CONTROL OF TRANSCRIPTION IN EMBRYONAL CARCINOMA CELLS

a thesis presented by

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To my parents
ABSTRACT

The aim of this thesis was to examine the mechanisms that regulate transcription during early murine embryogenesis. F9 embryonal carcinoma (EC) cells were used as a model system, since their in vitro differentiated derivatives, the parietal endoderm-like (PE) cells, are similar, by a number of criteria, to cells of the early mouse embryo.

Members of the ATF transcription factor family bind DNA through a domain rich in basic amino acid residues, adjacent to a leucine zip dimerization domain. They are required for unstimulated activity of ATF site-containing promoters but can also modulate transcription in response to diverse stimuli, such as viral trans-activators, intracellular cAMP, and Ca^{2+} ions.

In this study, six ATF site-binding activities were defined in F9 EC and PE cells. They could be distinguished by their electrophoretic mobilities, their regulation during differentiation, and the effect that point mutations in or flanking the ATF site had on their ability to bind DNA. Two promoters containing ATF sites, that of the adenovirus E4 and that of the human vasoactive intestinal polypeptide (VIP) genes, bound different subsets of these activities. This correlated with these promoters' distinct transcriptional activities in vivo, since E4 was active in F9 EC cells and was further activated during differentiation, while VIP was inactive in both cell types. In addition, the regulation of transcription from the E4 promoter in vivo was reflected in a differentiation-specific change in the pattern of ATF site-binding proteins that recognized this promoter in vitro.

Five human cDNAs derived from different members of the ATF family were used to measure ATF RNA levels in F9 EC and PE cells. ATF2, ATF4 and ATF6 RNA levels remained constant during F9 EC cell differentiation, whereas ATF3 RNA levels were higher in PE cells and ATFL transcripts were undetectable in both cell types. This regulation of ATF genes contrasted with that of members of the related API transcription factor family: junD RNA levels were constant during differentiation while cjun and cfos were only expressed in PE cells.
It is known that members of both the ATF and AP1 transcription factor families bind DNA as homodimers or heterodimers with members of their own or the related family. Here it was shown that heterodimerization can not only alter but also broaden DNA binding site specificity.

Finally, after an ATF2-specific anti-peptide rabbit serum was raised, ATF2 protein levels were also shown to remain constant during F9 EC cell differentiation, and a similar group of nuclear polypeptides, with apparent molecular weights of 41-45 kD, was found to be present in a number of different cell types.

This study has therefore shown that the regulation of ATF-dependent transcription during differentiation of EC cells can potentially occur at several levels, including ATF gene expression, as well as differential heterodimerization, binding site preference and promoter specificity of ATF proteins. The significance of these results with respect to transcriptional control during development is discussed.
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LIST OF ABBREVIATIONS

A : adenine ; alanine
Ad5 : adenovirus serotype 5
ADA2 : adaptor gene 2
AFP : α-foetoprotein
αGH : glycoprotein hormone α subunit
AP1 : activating protein 1
APoM : alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins
APoR : alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins
ATF : activating transcription factor
ATP : adenosine 5′-triphosphate
BBF-2 : box B binding factor 2
bHLH : basic domain/helix-loop-helix
bp : base pairs
BSA : bovine serum albumin
BS-KS : Bluescript-KS
bzip : basic domain/leucine zip

C : cytosine ; cysteine
°C : degrees Caelsius
CaM : calcium-calmodulin dependent
cAMP : adenosine 3′:5′-cyclic monophosphate
CAP : catabolite activator protein
CAT : chloramphenicol acetyl transferase
cDNA : complementary DNA
C/EBP : cellular enhancer binding protein
Cf1-a : C element binding factor 1a
CKII : casein kinase II
CMV : cytomegalovirus
CR : conserved region
CRE : cAMP-responsive element
CREB : CRE-binding protein
CREBP-1 : CRE-binding protein 1
CREM : CREB modulator
CTD : carboxy-terminal domain of RPBl
CTF : CCAAT box-binding transcription factor
CTP : cytidine 5'-triphosphate

D : aspartic acid
dbcAMP : N°,2°-O-dibutyryl cAMP
dCREB-A : Drosophila CREB A
DEPC : diethyl pyrocarbonate
DIF : de-ionised formamide
DMEM : Dulbecco's modified Eagle's medium
DNA : deoxyribonucleic acid
DNase I : deoxyribonuclease I
dNTP : deoxynucleotide 5'-triphosphate
dpc : days post coitum
Dr1 : TFIID repressor 1
DRTF-1 : differentiation-regulated transcription factor 1
DTT : dithiothreitol

E : glutamic acid
E1A : adenovirus early gene 1A
E2A : adenovirus early gene 2
E2F : E2A promoter binding factor
E4 : adenovirus early gene 4
E4BP4 : E4 promoter binding protein 4
E4F : E4 promoter binding factor
EivF : E4 promoter binding factor
EC : embryonal carcinoma
ECRE : EC CRE binding protein
EDTA : ethylene diamine tetra-acetate
ELISA : enzyme-linked immuno-sorbent assay

F : phenylalanine
FCS : foetal calf serum

G : guanine ; glycine
g : gramme
GAL4 : galactose catabolism gene activator 4
GATA-1 : GATA motif binding factor 1
GCN4 : general control of nitrogen metabolism gene 4
GHF-1 : growth hormone promoter binding factor 1
GTP : guanosine 5'-triphosphate

H : histidine
h : hours
HAP4 : haeme activator protein 4
HBV : hepatitis B virus
HNF : hepatic nuclear factor
Hox : murine homoeobox gene
HRPαM : horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins
HRPαR : horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins
HS : heparin Sepharose
HSF : heat shock factor
HSV : herpes simplex virus
HTLV-I : human T cell leukaemia virus I

I : isoleucine
ICM : inner cell mass
Id : inhibitor of differentiation
IE : immediate early gene
I-POU : inhibitor of POU
ITR : inverted terminal repeat

K : lysine
kD : kiloDaltons
KLH : keyhole limpet haemocyanin

L : leucine
LCR : locus control region

M : methionine ; molar
Met : methionine
mg : milligramme
μg : microgramme
min : minutes
ml : milliliter
μl : microliter
mM : millimolar
μM : micromolar
μm : micrometre
M_r : relative molecular weight

N : asparagine
NC1 : negative component 1
NC2 : negative component 2
NFkB : nuclear factor binding to the B element of the immunoglobulin κ light chain enhancer
ng : nanogramme
nM : nanomolar
nmol : nanomoles
NP40 : Nonidet P40

Oct : octamer motif binding protein
ORF : open reading frame

P : proline
PBS : phosphate-buffered saline
PCRE : PE CRE binding protein
PE : parietal endoderm-like
PEPCK : phosphoenolpyruvate carboxykinase
Pit-1 : pituitary-specific transcription factor 1
PKA : protein kinase A
PKC : protein kinase C
pmol : picomoles
POU : Pit-1, Oct and Unc86 DNA binding domain
pRb: retinoblastoma gene product
PrIE: pseudorabies virus immediate early protein
PVC: polyvinylchloride

Q: glutamine

R: arginine
RA: (all)trans-retinoic acid
RAP30: RNA polymerase II-associated protein of 30 kD
RAR: retinoic acid receptor
RNA: ribonucleic acid
RNase: ribonuclease
RPB: RNA polymerase B (II) subunit
rpm: revolutions per minute
r.t.: room temperature
RXR: retinoid X receptor

S: serine
SDS: sodium dodecyl sulphate
SDS-PAGE: SDS polyacrylamide gel electrophoresis
SNF: sucrose non-fermenting
Sp1: Sph motif binding protein 1
SRF: serum response factor
SSEA-1: stage-specific embryonic antigen 1
SV40: simian virus 40
SWI: switch

T: thymine; threonine
TAF : TBP-associated factor
TAT : tyrosine aminotransferase
Tax : HTLV-I \textit{trans}-activator protein
TBP : TATA box-binding protein
TFII : RNA polymerase II transcription factor
TFE3 : immunoglobulin enhancer \( \mu E3 \) motif-binding transcription factor
tk : thymidine kinase
TRE : 12-\textit{O}-tetradecanoylphorbol-13-acetate responsive element
TREB : Tax-responsive element binding protein
tRNA : transfer RNA

Y : pyrimidine (C or T) ; tyrosine
YY1 : Ying Yang 1

USF : upstream stimulating factor
UTP : uridine 5'-triphosphate

V : valine ; Volts
VIP : vasoactive intestinal polypeptide
VP16 : HSV virion protein 16

W : tryptophan ; Watts
CHAPTER 1

GENERAL INTRODUCTION
Development of the mouse, as of all higher eukaryotic organisms, relies on the temporally and spatially controlled expression of genes, the products of which determine cellular phenotype and dictate cellular interactions. A major control point of gene expression is the initiation of transcription and, indeed, a number of genes first identified through their importance for normal development of the *Drosophila* embryo, as well as their mouse homologues, are now known to encode proteins that regulate RNA synthesis by binding to the transcriptional control regions of genes in a sequence-specific fashion (reviewed by Biggin and Tjian, 1989; Kessel and Gruss, 1990).

This chapter reviews some aspects of current knowledge about eukaryotic RNA polymerase II-directed transcription and its regulation by transcription factors, through protein/DNA and protein/protein interactions. It also describes the reasons for using the F9 murine embryonal carcinoma cell system as a model for differentiation during early murine embryogenesis, and the relevance of studying the control of adenovirus transcription in this system. Finally, it introduces the ATF (activating transcription factor) transcription factor family, with which the main body of this thesis is concerned; properties of ATF family members are presented in further detail in the General Discussion (Chapter 11), where the experimental results of this thesis are discussed in the context of transcription factor families in general, and their role during murine development.

1.1 Regulation of eukaryotic RNA polymerase II-directed transcription

1.1.1 Cis-acting elements

Transcription of protein-encoding genes by eukaryotic RNA polymerase II usually depends on DNA regions that flank the coding sequences of the gene: these ensure correct initiation of the RNA and regulate its levels. Historically, these DNA sequences have been classified into promoter or enhancer elements. A promoter was originally defined as the
proximal upstream region of the gene that incorporated the transcriptional start site and was necessary for synthesis of uninduced levels of correctly initiated RNA molecules (reviewed by Wasylyk, 1988). An enhancer, on the other hand, could be positioned up to several hundred nucleotides away from the start site in either direction, and regulate the levels of transcription in a tissue-specific or signal-inducible manner (reviewed by Maniatis et al., 1987). However, these elements were soon discovered to be built of smaller nucleotide sequences which were often encountered, though in different combinations, in both promoters and enhancers (reviewed by Dynan, 1989). Excluding the region around the transcriptional start site which is only present in promoters, there therefore do not appear to be any major differences between their composition and that of enhancers; thus, a more valid distinction would be between the basal transcriptional elements and the regulatory elements.

The former are only found in promoters and are responsible for the basal level of correctly initiated transcripts. Two types of such mammalian cis-acting elements have been characterised in protein-encoding genes: the TATA box (nucleotide sequence consensus: TATA(A/T)A), located at approximately nucleotide -30 relative to the transcriptional start site, and the initiator (consensus: YAYTCYY, where Y may be C or T), centered around nucleotide +1 (reviewed by Roeder, 1991). Genes may contain one or the other, or both.

Regulatory elements, on the other hand, of which there is a greater variety, occur further upstream or downstream from the basal elements. They do not function independently of the basal elements but modulate their efficiency in initiating transcription, and thus regulate the levels of RNA transcripts synthesized (reviewed by Hatzopoulos et al., 1988). As discussed in the following sections, the distinct roles of these two classes of transcriptional control elements can be explained by the nature of proteins which bind to their DNA sequences and thereby direct RNA synthesis.
1.1.2 The basal trans-acting factors

Protein-encoding genes are transcribed by RNA polymerase II (pol II), a high molecular weight, multi-subunit enzyme. Genes for all ten subunits, RPBl to RPB10 (RNA polymerase B subunit), have been cloned from *Saccharomyces cerevisiae*: they are single copy genes and are conserved to 30-50%, at the predicted amino acid level, with respect to their homologues from other eukaryotes (reviewed by Woychik and Young, 1990). The two largest subunits, RPBl and RPB2, also bear regions of amino acid similarity to the β' and β subunits, respectively, of the *Escherichia coli* RNA polymerase. The carboxy-terminal domain (CTD) of RPBl, which lies outside these regions of similarity, contains a large number of repeats of the PTSPSTS heptapeptide consensus sequence. The CTD, which, due to its hydrophilicity is predicted to be exposed at the surface of the RPBl subunit, is essential for *S.cerevisiae* viability; its role in the initiation of transcription is discussed below. Interestingly, the three eukaryotic polymerases, I, II and III, share common subunits: for example, pol III contains RPB5, 6, 8 and 10 in common with pol II (reviewed by Gabrielsen and Sentenac, 1991). As will be discussed later, transcription by the three polymerases also requires at least one other common polypeptide, indicating that polymerase specificity relies on combinations of unique and shared components, rather than on three sets of distinct factors.

Pol II alone is not sufficient for correct initiation of transcription; for this, it also requires the general or basal pol II transcription factors (TFIIs) (reviewed by Roeder, 1991). *In vitro* reconstitution studies, with TFII fractions chromatographically purified from cell extracts, have shown that these assemble, in an orderly fashion, around the TATA box (Buratowski *et al.*, 1989; reviewed by Peterson and Tjian, 1992) (Fig.1.1). Binding of TFIID fraction to this DNA sequence is the first step in the formation of the initiation complex. TBP (TATA binding protein), the DNA binding component of TFIID, is the only basal transcription factor to bind DNA directly. TBP cDNAs have been cloned from *S.cerevisiae* (Horikoshi *et al.*, 1989), *Arabidopsis thaliana* (Gasch *et al.*, 1990), *Drosophila melanogaster*
(Hoey et al., 1990) and human cells (Peterson et al., 1990). All these organisms have a single TBP gene (with the exception of A.thaliana which has two closely related genes) that is predicted to encode a 22-39 kD polypeptide, depending on the species. There is 80-90% similarity in the 180 carboxy-terminal amino acids of the various TBPs (Hoffmann et al., 1990). This domain contains two amino acid repeats flanking a basic region; the second repeat overlaps with a region of homology to the E.coli σ70 initiation factor, mapping to the σ70 domain required for recognition of the bacterial TATA box (Horikoshi et al., 1989). TBP binds to the TATA box as a monomer via its two carboxy-terminal repeats (Horikoshi et al., 1990; Reddy and Hahn, 1991); specific amino acid positions in the second repeat determine its specificity for variants of the TATA consensus sequence (Strubin and Struhl, 1992). However, the amino-terminal region of TBP is species-specific in its composition and length; for example, human TBP contains 155 amino acids, whereas yeast only 61 (Hoffmann et al., 1990). Not surprisingly, the domains needed for TATA box binding and transcriptional initiation from TATA box-containing promoters co-localise, by deletion analysis, to the carboxy-terminus, emphasizing the importance of direct contact of TBP with the DNA (Horikoshi et al., 1990). Despite the conservation of the carboxy-terminal domain though, the human TBP carboxy-terminus fused to the yeast amino-terminus cannot support the growth of yeast cells that lack the endogenous molecule (Cormack et al., 1991; Gill and Tjian, 1991). This is most likely due to subtle differences between the two proteins, especially since their in vitro TATA box binding specificities are very similar but not identical (Wobbe and Struhl, 1990). Nevertheless, human TBP is able to activate transcription of at least one tested gene in yeast, and therefore its failure to support growth may be partly explained by the stringency of the viability assay (Strubin and Struhl, 1992).

TBP is also required for transcription by pol I and pol III (reviewed by Sharp, 1992). This has been shown for yeast in vivo and in vitro, using strains carrying temperature-sensitive mutant TBP alleles and assaying transcription of genes by pol I, II and III (Cormack and Struhl, 1992; Schultz et al., 1992). Furthermore, two different single amino acid substitution mutations in or next to the first DNA binding repeat display distinct polymerase
specificities: both of them support pol I but not pol II transcription, consistent with the fact that they do not bind to the TATA box, but only one functions in conjunction with pol III (Schultz et al., 1992). This implies that, though TBP is required by all three polymerases, there are differences in the way it interacts with them to promote transcriptional initiation. That TBP protein is important for pol III-directed RNA synthesis in vitro has also been demonstrated by White et al. (1992), while Comai et al. (1992) have shown that TBP is present in the pol I selectivity factor (SL1). How does TBP bind to pol I- and pol III-driven TATA box-less promoters? A clue to this question is provided by the fact that only SL1 but not TBP alone can restore activity of an extract immunodepleted by anti-TBP antibodies. This is because, in the SL1 fraction, TBP is complexed with three other polypeptides or TBP-associated factors (TAFs): only when these are added to TBP, is SL1 activity reconstituted (Comai et al., 1992). Similarly, TBP recognizes a TATA box-less pol II promoter in association with six TAFs (Pugh and Tjian, 1991). TBP's TATA box-binding ability is therefore not its main activity during the initiation process, but simply one of the mechanisms for tethering it to a promoter. When a TATA box is lacking, binding of TBP near the transcriptional start site presumably occurs indirectly, via its association with TAFs.

The next step in the assembly of the initiation complex is entry of TFIIA, which, though not absolutely necessary for initiation, stabilises TBP binding (Buratowski et al., 1989; Maldonado et al., 1990; Sumimoto et al., 1990). TFIIA is followed by TFIIB, whose presence is required before pol II can join the complex (Buratowski et al., 1989; Lu et al., 1991). Yeast TFIIB is essential for correct in vivo transcriptional initiation of at least two tested genes, since TFIIB− mutant strains produce transcripts initiated at sites downstream from those used in wild type strains (Pinto et al., 1992). TFIIB also contains a region of amino acid similarity to E.coli σ70, namely to the domain that is involved in contacting the bacterial TATA box (Ha et al., 1991). However, since TFIIB has no specific double-stranded DNA binding activity and, furthermore, requires the presence of sequences downstream of +1 in order to complex with TFIID, TFIIA and DNA in vitro (Maldonado et al., 1990), the biological significance of this similarity is unclear.
When pol II binds to the pre-assembled factors, it is already complexed to TFIIF, via the latter's small subunit, termed RAP30 (RNA polymerase II-associated polypeptide of 30 kD) in yeast (reviewed by Greenblatt, 1991). The similarities of the eukaryotic pol II transcriptional machinery with the prokaryotic one, already observed in the two largest pol II subunits, TFIID and TFIIB, are again apparent here, as RAP30 contains sequences with amino acid similarity to $\sigma^{70}$ regions responsible for binding to the polymerase. This sequence similarity reflects a functional similarity, since TFIIF can bind to both the prokaryotic and eukaryotic polymerases. The homology to $\sigma^{70}$ extends further: as $\sigma^{70}$ directs the bacterial RNA polymerase to the promoter by increasing its affinity for specific promoter sequences, so TFIIF prevents binding of pol II to non-specific sites. In addition, the TFIIF-containing initiation complex is also able to "melt" the double-stranded DNA around the start site (Greenblatt, 1991).

Two populations of pol II exist in cells, distinguished by whether or not they are phosphorylated at the CTD (Woychik and Young, 1990). The unphosphorylated form, IIA, preferentially joins the initiation complex; however, it is mostly its phosphorylated derivative, IIO, that is found associated with nascent RNA, suggesting that IIA complexes with the basal transcription factors and is converted to the IIO form before it starts synthesizing the transcript (Lu et al., 1991). In fact, an unphosphorylated CTD, but not its phosphorylated derivative, can bind directly to TBP (Usheva et al., 1992). A number of protein kinases, including $p34^{\text{cdk2}}$, which is involved in cell cycle regulation, are capable of phosphorylating the CTD in vitro (reviewed by Corden, 1990). However, the fact that CTD phosphorylation can occur even in the presence of purified basal factors suggested that an appropriate kinase was present in those fractions (reviewed by Peterson and Tjian, 1992). Indeed, TFIIH, which associates with the initiation complex after the addition of pol II and TFIIF, was shown to contain such an activity, which was enhanced in the presence of the TFIIE fraction (Lu et al., 1992). Phosphorylation of the CTD by TFIIH would therefore lead to release of pol II from the initiation complex after dissociation from the TBP (Usheva et al., 1992), and allow elongation of the transcript. Another component, TFIJI, is also required.
for initiation, but its precise function and polypeptide composition have not been defined (Flores et al., 1992).

The sequence of events leading to initiation from promoters containing an initiator rather than a TATA box have not been fully characterised yet, though two distinct proteins, YY1 (Ying Yang) and TFII-I, have been described that can bind to the initiator and direct transcription from it (Shi et al., 1991; Seto et al., 1991; Roy et al., 1991). TFII-I can function in conjunction with purified TFIID, B, E and F (Roy et al., 1991), and therefore may represent a TFIIA-equivalent for initiator-containing promoters, though its direct and specific DNA binding activity constitutes a distinct difference from TFIIA.

This relatively simple picture of the regular assembly of an initiation complex on a basal element leading to transcription, needs to be qualified in two ways. Firstly, though basal, the TFII factors do not seem to be general. For example, while in vitro transcription from the adenovirus major late promoter required TFIIE, this was dispensable for immunoglobulin heavy chain promoter activity (Parvin et al., 1992). In addition, TFIIG, while chromatographically distinct from TFIIA, could functionally substitute for it during initiation complex assembly (Sumimoto et al., 1990). Secondly, a number of factors are able to repress basal transcription. NCI (negative component I), for example, presumably exerts this effect by binding to TFIID, thus blocking its association with TFIIA, when the three factors are incubated together in vitro (Meisterernst et al., 1991), while NC2 can repress transcriptional initiation by actually displacing TFIIA from a pre-formed TFIID/TFIIA complex (Meisterernst and Roeder, 1991). By contrast, Dr1 (TFIID repressor I) is able to prevent both TFIIA and TFIIIB from complexing with TFIID, by binding to the latter, and thus repress basal transcription in a phosphorylation-dependent manner (Inostroza et al., 1992).

Basal transcription, therefore, occurs as a result of the physical association of pol II with the basal transcription factors and the DNA. Its level can be repressed by proteins that prevent formation of the initiation complex, but also activated by transcription factors which
bind to the DNA upstream or downstream from the initiation complex, at a distance of a few nucleotides to several hundred nucleotides away from it, as discussed in the following section.

1.1.3. The regulatory trans-acting factors

The importance of the regulatory cis-acting elements in transcriptional control lies in their ability to bind trans-acting factors which increase or decrease the activity of the promoter (reviewed by La Thangue and Rigby, 1988b; Levine and Manley, 1989; Renkawitz, 1990). The molecular mechanisms by which they achieve this are beginning to be understood, thanks to the fact that, in many cases, the protein regions that are required for transcriptional regulation, and especially activation, have been characterised, as have the regions necessary for DNA binding (reviewed by Mitchell and Tjian, 1989; Johnson and McKnight, 1989). It has become apparent that these two properties map to distinct and functionally independent parts of the polypeptides. Furthermore, such regions are often similar among different transcription factors, which can therefore be grouped according to the type of their DNA binding and activation domains. Inevitably, similarities in the DNA binding region usually reflect similarities in the DNA sequence recognized. This means that, at least in vitro, closely related DNA binding domains may be able to bind the same DNA motif, as do, for example, members of the ATF family (for example, Hai et al., 1989). The converse is also true: a single transcription factor may bind to more than one related DNA sequences (for example, Cowell et al., 1992). It is possible that this flexibility is exploited in vivo to enhance the repertory of responses mediated by a single cis-acting element, and increase the number of target genes that a single trans-acting factor can regulate.

Eukaryotic protein motifs that bind DNA include the three α-helices encoded by homoeoboxes (found, for example, in octamer binding proteins and transcription factor Pit-1 (pituitary-specific transcription factor 1)) (reviewed by Levine and Hoey, 1988), zinc ion-coordinated polypeptide structures (present, for example, in the glucocorticoid and oestrogen nuclear receptors, and transcription factor Sp1 (Sph motif binding protein 1)) (reviewed by
Vallée et al., 1991; Luisi, 1992), and the α-helix formed by basic domains (as in API (activator protein 1) and ATF transcription factors) (reviewed by Busch and Sassone-Corsi, 1990). It should be stressed that, though the DNA binding domains of transcription factors within the same family may have structural and amino acid similarities and recognize similar DNA elements, they still retain some specificity. This is dictated both by the non-conserved amino acids in the protein and by DNA determinants such as precise nucleotide sequence (for example, Blackwell and Weintraub, 1990), and spacing of the recognition motifs (Nåår et al., 1991; Luisi et al., 1991; reviewed by Rhodes and Schwabe, 1991).

In order to bind efficiently to their sites, some transcription factors require, apart from the domains described above that directly recognize the DNA, additional protein sequences, such as ligand binding and dimerization domains. Nuclear hormone receptors' recognition of their sites, for example, is enhanced by hormone binding which induces a conformational change leading to dimerization through the same ligand-binding region of the receptor (reviewed by Green and Chambon, 1988). CREB (adenosine 3':5'-cyclic monophosphate (cAMP) responsive element binding protein), on the other hand, an activator which contacts the DNA via a basic domain, relies on an adjacent dimerization domain of the "leucine zip" type (see section 1.6) for DNA binding (Dwarki et al., 1990). Furthermore, formation of a CREB dimer is promoted by phosphorylation (Yamamoto et al., 1988); post-translational modifications are therefore also important for transcription factor activity (discussed in detail in Chapter 11).

In addition, transcription factors can be grouped according to the amino acid content of their activation domains. Thus, acidic (eg. in the yeast factor GAL4) (galactose catabolism gene activator 4), glutamine-rich (eg. in Sp1) and proline-rich (eg. in CTF) (CCAAT box binding transcription factor) polypeptide sequences have been shown to activate basal transcription (Mitchell and Tjian, 1989). The significance of amino acid content probably lies in imposing a local structure capable of interacting with the initiation complex. It has been suggested, for example, that the activating function of acidic domains depends on the formation of an amphipathic α-helix, where charged and hydrophobic residues are exposed
on opposite sides, and that net negative charge per se is not important (reviewed by Ptashne, 1988).

This modular nature of transcriptional regulators has been repeatedly demonstrated by the construction of hybrids which combine the DNA binding specificity of one transcription factor with the activation domain of another (reviewed by Frankel and Kim, 1991; Ptashne, 1988), and by the existence of trans-activators, such as viral early gene products (reviewed by Nevins, 1991), which do not bind DNA but contain activation domains and exert their effect by interacting with both regulatory and basal factors (see section 1.1.4 and 1.4).

1.1.4 Mechanisms of trans-activation

A number of observations led to the conclusion that regulatory trans-acting factors modulate transcription levels by physically interacting with components of the initiation complex. For example, it was shown that mammalian ATF, when incubated with TFIID, was able to increase the latter's area of contact with the DNA, and promote the assembly of an initiation complex by stabilising its first step (Horikoshi et al., 1988a; Hai et al., 1988a). GAL4 also modified the interaction of TFIID with the DNA and, furthermore, this effect depended on the presence of a functional activation domain (Horikoshi et al., 1988b).

In other experiments, GAL4, though an activator under physiological conditions, could in fact inhibit the transcription of genes that lacked GAL4 binding sites, when over-expressed in cells (Gill and Ptashne, 1988). This effect was greater with wild type GAL4, and decreased with decreasing trans-activating potential of GAL4 mutants, indicating that both DNA-independent inhibition and DNA-dependent activation of transcription were mediated by the activation domain. Similar results were obtained with the non-DNA binding activator of herpes simplex virus type 1 (HSV), VP16 (virion protein 16) (Triezenberg et al., 1988). This effect, called "squelching", was attributed to the ability of activation domains to interact
with basal factors even in the absence of DNA, thus sequestering them away from a promoter and inhibiting the formation of an initiation complex.

Another characteristic of transcription factor activity that led to the same idea of physical association with basal factors is synergy, that is activation from multiple binding sites to levels higher than those predicted by the addition of single binding site-directed transcription levels. Steroid receptors, for example, bind DNA cooperatively to multiple hormone-responsive sites arranged in tandem, resulting in super-additive levels of activation (Tsai et al., 1989). However, synergistic trans-activation from multiple binding sites, for the same or two different factors, can also occur when the levels of DNA binding proteins are saturating (Lin et al., 1988; Lin et al., 1990; Carey et al., 1990), suggesting that, in this case, it is the activating functions of the factors, rather than cooperation at the DNA binding step, that are responsible for the effect. The proposed interpretation was that the proteins bound to the DNA could all simultaneously contact the initiation complex. Furthermore, a combination of "squelching" and synergy experiments suggested that different classes of activation domains interacted with distinct, though sometimes overlapping, basal components (Martin et al., 1990; Tasset et al., 1990).

Other experiments directly addressed the ability of trans-activators to bind basal transcription factors. Thus, both VP16 and the adenovirus trans-activator protein E1A (early gene 1A) can bind to bacterially expressed yeast TFIID (Stringer et al., 1990; Horikoshi et al., 1991). In addition, VP16 can bind to bacterially expressed human TFIIB, in an activation domain-dependent fashion (Lin et al., 1991). Given that both these non-DNA binding proteins can also interact with trans-regulatory factors, VP16 with Oct-1 (octamer binding protein 1) (Stern et al., 1989), and E1A with ATF2 (Liu and Green, 1990), for example, it was proposed that such viral activators could enhance transcription by contacting both classes of cellular factors. Association with DNA-bound regulatory proteins would bring them in the proximity of the transcriptional start site, to which they would recruit basal factors bound to their activation domains, thus promoting the formation of the initiation complex (Lillie and Green, 1989). DNA binding activators, like GAL4, on the other hand, capable of directly
binding to the promoter, would assist the assembly of the initiation complex by interacting with basal factors through their own activation domains.

The initially defined orientation-independent activity of enhancers can now be explained by the various modes of protein/protein interaction described above, from cooperative DNA binding of regulatory factors to interactions of activators with the initiation complex. Indeed, electron microscopy experiments have demonstrated that long sequences of DNA can be looped out between two transcription factors which are bound at positions hundreds of nucleotides apart but directly contact each other (Théveny et al., 1987; Su et al., 1991; Li et al., 1991). The DNA looping model had been proposed to explain the action of enhancers, in opposition to the sliding model, where transcription factors, once bound to the enhancer, would slide along the DNA and finally contact the initiation complex, and a third model which postulated DNA conformation changes being transmitted from the enhancer to the promoter (reviewed by Ptashne, 1986). The sliding model seemed unlikely to apply to eukaryotic DNA which is rarely totally naked of associated histones. The third model was shown to be inappropriate by the demonstration that an enhancer can function even when attached to a promoter via an avidin-biotin link rather than a phosphodiester bond (Müller et al., 1989). Therefore, proteins bound at a great distance from the initiation site could interact with proteins bound near it by looping out the intervening DNA; in that case, the precise orientation, 5' or 3', of the distal proteins would be irrelevant. A prediction of this model would be that the precise position and orientation of proximally bound proteins would be much more important, since there would be higher thermodynamic costs in introducing sharp bends in small stretches of DNA. Protein/protein interactions of proximally bound factors would be limited by protein and DNA flexibility, hence the importance of orientation for "promoter" cis-acting elements. The fixed orientation of such elements, together with the asymmetric nature of the initiation complex and TBP itself, would then determine the direction of transcription.

Regulatory factors, therefore, can activate transcription by directly associating with basal factors and recruiting them to the promoter. Protein/protein interactions between
transcription factors are also important both for cooperative DNA binding and for bringing a number of activation domains together near the initiation complex.

1.1.5 The effects of transcription factors on nucleosome DNA templates

The observations and conclusions described in the previous section were mostly derived from transcription experiments with DNA that was free of histones and other chromatin proteins. However, in the nucleus, DNA is tightly packed with histones into nucleosomes which are themselves compacted further into higher order structures. Transcriptional initiation \textit{in vitro} is inhibited by nucleosomes; conversely, in yeast cells where histone synthesis has been inhibited, a number of previously inactive genes become expressed (reviewed by Felsenfeld, 1992; Kornberg and Lorch, 1991). DNA binding proteins, therefore, must access their sites in direct competition with histones, before they can exert their effects on transcription. Two non-exclusive models can be envisaged for how such proteins activate transcription in the presence of histones. They could actively compete with, and displace histones in order to contact their binding sites (dynamic competition). Alternatively, binding of transcription factors, for example when the DNA becomes exposed during replication, would preclude histone binding (pre-emptive competition). Both models are supported by experimental data.

GAL4 can bind to its site when this is pre-assembled in a nucleosome, arguing for the possibility of dynamic competition. In contrast, the heat shock transcription factor (HSF) cannot, unless TBP is already bound to the TATA box in the pre-assembled nucleosome (Taylor \textit{et al}., 1991). Such cooperative protein/protein interactions are a recurring feature of transcription factor associations with nucleosomal templates, as seen in the following cases which provide examples illustrating how pre-emptive competition by one factor could promote dynamic competition by a second one.

For instance, if DNA is incubated with a TFIID fraction before nucleosome assembly, transcription can initiate upon subsequent addition of the other basal factors (Workman and
Roeder, 1987). However, addition of TFIID during nucleosome assembly can only lead to initiation if GAL4 is also allowed to bind to the DNA template during the assembly reaction, or if the pseudorabies virus immediate early activator protein (PrIE) is included in the reaction (Workman et al., 1991; Workman et al., 1988). Furthermore, this effect relies on a functional PrIE or a GAL4 derivative containing an activation domain, indicating that contact of the activator with the initiation complex is responsible. Interestingly, in vitro activation by GAL4 derivatives, Spl and VP16 is higher in the presence of histones than in their absence; this correlates with the suppressed basal levels of transcription from nucleosome templates, and may partly explain the fact that trans-activation effects observed in vivo are generally higher than those obtained in vitro (Laybourn and Kadonaga, 1991; Workman et al., 1991).

DNA regions that are hypersensitive to deoxyribonuclease I (DNase I) in vivo, and therefore presumably not packed into chromatin, do exist, like, for example, the β globin locus control region (LCR) (reviewed by Orkin, 1990; Felsenfeld, 1992). The LCR, positioned more than 20 kilobases upstream from the globin genes, contains binding sites for many transcription factors and, in transgenic mice, confers high level expression of the trans-gene independently of its position of integration. Presumably, proteins recognizing the LCR cause cooperative binding of other factors further downstream, leading to transcriptional activation. The LCR does not function as an "enhancer" in transient transfections; its influence is only observed after stable integration in the genome, arguing that it has a chromatin-mediated effect.

One of the mechanisms, therefore, by which trans-activators can enhance transcription, is by promoting the binding of basal factors on nucleosomal DNA templates.

1.1.6 Co-activators

As mentioned in section 1.1.4, some transcriptional activators have been shown to contact the initiation complex directly in direct binding assays. However, trans-activation
experiments using the products of cDNAs encoding basal and regulatory factors subsequently indicated that other proteins, in addition to the TFIIIs, may in fact be required to mediate the effects of the regulatory proteins (reviewed by Lewin, 1990).

In *in vitro* transcription studies, it was observed that recombinant yeast TBP, fractionated yeast TFIID, recombinant *Drosophila* TBP or recombinant human TBP, did not support binding site-specific *trans*-activation by purified human Sp1 (Hoey *et al.*, 1990; Peterson *et al.*, 1990; Pugh and Tjian, 1990). These were perfectly adequate for basal levels of transcription, but only human and *Drosophila* TFIID fractions were able to also mediate stimulation by Sp1. In addition, when a heat-treated nuclear extract, which lacks TFIID activity but contains all other necessary factors, was used, only a full length human TBP, but not a deletion mutant that lacked the species-specific amino-terminus, restored the response to Sp1. Two conclusions can be drawn from these and similar experiments with other transcription factors, including VP16 (Kelleher *et al.*, 1990; Berger *et al.*, 1990), USF (upstream stimulatory factor) (Hoffmann *et al.*, 1990) and CTF (Pugh and Tjian, 1990). Firstly, proteins, termed co-activators, mediators or adaptors, distinct from the previously characterised basal and regulatory factors, are required for stimulation of transcription above basal levels. Secondly, the interactions of co-activators with the initiation complex, and specifically with TBP, can be species-specific, and mediated by the TBP amino-terminus. It was clear, however, that whereas the co-activator(s) defined by the glutamine-rich activator Sp1 were associated with endogenous TBP (Peterson *et al.*, 1990), those required by acidic activators in yeast were not (Kelleher *et al.*, 1990; Flanagan *et al.*, 1991).

The discrepancy between the large chromatographic size of native TFIID fractions (Reinberg *et al.*, 1987) and the relatively small molecular weight of TBP (see section 1.1.2) had already suggested that native TBP was complexed with other cellular proteins. Indeed, two distinct cellular fractions of 300 and >700 kD molecular weight, contained TBP protein (Timmers and Sharp, 1991). However, though they could both reconstitute basal transcription, the 300 kD fraction responded extremely poorly to *trans*-activation by Sp1, USF or an acidic activator. A number of TAFs have now been characterised from human (Pugh and Tjian,
and Drosophila cells (Dynlacht et al., 1991) and shown to be required for stimulated but not basal transcription. TAFs, therefore, have multiple roles, including bringing TBP in the proximity of the initiation site (see Section 1.1.2 above), and mediating activation by regulatory factors. Consistent with the observation that co-activators for acidic domain transcription factors were not present in a TFIID fraction (see above), the isolated human TAFs could mediate trans-activation by the proline-rich CTF and the glutamine-rich Spl but not the acidic VP16 (Tanese et al., 1991). Instead, a genetic screen in yeast yielded a protein, ADA2 (adaptor 2), necessary for transcriptional response to VP16 and the also acidic GCN4 (general control of nitrogen metabolism gene 4) both in vivo and in vitro (Berger et al., 1992); interestingly, ADA2 was dispensable for activation by HAP4 (haeme activator protein 4), which also possesses an acidic activation domain. Finally, an activity that potentiated trans-activation co-fractionated with a separable activity that inhibited basal transcription, suggesting that negative control of unstimulated transcription may be a prerequisite for positive regulation (Meisterernst et al., 1991).

A number of bona fide co-activator genes, such as SWI1 (switch 1), SWI2/SNF2 (sucrose non-fermenting 2), SWI3, SNF5 and SNF6, that do not encode DNA binding proteins but are necessary for the transcription of many different genes, have been characterised in yeast (Peterson and Herskowitz, 1992; Laurent and Carlson, 1992). However, there seems to be some specificity in this effect since, for example, SWI1 is required for activity of a promoter containing a GAL4 but not a SWI4/SWI6 binding site (Peterson and Herskowitz, 1992). Furthermore, some of the encoded proteins act cooperatively, suggesting that they could be present in the same functional complex (Laurent and Carlson, 1992).

The requirement for protein/protein interactions for transcriptional regulation therefore seems to also involve molecules that act as a "bridge" between the DNA-bound basal and regulatory factors.
1.1.7 Repression of transcription

The term "repression" covers two distinct phenomena: repression of basal transcription (see section 1.1.2) and inhibition of trans-activation. The latter can in turn be due to a number of different mechanisms. For example, proteins that share a common binding site with an activator can compete with it for binding to the DNA and thus prevent trans-activation, as is the case for some Drosophila homoeodomain proteins (Jaynes and O'Farrell, 1988; Han et al., 1989). Alternatively, an activator, e.g. the thyroid hormone receptor, may bind to its own site but not activate transcription because it is in a non-active conformation, e.g. in the absence of thyroid hormone (reviewed by Levine and Manley, 1989). Finally, a DNA binding protein may simply be unable to interact productively with the initiation complex. This effect may be concentration-dependent, as for the Drosophila Krüppel protein, which activates at low and represses at high concentrations (Sauer and Jäckle, 1991), or position-dependent, as in the case of YY1, which is an activator when bound to the initiator but represses when bound upstream of a TATA box in a synthetic promoter (Shi et al., 1991; Seto et al., 1991). Protein domains specific for repression have not been as extensively characterised as activation domains, though an alanine-rich region in Krüppel has been implicated in its repressor function (Licht et al., 1990).

Finally, trans-activation can be prevented by sequestering activators into inactive complexes. Three such mechanisms have been described so far and are discussed in greater detail in Chapter 11. Briefly, IκB, the inhibitor of NFκB, a transcription factor initially described as binding to the B element of the immunoglobulin κ light chain enhancer, binds NFκB in a cytoplasmic complex and prevents it from reaching the nucleus and activating transcription (Bauerle and Baltimore, 1988). CREM (CREB modulator) proteins, on the other hand, bind to the ATF site as homodimers or heterodimers with CREB; some CREM isoforms prevent trans-activation presumably because they lack an activation domain (Foulkes et al., 1991). Finally, Id (inhibitor of differentiation) heterodimers with basic
domain/helix-loop-helix (bHLH) trans-activators are unable to bind DNA since Id lacks a basic domain (Benezra et al., 1990).

Thus, although some proteins with an inherent ability to repress transcription have been described, inhibition of trans-activation can clearly also occur by DNA-dependent and -independent antagonists of activating transcription factors.

Three classes of proteins are therefore necessary for regulated transcription: the general factors, including pol II, which assemble near the initiation site, activators or repressors that may or may not bind to the DNA at varying distances from the initiation complex, and co-activators which "mediate" between the two previous classes (reviewed by Lewin, 1990) (Fig. 1.2). Transcriptional control relies not only on the interactions of these proteins with the DNA but also on positive and negative interactions among them.

1.2 Early differentiation events during murine embryogenesis

The first two cell divisions after fertilisation of the mouse oocyte give rise to cleavage cells that remain loosely associated. These blastomeres are equipotent and totipotent: any one of them can give rise to a whole embryo. However, after the third cleavage, the eight blastomeres compact, resulting in a structure, the morula, which has a clear external surface and an internal core. In the morula, intercellular junctions are established and cells on the outside layer acquire distinct polar and basal membranes, accompanied by cytoskeletal and organelle re-organisation. This polarisation and the subsequent exposure of cells to different microenvironments, depending on their position, underlie the first differentiation event which occurs after the fourth cleavage. Cells on the inside become the inner cell mass (ICM), while cells on the outside form an epithelial layer, the trophoblast, surrounding the ICM and a fluid-filled cavity, the blastocoel (Fig. 1.3). These two new groups of cells have distinct developmental fates: the trophoblast will give rise to exclusively extra-embryonic, supporting structures while the ICM will contribute to both embryonic and extra-embryonic lineages.
After the 4.5 days post coitum (dpc) blastocyst has hatched out of the zona pellucida, and before it implants into the uterus, both ICM and trophoblast undergo further differentiation. Depending on their position, trophoblast cells become polar or mural trophectoderm, in contact with the ICM or the blastocoel, respectively. ICM cells exposed to the blastocoel are then organised into a layer of primitive endoderm, restricted to the extra-embryonic lineage, surrounding the rest of the ICM cells which differentiate into primitive ectoderm or epiblast cells, destined to form the embryo proper (Fig. 1.3). After implantation, cell movement and differentiation intensify. Primitive endoderm cells proliferate to coat the expanding primitive ectoderm and become visceral endoderm, or line the mural trophectoderm and become parietal endoderm (Fig. 1.3) (reviewed by Hogan et al., 1986; Jackson, 1989). Parietal and visceral endoderm have distinct functions and, consequently, morphological and biochemical properties (reviewed by Hogan et al., 1986). Visceral endoderm cells form an epithelial layer around the embryonic ectoderm, displaying microvilli on their apical surfaces, and other specialised morphological characteristics. Their main functions are to absorb substances from the maternal circulation and to secrete substances required by the foetus, such as α-foetoprotein (AFP). Parietal endoderm cells, on the other hand, are not in close contact with each other, are motile, and display a number of morphologies, from stellate to round (Hogan et al., 1983). Their most important function is to secrete Reichert's membrane which serves as a barrier between the foetal and the maternal environments. For this, they synthesize large amounts of basement membrane proteins, such as laminin and type IV procollagen.
1.3 F9 embryonal carcinoma cells

1.3.1 F9 embryonal carcinoma cell differentiation as a model for early differentiation events in the mouse embryo

F9 embryonal carcinoma (EC) cells are ultimately derived from a 6 dpc male embryo which had been transplanted into the testis to give rise to the transplantable tumour OTT6050 (Bernstine et al., 1973). They have a very low rate of spontaneous differentiation. However, when treated in vitro with (all)trans-retinoic acid (RA) in monolayers or as aggregates, they differentiate into primitive endoderm-like or visceral endoderm-like cells, respectively (Fig. 1.4). If compounds that elevate intracellular cAMP levels, such as N\textsuperscript{6},2\textsuperscript{-}O-dibutyryladenosine 3\textsuperscript{'}:5\textsuperscript{'}-cyclic monophosphate (dbcAMP), are also added to F9 EC cell monolayers being treated with RA, these differentiate further into parietal-endoderm-like (PE) cells. Though it is not clear whether undifferentiated F9 EC cells are ICM-like, the similarities between F9 PE cells and parietal endoderm cells found in vivo have been well established. Like them, they are flat cells, often with long processes, which do not contact each other. They secrete tissue plasminogen activator, type IV procollagen and laminin, have low levels of alkaline phosphatase and lactate dehydrogenase activity, and do not synthesize AFP (Strickland and Mahdavi, 1978; Strickland et al., 1980; Hogan et al., 1981). F9 EC cells, therefore, provide a good model system for studying the molecular mechanisms of early differentiation events in the mouse embryo.

1.3.2 Regulation of viral transcription in F9 EC cells

F9 EC cells are particularly suitable for the study of transcriptional regulation since the activities of a number of viral transcriptional control sequences are regulated during their differentiation. The advantage of using viral, instead of cellular, control elements to study transcription is that they are small and well defined. Since viruses use their hosts'
transcriptional apparatus, proteins important for viral transcription will also be responsible for controlling the expression of host genes (reviewed by Jones et al., 1989). Thus, viral transcriptional control regions whose activity is altered during the differentiation of EC cells can be used as probes for cellular transcription factors which are regulated during differentiation, and which will also affect the expression of cellular genes. For example, the simian virus 40 (SV40) and the polyoma virus A enhancers are inactive in undifferentiated EC cells but enhance transcription in their differentiated derivatives. This has been attributed both to the presence of enhancer-binding repressors of transcription in, and the absence of enhancer-binding transcriptional activators from, EC cells (Herbomel et al., 1984; Gorman et al., 1985; Sleigh and Lockett, 1985; Kryszke et al., 1987; La Thangue and Rigby, 1987; Sleigh et al., 1987; Wasylyk et al., 1988). Similarly, transcription of murine leukaemia and sarcoma viruses occurs only after differentiation of EC cells, and its repression in undifferentiated cells has been correlated with the binding of EC cell-specific proteins to its transcriptional control sequences (Linney et al., 1984; Gorman et al., 1985; Loh et al., 1988; Loh et al., 1990; Akgün et al., 1991). On the other hand, transcription from the adenovirus type 5 (Ad5) E2A (early gene 2A) promoter occurs in F9 EC cells (Impériale et al., 1984), in mouse oocytes and in pre-implantation embryos (Suemori et al., 1988; Dooley et al., 1989) in the absence of viral E1A, the trans-activator of adenovirus early genes, which is normally required in other cell types (Jones and Shenk, 1979). This led to the suggestion that a cellular "E1A-like" activity exists in these experimental systems; when assayed by Ad5 E2A gene expression, it is seen to be reduced after the differentiation of F9 EC cells or implantation of the mouse embryo (Impériale et al., 1984; Suemori et al., 1988; Dooley et al., 1989). The analogy between transcriptional regulation in F9 EC cells and cells expressing adenovirus E1A extends further. In F9 EC cells, the hsp70 gene, which is activated by E1A, is constitutively expressed (Impériale et al., 1984). In contrast, and again similarly to the situation in F9 EC cells, E1A represses the SV40 and polyoma enhancers but not their promoters (Velcich and Ziff, 1985; Velcich et al., 1986). However, it is unlikely that F9 EC cells contain an E1A "look-alike" molecule, since expression of E1A in these cells leads to
their differentiation, rather than inhibits it (Montano and Lane, 1987). It is more probable, therefore, that the "EIA-like" activity is the result of a number of biochemical activities specific to the undifferentiated state.

1.4 The adenovirus transcriptional regulator E1A

Trans-activation by E1A has been extensively studied (reviewed by Berk, 1986). The protein domain responsible for trans-activation was originally mapped to conserved region 3 (CR3), a region of homology among all adenovirus serotypes, and unique to the product of the largest, 13S, EIA RNA (reviewed by Moran and Mathews, 1987). CR3 has been shown to be necessary and sufficient for transcriptional stimulation by E1A, in vivo and in vitro, and in the absence of protein synthesis (Lillie et al., 1987; Green et al., 1988). Since E1A does not bind DNA in a sequence-specific fashion (Ferguson et al., 1985; Chatterjee et al., 1988), these results suggested that it regulates transcription by modifying the binding and/or activation properties of existing cellular factors. During the attempt to identify those cis-acting sequences in E1A-inducible promoters which were responsive to E1A, it emerged that a large number of DNA motifs, including some TATA boxes, could mediate this activation (Pei and Berk, 1989; Taylor and Kingston, 1990, and references therein). Furthermore, the E1A-responsive elements were also required for unstimulated transcription (Williams et al., 1989; Murthy et al., 1985), and the extent of trans-activation by E1A was inversely proportional to the strength of the uninduced promoter (Taylor and Kingston, 1990). These two observations suggested that either E1A was acting at a single general step in the transcription process, or by speeding up different rate-limiting steps through different mechanisms, depending on the promoter.

That E1A may in fact affect transcription by various pathways, seems to be supported by the variety of its reported direct or indirect interactions. Studies by Lillie and Green (1989) and Martin et al. (1990) have defined two sub-regions in the CR3 activation domain
(amino acids 139-189) and demonstrated the importance of the C-C type zinc finger that is found in this region. The amino-terminal end, including the zinc finger (amino acids 140-178) is required for activation proper and is thought to interact with the basal transcriptional machinery. The carboxy-terminal amino acids (179-189), on the other hand, serve to bring E1A to the proximity of an E1A-inducible promoter, presumably by protein-protein associations with DNA-bound transcription factors, such as ATF2 (Liu and Green, 1990). A complex between E1A and TBP over-expressed in vivo or in vitro, involving the basic domain of TBP which lies between the two DNA binding repeats, and the activation domain of E1A, has been defined (Lee, W.S. et al., 1991). Not all E1A molecules which are mutated in the amino-terminal activation sub-region affect formation of this complex, however, implying that the E1A/TBP interaction is necessary but not sufficient for trans-activation.

In addition, "squelching" experiments suggested that E1A interacted with a component of the transcriptional machinery not required by the VP16 acidic activator, perhaps a co-activator (Martin et al., 1990). The idea that E1A might be stabilising the interactions of transcription factors with co-activators, or act as a co-activator itself, was supported by studies of the interactions between E1A and the octamer motif binding protein, Oct-4, or the retinoic acid receptor β2 (RARβ2). The octamer motif activates transcription efficiently in F9 EC cells, which contain high levels of Oct-4, and in 293 cells, but not HeLa cells, transfected with an Oct-4 expression vector. 293 cells constitutively express E1A and F9 EC cells contain an "E1A-like" activity; similarly, HeLa cells responded to Oct-4 when co-transfected with an E1A expression vector. However, only specific combinations of Oct-4 and E1A concentrations optimally activated transcription, suggesting an equilibrium between "squelching" and productive interaction with the initiation complex. The Oct-4/E1A interaction and trans-activation required the activation domains of both molecules and could be reproduced in vitro (Schöler et al., 1991). In a similar experiment, expression of TBP and RARβ2 in embryonal carcinoma cells led to cooperative trans-activation, but simultaneous expression of E1A was needed in COS cells (Berkenstam et al., 1992). Thus, one mechanism
of transcriptional stimulation by E1A seems to consist of providing a "link" between the basal and regulatory factors.

Two cis-acting elements and their cognate binding proteins have received particular attention with respect to their ability to mediate activation by E1A. One is the E2F (E2A promoter binding factor) binding site (consensus TTTCGCGC; Mudryj et al., 1990), which was initially characterised as an E1A-inducible motif in the adenovirus E2A promoter recognized by the transcription factor E2F in adenovirus-infected HeLa cells (Kovesdi et al., 1986) and by DRTF-1 (differentiation-regulated transcription factor 1) in F9 EC cells (La Thangue et al., 1990). In the absence of E1A, E2F/DRTF-1 is bound to the retinoblastoma gene product (pRb) and cell cycle regulators, such as cyclin A, in a transcriptionally inactive complex (Bandara and La Thangue, 1991; Bandara et al., 1991). E1A, however, is able to dissociate this complex by binding to pRb, and release active E2F/DRTF-1 (Zamanian and La Thangue, 1992); E1A conserved regions 1 and 2 rather than CR3 are required for this (Bandara and La Thangue, 1991). After dissociation from pRb, DNA binding by E2F can be stabilised by complexing with the 19 kD product of the ORF6/7 transcript of the adenovirus E4 gene (Huang and Hearing, 1989; Bagchi et al., 1990).

Phosphorylation has been claimed to be the E1A-induced mechanism that activates a different transcription factor, E4F, binding to a site (consensus (A/T)CGTCA; Lee, K.A.W et al., 1987), known as the cAMP-responsive element (CRE) or the ATF site (Raychaudhuri et al., 1989). E4F is one of the many characterised proteins recognizing this site, and its binding is enhanced after adenovirus infection of HeLa cells (Raychaudhuri et al., 1987). Another ATF site-binding protein, ATF2, mediates E1A trans-activation apparently by interacting with E1A; for this, the E1A CR3 region and the ATF2 amino-terminus are required (Liu and Green, 1990).

Thus, the mechanisms by which E1A activates transcription seem to include direct protein/protein interactions, resulting in the formation of active complexes, as in the Oct-4/E1A case, or in the dissociation of transcriptionally inert ones, as with E2F/pRb, as well as induction of phosphorylation.
1.5 Transcriptional regulation through the ATF site

The ATF site, or CRE, is present in many cellular and viral promoters (reviewed by Roesler et al., 1988; Lee, K.A.W et al., 1987), and is required for both basal and induced transcription. For simplicity, it will be henceforward referred to solely as the ATF site, irrespective of its functional role.

Its importance for uninduced transcription has been demonstrated both in vivo, for the somatostatin and Ad5 E2A genes (Andrisani et al., 1987; Murthy et al., 1985), and in vitro, for the phosphoenolpyruvate carboxykinase (PEPCK) gene (Klemm et al., 1990). In addition, the ATF site is responsible for the activation of, for example, the somatostatin, the vasoactive intestinal polypeptide (VIP), and the glycoprotein hormone α subunit (αGH) genes by cAMP, and can act as a cAMP-responsive enhancer of a heterologous promoter (Montminy et al., 1986; Silver et al., 1987; Deutsch et al., 1987; Fink et al., 1988). A functional cAMP-dependent protein kinase A (PKA) is required for this response (Montminy et al., 1986). The same sequence in the context of the adenovirus E2A and E4 promoters contributes to their E1A-inducibility (Murthy et al., 1985; Lee and Green, 1987), while it is a Ca\(^{2+}\)-responsive element in the enkephalin and c-fos promoters (Sheng et al., 1988; Van Nguyen et al., 1990). Finally, the two closely spaced αGH ATF sites are part of a species- and tissue-specific enhancer which allows expression of the human αGH gene in the placenta as well as the pituitary; the bovine αGH gene, which lacks these ATF sites, is only expressed in the pituitary, both in the animal and in transgenic mice (Delegeane et al., 1987; Bokar et al., 1989).

Despite their sequence similarity, however, ATF sites are not functionally interchangeable. For example, an E1A-inducible E4 ATF site cannot respond to cAMP, nor is the cAMP-inducible VIP ATF site E1A-responsive (Lee et al., 1989). Furthermore, the protein binding properties of sites which are all cAMP-responsive can still differ. For
example, protein binding to the tyrosine aminotransferase (TAT) ATF site is enhanced in vivo by forskolin treatment, which increases intracellular cAMP levels, and in vitro by incubation with PKA, while binding to the somatostatin and PEPCK ATF sites remains unaltered; similarly, one of the TAT ATF site complexes is reduced when protein synthesis is blocked, while no such effect is seen on the somatostatin ATF site (Weih et al., 1990; Nichols et al., 1992). Given that not all ATF sites form the same protein/DNA complexes in vitro or are equally potent in their response to cAMP, functional differences are likely to be due to the binding of different proteins which would all recognize the ATF site but would be influenced in their precise specificities by flanking DNA sequences (Andrisani et al., 1988; Deutsch et al., 1988).

1.6 A family of ATF site-binding proteins and ATF genes

Polypeptide fractions with molecular weights ranging from 30 to 80 kD were able to form ATF site-specific protein/DNA complexes (Cortes et al., 1988), and indeed, a number of ATF site-binding proteins with distinct properties were soon purified. These included CREB, which has a molecular weight of 43 kD (Montminy and Bilezikjian, 1987), ATF (43 and 47 kD) (Hai et al., 1988b), E4F (50 kD) (Raychaudhuri et al., 1989), and EivF (65-72 kD) (Cortes et al., 1988). Apart from their relative molecular weights, other differences, such as precise binding specificities, also became apparent. For example, E4F bound exclusively to an adenovirus E4 ATF site, and with distinct nucleotide requirements from those of purified ATF (Raychaudhuri et al., 1987; Rooney et al., 1990). EivF, on the other hand, bound only to two out of three E4 ATF sites, though, contrary to E4F, it was also able to bind to ATF sites from cellular promoters (Cortes et al., 1988). Similarly, though the methylation interference patterns of ATF1 and ATF2 were indistinguishable, binding by ATF3 required an additional nucleotide outside the core ATF site (Hai et al., 1989).
The variety in functions of the ATF site and binding characteristics of purified ATF proteins was eventually reflected in the cloning of at least ten distinct mammalian cDNAs encoding ATF site-binding proteins: CREB (Hoeffler et al., 1988; Gonzalez et al., 1989), CREBP1/ATF2 (Maekawa et al., 1989; Hai et al., 1989), ATF1, ATF3, ATF4 and ATF6 (Hai et al., 1989), ATF4 (Gaire et al., 1990), TREB5 (Yoshimura et al., 1990), CREM (Foulkes et al., 1991) and E4BP4 (Cowell et al., 1992). Although these cDNAs were different from each other, they shared a region of homology, encoding a DNA binding domain rich in basic amino acids, adjacent to a "leucine zip" dimerization domain (Landschulz et al., 1988b). The ATF gene family was therefore shown to be a relative of the C/EBP (cellular enhancer binding protein) and the API basic domain-leucine zip (bzip) transcription factor gene families, whose members bind DNA as homo- or heterodimers (reviewed by Busch and Sassone-Corsi, 1990; Habener, 1990).

1.6.1 ATF protein domains

Studies on the Jun, Fos and C/EBP bzip families had already shown that both the basic domain and the "leucine zip" were required for DNA binding, and led to the hypothesis that, in a dimer, the leucine heptad repeats are aligned parallel to each other, with the leucine side chains interdigitating and the basic domains contacting the DNA (Landschulz et al., 1989; Turner and Tjian, 1989; Gentz et al., 1989). Crystallographic experiments confirmed that the "leucine zip" has a coiled coil conformation, where the α-helical leucine repeat regions of the two monomers coil around each other, in parallel fashion, held together by electrostatic and hydrophobic interactions (Fig. 1.5) (O'Shea et al., 1991). Modelling and physicochemical studies of the basic domain, on the other hand, have indicated that it undergoes a structural change upon binding to the DNA: the two basic regions of the dimer adopt an α-helical conformation and contact, in opposite orientations, the major groove of the DNA (Fig. 1.5) (Talanian et al., 1990; Shuman et al., 1990; O'Neill et al., 1990).
Domains of ATF transcription factors that are important for functions other than DNA binding have been most extensively studied for CREB, in which a number of regions important for activation have been defined. Its amino-terminus (amino acids 1-87) has a 20% glutamine content; its deletion reduces basal CREB activity to 15% of the wild type molecule but has no effect on its response to cAMP (Gonzalez et al., 1991). Interestingly, glutamine-rich regions are absent in the α and β CREM isoforms, which inhibit CREB function, but present in the τ isoform which is an activator (Foulkes et al., 1991; Foulkes et al., 1992).

CREB amino acids 87-151 are critical for both basal and cAMP-induced trans-activation (Lee et al., 1990; Gonzalez et al., 1991). They include peptide α, which is predicted to form an amphipathic α-helix (Yamamoto et al., 1990) and is one of the alternatively spliced CREB domains (Ruppert et al., 1992), and serine 133, which is phosphorylated by PKA, an event required for cAMP-dependent stimulation of transcription (Gonzalez and Montminy, 1989; Lee et al., 1990). However, serine 133 is necessary but not sufficient: residues lying amino-terminally to it are also required for CREB activity, presumably due to their ability for phosphorylation in vivo, as are carboxy-terminal sequences (Lee et al., 1990). Interestingly, the latter contain a tetrapeptide, DLSS, that is conserved in ATF1, which is also cAMP-responsive; mutation of the aspartic acid residue to asparagine resulted in a five-fold decrease in both basal and cAMP-induced activity (Gonzalez et al., 1991). Since these carboxy-terminal sequences do not contribute to phosphorylation (Lee et al., 1990), their role may be structural, especially as introduction of a triglycine spacer between the DLSS tetrapeptide and serine 133 totally abolishes CREB activity (Gonzalez et al., 1991).

Two regions that are important for the function of other ATF proteins have also been described. One is the ATF2 amino-terminus (amino acids 1-109), through which it mediates E1A trans-activation; it contains a putative zinc-binding region whose precise molecular involvement in the response to E1A, however, has not been studied (Liu and Green, 1990). The carboxy-terminus of E4BP4 is responsible for ATF site-dependent repression; this region does not bear any similarity to characterised repression domains but
encodes a putative second "leucine zipper", though, again, the importance of this structure has not been assessed (Cowell et al., 1992).

1.6.2 Diverse properties and responses of ATF family members

The complexity of the ATF gene family is not restricted to the large number of its members; it is discussed briefly here, and at greater length in Chapter 11, in the light of results presented in this thesis and in the context of other transcription factor families.

Heterodimerization increases the number of potential ATF site-binding protein complexes. Dimerization, however, is not promiscuous: ATF2, for example, can complex with ATF3, but ATF1 cannot (Hai et al., 1989). Nevertheless, members of the ATF family can also dimerize with proteins belonging to the related bZIP API family, and the choice of dimerization partner influences DNA binding specificity (Ivashkiv et al., 1990; Benbrook and Jones, 1990; Macgregor et al., 1990; Hai and Curran, 1991). Alternative splicing of transcripts from the same gene adds another level of complexity and regulation, as has been reported for ATFa (Gaire et al., 1990), CREM (Foulkes et al., 1991) and CREB (Ruppert et al., 1992).

Functional differences between the different ATF proteins also exist. ATF2, for example, responds to E1A trans-activation (Liu and Green, 1990), while ATF1 and CREB activate transcription in response to cAMP (Gonzalez et al., 1989; Liu and Green, 1990; Rehfuss et al., 1991).

The ATF family, therefore, contains transcriptional activators, like CREB (Yamamoto et al., 1990), repressors, like E4BP4 (Cowell et al., 1992), and antagonists, like CREM, which lack an activation domain and prevent the activators from stimulating transcription by forming inactive heterodimers with them (Foulkes et al., 1991). With different members of this family responding to distinct stimuli, a variety of regulatory functions can be fulfilled by a single cis-acting element and through a conserved DNA binding domain.
1.6.3 ATF "homologues" in non-mammalian organisms

ATF proteins are not restricted to mammalian organisms; a 66 kD ATF site-binding protein has been purified from yeast (Lin and Green, 1989a), and two cDNAs each from tobacco and wheat encoding bzip proteins have been isolated that bind to similar DNA sequences (Katagiri et al., 1989; Tabata et al., 1991). The versatility of "ATF" site-binding proteins was again apparent, since expression of the two tobacco cDNAs varies among different plant tissues and in its response to light (Katagiri et al., 1989). A Drosophila bzip trans-activator cDNA has also been cloned that bears 66% similarity to mammalian CREB in the basic region (Abel et al., 1992; Smolik et al., 1992). BBF-2 (box B binding factor 2)/dCREB-A (Drosophila CREB A) is expressed throughout Drosophila development (Abel et al., 1992), and the RNA is detected specifically in regions of the brain and the reproductive organs of both sexes in adult animals (Smolik et al., 1992). In addition, the protein binds to and activates fat body-specific enhancers (Abel et al., 1992); it has therefore been postulated that it may play a role in tissue-specific gene expression. Surprisingly, since the mechanisms of induction by cAMP are different in bacteria and higher eukaryotes, there are nucleotide similarities between some E.coli catabolite activator protein (CAP) binding sites and the ATF site, and the crp gene CAP binding site can function as a cAMP-inducible transcriptional control element in eukaryotic cells (Lin and Green, 1989b). These results suggest that there has been both structural and, in some respects, functional conservation of ATF genes in evolution.

1.7 Aim of the project

The main body of this thesis focuses on the regulation of ATF proteins during the differentiation of F9 EC cells. The adenovirus E4 promoter contains at least three consensus ATF sites which have been shown to be its most important cis-acting elements in vivo and
in vitro, both for basal expression and for E1A-mediated trans-activation (Handa and Sharp, 1984; Gilardi and Perricaudet, 1984; Gilardi and Perricaudet, 1986; Hanaka et al., 1987).

The E4 gene is the second viral gene to be expressed after E1A during the early stages of adenovirus infection. It contributes to the activation of the E2A gene, then its transcription is rapidly repressed by the 72 kDa E2A product (Nevins et al., 1979; Nevins and Jensen-Winkler, 1980; Goding et al., 1985). There were two reasons for studying its transcription and the ability of its promoter to bind transcriptional regulator proteins in F9 EC and PE cells. The first was that E4 expression, as mentioned above, activates E2A transcription (Goding et al., 1985). It was therefore possible that in the original experiments where E2A expression, from mutant viruses which were only defective in the E1A gene, was interpreted as an indication of a cellular "E1A-like" activity in F9 EC cells (Imperiale et al., 1984), E4 expression was also activating E2A, and thereby contributing to the so-called "E1A-like" activity. If this were true, it would be reasonable to postulate the existence of EC cell-specific transcription factors that activate E4 transcription in the absence of E1A. The second reason was that ATF site-binding activities, most likely to control E4 transcription through its ATF sites, had not been studied in the F9 EC cell system. Given that F9 EC cells can be differentiated, they offered an ideal system for studying the possible regulation of ATF-site binding proteins.

A different transcription factor, DRTF-1, contributes to the high transcriptional activity of the Ad5 E2A gene in F9 EC cells and is down-regulated during their differentiation, as assayed by gel retardation (La Thangue et al., 1990; Shivji and La Thangue, 1991; Zamanian and La Thangue, 1992). DRTF-1 binds to the previously characterised binding site of E2F, a transcription factor originally defined in adenovirus-infected HeLa cells (Kovesdi et al., 1986; Yee et al., 1989). The relationship of F9 DRTF-1 to HeLa E2F is, at the moment, unclear, though it is evident that there exist more than one cDNA products with E2F/DRTF1 properties (Helin et al., 1992; Kaelin et al., 1992; R.Girling et al., submitted). Chapter 10 describes an attempt to generate monoclonal
antibodies against DNA binding site affinity-purified DRTF-1, the results of which were inconclusive.

The diversity of ATF transcription factors in other biological systems, and of responses mediated by the ATF site, prompted the present study of the number and regulation of ATF site-binding proteins during the differentiation of embryonal carcinoma cells. The results described here suggest that the regulation of ATF-dependent transcription during differentiation of embryonal carcinoma cells can potentially occur at several levels, including $ATF$ gene expression, as well as differential heterodimerization, binding site preference, and promoter specificity of ATF proteins.
FIG. 1.1: Assembly of the initiation complex (adapted from Roeder, 1991)

a) Order of assembly of basal transcription factors; it is not clear whether TFIIA is required for assembly of all initiation complexes or whether it remains in the complex throughout the process (Buratowski et al., 1989; Maldonado et al., 1990; Sumimoto et al., 1990). A fraction called TFIIJ has also been purified, but is not included in this diagram, since its polypeptide composition has not been characterised (Flores et al., 1992).

b) Schematic representation of the initiation complex.
a

Promoter → D → A → B → Pol II/F → E+H → Initiation Complex

b

TATA

D

A

E

H

F

POL II

B

→
FIG. 1.2: Protein-DNA and protein-protein interactions on the promoter of a pol II-transcribed gene

The DNA is depicted as a single line and the transcriptional start site is indicated by an arrow. TF: DNA binding transcription factor; CO-ACT: co-activator; TA: non-DNA binding trans-activator; IC: initiation complex.
FIG. 1.3: Early development of the mouse embryo (adapted from Jackson, 1989)
- **zona pellucida**
- **♀ pronucleus**
- **♂ pronucleus**
- **early cleavage**: cells loosely associated
  - ~8 hours p.c.
  - ~24 hours p.c.
  - ~40 hours p.c.
- **8 cell embryo**
  - ~48 hours p.c.
- **trophoblast**
- **compacted morula**
  - tight junction formed
  - ~54 hours p.c.
- **early blastocyst**
  - 64 cells
  - ~96 hours p.c.
- **~108 hours p.c.**
  - hatching blastocyst

- **polar trophectoderm**
- **epiblast**
- **mural trophectoderm**
  - 4.5 dpc
- **epiblast**
  - (primitive ectoderm)
- **parietal endoderm**
- **Primitive Endoderm**
- **6 dpc (egg cylinder)**
  - embryonic ectoderm
  - proamniotic cavity
  - visceral endoderm
FIG. 1.4: Comparison of F9 EC cell differentiation with differentiation events in the early mouse embryo (adapted from Kurkinen et al., 1983)

"L"s indicate the "leucine zip" helices, "+"s indicate the basic domains. The DNA is represented as a double helix, with the basic domains of the dimer contacting its major groove.
CHAPTER 2

MATERIALS AND METHODS
DNA preparation and manipulations were performed as described in Sambrook et al. (1989). Any deviations from the procedures described here, are indicated in the figure legends.

Cell culture

F9 EC cells were cultured in Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (Imperial Laboratories (Europe)), 2-4 mM L-glutamine (GIBCO), 100 units/ml penicillin (GIBCO) and 10 mg/ml streptomycin (GIBCO) (= EC medium), at 37 °C in a 5% CO₂ atmosphere; they were re-plated at a density of approximately 4.5x10⁴ cells/ml every 2-3 days. Differentiation to F9 PE cells was obtained by plating F9 EC cells at a density of 10⁶/ml in EC medium supplemented with 5x10⁻⁸ M (all)trans-retinoic acid (RA) (Sigma), 10⁻³ M N⁶,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dbcAMP) (Sigma) and 10⁻⁴ M isobutylmethylxanthine (Sigma) for 5 days. The undifferentiated F9 EC cell phenotype was assessed by immunoperoxidase staining with an α-SSEA1 monoclonal antibody (MC480) (Solter and Knowles, 1978), according to the procedure described by Harlow and Lane (1988), after fixing the cells in 4% formaldehyde for 10 min and treating with 0.2% Triton X-100 in PBS for 10 min. F9 EC cells and F9 EC cells treated with dbcAMP (see "Transfection and CAT assays") were morphologically indistinguishable and both stained for SSEA1; F9 PE cells had a distinct morphology and had lost the SSEA1 surface marker (see Fig.4.5). Growth conditions for all other cells was as for F9 EC cells, except for NIH 3T3 cells which were grown in EC medium containing 1% FCS and transferred to 10% FCS for 17 h prior to harvesting.

Plasmids for transfections, gel retardation probes and oligonucleotides

pBLcat2 contains nucleotides -105/+51 of the HSV thymidine kinase (tk) gene, and pBLcat3 is a promoterless vector (Luckow and Schütz, 1987). pE2A-E-cat (designated pE2A) contains the adenovirus type 5 E2A promoter and has been previously described (Murthy et al., 1985).
pIEPIcat (designated pCMV) contains nucleotides -301/+72 as well as the polyadenylation signal of the major immediate early gene of human cytomegalovirus (CMV) and has been previously described (La Thangue and Rigby, 1988a). VIPcat4 (designated pVIP) contains nucleotides -94/+146 of the human vasoactive intestinal polypeptide (VIP) gene (Tsukada et al., 1987). Plasmids E4Δ240 and E4Δ138 contain nucleotides -240/+35 and -138/+35, respectively, of the adenovirus 5 E4 gene (Lee and Green, 1987). Plasmids pE4 and pE4Δ were constructed by ligating BgIII linkers to the EcoRI/TaqI E4 promoter fragments from E4Δ240 and E4Δ138, respectively, and cloning into the BgIII site of the pBLcat3 polylinker. Plasmid pCMV-βgal (constructed by D.Stott) contains nucleotides -301/+72 of the human CMV immediate early gene cloned upstream of the E.coli lacZ gene followed by the simian virus 40 (SV40) small t intron and polyadenylation signal. Gel retardation probes E4 and E4Δ were the BgIII inserts from pE4 and pE4Δ, respectively, and probe VIP was a HindIII/NcoI fragment from VIPcat4, containing nucleotides -94/+146 of the VIP gene. The HSV tk promoter probe was a BamHI/BgIII fragment from pBLcat2, containing nucleotides -105/+51 of the HSV tk gene. The SV40 origin of replication probe was a 60 base pairs (bp) long synthetic oligonucleotide, containing nucleotides 5175 to 37 of the SV40 genome (Tooze, 1981). Synthetic single stranded oligonucleotides were annealled in ligase buffer (Sambrook et al., 1989).

Transfection and CAT assays

Differentiation of F9 EC cells to F9 PE cells was obtained by plating F9 EC cells at a density of 10^5/ml in EC medium supplemented with 5x10^-8 M RA, 10^-3 M dbcAMP and 10^-4 M isobutylmethylxanthine for 3 days before transfection. By this time, the cells were morphologically indistinguishable from F9 PE cells after 5 days of treatment. Both F9 EC and PE cells were re-plated at 10^5/ml approximately (in EC medium without any additives, or containing RA and dbcAMP, or dbcAMP alone) 4 h before transfection with a DNA (20 μg of construct)/calcium phosphate co-precipitate, as previously described (Gorman et al.,
Transfections were performed in parallel: two identical precipitates were pooled, divided in two equal parts and transfected into F9 EC and F9 PE cells, or untreated F9 EC and F9 EC cells treated with dbcAMP. The cells were washed with DMEM approximately 18 h after transfection, re-fed with EC medium containing the appropriate additives, and harvested after a further 24 h. By that time, F9 PE cells had been treated with differentiation agents for 5 days. Chloramphenicol acetyl transferase activity was assayed as previously described (Gorman et al., 1982); approximately 50 μg of extract were incubated with substrate at 37 °C for 1-1.5 h. The results were quantitated by cutting out converted substrate from the silica gel thin layer chromatography plates (Merck) and counting in a scintillation counter. Cells transfected with pCMV-βgal, were fixed and stained for β-galactosidase activity at the time of harvest, essentially as previously described (Dannenberg and Suga, 1981).

Protein extracts

Whole cell Manley extracts were prepared as previously described (Manley et al., 1980). dbcAMP-treated F9 EC cells were harvested after 42 h of treatment, F9 PE cells after 5 days of treatment with differentiation agents. Whole cell micro-extracts were prepared from approximately 10^7 cells, as described by Schöler et al. (1989) except that sonication was replaced by freeze-thawing three times. Protein concentration was measured according to the Bradford method, using the Bio-Rad protein assay. Dignam nuclear and cytoplasmic extracts were prepared as previously described (Dignam et al., 1983).

Gel retardation assay

The binding reaction was performed in buffer containing 50 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 40 mM (NH₄)₂SO₄, 0.2 mM ethylene diamine tetra-acetate (EDTA), 1 mM dithiothreitol (DTT) and 15% glycerol. Each reaction (20 μl) contained 0.5 ng of a promoter
fragment or 1.3 ng of a synthetic oligonucleotide probe end-labelled with the appropriate α-\(^{32}\)P-labelled deoxynucleotide triphosphate (α-\(^{32}\)P-dNTP) (Amersham), 5 µg protein extract or 2 µl of an in vitro translation reaction, and 2 µg denatured salmon sperm DNA as non-specific competitor. All reaction components, including oligonucleotide competitors where applicable, were incubated at 30 °C for 10 min in the absence of probe; this was followed by probe addition and a further incubation at 30 °C for 10 min. Where anti-DRTF-1 serum or hybridoma tissue culture supernatant was included in the assay, the antigen was first incubated with the antibody for a minimum of 2 h at 4 °C, then denatured salmon sperm DNA was added and the reaction continued as above. The reaction was then loaded onto a non-denaturing 4% polyacrylamide gel (acrylamide : bis-acrylamide = 20 : 1) and run in Tris-Acetate-EDTA buffer (Sambrook et al., 1989), at +4 °C and with buffer recirculation, for 3 h (VIP promoter fragment), 2.5 h (E4, E2A and HSV tk promoter fragments, and SV40 origin oligonucleotide) or 1.5 h (other oligonucleotides) at 150 Volts (V) after an initial pulse of 500 V for 2 min. The gels were vacuum-dried and exposed to X-ray film (Fuji) at -70 °C.

"Super-shifts" with anti-peptide sera

2 µl of serum were pre-incubated with all gel retardation components except for the probe, for 1 h at +4°C, in the presence or absence of 400 µM peptide in phosphate-buffered saline (PBS). The reaction was then continued as above.

Construction of BS-ATG vectors

Bluescript-KS (Stratagene) was modified according to the scheme presented in Chapter 8.
In vitro transcription and translation

ATF1S (Hai et al., 1989) was linearized with Ncol and transcribed by T7 RNA polymerase (Stratagene). ATF2S (Hai et al., 1989) was linearized with BglII and transcribed by SP6 RNA polymerase (Stratagene). ATF2L (Hai et al., 1989) was linearized with XhoI and transcribed by T7 RNA polymerase. BA1-CREBP1 was constructed by digesting pUCCRE-BP1Eco3.2 (Maekawa et al., 1989) with EcoRI and cloning the CREBP1 cDNA fragment into BS-ATG1. It was linearized with BamHI and transcribed by T3 RNA polymerase (Stratagene). ATF3S (Hai et al., 1989) was linearized with Ncol and transcribed by T7 RNA polymerase. ATF3L (Hai et al., 1989) was linearized with Ncol and transcribed by T7 RNA polymerase. BA0-ATF4 was constructed by digesting pGEM3-ATF4 (Hai et al., 1989) with EcoRI and cloning the ATF4 cDNA into BS-ATG0. It was linearized with BamHI and transcribed with T3 RNA polymerase. BA1-ATF6 was constructed by digesting pGEM3-ATF6 (Hai et al., 1989) with EcoRI and cloning the ATF6 cDNA into BS-ATG1. It was linearized with BamHI and transcribed with T3 RNA polymerase. BS-cjun was constructed by digesting AH119 (Ryseck et al., 1988) with EcoRI and PstI and cloning the cjun cDNA into Bluescript-KS (Stratagene). It was linearized with PstI and transcribed with T3 RNA polymerase. BS-JunB was constructed by digesting RSVJunB (Ryder et al., 1988) with EcoRI and PstI and cloning the cjun cDNA into Bluescript-KS (Stratagene). It was linearized with PstI and transcribed with T3 RNA polymerase. BS-JunD was constructed by digesting RSVJunD (Hirai et al., 1989) with EcoRI and PstI and cloning the cjun cDNA into Bluescript-KS (Stratagene). It was linearized with PstI and transcribed with T3 RNA polymerase. BA1-cfos was constructed by digesting pGEM3-ATF5 (Hai et al., 1989) with EcoRI and cloning the ATF5 ( = cFos ; M.Green, pers. comm.) cDNA into BS-ATG0. It was linearized with BamHI and transcribed by T3 RNA polymerase. In vitro transcription in the presence of m7G(5')ppp(5')G (Pharmacia) and in vitro translation in rabbit reticulocyte lysate (Promega) were performed according to the manufacturers' instructions. A standard in vitro translation reaction was in a volume of 20 μl.
Isolation of total cellular RNA with guanidine isothiocyanate
(adapted from Sambrook et al., 1989)

Approximately $10^8$ tissue culture cells were lysed by vortexing with 8 ml homogenising solution (4.25 M guanidine isothiocyanate, 0.5% sodium lauroylsarcosine, 5mM sodium citrate pH7, 0.1 M β-mercaptoethanol) and overlaid on 3.5 ml CsCl solution (5.7 M CsCl, 5 mM sodium citrate pH7, 50 mM EDTA) in a centrifugation tube. They were centrifuged in an SW40-Ti (Beckman) rotor at 30,000 rpm at 22 °C for 18-20 hr. The RNA pellet was resuspended in water treated with diethyl pyrocarbonate (DEPC), extracted and precipitated. It was finally resuspended at 20 mg/ml in DEPC-treated water and stored at -70 °C.

Templates for synthesis of anti-sense RNA probes

pSP64γ-actin (Enoch et al., 1986) was linearized with HindIII and transcribed by SP6 RNA polymerase. pGEM3-ATF1 (Hai et al., 1989) was linearized with XhoI, which cuts in the middle of the basic domain, and transcribed by T7 RNA polymerase. ATF2L was linearized with XhoII, which cuts at the N-terminus of the DNA binding domain, and transcribed by SP6 RNA polymerase. pGEM3-ATF3/X was constructed by digesting pGEM3-ATF3 (Hai et al., 1989) with Xbal and religating. It was linearized with PvuII, which cuts in the middle of the basic domain, and transcribed by SP6 RNA polymerase. BA0-ATF4 was linearized with XhoI, which cuts in the plasmid polylinker, and transcribed by T7 RNA polymerase. BA1-ATF6 was linearized with ClaI, which cuts in the plasmid polylinker, and transcribed by T7 RNA polymerase. BS-cjun was linearized with NarI, which cuts after the end of the coding sequences, and transcribed by T7 RNA polymerase. BS-junD/HI was constructed by digesting BS-junD with BamHI and religating. It was linearized with PvuII, which cuts 3' to the DNA binding domain, and transcribed by T7 RNA polymerase. BA1-cfos/S was constructed by digesting BA1-cfos with SmaI and StuI and religating. It was linearized with...
EcoRI, which cuts in the plasmid polylinker, and transcribed by T7 RNA polymerase. afra1 was constructed by inverting the orientation of the EcoRI fra1 cDNA insert of pSP65fra1 (Cohen and Curran, 1988) in the same vector. It was linearized with XbaI, which cuts after the end of the coding sequences, and transcribed by SP6 RNA polymerase. afosB was constructed by cloning the EcoRI/BamHI fosB cDNA fragment from pTZfosB (Zerial et al., 1989) into Bluescript-KS (Stratagene) digested with EcoRI and BamHI. It was linearized with NcoI, which cuts 5’ to the DNA binding domain, and transcribed by T7 RNA polymerase. pPX490, containing a mouse c-myc PvuII/XhoI exon I fragment (Roberts et al., 1992), was linearized with EcoRI and transcribed with T3 RNA polymerase.

Synthesis of radiolabelled anti-sense RNA probes
(adapted from Sambrook et al., 1989)

2 µl template plasmid (approximately 0.2 pmol) were mixed with 0.5 µl ribonucleotide mix (ATP, CTP, GTP at 10 mM, UTP at 0.5 mM) (all from Stratagene), 0.5 µl DTT 750 mM, 0.2 µl "RNA guard" (Pharmacia), 2 µl 5x transcription buffer (Stratagene), and 5 µl α-32P-UTP (800 Ci/mmol) (NEN Du Pont). The mixture was incubated with 12 units of the appropriate phage RNA polymerase for 30 min at 37 °C. 1 µl ribonuclease-free DNase (1 mg/ml) (Stratagene) was added and the incubation continued for 15 min at 37 °C. The probe was extracted and ethanol-precipitated with 20 µg yeast transfer RNA (tRNA) as carrier. It was pelleted, dried and resuspended in 25 µl DEPC-treated water.

Ribonuclease protection assay
(adapted from Williams et al., 1988)

1 µl total cellular RNA (20 mg/ml, unless otherwise indicated in the figure legends) was mixed with 1 µl anti-sense RNA probe, 3 µl 7x hybridization buffer (1x final concentration: 40 mM piperazine-N,N'-bis[2-ethane-sulfonic acid], 1 mM EDTA, 400 mM NaCl) and 16.8
μl de-ionised formamide (DIF) in a 1.5 ml Eppendorf tube. The tubes were heated at 90 °C for 5 min and quickly transferred to the hybridization bath set at 55 °C where they were incubated overnight. The tubes were then chilled on ice and 300 μl of ice-cold ribonuclease (RNase) mix (8 μg/ml ribonuclease A (Boehringer), 40 units/ml ribonuclease T1 (Boehringer), 300 mM NaCl, 50 mM Tris, 25 mM EDTA) were added to each tube. The tubes were incubated for 1 h at 37 °C, then each received 20 μl 10% sodium dodecyl sulphate (SDS) and 5 μl proteinase K (BCL) (10 mg/ml) and were further incubated for 15 min at 37 °C. The contents were extracted once with phenol:chloroform and precipitated with 750 μl ethanol and 20 μg yeast tRNA for 20 min on dry ice. The precipitates were pelleted, dried, resuspended in 5 μl DIF loading buffer (80% DIF, 0.05% bromophenol blue (BDH), 0.15% xylene cyanol FF (BDH), 200 μM EDTA, 10% glycerol), denatured for 5 min at 90 °C, quenched on ice for 3 min, and loaded on a 4% or 6% polyacrylamide/Tris-borate-EDTA/urea gel run at 40 Watts (W) constant power. The gels were dried and exposed to Kodak XR film, with intensifying screens.

Computer-assisted sequence comparisons, manipulations and predictions

Hydrophilicity and hydropathy predictions, and prediction of amino acid sequences from cDNA sequences were performed using the MGS package, written by P.Gillett at N.I.M.R.. Sequence comparisons and predictions of modification sites were performed using the GCG Wisconsin package (Devereux et al., 1984).

Production of anti-peptide sera

All procedures were according to Harlow and Lane (1988). Peptides ATF2D, ATF3C, PEP1 and PEP2 were conjugated to keyhole limpet haemocyanin (KLH) (Sigma) with glutaraldehyde, P10 with m-maleimidobenzoyl-N-hydroxysuccinimide ester (Sigma). Sandylop rabbits were immunised subcutaneously with 0.5 ml of a peptide-KLH conjugate
emulsion, containing approximately 180-1000 μg (approximately 100-250 nmol) of peptide, assuming 100% coupling efficiency, in Freund's complete adjuvant (GIBCO) for the first injection, and the same amount of peptide-KLH conjugate in Freund's incomplete adjuvant (GIBCO) for subsequent injections, spaced by approximately 6 week periods. Test bleeds were collected and treated by standard procedures.

**Anti-peptide enzyme-linked immuno-sorbent assay (ELISA)**

Flat-bottomed 96-well polyvinylchloride (PVC) plates (Falcon) were coated with 50 μl of peptide (400 pmol/ml) in 0.1 M sodium carbonate buffer pH 9.6, at 4 °C overnight; all subsequent manipulations were performed at room temperature (r.t.). The plates were washed twice for 2 min in PBS. They were blocked in 10% bovine serum albumin (BSA; Sigma) for 70 min, washed twice for 2 min with 0.1% polyoxyethylene-sorbitan monolaurate (Tween-20; Sigma) in PBS (=PBS/T), and incubated with sera diluted 1:100 in blocking buffer for 80 min. They were then washed three times for 2 min in PBS/T, and incubated with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (HRPαR; DAKOPATTS) diluted 1/2500 in blocking buffer, for 90 min, washed 3x2 min in PBS/T, and incubated with a 67 mg/l solution of 1,2-phenylenediamine dihydrochloride (DAKOPATTS) in 0.1 M citrate-phosphate buffer pH 5.0, for 5 min. The reaction was stopped with an equal volume of 1M sulphuric acid.

**Anti-ATF/API Immunoblots**

20 μg of protein extract or 5 μl of an in vitro translation reaction were loaded in each lane of a 7.5% or 10% SDS-polyacrylamide mini-gels (Bio-Rad Mini Protean II) alongside "Rainbow" protein molecular weight standards (Amersham). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the manufacturers' instructions. The polypeptides were transferred onto 0.45 μm pore nitrocellulose membrane (Schleicher &
Schüll) using a Bio-Rad Mini Protean Trans-Blotter, according to the manufacturers' instructions. The polypeptides were visualised by staining the membrane with a Ponceau-S solution. After blotting, each lane was divided in two halves, to be incubated with pre-immune and immune sera, or in the presence or absence of peptide competitor (20 μM). The membrane was rinsed in PBS, blocked in blocking buffer (10% BSA in PBS) for 1 h, and incubated with sera diluted 1/200 in blocking buffer for 3 h. It was then washed twice for 10 min in PBS/0.1% Nonidet P40 (BDH) (PBS/NP40), incubated with alkaline phosphatase conjugated swine anti-rabbit immunoglobulins (APaR) (Promega) (diluted 1/5000 in blocking buffer) for 2 h at r.t., washed twice for 10 min in PBS/NP40 and incubated with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma), according to the manufacturers' instructions. The reaction was stopped by immersing the membrane in 1% acetic acid.

Immunoprecipitations

Cells which had been labelled with 20 μCi/ml of 35S-methionine for 17 h were lysed in buffer A (50 mM Tris pH 7.9, 125 mM NaCl, 0.2 mM EDTA, 1mM DTT, 1% NP40) by rotation for 1 h at +4°C. 50 μg of cell lysate or 3 μl of an in vitro translation reaction were incubated with 5 μl of serum, in the presence or absence of peptide competitor (200 μM) made up to a total volume of 40 μl with buffer A or C (= A + 1% sodium deoxycholate, 0.1% SDS), rotating for 1 h at +4°C. After addition of 10 μl of protein A-Sepharose beads (Sigma) : buffer A (1 : 5 vol/vol), the reaction was incubated for 1 h, with rotation, at +4°C. The pellet was washed with 180 μl of the appropriate buffer, three times for cell lysates, four for in vitro translations, washed once with the appropriate buffer without NP40, denatured and used for SDS-PAGE alongside 14C-labelled protein molecular weight standards (Amersham). The gels were dried and exposed to Kodak XR film, with intensifying screens.
Purification of DRTF-1

DNA binding site affinity-purified DRTF-1 was kindly provided by M.K.K. Shivji and N.B. La Thangue; its purification has been described elsewhere (Shivji and La Thangue, 1991).

Production of hybridomas

All immunological and immunochemical procedures were as described (Harlow and Lane, 1988), unless otherwise indicated. Four BALB/c female mice (referred to as mouse 1, 2, 3 and 4) were injected with six doses of affinity purified DRTF-1 (mice 1 and 2: 50 μl/injection; mice 3 and 4: 25 μl affinity fraction mixed with 25 μl of affinity matrix), as described, at a rate of approximately one injection every 2–3 weeks. Tail bleeds were taken one week after the 3rd, 5th and 6th injections and the serum was extracted. A booster injection was given to mouse 2 on the 35th week after the first injection. Five days later, the mouse was sacrificed, its spleen cells were fused, by stirring, to SP2 myeloma cells, and the fusion products were plated out in sixteen 96-well plates (Falcon). They were grown in DMEM supplemented with 20% FCS (Imperial), 5.8 μM azaserine (Sigma), 100 μM hypoxanthine (Sigma), and 1 ng/ml recombinant interleukin-6 (British Biotechnology), at 37 °C in 8% CO₂. Medium supernatants (140 μl) were collected at 9, 11 and 14 days after the fusion and the wells replenished with an equal volume of fresh medium. The contents of wells that were positive in the ELISA assay were transferred into 24-well plates (Falcon) and diluted 1:5 into a total volume of 0.5–1 ml. Medium supernatants were collected when the cells reached confluence. All positive wells were eventually frozen down, after 1–3 passages, and stored in liquid nitrogen.
**Anti-DRTF-1 ELISA**

Flat-bottomed 96-well PVC plates (Falcon) were coated with 35 \( \mu l \) of a heparin Sepharose DRTF-1 fraction (17 \( \mu g/ml \)), unless otherwise stated, at 4 °C overnight; all subsequent manipulations were performed at r.t. The plates were washed 2x2 min in PBS. They were blocked in 10% BSA, unless otherwise stated (= blocking buffer) for 1 h, washed 2x2 min with PBS/T, and incubated with sera diluted in blocking buffer or neat hybridoma supernatants for 1-3 h. They were then washed 3x2 min in PBS/T, and incubated with horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins (HRPaM; DAKOPATT) diluted 1/2500 in blocking buffer, for 1-3 h, washed 3x2 min in PBS/T, and incubated with a 67 mg/l solution of 1,2-phenylenediamine dihydrochloride (DAKOPATT) in 0.1 M citrate-phosphate buffer pH 5.0, for 45 min. The reaction was stopped with an equal volume of 1M sulphuric acid.

**Anti-DRTF-1 Immunoblots**

Protein samples were loaded alongside protein molecular weight markers (Sigma) onto 7.5% or 10% SDS-polyacrylamide mini-gels (Bio-Rad Mini Protean II) prepared and run according to the manufacturers’ instructions. The polypeptides were transferred onto 0.45 \( \mu m \) pore nitrocellulose membrane using a Bio-Rad Mini Protean Trans-Blotter, according to the manufacturers’ instructions. The polypeptides were visualised by staining the membrane with a Ponceau-S solution. The membrane was rinsed in PBS, blocked in blocking buffer (10% BSA in PBS, unless otherwise indicated) for 1 h, washed 2x10 min in PBS/T, and incubated with sera diluted in blocking buffer or neat hybridoma supernatants for 1-3 h. It was then washed 2x10 min in PBS/T, incubated with either HRPaM (diluted 1/500 in blocking buffer) or alkaline phosphatase-conjugated rabbit α-mouse immunoglobulins (APaM; DAKOPATT), diluted 1/1000 in blocking buffer, as indicated in the figure legends, washed
2×10 min in PBS/T and incubated with the appropriate substrate: 3,3' diaminobenzidine tetrahydrochloride (Sigma) for HRP or nitro blue tetrazolium dimethylformamide and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) for AP, according to the manufacturers' instructions. The HRP reaction was stopped by rinsing the membrane with distilled water; the AP reaction was stopped by immersing the membrane in 1% acetic acid.
CHAPTER 3

REGULATION AND NUCLEOTIDE SPECIFICITY OF ATF SITE-BINDING ACTIVITIES IN F9 EC AND PE CELLS
3.1 Sequence specificity and regulation during differentiation of F9 ATF site-binding activities

Given that a multitude of different ATF site-binding proteins had been described in other cell types, it was decided to investigate the number and regulation of ATF site-binding activities in F9 EC and PE cells. For this purpose, the panel of oligonucleotides shown in Table 3.1 was designed. The wild type ATF site (P) and a double point mutation already shown to abolish ATF binding (Pm1) (Hurst and Jones, 1987), together with eight other oligonucleotides were used to assess the specificity of F9 EC and PE cell ATF site-binding activities. Four of these oligonucleotides were designed to distinguish between ATF and E4F, the latter being a cellular factor whose binding site overlaps that of ATF but also extends over neighbouring nucleotides (Raychaudhuri et al., 1987). Oligonucleotides PATFm and Pm2 are mutated in the core ATF site, and oligonucleotides PE4Fm and Pm3 are mutated within the additional nucleotides required by E4F; these oligonucleotides were therefore used in order to distinguish between ATF and E4F complexes. A TRE (12-O-tetradecanoylphorbol-13-acetate responsive element) was also included, since its cognate binding activity, AP1 (Lee, W. et al., 1987), has been reported to bind to the ATF site (Hai et al., 1988). Similarly, another site whose cognate binding protein, C/EBP, is a member of a distantly related bzip family (Landschulz et al., 1988a), was included in the panel, as the complexes it forms in liver nuclear extracts have been shown to be competed by an E4 ATF site (Bakker and Parker, 1991). Finally, two sites, one that is located 3' to the E2A transcriptional initiation site, E2A-3', bears some homology to an ATF site, and is protected from DNase I cleavage in F9 EC cell extracts (N.B. La Thangue, unpublished observation), and one that is a distal ATF site, DA, from the Ad5 inverted terminal repeat (ITR) situated immediately upstream of the E4 promoter, were also included in the panel of variant ATF site oligonucleotides.

The above oligonucleotides were used as binding sites in a gel retardation assay with F9 EC and PE whole cell extracts. As expected, no ATF site-specific complexes formed on
mutant Pm1 either in F9 EC or PE extracts (Fig.3.1a: lanes 1-2). Oligonucleotide PATFm produced one complex, ECRE-3 (for EC CRE binding activity), the abundance of which was down-regulated during differentiation (Fig.3.1a: compare lane 10 to lane 9). Probe E2A-3' produced the same complex, though more efficiently (Fig.3.1a: compare lane 11 to lane 9). Mutant Pm2 produced two complexes in F9 EC extracts: ECRE-1, of slower mobility, and ECRE-4, a diffuse complex of faster mobility than ECRE-3, and complex PCRE-1 in F9 PE extracts (Fig.3.1a: lanes 13-14; ECRE-4 is resolved better in the experiment shown in Fig.3.2B: lane 6, where the two distinct complexes are labelled ECRE-4a and ECRE-4b). The distinction between ECRE-4 and PCRE-1 was made on the basis of a difference in mobility as well as the number of retarded bands in each complex, which are clearer when ECRE-4 and PCRE-1 are resolved on E4Δ, a fragment of the Ad5 E4 promoter containing one ATF site (see Chapter 5). There was no apparent qualitative difference between the protein binding patterns of P, PE4Fm and Pm3 (Fig.3.1a: lanes 3-8) since they all bound an activity, ECRE-2, equally abundant in F9 EC and PE cell extracts and more abundant than any of the other ECRE activities, as well as ECRE-1, ECRE-3, ECRE-4 and PCRE-1 (see Fig.3.1d and e, and Chapter 5). There was, however, a quantitative difference in the efficiency of binding to these oligonucleotides: P bound ECRE-2 more efficiently than PE4Fm and Pm3. That there was no qualitative difference between the complexes bound by P, PE4Fm and Pm3 was confirmed when these oligonucleotides were used to compete for complexes formed on E4Δ, since they competed for the binding of all ATF site-specific activities (Chapter 5). This suggested that there was no detectable E4F-like activity in F9 EC whole cell extracts, given that PE4Fm and Pm3 were designed so that they would not bind E4F. Rooney et al. (1990) have indeed shown that Pm3 (called E4.pm4 by them) does not bind E4F purified from HeLa cells. The intensity of the ECRE-2 complex formed on P presumably masked the other ATF-site specific complexes, ECRE-3, ECRE-4 and PCRE-1 that migrated to a similar position. The ECRE-2 activity also served as a control for extract integrity since it was consistently of equal abundance in all pairs of F9 EC and PE extracts tested.
The TRE formed one complex common to F9 EC and PE extracts and one slower migrating complex which was induced during differentiation (Fig.3.1a: lanes 15 and 16). The DNA binding properties of this slower migrating complex were those of API which is undetectable in F9 EC cells but is induced during differentiation (Kryszke et al., 1987), and its mobility as well as its specificity were distinct from those of ECRE-1, -2, -3, -4 and PCRE-1 (Fig.3.1d and e).

One possibility that needed to be eliminated was that the different ECRE complexes were created by sequences in the mutated binding sites unrelated to the ATF site. This was eliminated by showing that they could be competed only by a wild type site (P) but not by one where the central CG nucleotides had been mutated (Pm1) (Fig.3.1b and c), indicating that all these activities bound to the wild type ATF site, P. The API complex was also competed by P (Fig.3.1b and c: lane 14) and therefore must have also been capable of binding when P was used as a probe, but was then presumably masked by ECRE-2.

The precise DNA binding specificities of the different activities were then confirmed by assessing how the mutated oligonucleotides competed for each other's complexes (Fig.3.1d and e). These studies demonstrated that while all the defined activities bound to the wild type ATF site, they did so with distinct nucleotide requirements. For example, complexes ECRE-1, ECRE-4 (Fig.3.1d: lane 6) and PCRE-1 (Fig.3.1e: lane 6) bound to Pm2 but not E2A-3' (Fig.3.1d and e: compare lane 8 to lane 9), while both Pm2 and E2A-3' were unable to bind ECRE-2 (Fig.3.1d and e: lanes 3 and 4; note that Pm2 did compete for ECRE-1), confirming that ECRE-2 was not just composed of the other comigrating ECRE complexes, but was indeed a complex distinct from them. Significantly, neither the TRE (Fig.3.1d and e: compare lanes 5 to 1, 10 to 6, 15 to 11, 20 to 16) nor the C/EBP site (Fig.3.2B: compare lanes 5 to 1, 10 to 6, 15 to 11) bound any of the ECRE activities, emphasizing that, although they have distinct requirements for nucleotides within the ATF site, the primary specificity of the ECRE activities is for the ATF site, rather than the related TRE and C/EBP sites.

When the same set of experiments was conducted with oligonucleotide DA, this probe bound ECRE-1 (Fig.3.2A: lane 1), albeit more weakly than either Pm2 (Fig.3.2B: compare...
lane 4 to lane 3; Fig. 3.2A: compare lane 1 to lane 7) or P (Fig. 3.2B: compare lane 4 to lane 2; Fig. 3.2A: compare lane 1 to lane 5). DA also formed a faster-migrating complex in both EC and PE extracts (indicated by brackets in Fig. 3.2A: compare lanes 9 and 10) which was also ATF site-specific (Fig. 3.2A: compare lane 3 to lane 2). The composition of this latter complex was elucidated by using DA to compete for the previously defined ECRE complexes (Fig. 3.2B). When DA was used as a competitor against complexes formed on probe Pm2, it bound ECRE-1 and the slower migrating of the two ECRE-4 complexes, ECRE4a, but not the faster one, ECRE-4b (Fig. 3.2B: compare lane 9 to lane 6). When E2A-3′ was used as the probe, DA competed for ECRE-3 as efficiently as either P or E2A-3′ (Fig. 3.2B: compare lane 14 to lanes 12 and 13). Finally, when P was used as the probe, DA competed for ECRE-1, albeit weakly, ECRE-3, and ECRE-4a, but also for ECRE-2, so that only ECRE-4b and some residual ECRE-1 remained bound to P (Fig. 3.2B: compare lane 4 to lane 1). Therefore, although DA did not define any new ECRE activities, it did have binding characteristics that were distinct from those of P, Pm2 or E2A-3′ (summarized in Table 3.2), and it distinguished between ECRE-4a and ECRE-4b.

3.2 Discussion

In summary, these oligonucleotides, which contained wild type and variant ATF sites, defined six ATF site-binding activities in F9 EC and PE cell extracts which could be distinguished from each other by their electrophoretic mobility, their precise DNA sequence specificity and their regulation during F9 EC cell differentiation (summarized in Table 3.2). For example, the abundance of ECRE-2 was not altered during differentiation of F9 EC cells, whereas ECRE-1 and ECRE-3 were down-regulated during differentiation.

Because oligonucleotides that bound ECRE-2 all had an intact core ATF site, ECRE-2 would appear to have the DNA binding specificity of purified ATF as defined by others (Hurst and Jones, 1987; Lee et al., 1987). Their "ATF-43" and "ATF-47" were eventually identified as the products of the ATF1 and CREB genes, respectively (Hurst et al., 1991;
Hurst et al., 1990). This would, at first sight, imply that ECRE-2 was encoded by CREB or ATFI. However, there is no detectable ATFI RNA in F9 EC and PE cells (Chapter 7) and therefore ATFI is unlikely to be encoding ECRE-2. On the other hand, both ATF2 and ATF3, both products of genes which are expressed in F9 EC cells, bind to the wild type ATF site, P, (Chapter 6) and would therefore also be potential candidates for ECRE-2.

Failure to detect E4F, which was reported to be present in EC cells (Raychaudhuri et al., 1989), may have been due to differences in experimental conditions. E4F was detected in fractionated extracts whereas whole cell extracts were used in this study. Although C/EBP has been shown to bind to the ATF site (Bakker and Parker, 1991), the converse was not true in the experiments presented here, although it should be mentioned that the C/EBP site used was not identical to the one used by Bakker and Parker (1991).

The failure of an API site to compete for ECRE complexes does not contradict the results presented by Hai et al. (1988). Before anything was known about heterodimerization between ATF and API proteins, these workers had shown that some of the proteins purified by ATF binding site affinity purification cross-reacted with anti-cJun antibodies, and some of the proteins purified by API binding site affinity purification cross-reacted with anti-ATF antibodies. At the time, when none of the ATF genes had been cloned, it was thought that "ATF" was a single and homogeneous DNA binding activity, and those results were interpreted as indicating that "ATF" and "API" were immunologically related and could bind to each other's site. Today, of course, we know that, as illustrated in Chapter 7, members of the ATF and API families can heterodimerize to produce DNA binding activities specific either for the ATF site, or the API site, or both. It is therefore possible that Hai et al. (1988) were detecting ATF/Jun heterodimers in their affinity-pure ATF and API preparations.

In conclusion, the data presented here constitute a novel demonstration of a family of DNA binding proteins with distinct nucleotide specificities and modes of regulation during F9 EC cell differentiation (summarized in Table 3.2) (Tassios and La Thangue, 1990).
TABLE 3.1: Sequences of ATF sites and variant oligonucleotides used in this study

Complete sequences of the oligonucleotides used in this study, including GATC or other 5' overhangs. The position of these sequences relative to the transcriptional start sites of the indicated genes are shown where appropriate. Deviations from the ACGTCA ATF site consensus are double-underlined and in bold. Sequences from: E4 (Steenbergh and Sussenbach, 1979), E2A (Murthy et al., 1985), c-fos TRE (Krysze et al., 1987), C/EBP (Vinson et al., 1988).
Sequences of ATF sites and variant oligonucleotides

<p>| | | | |</p>
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FIG. 3.1: Distinct but ATF site-specific protein complexes are formed on different variants of the ATF site and on a TRE

a) Complexes formed on the indicated oligonucleotide probes in F9 EC and PE whole cell extracts are indicated by arrows.

b) Competition of the F9 EC complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of wild type (P) or mutant (Pm1) ATF site oligonucleotide. The fastest migrating complex is non-specific.

c) Competition of the F9 PE complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of wild type (P) or mutant (Pm1) ATF site oligonucleotide.

(continued opposite next page)
FIG. 3.1 (cont'd): Distinct but ATF site-specific protein complexes are formed on different variants of the ATF site and on a TRE

d) Competition for the F9 EC complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of the indicated ATF site variant and TRE oligonucleotides. The fastest migrating complex is non-specific.

e) Competition for the F9 PE complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of the indicated ATF site variant and TRE oligonucleotides.
FIG. 3.2: Specificity of probe DA for ECRE activities

A) Lanes 1-4: Competition for the F9 EC complexes formed on probe DA with 50-fold molar excess of the indicated ATF site variant oligonucleotides. Lanes 5-10: Comparison of protein complexes formed on probes P, Pm2 and DA in F9 EC and PE whole cell extracts. All lanes are from the same experiment. The fastest migrating complex is non-specific.

B) Competition for the F9 EC complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of the indicated ATF site variant oligonucleotides. Lanes containing the same probe are from the same experiment.
A

Probe: DA
Extract: EC
Competitor: Pm1 P DA

ECRE-1
ECRE-2
ECRE-3
ECRE-4a

B

Probe: P
Extract: Pm2 EC
Competitor: Pm1 P Pm2 DA C

ECRE-1
ECRE-2
ECRE-3
ECRE-4a
ECRE-4b
ECRE-4c

TABLE 3.2: Regulation and binding site specificity of F9 ATF site-binding activities

The regulation of ECRE activities during differentiation of F9 EC cells to PE cells and their relative binding to the various oligonucleotides are shown. The TRE and C/EBP sites, like Pm1, also did not bind any of the ECRE activities. NT = not tested.
## F9 ATF site Binding Activities

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CHAPTER 4

TRANSCRIPTIONAL REGULATION OF ATF SITE-CONTAINING PROMOTERS IN F9 EC AND PE CELLS
4.1 Introduction

The ATF site-binding activities which are regulated during differentiation of F9 EC cells are presumably responsible for regulating transcription from ATF site-containing promoters. It was therefore of interest to study the transcription of such promoters in F9 EC cells, and its regulation during differentiation. The promoters of the E1A-inducible Ad5 E4 and E2A genes, and that of the cAMP-inducible human VIP gene were chosen for this study, since it is known that their ATF sites are important cis-acting elements both for their basal transcription and their induction by E1A and cAMP (Lee and Green, 1987; Murthy et al., 1985; Tsukada et al., 1987).

4.2 Regulation of ATF site-containing promoters in F9 EC and PE cells

Transcription was assayed by transflecting promoter-driven chloramphenicol acetyl transferase (CAT) reporter constructs (Fig.4.1) in F9 EC and PE cells and measuring the CAT activity of protein extracts from transfected cells, as previously described (Gorman et al., 1982). Construct pE4 contained nucleotides -240 to +35 (four ATF sites), and pE4Δ, nucleotides -138 to +35 (one ATF site) of the Ad5 E4 gene (Lee and Green, 1987). Sequences up to -240 had already been shown to be sufficient for wild type expression in vivo and in vitro (Lee and Green, 1987). The transcriptional activity of the E2A promoter (nucleotides -96 to +68), containing one ATF site (Murthy et al., 1985; La Thangue et al., 1990), and the VIP promoter (-94 to +146), containing two ATF sites (Tsukada et al., 1987), were also tested in these cells; the VIP promoter has previously been shown to be activated by cAMP via its ATF sites (Fink et al., 1988). Several control constructs were used to determine transfection efficiency. Construct pCMV, containing nucleotides -301 to +72 of the immediate early (IE) human cytomegalovirus (CMV) gene, was used as a positive control for transcription as these transcriptional control sequences are exceptionally active in the CV1 cell line (Boshart et
al., 1985). The promoterless vector pBLcat3 and the vector pBLcat2, which contains the HSV tk promoter (-105/+51), were chosen in order to assess basal transcription. Transfection efficiency was determined by transfecting cells with an expression vector containing nucleotides -301 to +72 of the CMV IE gene cloned upstream of the E.coli lacZ reporter gene: cells that take up the plasmid should express the enzyme, allowing the number of cells transfected in cultures of F9 EC and PE cells to be determined by assaying for β-galactosidase activity in situ. The proportion of β-galactosidase-positive cells in each transfected culture ranged from 0.5% to 6%. Although pBLcat3 has no recognizable promoter elements, extracts prepared from either F9 EC or PE cells transfected with it did have low CAT activity, presumably resulting from initiation of transcription at sites in the vector sequence. However, the ratio of pBLcat3 activity in F9 EC and PE cells generally reflected the ratio of transfection efficiency in the two cell types, suggesting that quantitative differences in transcriptional activity of pBLcat3 resulted from expression in different numbers of cells, rather than differences in the rate of transcriptional initiation in the two cell types. For this reason, the activity of the test promoters was normalised with respect to pBLcat3 activity, thus eliminating differences that arose from the different efficiencies of DNA uptake by these two cell types (Rickles et al., 1989).

For any of the constructs studied, a degree of variation in absolute CAT activity values was observed, although the trend for each construct was clear (Fig.4.2). As discussed above, pBLcat3 activity reflected the difference in transfection efficiencies between the two cell types (Fig.4.2: lanes 11 and 12) and was therefore set to an arbitrary value of 1 in both cell types. The positive control, pCMV, was active in F9 EC cells but was induced further during differentiation (Fig.4.2: compare lanes 13 and 14). pBLcat2, which had low activity, was also activated during differentiation (Fig.4.2: compare lanes 9 and 10). Of the four test promoters, E4 was the most active in F9 EC cells (Fig.4.2: compare lane 1 to lanes 3, 5 and 7), whereas a deletion of 102 bp (nucleotides -240/-139) reduced its activity 36-fold (Fig.4.2: compare lane 3 to 1). This was in agreement with the results of Lee and Green (1987) who demonstrated that these sequences are required for full promoter activity in 293 cells and
HeLa cell extracts; both E4 promoter constructs were nevertheless activated further during differentiation (Fig. 4.2: compare lane 2 to lane 1, 4 to 3). In contrast, and in agreement with previous in vitro results (La Thangue et al., 1990), pE2A activity was reduced during differentiation (Fig. 4.2: compare the average CAT activity of lane 6 to that of lane 5). Surprisingly, the VIP promoter was inactive in both cell types (Fig. 4.2: compare lanes 7 and 8), although this construct was transcriptionally active as well as forskolin- and cAMP-inducible in PC12 cells (Tsukada et al., 1987), indicating that its lack of activity in F9 cells was cell-specific and not due to a generally transcriptionally inert construct.

In summary, the -240 E4 promoter deletion (containing four ATF sites) was active in F9 EC and PE cells, whereas the -138 deletion (containing one ATF site) was less active in both cell types, and both promoters were induced during differentiation. In contrast, Ad5 E2A promoter activity was down-regulated (see summary in Fig. 4.4). The VIP promoter, on the other hand, which contains two ATF sites, was inactive in both cell types. These results demonstrate that different ATF site-containing promoters have distinct transcriptional activities in F9 EC cells.

4.3 dbcAMP treatment of F9 EC cells does not affect in vivo transcription

Since one of the agents used to differentiate F9 EC cells was dbcAMP, it was important to determine whether the increase in E4 promoter activity during differentiation was due to a cAMP-dependent stimulation via its ATF sites rather than a response to differentiation per se. However, when F9 EC cells were treated with dbcAMP alone, no increase in transcription was apparent with either E4, E4A or the cAMP-inducible promoter VIP (Fig. 4.3), suggesting that activation was not caused by dbcAMP treatment alone, but rather that it was dependent upon differentiation (summarized in Fig. 4.4). In addition, dbcAMP treatment of F9 EC cells did not alter their morphology or reactivity with α-SSEA-1 (Solter and Knowles, 1978), which recognizes an early embryonic antigen that is present in EC cells but disappears after differentiation (Fig. 4.5; see also Strickland et al., 1981), nor
did it alter the pattern of proteins binding to the $E4\Delta$ promoter assayable by gel retardation (see Chapter 5). This result is consistent with those of Rickles et al. (1989) who demonstrated that cAMP-dependent trans-activation of the tissue plasminogen activator gene cannot occur in F9 EC cells, Gonzalez and Montminy (1989), who reported very low activity of the somatostatin ATF site in F9 EC cells, and Imperiale et al. (1984) who found that $E2A$ expression was not altered during treatment of F9 EC cells with cAMP.

4.4 Discussion

The first conclusion to be drawn from these experiments is that different ATF site-containing promoters have distinct transcriptional activities in F9 EC and PE cells. Down-regulation of transcription from the $E2A$ promoter is explained by the fact that DRTF-1, a transcription factor that binds to a cis-acting element adjacent to the ATF site and is transcriptionally active in F9 EC cells (La Thangue et al., 1990; Shivji and La Thangue, 1991), is also down-regulated during F9 EC cell differentiation (La Thangue and Rigby, 1987). The DRTF-1 site has been shown to be important for $E2A$ transcription in vivo and in vitro (Murthy et al., 1985; La Thangue et al., 1990; Murray et al., 1991; Zamanian and La Thangue, 1992). However, in the $E4$ and VIP promoters, the ATF site is the most important cis-acting element for in vivo and in vitro transcription (Gilardi and Perricoudet, 1984; Gilardi and Perricoudet, 1986; Lee and Green, 1987; Tsukada et al., 1987; Fink et al., 1988). The fact, therefore, that one ATF site-containing promoter, VIP, is inactive while another, $E4$, is active in F9 EC cells suggests a difference in the activity of their ATF sites, which may result from them being recognized by different ATF site-binding proteins. Functional differences between the $E4$ and VIP ATF site have also been demonstrated by Lee et al. (1989), who showed that a VIP ATF site is cAMP-, but not E1A-inducible in HeLa cells, while the converse is true for an $E4$ ATF site. Another possible explanation would be that a VIP-specific repressor inhibits VIP transcription in these cells. Alternatively, other transcription factors could be important for $E4$ promoter activity in F9 EC cells and its
increase in PE cells. With respect to that last possibility, it is worth noting that no similarity to the defined retinoic acid responsive elements (Vasios et al., 1989; de Thé et al., 1990; Lucas et al., 1991), which might be expected to be induced during differentiation of F9 EC cells with retinoic acid and dbcAMP, was revealed by visual inspection of the E4 promoter sequences. These two possibilities are addressed in the next chapter, where in vitro protein binding to the E4 and VIP promoters is investigated.

The second conclusion, that transcription in F9 EC cells is insensitive to dbcAMP treatment, is in agreement with the findings of Rickles et al. (1989) as well as Gonzalez and Montminy (1990). The latter attributed this to a lack of functional cAMP-dependent protein kinase A and transcription factor CREB, since the somatostatin ATF site was transcriptionally active only on the presence of exogenous PKA catalytic subunit and CREB. However, recent reports from Masson et al. (1992) and Ruppert et al. (1992) indicate that both CREB RNA and protein are present in F9 EC cells even though the somatostatin and VIP promoters are inactive. In addition, there is measurable cAMP-stimulated kinase activity in F9 EC cells which furthermore increases during differentiation (Plet et al., 1982). Lack of cAMP inducibility, therefore, may be due to insufficiently high endogenous PKA and CREB levels, or factors that inhibit their productive interaction and subsequent ATF site-dependent trans-activation.

Finally, this study demonstrated that differentiation affects the level of transcription from the ATF site-containing promoter of the adenovirus E4 gene. Given that ATF site-binding activities are themselves regulated during differentiation of F9 EC cells, one could speculate that this transcriptional regulation results from different or modified ECRE activities regulating the E4 promoter in the two cell types; this possibility is examined in the next chapter.
Some of the known transcription factor binding elements are indicated. Sequences and nucleotide numbering from: HSV tk (McKnight and Kingsbury, 1982), E4 and E4Δ (Steenbergh and Susenbach, 1979), E2A (Murthy et al., 1985), VIP (Tsukada et al., 1985), CMV (Akrigg et al., 1985).
Transcriptional Control Regions used for Transfection

BLcat2

\[-105 \quad \text{Sp1} \quad \text{CAAT} \quad \text{Sp1} \quad +51\]

(HSV 1 thymidine kinase gene)

E4

\[-240 \quad \text{ATF} \quad -160 \quad -140 \quad \text{ATF} \quad -50 \quad +35\]

(Ad5 E4 gene)

E4 ∆

\[-138 \quad \text{ATF} \quad -50 \quad +35\]

(Ad5 E4 gene)

E2A

\[-96 \quad \text{ATF} \quad \text{DRTF 1} \quad +68\]

(Ad5 E2A gene)

VIP

\[-94 \quad \text{ATF} \quad \text{ATF} \quad +146\]

(human vasoactive intestinal polypeptide gene)

CMV

\[-301 \quad \text{AP1} \quad -140 \quad \text{ATF} \quad +72\]

(human cytomegalovirus immediate early gene)
FIG. 4.2: Transcriptional activity of ATF site-containing promoters in F9 EC and PE cells

Activities of CAT constructs transfected into F9 EC and PE cells. * "Ave. CAT Act." represents the average CAT activity for each construct from the number of experiments indicated below, after normalisation with respect to pBLcat3 activity (lanes 11 and 12) in order to take transfection efficiency differences into account (see text). Construct : number of different plasmid preparations used for transfection / number of experiments performed / number of experiments quantitated and averaged = pE4 : 3/5/2, pE4A : 3/5/5, pE2A : 3/5/5, pVIP : 2/3/3, pBLcat2 : 2/3/3, pBLcat3 : 3/5/5, pCMV : 1/4/3 ; not all experiments were quantitated since in some cases the substrate became limiting. Two contaminants of the chloramphenicol preparation can be seen, one migrating in front of, and one behind the 1-acetate chloramphenicol product (the slowest of the two mono-acetylated forms), most clearly in lane 10, for example.
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FIG. 4.3: *dbcAMP treatment of F9 EC cells has no effect on transcriptional activity*

Activities of CAT constructs transfected into F9 EC cells and F9 EC cells treated with $10^{-3}$ M *dbcAMP (cA)*. "Ave. CAT Act." represents the average CAT activity for each construct from the number of experiments indicated below, after normalisation with respect to pBLcat3 activity, in order to take into account transfection efficiency differences between the two cell types (see text). Construct: number of different plasmid preparations used for transfection / number of experiments performed / number of experiments quantitated and averaged = pE4: 2/3/3, pE4Δ: 2/2/2, pVIP: 2/2/2, pBLcat3: 2/2/2. A contaminant of the chloramphenicol preparation can be seen migrating between the two acetylated products.
Cell Type: EC cA EC cA EC cA EC cA

Ave. CAT Act.*: 99 96 2.5 1.8 0.7 0.6 1 1

Construct: pE4 pE4Δ pVIP pBLcat3
FIG. 4.4: Summary of results

Ratio of CAT activities of the indicated constructs: in F9 PE relative to EC cells (PE/EC), and in dbcAMP-treated (cA) relative to untreated F9 EC cells (cA/EC); n.d.: dbcAMP-treated EC cells were not transfected with pE2A.
cAMP Treatment Differentiation
FIG. 4.5: *Immunochemical staining of F9 EC cells* (a, b), *F9 EC cells treated with dbcAMP* (c, d) and *PE cells* (e, f) *for stage-specific embryonic antigen 1*

a), c) and e) no primary antibody; secondary antibody: horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins.

b), d) and f) primary antibody: α-SSEA1 MC480; secondary antibody: horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins.
CHAPTER 5

ATF SITE-DEPENDENT PROTEIN BINDING TO THE E4 AND VIP PROMOTERS
5.1 Introduction

This chapter describes the results from experiments that investigated the DNA binding activities which recognize the E4 and VIP promoters in vitro. If the difference in their levels of transcription in vivo were indeed due to differences in the activity of their ATF sites, two models could be envisaged to explain it. In the first model, each promoter would bind different ATF site-binding activities: those binding to E4 would activate transcription while those binding to VIP would inhibit it. In the second model, the two promoters would bind common ECRE activities but these would not activate transcription from the VIP promoter by failing, for example, to interact productively with the initiation complex. By a similar reasoning, the increase in E4 transcriptional activity during differentiation would be caused by ECRE species of higher trans-activation potential binding to this promoter in F9 PE than in EC cells.

5.2 E4 promoter ATF site-binding activities are regulated during differentiation

To test whether the regulation of E4 transcription during differentiation correlated with regulated binding of ATF site-binding activities to this promoter, gel retardation experiments were performed, in which the oligonucleotides used previously to define the ECRE activities (Chapter 3) were used to compete for complexes formed on probe E4Δ. This allowed the assignment of the E4Δ complexes to the most appropriate ECRE activities. The E4Δ rather than the E4 promoter was used because it afforded better resolution, though the full length E4 promoter, as expected, also bound ATF site-specific activities in both EC (Fig.5.1: compare lane 3 to lane 4) and PE (Fig.5.1: compare lane 6 to lane 7) cells.

Three ATF site-specific complexes, ECRE-1, ECRE-3 and ECRE-4, formed in F9 EC cell extracts on the E4Δ promoter (Fig.5.2a: lane 2) and two, ECRE-3, much reduced with respect to its F9 EC cell levels, and PCRE-1, in F9 PE cell extracts (Fig.5.2b: lane 2).
As expected, they were all competed by the wild type ATF site (P) but not by the mutant site Pm1 (Fig. 5.2 a and b: compare lanes 5 and 6 to lanes 3 and 4) indicating that they were all ATF site-specific. However, each single point mutation, in or around the ATF site, affected the formation of each complex differently. Mutant Pm2 competed preferentially for complexes ECRE-1 (composed of a single band) and ECRE-4 (composed of at least two closely spaced bands) in F9 EC cells, and PCRE-1 (composed of a single band) in F9 PE cells (Fig. 5.2 a and b: lanes 15 and 16). The mobility difference between the ECRE-1 and ECRE-4 complexes on E4Δ was similar to the mobility difference of the two complexes formed in the same extract on the Pm2 oligonucleotide; this correlation, combined with their sequence specificity and regulation, established their identity as ECRE-1 and ECRE-4. Similarly, oligonucleotides PATFm and E2A-3’ competed for a complex which was unaffected by competition with Pm2 (Fig. 5.2 a: compare lanes 11-14 to 15 and 16), and down-regulated during F9 EC cell differentiation (compare lane 2 in Fig. 5.2a to lane 2 in Fig. 5.2b), suggesting it was caused by ECRE-3. Again, competition for promoter-bound ECRE-3 reflected the already observed binding activity of the oligonucleotides, since E2A-3’ showed higher affinity than PATFm (Fig. 5.2 a: compare lane 13 to 11). When a similar competition was performed in F9 PE cell extracts, PATFm and E2A-3’ competed for the ECRE-3 complex, but this was less apparent because ECRE-3 was down-regulated during differentiation of F9 EC to PE cells (Fig. 5.2 b: lanes 11-14); they did not, however, affect the PCRE-1 complex. On the other hand, mutant sites PE4Fm and Pm3, though initially designed to bind ATF in preference to E4F, competed equally for all complexes in both cell types, albeit to a weaker extent than did P (Fig. 5.2 a and b: lanes 5-10), suggesting that E4F could not be detected as binding to the E4Δ promoter in F9 EC whole cell extracts.

These experiments allowed the relationship of all the complexes formed on the E4Δ promoter with those formed on the isolated variant ATF binding sites to be established. From the activities defined, ECRE-1, -3 and -4 bound to the E4Δ promoter in F9 EC cell extracts, whereas PCRE-1 and reduced levels of ECRE-3 bound in F9 PE extracts. The regulation of ECRE-1 and ECRE-3 promoter complexes reflected their regulation as defined with the
oligonucleotide probes. In contrast, the most abundant binding activity, ECRE-2, defined with the isolated ATF site in both F9 EC and PE extracts, did not bind to the E4Δ promoter. This was somewhat surprising and suggests that the P oligonucleotide, which contains E4 promoter sequences from -58 to -39, has different binding characteristics in the context of the promoter, indicating that other parameters, apart from sequence specificity, must influence binding efficiency.

There are two conclusions to be made from these experiments. First, the in vivo differentiation-regulated activity of the E4Δ promoter is reflected in a differentiation-regulated change in the ATF site-binding activities binding to it. Second, the promoter binds a subset of the total available ECRE activities.

5.3 dbcAMP treatment of F9 EC cells does not affect the ECRE activities

Consistent with its lack of effect on transcriptional activity (Chapter 4), dbcAMP treatment of F9 EC cells did not affect the pattern of ECRE activities binding to the E4Δ promoter (Fig.5.3: compare lane 2 to lane 1). This suggests that, as for its transcriptional regulation, the regulation of protein binding to the E4Δ promoter was due to differentiation per se and not simply to an increase of intracellular cAMP. It also reinforces the correlation between in vivo transcriptional activity of, and in vitro protein binding to promoters: a change in E4Δ promoter activity was accompanied by a change of ECRE complexes formed on this piece of DNA during differentiation, while identical rates of transcription in dbcAMP-treated and untreated F9 EC cells were reflected in the unaltered pattern of ECRE activities binding to E4Δ.

5.4 Promoter-specific and common ATF site-dependent complexes

The complexes formed on the VIP promoter which, in contrast to E4Δ, was inactive during differentiation, were investigated next. Three ATF site-specific complexes were
formed on this promoter in F9 EC extracts and two in PE extracts (Fig. 5.2c and d: lane 2). 

As for the E4Δ promoter, their identity was established by competing with the panel of variant ATF sites. In F9 EC extracts, two complexes (jointly labelled as ECRE-2) were only competed by P, PE4Fm and Pm3 (Fig. 5.2c: lanes 4, 6 and 9) indicating an ECRE-2 specificity, whereas the slower migrating complex (labelled ECRE-3), which migrated just above a non ATF site-specific complex, was competed by PATFm and E2A-3' (Fig. 5.2c: lanes 5 and 10). Pm2 did not compete efficiently for any of the complexes in F9 EC or PE extracts, in contrast to its effect on complexes formed on the E4Δ promoter (Fig. 5.2c and d: lanes 7 and 8), indicating that ECRE-1 and ECRE-4 did not bind to the VIP promoter.

In F9 PE extracts, the two ATF site-specific complexes were, again, only competed by P, PE4Fm and Pm3 (Fig. 5.2d: lanes 4, 6 and 9) arguing that the VIP promoter binds only one activity, ECRE-3, in common with the E4Δ promoter, and another activity, of ECRE-2 specificity, present in both extracts and not bound by E4A. The lack of regulation of the VIP ECRE2 complex was consistent with the constitutive presence of ECRE2 during differentiation (see Chapter 3).

5.5 Discussion

The data presented here could be accommodated within a combination of the two models described in the Introduction (5.1), which proposed either that distinct ATF activities bound to E4 and VIP, or that common ATF site-binding activities had different functions in the context of each promoter. The E4Δ and VIP promoters, which had different in vivo transcriptional activities, bind distinct but overlapping subsets of the total available ATF site-binding activities in F9 cells. They both bind ECRE-3, but ECRE-1, ECRE-4 and PCRE-1 bind exclusively to E4Δ, whilst ECRE-2 binds exclusively to VIP (summarized in Table 5.1). While it cannot be excluded that non-ATF-specific complexes binding to the VIP promoter, such as can be seen in Fig. 5.2c and d, inhibited its transcription, it is likely that ECRE-2 and ECRE-3 are inactive in the context of this promoter. The hypothesis that
ECRE-2 is not an ATF site-binding *trans*-activator is supported by the fact that it does not bind to the transcriptionally active *E4Δ* promoter. On the other hand, because ECRE-3 binds to both the transcriptionally active *E4Δ* and the transcriptionally inactive *VIP*, its *trans*-activating potential cannot be safely surmised.

The *E4Δ* promoter experiments demonstrated that a correlation exists between *in vivo* transcription and *in vitro* protein binding. Its transcriptional induction during differentiation is reflected in a change in its ATF site-dependent complex formation between F9 EC and PE cells: binding of ECRE-1, ECRE-3 and ECRE-4 in EC cells was replaced by binding of PCRE-1 in PE cells. PCRE-1 may be related to ECRE-4, since they both have the same specificity, and may result from a PE cell-specific post-translational modification of ECRE-4. This would explain its reduced electrophoretic mobility compared to ECRE-4 (Fig.5.3: compare lane 3 to lane 1). Therefore, ECRE-1, ECRE-4 and possibly ECRE-3 in F9 EC cells, and PCRE-1 in PE cells, are probably responsible for *E4Δ* transcription and its activation during differentiation.

The correlation between *in vivo* transcription and *in vitro* protein binding is further supported by the observation that the same ECRE activities bind to the *E4Δ* promoter in EC cells and dbcAMP-treated EC cells reflecting the fact that, as was shown in Chapter 4, dbcAMP treatment of F9 EC cells does not affect the transcriptional activity of this promoter.

Another interesting point arising from these experiments is that ECRE-2 did not bind to *E4Δ* although it bound P, whose sequence is taken from within the *E4Δ* sequence. Two possible models may explain this. According to the first, ECRE-2 would be excluded from its binding site by other DNA binding proteins; according to the second, ECRE-2 would be unable to bind to its site when this is present in the context of this promoter. The first model seems unlikely since, in the binding reaction, the probe was in excess over the protein extract. However, the possibility cannot be excluded that the promoter-specific binding of ECRE-1, ECRE-2 and ECRE-4 is determined by "auxiliary" DNA binding proteins, which do not form discrete complexes on their own but selectively promote the
binding of specific ECRE activities; this is presented, in cartoon form, in Fig. 5.4. According to this model, "auxiliary" proteins would promote binding of ECRE-1 and ECRE-4, but not ECRE-2 to E4Δ. Different "auxiliary" proteins would promote binding of ECRE-2 to VIP. Alternatively, and according to the second model, in the context of the larger DNA fragment, E4Δ, the P DNA sequence would adopt a conformation that precludes binding of ECRE-2. This second model could also incorporate a modification of the first, since the E4Δ-induced conformation of P could exclude ECRE-2 indirectly by increasing the affinity of the P sequence for other ECRE activities. The same arguments can be applied to AP1, whose complex with the TRE in F9 PE cells could be competed by P but which did not bind to the E4Δ promoter.

In conclusion, ATF site-containing promoters that have different transcriptional activities bind distinct ATF site-binding proteins in F9 EC and PE cells (summarized in Table 5.1). In addition, a change in a promoter's in vivo transcriptional activity during differentiation is reflected by a change in the pattern of ECRE proteins that this promoter binds in vitro. These results imply that ECRE activities differ, not only in their precise nucleotide specificity and their regulation during differentiation, but also in their affinities for different promoters and, presumably, their ability to activate transcription.
FIG. 5.1: Complexes formed on the full length E4 promoter in F9 EC and PE whole cell extracts

Gel retardation of the full length E4 promoter with F9 EC and PE whole cell extracts in the presence or absence of 770-fold molar excess wild type (wt)(ACGTCA) or mutant (mut) (AAATCA) ATF oligonucleotides. The ATF site-specific complexes are indicated by arrows. All lanes are from the same experiment.
FIG. 5.2: Specificity of protein complexes formed on the E4Δ and VIP promoters in F9 EC and PE cell extracts

Gel retardation with probe E4Δ in F9 EC (a) and PE (b) whole cell extracts, in the presence of the indicated oligonucleotide competitors. Lanes 3, 5, 7, 9, 11, 13, 15, and 17: 100-fold molar excess competitor; lanes 4, 6, 8, 10, 12, 14, 16 and 18: 1000-fold molar excess competitor. The fastest migrating complex, which is competed both by P (lane 4) and Pm1 (lane 6), is non-specific.

(continued opposite next page)
FIG. 5.2 (cont'd.): Specificity of protein complexes formed on the E4Δ and VIP promoters in F9 EC and PE cell extracts

Gel retardation with probes VIP and E4Δ in F9 EC (c) and F9 PE (d) whole cell extracts in the presence of the indicated oligonucleotide competitors. All lanes contained 1000-fold molar excess competitor, except lanes 8 and 18 which contained 2000-fold excess. All lanes are from gels run simultaneously.
FIG. 5.3: *dbcAMP treatment of F9 EC cells has no effect on protein/DNA complex formation*

Gel retardation with probe $E4\Delta$ in F9 EC cells (EC), F9 EC cells treated with dbcAMP (cA), or F9 PE (PE) whole cell extracts.
FIG. 5.4: Schematic representation of a possible model for promoter-specific binding of ECRE activities

Two different "auxiliary" proteins, which do not form discrete DNA complexes on their own, direct binding of ECRE-2 only to the VIP promoter and binding of ECRE-4 only to E4; ECRE-3, on the other hand, can bind to both promoters without the aid of an "auxiliary" protein.
TABLE 5.1: Promoter and oligonucleotide binding specificity of the regulated ATF site binding activities in F9 EC and PE cells

The regulation of F9 ATF site-binding activities during differentiation of F9 EC cells to PE cells, and their relative ability to bind to the listed oligonucleotides and promoters are shown.
### F9 ATF site Binding Activities

<table>
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<th>Promoter Specificity</th>
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</thead>
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</tr>
<tr>
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<td>- +++ ++ + - ++ - - - ✓ ✓</td>
<td></td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>PCRE-1</td>
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</table>
CHAPTER 6

ATF RNA LEVELS IN F9 EC AND PE CELLS
6.1 Introduction

Having identified a number of ATF site-binding proteins in F9 EC and PE cells that had distinct sequence and promoter preferences and were regulated during differentiation, it was of interest to address the mechanisms of their regulation and sequence selectivity, as well as to determine their different effects on transcription. To achieve this, it would be necessary to study each activity in a pure state, preferably as the product of a cDNA. Two approaches were available. One would involve purification of each activity in turn by DNA binding site affinity purification, and cloning of the corresponding cDNA(s) on the basis of the protein sequence; the other approach would be to assess whether any of the ECRE activities were encoded by any of a growing number of cDNAs that were being isolated by other laboratories. The first approach had two main disadvantages: DNA affinity purification would not be entirely practical since some activities, for example ECRE-1 and ECRE-4a, shared the same DNA specificity, and such an effort would be extremely time-consuming. The second approach, on the other hand, offered the hope of a relatively rapid identification, by comparing the regulation and specificity of known cDNA products to those of the ECRE activities. This approach was therefore chosen, especially since, even in the case that its primary aim were to fail, it would still yield pertinent information on the regulation of the ATF transcription factor gene family during cellular differentiation.

During the course of the identification of ATF site-binding activities in F9 cells, a number of laboratories had isolated cDNAs encoding distinct ATF site-binding proteins (Hoeflper et al., 1988; Gonzalez et al., 1989; Maekawa et al., 1989; Hai et al., 1989), using a variety of cloning techniques including screening an expression library with an oligomerized E4 ATF site (Hai et al., 1989). The predicted polypeptides were highly homologous across a region rich in basic amino acids followed by a "leucine zip" region, similar to the DNA binding domains of the Jun, Fos and C/EBP families of transcription factors (reviewed in Busch and Sassone-Corsi, 1990).
Since the proteins that bind to the ATF site were encoded by a gene family, an investigation of their RNA levels in F9 EC and PE cells was carried out. This would determine whether there were any instances where ATP RNA regulation reflected the regulation of ECRE activities and, therefore, if there were any potential candidates among the ATP genes for encoding ECRE activities.

6.2 Results

The available ATP cDNAs were all derived from human cells. Therefore, two prerequisites regarding the specificity of mouse F9 EC and PE cell RNA detection needed to be satisfied: that experimental conditions allow only the closest mouse homologue of each human cDNA to be detected, and that cross-hybridization across the highly homologous DNA binding domain be avoided. These two prerequisites seemed most efficiently met by excluding, wherever possible, the DNA binding domain from probe sequences and by using the ribonuclease (RNase) protection assay. RNase protection affords maximum specificity of detection by eliminating weak cross-hybridization at the RNase digestion step. This is because very few consecutive base-pairing mismatches are tolerated in each protected band, since RNase A detects and cleaves approximately 50% of possible base pair mismatches (Sambrook et al., 1989). With the exception of ATP2, ATP4, ATP6, cfos and fosB, no probe contained sequences coding for a complete DNA binding domain (see Fig. 6.1 and Chapter 2). Furthermore, when a selection of the probes used in this study were hybridized to in vitro transcribed RNA from the same cDNAs, under the same conditions which were used subsequently to assay F9 cellular RNA, no cross-hybridization was observed between, for example, human ATP1 and human ATP2 sequences (Fig. 6.2: compare lanes: 4 to 3, 6 to 7), human ATP4 and human ATP6 (Fig. 6.2: compare lanes: 10 to 9, 12 to 13), or mouse cj un and mouse JunD (Fig. 6.2: compare lane 16 to 15). The lack of a distinct protected fragment in this control experiment was due to the presence of RNA molecules that were smaller than full length, due to premature termination by RNA polymerase in vitro, and gave rise to a
continuous series of protected probe fragments appearing as a smear. This lack of cross-hybridization confirmed that, though different ATF and API family members share some amino acid similarity in their DNA binding domains, they lack significant overall nucleotide similarity.

As a control for RNA amounts, a probe for the human $\gamma$-actin gene was used because levels of $\gamma$-actin RNA are constant in different human cell lines and mouse tissues (Enoch et al., 1986; Dzierzak et al., 1988).

This study also addressed the RNA induction of members of the API (jun and fos) families during F9 EC cell differentiation to PE cells with dbcAMP and retinoic acid, as this had previously been studied only during retinoic acid treatment of EC cells (Yang-Yen et al., 1990), which does not give rise to parietal endoderm-like cells (Strickland et al., 1980).

A second control probe was derived from the proliferation-regulated c-myc mouse cellular oncogene (reviewed in Marcu, 1987) whose expression was known to be down-regulated as the proliferation rate decreases during F9 EC cell differentiation (Dony et al., 1985).

The $\gamma$-actin signals from F9 EC and PE total RNA were indeed equal in equal amounts of total cellular RNA (Fig.6.3: compare lanes 3 and 4), and hybridizations with this probe were always included in every experiment.

No specific protected probe fragments were detected with the $ATF1$ probe (Fig.6.3: compare lanes 7 and 8 to lane 6) in F9 EC or PE cell RNA, although HeLa RNA did give rise to a protected probe fragment (Fig.6.5: lane 11), as expected since the $ATF1$ cDNA was cloned from HeLa cells (Hai et al., 1989). On the other hand, multiple protected probe fragments, indicated by arrows and brackets, whose intensity did not alter during differentiation, were observed with the $ATF2$, $ATF4$ and $ATF6$ probes (Fig.6.3: compare lane 12 to lane 11, 20 to 19, 24 to 23). In contrast, the intensity of the probe fragments protected with the $ATF3$ probe was higher in PE cells (Fig.6.3: compare lane 16 to lane 15). The multiplicity of mouse RNA-specific bands was presumably due to species-specific nucleotide differences giving rise to imperfect base pair matching and subsequent RNase cleavage, and
possibly also to alternative splicing, which has been documented for \textit{ATF2} \cite{Georgopoulos1992} and two other members of the \textit{ATF} family, \textit{CREB} \cite{Berkowitz1990, Ruppert1992} and \textit{CREM} \cite{Foulkes1991, Foulkes1992}.

In agreement with results published by others, \textit{junD} was constitutively transcribed (Fig.6.4 : compare lanes 7 and 8), while \textit{jun} and \textit{c-fos} transcripts were detectable only in PE cells (Fig.6.4 : compare lane 4 to lane 3, 12 to 11) \cite{Yang-Yen1990}. Yang-Yen \textit{et al.} (1990) also found that, similarly to \textit{cjun}, \textit{junB} expression is transiently induced by RA treatment of F9 EC cells. \textit{FosB} transcripts were undetectable in both cell types (Fig.6.4 : compare lanes 19 and 20 to lane 18) \cite{Mumberg1991}, and \textit{fra-1} transcripts were similarly not detected in either cell type (Fig.6.4 : compare lanes 15 and 16 to lane 14), though in this case, because the probe only contained 3' untranslated RNA, the possibility cannot be excluded that lack of hybridization was due to lack of conservation between rat and mouse 3' untranslated sequences.

Finally, transcripts initiating at both \textit{c-myc} promoters (reviewed by Fahrlander and Marcu, 1986) were only present in the rapidly proliferating EC cells and not in the terminally differentiated PE cells (Fig.6.4 : compare lane 23 to lane 24), consistent with previous results which have shown that the decrease in \textit{c-myc} RNA during differentiation is due to a post-transcriptional mechanism \cite{Dony1985}.

The occasionally high non-specific hybridization background was due to the fact that all probes were hybridized to the RNA synchronously and conditions were not optimized for any one specific probe.

In conclusion, most \textit{ATF} genes were expressed to the same levels in F9 EC and PE cells, with the exception of \textit{ATF1}, whose RNA was undetectable, and \textit{ATF3}, whose RNA levels increased during differentiation (Table 6.1).
6.3 Discussion

Two important conclusions can be drawn from this study of ATF RNA levels in F9 EC and PE cells. First, it has shown that the ATF gene family is not coordinately regulated during differentiation. ATF2, ATF4 and ATF6 are constitutively expressed while ATF3 RNA levels increase during differentiation, and ATF1 transcripts are not detected in either cell type. These distinct modes of regulation during differentiation provide one more example of the diverse responses and functions of ATF proteins, as discussed in Chapters 1 and 11.

Second, the RNA regulation of some ATF genes was consistent with their protein products contributing to an ECRE activity. For example, ATF2, ATF4 or ATF6 could be involved in ECRE-2 or ECRE-4/PCRE-1, since their levels of expression did not change during differentiation. ATF3, whose RNA levels increased during differentiation, could nevertheless also be a potential candidate, since RNA and protein levels need not necessarily reflect DNA binding activity. This can be modulated by post-translational modifications (reviewed by Berk, 1989), and by the choice of dimerization partner. This latter possibility will be illustrated in the next chapter, but it is worth noting here that, in a theoretical equilibrium of homodimers and heterodimers displaying distinct DNA binding properties, it would suffice for the levels of one monomer to be altered in order to produce a new set of dimers with different properties. In this light, it is interesting to note that expression of jun/fos family members was also non-coordinately regulated during F9 EC cell differentiation. This could allow, for example, the substitution of an ATF3/JunD dimer in EC cells by an ATF3/cJun dimer in PE cells. ATF1, on the other hand, was ruled out as a possible ECRE candidate since its transcripts were not detected in F9 EC or PE cells.

As will be seen in the next chapter, the list of ECRE gene candidates was narrowed down by assaying the DNA binding activity of in vitro translated polypeptides. However, no ATF RNA was down-regulated as sharply as ECRE-1 or ECRE-3 during differentiation, and therefore the possible identity of the genes encoding these activities remained elusive.
Finally, this study guided the choice of two *ATF* members for study at the protein level: *ATF2*, whose expression remains constant during F9 EC cell differentiation, and *ATF3*, whose RNA levels increase during differentiation.
FIG. 6.1: Schematic representation of sequences contained in the anti-sense RNA probes

Arrows indicate the sequences contained in the anti-sense RNA probes synthesized from the indicated cDNAs. Boxes indicate coding cDNA regions (open-sided boxes indicate that the cDNA was partial), lines indicate non-coding cDNA regions. B = basic domain, LZ = "leucine zipper".
FIG. 6.2: Specificity of anti-sense RNA probes

RNase protections of the indicated probes by the indicated *in vitro* translated RNAs (1 = *ATF1*, 2 = *ATF2*, 4 = *ATF4*, 6 = *ATF6*, jD = *junD*, cj = *cjun*). The lack of distinct protected fragments was due to the presence of prematurely terminated RNA molecules, a common occurrence during *in vitro* transcription, which gave rise to a continuous series of protected probe fragments appearing as a smear.
<table>
<thead>
<tr>
<th>Probe</th>
<th>ATF1</th>
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<td>1</td>
<td>2</td>
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|         | -    | 4    | 6    | -    | jD   |

- 998 -
- 634 -
- 517/506 -
- 396 -
- 344 -
- 298 -
- 220/1 -
- 154 -

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
FIG. 6.3 : γ-actin and ATF RNA levels in F9 EC and PE cells

RNase protections of the indicated probes (h = human) by the indicated RNAs (20 μg, except for lanes 1-4 : 2 μg) (t = yeast tRNA). Specifically protected fragments are indicated by arrows and brackets. Lanes 1, 5, 9, 13, 17 and 21 contained 1 μl of a 1:1000 dilution of the indicated undigested probe. Positions and sizes, in nucleotides, of denatured DNA size standards are shown to the left of each panel. The following groups of lanes are from the same experiment (autoradiography times indicated in brackets) : 1-4 (overnight) and 21-24 (5 days) ; 5-12 (4 days) ; 13-16 (5 days) and 17-20 (overnight).
FIG. 6.4: RNA levels of AP1 family members and c-myc in F9 EC and PE cells

RNase protections of the indicated probes (m = mouse, h = human, r = rat) by the indicated RNAs (20 μg) (t = yeast tRNA). Specifically protected fragments are indicated by arrows and brackets. Lanes 1, 5, 9, 13, 17 and 21 contained 1 μl of a 1:1000 dilution of the indicated undigested probe. Positions and sizes, in nucleotides, of denatured DNA size standards are shown to the left of each panel. The following groups of lanes are from the same experiment (autoradiography times indicated in brackets): 1-4 (overnight); 5-8 (overnight) and lanes 5-12 of Fig.6.3; 9-20 (10 days); 21-24 (2 days).
FIG. 6.5: ATF1 is expressed in HeLa but not in F9 EC and PE cells

RNase protections of the indicated probes (h = human) by the indicated RNAs (20 µg) (t = yeast tRNA). Specifically protected fragments are indicated by arrows. Lanes 2 and 7 contained 1 µl of a 1:1000 dilution of the indicated undigested probe. Lane 1: denatured DNA size standards whose sizes, in nucleotides, are indicated to the left. Autoradiography time: 2 days.
Probe: (h)yA (h)ATF1

RNA:

(h)yA

1 2 3 4 5 6 7 8 9 10 11

(h)ATF1

(m)yA

75
TABLE 6.1: Regulation of ATF and API family members’ RNA levels during differentiation

NT: not tested
## Regulation of RNA levels

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</tr>
<tr>
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CHAPTER 7

DNA BINDING SPECIFICITY OF *IN VITRO* TRANSLATED ATF AND AP1 FAMILY PROTEINS
7.1 Introduction

As discussed in the previous chapter, the study of ATF transcript levels suggested some candidate genes that could, by virtue of their regulation during differentiation, encode ECRE activities. ATF2, ATF4 and ATF6 could contribute to ECRE-2 and/or ECRE-4/PCRE-1. So could ATF3, because its induction, at the RNA level, during differentiation may not directly reflect an increase in DNA binding activity. No ATF gene, on the other hand, was down-regulated at the RNA level during differentiation, which made it difficult to speculate on the relation between ATF proteins and ECRE-1 or ECRE-3.

Since ECRE activities were distinguishable in their ability to bind variant ATF sites (Table 3.2), another way of relating them to known ATF proteins was to assess the binding of in vitro translated ATF polypeptides to the panel of ATF probes.

7.2 Construction of in vitro expression vectors

Such a study required in vitro transcription and translation of the ATF cDNAs, some of which did not contain an translational initiation codon, while the complete sequence of others was not available. For this reason, a set of three vectors was constructed which would allow in vitro transcription and translation of an inserted cDNA in any of the three possible translational frames. The Stratagene vector Bluescript-KS (BS-KS) was modified by the insertion, 3' to the T3 promoter, of an oligonucleotide containing the Kozak consensus for vertebrate initiation of translation (Kozak, 1987) followed by an ATG initiation codon and an EcoRI cloning site. The three vectors differed in the number of nucleotides inserted after the ATG codon, thus allowing translation in all three translational frames (Fig.7.1). They also differed from each other and from the parent vector, BS-KS, in their restriction maps (Fig.7.1 and Fig.7.2). These vectors did indeed fulfill their purpose since, when the ATF6 cDNA, whose reading frame was unknown because of partial sequence information, was cloned in all three, only one construct produced a polypeptide after in vitro transcription by
phage T3 RNA polymerase and translation in a rabbit reticulocyte lysate, and this polypeptide had the correct molecular weight as predicted from the size of the cDNA (Fig. 7.2B: compare lane 2 to lanes 1 and 3; see Fig. 7.3B for predicted molecular weight of ATF6).

7.3 DNA binding specificity of in vitro translated ATF dimers

Having engineered constructs that could be used for in vitro expression of the ATF cDNAs which are expressed in F9 EC and PE cells, the apparent relative molecular weight (M_r) of the resulting polypeptides was determined by ^35S-methionine labelling and SDS-PAGE (Fig. 7.3). In all cases, the predominant translated product matched the expected M_r within an acceptable margin.

The DNA binding specificity of the polypeptides was then tested by gel retardation experiments using the oligonucleotide binding sites that define the ECRE activities. This study was restricted to the ATF and API family members which are expressed in F9 EC and PE cells and were available in cDNA form. For each experiment, synthesis of the polypeptides was checked by performing parallel translations with either unlabelled L-methionine or ^35S-labelled methionine and examining the products of the latter after SDS-PAGE and autoradiography (see, for example, Fig. 7.3A). The rabbit reticulocyte lysate itself did contain weak P-binding activity (Fig. 7.4A: lane 1) but this did not mask the binding of ATF2 (Fig. 7.4A: lanes 2-4) or ATF3 (Fig. 7.4A: lanes 5 and 6) polypeptides to a wild type ATF site. Neither ATF2 nor ATF3 bound to the Pm1 mutant (Fig. 7.4A: compare lanes 8, 9, 10, 11 to lanes 2, 3, 5, 6, respectively), or to the E2A-3' site (Fig. 7.4A: compare lanes 13, 14, 15, 16 to lanes 2, 3, 5, 6, respectively), though they did bind to the Pm2 mutant, albeit less efficiently than to P (Fig. 7.6B: compare lanes 9, 10, 11, 12, 13, 14 to lanes 1, 2, 3, 4, 5, 6, respectively). However, as shown in Fig. 7.4B, contrary to ATF3 (lane 1) and ATF2 (lane 6), ATF4 (lane 2) and ATF6 (lane 8) showed only negligible binding, indistinguishable from cFos (lane 3) or the endogenous activity in reticulocyte lysate (lane 5). In order to investigate
the possibility of ATF4 and ATF6 binding only as heterodimers, they were co-translated with either ATF2 or ATF3. The complexes formed by these co-translations on the DNA were, however, indistinguishable from those seen with ATF2 or ATF3 alone (Fig.7.4B: compare lanes 9 and 10 to 6, lane 11 to 7). Similarly, ATF4 and ATF6 did not bind to the E2A-3' site, either alone or in a co-translation (Fig.7.4B: lanes 12-14; Fig.7.5: lanes 8-11).

These results confirmed the RNA studies by indicating that none of the ATF cDNA products tested is likely to participate in ECRE-3, defined by the E2A-3' sequence. ATF2 and ATF3 could, however, contribute to ECRE-1 and ECRE-4, since they bound both to the Pm2 mutant and the wild type site P. Finally, the inability of ATF4 and ATF6 to bind to the wild type P probe seems to preclude them from being related to the ECRE activities.

7.4 ATF/API heterodimerization causes changes in DNA binding specificity

While this study was in progress several reports were published showing heterodimerization between members of the ATF and API transcription factor families (Benbrook and Jones, 1990; Hai and Curran, 1990; Ivashkiv et al., 1990; Macgregor et al., 1990). Co-translation of ATF and API family RNAs can yield DNA binding heterodimers so that, for example, ATF2 can heterodimerize with cJun or JunD to form DNA-binding complexes which migrate to a position between the ATF2 and Jun homodimers (Fig.7.6A: compare lane 2 to lanes 1 and 3; Fig.7.6B: compare lane 7 to lanes 6 and 8).

Importantly, the choice of heterodimerization partner affects DNA binding specificity. The cJun/cFos heterodimer, for example, bound to the TRE and not to the ATF site (Fig.7.7A: compare lane 7 to lane 1), while the cJun/ATF2 heterodimer bound exclusively to the ATF site (Fig.7.7A: compare lane 9 to lane 3). ATF3, on the other hand, as a homodimer, bound only to the ATF site (Fig.7.7B: compare lane 2 to lane 8); as a heterodimer with cJun or JunD, however, it bound both to the ATF site and to the TRE, albeit more efficiently to the former (Fig.7.7B: compare lane 1 to lane 7; Fig.7.7A: compare lane 2 to lane 8). ATF2/cJun, ATF3/cJun and ATF3/JunD also bound to Pm2 (Fig.7.7A: 

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lanes 5 and 6; Fig. 7.7B: lane 4). Again, no ATF2/Jun or ATF3/Jun heterodimer bound to the E2A-3' probe (Fig. 7.5: compare lanes 4-7 to the negative control in lane 3, and the positive control in lane 2).

Therefore, heterodimerization can occur between members of the ATF and API families as well as within each family (Hai et al., 1989; Nakabeppu et al., 1988). Furthermore, the resulting heterodimers can display a specificity for either an ATF site (ATF2/cJun), or an API site (cJun/cFos), or both (ATF3/JunD).

In conclusion, this part of the study increased the possible contributors to ECRE activities to include JunD in F9 EC and PE cells and cJun in PE cells, since they too bound to the ATF site, as heterodimers with ATF2. ATF3/Jun dimers, on the other hand, would seem unlikely to correspond to an ECRE activity, since they also bound to the TRE, in contrast to all ECRE activities. These results are summarized in Table 7.1.

7.5 Discussion

The DNA binding specificity of in vitro translated ATF proteins and their RNA levels during F9 EC cell differentiation suggested that ATF2, ATF3, JunD and cJun could be components of the ECRE activities (summarized in Table 7.1). ATF2, whose RNA levels remained constant during differentiation, bound to both P and Pm2 but not E2A-3'. ATF3, whose RNA levels are higher in PE cells also bound both P and Pm2 but not E2A-3'. Finally, JunD and cJun also bound to both P and Pm2 as heterodimers with ATF2 and ATF3. ATF4 and ATF6, on the other hand, which were constitutively expressed at the RNA level during differentiation, did not bind to the ATF site in solution; this is in agreement with Hai and Curran (1990), who showed that ATF4 binds to the ATF site only as a heterodimer with Fos proteins. It cannot be excluded, however, that the inability of ATF4 and ATF6 to bind to the ATF site was due to partial cDNA sequences. Nonetheless, these were obviously able to bind to a multimerized ATF site when expressed as fusion proteins in bacteria and immobilized on a membrane, since this is how they were isolated (Hai et al., 1989). The DNA binding
domain alone, however, may not allow stable binding to a monomeric site in solution, although it has been shown to be sufficient for DNA binding of Jun and Fos (Abate et al., 1990). None of the ATF proteins examined bound to the E2A-3′ site; this was consistent with the lack of any ATF gene that would reflect the regulation of ECRE-3, by being transcribed in EC cells and not in PE cells. This is not too surprising since the E2A-3′ site, despite its similarity, is quite unlike, in terms of nucleotide sequence and position relative to the transcriptional start site, any other ATF site. Significantly, a detailed analysis of this site by point mutation revealed that ECRE-3 binding requires not only the CG nucleotides of the ATF core site but also the CG nucleotides further downstream, at positions +23 and +24 relative to the E2A transcriptional start site (M. Zamanian, unpublished observations).

Although it was clear, therefore, that neither ATF2 nor ATF3 could be involved in ECRE-3, it was not possible to assign them or their heterodimers with Jun proteins to specific ECRE activities. They all bound to both P and Pm2 and could therefore equally contribute to ECRE-1 and ECRE-4/PCRE-1. However, the common binding properties of these different ATF and Jun complexes could be due to a relaxed specificity of in vitro synthesized polypeptides, since they were often translated from incomplete cDNAs. It is therefore possible, for example, that one of these complexes contributes to ECRE-2, which, when produced in the cell, only binds to P, but under the conditions used in these experiments also binds more weakly to the Pm2 site.

Furthermore, although the sequence specificity of ATF2 and ATF3 is consistent with either or both being present in an ECRE-1 complex, the down-regulation of ECRE-1 during differentiation is in contrast to the unaltered RNA levels of ATF2 and ATF3. RNA levels, however, need not always reflect levels of protein (Mueller et al., 1990; Cusella-De Angelis et al., 1992) and even the presence of protein does not always guarantee DNA binding activity, since association with other proteins may sequester it away from the DNA (Bauerle and Baltimore, 1988). Furthermore, even if, for example, ATF3 protein levels increased during differentiation, and an ATF3 homodimer were binding to the ATF site in
EC cells, heterodimerization with a PE cell-specific bZIP protein could change its specificity and prevent it from binding to the ATF site in F9 PE cells.

In conclusion, therefore, although no clear assignations of ATF proteins to ECRE activities could be made, these DNA binding experiments have suggested three ECRE activities that may be related to the examined ATF polypeptides: ECRE-1, ECRE-2 and ECRE-4/PCRE-1 may be encoded, solely or jointly, by \textit{ATF2}, \textit{ATF3}, \textit{cjun} and \textit{junD}.

The combinations of possible ATF/AP1 dimers that might bind DNA are numerous, and even if this study were exhaustive, it could not hope to be conclusive, given the limitations described above. It has, nevertheless, served as a pointer to potential candidates, but it has also illustrated the importance of differential dimerization for determining DNA binding specificity, a mechanism that has important implications for the regulation of transcription during differentiation. Taking ATF3 as an example, let us assume that, in EC cells, several dimers exist in equilibrium, say ATF3/ATF3, ATF3/ATF2, ATF3/JunD. In PE cells, two kinds of changes known to occur will upset this equilibrium: the increase in ATF3 levels and the appearance of cJun and cFos. Not only is there now more ATF3 to complex with JunD, but cJun is also available for dimerization; on the other hand, both cJun and JunD have an increased choice in partners with the appearance of cFos (presented in cartoon form in Fig. 7.8). Thus, both the levels of existing dimers can change and new dimers can be formed after differentiation. Given that different dimers have an ATF site and/or a TRE specificity, the ability of the cell to regulate transcription \textit{via} these sites will be modified as the levels of \textit{trans}-regulating dimers of the appropriate specificity change. Therefore, changes in transcription during differentiation could be partly caused by changes in the levels of heterodimers that exhibit a given DNA binding specificity. Of course, not only would this mechanism be combined with others, such as differential modification of activation domains of transcription factors, but also influenced by them, since dimerization itself can be controlled by post-translational modifications (discussed in Chapter 11).
FIG. 7.1: Design of the BS-ATG vectors
Construction of BS-ATG Vectors

1. Digest BS-KS with HindIII and EcoRI

2. Insert oligonucleotides containing Kozak sequence - ATG - EcoRI cloning site, as follows:

   - BS-ATG0 → EcoRV site deleted from BS-KS
     \[
     \text{AGCTTGCCACC}{}^{\text{ATG}} \text{CGGGTGGTACCTTAA}
     \]

   - BS-ATG1 → EcoRV site deleted from BS-KS
     HindIII site destroyed after re-ligation
     \[
     \text{AGCTCGCCACC}{}^{\text{ATGG}} \text{GCGGTGGTACCTTAA}
     \]

   - BS-ATG2 → EcoRV site deleted from BS-KS
     HindIII site destroyed after re-ligation
     NruI site created
     \[
     \text{AGCTCGCGAGCGACC}{}^{\text{ATGGG}} \text{GCGGTGGTACCTTAA}
     \]

<table>
<thead>
<tr>
<th>Vector</th>
<th>Expect linearisation by:</th>
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<tr>
<td></td>
<td>EcoRI</td>
</tr>
<tr>
<td>BS-KS</td>
<td>✓</td>
</tr>
<tr>
<td>BS-ATG0</td>
<td>✓</td>
</tr>
<tr>
<td>BS-ATG1</td>
<td>✓</td>
</tr>
<tr>
<td>BS-ATG2</td>
<td>–</td>
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</table>
FIG. 7.2: Properties of the BS-ATG vectors

A) Diagnostic restriction digests of the three BS-ATG vectors and the parent vector BS-KS (refer to Fig. 7.1 for predicted patterns). BS-KS = Bluescript-KS, BA0 = BS-ATG0, BA1 = BS-ATG1, BA2 = BS-ATG2. The conformations of plasmid DNA are indicated to the left of lane 1.

B) The ATF6 cDNA was cloned into, and transcribed from, all three vectors but was only translated from the one which provided the correct translational frame. The positions and sizes, in kD, of protein molecular weight standards (M_p) are shown to the left of lane 1.
A

Vector: BS-KS, BAO, BA1, BA2
Enzyme: -R1, RV, H, N

open circular
linear
super-coiled

B

Mr/kD

66 -
45 -
29 -
20 -

BA2 - ATF6
BA1 - ATF6
BA0 - ATF6

1 2 3
FIG. 7.3: In vitro translated and co-translated $^{35}\text{S}$-Met-labelled polypeptides

A) SDS-PAGE of the indicated $^{35}\text{S}$-Met-labelled in vitro translations (2 μl); lane 8: the reticulocyte lysate was incubated with $^{35}\text{S}$-metionine in the absence of any RNA. The positions and sizes, in kD, of protein molecular weight standards are shown to the left of each panel.

B) Table of vectors used to obtain the polypeptides shown in (A), observed and expected relative molecular weights ($M_r$), in kD. Vectors ATF2S, ATF2L, ATF3S and ATF3L were constructed by T.Hai. The following expected molecular weights were taken from the references listed in brackets: CREBP1 (Maekawa et al., 1989), cJun (Ryseck et al., 1988), ATF2L, ATF2S, ATF3L, ATF3S (Hai et al., 1989); the others were calculated by multiplying the number of codons in each cDNA by the weighted average molecular weight of an amino acid, 0.126 kD (calculated from Harlow and Lane, 1988).
### A

**RNA**

- ATF6
- ATF4
- ATF3S
- ATF2L
- ATF2S
- CREBP1

**Gel Analysis**

- Lanes 1-8
- Lanes 9-13

### B

<table>
<thead>
<tr>
<th>cDNA Expression Vector</th>
<th>Approx. Observed Mr/kD</th>
<th>Expected Mr/kD</th>
</tr>
</thead>
<tbody>
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<td>BA1-CREBP1</td>
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<td>51.4</td>
</tr>
<tr>
<td>ATF2L</td>
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<td>31</td>
</tr>
<tr>
<td>ATF2S</td>
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<td>ATF3L</td>
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</tr>
<tr>
<td>BA0-ATF4</td>
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</tr>
<tr>
<td>BA1-ATF6</td>
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<td>22</td>
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<tr>
<td>BS-cjun</td>
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<td>35.9</td>
</tr>
<tr>
<td>BS-junB</td>
<td>40</td>
<td>43</td>
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<tr>
<td>BS-junD</td>
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<td>42</td>
</tr>
<tr>
<td>BA1-cfos</td>
<td>32-37</td>
<td>37</td>
</tr>
</tbody>
</table>
FIG. 7.4: Binding of in vitro translated ATF proteins to probes P, Pm1 and E2A-3'

Gel retardation of the indicated probes with the indicated in vitro translated polypeptides (rl = reticulocyte lysate unprogrammed with RNA, or programmed with RNA for: c1 = CREBP1, 2L = ATF2L, 2S = ATF2S, 3L = ATF3L, 3S = ATF3S, 4 = ATF4, 6 = ATF6; CREBP1 is a longer cDNA for ATF2, isolated from a human brain, rather than a HeLa cell, cDNA library). This figure is a composite of five experiments from which lanes that were not relevant have been omitted; the following groups of lanes are from the same gels: A) 1-6, 7-16: the two gels were run simultaneously but lanes 7-16 were exposed for three times as long as lanes 1-6, B) 1-3, 4-8, 9-14.
FIG. 7.5: No ATF polypeptides bind to the E2A-3' probe

Gel retardation of the E2A-3' probe with the indicated *in vitro* co-translated polypeptides. Lane 2 contained F9 EC whole cell extract as a positive control for binding; lane 3 contained reticulocyte lysate incubated in the absence of RNA, as a negative control.
FIG. 7.6: Binding of in vitro translated ATF homodimers and ATF2/Jun heterodimers to probes P and Pm2

A) Gel retardation of probe P with in vitro translated c-Jun (cJ) and CREBp1 (Cl) homodimers and the intermediate mobility co-translated CREBp1/c-Jun (Cl+cJ) heterodimer.

B) Gel retardation of the indicated probes with the indicated in vitro translated polypeptides (3S = ATF3S, Cl = CREBp1, 2S = ATF2S, 2S+JD = ATF2S and JunD co-translated) (lanes 1, 3, 5, 7, 8, 9, 11, 13 : 4 μl; lanes 2, 4, 6, 10, 12, 14 : 2 μl). The arrows indicate the JunD and ATF2S homodimers (JD/JD, 2S/2S) and the intermediate mobility ATF2S/JunD (2S/JD) heterodimer. All lanes are from the same experiment.
FIG. 7.7: Dimerization controls DNA binding specificity

DNA binding of the indicated in vitro co-translated dimers to the indicated probes. All lanes in (A) are from the same experiment, as are all lanes in (B).
FIG. 7.8: Regulation of possible ATF3 dimers during F9 EC cell differentiation

This cartoon illustrates the levels of some ATF and API family proteins in F9 EC and PE cells, possible homo- and hetero-dimers and their DNA binding specificity.
TABLE 7.1: Summary of DNA binding specificities of in vitro translated polypeptide complexes

Probe names listed in boxes indicate that the corresponding translated or co-translated polypeptides were tested for binding to these probes; probe names in circles indicate that they bound the relevant dimer.
DNA Binding of In Vitro Translated Polypeptides

<table>
<thead>
<tr>
<th></th>
<th>ATF2</th>
<th>ATF3</th>
<th>ATF4</th>
<th>ATF6</th>
<th>cJun</th>
<th>JunB</th>
<th>JunD</th>
<th>cFos</th>
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<tr>
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<td></td>
<td></td>
<td>P</td>
<td>P</td>
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<td>ATF6</td>
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<td>cJun</td>
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<td>cFos</td>
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CHAPTER 8

GENERATION AND CHARACTERISATION OF ANTI-PEPTIDE POLYCLONAL ANTIBODIES AGAINST MEMBERS OF THE ATF AND AP1 TRANSCRIPTION FACTOR FAMILIES
8.1 Introduction

The next stage in this study involved preparing antibodies against particular ATF proteins: these would be useful reagents for three purposes. Firstly, they would allow a direct study of the regulation of ATF protein levels during F9 EC cell differentiation. Secondly, they should, in principle, provide a means of relating the ECRE activities to specific ATF proteins through a study of the effects of anti-ATF antibodies on the ECRE/DNA complexes. As shown elsewhere in this study (Chapter 10) and by others (Hay et al., 1989; Huang and Hearing, 1989), antibodies that react with a given DNA binding protein will often inhibit DNA binding or further retard the protein/DNA complex in a gel retardation assay. Thirdly, they could be used to study in vivo dimerization. The analysis of ATF RNA levels during the differentiation of F9 EC cells (Chapter 6) combined with the study of the DNA binding properties of in vitro translated ATF polypeptides (Chapter 7) suggested that two ATF species should be studied further at the protein level: namely, ATF2, whose RNA levels remain constant during differentiation, and ATF3, whose RNA levels are higher in PE cells. ATF2 and ATF3 were the only members of the family that were both expressed in F9 cells and bound efficiently to the ATF site in vitro. Finally, both polypeptides were also of intrinsic interest because of their heterodimerization properties. ATF2/ATF3 dimers bind to the ATF site (Hai et al., 1989) but heterodimerization with members of the API transcription factor family alters or broadens the DNA binding specificity of the constituent proteins (Chapter 7). Furthermore, cJun and JunD, which both can dimerize with ATF2 and ATF3 in vitro, differ in their RNA regulation during F9 EC cell differentiation: cjun RNA is detectable only in PE cells, while the levels of junD are identical in both EC and PE cells. Thus, antibodies against ATF2, ATF3 and Jun proteins should allow a study of in vivo heterodimerization and its regulation during F9 EC cell differentiation.
8.2 Choice of peptide immunogens

It was therefore decided to raise antibodies against ATF2 and ATF3. However, in order to avoid cross-reactivity to the similar DNA binding domains of other ATF proteins, it was considered advisable to choose potential epitopes outside this region.

A computer-assisted prediction of hydrophobic (Kyte and Doolittle, 1982) and hydrophilic (Hopp and Wood, 1983) regions in ATF2 and ATF3 was performed, since it is thought that hydrophilic regions are immunogenic, based on the general belief that they are exposed to water and therefore also at the surface of the protein. Not surprisingly, the most hydrophilic region in both molecules was in the conserved highly charged basic domain (Fig. 8.1). Two other regions were therefore chosen, from which the peptides ATF2D and ATF3C were derived, based on their relatively high hydrophilicity value (Fig. 8.1) and their position, flanking the DNA binding domain (Fig. 8.2A). It was speculated that this might make them accessible to antibodies since they would be adjacent to the two α-helices of the basic region and the "leucine zip" (Talanian et al., 1990; Shuman et al., 1990; Patel et al., 1990), and therefore relatively near the surface of the protein that was exposed and available for DNA binding. The lack of regions of significant amino acid similarity among other members of the ATF and AP1 families was checked by a computer-assisted comparison, which showed that no more than three consecutive amino acids of the peptide were identical in any of these related proteins (Fig. 8.2B and C).

For a comparative study of AP1 family proteins, peptides from the basic domain (PEP1) and a region near the middle of the amino acid sequence (PEP2) of cJun, as well as a peptide from the DNA binding domain of cFos (P10), were also used for immunisation. Previously, PEP1 and PEP2 had been successfully used by Bohmann et al. (1987) to obtain rabbit antisera that recognized affinity purified human AP1, as well as in vitro translated and bacterially expressed vJun. Anti-PEP1 sera were expected to cross-react with JunB and JunD, due to the similar amino acid sequences of their DNA binding domains, while anti-PEP2 sera were expected to be cJun-specific, as the PEP2 region lies outside the DNA binding domain.
binding domain (Fig. 8.3). P10 had also been successfully used by Adamkiewicz et al. (1990) to obtain rabbit antisera that recognized native in vitro translated Fos and inhibited DNA binding of HeLa cell API. Anti-P10 serum might also be expected to cross-react with ATF3, given that the ATF3 DNA binding domain is the most similar to that of cFos among all members of the ATF/API families (Fig. 8.4). The positions of the chosen peptide epitopes are schematically summarized in Fig. 8.5.

8.3 Immunisation

All peptides were coupled to keyhole limpet haemocyanin, and used to immunise rabbits subcutaneously, as described in Chapter 2. Pre-immune sera are referred to by the rabbit identification number (see Table 8.1) preceded by "R" (for example, the pre-immune serum from rabbit 42 is referred to as serum R42). Sera from test bleeds were numbered with the rabbit identification number to which a last digit was added, indicating the serial number of the test bleed (for example, serum from the first bleed of rabbit 42 is identified as serum 421).

8.4 ELISAs with immobilised peptides

The specificity of the rabbits' immune response was first assayed by testing the sera against immobilised peptide in an ELISA. As shown in Fig. 8.6, each immune serum recognized only the peptide against which it had been raised, and none of the pre-immune sera showed any anti-peptide reactivity.

8.5 Immunoblotting of in vitro translated polypeptides

Each serum was then tested for reactivity against the denatured in vitro translated polypeptide which contained the peptide used as immunogen. As a control for antigen
specificity, reactivity with *in vitro* translated ATF IS was used in all cases. As can be seen in Fig.8.7, sera from rabbits immunised with cFos-derived P1O reacted specifically only with a polypeptide (indicated by arrows) that was only present in a cFos *in vitro* translation and not in an ATF IS *in vitro* translation (compare lanes 2 to 1 and 5 to 4, and lanes 3 to 2, 6 to 5). Sera from rabbits immunised with cJun-derived PEP2, on the other hand, recognized *in vitro* translated cJun (indicated by arrows) but not ATF IS (Fig.8.7: compare lanes: 26 to 25 and 29 to 28, 27 to 26 and 30 to 29). The high background of cross-reacting bands in the reticulocyte lysate is presumably due to the high amounts of lysate (200-300 mg per lane) and serum (1/20 dilution) that were used, the long incubation time (overnight) and the fact that the sera were not purified. It is clear, however, that the above sera recognized their cognate antigens above this high background, since the bands indicated by arrows were present only in reticulocyte lysate that had been programmed with the appropriate *in vitro* transcribed RNA rather than ATF IS RNA. Unfortunately, none of the sera raised against ATF2D, ATF3C or PEP1 were able to specifically recognize denatured *in vitro* translated ATF2, ATF3 or cJun, respectively (Fig.8.7: compare lanes: 9 to 8 and 12 to 11, 15 to 14 and 18 to 17, 21 to 20 and 24 to 23), despite the fact that they did specifically react with their cognate peptides (see Fig.8.6). Later test sera likewise did not show any reactivity either (not shown). This lack of reactivity could be due either to the sensitivity of the antibodies, since only small amounts of antigen were present in the *in vitro* translation reactions, or to denaturation-sensitive epitopes. However, when the *in vitro* translated polypeptides were labelled by $^{35}$S-methionine incorporation, thus enhancing detection, and used in the native state, they were all recognized by the appropriate sera (see Section 8.6).

In conclusion, only anti-P1O and anti-PEP2 sera could react, in an immunoblotting experiment, with the denatured *in vitro* translated polypeptides which contained the P10 and PEP2 sequences, respectively.
8.6 Immunoprecipitation of $^{35}\text{S}$-labelled \textit{in vitro} translated polypeptides

Detection of \textit{in vitro} translated polypeptides was improved when these were labelled by $^{35}\text{S}$-methionine incorporation and used in an immunoprecipitation assay. Indeed, ATF2, either as ATF2L or as the longer \textit{ATF2} cDNA product CREBP1, could be immunoprecipitated specifically by an anti-ATF2D serum but not by the corresponding pre-immune serum (Fig.8.8A: compare lanes 6 to 5 and 12 to 11; Fig.8.9A: compare lanes 6 to 5); an unrelated immune serum had the same effect as the pre-immune (Fig.8.8A: compare lanes 7, 8 and 5 to lane 6; Fig.8.9A: compare lanes 11-14 and 5 to lane 6). The immunoprecipitation was specific for ATF2 since when ATF3 was used as the antigen in the same experiment, only background bands were observed (Fig.8.8 : compare lane 9 in panel A to 15 in panel B). It was clear, nevertheless, that the efficiency of immunoprecipitation was low (Fig.8.8A: the band in lane 6 results from immunoprecipitation of three times the amount of neat antigen loaded in lane 2) when compared with that of anti-ATF3C sera (Fig.8.8B: the band in lane 16 results from immunoprecipitation of three times the amount of neat antigen loaded in lane 10), and that not all animals immunised with ATF2D were capable of immunoprecipitating \textit{in vitro} translated ATF2 (Fig.8.9A: compare lanes 4, 8 and 10 to lane 6; the first test serum from animal 1034 also seemed to be reacting weakly with ATF2L - subsequent test sera from this animal were not tested and therefore it could not be confirmed that this was a specific response). All anti-ATF2D sera were, however, capable of detecting with equal efficiency a cluster of ATF2 proteins in cell extracts, both by immunoblotting and by immunoprecipitation (see Chapter 9). This "inconsistency" may be due to post-translational modifications of the peptide region occurring in the reticulocyte lysate but not in the cell, or association with endogenous reticulocyte lysate proteins that prevented the antibody from recognizing its epitope. Conversely, the epitope may adopt a non-reactive conformation in the \textit{in vitro} translated polypeptide but may be exposed in the cellular protein polypeptide via a post-translationally induced conformational change. Potential phosphorylation sites for protein kinase C (PKC) and casein kinase II (CKII) in or around
the immunogen peptide sequences in ATF2 (PKC : S^{464}; CKII : S^{437}, S^{466}) and ATF3 (PKC : S^{109}; CKII : S^{101}, T^{107}, T^{118}) were in fact identified by a computerized search for modification sites.

All anti-ATF3C sera, on the other hand, specifically immunoprecipitated ATF3 as compared to the corresponding pre-immune sera (Fig.8.9B : compare lanes 4 to 3, 6 to 5, 8 to 7, and 10 to 9) or an unrelated immune serum (Fig.8.9B : compare lane 11 to lane 10). The immunoprecipitating efficiency of the antiserum increased with successive booster immunisations (Fig.8.9B : compare lane 7 (third test bleed) to lane 6 (second test bleed)). Immunoprecipitation by anti-ATF3C sera was also antigen-specific since they failed to immunoprecipitate ATF2 (Fig.8.8B : lanes 4 and 8). An anti-P10 serum, directed against the cFos DNA binding domain, immunoprecipitated ATF3 (Fig.8.8B : compare lane 14 to lane 13) as well as cFos (Fig.8.8B : compare lane 14 to lane 12) and with the same efficiency as an anti-ATF3C serum (Fig.8.8B : compare lane 14 to lane 16). This was expected, since the ATF3 DNA binding domain is very similar to that of cFos (see Fig.8.4). Anti-ATF3C serum, on the other hand, did not cross-react with cFos (Fig.8.8B : lane 17) confirming the lack of cFos/ATF3 similarity outside the DNA binding domain.

Both anti-P10 sera immunoprecipitated cFos specifically when compared to the corresponding pre-immune sera (Fig.8.10 : compare lanes 4 to 3, 6 to 5) or a serum raised against a similar region of cJun (Fig.8.10 : compare lanes 4 and 6 to lane 7). This reaction was antigen-specific, since neither ATF2 nor cJun were recognized by an anti-P10 serum (Fig.8.10 : lanes 8 and 18).

Both anti-PEP2 sera immunoprecipitated cJun specifically when compared to the corresponding pre-immune sera (Fig.8.10 : compare lanes 11 to 10, 13 to 12) or a serum raised against the DNA binding domain of cFos (Fig.8.10 : compare lanes 11 and 13 to lane 18). Both anti-PEP2 sera cross-reacted with JunD (Fig.8.10 : compare lanes 31 to 30, 33 to 32), though not with JunB (Fig.8.10 : compare lanes 24 to 23, 26 to 25). This was unexpected, since PEP2 is not in the conserved DNA binding domain. However, when a computer-assisted comparison was performed, JunD, but not JunB, was seen to contain a sequence showing
extensive similarity (9/11 identical amino acids, compared with 4/11 for JunB) to PEP2 (see Fig.8.3), which may explain its cross-reactivity with anti-PEP2.

Only one of the anti-PEP1 sera, 333, immunoprecipitated cJun specifically (Fig.8.10: compare lane 15 to 14), though they both reacted against immobilised PEP1 peptide in an ELISA assay (Fig.8.6). 333 also immunoprecipitated JunD (Fig.8.10: compare lane 29 to lane 28), but not JunB (Fig.8.10: compare lane 22 to lane 21), or the unrelated ATF3 (Fig.8.10: lane 19; the background levels of ATF3L present in this lane are not higher than those in lane 5 of Fig.8.9, where a pre-immune serum was used). Cross-reactivity with JunD but not JunB is presumably due to the longer uninterrupted sequences of amino acid identity between JunD and PEP1 than between JunB and PEP1 (Fig.8.3).

In conclusion, in an immunoprecipitation assay, at least one of the sera raised against each peptide specifically recognized the polypeptide from which the peptide sequence was derived. All of the observed cross-reactions could be explained by the presence of similar amino acid sequences in the cross-reacting polypeptides.

8.7 Effects of antisera on antigen/DNA complexes

The antisera that were capable of immunoprecipitating should also be able to react with the native antigen in a gel retardation assay. Depending on the epitope they recognize, they should either inhibit the formation of the antigen/DNA complex, by interfering with the antigen’s ability to bind DNA, or retard that complex even further, by forming an antibody-antigen/DNA complex.

Indeed, both anti-ATF3C sera were able to "shift the shift" when incubated with ATF3 and its DNA recognition site, the P oligonucleotide, as compared to the corresponding pre-immune sera (Fig.8.11A: compare lanes 3 to 2, 5 to 4) or an anti-ATF2D serum (Fig.8.11A: compare lanes 3 and 5 to 6). This "super-shift" was abolished when ATF3 was incubated with antibody in the presence of ATF3C, but not ATF2D, peptide (Fig.8.11A: compare lanes 12 and 13 to lane 11). Consistent with the immunoprecipitation experiments,
anti-P10 could also react with ATF3: in fact, and as expected, anti-P10, which recognizes the DNA binding domain, inhibited the ATF3/DNA complex (Fig.8.11A: compare lane 9 to lane 8). In contrast, anti-ATF3C, which reacts with a region outside the DNA binding domain, did not inhibit DNA binding but rather, by reacting with ATF3, caused a larger antibody-ATF3/DNA complex which migrated more slowly than the ATF3/DNA complex. This reaction was antigen-specific, since anti-ATF3C had no effect on an ATF2/DNA complex (Fig.8.11B: compare lane 9 to lane 5).

Under the same conditions though, no effect was seen with anti-ATF2D on an ATF2/DNA complex (Fig.8.11B: compare lanes 7 to 6, 12 to 11); this lack of effect did not respond to titration (not shown). When, however, the binding reaction was carried out in immunoprecipitation buffer, rather than "Manley C" (Manley et al., 1980) buffer used in all previous gel retardation experiments, the anti-ATF2D sera responded as predicted from the immunoprecipitation experiments. Namely, anti-ATF2D serum 423 "shifted" the ATF2/DNA complex to a slower mobility complex (Fig.8.12: compare lanes 8 to 7, 13 to 12). This effect was specific since it could be inhibited by excess ATF2D, but not ATF3C, peptide (Fig.8.12: compare lane 8 to lanes 9 and 10). The efficiency of this "super-shifting" was lower than that of anti-ATF3C sera 433 or 443 on the ATF3/DNA complex (see Fig.8.11), reflecting the relative efficiencies of immunoprecipitation previously observed. Similarly, consistent with the inability of anti-ATF2D serum 314 to immunoprecipitate ATF2, its small inhibitory effect on the ATF2/DNA complex was non-specific since it was not affected by the presence of ATF2D peptide (Fig.8.12: compare lanes 5 and 6 to lane 4). The same was true of an unrelated anti-PEP2 serum (Fig.8.12: compare lanes 16 and 17 to lane 15).

Anti-P10, anti-PEP1 and anti-PEP2 sera also interfered with the binding of a cJun/cFos complex to DNA whether the proteins were in vitro translated (Fig.8.13: compare lanes 4 to 3, 6 to 5, 8 to 7) or present in the AP1 complex from NIH 3T3 cells (Fig.8.13: compare lanes 11 to 10, 13 to 12, 15 to 14).
Finally, it was also apparent that sera which recognize their antigen in a given dimer may not necessarily do so when the dimerization partner is altered. For example, anti-P10 inhibited ATF3 binding to DNA (Fig.8.14: compare lane 14 to 13) but had no such effect on co-translated ATF3/JunD (Fig.8.14: compare lane 11 to 10) or co-translated ATF3/CREBP1 heterodimers (Fig.8.14: compare lane 8 to 7). Both anti-ATF2 and anti-ATF3 sera, on the other hand, "super-shifted" an ATF2/ATF3 heterodimer (Fig.8.14: compare lanes: 4 to 3, 6 to 5) as they did ATF2 or ATF3 homodimers (see above). This difference may have arisen from the unequal stabilities of different dimers. The ATF3/CREBP1 and ATF3/JunD heterodimers, for example, were presumably more stable than the ATF3/ATF3 homodimer and prevented the anti-P10 antibodies from reaching their epitope which is in the basic domain, adjacent to the dimerization domain. Anti-ATF2D and anti-ATF3C, on the other hand, react with epitopes outside the DNA binding domain and for this reason did not disrupt the protein/DNA complexes but rather "super-shifted" them.

In conclusion, all antisera capable of immunoprecipitating were also able to interact with their antigens in a gel retardation assay, causing either an inhibition of antigen/DNA complexing or a "super-shift" of the antigen/DNA "shift".

8.8 Discussion

The peptide immunisation strategy was successful in producing antisera that recognized the polypeptides from which the immunogen peptide sequences were derived, in at least two different assays, as summarized in Table 8.1. It was also successful in avoiding extensive cross-reactivity with \textit{in vitro} translated polypeptides other than the parent one, unless such cross-reactivity could be explained by extensive amino acid similarity. Finally, that the antisera reacted with the native polypeptides should allow them to be used for the study of protein levels during differentiation of F9 EC cells and the investigation of the relation of ECRE activities to ATF proteins.
FIG. 8.1: Hydropathy and hydrophilicity plots of regions within ATF2 and ATF3

Computer-generated hydropathy and hydrophilicity plots of ATF2 and ATF3 regions. The DNA binding domains are underlined; the peptide antigen sequences are boxed.
FIG. 8.2 : The ATF2D and ATF3C sequences are unique

A) Alignment of ATF2 and ATF3 amino acid sequences (incomplete, due to partial cDNAs). Similarity between amino acids is indicated, in decreasing order, by a vertical line, two dots, one dot. Gaps have been introduced in order to maximize alignment of similar sequences. The broken line indicates the DNA binding domain. The antigen peptide sequences are underlined and in bold.

B) Alignment of ATF2D peptide with other ATF/AP1 family members. Identical amino acids are indicated by a single dot. (h) = human, (m) = mouse.

C) Alignment of ATF3C peptide with other ATF/AP1 family members. Identical amino acids are indicated by a single dot. (h) = human, (m) = mouse.

A) Alignment of ATF2 and ATF3 amino acid sequences

ATF2 101 PIVPVPGPFLLLHLPQGTMVPAVIPASITSSNVHVPAAVPILVYRPMV 150
ATF3 1 ..........EFRAHPARAPAYSPAPCFPPGTSNPMPPDVPIL ..... AIP 36

151 SVPGIPGSSQPQQSEQMKRLKALTQHPPVNTGKGVHHGSGLVRQ 200
37 SELQONDASTPRPGCLG.... SECCHRPLVPPGSLVFEDFA LTPFV 82

201 SEERQPQSLQQPATSTETPSAHTTPQTSQSGRRRAANEDPEKR 250
83 KEEIPF.AIQKHKLCHRMSALESVTSSDRPLGVSITKAEVAPEEGERKK 131

[ ATF3C ]

DNA binding domain

251 K vertebral muscle, Raf1R consuming CRK growth factor 300
132 RRERKMAAKCRNKKKEKTECLQKESEKLESVNAELQAEELNKEQ 181

[ ATF2 ]

301 QLQDLLLAHDCPMTAMOICKSGYHTADKDDS 350
351 VSTSGVSS 359
217 EGTLQS ... 222

B) Alignment of ATF2D peptide with other ATF/API family members

(h)ATF2 319 KKSQHYTADKDDS 331
(h)CREB 237 SNOQVQVQASGQV 249
(h)ATF3 157 KEKLESVNAEL 169
(h)ATF4 25 KKRAEQALGEC 37
(h)ATF6 16 RESACQSRKKKE 28
(m)cJun 46 QSMTLNLADPVGS 58
(m)JunB 201 TGSSYPATISY 213
(m)JunD 149 KQSQLGAATAATS 161
(m)cFos 223 LTGGLPGEASTPES 235

(h)ATF3 110 DRPLGVSITKAEV 122
(h)CREB 276 TQPAEAARKREV 288
(h)ATF2 122 PVAIPASITSSNV 134
(h)ATF4 2 GEKLDKXXKMQE 14
(h)ATF6 6 LRRQRMKNRES 18
(m)cJun 230 EEEQTVPEMPGET 242
(m)JunB 75 DTGASLKLAST 87
(m)JunD 52 KDALTLSLAEQGA 64
(m)cFos 291 DVLDSLGSFYAADW 303

C) Alignment of ATF3C peptide with other ATF/API family members
FIG. 8.3: Alignment of cJun PEP1 and PEP2 sequences with other members of the
ATF/AP1 families

The complete amino acid sequence of mouse cJun is shown, with the antigen peptide
sequences in bold and indicated by a broken line. Identical amino acids are indicated by a
single dot. (h): human, (m): mouse.

(continued opposite next page)
cJun amino acid sequence: Homologies of other ATF/AP1 family members to peptides PEP1 and PEP2

1 MTAKMETTFY DDALNASFLQ SESGAYGYSN PKILKQSMTL NLADPVGSLK

PEP2

51 PHLRAKNSDL LTSPDVGLLK LASPELERLI IQSSNGHITT TPTPTQFLCP

(m)JunB 102 TPTPPGQYFY

(m)JunD 117 TPTSTQFLYP

(h)CREBP1 69 TPTPTRFLKN

(h)ATF3 203 TPEDERNLF

101 KNVTDEQEGF AEGFVRALAE LHSQNTLPST PSAQPVSGA GMVAPAVASV

P 112

K 127

C 79

Q 217

151 AGAGGGGGYS ASLHSEPPVY ANLSNFPYA LSCGGGAPSY GAAGLAFPSQ
FIG. 8.3 (cont'd.): Alignment of cJun PEP1 and PEP2 sequences with other members of the ATF/API families

The complete amino acid sequence of mouse cJun is shown, with the antigen peptide sequences in bold and indicated by a broken line. Identical amino acids are indicated by a single dot. (h): human, (m): mouse.
cJun amino acid sequence: Homologies of other ATF/AP1 family members to peptides PEP1 and PEP2 (cont’d.)

201  PQQQQQPQP PHHLPPQPIPV QHPRLQALKE EPQTVPEMPG ETPPLSPIDM
     (h)ATF2 RRAA
     (h)ATF3 AEVH
     (h)ATF4
     (h)ATF6
     (m)JunB PINM
     (m)JunD PIDM
     (m)cFos VEQL

PEP1

421  ESQERIKAER KRMNRKLAAS KCRKRLERI ARLEEKVT KANSELAST
     NEDPDKRRK VLE
     APEQDERKRR RE
     KGEKDLKKL KME
     SDEAVLRRQQ RMI
     EDQERIKVER KRL
     DTQERIKAEK RKL
     SPEEEKRRRI RE

301  ANMLREQVAQ LKQKVMNHVN SGCQLMLTQQ LQTF
FIG. 8.4: Alignment of cFos P10 sequence with other members of the ATF/API families

The complete amino acid sequence of mouse cFos is shown, with the antigen peptide sequence in bold and indicated by a broken line. Identical amino acids are indicated by a single dot. (h): human, (m): mouse.
cFos amino acid sequence: homologies of ATF/AP1 family members to peptide P10

<table>
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<tr>
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<th>MMFSGFNADY EASSSRCSSA SPAGDLSYY HSPADSFSSM GSPVNTQDFC</th>
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<td>51</td>
<td>ADLSVSSANF IPTVTAISTS PDLQWLVQPT LVVSAQSQT RAPHYGLPT</td>
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P10

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<tr>
<td>101</td>
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</tr>
<tr>
<td></td>
<td>(h)ATF3 EEDREK KRRERNKIA</td>
</tr>
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<td></td>
<td>(h)ATF4 EKLDKK LKKMEQNKRA</td>
</tr>
<tr>
<td></td>
<td>(h)ATF6 IAVLRK QRMIKNRES</td>
</tr>
<tr>
<td></td>
<td>(m)cJun QERlKA ERKMRlNlRA</td>
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<tr>
<td></td>
<td>(m)JunB QERlKV ERKlRlNRLA</td>
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<tr>
<td></td>
<td>(m)JunD QERlKA ERKlRlNRLA</td>
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<td>ASRCRQKRKV WV</td>
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<tr>
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<td>AAKCRKKKE KT</td>
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<tr>
<td></td>
<td>FPSCAAHRK GSSSNEPSSD SLSSPTLLAL</td>
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</tbody>
</table>
FIG. 8.5: Position of the peptide epitopes in the polypeptides

Schematic representation of the peptides' position within the proteins. Stipled boxes: basic domain, dotted boxes: "leucine zipper", black boxes: peptide epitopes; numbers indicated underneath refer to the amino acid boundaries of the peptides.
Location of Epitopes

ATF2

ATF3

cFos

cJun
FIG. 8.6: *Specificity of the antisera tested by ELISA*

ELISA with the indicated immobilised peptides and the indicated sera.
<table>
<thead>
<tr>
<th>Immuneogen</th>
<th>Serum</th>
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<tbody>
<tr>
<td>P10</td>
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FIG. 8.7: Specificity of the antisera tested by immunoblotting against in
vitro translated polypeptides

Immunoblotting experiment of the indicated in vitro translations (5 µl) by the indicated sera (diluted 1:20 in 10% BSA). Incubation with the antisera was overnight at +4°C. Positions and sizes, in kD, of protein molecular weight standards are indicated to the left of lanes 1 and 16. Arrows indicate the specifically recognized bands that were only present in the appropriate in vitro translation.
FIG. 8.8: Immunoprecipitation of $^{35}$S-labelled in vitro translated polypeptides by anti-ATF2D and anti-ATF3C sera

Immunoprecipitation of the indicated $^{35}$S-labelled in vitro translated polypeptides (3 μl) by the indicated sera. Lanes A2, A10, B10 and B11 were loaded with 1 μl of the indicated in vitro translated polypeptide. The sizes, in kD, of $^{14}$C-labelled protein molecular weight standards loaded on lanes A1, B1 and B9, are indicated to the left of these lanes.
FIG. 8.9: Immunoprecipitation of $^{35}$S-labelled in vitro translated polypeptides by anti-ATF2D and anti-ATF3C sera

Immunoprecipitation of the indicated $^{35}$S-labelled in vitro translated polypeptides (3 µl) by the indicated sera. Lanes A2 and B2 contain 1 µl of the indicated in vitro translated polypeptide. The sizes, in kD, of $^{14}$C-labelled protein molecular weight standards loaded on lanes A1 and B1 are indicated to the left of these lanes.
FIG. 8.10: Immunoprecipitation of in vitro translated polypeptides by anti-P10, anti-PEP1 and anti-PEP2 sera

Immunoprecipitation of the indicated $^{35}$S-labelled in vitro translated polypeptides (3 μl) by the indicated sera. Lanes 2, 9, 20 and 27 contain 1 μl of the indicated in vitro translated polypeptide. The sizes, in kD, of protein molecular weight standards loaded on lane 1 are indicated to its left. The following lanes come from a single experiment: 1-19; 20-33.
FIG. 8.11: Effects of anti-ATF3C and anti-P10 sera on ATF3/DNA complexes

A) Gel retardation of oligonucleotide P with the indicated in vitro translated proteins in the presence or absence of the indicated sera and peptide competitors.

B) Anti-ATF2D sera do not recognize in vitro translated ATF2D in "Manley C" buffer (Manley et al., 1980). The asterisk indicates a DNA binding activity present in rabbit serum.
FIG. 8.12: Effects of anti-ATF2D sera on ATF2/DNA complexes

Gel retardation of oligonucleotide P with the indicated *in vitro* translated proteins in the presence or absence of the indicated sera and peptide competitors. CREBP1 is a longer ATF2 cDNA product than ATF2L.
Protein:

Serum:

Peptide Comp.:

ATF2L

CREBP1

Peptide Comp.:
FIG. 8.13: Effects of anti-P10, anti-PEP1 and anti-PEP2 sera on in vitro co-translated cJun-cFos/DNA complexes and 3T3 AP1/DNA complexes

Gel retardation of oligonucleotide TRE with in vitro co-translated cJun/cFos or 3T3 AP1 in the presence or absence of the indicated sera. The arrow indicates a DNA binding activity present in rabbit serum.
FIG. 8.14: *Differential effects of sera on DNA binding dimers*

Gel retardation of oligonucleotide P with the indicated *in vitro* translated or co-translated polypeptides in the presence or absence of the indicated sera. Notice that the mobility of the CREBP1/ATF3L dimer (lane 2) is intermediate between that of the CREBP1 homodimer (lane 1) and the ATF3L homodimer (lane 12), and that of the ATF3L/JunD (lane 9) dimer slower than that of the ATF3L homodimer (lane 12). Pairs of lanes are labelled with a rabbit number: the left lane contains pre-immune serum, the right lane contains serum from the third test bleed. All lanes are from the same experiment; lane 1 and lanes 2-14 are taken from two gels run simultaneously for the same length of time.
TABLE 8.1: Summary of effects of antisera on in vitro translated polypeptides

IP: immunoprecipitation; NT: not tested.
### Summary of Effects of Antisera on *in vitro* Translated Polypeptides

<table>
<thead>
<tr>
<th>Immunogen (antigen)</th>
<th>Rabbit No.</th>
<th>ELISA</th>
<th>Immuno blotting</th>
<th>IP</th>
<th>Gel Retardation</th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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CHAPTER 9

IMMUNOCHEMICAL STUDY OF ATF AND API PROTEINS IN F9 EC AND PE CELLS
9.1 Introduction

Since the anti-ATF and -AP1 sera reacted with the appropriate *in vitro* translated antigens, it was possible to use them for the purposes for which they were originally raised, namely, to study the regulation during differentiation of ATF2 and ATF3 at the protein level, and to assess whether the ECRE activities were immunologically related to known ATF family members.

The antisera were again tested in immunoblotting and immunoprecipitation assays with F9 EC and PE cell extracts, and for their ability to affect ECRE/DNA complexes in a gel retardation assay in crude cell extracts.

9.2 ATF2 protein levels remain constant during differentiation

All anti-ATF2D sera specifically immunoblotted the same cluster of four polypeptides with approximate relative molecular weights ranging from 41 to 45 kD in EC whole cell extracts (Fig.9.1A : compare lane 2 to lane 1, 8 to 7, 10 to 9, 12 to 11). The same polypeptides, qualitatively and quantitatively, were specifically recognized in PE whole cell extracts (Fig.9.1A : compare lane 5 to lane 4 and to lane 2). Furthermore, these bands were present exclusively in nuclear and not in cytoplasmic extracts (Fig.9.1B : compare lane 1 to lane 3). The exclusive presence of ATF2 in the nucleus was consistent with nuclear, rather than cytoplasmic, proteins binding to an ATF site (Fig.9.1C : compare lane 2 to lane 1) and with the function of ATF2 as a transcription factor. Recognition by the antisera was blocked by ATF2D peptide but not by ATF3C (Fig.9.1A : compare lane 3 to lane 2, 6 to 5 ; Fig.9.1B : 2 to 1). The same cluster of bands was immunoprecipitated from ³⁵S-methionine-labelled EC and PE cell extracts, only by an anti-ATF2D immune serum (Fig.9.2A : compare lane 3 to lane 2, 6 to 5) and only in the absence of ATF2D peptide (Fig.9.2A : compare lane 3 to lane 4, 6 to 7). The ATF2 cluster migrated faster in these experiments due to the distortion caused in the 45 kD region by the presence of large amounts of immunoglobulins in the
immunoprecipitate; this was confirmed when anti-ATF2D immunoprecipitates were
immunoblotted with anti-ATF2D after electrophoresis (not shown). In addition to the 41-
45 kD bands, however, anti-ATF2D immunoprecipitates contained two polypeptides of
molecular weights 48 and 52 kD (corrected for distortion) from both EC and PE cell extracts
(indicated by an asterisk in Fig.9.2A: compare lane 3 to lane 2, 6 to 5) which were not
recognized in the immunoblotting assays (see Fig.9.1). Their precipitation was also specific,
since it was competed by ATF2D peptide (Fig.9.2A: compare lane 3 to lane 4, 6 to 7), but,
contrary to the 41–45 kD cluster, they were not recognized by anti-ATF2D under harsher
conditions of immunoprecipitation (Fig.9.2B: compare ATF2 and polypeptides indicated by
an asterisk in lanes 5 and 3). These 48 and 52 kD polypeptides could therefore be ATF2-
related, cross-reacting with anti-ATF2D only in the native state and under mild conditions.
Alternatively, they could be immunologically unrelated proteins that co-immunoprecipitated
with ATF2, and whose complexing to ATF2 was disrupted by stringent immunoprecipitation
conditions.

ATF2, therefore, appeared as a population of nuclear polypeptides, of approximate
molecular weights 41-45 kD, that were present at the same levels in F9 EC and PE cells. Two
other constitutively expressed polypeptides, of approximate molecular weights 48 and 52 kD,
were either related to ATF2 or complexed with it.

9.3 ATF2 is expressed in a variety of cell types

When an anti-ATF2D serum was used to immunoblot whole cell extracts from a
number of different cell types, similar clusters of polypeptides were specifically recognized
(Fig.9.3A: compare the odd-numbered to their adjacent even-numbered lanes; Fig.9.4A:
compare lane 6 to lanes 5 and 7; ATF2 polypeptides indicated by brackets). The similar
abundance of these polypeptides in the different cell lines tested correlated with similar levels
of ATF site binding activity (Fig.9.3B and Fig.9.4C). In fact, those extracts in which
immunoblotting detected polypeptides that were migrating faster than the 41-45 kD cluster,
and which were presumably ATF2 degradation products, also showed a pattern of partly
degraded, faster migrating ATF site complexes in the gel retardation experiment (compare,
for example, lane 12 in Fig.9.3A with lane 4 in Fig.9.3B; the presumed ATF2 degradation
products and the corresponding DNA complexes are indicated by asterisks). It was also
noticeable that there were fewer ATF2 species in, for example, HeLa cells than in F9 EC
cells (Fig.9.4A: compare lane 6 to lane 3). To test the possibility that different populations
of ATF2 molecules were phosphorylated to different extents and consequently not migrating
as a single band, extracts were incubated with alkaline phosphatase prior to SDS-PAGE and
immunoblotting: the number of bands was not, however, reduced by this treatment (Fig.9.4B
: compare lane 2 to lane 1, 4 to 3, 6 to 5), suggesting that phosphorylation was unlikely to be
responsible for the multiplicity of ATF2 polypeptides observed.

ATF2 polypeptides were therefore present in various cell types, as initially suggested
by ATF2 RNA distribution in different animal tissues (Maekawa et al., 1989; Kara et al.,
1990), and in all cases migrated as a cluster with approximate molecular weights 41-45 kD.

9.4 Reaction of anti-ATF3 sera with F9 EC and PE polypeptides

When anti-ATF3C sera were used to immunoblot F9 EC and PE whole cell extracts,
only one polypeptide, of 35 kD, was detected that was specific for the immune serum and
the peptide epitope (indicated by an arrow in Fig.9.5A: compare lane 2 to lanes 1 and 3), and
which was down-regulated during differentiation (Fig.9.5A: compare lane 5 to lane 2). All
four anti-ATF3C sera raised shared this reactivity (indicated by an arrow in Fig.9.5A:
compare lanes 8, 10, 12, 14). Contrary to ATF2 (Fig.9.1B), the 35 kD polypeptide recognized
by anti-ATF3C was cytoplasmic (Fig.9.5A: compare lane 17 to lane 15). Given that the
ATF3 cDNA was isolated from a HeLa cell cDNA library (Hai et al., 1989), immunoblotting
against extracts from HeLa cells and another human cell line, JM, was performed: neither
the 35 kD polypeptide (indicated by an arrow) nor any other specific polypeptides were
detected in either (Fig.9.6: compare lane 2 to lane 1, 6 to 5), while ATF2 could be detected

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in the same extracts during the same experiment (Fig.9.6: compare lane 4 to lane 3, 8 to 7).

Three pieces of evidence, therefore, suggested that the 35 kD polypeptide detected in F9 EC cells was not ATF3: its absence in HeLa cells, which are known to express the \textit{ATF3} gene, its down-regulation during differentiation of F9 EC cells, which contradicted the increased \textit{ATF3} RNA levels found in F9 PE cells (Chapter 6), and its absence from a nuclear protein fraction, which was inconsistent with it being a DNA binding protein. The possibility that F9 PE cells contained the 35 kD "ATF3" protein but that a PE cell-specific modification was blocking antibody recognition was addressed by pre-incubating equal amounts of F9 EC with PE extracts prior to SDS-PAGE and immunoblotting. The 35 kD polypeptide was still detected in the mixed extract as it was in the F9 EC extract (Fig.9.6: compare lane 14 to lane 10), suggesting that no PE cell-specific modification that could be reproduced \textit{in vitro} under those conditions could be responsible for the lack of the 35 kD polypeptide in F9 PE cells.

It was possible that cellular ATF3, like \textit{in vitro} translated ATF3, was not being detected by immunoblotting because the ATF3C epitope was denaturation-sensitive. However, contrary to its strong reactivity with native \textit{in vitro} translated ATF3, anti-ATF3C did not reproducibly immunoprecipitate any specific polypeptides from \textsuperscript{35}S-methionine labelled F9 EC and PE cell extracts (Fig.9.5B: compare lane 4 to lanes 5 and 2, 8 to 9 and 6), suggesting perhaps that, although ATF3 transcripts were present in both cell types, the protein levels were very low.

Anti-ATF3C sera, therefore, failed to detect ATF3 by immunoblotting in F9 EC, PE or HeLa cells, and by immunoprecipitation from F9 EC and PE cell extracts, despite the fact that they recognized \textit{in vitro} translated ATF3. This could be due to a denaturation-sensitive epitope and low levels of ATF3 protein.
9.5 Reaction of anti-P10 sera with cellular proteins

Anti-P10 sera were first tested for their ability to recognize cFos in $^{35}$S-methionine-labelled extracts from serum-stimulated NIH 3T3 cells which are known to express c fos (Almendral et al., 1988). The anti-P10 serum 303 immunoprecipitated specifically a polypeptide of approximate molecular weight 55 kD, which would be compatible with it being cFos (indicated by an arrow in Fig.9.7A: compare lane 3 to lane 2) (Curran et al., 1984). This reaction was competitively inhibited by excess P10 peptide but not by the related cJun basic domain, PEP1, or the unrelated ATF2D (Fig.9.7A: compare lane 6 to lanes 5 and 4). However, no similar polypeptide was recognized in c fos-expressing F9 PE cells, though a number of polypeptides were specifically immunoprecipitated and were competed by excess P10 in both EC and PE cells (indicated by arrows in Fig.9.7B: lane 8), presumably due to cross-reactivity with anti-P10. The 412 serum, on the other hand did not immunoprecipitate a 3T3 polypeptide of a molecular weight compatible with that of cFos, though it did specifically recognize a 46 kD polypeptide (indicated by an arrow in Fig.9.7A: lane 8), and was therefore not tested in F9 PE cells. Neither 303 nor 412 immunoblotted a PE-specific polypeptide of the appropriate molecular weight for cFos in whole cell extracts, though, again, a number of polypeptides specifically cross-reacted in both EC and PE extracts (indicated by arrows in Fig.9.8: compare lanes: 3 to 2, 6 to 5, 9 to 8, 12 to 11).

Anti-P10 sera, therefore, though capable of immunoblotting and immunoprecipitating in vitro translated cFos, were not capable of recognizing cFos in a crude F9 PE whole cell extract. This may have been due to low levels of cFos protein rather than weak antibody affinity, especially since a protein of the appropriate molecular weight was recognized by immunoprecipitation from 3T3 cells.
9.6 Reactivity of anti-Jun sera with polypeptides in whole cell extracts

Anti-PEP1 and anti-PEP2 sera presented a similar picture as the anti-P10 sera: neither immunoprecipitated a 3T3- or PE-specific polypeptide of a size consistent with that of cJun, which varies from 39-47 kD, depending on cell type (Curran et al., 1985; Bohmann et al., 1987; Angel et al., 1988) though they did specifically immunoprepitate polypeptides (indicated by arrows) common to EC and PE extracts (anti-PEP2: Fig.9.9A: compare lane 5 to lane 4, Fig.9.9B: compare lane 7 to 3, 16 to 12; anti-PEP1: Fig.9.9A: compare lanes 3, 8, 10). Immunoblotting of F9 EC or PE cell extracts did not yield any PE-specific bands either (Fig.9.8: compare lane 20 to lane 14, 23 to 17).

Neither anti-PEP1 nor anti-PEP2, therefore, recognized any polypeptides whose presence in PE but not EC cells would be consistent with them being cJun, either by immunoblotting or immunoprecipitation. As shown in Chapter 8, anti-PEP1 did not immunoblot in vitro translated cJun but anti-PEP2 did, and both immunoprecipitated in vitro translated cJun and JunD. Therefore, the specifically immunoprecipitated polypeptides that were common to EC and PE cells and migrated just above (Fig.9.9A: lanes 8 and 10; indicated by arrows) or below (Fig.9.9B: lanes 3 and 7) the 46 kD marker could conceivably correspond to JunD, which is expressed, at the RNA level, in both cell types (Chapter 6). The size of in vitro translated JunD is 42.6 kD (Chapter 7); however, post-translational modifications in the cell may increase its apparent molecular weight, much as, although the predicted molecular weight of human cJun is 37 kD, the cellular protein migrates as a 44-45 kD polypeptide (Angel et al., 1988). The failure of these sera, however, to recognize cellular cJun may reflect its low levels in crude whole cell extracts as well as the fact that unpurified sera, rather than purified immunoglobulins, were used.
9.7 Effects of anti-ATF and anti-API sera on ECRE/DNA complexes

Given that all antisera raised were capable of affecting *in vitro* translated protein/DNA complexes, either by inhibiting DNA binding (anti-P10, anti-PEPL, anti-PEP2) or by further retarding the complexes (anti-ATF2D, anti-ATF3C), with varying efficiencies, they should also, in principle, have the same effects on the corresponding cellular DNA binding proteins. It was therefore interesting to test whether they inhibited or "super-shifted" any of the ECRE/DNA complexes. Such effects would argue for an immunological, at least, relationship between ECRE activities and ATF/API proteins.

The wild type ATF site, P, was used as probe in these experiments, since it bound all ECRE activities very strongly. No inhibition of DNA binding or "super-shifting" was seen with anti-ATF2D (Fig.9.10B : compare lane 9 to lane 8), anti-ATF3C (Fig.9.10B : compare lane 11 to lane 10), anti-P10 (Fig.9.10A : compare lane 14 to lane 13), or anti-PEPL (Fig.9.10A : compare lane 12 to lane 11). Surprisingly, anti-PEP2, which is directed against a sequence in cJun that lies N-terminal to the DNA binding domain, was the only antiserum that had a detectable effect in this assay. Both 343 and 501 "super-shifted" ECRE-1 (indicated by an open arrow in Fig.9.10A : compare lane 6 to lane 5, 10 to 9). It was confirmed that the slower migrating complex that was generated contained both ECRE-1, since it was specific for the ATF site and could be competed by wild type P but not by mutant Pm1 (Fig.9.10A : compare lane 7 to lane 8), and anti-PEP2 antibody, since its formation was inhibited by PEP2 peptide but not by PEPL or ATF2D (Fig.9.10B : compare lane 5 to lanes 6 and 7). As can be seen in Fig.9.12, there is considerable similarity between PEP2 and an amino acid sequence in JunD, and, as shown in Chapter 8 (Fig.8.10), anti-PEP2 immunoprecipitated *in vitro* translated JunD but not JunB, which has a less extended region of similarity. 343 also specifically immunoprecipitated a a polypeptide from F9 EC cells, of a size compatible with it being JunD (Fig.9.9B). This reaction of anti-PEP2 with ECRE-1 therefore suggested that JunD, which is expressed in F9 EC cells (Chapter 6 : Fig.6.4), or an unidentified protein cross-reacting with anti-PEP2, was present in the ECRE-1 complex.
comparison of the PEP2 sequence with other ATF/AP1 family members also yielded a region of similarity at the N-terminus of ATF2 (Fig.9.12). To test whether this similarity was immunologically significant, an immunoprecipitation experiment was performed where \textit{in vitro} translated ATF2, ATF3 and JunD were incubated with their cognate antisera or with anti-PEP2. Neither the longest ATF2 cDNA, CREBP1, which contained the region of amino acid similarity, nor the truncated polypeptide ATF2L which did not, were immunoprecipitated by anti-PEP2 (Fig.9.11A : compare lane 3 to lane 5, 8 to 10). ATF3L was also not immunoprecipitated by anti-PEP2, as expected from the lack of extensive amino acid similarity (Fig.9.11A : compare lane 13 to 15). JunD, therefore, was the only polypeptide with amino acid similarity to PEP2 that could be immunoprecipitated by anti-PEP2 (Fig.9.11A : lane 18), making it the only known member of the ATF/AP1 families that could be involved in ECRE-1. Since JunD binding to the ATF site was almost undetectable, while ATF2/JunD or ATF3/JunD heterodimers bound very efficiently (Chapter 7 : Fig.7.6B), it was possible that ECRE-1 contained such a heterodimer. This possibility was tested by incubating \textit{in vitro} translated or co-translated polypeptides with anti-PEP2 in the presence of the ATF site, P, and assaying for a "super-shift". Anti-PEP2, however, had no specific effect on either CREBP1/JunD (Fig.9.11B : compare lanes 8-10 to lane 7), or ATF3/JunD (Fig.9.11B : compare lanes 13-15 to lane 12), under conditions that "super-shifted" ECRE-1 ; as expected from the lack of immunoprecipitation, anti-PEP2 did not have any effect on a CREBP1 homodimer (Fig.9.11B : compare lanes 3-5 to lane 2) or a CREBP1/ATF3 heterodimer either (Fig.9.11B : compare lanes 18-20 to lane 17). Presumably therefore, although anti-PEP2 did recognize JunD in isolation, in an immunoprecipitation assay, \textit{in vitro} heterodimerization with ATF2 or ATF3 in the presence of DNA blocked the anti-PEP2 cross-reacting epitope. Unless \textit{in vivo}, rather than \textit{in vitro}, heterodimerization of JunD with full length, rather than truncated, ATF2 or ATF3 did not block the PEP2-like epitope, these results would suggest that it is unlikely that ECRE-1 is a JunD/ATF2 or /ATF3 heterodimer. Nevertheless, of the known ATF/AP1 family members, JunD is the only one that may be
involved in ECRE-1, possibly complexed with other, unidentified proteins; alternatively, ECRE-1 may contain an unrelated, unknown protein that cross-reacts with anti-PEP2 serum.

9.8 Discussion

The study of ATF and API protein levels proved most fruitful in the case of ATF2. As with its RNA levels, ATF2 protein levels remained constant during differentiation: a cluster of four polypeptides, with molecular weights ranging from 41 to 45 kD, was recognized by anti-ATF2D serum in both F9 EC and PE cells. ATF2 was found exclusively in the nuclear fraction of F9 EC cells, an observation consistent with its function as a DNA binding protein. Similar clusters of polypeptides reacted with anti-ATF2D in a number of other cell types, indicating that ATF2 is widely expressed. The different ATF2 polypeptides within each cluster could be products of alternatively spliced ATF2 RNA, as reported by Georgopoulos et al. (1992), or differentially modified products of the same transcript. Although a computerized search for modification sites in ATF2 revealed a number of potential protein kinase A, protein kinase C and casein kinase II phosphorylation sites, it is unlikely that the ATF2 polypeptides are differentially phosphorylated products of the same transcript, since treatment of the extract with alkaline phosphatase did not reduce the number of polypeptides recognized by the antiserum. The same search, nevertheless, also yielded two potential N-glycosylation sites near the amino terminus; whether they are used in vivo, however, and whether glycosylation at these sites would affect electrophoretic mobility is unclear.

None of the anti-ATF3C, anti-Fos and anti-Jun sera reacted specifically with F9 EC and PE polypeptides that were consistent with being their correct protein targets, and therefore no information about the protein levels of ATF3, cFos and Jun proteins in these cells could be obtained. This lack of detection could be due to a number of reasons, including low serum affinity, low amounts of the cognate polypeptides in whole cell extracts, and denaturation-sensitive epitopes.
Unfortunately, anti-ATF2D, which did recognize native F9 EC and PE ATF2 polypeptides in an immunoprecipitation assay, and was able to "super-shift" in vitro translated ATF/DNA complexes (Chapter 8), did not affect the ECRE complexes formed on the P probe in a gel retardation assay. This would suggest either that ATF2 is not involved in those ECRE activities or that binding of cellular, as opposed to in vitro translated, ATF2 to DNA prevented antibody recognition. The anti-ATF3C sera also did not affect the ECRE complexes formed on P; this was consistent with their inability to react with native F9 EC cell proteins in an immunoprecipitation assay.

However, the anti-PEP2 sera, one of which, 343, also immunoprecipitated a polypeptide of approximately 46 kD molecular weight, in F9 EC and PE cell extracts, did specifically "super-shift" the ECRE-1/DNA complex in a gel retardation assay. A comparison of the cJun PEP2 amino acid sequence with those of other ATF and API family members, and immunoprecipitation assays with anti-PEP2 against various in vitro translated ATF and API family members, revealed that only JunD cross-reacted with anti-PEP2. This would suggest that JunD may be involved in ECRE-1, which would be compatible with the presence of JunD transcripts in F9 EC cells. However, the possibility that an unknown DNA binding protein may be cross-reacting with anti-PEP2 cannot, at present, be excluded.
FIG. 9.1: Immunoblotting of F9 EC and PE whole cell extracts with anti-ATF2D sera

A) Immunoblots of the indicated whole cell extracts with the indicated sera, in the presence or absence of the indicated peptide competitors. The specific ATF2 polypeptides are indicated by brackets on the right of lanes 6 and 12. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

B) Immunoblots of F9 EC nuclear (NUC.) or cytoplasmic (CYT.) extracts with the 314 anti-ATF2D serum, in the presence of the indicated peptide competitors. The specific ATF2 polypeptides are indicated by brackets on the right of lane 4. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

C) Gel retardation of probe P by F9 EC nuclear (NUC.) or cytoplasmic (CYT.) extracts. The positions of the ECRE complexes are indicated.
FIG. 9.2: Immunoprecipitation with anti-ATF2D sera from $^{35}$S-Met-labelled F9 EC and PE lysates

A) Immunoprecipitations with the indicated extracts and sera in the presence or absence of the indicated peptide competitors. The specific ATF2 polypeptides are indicated by brackets; the asterisk denotes the co-precipitating polypeptides that were not recognized by immunoblotting with anti-ATF2D. All lanes are from the same experiment. Lane 1: $^{14}$C-labelled protein molecular weight standards whose sizes, in kD, are shown to the left of the lane. Background bands in the pre-immune serum lanes were largely due to non-specific binding of proteins to the protein A beads, as can be seen in (B): compare lane 2 to lane 1.

B) Immunoprecipitations from F9 EC whole cell extracts with the indicated sera in buffer A or C ( = A + 1% DOC, 0.1% SDS). The specific ATF2 polypeptides are indicated by brackets; the asterisk indicates the polypeptides that are only precipitated in the milder buffer, A. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1. All lanes are from the same experiment.
FIG. 9.3: Detection of ATF2 polypeptides and ATF site-binding activity in various cell types

A) Detection of ATF2 (brackets) by immunoblotting against whole cell extracts (3T3, F9 EC, 293 Man., cl.38 Man., C2MT = C2 myotubes) or nuclear extracts (293 Dig., cl.38 Dig.) in the presence of ATF2D (+) or ATF3C (-) peptide competitor; the asterisk indicates presumed ATF2 degradation products. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

B) Gel retardation of probe P with the indicated extracts (see A). The position of ECRE complexes is shown to the left of lane 1. Asterisks indicate diffuse complexes presumably due to protein degradation products. All lanes are from the same experiment.
A

Serum: 314

Extract: 3T3 EC 293 293 cl.38 cl.38 C2

ATF2D comp.: - + - + - + - + - + - +

B

Probe: P

Extract: - EC 3T3 C2 Man. cl.38 Dig. Man. Man. MT

ECRE-1

ECRE-2, -3, -4a/b

1 2 3 4 5 6 7 8
FIG. 9.4: Detection of ATF2 in HeLa cells

A) Immunoblots of F9 EC and HeLa whole cell extracts with the indicated sera and peptide competitors. The ATF2 polypeptides are indicated by brackets to the right of lane 7. Positions and sizes, in kD, of protein molecular weight standards in lane 1 are shown to the left of the lane.

B) Immunoblots of F9 EC, PE and HeLa whole cell extracts with the anti-ATF2D serum, 314. Where indicated (+), the extracts were pre-treated with 1 unit of calf intestinal alkaline phosphatase (Stratagene) for 30 min at 37°C. The ATF2 polypeptides are indicated by brackets to the right of lane 6. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

C) Gel retardation of probe P with F9 EC or HeLa whole cell extracts.
FIG. 9.5: Immunoblotting and immunoprecipitation experiments with anti-ATF3C sera
in F9 EC and PE whole cell extracts

A) Immunoblots of the indicated extracts with the indicated sera in the presence or absence of the indicated peptide competitors. (nuc.): F9 EC cell nuclear protein extract, (cyt.): F9 EC cell cytoplasmic protein extract. The arrows indicate the specifically recognized 35 kD polypeptide. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lanes 1, 7 and 15. Lanes 1-6 are from the same experiment.

B) Immunoprecipitations with the indicated sera in the presence or absence of the indicated peptide competitors from the indicated $^{35}$S-methionine-labelled cell lysates. The sizes, in kD, of the $^{14}$C-labelled protein molecular weight standards (lane 1), are shown to the left of that lane.
FIG. 9.6: Lack of anti-ATF3C reactivity in HeLa cell extracts

Immunoblots of the indicated whole cell extracts with the indicated sera. The extracts electrophoresed in lanes 9-14 were incubated for 30 min at 30°C prior to SDS-PAGE. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.
FIG. 9.7: Immunoprecipitation from $^{35}$S-Met-labelled cell lysates with anti-cFos sera

Immunoprecipitations from the indicated $^{35}$S-Met-labelled cell lysates with the indicated sera in the presence or absence of the indicated peptide competitors. Lane 1: $^{14}$C-labelled protein molecular weight standards; their sizes, in kD, are indicated to the left.
A

Extract: 3T3
Serum: R30 303 R41 412
Peptide Comp.: ATF2D PEP1 P10

B

Extract: EC PE
Serum: R30 303 R30 303
Peptide Comp.: ATF2D PEP1 P10 ATF2D PEP1 P10
FIG. 9.8: Immunoblotting with anti-cFos and anti-cJun sera against F9 EC and PE whole cell extracts

Immunoblotting with the indicated sera against the indicated extracts in the presence or absence of the indicated peptide competitors. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.
FIG. 9.9: Immunoprecipitation from 3T3, F9 EC and PE $^{35}$S-Met-labelled cell lysates with anti-cJun sera

A) Immunoprecipitations from $^{35}$S-Met-labelled 3T3, F9 EC and PE cell lysates with the indicated sera in the presence or absence of the indicated peptide competitors. Lanes 1 and 6: $^{14}$C-labelled protein molecular weight standards; their sizes, in kD, are shown to the left of the lanes.

B) Immunoprecipitations from $^{35}$S-Met-labelled F9 EC and PE cell lysates with the indicated sera in the presence or absence of the indicated peptide competitors. Lanes 1 and 10: $^{14}$C-labelled protein molecular weight standards; their sizes, in kD, are shown to the left of the lanes.
FIG. 9.10: Effects of the anti-peptide sera on ECRE complexes

A) Gel retardation of probe P with F9 EC whole cell extract in the presence of the indicated sera and oligonucleotide competitors. Where only a rabbit number is given over a pair of lanes, the left lane contains pre-immune serum, the right one serum from the third test bleed - except for lane 10 which contains serum from the first test bleed. The position of the ECRE-1 complex is indicated; the open arrow indicates the position of the "super-shifted" ECRE-1 complex. All lanes are from the same experiment.

B) Gel retardation of probe P with F9 EC whole cell extract in the presence of the indicated sera and peptide competitors. Where only a rabbit number is given over a pair of lanes, the left lane contains pre-immune serum, the right one serum from the third test bleed.
FIG. 9.11: Anti-PEP2 serum cross-reacts with JunD but not ATF2 or ATF3

A) Immunoprecipitations of the indicated $^{35}$S-Met-labelled in vitro translated polypeptides with the indicated sera. For each pair of bands labelled with a rabbit number, the left lane contains pre-immune serum, the right one contains serum from the third test bleed. Lane 19: $^{14}$C-labelled protein molecular weight standards; their sizes, in kD, are shown to the right of the lane. All lanes are from the same experiment.

B) Gel retardation of probe P with the indicated in vitro translated and co-translated polypeptides, in the presence or absence of the indicated sera and peptide competitors. This experiment was performed simultaneously with the experiment in Fig.8.14, where supershifts were observed as predicted.
A

Protein: CREBP1  ATF2L  ATF3L  JunD
Serum: 34 42 34 42 34 43 34

B

Extract: CREBP1  CREBP1+JunD  ATF3L+JunD  CREBP1+ATF3L
Serum: R34 343  R34 343  R34 343  R34 343
Pep. comp.: FEP2 FEP1 FEP2 FEP1 FEP2 FEP1 FEP2 FEP1 FEP2 FEP1
FIG. 9.12: cJun amino acid sequence: amino acid similarities of other ATF/API family members to peptide PEP2

Identical amino acids are indicated by a single dot. (h) = human, (m) = mouse.
**cJun amino acid sequence : Homologies of other ATF/AP1 family members to peptide PEP2**

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<th>Sequence</th>
<th>Homologs</th>
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<tr>
<td>MTAKMETTFY DDALNASFLQ SESGAYGYSN PKILKQSMTL NLADPVGSLK</td>
<td><strong>PEP2</strong></td>
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<tr>
<td>PHLRAKNSDL LTSPDVGLLK LASPELERLI IQSSNGHITT TPTPTQFLCP</td>
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<td></td>
<td>(h)ATF3 203 TPEDERNLFI</td>
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<tr>
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</tr>
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</tr>
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CHAPTER 10

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST THE DIFFERENTIATION-
REGULATED TRANSCRIPTION FACTOR DRTF-1
10.1 Introduction

As discussed in Chapter 1, the Ad5 E2A gene is transcriptionally regulated during differentiation of F9 EC cells (La Thangue et al., 1990; Murray et al., 1991). In F9 EC cells, its promoter is trans-activated by transcription factor DRTF-1, which had been purified in the laboratory by DNA binding site affinity chromatography (Shivji and La Thangue, 1990). It was decided to raise monoclonal antibodies against the purified factor in order to screen an expression library and isolate a cDNA encoding DRTF-1.

10.2 Immunisation

Four BALB/c female mice (referred to as mouse 1, 2, 3, and 4) were immunised, according to standard procedures, with six doses of antigen. The antigen consisted of an affinity-purified fraction containing DRTF-1 activity (purified by Mahmud K.K.Shivji and Nicholas B. La Thangue); when this fraction was analysed by SDS-PAGE, two polypeptides were prominent after silver staining: one in the region of 50 kD, the other, less intense, in the region of 30 kD (Shivji and La Thangue, 1990). Two of the mice were immunised with the affinity-pure fraction alone, while the other two were immunised with aliquots of the same fraction which had been mixed with the oligonucleotide-conjugated resin, since the resin beads might serve as an adjuvant to enhance the immune response. Three tail bleeds were taken from each mouse after the third, fifth and sixth immunisations (referred to as test sera TX/1, TX/2 and TX/3, respectively, where X is the number assigned to each mouse) and compared with the pre-immune sera (referred to as PX, where X is the number assigned to each mouse) from the same mice.

Three assays were used to monitor the immune response of the mice to the injected antigen: 1) an ELISA, 2) the gel retardation assay, and 3) immunoblotting.
10.3 ELISA

96-well PVC plates were coated with F9 EC whole cell extract and the rabbit anti-mouse immunoglobulins were conjugated to horseradish peroxidase. When the first immune sera were compared to the pre-immune sera, there was a clear difference in the intensity of the colour reaction produced by the enzyme. However, the absolute value of the signal was low; a photograph of a representative plate is shown in the top half of Fig.10.1. Subsequent test sera were compared to the immediately preceding ones. When the second test serum was compared to the first, a low signal appeared to persist at lower antigen and serum concentrations; this was interpreted as indicating the appearance of high affinity but low titre DRTF-1-specific antibodies. Such antibodies, being of low titre, would not cause a large signal but, being of high affinity, they would be able to recognize low amounts of antigen efficiently. A similar modest increase in conversion of the chromogenic substrate was apparent when the third test serum was compared to the second (bottom half of Fig.10.1; compare, for example, column 8 to column 3).

Thus, it appeared that the mice were responding weakly to the injected antigen. The next assay was designed to assess the specificity of this response.

10.4 Gel retardation assays

The use of this assay to detect an antigen/antibody complex was based on the rationale explained in Chapter 8.

Given the limited amounts of only partially pure DRTF-1 available, it was clear that the most sensitive and specific method available for assaying for an interaction of an antibody with DRTF-1 was the gel retardation assay. This is because this assay not only allows detection of very small amounts of antigen but also the specificity of any complex can be assessed by competition with the appropriate DNA sequence, in the form of an oligonucleotide, as described in Chapter 3.
That an antibody/DNA binding protein interaction could be detected under the normal conditions of this assay, was demonstrated by incubating an F9 EC whole cell extract with a polyclonal antibody raised against transcription factor Sp1 (Dynan and Tjian, 1983), expressed as a β-galactosidase fusion protein in E.coli, or a non-immune serum, before adding a DNA probe which contained two Sp1 binding sites. A "super-shift" formed with as little as 0.5 µl of α-Spl serum but not with the same amount of non-immune serum (Fig.10.2 : compare lanes 5, 7, 9 to lanes 4, 6, 8, respectively).

The effects of the third test serum from the mice that had been injected with affinity-purified DRTF-1, and the non-immune serum from the same mice on the complex formed between affinity purified DRTF-1 and the E2A promoter were then compared. An increasing "super-shifting" of the complex was apparent with increasing amounts of T2/3 but not P2 (Fig.10.3a : compare lanes 5 and 7 to 3 and 4, respectively). Although the "super-shift" was not as clearly resolved as that with the α-Spl serum (Fig.10.2), and the non-immune serum seemed to enhance the intensity as well as slightly affect the mobility of the complex (Fig.10.3a : compare lane 3 to 2), possibly through non-specific interactions of the proteins in the complex with serum proteins which were in large excess, the difference between the effect of the test serum and the non-immune serum on the mobility of the DRTF-1/E2A complex was clear. Test sera from all four mice gave similar results (Fig.10.3b : compare lanes 4 to 3, 8 to 7, 12 to 11, 16 to 15). That this "super-shift" involved DRTF-1 was demonstrated by including oligonucleotide competitors in the reaction. The "super-shift" was competed by the wild type DRTF-1 site (19/20 ; see legend to Fig.10.3 for sequence) but not by a point mutated site (63* ; see legend to Fig.10.3 for sequence) that fails to bind DRTF-1 (La Thangue et al., 1990) (Fig.10.3b : compare lanes 5 to 6, 9 to 10, 13 to 14, 17 to 18).

In conclusion, there was a specific "super-shifting" of the DRTF-1 complex with the third test sera, compared to the non-immune sera, although it was poorly resolved. This indicated that antibodies recognizing DRTF-1 were present in the test sera, but were presumably of low titre and/or affinity.
10.5 Immunoblots

The evidence from the ELISAs and the gel retardation assays suggested that the mice were producing specific antibodies against DRTF-1. An immunoblot would indicate whether this immune response was directed against the 50 kD and 30 kD DRTF-1 polypeptides.

Because of the limited availability and low protein concentration of affinity purified DRTF-1, it was decided to use heparin Sepharose (HS) fractions which contained DRTF-1, for immunoblotting. When the third test sera were compared to the non-immune sera, a specific quantitative increase in reaction against a 50 kD protein was observed with T1/3 (Fig.10.4a : compare lanes 4 to 3, 5 to 6) and a similar increase in recognition of a 25 kD protein was observed with T2/3 (Fig.10.4b : compare lane 2 to 1). That the response appeared quantitative rather than qualitative may have been due to inefficient blocking. In addition, more than one protein is likely to migrate to the region of 50 kD; different but co-migrating proteins could therefore be recognized by the pre-immune and the test sera. Test sera from mice 3 and 4 did not show any difference in the pattern of bands they recognized, compared with the corresponding non-immune sera (Fig.10.4a : compare lane 2 to 1 ; Fig.10.4b : compare lane 4 to 3).

Therefore, the combined results from the three types of assay (ELISA, gel retardation and immunoblotting) suggested that mice 1 and 2 had mounted the most readily detectable immune response against DRTF-1.

10.6 Production of hybridomas

During the interval between the sixth injection and the fusion, mouse 1 died. After a booster injection of antigen, mouse 2 was sacrificed and its spleen cells were fused, by stirring, to Sp2 myeloma cells (Köhler and Milstein, 1976). Tissue culture medium supernatants from the resultant hybridomas were tested by ELISA, in duplicate, at 9, 11 and 14 days after the fusion. The ELISA plates were coated with a HS fraction enriched in
DRTF-1 activity; the same fraction was used in gel retardation assays and for immunoblots. Cells from duplicated positive wells were transferred to 24-well plates and supernatants from these were also subsequently tested when the cells reached confluence. From the sixteen 96-well plates screened, approximately 1.5% of the wells were identified as positives (data not shown); due to fungal contamination, cells from only 20 positive wells survived until the conclusion of the experiments.

The initial 96-well supernatants, as well as the 24-well supernatants, were also tested for their ability to affect the complex formed between a HS DRTF-1 fraction and the E2A promoter in a gel retardation assay. None, however, "super-shifted" or abolished the DRTF-1 "shift" (an example is shown in Fig.10.5: compare lanes 7, 8 (positive supernatants) to 9, 10 (negative supernatants)), though a control experiment using the SV40 origin of DNA replication, purified SV40 large T antigen and a supernatant from a hybridoma clone producing α-T monoclonal antibody, did produce a "super-shift" under identical conditions (Fig.10.5: compare lane 13 to 12). Using an affinity purified DRTF-1 fraction instead of a HS fraction did not alter the result (data not shown). This indicated that either the α-DRTF-1 antibodies produced were unable to bind to DRTF-1 under gel retardation conditions, or that the antibodies reacting in the ELISA assay recognized antigens present in an affinity purified DRTF-1 fraction but different from DRTF-1 itself.

Immunoblots performed with supernatants from the 24-well plates gave a variety of results (Fig.10.6b): some ELISA-positive supernatants did not recognize any protein under these conditions (lanes 4, 6, 8, 12, 14-17), several reacted strongly with a group of proteins in the region of 140 kD (lanes 5, 7, 9-11, 13), and strongly (lane 7) or weakly (lanes 5, 9-11, 13) with proteins in the region of 100 kD. One (lane 7) also reacted strongly with a 50 kD and weakly with a 30 kD protein, while another (lane 11) reacted strongly with a different 50 kD protein. Supernatants 7G3 (lane 7) and 15C2 (lane 11) were therefore chosen for an immunoblot against affinity purified DRTF-1, because they recognized proteins in the regions of 50 and 30 kD. By that time, the cells had already been re-plated at low density once and reactivity of 15C2 was lost (Fig.10.7b: lanes 5 and 7), presumably because the
antibody producing clone(s) had been outgrown by faster growing clones. 7G3, however, still recognized a 50 kD protein in the HS fraction (Fig. 10.7b: lane 6), but not in the affinity column fraction (lane 8). This could be explained either by the low amount of antigen in the affinity fraction or by the antibodies in 7G3 being auto-antibodies against a 50 kD protein present in the HS fraction but absent from the affinity fraction. Neither 7G3 nor 15C2 reacted positively in an ELISA assay where the antigen was the same affinity fraction (data not shown). A supernatant (6C3) that did react positively in that assay, was also negative by immunoblotting against an affinity fraction although it did weakly recognize a 50 kD protein in a HS fraction under the same conditions (data not shown).

In summary, the gel retardation assay failed to identify an ELISA-positive hybridoma population producing α-DRTF-1 antibody able to affect the DRTF-1/E2A complex, and thus the most sensitive and specific assay available was proven to be unusable. On the other hand, two ELISA-positive supernatants contained antibody that recognized, by immunoblotting, two different 50 kD proteins in a HS but not in an affinity fraction. However, given that affinity purified DRTF-1 was of low concentration and limited in amount, it was not possible to determine the identity of these proteins with certainty, in order to proceed to the cloning of the α-DRTF-1 antibody producing clones. Although other ways of identification were considered (for example, testing the positive supernatants by immunoprecipitation from F9 EC and PE extracts), none seemed to offer a practical basis for cloning to homogeneity the appropriate antibody producing cells. Thus, all positive cells were frozen down and stored until the time when greater availability of purer antigen might offer a feasible means of identifying the clones of interest.

10.7 Discussion

The ELISAs and the immunoblots performed with test sera from the immunised mice indicated that the animals were responding to the injected antigen and producing
antibodies that recognized proteins of molecular weights consistent with them being components of DRTF-1 activity as characterised previously (Shivji and La Thangue, 1991). The same sera, when incubated with affinity-purified DRTF-1 and the E2A promoter, also "super-shifted" the DRTF-1/E2A complex.

Thus, although no single type of assay revealed an impressively strong immune response, their combined results suggested that the mice, especially 1 and 2, had responded.

None of the ELISA-positive supernatants of hybridomas derived from mouse 2 "super-shifted" or abolished the DRTF-1 "shift". And, although some of them recognized, by immunoblotting of a HS fraction, proteins whose molecular weights were consistent with them being components of DRTF-1 activity, none of them reacted, under the same conditions, with the affinity fraction. This failure to identify a specific reaction between the antibodies produced and DRTF-1, may originate from the weakness of the immune response. The number of α-DRTF-1 antibody producing cells in the animal may not have been high enough to give rise to a detectable number of the appropriate hybridomas. On the other hand, this failure may simply be a problem of detection. For example, the epitopes recognized on the partly denatured antigen on an ELISA plate may not be available for binding in the native protein assayed by gel retardation. At the same time, the antigen concentration in the affinity fraction may be below the level of detection by immunoblotting. A third explanation may be that DRTF-1 is not immunogenic and the antibodies produced were against unrelated contaminating antigens or even auto-antibodies against antigens that happened to be present in the HS fraction used for the screening, but not in the injected affinity fraction. However, given that the test sera indicated that the mice were responding against the right antigen and that at least one supernatant reacted against an affinity fraction in an ELISA assay, the second explanation seems more likely: i.e. that the limiting factor is detection. Since the only functional assay, i.e. gel retardation, failed, there was no way of confirming that any of the proteins detected by immunoblotting was the right one, and therefore it was not judged advisable to continue the analysis. Hopefully, greater availability and purity of antigen in the
future, for example after the already successful isolation of a DRTF-1 cDNA (Girling et al., submitted), will provide the means for identifying any α-DRTF-1 antibody producing clones with ease and certainty.
FIG. 10.1: ELISAs with test sera from mouse 2

Examples of ELISAs with test sera from mouse 2. Rows A→H: serial two-fold dilutions of F9 EC whole cell extract (WCE), ranging from 4.5 μg/well (90 μg/ml) to 33 ng/well (660 ng/ml). Columns 1→5 and 6→10: serial two-fold dilutions of the pre-immune and test sera indicated, ranging from 1/100 to 1/1600 (top), or 1/400 to 1/6400 (bottom). Column 11: no serum. Column 12: peroxidase substrate only. The blocking buffer was 3% BSA in PBS.
FIG. 10.2: Control "super-shift" experiment with α-Sp1 polyclonal antibody

Gel retardation assay in the presence of polyclonal sera; Probe: HSV tk promoter (lanes 1-9); Extract: F9 EC whole cell extract (lanes 2-9); PI: pre-immune rabbit serum; (2892-pl; lanes as indicated); α: α-Sp1 rabbit polyclonal serum raised against bacterially synthesized Sp1 (2893-E; lanes as indicated). Volume/µl of serum used: 0.2 (lanes 2,3), 0.5 (lanes 4,5), 1 (lanes 6,7), 2 (lanes 8,9).
FIG. 10.3: a) Titration of test sera in a gel retardation assay

Gel retardation assay in the presence of pre-immune (P2) and test (T2/3) sera from mouse 2 (volumes/µl as indicated). Probe: E2A (lanes 1-7); Protein: affinity purified DRTF-1 (0.5 µl; lanes 2-7).

b) Specificity of "super-shifts" produced by test sera

Gel retardation assay in the presence of pre-immune and test sera (1.5 µl; origin as indicated). Probe: E2A (lanes 1-18); Protein: affinity purified DRTF-1 (0.5 µl; lanes 2-18); Oligonucleotide competitors (650-fold molar excess): 19/20 or 63* (lanes as indicated; sequences shown below):

19/20  TAGTTTTTCGCGCTTAATTTGA
63*  TAGTTTTTCTCGCTTTAAATTTGA
FIG. 10.4: *Immunoblots with test sera*

a) Protein: heparin Sepharose column fraction (30 μg/lane). The membrane was blocked in 3% BSA. It was first incubated with the indicated pre-immune and test sera (1/100 dilution) for 1 h at r.t., and then with horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins (1/500 dilution) for 1 h at r.t.. The enzyme reaction was stopped after 20 min. Positions of migration and sizes, in kD, of protein molecular weight standards are indicated to the left of the blot.

b) Protein: heparin Sepharose column fraction (30 μg/lane). The membrane was blocked in 3% BSA. It was first incubated with the indicated pre-immune and test sera (1/100 dilution) for 75 min at r.t., then with horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins for 1 h at r.t.. Positions of migration and sizes, in kD, of protein molecular weight standards are indicated to the left of the blot.
FIG. 10.5: Gel retardation assay in the presence of supernatants from the first screen of hybridomas in 96-well plates

Probes: E2A and SV40 origin of replication (ori; nucleotides 5175/37), as indicated; Protein: heparin Sepharose column fraction (HS; 200 ng) and purified SV40 large T antigen (T; 150 ng), as indicated (lanes 11–13 also contain 5 mM ATP and 100 µg BSA); Supernatants: 419 (lanes 11 and 13: 2 µl) contains α-T monoclonal antibody 419 (L19 in Harlow et al., 1981); 1C2 and 3G6 (lanes 2, 7 and 3, 8, respectively; 10 µl) came from wells that were positive, 1F2 and 3B6 (lanes 4, 9 and 5, 10, respectively; 10 µl) from wells that were negative, in the ELISA.
a) Ponceau-S stained protein blot used for immunoblots shown in (b). Protein: heparin sepharose column fraction (5 μg/lane shown in (b)). Protein molecular weight standards are on the right side of the blot. (The "bubbles" seen towards the left of the blot are an artefact which occurred during photocopying of the wet nitrocellulose filter, and do not reflect irregularities in protein transfer.)

b) Immunoblot. The nitrocellulose strips were incubated overnight at 4 °C with the indicated supernatants (419: α-T monoclonal antibody; 6A7: monoclonal antibody against the 70 kD subunit of human DNA polymerase α; all others are supernatants from 24-well plates), then for 4 h at r.t. with alkaline phosphatase-conjugated rabbit α-mouse immunoglobulins. The enzyme reaction was stopped after 15 min. Sizes, in kD, of protein molecular weight standards (M) in lane 1 are indicated to the left of the figure. The membrane was blocked with foetal calf serum. [This experiment was performed jointly with R.Girling].
FIG. 10.7: Immunoblot with 24-well plate second supernatants

a) Ponceau-S stained protein blot used for immunoblots shown in (b). Protein: heparin sepharose column fraction (HS; 5 µg/lanes 1–6 in (b)) and affinity column fraction (A; 15 µl/lanes 7, 8 in (b)). M: protein molecular weight standards.

b) Immunoblot. The nitrocellulose strips were incubated overnight at 4 °C with the indicated supernatants (for description of 6A7 and 419 see Fig.10.6; T2/3 test serum is diluted 1/200; all others are supernatants from 24-well plates), then for 3 h at r.t. with alkaline phosphatase-conjugated rabbit α-mouse immunoglobulins. The enzyme reaction was stopped after 20 min. The sizes, in kD, of protein molecular weight standards (M) in lane 9 are indicated to the right of the figure.
CHAPTER 11

GENERAL DISCUSSION
11.1 Summary and perspectives

The aim of this study was to examine aspects of transcriptional regulation in the context of a differentiating cell system which models early events of murine embryogenesis. A number of interesting observations were made, illustrating the important role that transcription factor families can play during development.

The results presented here have demonstrated that a family of six DNA binding activities, the ECRE family, that recognize the ATF site, exist in murine F9 EC cells and their differentiated derivatives. Members of this family differ in the electrophoretic mobility of their DNA complexes and in their regulation during differentiation of EC cells: ECRE-2, for example, is detectable in both cell types, while DNA binding by ECRE-3 is down-regulated as EC cells differentiate. These ATF site-binding activities are distinguished further by their ability to bind DNA which is mutated within or near the ATF site. ECRE-2, for example, only binds the wild type site, ACGTCA; ECRE-1 and ECRE-4, on the other hand, also bind the point-mutated site, ACATCA. Differences in binding site preference between ECRE activities extend to their selective binding to promoters which contain ATF sites: ECRE-2 binds to the VIP but not the E4 promoter, while ECRE-1 and ECRE-4 exhibit the converse specificity. Since the most important cis-acting elements of these promoters are their ATF sites, it is presumably their differences in ECRE binding that are responsible for their different transcriptional activities in vivo: in contrast to E4, VIP is transcriptionally inactive in F9 EC and PE cells. Similarly, the increased transcription from the E4 promoter after differentiation correlates with a change in the ECRE activities that bind to it in PE cells compared to EC cells.

The existence of a multiplicity of ATF site-binding activities in F9 EC and PE cells reflects the existence of a mammalian ATF gene family, which so far comprises of ten cDNAs isolated by various laboratories. In an attempt to relate the products of these cDNAs to the ECRE activities, the expression of five ATF genes was tested. ATF2, ATF4 and ATF6, are expressed to the same extent in both EC and PE cells, while ATF3 RNA levels increase.
during differentiation, and no *ATF1* RNA is detected in either cell type. These results therefore suggested three candidate genes, *ATF2, ATF4* and *ATF6*, that could encode ECRE-2 and ECRE-4/PCRE-1, but no obvious candidates for the down-regulated ECRE activities. However, as experiments with *in vitro* translated ATF polypeptides demonstrated, dimerization can affect DNA binding specificity and, consequently, constant RNA or even protein levels need not necessarily result in constant DNA binding activity. For example, *junD* is expressed in both F9 EC and PE cells. However, the JunD/ATF2 dimer binds to the ATF site, while JunD/cFos binds to the TRE. Assuming, for the sake of argument, that JunD/ATF2 represents an ECRE activity, it is therefore conceivable that cFos, which is synthesized only after differentiation, sequesters JunD away from the ATF site and onto the TRE. This would result in the down-regulation of the ECRE activity, even though neither *ATF2* nor *junD* RNA levels decrease during differentiation. Anti-peptide polyclonal antibodies were therefore raised against regions of ATF2 and ATF3 in order to investigate further the relationship between these ATF proteins and the ECRE activities. Unfortunately, using a gel retardation assay, no cross-reactivity between unpurified anti-ATF2 or anti-ATF3 sera and the ECRE activities in whole cell extracts was detected. An anti-cJun serum that also reacted with JunD, which is expressed in both EC and PE cells, and can bind to an ATF site as a heterodimer with ATF2 or ATF3, did recognize ECRE-1, however. It is possible, therefore, that JunD is contained in ECRE-1. Despite its failure to react with any of the ECRE activities, anti-ATF2 serum was, nevertheless, successfully used to characterise ATF2: it is a cluster of nuclear polypeptides, of 41-45 kD, whose levels remain constant during F9 EC cell differentiation, thus reflecting the unchanging *ATF2* RNA levels.

This study has therefore described the regulation and DNA binding specificity of ATF site-binding activities in the F9 EC cell system, and related them to the transcription of ATF site-containing promoters. However, the intended identification of these activities with defined members of the ATF family proved unsuccessful, since neither the anti-ATF2 nor the anti-ATF3 sera affected the ECRE/DNA complexes in a gel retardation assay. Perhaps the sensitivity of detection could be improved by partial purification of both extracts.
and sera. It should be noted, though, that an anti-Jun serum did "super-shift" ECRE-1 under the conditions used. An additional problem was that the chosen ATF3 epitope appeared to be denaturation-sensitive, since anti-ATF3 sera reacted with native in vitro translated ATF3 but not with denatured in vitro translated or cellular ATF3. This problem might perhaps be overcome by raising antisera against the appropriate fusion proteins expressed in bacteria; such sera should react with a greater variety of epitopes. Alternatively, sera against a number of peptide epitopes from each protein could be raised, in the hope of obtaining some that would recognize the native and/or the denatured polypeptides. The failings of the antisera did not allow the in vivo heterodimerization hypothesis to be tested, although the presence of polypeptides that co-immunoprecipitated with ATF2 but were not recognized by anti-ATF2 in an immunoblotting experiment suggests that ATF heterodimerization may indeed occur in vivo.

Even though these specific antisera had the mentioned shortcomings, the immunochemical approach still remains the most valid single method for establishing the relationship between ECRE activities and ATF proteins. DNA binding site affinity purification could perhaps be used as an alternative method for eventually obtaining cDNAs for the ECRE activities, though this method may not necessarily be appropriate in all cases, as exhaustive but unsuccessful attempts to purify ECRE-3 have suggested (M.Zamanian, unpublished observations).

Regardless of these problems, the present study has yielded some results that can be readily followed up. For example, it should be feasible to screen a λgt11 expression library with anti-PEP2 in order to clone the cDNA for the ECRE-1 protein that is recognized by that serum. The anti-ATF2 sera, which exclusively stain the nuclei of cells (Tassios and La Thangue, submitted), could also be used in further immunocytochemical experiments, to examine, for example, whether ATF2 and E1A co-localise after adenovirus infection, given that E1A is thought to interact with ATF2 (Liu and Green 1990). The anti-P10 serum, on the other hand, could be used to investigate the differences in structure and stability of different dimers, since it inhibited the DNA binding of ATF3 homodimers but not
heterodimers. This inhibition could be due to disruption either of homodimerization or of contact to the DNA. These two possibilities could be distinguished by assessing the efficiency of co-immunoprecipitation of an epitope-lacking but dimerization-competent ATF3 polypeptide with a wild type ATF3 polypeptide. Co-precipitation of the epitope-lacking ATF3 with wild type ATF3 would mean that dimerization was not disrupted by antibody binding and that therefore the inhibitory effect of the anti-serum was on DNA binding. On another level, it would be interesting to establish whether expression of the ATF2 and ATF3 genes, whose steady state RNA levels are regulated in distinct fashions during differentiation of F9 EC cells, those of ATF2 remaining constant while those of ATF3 are induced, is controlled at the level of transcription, and if so, to make a comparative study of the two genes' transcriptional elements and activities.

In conclusion, ATF site binding activities in F9 EC and PE cells have been shown to constitute a model family of DNA binding proteins, with respect to their complex DNA binding specificities, heterodimerization, regulation during differentiation and binding to diverse promoters.

Such characteristics are shared by transcription factor families in general; they, and their relevance to transcriptional control during development, are discussed in the sections that follow.

11.2 The versatility of transcription factor families

It is now recognized that transcription factors can be grouped into families, according to sequence similarities in their DNA binding domains and recognition sites. Members of such families are thought to be used during mouse development, for example in transcriptional "cascades" (the myogenic transcription factors; see below), or for providing an identity code for similar components of an organism (the Hox (murine homoeobox) genes and the vertebral column; Kessel and Gruss, 1991), or fulfilling presumably similar roles in
different parts of the organism where they are differentially expressed (the retinoic acid receptors; see below). Of course, transcription factor families have been extensively studied in other developmental systems, from σ factors during sporulation of *Bacillus subtilis* (reviewed by P.Stragier, 1991), and proteins directing the mating type switch in yeast (reviewed by Herskowitz, 1989), to those *D.melanogaster* homoeodomain proteins which set up the antero-posterior embryonic axis (reviewed by Nüsslein-Völlhard, 1991) or define segment polarity and identity (reviewed by Levine and Harding, 1989). However, there is now such a wealth of examples from murine development that this discussion will mostly focus on them, and specifically on those that display similarities to aspects of the ATF family.

11.2.1 Alternative splicing

Indeed, it seems that all the mechanisms for generating even more diversity from transcription factor families than that already provided by their different member genes, are encountered in the ATF family. One such mechanism is alternative splicing of primary transcripts. Six distinct mouse *CREB* transcripts have been characterised in F9 EC cells (Ruppert et al., 1992), arising from alternative splicing, promoter usage and polyA sequences. The rat and human equivalents of two of these transcripts, *CREB*α, the longest known *CREB* transcript, and *CREB*Δ, lacking the 14 amino acid α exon, have also been characterised (Yamamoto et al., 1990; Berkowitz and Gilman, 1990). Reports are conflicting as to their relative trans-activating potentials, Yamamoto *et al.* reporting that *CREB*Δ is 10 times less efficient than *CREB*α in activating transcription, while the other two groups observe no such difference. In addition, two other transcripts, *CREB*αγ and *CREB*γ, which both lack the DNA binding domain, are present in the primary spermatocytes of the adult testis at much higher levels than in any other tissue (Ruppert *et al.*, 1992); their function is unclear. An alternative transcript of *ATF2* is also preferentially expressed in the adult, but not the newborn, thymus or the adult spleen (Georgopoulos *et al.*, 1992). Furthermore, three
characterised *ATF2* transcripts have distinct abilities to stimulate transcription upon co-transfection with a T-cell-specific enhancer to which they can all bind *in vitro* (Georgopoulos *et al.*, 1992), a situation reminiscent of the distinct ligand binding and *trans*-activation properties of different thyroid hormone receptor α isoforms (Izumo and Mahdavi, 1988). Similarly, an alternatively spliced form of Pit-1/GHF1, GHF2, which contains an additional twenty-six amino acids in the activation domain, retains the same binding specificity as GHF1, but only activates a subset of the GHF1-inducible genes (Theill *et al.*, 1992). Alternative splicing has also been reported for *ATFa* (Gaire *et al.*, 1990), and *fosB* transcripts (Mumberg *et al.*, 1991; Dobrzanski *et al.*, 1991). No functional comparison has been made of the two *ATFa* proteins; however, the short isoform of FosB, lacking the 101 carboxy-terminal amino acids of the full length protein, is unable to transform cells and repress the *cfos* promoter, though both long and short isoforms *trans*-activate through a TRE. Distinct mRNA molecules, arising not only from alternative splicing and the use of more than one promoters but also from different translational initiation codons, also give rise to multiple isoforms of the three retinoic acid receptors (RARs) (Zelent *et al.*, 1991; Nagpal *et al.*, 1992). Alternative transcripts of transcription factor genes can therefore exhibit not only different temporal and spatial expression patterns and *trans*-activation potentials during development, but also distinct DNA binding specificities, as shown for the two *Drosophila* Tramtrack isoforms which contain alternative sets of zinc fingers (Read and Manley, 1992).

**11.2.2 Antagonists of transcriptional activators**

Alternatively spliced transcripts of transcriptional activator genes can also give rise to specific transcriptional antagonists, as in the case of the basic domain/helix-loop-helix (bHLH) protein gene *TFE3* (immunoglobulin enhancer μE3 motif-binding transcription factor): exclusion of an exon generates a shorter form of this transcription factor, which is expressed to varying levels in different mouse tissues (Roman *et al.*, 1991). The resulting polypeptide binds DNA, since it has a fully functional DNA binding domain, but does not
stimulate transcription, since it lacks an activation domain. On the other hand, an inhibitor of CREB activity, CREM, is encoded by a separate gene containing two bzip regions. Alternatively spliced transcripts, $CREM\alpha$, containing both bzip's, $CREM\beta$ containing only the carboxy-terminal one, and $CREM\gamma$, differing from $CREM\beta$ by a deletion of 36 nucleotides near the amino-terminus, are differentially expressed in adult mouse tissues (Foulkes et al., 1991). All three resulting CREM proteins contain a fully functional DNA binding domain through which they bind to the ATF site as CREM homodimers or CREM/CREB heterodimers. Co-expression of $CREB$ and $CREM$ inhibits ATF site-dependent transcription, presumably because CREM lacks an activation domain (Foulkes et al., 1991). However, a different temporally and spatially regulated $CREM$ transcript encodes a transcriptional activator, CREMr, which incorporates two relatively glutamine-rich amino acid stretches in the $CREM\beta$ protein and appears in the testis at the onset of spermatogenesis (Foulkes et al., 1992). Conversely, a 21 kD antagonist of CREMr, lacking one of these two insertions, is produced by translational initiation from an internal AUG in $CREM\gamma$ RNA (Delmas et al., 1992). Most CREM isoforms, therefore, inhibit transcription by sequestering CREB activator molecules into complexes that can bind DNA but cannot trans-activate. Id (inhibitor of differentiation) proteins, on the other hand, prevent some bHLH proteins from activating transcription by a different mechanism: they can heterodimerize with, for example, the activator E12, but the resulting dimer cannot bind to a bHLH recognition site ("E-box") since Id lacks the basic domain that is required for DNA binding (Benezra et al., 1990; Sun et al., 1991). Id RNA levels are regulated during differentiation of a variety of cell types (Benezra et al., 1990; Sun et al., 1991). Forced constitutive expression of Id can block both differentiation of a myeloid precursor cell line and the consequent appearance of "E-box" binding activity (Kreider et al., 1992). Id also forms inactive complexes with E12 and E47, thus inhibiting the formation of transcriptionally active dimers of, for example, MyoD and E12, and, consequently, not allowing muscle differentiation to proceed (Jen et al., 1992).
Transcription factor families, therefore, increase their versatility by super-imposing on the multiplicity of their member genes alternative splicing, transcriptional and translational starts. Alternative transcripts can include or exclude transcriptional activation or DNA binding domains, giving rise, in the latter case, to transcriptional antagonists belonging to the same family. The expression of both activators and their antagonists is under temporal and spatial control, thus rendering the regulation of transcription from the cognate DNA binding site during development even more flexible.

11.2.3 Different members of transcription factor families display distinct expression patterns and responses to stimuli

As was shown in Chapter 6, RNA levels of ATF3, cjun and cfos differ between undifferentiated and differentiated EC cells. In the mouse embryo also, cjun and junB show distinct patterns of in situ RNA hybridization (Wilkinson et al., 1989), as do members of the RAR gene family and the related RXR (retinoid X receptor) gene family (Ruberte et al., 1991; Mangelsdorf et al., 1992). Such differential expression may have functional consequences, since, for example, cJun activates transcription through the cjun and collagenase TREs whereas JunB does not (Chiu et al., 1989; Schütte et al., 1989). This is presumably due to the lower affinity of the JunB/cFos dimer for the TRE, compared to cJun/cFos (Ryseck and Bravo, 1991), as well as to differences in their activation domains (Chiu et al., 1989). Similarly, DNA binding and RNA levels of different proteins recognizing the octamer motif have overlapping but distinct spatial and temporal distributions in the mouse embryo (Schöler et al., 1989; Rosner et al., 1990; Stoykova et al., 1992). Therefore, distinct patterns of expression of different family members in the adult organism or during embryogenesis presumably provide another way of expanding the repertoire of trans-regulatory effects that can be achieved through a single cis-acting element.

Different members of a transcription factor family can respond to different signals. In the ATF family, CREB and ATF1, but not ATF2, positively regulate transcription in
response to protein kinase A activation (Yamamoto et al., 1988; Gonzalez et al., 1989; Liu and Green, 1990; Rehfuss et al., 1991). On the other hand, ATF2, but not ATF1 or ATF3, binds to the retinoblastoma gene product (pRb), a negative regulator of proliferation (reviewed by La Thangue, 1992), and activates the transforming growth factor β2 promoter through a pRb-inducible ATF site (Kim et al., 1992). In the AP1 family, cfos and junB but not cjun RNA levels increase with the rise of intracellular Ca\(^{2+}\) ions during membrane depolarization of PC12 cells (Bartel et al., 1989). Furthermore, induction of cfos transcription under these conditions has been shown to be mediated by phosphorylated CREB, which is therefore a substrate of calcium-calmodulin-dependent (CaM) protein kinases I and II, as well as of protein kinase A (Sheng et al., 1990). The CaM kinases are activated in response to elevated intracellular calcium concentration after membrane depolarization of neuronal cells (Nairn and Greengard, 1987) and ATF sites have been shown to be responsible for the transcriptional induction of the cfos and proenkephalin genes by intracellular calcium and membrane depolarization (Sheng et al., 1988; Van Nguyen et al., 1990). Such selective responses to extracellular and intracellular stimuli presumably determine the involvement of transcription factors in specific developmental processes, since, for example, membrane depolarization, by altering short and long term gene expression in neuronal cells, is thought to contribute to the establishment of their identity and position in the synaptic network of the nervous system (reviewed by Sheng and Greenberg, 1990; He and Rosenfeld, 1991). The ATF site also plays an important role in a different regulatory network, namely the positive autoregulation of G protein-coupled receptors, such as the β₂ adrenergic receptor, which activate adenylyl cyclase, thus generating high intracellular cAMP levels (reviewed by Collins et al., 1992).

As well as being regulated by different kinases, ATF proteins respond to a number of viral trans-activators. E1A can trans-activate via ATF2 but not ATF1 (Liu and Green, 1990). The human T cell leukaemia virus I (HTLV-I) Tax (transcriptional activator) protein also trans-activates its own promoter via an ATF site (Fujisawa et al., 1989) by binding to at least three ATF proteins (Yoshimura et al., 1990), while the hepatitis B virus (HBV) X
protein can induce binding of ATF2 and CREB to a non-consensus ATF site in the HBV enhancer (Maguire et al., 1991). Proteins binding to the ATF site, therefore, can respond to a variety of stimuli and participate in a number of regulatory networks.

11.2.4 Transcription factor dimerization

Members of the same transcription factor family can also deliver diverse regulatory effects by displaying subtle differences in binding site-, and therefore target gene-specificity, as exemplified by the ECRE activities described in this study. An efficient way of achieving this is through heterodimerization, as demonstrated by the cJun/ATF2 dimer, which binds to the ATF site, compared to cJun/cFos, which binds to the TRE. Similarly, a comparative study of affinity chromatography-purified, SDS-PAGE-separated and renatured CREB/CREB, CREB/ATF1 and ATF1/ATF1 dimers, suggested that they differ in their binding affinities as well as their trans-activation efficiencies in vitro (Wada et al., 1991). Heterodimers that display increased binding efficiency with respect to homodimers include Jun/Fos compared to Jun/Jun (Halazonetis et al., 1988), and NFκB p50/p65 compared to p50/p50 (Urban and Bauerle, 1991). Significantly, Fos/Jun and Jun/Jun dimers bend DNA in opposite orientations (Kerppola and Curran, 1991). Thus, different dimers could make different contacts with the transcriptional initiation complex, presumably affecting transcriptional initiation in a non-identical fashion. All these aspects of transcription factor activity regulation by heterodimerization are also encountered in the bHLH family. E12, for example, binds DNA only as a heterodimer (Sun and Baltimore, 1991) and different bHLH protein dimers have distinct preferences for variations of the CANNTG (where N is any nucleotide) consensus binding site (Blackwell and Weintraub, 1990). In addition, the full trans-activation and myogenic effects of MyoD depend on dimerization with the products of the ubiquitously expressed E2A gene (Lassar et al., 1991), while two alternative transcripts of the max gene, whose protein products dimerize with Myc (Blackwood and Eisenman, 1991; Blackwood et al., 1992), have opposite effects on myc-ras cotransformation of primary
cells (Mäkelä et al., 1992). Furthermore, DNA binding of Max homodimers but not that of Myc/Max heterodimers is inhibited by casein kinase II in vitro phosphorylation (Berberich and Cole, 1992). Thus, heterodimerization, a mechanism that regulates transcription factor activity by controlling the efficiency and specificity of DNA binding, is itself regulated by post-translational modifications. It is also clear that, although heterodimerization is, in principle, an efficient way of enhancing the repertoire of transcriptional regulators and integrating different stimuli, it is not promiscuous (Hai et al., 1989; Hai and Curran, 1991; Sun et al., 1991): heterodimerization increases the number of specific responses rather than abolishes specificity altogether. Dimerization between ATF and API proteins, for example, provides one more "cross-talk" point between the PKA and the PKC pathways (Otte et al., 1989), since members of the ATF family respond to PKA (Gonzalez et al., 1991; Rehfuss et al., 1991), while members of the API family respond to PKC activation (Lee, W. et al., 1987).

11.2.5. Distinct DNA binding specificities of transcription factor family members

All members of a transcription factor family have, by definition, overall highly similar DNA binding domains and, as a result, can bind to the same DNA site under appropriate conditions in vitro. However, it is the differences in these homologous amino acid sequences which determine individual preferences for nucleotides deviating from the consensus recognition site and which will therefore presumably also define distinct in vivo targets for each member. In the absence of other cooperative factors, such individual preferences will presumably be guided by the compatibility between protein structure and DNA structure, as well as optimum electrostatic attraction and hydrogen bond formation. It has been shown, for example, that each of the three Krox20 zinc fingers recognizes either a GGG or a GCG triplet within the nonamer recognition site, depending on the amino acids present in two critical positions of each finger (Nardelli et al., 1991). The functional importance of a few amino acids in the interaction of a transcription factor with other
proteins and the DNA is borne out most spectacularly in the case of I-POU (inhibitor of POU), a Drosophila member of the POU transcription factor family (including Pit-1, Oct proteins and Unc-86) (Herr et al., 1988), which forms non-DNA binding heterodimers with the transcriptional activator Cfl-a (C element binding factor 1a), thus preventing the activation of a neuron-specific gene (Treacy et al., 1991). An alternatively spliced form, "twin of I-POU", however, which differs from I-POU only by two amino acids in the homoeodomain, does not dimerize with Cfl-a but can act independently as a transcriptional activator, though through a different target sequence than Cfl-a (Treacy et al., 1992). By contrast, ECRE-2 presents the converse situation: a single protein surface binds to an isolated ATF site but not to that same site when it is in the context of the E4 promoter. Though the linear nucleotide sequence remains identical, it is possible that its structure is altered by the presence of neighbouring sequences, rendering it incapable of recognizing a previously compatible protein structure, in an analogous fashion, perhaps, that identical Pit-1 binding site core sequences flanked by different nucleotides differentially bind phosphorylated Pit-1 (Kapiloff et al., 1991).

Starting and evolving from a common DNA binding protein domain and its cognate DNA recognition sequence, a transcription factor family can therefore generate and accommodate diversity of transcriptional regulation through its members. A repertoire based on a number of related but distinct activator genes can be enhanced further by alternative transcripts produced from a single gene, according to a temporal or cell-specific programme, and counter-balanced by the similarly complex expression of antagonists. The differences in the precise amino acid sequence of different members' DNA binding domains presumably dictate precise DNA binding specificities that vary from the consensus, and these can be modified by heterodimerization of activator and inhibitor proteins. Thus, different signals, mediated by the monomers, can be integrated, and specific promoters activated or repressed, according to the affinity of their sites for the expressed transcription factor complexes.
11.3 Transcription factors as instruments of development

Development of the mouse, as of any multicellular organism, relies on the stage-specific expression of genes according to cell lineage and in response to intercellular and hormonal stimuli. Transcription factors, precisely because of their ability to regulate transcription, are the primary instruments of gene expression programmes, and their own expression precedes or coincides with, and reflects the developmental events which they direct.

One of the most interesting characteristics of the ECRE activities and the ATF genes is that their DNA binding and RNA levels are regulated during differentiation of EC cells. Similarly, as was mentioned in the previous sections, the expression of other transcription factor genes is regulated both temporally and spatially during embryogenesis proper. This developmental control extends, via post-transcriptional and post-translational mechanisms, to their DNA binding and trans-activation activities.

11.3.1 Transcription factors as developmental "switches"

The regulated expression of transcription factors can take the form of a transcriptional activation cascade during development, as has been demonstrated for Drosophila (reviewed by Nüsslein-Volhard, 1991; Levine and Harding, 1989). In the mouse, and on a smaller scale, the sequential though partly overlapping expression of myogenic transcription factors in the developing mouse embryo, culminating with skeletal α-actin expression, may represent a similar phaenomenon (Sassoon et al., 1989; Hinterberger et al., 1991; Ott et al., 1991). Expression of an exogenous copy of a myogenic gene in muscle cell cultures activates the expression of the endogenous copy as well as other myogenic and muscle structural protein genes (Thayer et al., 1989; Braun et al., 1989; Yutzey et al., 1990).
Given that the promoters of such myogenic genes as have been characterized contain consensus bHLH protein binding sites (Salminen et al., 1991; S.-P. Yee, pers. comm.), it is possible that myogenic factors bind to and activate each other's promoters, and that this transcriptional cascade plays an important role in muscle differentiation. A similar transcriptional activation hierarchy exists in hepatocytes, where the steroid receptor superfamily transcription factor HNF4 (hepatic nuclear factor 4) is required for expression of the homoeodomain transcription factor HNF1α (hepatic nuclear factor 1α) (Kuo et al., 1992). Of course, the question arises: if development relies on a cascade of tissue-specific transcription factors, how is this cascade itself put into motion? In the case of the pituitary-specific transcription factor Pit-1, it has been suggested that CREB, a less tissue-specific protein (Berkowitz and Gilman, 1990), activates the Pit-1 gene, which thereafter is autoregulated and is required for the maintenance of three anterior pituitary cell types (McCormick et al., 1990; Li et al., 1990). In addition, it is not unreasonable to expect that, as with Drosophila (reviewed by Nüsslein-Volhard, 1991), maternally expressed transcription factors that are stored in the oocyte, in RNA or protein form, such as murine Oct-3 (Rosner et al., 1990), also initiate transcriptional cascades during murine development.

Transcription of any given gene during development can be modulated by the stage-specific binding of different transcription factors on the same or overlapping sites. The increase in E4A promoter activity during F9 EC cell differentiation, for example, coincides with a change in the ECRE activities binding to its ATF site. Similarly, in the skeletal α-actin promoter, the binding site of SRF (serum response factor), a transcriptional activator necessary for muscle-specific expression of α-actin, is occupied by an inactive protein, F-ACT1, in non-muscle cells and proliferating myoblasts. It is only after myoblasts have differentiated to myotubes that F-ACT1 is replaced by SRF and α-actin expression occurs (Lee, T.C. et al., 1991). Even a modulation of transcription factor levels can result in specific activation of genes which respond above a given threshold of transcription factor concentration, as has been demonstrated for Bicoid-dependent activation of hunchback expression in Drosophila (Struhl et al., 1989). For example, the down-regulation of p-globin
gene expression during chicken erythroblast differentiation correlates with a ten-fold decrease in Sp1 and GATA1 (GATA motif binding protein 1), whose binding sites are important for \( \rho \)-globin transcription (Minie et al., 1992).

### 11.3.2 Post-translational regulation of transcription factor activity

Alternatively, rather than appear at a specific developmental stage, pre-existing transcription factors can be maintained in an inactive state by post-translational mechanisms. The p50 subunit of NF\( \kappa \)B, for example, is translated as a non-DNA binding, cytoplasmic polypeptide of 110 kD molecular weight (Ghosh et al., 1990; Kieran et al., 1990). Only after ATP-dependent proteolytic cleavage can the active p50, complexed to the p65 NF\( \kappa \)B subunit, migrate to the nucleus and activate transcription (Fan and Maniatis, 1991). However, contrary to mature B cells, where NF\( \kappa \)B is nuclear and binds DNA (Sen and Baltimore, 1986), in pre-B cells and other cell types, it is sequestered in the cytoplasm as a non-DNA binding complex with I\( \kappa \)B: this complex can be dissociated in vitro after phosphorylation of I\( \kappa \)B (Bauerle and Baltimore, 1988; Ghosh and Baltimore, 1990). It is therefore likely that NF\( \kappa \)B becomes activated by a similar post-translational mechanism during B-cell maturation.

Clearly, mechanisms used for the activation of transcription factors during differentiation may be shared with other processes where such activation is required: another instance where phosphorylation is a prerequisite for transcription factor activity is CREB-mediated trans-activation (Gonzalez and Montminy, 1989; Lee et al., 1990). Phosphorylation can also provide a link between a developmentally regulated transcription factor like DRTF-1 (Partridge and La Thangue, 1991), and the cell cycle. DRTF-1 has the ability to complex with the underphosphorylated form of pRb, the form that is most likely to suppress proliferation (Bandara and La Thangue, 1991; Ludlow et al., 1990). In the rapidly proliferating F9 EC cells, the levels of DRTF-1 which is not complexed with pRb are high and DRTF-1 binding site-dependent transcription is very efficient. After differentiation, however, the majority of DRTF-1 molecules are complexed with pRb and DRTF-1-
dependent transcription decreases, as a direct consequence of this association (Partridge and La Thangue, 1991; La Thangue et al., 1990; Zamanian and La Thangue, 1992). Phosphorylation of pRb, and consequently its ability to complex with DRTF-1, is cell cycle-regulated and differentiation-dependent (Chen et al., 1989). Thus, pRb, a suppressor of proliferation, regulates, in a differentiation-dependent manner, the activity of a transcription factor, DRTF-1, binding sites for which are present in a number of promoters which are active in rapidly growing cells (Mudryj et al., 1990). There is evidence that cJun activity is also regulated in a cell cycle-specific manner, since mitogen-activated serine kinases can phosphorylate the amino-terminus of cJun, thus enhancing its trans-activation potential (Pulverer et al., 1991). This phosphorylation domain of cJun is an activation domain which is the target for cell-specific repression of cJun activity (Baichwal and Tjian, 1990; Baichwal et al., 1992): as in the interaction of DRTF-1 with pRb, the mechanisms of cell-specific and cell cycle regulation seem to converge.

Another, as yet unstudied, mechanism through which the activity of transcription factors could be regulated during development is the expression of transcriptional co-activators, proteins that do not bind DNA but are required for trans-activation above the levels of transcription achieved by the basal initiation complex, and are thought to act as "bridges" between it and DNA bound activators. Their regulation may be particularly relevant to transcriptional control in F9 EC cells and the mouse blastocyst which both contain an "E1A-like" activity, since adenovirus E1A seems to trans-activate by "mediating" between specific DNA binding proteins and the initiation complex (Martin et al., 1990; Schöler et al., 1991; Lillie and Green, 1989). Importantly, activation by transcription factors Oct-4 and RARβ2 can occur in the absence of E1A in F9 EC cells but requires E1A in other cell types (Schöler et al., 1991; Berkenstam et al., 1992). Therefore, if adenovirus E1A does indeed function as a co-activator, or if it stabilises the interaction of cellular co-activators with DNA binding transcription factors, it is likely that the "E1A-like" activity of F9 EC cells will function in a similar way. In that case, transcriptional control during differentiation would
rely as much on the activity of DNA binding proteins as on that of transcriptional co-activators.

11.4 Conclusions

In conclusion, ATF site binding proteins constitute a model family of transcription factors whose activity is controlled at various levels and which respond to a variety of stimuli. Transcripts of different ATF members, as well as alternative transcripts of the same gene, are differentially distributed in different tissues and their levels are regulated during the differentiation of EC cells. Alternative splicing gives rise to ATF isoforms lacking a DNA binding or an activation domain, thus increasing the repertoire of ATF trans-activation and DNA binding properties. This functional multiplicity is enhanced further by dimerization. ATF proteins bind DNA as homodimers or heterodimers: the choice of dimerization partner, which can be a member of the ATF or the related API family, determines DNA binding specificity and therefore presumably trans-activation potential as well. These are also regulated by phosphorylation and can be further modified by interaction with viral transcriptional activators. It is, presumably, such mechanisms, as well as specific amino acid differences in their DNA binding domains, that result in the ability of distinct ATF site-binding activities to bind preferentially to specific variants of the ATF site and ATF site-containing promoters. Finally, ATF transcription factors seem to be involved in various developmental processes. They activate, for example, the expression of a cell-specific transcription factor in the pituitary, they participate in the electrically induced gene expression changes required for neuronal development, and their DNA binding levels are regulated during differentiation.

The present study has explored the regulation of the ATF family at the level of expression and DNA binding, and related it to the transcriptional activities of ATF site-containing promoters in a cell system that models early differentiation events in the mouse
embryo. The results presented, emphasizing the complexity and versatility of this family, are compatible with, and contribute to, the accumulating evidence which indicates that the activity of ATF proteins is under multiple controls and responds to various stimuli, characteristics which make it a useful agent for organising gene expression programmes during development.
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