1 cells were plated in triplicate at 40% density into 96 well tissue culture plates with normal media or media supplemented with 5 µg ml\(^{-1}\) ganciclovir. Media was changed daily and at specified time intervals the number of surviving cells was determined by counting in a haemocytometer.

g) Preparation of genomic DNA from tissue culture cells

A confluent plate of adherent cells (~10^7 cells) were trypsinised and washed twice in ice-cold PBS. Cells were resuspended in 1 ml of digestion buffer (100 mM NaCl, 10 mM Tris.HCl [pH8], 25 mM EDTA [pH8], 0.5% SDS and 0.1 mg ml\(^{-1}\) proteinase K added fresh) and incubated with shaking at 50\(^{\circ}\)C for 16 h. Samples were extracted twice with an equal volume of
phenol/chloroform and centrifuged at 3000 rpm for 15 min in a Beckman J6 centrifuge. The aqueous layer was transferred to an Eppendorf microcentrifuge tube to which was added 0.5 vol of 7.5 M ammonium acetate and 2 vol of 100% ethanol. The DNA formed as a stringy precipitate and was recovered by centrifugation at top speed in an Eppendorf microcentrifuge. The pellet was rinsed with 70% ethanol, re-dissolved in TE buffer with gentle shaking at 37°C and then stored at 4°C.
CLONING AND CHARACTERISATION OF AN ALTERNATIVELY SPliced FORM OF ATF1

3.1 Introduction

The objective of this section was to clone cDNAs encoding proteins capable of interacting directly with CREB. CREB binding proteins (CBPs) are important for conferring gene specific regulation (see section 1.14b) and for coupling CREB to the general transcriptional machinery (see section 1.11 and 1.12).

The mouse undifferentiated F9 (UF9) embryonal carcinoma cell line was specifically chosen for the screen. UF9 cells, which resemble pluripotent stem cells of the pre-implantation embryo (Strickland, 1978; Strickland et al., 1980) are refractory to cAMP. Evidence suggests that this is at least partly due to a CREB repressor referred to as ICR (Masson et al., 1992) which interacts with CREB via the leucine zipper to form an inactive heterodimer (Ellis et al., unpublished observations).

Several screening strategies were devised and evaluated to directly clone CBPs from a UF9 λ ZAP cDNA library (see Chapter 2, section 2.2b)

The first was based on PCR using degenerate oligonucleotide primers. The strategy relied on the premise that proteins interacting with CREB through the leucine zipper would, like CREM and ATF1 (see Chapter 1, Figure 3), share high amino acid sequence homology in this domain. The degenerate primers worked well for amplification of positive control CREB phage (data not shown). However with the library non-specific amplification was a problem so the approach was abandoned.

The second strategy involved the protein blotting or Far Western technique. The principle of this method originates from a report which identified a gene encoding a protein antigen following a screen of an expression library with a specific antibody (Young and Davies, 1983). In the Far Western,
the probe is the specific protein of interest (in this case CREB) rather than an antibody and is labelled or else detected indirectly using a specific antibody. The Far Western approach to detect protein-protein interactions and as a basis for cloning has proved highly successful (for review see Phizicky and Fields, 1995) particularly for leucine zipper mediated interactions (MacGregor et al., 1990; Hoeffler et al., 1991; Blanar and Rutter, 1992).

Initially the possibility of using a biotinylated CREB leucine zipper peptide probe was tested. The probe was detected with a streptavidin-alkaline phosphatase conjugate but the system was limited due to excessive non-specific signal.

Ultimately bacterially over-expressed CREB, $^{32}$P-labelled \textit{in vitro} with the catalytic subunit of PKA at the native PKA phosphoacceptor site was used as a probe (see Chapter 2, Figure 1). The details and outcome of the Far Western screen are described in this chapter.

3.2 RESULTS

a) Far Western screen to clone CBPs

Prior to screening the UF9 cDNA expression library, the labelled CREB probe was tested for its ability to detect bacterial plaques derived from a $\lambda$ ZAP phage expressing CREB. This effectively represented a positive control since CREB molecules interact through the leucine zipper to form homodimers. Strong discrete signals were obtained with the positive control but not with a selection of library derived plaques (Figure 1A).

The specificity of the interaction was assessed further with a leucine zipper competition (ZC) assay in which an excess of either CREB or JUN leucine zipper peptide was included during hybridisation of the nitrocellulose filters with probe. Incubation with CREB zipper peptide effectively eliminated the signal obtained with the positive plaques whilst in contrast, incubation with
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the same quantity of JUN zipper, which is unable to dimerize with CREB, had no effect (Figure 1B). Together these results indicate that the interaction detected is highly specific.

With the efficacy of the Far Western protocol using radio-labelled CREB established, the screen of the U937 cDNA expression library was initiated. Approximately 20 000 plaques were screened and 100 positives were identified. Approximately 50 plaques were selected for the phage re-screening and 15 of these were successfully re-amplified.

b) Characterisation of positive clones

To proceed for sequence analysis, a preliminary feature of the positive clones was established.

Firstly, clones were probed with all clones the radiolabelled CREB antibody and this is demonstrated for two clones in Figure 1A. This strongly suggested that the interactions were mediated via the leucine zipper.

Secondly it was established whether any clones encoded ATF1, CREM or indeed CREB, since all could potentially be cloned by the Far Western screen. Phage DNA containing the cloned cDNA, was prepared from λZAP phage using the Stratagene in vitro excision protocol. This was analysed by PCR with primers specific to CREB, CREM and ATF1. Of the 11 positive clones 7 gave a positive PCR product with λZAP phage DNA. However, it was demonstrated that they encoded ATF1 by screening the λZAP phages for a chloramphenicol resistance cassette, which is present in all λZAP phage vectors.

Figure 1. Specificity of the Far Western screening protocol

(A) Nitrocellulose filters lifted from plaques generated by λ ZAP phage encoding either U9 cDNA library or CREB as indicated, were probed with 32P labelled CREB. (B) Zipper competition assay. Nitrocellulose filters lifted from plaques generated by λ ZAP phage encoding CREB were probed with 32P labelled CREB in the presence of 6 μg ml⁻¹ CREB or JUN leucine zipper peptide as indicated.
the same quantity of JUN zipper, which is unable to dimerise with CREB, had no effect (Figure 1B). Together these results indicate that the interaction detected is highly specific.

With the efficacy of the Far Western protocol using radio-labelled CREB established, the screen of the λ ZAP UF9 cDNA expression library was initiated. Approximately $2 \times 10^5$ plaques were screened from which a total of 16 positives were identified. For cloning, positive plaques were isolated and the phage re-screened at low density. Of the original 16 positives 11 were successfully re-amplified (Figure 2).

b) Characterisation of positive clones

To prioritise for sequencing some preliminary features of the positive clones were established.

Firstly, clones were analysed using the ZC assay described earlier. For all clones the radioactive signal was eliminated if an excess of CREB leucine zipper peptide was included during hybridisation with the CREB probe and this is demonstrated for two clones in Figure 3A. This strongly suggested that the interactions were mediated via the leucine zipper.

Secondly it was established whether any clones encoded ATF1, CREM or indeed CREB, since all could potentially be cloned by the Far Western screen. pBluescript plasmid DNA containing the cloned cDNA, was prepared from λ ZAP phage using the Stratagene in vivo excision protocol. This was analysed by PCR with primers designed to distinguish CREB, CREM and ATF1. Of the 11 positive clones, 6 generated a characteristic PCR product of 537 bp using primers specific to ATF1 (ATF IDP and ZIP P, Chapter 2, Diagram 2) suggesting that they encoded ATF1 or were at least ATF1 related. Data for 5 of these clones is shown in Figure 3B).

The remaining 5 clones were not amplified by any of the primers (data not shown). One obvious possibility was that they corresponded to ATF1,
Figure 2: A representative selection of plaque purified clones
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CREM or CREB but the clones were too short to be amplified using the specific primers. An approximate restriction enzyme map supported this possibility as the shortest in the polyclones were about 500 nucleotides. Specificities above 50% were therefore determined by sequencing DNA sequences clones on the basis of PCR (Figure 3B). The sequences were determined using ATFL, which includes the region containing 3' leader nucleotides of an alternative sequence containing three stop codons (Figure 3B).

Given known the multi-exonic structure of both the CREB and CREM genes (Ruppert et al., 1992; Lehn et al., 1993) it was assumed to give rise to numerous protein isoforms by alternative splicing (Gomma et al., 1996) and Laude et al., 1993). The variant form of ATFL, although its gene is unknown, several non-overlapping ATFL genomic fragments had been cloned by Tony Cole in the GCRF, Heidelberg, in a collaborative effort using two primers designed to anneal to the alternative ATFL sequence (VT1 and VT2, see Chapter 2, Diagram 3).

Figure 3  Preliminary characterisation of positive clones

(A) Zipper competition assay. Nitrocellulose filters lifted from plaque purified clones were probed with 32-P labelled CREB either in the presence or absence of 6 μg ml⁻¹ CREB leucine zipper peptide as indicated. Numbers identify individual clones and were arbitrarily assigned. (B) PCR analysis. pBluescript plasmid DNA prepared from λ ZAP phage clones was amplified with primers specific to ATFL and the products analysed by agarose gel electrophoresis alongside DNA size markers (m). As above numbers identify individual clones.
CREM or CREB but the cDNAs were too short to be amplified using the specific primers. An appropriate restriction enzyme digest supported this possibility as the shortest cDNA inserts were present in the 5 unidentified clones (data not shown). Detailed restriction enzyme digests indicated that the 5 remaining clones corresponded to ATF1 and this was later confirmed by sequencing. DNA sequence analysis of the 6 clones suspected to encode ATF1 on the basis of PCR (Figure 3B), revealed that only three were identical to ATF1 (Lee et al., 1992). The others differed from ATF1 towards the N-terminus. Specifically, the sequence which normally encodes the first 64 amino acids of ATF1, which includes the NTR and most of the KID, was replaced by 113 nucleotides of an alternative sequence containing three in frame stop codons (Figure 4).

Given how the multi-exonic structure of both the CREB and CREM genes (Ruppert et al., 1992; Laoide et al., 1993), has been exploited to give rise to numerous protein isoforms by alternative splicing (Yamamoto et al., 1990; Waeber et al., 1991; Rupert et al., 1992; Foulkes et al., 1991a; Delmas et al., 1992 and Laoide et al., 1993) it seemed likely that this mechanism might account for the variant form of ATF1. Although the structure of the ATF1 gene is unknown, several over-lapping ATF1 genomic fragments had been cloned by Tim Cole at the GCRC, Heidelberg. In a collaborative effort using two primers designed to anneal to the alternative ATF1 sequence (VP1 and VP2, see Chapter 2, Diagram 2), a characteristic 90 bp PCR product was amplified from genomic ATF1 fragments (data not shown). Furthermore sequence analysis identified consensus AG and GT splice sites indicating that the alternative 113 bp sequence was derived from a distinct exon within the ATF1 gene (Figure 4). The exon is located between two that encode the KID domain (analogous to the two exons that encode the KID in CREB and CREM, see Chapter 1, Diagram 6) and is actually 117 bp long, with an additional 4 bp lying 3' to the alternative sequence which are identical to the sequence they replace in normal ATF1 (as
Figure 4 The alternative sequence present in the variant form of ATF1 corresponds to a distinct exon in the ATF1 gene. The switch from normal ATF1 sequence (□) to 113 bp of alternative sequence (■) in the variant form of ATF1 replaces 190 nucleotides which normally encode the first 64 amino acids of ATF1. The alternative sequence contains three in-frame stop codons (*) and is derived from a distinct 117 bp exon in the ATF gene as defined by consensus AG and GT splice sites. Functional domains of ATF1 are as described previously.
c) Variant ATF1 encodes an N-terminally truncated form of ATF1.

To determine the structure of the variant ATF1 (vATF1) protein, RNA generated \textit{in vitro} from a vATF1 cDNA clone was translated in rabbit reticulocyte lysate in the presence of $^{35}$S methionine. The labelled products were resolved by SDS PAGE identifying vATF1 as a polypeptide of ~20 kDa (Figure 5B, lane 2).

Since no ATG translation initiation codons are present in the alternative sequence of the cDNA encoding vATF1 (Figure 4), translation must be directed from one of two ATG codons present within the remaining 618 bp of sequence as indicated in Figure 5A. To identify which constitutes the translation initiation site, two constructs, pBV-ATF1-ATG1 and pBV-ATF1-ATG2 were generated in which either the first ATG corresponding to nucleotides 203-205 or the second between 326-328 respectively was changed to ATG. The effect of these changes on the translation of vATF1 revealed that only mutation of the second ATG, corresponding to nucleotides 326-328 prevented translation (Figure 5B, lanes 3 and 4) identifying it as the translation initiation codon. Notably this ATG lies in a context which most closely resembles the nine nucleotide consensus sequence CCACC\textbf{ATGG} (Figure 5A) which is optimal for eukaryotic translation initiation (Kozak, 1986).

vATF1 is therefore an N-terminally truncated form of ATF1 composed of 135 amino acids. The protein retains an intact basic region and leucine zipper together with part of the glutamine rich transcriptional activation domain but lacks all of the KID domain and NTR (Figure 5C).
Figure 5  Translation of vATF1 (A) Potential translation initiation codons. Within the 730 bp of sequence representing vATF1, composed of alternative (■) and normal ATF1 sequence (□) the position of 2 potential ATG translation initiation codons are indicated together with details of surrounding nucleotides. (B) In vitro translation of vATF1. 35S-methionine labelled products from a rabbit reticulocyte lysate translation either in the absence of RNA (lane 1) or else programmed with RNA derived from pBV-ATF1 (lane 2), pBV-ATF1-ATG1 (lane 3) or pBV-ATF1-ATG2 (lane 4) were resolved by SDS PAGE and autoradiographed (see text for plasmid details). (C) Functional regions of vATF1. Functional regions present within the 135 amino acid vATF1 protein are shown along with those in ATF1 for comparison.
d) Expression of vATF1

The expression pattern of vATF1 was analysed initially by the highly sensitive yet relatively simple technique of rtPCR. First an antisense oligonucleotide (ATF COM PI) was used to prime the synthesis of vATF1 and ATF1 (positive control) cDNA from total RNA. The cDNA was then analysed by PCR with a common primer (ATF COMP2) and one specific for either ATF1 (ATF NP) or vATF1 (ATF VP) (Chapter 2, Diagram 2). Analysis of UF9 total RNA in this way generated PCR products of 146 bp and 223 bp corresponding to vATF1 and ATF1 respectively (Figure 6).

Next total RNA from differentiated F9 (DF9) cells was analysed. DF9 cells are derived from UF9 cells following retinoic acid treatment and this event is accompanied by a gain in cAMP inducibility (Rickles et al., 1989; Masson et al., 1992). rtPCR revealed that vATF1 was also expressed in these cells (Figure 6).

With no obvious rationale to preferentially select other samples for analysis, various stages of the developing mouse embryo and a selection of differentiated adult mouse tissues were examined for expression of vATF1. In contrast to ATF1, which was detected in all samples examined, vATF1 was detected only in RNA derived from adult testis indicating that the expression pattern of vATF1 is highly restricted (Figure 6).

Although highly sensitive, rtPCR is difficult to quantitate, therefore samples were subsequently analysed by RNAse protection using a ^32P-labelled antisense RNA probe generated from HindIII linearised pGEM3-VATF1. The probe corresponded to the first 279 nucleotides of vATF1 so both vATF1 and ATF1 mRNA could be identified the former as a protected 279 bp band, the latter of 166 bp. Whilst ATF1 was detected in all samples analysed, a protected fragment of 279 bp, indicative of vATF1, was not observed in any of the RNA samples analysed including those where expression had previously been indicated by rtPCR (Figure 7).
Figure 6. **rtPCR analysis of RNA.** ATF COMP1 was used to generate cDNA from mouse cell lines, adult tissues and stages of embryonic development as indicated. cDNA was subsequently analysed by PCR using ATF COMP2 in combination with either ATF NP (a) or ATF VP (b) to detect ATF1 and vATF1 respectively (see text for primer details). Products of the reaction were analysed by agarose gel electrophoresis alongside DNA size markers (sm). Arrows highlight a 223 bp product corresponding to ATF1 (n) and another of 146 bp corresponding to vATF1 (v). H2O represents water controls.
Figure 7  RNAse protection analysis. RNA samples derived from mouse cell lines, adult tissues and stages of embryonic development as indicated together with tRNA control samples were analysed by RNAse protection using a $^{32}$P-labelled antisense RNA probe, generated from pGEM3-VATF1 (see text for plasmid details). A protected 166 bp band corresponding to ATF1 is indicated by an arrow and was resolved by denaturing PAGE and visualised by autoradiography. Labelled size markers (sm) are indicated to the right.
Together these results suggest that vATFl expression in UF9, DF9 and testis is at a considerably lower level than ATF1.

e) **Transcriptional activity of homodimeric vATFl.**

The transcriptional activity of homodimeric vATFl was analysed using the Triple Mutant (TM) assay which has previously been used to study ATF1 (Ribeiro et al., 1994). The assay overcomes the problem that most cells contain endogenous ATF1 and/or CREB which prevents analysis of exogenous proteins in their native form (see Chapter 1, section 1.11). In the TM assay the original bZIP domain is exchanged for a heterologous, hybrid bZIP domain, either Z3 or Z12. In both bZIP replacements the basic region is derived from the Epstein-Bar virus activator ZTA (Chang et al., 1990) whilst the leucine zipper corresponds to mutant versions of the CREB leucine zipper referred to as ZIP3 and ZIP12 (Loriaux et al., 1993). Whilst the ZTA basic region effectively reprogrammes the DNA binding specificity, the zipper mutations disrupt homodimerisation but allow heterodimerisation of mutated partners.

For the TM assay of vATFl two constructs pZ-VA3 and pZ-VA12 were generated, that express vATFl under the control of the SV40 early promoter but in which the native bZIP domain is replaced with either Z3 or Z12 respectively. To determine the activity of the vATFlZ3 - vATFlZ12 heterodimer, essentially representing homodimeric vATFl, the plasmids pZ-VA3 and pZ-VA12 were co-transfected with a reporter (Z7CAT), containing 7 ZTA binding sites, into JEG3 cells. Homodimeric EWS-ATF1, a transcriptional activator (Chapter 4, Figure 4), generated from expression of the plasmids pSV-EA3 and pSV-EA12 (see Chapter 2, section 2.3a) represented a positive control. In contrast to homodimeric EWS-ATF1 (lane 5), vATFl was unable to activate transcription in this assay (Figure 8).
Figure 8. Transcriptional activity of vATF1.

JEG3 cells were transfected with the Z7CAT reporter plasmid (5 µg) either alone or with a combination of pZ-VA3 and/or pZ-VA12 or pSV-EA3 and pSV-EA12 as indicated (see text for plasmid details). CAT assays were performed 36h post transfection.
3.3 DISCUSSION

a) Far Western Screen to clone CBPs

To clone proteins capable of interacting directly with CREB a protocol based on the Far Western technique using a radio-labelled full length CREB protein probe was employed.

The screening protocol adopted was relatively straightforward particularly since a denaturation/renaturation step was not essential. Moreover the screen proved effective, being both highly sensitive and specific with a very high signal to noise ratio. This was reflected by the relatively few false positives selected from the primary screen of a UF9 cDNA expression library (see section 3.2a). Indeed all successfully re-amplified clones corresponded to bona fide CBPs which interacted through a leucine zipper, a feature suggested first by ZC analysis (Figure 3A) and later confirmed by DNA sequencing.

b) vATFl

Sequence analysis revealed that the clones encode two distinct cDNAs. The first corresponds to the previously described murine version of ATFl (Lee et al., 1992). The remaining cDNA species differed from ATFl in that the sequence which encodes the N-terminal 64 amino acids of ATFl is replaced by 113 bp of sequence derived from a distinct exon within the ATFl gene (Figure 4). The exon maps to an homologous exon boundary in CREB and CREM, lying between the two exons that encode the KID domain (see Chapter 1, Diagram 6) lending support to the notion that CREB, CREM and ATFl arose from a common ancestral gene which has since diverged and acquired unique features. The novel cDNA represents the first example of alternatively spliced form of ATFl and encodes an N-terminally truncated ATFl isoform termed vATFl. vATFl retains the bZIP domain and part of the glutamine rich region but lacks the KID and NTR (Figure 5).
Structurally analogous transcription factors, possessing DNA binding and dimerisation domains but lacking regions required for transcriptional activation and/or regulation have been characterised as repressors. Examples have been described within the bZIP group of transcription factors (Descombes and Schibler, 1991; Mumberg et al., 1991; Nakabeppu and Nathans, 1991) and indeed within the ATF family itself (Foulkes et al., 1991a, b; Delmas et al., 1992; Laoide et al., 1993). Consistent with the predicted role as a repressor, homodimeric vATF1 fails to activate transcription (Figure 8).

In contrast to ATF1 which was expressed in all cells and tissues examined, vATF1 was expressed in a highly restricted manner (Figure 6) and at a far lower level than ATF1 (Figure 7) indicative of a specialised role. Possible functions of vATF1 are discussed later (see Chapter 6).

Although the variant sequence identified in vATF1 was shown to correspond to a distinct exon in the ATF1 gene (see Figure 4), the 5' end of the vATF1 transcript up to and including the transcription initiation site is unknown. Indeed since the variant exon contains three in-frame stop codons it is conceivable that the full length vATF1 transcript incorporates sequence from additional 5' exons and encodes a C-terminally truncated form of ATF1. In this respect it would be similar to the four exons in the CREB gene $\xi$, $\gamma$, $W$ and $\Omega$ which give rise to C-terminally truncated CREB isoforms (Ruppert et al., 1992; Waeber and Habener, 1992).

To identify the 5' end of the vATF1 transcript a rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) cloning strategy should be employed with cDNA primed from a site within the vATF1 exon. Isolation of ATF1 genomic clones will enable the location of the 5' regulatory region of vATF1 to be elucidated. This may correspond to that which also regulates transcription of ATF1. Alternatively it may form a distinct intronic promoter analogous to that in the CREM gene which gives rise to the ICER family of CREM repressors (Molina et al., 1993).
IN VIVO ASSAYS TO REVEAL THE TRANSCRIPTIONAL PROPERTIES OF EWS-ATF1

4.1 Introduction

As outlined in Chapter one (section 1.17) the human tumour MMSP exhibits the specific chromosomal translocation t(12; 22) (q13; q12). This chromosomal rearrangement generates the putative transforming protein EWS-ATF1, a hybrid protein in which the N-terminal 65 amino acids of the transcription factor ATF1 are replaced by the N-terminal 325 amino acids of EWS (see Chapter 1, Diagram 8). EWS-ATF1 is predicted to behave as an aberrant transcription factor de-regulating genes containing an ATF binding site(s), thus leading to transformation. In the simplest scenario the critical genes would be those that are normally regulated by ATF1. Here this possibility is addressed using in vivo assays to characterise the transcriptional properties of EWS-ATF1.

4.2 Results

a) EWS-ATF1 can function as a constitutive transcriptional activator of promoters with ATF binding sites

The EWS-Fli1 fusion protein, composed of the N-terminal region of EWS linked to the ETS domain family member Fli1 acts as an efficient, sequence specific transcriptional activator of target promoters containing ETS binding sites (May et al., 1993b; Ohno et al., 1993; Bailly et al., 1994). To test the prediction that EWS-ATF1 might transcriptionally activate promoters that contain ATF binding sites, a co-transfection assay in JEG3 cells was employed. Initially, a reporter Δ(-71)som containing the somatostatin promoter to position -71 linked to CAT was used. Δ(-71)som contains a single ATF binding site that is critical for transcriptional activation by members of the ATF family.
Transfection of $\Delta(-71)\text{som}$ alone gives very low activity, however co-transfection with increasing amounts of the vector pSVEWS-ATF1 that expresses EWS-ATF1 driven by the SV40 early promoter, results in strong activation (average of 230 fold) in the linear range (Figure 1A).

To test whether activation by EWS-ATF1 is dependent on the ATF binding site, the effect of mutations that eliminate or decrease ATF1 binding was examined (Figure 1B). $\Delta(-42)\text{som}$ lacks the ATF binding site present in $\Delta(-71)\text{som}$ and is not significantly activated by EWS-ATF1, having only 3% of the activity obtained with $\Delta(-71)\text{som}$. A similar result was obtained with a synthetic promoter (IE4WT) which possesses two ATF binding sites. Whilst IE4WT is activated by EWS-ATF1, corresponding promoters IE4PM1 and IE4PM2 containing point mutations that decrease ATF1 binding (Jones and Lee 1991), are not activated (Figure 1C). In conclusion exogenously expressed EWS-ATF1 in JEG3 cells, can act as a constitutive transcriptional activator of promoters that contain ATF sites.

b) Transcriptional activity of EWS-ATF1 deletion mutants

To identify regions in EWS-ATF1 that might be important for transcriptional activity the JEG3 assay was employed to determine the activity of a series of deletion mutants. The structure of deletion mutants are shown in Figure 2.

EWS-ATF1 and all mutants possessed an extended C-terminus containing a seven amino acid T-antigen epitope for the KT3 monoclonal antibody (McArthur and Walter, 1984). Western blot analysis of the epitope tagged mutants in nuclear extracts prepared from transfected JEG3 cells revealed differences in the levels of expression between the different constructs. This may be due to instability associated with the EWS sequence since mutants expressed most highly were those with the least EWS sequence (data not shown). Clearly this phenomenon provided an initial obstacle to accurately
Figure 1 Transcriptional activation by EWS-ATF1 in JEG3 cells

(A) Cells were transfected with Δ(-71)som (5 µg) and increasing amounts (0, 0.3, 1 and 5 µg) of pSVEWS-ATF1 as indicated above the autoradiogram, by calcium phosphate co-precipitation. CAT assays were performed 40 h post transfection. (B) For analysis of promoter mutants, transfections were performed with 5 µg of pSVEWS-ATF1 and 5 µg of reporters containing (Δ(-71)som and IE4WT) or lacking (Δ(-42)som, IE4PM1 and IE4PM2) functional ATF binding sites as indicated above the autoradiogram.
comparing the activity of individual mutants. To circumvent this the absolute quantity of activator plasmid transfected for each mutant was adjusted to obtain uniform expression (Figure 3C).

Regarding the strategy for designing mutants, in the first instance the contribution of the EWS sequence to the transcriptional activity of EWS-ATF1 was determined. Significantly, deleting the entire 325 amino acids of EWS sequence from EWS-ATF1 (Δ325) results in a complete loss of activity, (a representative CAT assay for this and all other mutants is shown in Figure 3A along with a graphical representation of the mean results from triplicate experiments in Figure 3B). Thus the EWS sequence is required for transcriptional activation by EWS-ATF1.

Previous studies have suggested that the N-terminal region of EWS can activate transcription directly when linked to a DNA binding domain (May et al., 1993b; Bailly et al., 1994) or indirectly by allosterically regulating an activation domain present in the fusion partner (Ohno et al., 1993). Since the ATF1 section of EWS-ATF1 contains a previously defined transcriptional activation domain (Q2) (Brindle et al., 1993), it was possible that EWS sequences act allosterically to alter the activity of this domain. However a mutant (ΔQ2), in which this glutamine rich activation domain is deleted, retains much of the activity of the intact fusion protein. Since Q2 is not required for efficient activation by EWS-ATF1 this strongly suggests that the EWS region activates transcription directly.

The amino acid sequence of EWS does not suggest any particularly discrete region that might be important for transcriptional activation. Consequently the effect of making progressive deletions from the N-terminus was examined as a starting point for the functional analysis of this region. Deletion of the N-terminal 77 residues of EWS-ATF1 (Δ77) results in a 21.4 fold reduction in transcriptional activity the mutant retaining only 4.6% of the activity associated with EWS-ATF1. A further deletion of 90 amino acids (Δ166)
Figure 2  Structure of EWS-ATF1 deletion mutants.

N-terminal deletions are named according to the number of EWS amino acids deleted. N1 retains only the N-terminal 86 residues of EWS in addition to ATF1 sequence. I1 harbors an internal deletion between residues 86 and 167. In ΔQ2 amino acids 402-441 of EWS-ATF1 are deleted thus removing the Q2 activation domain of ATF1.
reduces activity only marginally whilst removal of the next 43 residues (Δ209) results in a 5.25 reduction, the mutant having only 0.8% of the activity associated with the intact fusion protein. Δ209 remains 2.5 fold more active than Δ325 which as described above is essentially inactive.

The significant loss of activity resulting from deletion of the most N-terminal 77 residues of EWS sequence (Δ77), suggested that this small region might be responsible for much of the activity associated with the fusion protein. However, a mutant in which all EWS sequence with the exception of the first 86 residues was deleted (N1), retained only 3.5% of the activity obtained with EWS-ATF1. Therefore, whilst this region is clearly critical for transcriptional activity, as indicated by the mutant Δ77, the low activity associated with N1 suggests a requirement for additional regions of EWS. Since residues 167-325 of EWS were implicated as being responsible for essentially all of the remaining transcriptional activity an internal mutant (II) was constructed in which EWS residues 87-166 were deleted. This mutant was 11 fold more active than N1, retaining 39% of the activity associated with EWS-ATF1. Clearly the 89 amino acid region deleted from EWS II, although not contributing to the transcriptional activity of Δ166, is required at least to some extent to produce the full activity associated with EWS-ATF1.

c) Homodimeric EWS-ATF1 can activate transcription

In addition to forming homodimers CREB, ATF1 and CREM can form heterodimers with each other (Foulkes et al., 1991b; Rehfuss et al., 1991). Since JEG3 cells contain endogenous CREB, CREM and ATF1 (Foulkes et al., 1991a; Hurst et al., 1990) exogenously expressed EWS-ATF1 could potentially form several heterodimeric complexes. This makes it difficult to determine the activity of homodimeric EWS-ATF1. To address this question the TM assay was employed, the details of which were described in Chapter 3 where it was utilised to analyse the transcriptional activity of vATF1 (see section 3.2 e). In
Figure 3  Transcriptional activity of EWS-ATF1 deletion mutants

(A) CAT assays. JEG3 cells were transfected with Δ(-71)som (5 μg) and an appropriate quantity of activator plasmid as indicated to obtain uniform levels of expression for each construct (see part C). CAT assays were performed 40 h post transfection and a representative autoradiogram is shown. (B) Quantitation. Graphical representation of the mean results from triplicate experiments with CAT activity for each mutant expressed as a % of that for EWS-ATF1. (C) Expression of mutant proteins. Nuclear extracts were prepared from the same cells used to measure CAT activity and epitope tagged proteins detected by Western blotting using the KT3 monoclonal antibody. Molecular weight standards are indicated to the left.
this case the plasmids pSV-EA3 and pSV-EA12 were constructed which express EWS-ATF1 but in which the native bZIP domain is replaced with either the Z3 or Z12 heterologous, hybrid bZIP domain respectively. To determine the activity of an EWS-ATF1 ZIP3 - EWS-ATF1 ZIP12 heterodimer, (essentially representing homodimeric EWS-ATF1), the plasmids pSV-EA3 and pSV-EA12 were co-transfected with a reporter (Z7CAT) containing seven ZTA binding sites, into JEG3 cells. As expected EWS-ATF1 ZIP3 and EWS-ATF1 ZIP12 alone failed to significantly activate Z7CAT (Figure 4). The slight activity obtained with ZIP3 alone compared to ZIP12 reflects the greater efficiency with which the leucine zipper mutations present in ZIP12 prevent homodimerisation (Loriaux et al., 1993). In contrast, when EWS-ATF1 ZIP3 and EWS-ATF1 ZIP12 are co-expressed Z7CAT is strongly activated (200 fold in the linear range). In conclusion homodimeric EWS-ATF1 is able to function as a transcriptional activator in the TM assay.

d) Promoter specific effects of EWS-ATF1 in JEG3 cells

Using the co-transfection assay in JEG3 cells the ability of EWS-ATF1 to activate other promoters containing ATF binding sites was examined (Figure 5). The VIP (Tsukada et al., 1987) and c-fos (Sassone-Corsi et al., 1988; Berkowitz et al., 1989; Fisch et al., 1989) promoters are regulated by well characterised CREs that contain ATF binding sites. Like Δ(-71)som and IE4WT (Figure 1B) the intact VIP promoter (data not shown) and a synthetic promoter (25CAT) composed of the VIP CRE fused to the RSV promoter from positions -50 to +39, are both strongly activated by EWS-ATF1 (165 fold activation). The c-fos promoter is also significantly activated by EWS-ATF1 (10 fold activation) over and above the high basal activity. In marked contrast to the other promoters examined, EWS-ATF1 represses (activity reduced to 27%) the relatively high basal activity of the ATF dependent adenovirus E4 promoter (Lee and Green, 1987). Two promoters that are not thought to be directly
Figure 4. Transcriptional activation by homodimeric EWS-ATF1
JEG3 cells were transfected with the Z7CAT reporter plasmid (5 μg), pSV-EA3 (ZIP3) or pSV-EA12 (ZIP12) alone or together as indicated. CAT assays were performed 36 h post transfection.

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Figure 5  Differential effects of EWS-ATF1. (A) Cells were transfected with 5 μg of pSVEWS-ATF1 and 5 μg of a specific reporter as indicated above the autoradiograph. CAT assays were performed 40 h post transfection. (B) Cells were transfected with Δ(-71)som or E4CAT with increasing amounts (0, 0.3, 1 and 5 μg) of pSVEWS-ATF1. CAT assays were performed 40 h post transfection.
activated by ATF1 (the c-myc and growth hormone promoter (hGH)) are not significantly activated by EWS-ATF1, further indicating the specificity of activation. In conclusion EWS-ATF1 has distinct effects on promoters containing ATF binding sites, ranging from strong activation to repression.
4.3 DISCUSSION

As a first step towards elucidating the mechanism of transformation in MMSP, some of the transcriptional properties of the presumptive transforming protein EWS-ATF1 have been determined.

Expression of exogenous EWS-ATF1 in JEG3 cells results in the activation of several test promoters that contain intact ATF binding sites (Figures 1 and 5). This conclusion is supported in a recent study which also demonstrates that EWS-ATF1 behaves as a constitutive transcriptional activator (Fujimura et al., 1996).

Transcriptional activation by EWS-ATF1 is strongly dependent on the EWS region of the fusion protein (Figure 3). Previous studies involving other EWS fusion proteins suggest that the N-terminal region of EWS can activate transcription directly by providing a novel transcriptional activation domain (May et al., 1993b; Bailly et al., 1994) or alternatively functions indirectly via the allosteric regulation of an activation domain present in the fusion partner (Ohno et al., 1993, 1994). In the case of EWS-ATF1, the results strongly suggest that this region functions directly since the previously defined glutamine rich transcriptional activation domain present in the ATF1 region of the fusion protein (Brindle et al., 1993), can be deleted without any significant reduction in transcriptional activity (Figure 3). The amino acid sequence of EWS present in EWS-ATF1 reveals features which are typical of several classes of transcriptional activation domains. Notably, this region is particularly glutamine (13.8%) and proline (9.8%) rich and also contains a high proportion of serine (14.8%) and threonine (11.4%) residues all of which are characteristic features of activation domains (see Chapter 1, section 1.4 hi). However whilst the relatively homogenous distribution of these residues does not suggest any particularly discrete region(s) that might be important for transcriptional activity deletion analysis revealed that certain regions within EWS were in fact
significantly more important than others (Figure 3). For instance whilst deletion of the N-terminal 77 residues, results in a major (21.4 fold) reduction in transcriptional activity, deletion of a further 90 amino acids reduces activity only marginally (Figure 3). Another feature of the EWS sequence is the presence of a degenerate motif with the consensus sequence SYGQQS which is repeated 31 times (Delattre et al., 1992). Once again however the relatively homogeneous distribution of this repeat motif argues against any obvious role in activation. The precise nature of the EWS activation domain therefore remains obscure. Interestingly some recent data suggest that EWS mediated allosteric regulation of the section of the ATF1 KID domain retained in EWS-ATF1 may be important for transcriptional activity of EWS-ATF1 (Fujimura et al., 1996). The effect of deleting this region should be examined using the JEG3 assay described here.

The ability of ATF1 to form homodimers and heterodimers with CREB and CREM is a property that is also expected of EWS-ATF1 due to the presence of the ATF1 leucine zipper in the fusion protein. Consequently it is difficult to determine the transcriptional properties of a particular dimeric complex containing EWS-ATF1 in isolation. However using the TM assay originally employed to study ATF1 (Ribeiro et al. 1994) homodimeric EWS-ATF1 was demonstrated to be a constitutive activator (Figure 4). Activation by EWS-ATF1 therefore is not dependent on heterodimerisation with endogenous members of the immediate CREB family. Depending on the complement of potential EWS-ATF1 dimerisation partners in transformed cells (addressed in Chapter five) it might be particularly important to utilise the TM assay to examine the transcriptional properties of the complete repertoire of potential EWS-ATF1 dimeric complexes. This detail might be critical for a precise understanding of the role of EWS-ATF1 in transformation.

In addition to activating promoters containing ATF binding sites exogenous expression of EWS-ATF1 in JEG3 cells reduces the relatively high
basal activity of the adenovirus E4 promoter by 77% (Figure 5B). The reduction in E4 promoter activity appears to be specific given that under the same conditions EWS-ATF1 increases the relatively high basal activity of the c-fos promoter (Figure 5A).

Taken together the results presented in this section indicate that both transcriptional activation or repression could play a role in determining the effects of EWS-ATF1 which ultimately lead to transformation.
Chapter five

ANALYSIS OF MMSP DERIVED CELL LINES

5.1 Introduction

In this chapter the two available MMSP derived cell lines, SU-CCS-1 (Epstein et al., 1984) and DTC1 (Brown et al., 1995) are characterised. The availability of these cell lines presented the opportunity to test some predictions based on the properties of EWS-ATF1 revealed in Chapter 4 and to initiate in vitro experiments to begin testing a potential therapeutic strategy for MMSP.

5.2 Results

a) Detection of EWS-ATF1, ATF1 and CREB in tumour derived cell lines

Despite harbouring the t(12;22) chromosomal translocation characteristic of MMSP and expressing RNA corresponding to the EWS-ATF1 fusion, the presence of the EWS-ATF1 fusion protein had not yet been confirmed in either the SU-CCS-1 or DTC1 cell line. Additionally the expression of proteins such as ATF1 and CREB, which might dimerise with EWS-ATF1 and impact on its properties had also not been analysed in transformed cells.

To start to address this latter issue a previously described assay (Masson et al., 1993a) was employed. Crude nuclear extracts were prepared from DTC1, SU-CCS-1 and also from JEG3 cells and these were ³²P-labelled with protein kinase A (PKA) and then immunoprecipitated with either anti-CREB or anti-ATF1 antibodies (Figure 1A, reproduced with permission of K. Lee). Anti-CREB immunoprecipitates CREB homodimers and CREB-ATF1 heterodimers but not ATF1 homodimers (Hurst et al., 1990) whilst anti-ATF1 is specific for ATF1 (Hurst et al., 1991). As expected both CREB and ATF1 were detected in JEG3 cells and a proportion of CREB was evidently heterodimerised with ATF1 since some ATF1 was co-immunoprecipitated by the anti-CREB antibody.
Similarly, CREB and ATF1 are expressed in both tumour derived cell lines however the ratio of CREB to ATF1 appears to be greater than in JEG3 cells. There is no evidence for the existence of CREB-ATF1 heterodimers in either DTC1 or SU-CCS-1 cells since no ATF1 was co-immunoprecipitated by the anti-CREB antibody.

Since EWS-ATF1 lacks the PKA phosphoacceptor site it could not be detected using the immunoprecipitation assay described above. Instead EWS-ATF1 was detected by purification on DNA affinity resins containing ATF binding sites followed by Western blotting using the anti-ATF1 antibody (Figure 1B). Sequence specific DNA binding is indicated by competition with an excess of oligonucleotide (WT) containing an ATF binding site but no competition by an oligonucleotide (M) containing a mutated ATF binding site. In line with the immunoprecipitation assay (Figure 1A) ATF1 is detected in all three cell lines. In addition to ATF1 an ~ 80 kDa polypeptide reacts with anti-ATF1 and is specific for SU-CCS-1 and DTC1 cells. The following properties demonstrate that this polypeptide corresponds to EWS-ATF1: (1) reaction with anti-ATF1 antibody (2) co-migration on SDS gels with EWS-ATF1 made by \textit{in vitro} translation in rabbit reticulocyte lysate (3) sequence-specific binding to an ATF DNA-affinity resin and (4) recognition by an antibody to EWS (data not shown). The above result also reveals that EWS-ATF1 protein is present in SU-CCS-1 and DTC1 cells at a level that is similar to ATF1.
Figure 1  **Protein analysis in transformed cells.** (A) Detection of ATFl and CREB. Nuclear extracts, prepared from monolayers of different cell lines (indicated at the top) were $^{32}$P-labelled with protein kinase A, immunoprecipitated using anti-CREB (C), anti-ATFl (A) or pre-immune (P) antibodies (as indicated at the bottom), resolved by SDS-PAGE and autoradiographed. CREB and ATFl are indicated to the right of the autoradiogram and molecular weight standards (Biorad pre-stained, low molecular weight range) to the left. (B) Detection of EWS-ATFl. The left hand side shows total $^{35}$S-labelled products from a rabbit reticulocyte lysate translation programmed with EWS-ATFl RNA (+) or minus RNA (-). Molecular weight standards are to the left (Biorad pre-stained, low molecular weight range) and the position of EWS-ATFl is marked with an arrow head. On the right hand side, proteins were purified from nuclear extracts of JEG3, SU-CCS-1 and DTC1 cells by ATFl sequence specific DNA affinity chromatography, in the presence of 1 μM oligonucleotide containing a mutated ATFl binding site (M) or a consensus ATFl binding site (WT) as indicated at the bottom. Western blot analysis was performed using anti-ATFl. ATFl and EWS-ATFl are indicated to the left and molecular weight standards to the right.
b) Activity of ATF-dependent promoters in DTC1 and SU-CCS-1 cells.

In Chapter four it was concluded that the expression of exogenous EWS-ATFI in JEG3 cells has distinct effects on promoters containing ATF binding sites ranging from strong activation to repression. Since tumour derived cell lines express endogenous EWS-ATFI (Figure 1B) the prediction is that promoters that are activated by EWS-ATFI in JEG3 cells should be constitutively active in SU-CCS-1 and DTC1 cells.

To examine this the activity of three promoters previously tested for response to EWS-ATFI in JEG3 cells (Δ(-71)som, 25CAT and E4CAT) was compared with the RSV and SV40 early promoters that are known to be highly active in a variety of cell types (Figure 2). Representative autoradiograms are shown in Figure 2A and quantified results in Figure 2B.

In JEG3 cells (not transfected with EWS-ATFI), Δ(-71)som and 25CAT are expressed at near background levels. (Δ(-71)som has 0.03% and 25CAT has 0.13% of the activity of RSVCAT. E4CAT has a relatively high basal activity (4.9% of RSVCAT) but is further induced by co-expression of the adenovirus E1A protein (5-fold induction) as expected from studies in several other cell types (Jones & Lee, 1991).

The relative level of activity of the same promoters in DTC1 and SU-CCS-1 cells is distinctly different compared with JEG3 cells. In DTC1 cells, Δ(-71)som is the most active promoter (260% of RSVCAT) and 25CAT has relatively high activity (40% of RSVCAT). For both Δ(-71)som and 25CAT, deletion of promoter elements containing the ATF binding site (Δ(-42)som and ΔE respectively) reduces promoter activity to basal levels. This indicates that a factor(s) that interacts with the ATF binding site is required for the high constitutive activity. In contrast to Δ(-71)som and 25CAT, the basal activity of E4CAT in DTC1 cells is similar to that in JEG3 cells (8.6% of RSVCAT) but is strongly induced by E1A (26-fold induction). For SU-CCS-1 cells, the relative activity of the above panel of promoters is remarkably similar to that observed
in DTC1 cells (Figure 2B). Taken together these results demonstrate that two inducible promoters that can be activated when EWS-ATFl is expressed in JEG3 cells (Δ(-71)som and 25CAT) are relatively active in both SU-CCS-1 and DTC1 cells, while another promoter (E4CAT) that is not activated by EWS-ATFl exhibits only basal activity in these tumour derived cell lines.

The combined results strongly suggest that endogenous EWS-ATFl is responsible for the constitutive activity of Δ(-71)som and 25CAT in SU-CCS-1 and DTC1 cells.

c) An approach to a therapeutic strategy to selectively destroy MMSP derived tumour cells

Currently numerous gene therapy strategies are being devised with the aim of attaining the selective destruction of tumour cells (for reviews see Gutierrez et al., 1992; Rosenberg, 1992; Russell, 1993; Salmons and Gunzburg, 1993; Culver and Blaese, 1994). The principle of selective destruction is of core importance and can be attained at the actual transduction event where the gene delivery vehicle (eg retrovirus or liposome), is designed to preferentially recognise tumour cells. Further targeting can be obtained by exploiting the transcriptional differences between normal and neoplastic cells such that the therapeutic gene is predominantly expressed in tumour cells. The extremely high and selective activity of the somatostatin promoter in both DTC1 and SU-CCS-1 cells (Figure 2) which is almost certainly due to EWS-ATFl, meant that this promoter might successfully be used to preferentially direct the expression of a therapeutic gene in these cells.

A Directed Enzyme Prodrug Therapy (DEPT) based strategy was developed. This essentially entails the introduction of a chimeric gene composed of tissue or preferably tumour specific transcriptional regulatory sequences linked to a drug susceptibility ('suicide') gene. The suicide gene encodes an enzyme capable of converting a non-toxic pro-drug into a cytotoxic
Figure 2. Analysis of promoter activity in SU-CCS-1 and DTC1 cells. The activity of test promoters was assayed following transfection of JEG3, DTC1 and SU-CCS-1 cells. (A) Representative CAT assays. Note the absolute amount of activity for different cell types is not comparable due to different transfection efficiencies. (B) Quantitation. For JEG3 cells relative promoter activities are compared with RSVCAT set at 100%. For SU-CCS-1 cells relative promoter activities are compared with A(-71)som set at 100%. For DTC1 cells relative promoter activities are compared with A(-71)som set at 300%. The activity of E4CAT is shown in the absence (E4CAT) and presence (E4 + E1A) of the adenovirus E1A protein. Refer to text for promoter details.
derivative. Some strategies use cytosine deaminase which converts non-toxic 5’ fluorocytosine to the toxic derivative 5-flourouracil (Mullen et al., 1992; Harris et al., 1994). The strategy described here employed the more widely utilised system involving Herpes Simplex Virus thymidine kinase (HSV-tk). HSVtk converts the nucleoside analogue ganciclovir, to a monophosphate derivative which is subsequently phosphorylated to a triphosphate form by cellular kinases. This anabolite is incorporated into newly synthesised DNA resulting in strand breaks and inhibition of DNA polymerase activity which ultimately leads to cell death.

To test the possibility that the somatostatin promoter can be used to express HSVtk in MMSP derived tumour cells and thereby render them susceptible to ganciclovir the plasmid psomTK was constructed. psomTK contains the somatostatin promoter to position -71 linked to the sequence encoding HSVtk within a Polyneo vector conferring neomycin resistance. Stable DTC1 cell lines incorporating psomTK were obtained by transfection of psomTK followed by G418 selection. The selection procedure yielded 37 macroscopic colonies which were pooled and the genomic integration of psomTK confirmed by PCR. (SU-CCS-1 cells were also transfected but no G418 resistant colonies were selected reflecting the observed greater transfection efficiency of DTC1- see Figure 2). The mixed population of stably transfected DTC1 cells was then examined for sensitivity to ganciclovir in vitro (Figure 3). Cells were plated into either normal medium or medium supplemented with 5 μg ml⁻¹ ganciclovir. Control DTC1 cells not transfected with psomTK displayed no adverse effects to ganciclovir, exhibiting indistinguishable growth characteristics in the absence (Figure 3A) and presence (Figure 3B) of the pro-drug. In marked contrast, stably transfected DTC1 cells were clearly sensitive to ganciclovir (Figure 3C and D). Unlike cells in the control groups which grew steadily both in the presence and absence of ganciclovir until confluence was attained at around day 8, stably transfected DTC1 cells treated
For transfections to generate stable cell lines psomTK was not linearised.
Figure 3. Drug sensitivity assays. DTC1 control cells (Figures A and B) or DTC1 cells stably transfected with psomTK (Figures C and D) were plated into medium in the presence or absence of ganciclovir (5μg/ml) as indicated. Cell numbers were counted from triplicate cultures at various time points up to 11 days. Points correspond to mean values and bars represent standard deviations.
with the pro-drug had ceased increasing in number by day six after which numbers decreased progressively so that by day 11 there were fewer cells than had originally been plated.
5.3 DISCUSSION

a) Activity of test promoters in MMSP derived cell lines

In the preceding chapter *in vivo* assays revealed the ability of exogenously expressed EWS-ATF1 to influence the transcriptional activity of test promoters containing ATF binding sites in JEG3 cells. To complement these studies the activity of test promoters was determined in two MMSP derived cell lines (SU-CCS-1 and DTC1), which express endogenous EWS-ATF1 (Figure 1b).

The activity profile of transfected ATF dependent promoters in both tumour cell lines is remarkably similar and significantly correlates closely with the ability to be activated following expression of exogenous EWS-ATF1 in JEG3 cells (Figure 2). This strongly suggests that endogenous EWS-ATF1 is actively de-regulating transcription in these tumour derived cell lines and in the manner which we would predict based on the results obtained using the JEG3 assay in Chapter four. The available evidence thus vindicates that SU-CCS-1 and DTC1 cell lines will serve as useful models in conjunction with the JEG3 assay for understanding transcriptional de-regulation by EWS-ATF1.

b) Expression of CREB and ATF1 in MMSP derived cell lines

Although homodimeric EWS-ATF1 can function as a transcriptional activator (Chapter 4, Figure 4), heterodimeric complexes involving EWS-ATF1 may also exist *in vivo* and might participate in the process of transcriptional de-regulation. To begin to assess this possibility tumour derived cell lines were analysed for expression of CREB and ATF1 which represent potential dimerisation partners for EWS-ATF1. Both factors are present (Figure 1A) with ATF1 apparently expressed at a lower level (although the assay employed is not necessarily quantitative being affected by the degree to which the protein is...
already phosphorylated at the PKA site) and so may form heterodimeric complexes with the fusion protein.

c) Initial in vitro testing of a potential therapeutic strategy for MMSP

The development of DEPT strategies utilising either tissue specific (Vile and Hart, 1993a, b; DiMaio et al., 1994; Smith et al., 1994) or more preferably tumour specific promoters (Harris et al., 1994; Macri and Gordon, 1994), to direct expression of a 'suicide gene' to tumour cells have produced some encouraging results both in vitro and in vivo. Since the somatostatin promoter exhibited extremely high activity in MMSP derived cell lines (Figure 2) and critically, that this activity was almost certainly dependent on the tumour specific factor EWS-ATF1, it seemed likely that a DEPT strategy based on this promoter might be effective against these tumour cells.

To investigate the feasibility of this approach DTC1 cells stably transfected with HSVtk under the control of the somatostatin promoter were selected and the sensitivity of these cells to ganciclovir determined. In complete contrast to non-transfected control DTC1 cells which grew normally, stable transfectants displayed an acute cytotoxicity to the pro-drug (Figure 3). Encouragingly the sensitivity conferred on DTC1 cells is comparable with that attained in other tumour types employing HSVtk DEPT strategies. For example, utilisation of the tyrosinase promoter to direct HSVtk expression to murine melanocytes effected 100% cell death of a stably transfected B16 melanoma clone after 12 days in culture at 1 μg ml⁻¹ ganciclovir (Vile and Hart, 1993b). Significantly the results from the above study were obtained with a single clone which was chosen in preference to 47 others as it was the most sensitive to ganciclovir. If, as is likely, equivalent variations in sensitivity are mirrored in the selected DTC1 clones then the results presented here, which effectively represent the mean response of all 37 colonies, therefore compare even more favourably. To aid future experiments individual clones have since
been isolated which should enable more comparable sensitivity assessments to be made.

The apparent sensitivity of DTC1 cells incorporating psomTK to ganciclovir must be qualified through certain controls to confirm that the effect observed is occurring for the expected reason. Firstly HSVtk expression should be formally demonstrated. Expression could be confirmed at the RNA level by rtPCR, Northern or RNAse protection analysis or ideally at the protein level by Western blotting using a HSVtk specific antibody. Next critically, HSVtk expression (and cellular sensitivity to ganciclovir) must be shown to be dependent on the ATF site present in the promoter of the psomTK 'suicide gene'. An appropriate experiment would be to compare DTC1 cells incorporating psomTK with those incorporating an equivalent plasmid in which the ATF promoter element is deleted.

For successful clinical application the anticipated specificity of this strategy must be rigorously demonstrated. Initially it would be useful to examine the relative cytotoxic effect of ganciclovir on a variety of cell lines incorporating psomTK but which do not express EWS-ATFI. Cell lines derived from particularly sensitive organs such as liver or kidney may serve as especially helpful indicators to evaluate the potential success of this strategy. Ultimately however in vivo experiments should be performed, possibly upon established xenografts in nude mice. The absence of any systemic toxic effects in such an animal model would serve to verify the specificity and therefore potential therapeutic efficacy of the system.
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GENERAL DISCUSSION

The expression of a vast and diverse array of genes with varied biological functions are specifically regulated through a common promoter element - the ATF site. Recent studies have yielded insights into the mechanisms which enable this to be achieved. Two key points to emerge are particularly pertinent to the work presented within this thesis. Firstly the ATF element can be recognised by multiple transcription factors, with diverse functions. Secondly the expression and/or activity of these transcription factors is normally subject to numerous and diverse regulatory controls, critical for the appropriate regulation of target genes.

6.1 Alternative splicing increases the transcriptional versatility of ATF1

a) Cloning of vATF1

Characterisation of the functional elements of ATF1, CREB and CREM reveals a highly modular structure. Whilst some modules share extensive homology, others diverge considerably and confer distinct functional and/or regulatory properties (see Chapter 1, section 1.15 and 1.16). In the case of CREB (Ruppert et al., 1992) and CREM (Laoide et al., 1993) the modular structure and multi-exonic gene organisation, has allowed differential splicing, alternative translation and alternative promoter usage to produce multiple CREB and CREM isoforms with functions ranging from activators to repressors (see Chapter 1, Diagram 6). Expanding the functional repertoire of genes via these mechanisms serves to provide an additional level of complexity and versatility in the regulation of gene control, whilst minimising the proportion of the genome dedicated to the execution of transcriptional regulation. In the case of ATF1 only differential phosphorylation has previously been found to increase the repertoire of factors that might participate in specific transcriptional
responses (see Chapter 1, section 1.16). However in Chapter 3 cDNA encoding an alternatively spliced ATF1 isoform termed vATF1 was cloned.

b) Structure / function of vATF1

vATF1 is an N-terminally truncated form of ATF1 which retains only the bZIP domain and part of the glutamine rich region (see Chapter 3, Figure 5). As discussed in Chapter 3 transcription factors structurally analogous to vATF1, possessing DNA binding and dimerisation domains but lacking regions required for transcriptional activation and/or regulation have been characterised as repressors. Consistent with this homodimeric vATF1 fails to activate transcription (see Chapter 3, Figure 6). The ATF1 gene may therefore be added to the growing list of genes which encode both transcriptional activators and repressors (for review see Foulkes and Sassone-Corsi, 1992).

Could vATF1 correspond to ICR, the predicted dominant inhibitor of CREB in UF9 cells? Evidence indicates that ICR represses CREB through the formation of a leucine zipper mediated inactive heterodimer rather than by competition for promoter element occupancy. Specifically it is found that in the context of a GAL4-CREB fusion, deletion of the CREB leucine zipper causes a dramatic increase in activity in UF9 but not DF9 cells (M. Ellis, unpublished data). In view of the low expression level of vATF1 in UF9 cells and that expression is detected in DF9 cells which are cAMP responsive (see Chapter 3, Figures 7 and 8) it seems unlikely that vATF1 could correspond to ICR. In line with this conclusion evidence has since implicated ATF1 as a candidate for ICR (Masson et al., 1993a). Firstly most if not all CREB in UF9 cells is complexed with ATF1 which is also shown to be the most abundant CBP in these cells. Secondly ATF1 levels decrease during differentiation and this is accompanied by significant changes to the dimer population with CREB/ATF1 heterodimers and ATF1 homodimers predominating in UF9 whilst CREB homodimers predominate in DF9 (Masson et al., 1993a).
In addition to ATF1 Masson et al., (1993a) detected several unknown CBPs in UF9 cells including CBP 100 which interacts independently of the leucine zipper (Masson et al., 1993a). Consequently a larger Far Western screen of the UF9 cDNA expression library, perhaps incorporating a denaturation/renaturation step could be initiated and may result in the cloning of further CBPs.

So what might be the role of vATF1 in UF9 cells? In view of the low expression level (Chapter 3, Figure 7) one possibility is that vATF1 provides an additional, and perhaps more promoter specific level of repression to that imparted by the direct interaction of CREB with ICR. Indeed additional levels of repression are predicted to exist (Masson et al., 1992).

vATF1 expression is also detected in testis (see Chapter 3, Figure 7). Intriguingly various CREB and CREM isoforms also exhibit testis specific expression patterns indicative of an important role for these factors during spermatogenesis. In pre-meiotic germ cells CREM is expressed at low levels in the antagonist α and β forms while from the pachytene spermatocyte stage onward, a splicing event exclusively generates the CREM activator isoform which accumulates at high levels (Foulkes et al., 1992). At the same time CREB expression is switched from CREB α and CREB Δ to CREB αγ and CREB w isoforms which lack the DNA-binding domain and nuclear translocation signal (Yamamoto et al., 1990; Waeber et al., 1991; Ruppert et al., 1992; Waeber and Habener, 1992). In-situ hybridisation analysis should reveal the finer details of vATF1 expression in testis and may provide clues to the role of this factor in spermatogenesis.

Given the evident overlap in structure and function of CREB, CREM, ATF1 and related isoforms it is pertinent to note the significant homology of vATF1 to S-CREM, (see Chapter 1, Diagram 6), the transcriptional repressor expressed specifically in brain (Delmas et al., 1992). Despite the high degree of homology the highly restricted and non-overlapping expression patterns
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associated with vATF1 (see Chapter 3, Figure 6) and S-CREM (Delmas et al., 1992), suggest that specific roles for each factor can be anticipated.

c) Additional alternatively spliced ATF1 isoforms?

Given the extent to which alternative splicing generates novel CREB and CREM isoforms the same process might be anticipated to generate additional ATF1 isoforms. In this respect it may be significant that the novel exon identified in vATF1 contains in frame stop codons (see Chapter 3, Figure 4) similar to the four exons in the CREB gene $\psi$, $\gamma$, $W$ and $\Omega$ which give rise to the C-terminally truncated $\psi$, $\alpha \gamma$, $\gamma$, $\Omega$, $W$ and $\alpha W$ CREB isoforms (Ruppert et al., 1992; Waeber and Habener, 1992). Since the novel exon is located between two exons which encode the ATF1 KID region similar C-terminally truncated isoforms of ATF1 might exist. These could be detected in the first instance by rtPCR using appropriate primers.

Ultimately, the ATF1 gene structure should be determined. This may identify additional novel exons and lead to the identification of further ATF1 isoforms.

6.2 Transformation by EWS-ATF1

a) EWS-ATF1 behaves as an aberrant transcription factor

The combined results presented in Chapters 4 and 5 indicate that the chimeric transcription factor EWS-ATF1 can directly influence the activity of test promoters that contain ATF binding site(s). Specific effects range from strong activation to repression and are consistent with the hypothesis that EWS-ATF1 behaves as an aberrant transcription factor which de-regulates genes that contain ATF binding site(s), thus leading to transformation. A more recent study also demonstrates that EWS-ATF1 can behave as constitutive transcriptional activator (Fujimura et al., 1996).
Similarly to EWS-ATF1, fusion proteins in which EWS is linked to either Fli1 (containing an ETS DNA binding domain) or to the DNA binding domain of the yeast transcriptional activator GAL4, can also activate transcription of promoters containing appropriate binding sites (May et al., 1993b; Ohno et al., 1993; Bailly et al., 1994). Furthermore the chimeric transcription factor TLS/FUS-CHOP involving the EWS homologue TLS/FUS also functions as a transcriptional activator (Sanchez-Garcia and Rabbitts, 1994; Zinsner et al., 1994). Cumulatively this suggests that EWS fusion proteins are relatively promiscuous activators.

Although EWS is believed to be involved in RNA processing rather than transcription, most studies including this one (Chapter 4, Figure 3) indicate that the EWS sequence present in the chimeric transcription factors activates transcription directly (May et al., 1993b; Bailly et al., 1994). This in itself is not too surprising since many proteins, including those not normally involved in transcription are able to activate when fused to a DNA binding domain (Ma and Ptashne, 1987). The precise nature of the activation domain in EWS however remains obscure since the potency of activation, as revealed by the analysis of EWS-ATF1 deletion mutants (Chapter 4, Figure 3), does not simply correlate with the distribution of features typical of several classes of activation domains (for discussion see Chapter 4, section 4.3). Further studies are therefore required to determine the structural features of EWS-ATF1 that enable it to act as a transcriptional activator. Crystallographic analysis might for example reveal the precise structure of the region between amino acids 157 and 262 of EWS. This region shares 40% homology with the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II which interacts directly with the TATA-binding protein (TBP) (Usheva et al., 1992). It has been suggested that this region of EWS might similarly interact with TBP and by doing so promote the formation of a pre-initiation complex (PIC) (Bailly et al., 1994). Additionally, in vitro binding assays should be employed to test for
interactions between EWS-ATF1 and general transcription factors/coactivators. This could be supplemented by a screen based on the two hybrid system which may identify novel EWS-ATF1 binding proteins (for review see Phizicky and Fields, 1995).

As mentioned above, EWS-ATF1 can reduce the transcriptional activity of the E4 promoter (see Chapter 4, Figure 5). Chimeric transcription factors involving EWS have not previously been shown to directly result in transcriptional repression. The mechanism by which EWS-ATF1 reduces the activity of the E4 promoter has not yet been explored however several possibilities can be envisaged. In the context of the E4 promoter EWS-ATF1 might specifically interfere with PIC assembly. Although few distinct repression domains that interfere with this process have been identified one such transferable domain, present in the N-terminus of WT1 (the product of the Wilms tumour gene), is proline and glutamine rich (Madden et al., 1991) and in this respect is similar to EWS. Another possibility is that EWS-ATF1 sequesters or 'squelches' co-factors required for the basal activity of the E4 promoter. If this is the case then one significant implication is that genes need not necessarily possess ATF promoter elements in order to be repressed by the fusion protein.

Although homodimeric EWS-ATF1 can activate transcription (Chapter 4, Figure 4), heterodimeric complexes involving EWS-ATF1 may also exist in vivo and participate in the process of transcriptional de-regulation. Indeed both CREB and ATF1 which represent potential dimerisation partners for EWS-ATF1 are present in tumour derived cell lines (see Chapter Five, Figure 1A). The existence of CREB-EWS-ATF1 heterodimers could be confirmed using the assay described by Masson et al., (1993a) in which dimeric complexes containing CREB are first immunoprecipitated with an anti-CREB antibody, transferred onto nitrocellulose and then detected using a $^{32}$P-labelled CREB probe. Next the TM assay could be employed to determine the transcriptional
activity of these heterodimeric complexes. The TM assay has previously been used to study the transcriptional activity of homodimeric ATF1 (Ribeiro et al., 1994), EWS-ATF1 (Chapter 4, Figure 4) and vATF1 (Chapter 3, Figure 8).

Through the generation of heterodimeric complexes with CREB and ATF1, expression of the fusion protein would also effectively disrupt the normal cellular complement of functional dimers. Even subtle differences in the dimer population might be expected to have a dramatic effect on cellular transcription and this is reflected in the fact that specific mechanisms seem to exist in vivo to establish and maintain a particular dimer population (Lee, 1992). Given that EWS-ATF1 is expressed at a similar level to ATF1 in DTC1 and SU-CCS-1 cell lines (Chapter five, Figure 1B), (even though directed by distinct promoters), the potential impact could be significant. Furthermore it is possible that disruption of one copy of the ATF1 gene by the t(12;22) chromosomal translocation might effectively halve the cellular abundance of ATF1 and thereby impact on the dimer population.

b) Development of a transformation assay for EWS-ATF1

A transformation assay is essential if significant further progress into the process by which EWS-ATF1 results in tumourigenesis is to be gained. Current understanding of EWS-ATF1 mediated gene de-regulation is based on assays utilising exogenous reporter genes. Critically a transformation assay would enable the actual genes that are de-regulated (both directly and indirectly) as a consequence of EWS-ATF1 expression to be identified using techniques such as Representational Difference Analysis (RDA). RDA has recently been used to identify genes de-regulated by EWS-Fli1 (Braun et al., 1995). A transformation assay would also allow identification of the critical regions of EWS-ATF1 required for transformation. One key question is whether the only function of ATF1 is the provision of an appropriate DNA binding domain and if so could the bZIP domain of CREB or CREM effectively substitute for that of ATF1? In
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this respect it may be significant that in Ewing's sarcoma the N-terminal region of EWS can be fused to the DNA binding domain of the Ets family transcription factor FLI-1 (Delattre et al., 1992; May et al., 1993a; Zucman et al., 1993b) or alternatively (though less commonly) to other members of this family namely ERG-1 (Sorenson et al., 1994), ETV-1 (Jeon et al., 1995) and E1AF (Kaneko et al., 1996).

Unfortunately initial attempts to develop a transformation assay for EWS-ATF1 have been unsuccessful. In contrast to EWS-FLI1 which can efficiently transform NIH3T3 cells (May et al., 1993a,b), EWS-ATF1 appears toxic to both NIH3T3 and Rat-1 cells (Chris Denny, Pers. Comm.). This apparent toxicity might contribute to the cell type specific effects of EWS-ATF1 which is not known to be associated with any other malignancies.

Future progress will depend on the identification of cells that are susceptible to transformation by EWS-ATF1. One obvious possibility would be primary cells of neuroectodermal origin which would be similar to those that are affected in MMSP (Chung and Enzinger, 1983; Epstein et al., 1984). Others might include cells derived from tissues in which cAMP enhances or initiates proliferation (for reviews see Boynton and Whitfield, 1983; Dumont et al., 1989). Cells derived from the thyroid and pituitary might prove particularly useful given that subsets of both thyroid (O'Sullivan et al., 1991; Suarez et al., 1991; Parma et al., 1993; Duprez et al., 1994; Michiels et al., 1994) and pituitary tumours (Landis et al., 1989) are associated with situations where specific mutations up-regulate the adenyl cyclase-cAMP pathway (see Chapter 1, section 1.18).

c) Possible target genes de-regulated by EWS-ATF1

Although the actual genes de-regulated by EWS-ATF1 await identification, several key growth control genes are normally regulated through
ATF binding sites by members of the CREB/ATF family and therefore represent potential targets.

One obvious possibility is the cAMP inducible c-fos promoter which as already demonstrated, can be activated by exogenous EWS-ATF1 in JEG3 cells (see Chapter four, Figure 5). C-fos is an immediate early gene, which is induced rapidly and transiently in response to numerous proliferative signals (Muller et al., 1984). The transient nature of the response appears to be critical since continuous high level expression can transform fibroblasts in culture and induce bone tumours in transgenic mice (Miller et al., 1984; Meijlink et al., 1985; Grigoriadis et al., 1993; Miao and Curran, 1994).

Another possible target is the c-jun proto-oncogene promoter which can be regulated by CREB (Lamph et al., 1990). The product of this immediate early gene is a transcription factor which can heterodimerise with Fos and forms part of the AP-1 transcriptional complex (for review see Angel and Karin, 1991). Similarly to Fos, over-expression of Jun results in transformation of rat fibroblasts (Schutte et al., 1989).

The observation that the cyclin D1 promoter is suppressed by cAMP (Sewing et al., 1993) and can be induced by c-jun in a manner that is dependent on an ATF binding site in the cyclin D1 promoter (Herber et al., 1994) indicates that this might represent an important target for EWS-ATF1. Cyclin D1 is thought to be a key player in driving the cell cycle through the G1 restriction point, integrating extracellular signals with the cell cycle clock (for reviews see Sherr, 1993; Hunter and Pines, 1994). Moreover growing evidence highlights cyclin D1 as a putative oncogene with de-regulation of cyclin D1 expression suspected of contributing to several types of neoplasms (Seto et al., 1992; Lammie et al., 1992; Jiang et al., 1992; Wang et al., 1994; Chang et al., 1996; Jacks and Weinberg, 1996).

Another potential target is the proliferating cell nuclear antigen (PCNA) promoter which is transcriptionally regulated through an ATF binding site
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(Labrie et al., 1993; Labrie et al., 1995). PCNA is a critical component of the DNA replication machinery and elevated expression of this factor has been implicated in transformation (Zerlar et al., 1987; Morris and Mathews, 1990; Waga et al., 1994).

Finally, in light of these results, it is necessary to consider a role for the repression ability of EWS-ATF1. In this regard the promoter of the retinoblastoma (RB) gene which encodes the tumour suppressor pRB, contains an ATF binding site which is important for transcription (Sakai et al., 1991) and might be a critical target for the action of EWS-ATF1. Dominant repression of Rb expression could lead to proliferation as a result of lack of activation of inhibitory factors such as TGF-β2 (Kim, et al., 1992) or reduced repression of stimulatory factors such as c-fos (Robbins et al., 1990) or E2F (Chellapan et al., 1991). The idea that repression of the Rb promoter through the ATF pathway might cause transformation is substantiated by the occurrence of oncogenic germ line point mutations of the ATF binding site in the Rb promoter which inhibit binding of CREB/ATF factors (Sakai, et al., 1991).

6.3 Therapy for MMSP

a) Conventional therapy

Recommended treatment for MMSP consists of radical local tumour excision and removal of regional lymph nodes, accompanied by radiotherapy and chemotherapy. Prognosis with these conventional cancer therapies is however poor with many patients developing recurrence or metastasis (Chung and Enzinger, 1983).

b) Molecular Therapy

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Understanding the molecular genetic basis of MMSP offers opportunities to develop novel treatments to supplement existing conventional methods.

i) A DEPT based strategy

In chapter five a molecular therapeutic strategy designed to effect selective destruction of MMSP tumour cells is described. The strategy exploits the transcriptional properties of EWS-ATF1 which were characterised in earlier sections and significantly does not require additional knowledge of the precise molecular mechanism underlying tumourigenesis by EWS-ATF1. Essentially the sequence encoding HSVtk was linked to the somatostatin promoter. This created a potential tumouricidal 'suicide gene' as the somatostatin promoter can be strongly activated by EWS-ATF1 (Chapter 4, Figure 1A) and as predicted, is highly active in MMSP tumour derived cell lines (Chapter 5, Figure 2) which express endogenous EWS-ATF1 (Chapter 5, Figure 1B).

An initial experiment to test the feasibility of this approach produced encouraging results. Specifically, MMSP derived DTC1 cells, stably transfected with the 'suicide gene' acquired significant sensitivity to ganciclovir (Chapter 5, Figure 3). As an immediate priority it will be necessary to confirm that the effect observed in this preliminary experiment is occurring for the reason expected. Thus promoter element dependence and HSVtk expression must be demonstrated. Assuming this is so then it is anticipated that by exploiting the transcriptional properties of EWS-ATF1 in this manner, cytotoxic targeting of MMSP tumour cells should be possible. Critically this means that non-specific delivery of the 'suicide gene' to the tumour site, perhaps using amphotrophic retroviral particles or direct DNA injection, may be tolerated. In vivo experiments using an animal model should now be initiated. The absence of any systemic toxic effects would serve to verify the specificity of the system.
In addition to achieving appropriate expression, any effective future clinical use of this strategy requires that the therapeutic gene is delivered to as many tumour cells as possible, ideally 100%. This in itself represents a major technical challenge (for discussion see Russell, 1993). In this respect however DEPT strategies based on HSVtk mediated conversion of ganciclovir appear to have a unique advantage because of an associated phenomenon termed the 'bystander effect' (Kolberg, 1992). This describes the observation first made by Freeman et al., (1992), that not all cells need be transduced with the HSVtk 'suicide gene' for eradication of the cell population with ganciclovir and has since been reported by others (Ram et al., 1993; Bi et al., 1993; Vile and Hart, 1993b; Barba et al., 1994). The precise mechanism involved is poorly understood however the magnitude of the bystander effect can be significant. Freeman et al., (1993), demonstrated that even if just 10% of a mixed population of cells in vitro or in vivo were HSVtk positive the majority of cells could still be eradicated with similar results reported by Vile and Hart, (1993b). The isolation of stably transfected DTC1 clonal cell lines should permit similar 'mixing' experiments to assess the magnitude of any bystander effect in this system.

Recurrence and metastasis following conventional treatments are major problems associated with MMSP (Chung and Enzinger, 1983). Consequently evidence indicating that HSVtk mediated killing can promote development of a significant anti-tumour immune response (Barba et al., 1994) may be of immense importance. Furthermore it might be possible to enhance any tumour immunogenicity by utilising the DEPT system to express viral antigens or allogeneic MHC molecules on the tumour cell surface. Indeed the potential benefit of such an approach was recently demonstrated in a human clinical trial (Nabel et al., 1993).

In addition to MMSP several other tumours are associated with specific chromosomal translocations that link the N-terminal region of EWS to the DNA binding domain of a particular transcripion factor (see Chapter one, section
1.17). Available evidence indicates that these chimeric transcription factors can strongly activate promoters containing appropriate binding sites (May et al., 1993; Ohno et al., 1993; Bailly et al., 1994; Fujiimura et al., 1996 and this study). Consequently it can be envisaged that the DEPT strategy described here for MMSP may be applicable to these other tumours providing the 'suicide gene' is placed under the control of a suitable promoter.

ii) Other strategies

Numerous studies have demonstrated the anti-proliferative activity associated with specific antisense oligomers which inhibit translation of a gene crucial to the disease process. For example the growth of two Burkitt's lymphoma cell lines was significantly inhibited using a 21 base antisense oligonucleotide directed against c-myc (McManaway et al., 1990). The primary 'Achilles heel' in MMSP is presumably EWS-ATF1 however there is obviously only limited potential to disrupt expression of this factor without affecting expression of the normal copies of the EWS and ATF1 genes. Progress in identifying the genes that are de-regulated in MMSP should reveal other key targets for disruption using antisense strategies (see section 6.2 b and c).

EWS-ATF1 also represents a key tumour specific target for drug discovery programs. The objective here is to identify a molecule capable of blocking the predicted transforming ability of EWS-ATF1 without impairing the normal biological function of EWS and ATF1. Recent technological developments in drug design methods and random screening protocols will facilitate such a project (for review see Dean, 1994).
Published Results


References


References


References


References


Chang, Y.-N., Dong, D. L. Y., Hayward, G. S. and Hayward, S. D. (1990). The Epstein-Barr Virus Zta transactivator: a member of the bZIP family with unique DNA-binding specificity and a dimerisation domain that lacks the characteristic heptad leucine zipper motif. J. Virol. 64, 3358-3369.


References


References


References


References


References


References


References


157
References


References


References


References

family gene, E1AF, to EWS by t(17;22)(q12;q12) chromosome translocation in an undifferentiated sarcoma of infancy. Genes Chromosomes Cancer 15, 115-121.


References


References


References


References


References


References


References


References


References


Ron, D. and Habener, J. F. (1992). CHOP, a novel developmentally regulated nuclear-protein that dimerizes with transcription factors C/EBP and LAP and
functions as a dominant-negative inhibitor of gene-transcription. Genes Dev. 6, 439-453.


References


References


References


References


References


Yoshimura, T., Fujisawa, J. and Yoshida, M. (1990). Multiple cDNA clones encoding nuclear proteins that bind to the tax-dependent enhancer of HTLV-1: all contain a leucine zipper structure and basic amino acid domain. EMBO J. 9, 2537-2542.


