Transcriptional Regulation and Cell Transformation by v-Jun.

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Abstract

v-Jun, a mutated derivative of the c-Jun transcription factor, is the transforming oncoprotein of an avian sarcoma virus. v-Jun is thought to cause cell transformation and tumorigenesis by the mis-regulation of certain target gene promoters. v-Jun can both activate and repress gene transcription compared to c-Jun, however little is known about the underlying mechanisms and the identity of the critical "effector" target gene(s) responsible for cell transformation and tumorigenesis by v-Jun.

To investigate the mechanisms of transcriptional regulation by v-Jun, a comparative study was undertaken of two gene promoters, *bkj* and collagenase, which are respectively activated or repressed by v-Jun. Promoter mutagenesis experiments were performed to investigate the effects of Jun binding site position and core promoter element sequences on transcriptional regulation by v-Jun. The primary conclusion was that these factors alone did not determine whether target promoters were activated or repressed by v-Jun. However, alterations in the level of transcriptional activation and fold induction of the variant promoters by v-Jun implied that binding site position and core promoter sequences did influence transcriptional regulation by Jun proteins. This analysis also suggested that v-Jun regulated transcription by different mechanisms at different target promoters.

Further work investigated the relationship between transcriptional activation of v-Jun target promoters and cell transformation using ΔvJ-hER, an amino-terminally truncated v-Jun protein fused to the hormone-binding domain of estrogen receptor-α. This chimaeric protein was previously shown to induce activation of v-Jun target genes and cell transformation in an estradiol-dependent manner, despite lacking the v-Jun transcriptional activation domain. The estrogen receptor activating function-2 (AF-2) domain was proposed to substitute for this v-Jun domain, implying that estradiol-dependent transcriptional activation of v-Jun target gene promoters by ΔvJ-hER was required for cell transformation.

To test this hypothesis, an inactivating mutation was introduced into helix 12 of the AF-2 domain, which mediates estrogen receptor binding to co-activator proteins. The mutant ΔvJ-hER protein was inactive in transcription and cell transformation assays, confirming that these processes required AF-2 function. Many estrogen receptor co-activator proteins have histone acetyltransferase activity, however the p300 histone acetyltransferase domain

was unable to substitute for the estrogen receptor AF-2 domain function to induce either transcriptional activation or cell transformation.

In conclusion, while the mechanisms responsible for transcriptional activation and repression by v-Jun remain unclear, these results support the hypothesis that transcriptional activation of positive v-Jun target gene promoters is required for cell transformation.

Declaration

I am the sole author of this thesis. All the work presented in this thesis was performed by myself, unless otherwise stated.

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Abbreviations

ACTR	Activator of thyroid and retinoic acid receptors
AF-2	Activating function-2
AP-1	Activator protein-1
ASV17	Avian sarcoma virus 17
ATF	Activating transcription factor
bkj	β-keratin in *jun*-transformed cells
bZip	Basic region with leucine zipper
cAMP	Cyclic AMP
CAT	Chloramphenicol acetyltransferase
CBP	CREB binding protein
cdk	Cyclin-dependent kinase
CEF	Chicken embryo fibroblast
ChIP	Chromatin immunoprecipitation
c-Jun	Cellular Jun
CRE	cAMP response element
CREB	CRE binding protein
DBD	DNA binding domain
DPE	Downstream promoter element
EMSA	Electrophoretic mobility shift assay
ER-α	Estrogen receptor-α
Fra	Fos-related antigen
H	Histone

HAT	Histone acetyltransferase
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
HDAC	Histone deacetylase
IL-6	Interleukin-6
ILS	Initiator-like sequence
Inr	Initiator
JAB1	Jun-activation-domain binding protein
JDP	Jun dimerisation partner
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
mt	Mutant
N-CoR	Nuclear hormone receptor co-repressor
NFAT	Nuclear factor of activated T cells
NF-IL6	Nuclear factor for interleukin-6 expression
PAGE	Polyacrylamide gel electrophoresis
PCAF	p300/CBP associated factor
PCR	Polymerase chain reaction
QEF	Quail embryo fibroblast
Rb	Retinoblastoma
RCAS	Replication competent avian retroviral expression vector
RT	Room temperature
SBE	Smad binding element
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SPARC	Secreted protein, acidic and rich in cysteine

SRC-1	Steroid receptor co-activator-1
SSeCKS	Src-suppressed C kinase substrate
Stat	Signal transducer and activator of transcription
SWI/SNF	Mating type switch / sucrose non-fermenter
TAD	Transcriptional activation domain
TAF_{II}	TBP-associated factor
TBP	TATA binding protein
TFII	General transcription factor for RNA polymerase II
TGF-β	Transforming growth factor-β
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TRE	TPA response element
TSA	Trichostatin A
UTR	Untranslated region
UV	Ultra-violet
v-Jun	Viral Jun
v/v	Volume per unit volume
WCE	Whole cell extract
wt	Wild-type
w/v	Weight per unit volume

1 Introduction

1.1 Discovery and characterisation of the Jun proteins.

1.1.1 Background

Avian sarcoma viruses are retroviruses which induce tumour formation upon infection. The viral genes responsible for tumorigenesis were found to be similar to sequences found in avian genomic DNA (Stehelin *et al.*, 1976), suggesting that these viral oncogenes were derived by transduction of cellular genes. Identification of some of these cellular proto-oncogenes revealed that many are involved in the regulation of normal cell growth. Transforming retroviruses such as avian sarcoma viruses were used in the search for more cellular proto-oncogenes, in the hope that this would reveal more information about the control of cell growth and the mechanisms of tumorigenesis.

1.1.2 Discovery of v-Jun.

In one such search for novel proto-oncogenes, avian sarcoma virus 17 (ASV17) was isolated from a spontaneous chicken sarcoma (Cavalieri *et al.*, 1985). The virus caused the formation of fibrosarcomas *in vivo*, and transformed chicken embryo fibroblasts (CEFs) in culture. The ASV17 transformed phenotype is defined by a characteristic cell morphology in culture (Cavalieri *et al.*, 1985; Maki *et al.*, 1987); anchorage independence and the formation of foci on solid substrate (Bos *et al.*, 1990); enhanced cyclin E-cdk2 activity, resulting in an increased rate of cell growth, and continued cell cycle progression accompanied by high levels of apoptosis in the absence of serum growth factors (Clark *et al.*, 2000; Clark and Gillespie, 1997). The viral genome did not contain sequences similar to known proto-oncogenes (Cavalieri *et al.*, 1985; Maki *et al.*, 1987), indicating that the transforming ability of the virus was due to the activity of a novel oncoprotein. Subsequent analysis of the ASV17 genome identified a putative oncogene with no sequence homology to any previously identified proto-oncogene. The novel gene was named *jun*, from the Japanese word for 17 (Maki *et al.*, 1987).

Viral Jun (v-Jun) is expressed as a fusion with the viral Gag protein (Bos *et al.*, 1988). Expression of Gag-v-Jun, or the v-Jun sequences alone, transformed CEFs in culture (Ball *et al.*, 1988; Bos *et al.*, 1990) and caused tumour formation (Wong *et al.*, 1992), confirming that v-*jun* is the transforming oncogene of ASV17.

1.1.3 Discovery of the cellular Jun proteins.

During initial analysis of the ASV17 genome, sequences similar to v-*jun* were detected in genomic DNA from chickens and other vertebrate species (Maki *et al.*, 1987), indicating that v-*jun* was, like other viral oncogenes, derived from a cellular proto-oncogene. Chicken cellular *jun* (c-*jun*) was subsequently cloned and sequenced (Ball *et al.*, 1988; Nishimura and Vogt, 1988). c-Jun is essential for embryonic development and cell proliferation in culture (Johnson *et al.*, 1993). Additional members of the *jun* family, *jun*B and *jun*D, have also been identified (Hirai *et al.*, 1989; Ryder *et al.*, 1988); however, based on sequence homology, c-*jun* is the cellular progenitor of the v-*jun* oncogene.

Comparison of the c-*jun* and v-*jun* sequences revealed structural differences between the genes (see Figure 1.1). As well as the fusion of v-*jun* to *gag*, the viral oncogene lacks the c-*jun* 3' untranslated region (UTR), which is thought to destabilise c-*jun* mRNA. Deletion of this region from v-*jun* contributes to the high levels of expression of the viral protein (Bos *et al.*, 1990). Amino acids 32 to 58 of c-Jun are absent from v-Jun; this 27 amino acid sequence is known as the delta domain. v-Jun also contains two amino acid substitutions compared to c-Jun: a serine to phenylalanine substitution at c-Jun amino acid position 222, and a cysteine to serine substitution at position 248.

Over-expression of c-Jun causes transformation of CEFs in culture, albeit at a lower efficiency than v-Jun (Bos *et al.*, 1990; Wong *et al.*, 1992). However, only v-Jun is tumorigenic *in vivo* (Morgan *et al.*, 1994; Wong *et al.*, 1992). Therefore there are differences in the effects of the two proteins which are not due solely to the higher level of expression of v-Jun. The contributions of the mutations within the v-Jun protein to cell transformation and tumorigenesis have been studied in some detail. Deletion of the delta region had the most significant effect on the induction of cell transformation, with the double amino acid substitution of secondary importance (Bos *et al.*, 1990). The minor effect of the double substitution may be due to neutralisation of the opposing effects of the individual mutations; introduction of the serine to phenylalanine mutation into c-Jun decreased, while the cysteine to serine substitution increased, the efficiency of focus formation (Morgan *et al.*, 1993). The delta deletion and the double amino acid substitution both contributed to tumour formation by v-Jun (Morgan *et al.*, 1994). These results imply that the mutations within v-Jun are responsible for its altered function and oncogenic activity.

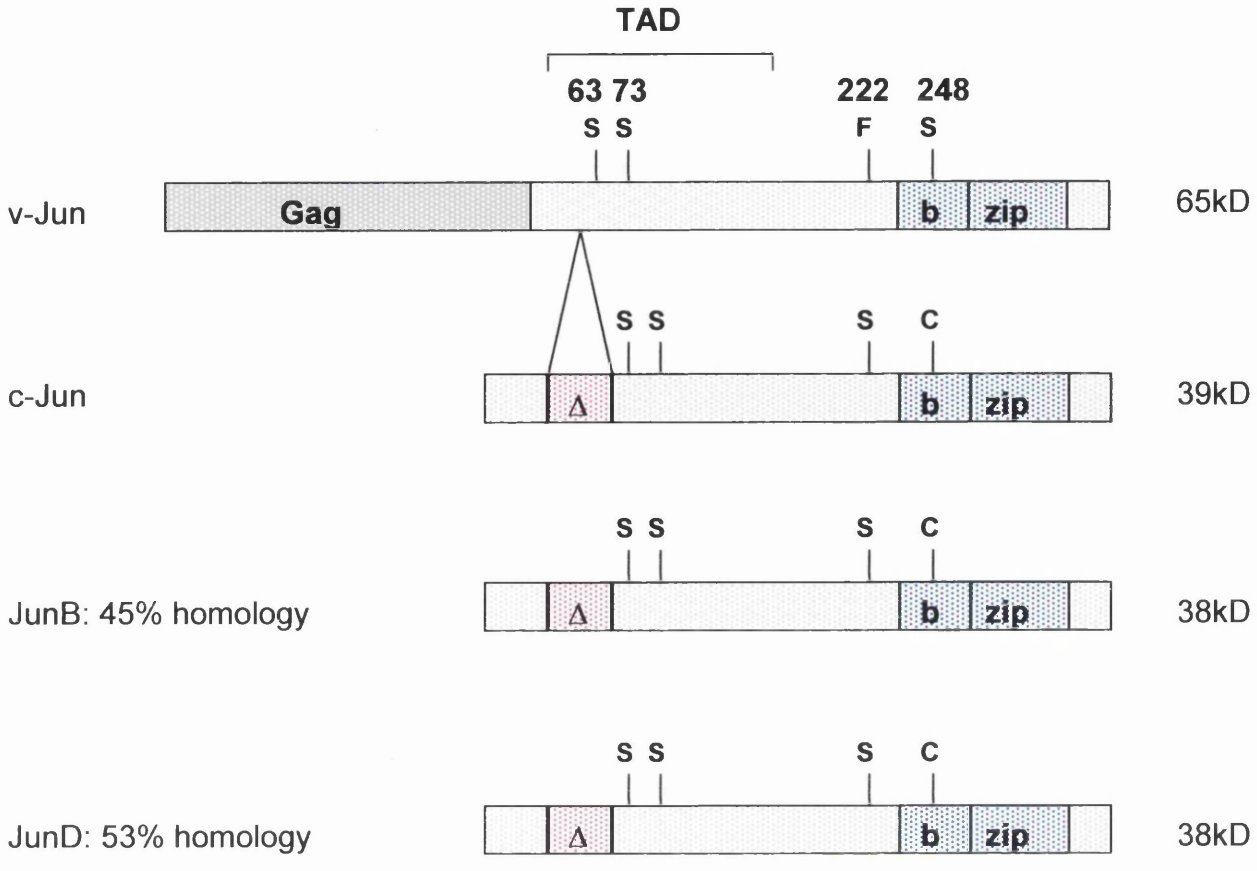


Figure 1.1

Comparison of the Jun family proteins.

The Jun transcriptional activation domain (TAD), delta (Δ) domain, basic (b) DNA binding domain and leucine zipper (zip), and the ASV17 Gag domain, are shown.

S: serine; F: phenylalanine; C: cysteine.

Amino acid positions are derived from the c-Jun sequence.

Protein molecular weights and amino acid homologies to c-Jun are shown.

The mutations within v-Jun compared with c-Jun alter the post-translational regulation of the viral protein and contribute to the mis-regulation of transcription and cell transformation – see text for details.

1.1.4 v-Jun as a transcription factor.

The function of the Jun proteins was first suggested by the observation that the C-terminal region of v-Jun shares 45% sequence homology with the sequence-specific DNA binding domain (DBD) of Gcn4, a yeast transcription factor (Vogt *et al.*, 1987). The implication that v-Jun could also bind specifically to DNA was confirmed in experiments which demonstrated that replacement of the Gcn4 DBD with the homologous region of v-Jun complemented a *gcn4* null phenotype (Struhl, 1987). Complementation required a functional Gcn4 binding site in a target promoter, suggesting that the DNA recognition sequence of v-Jun was similar to that of Gcn4. Later observations that full-length v-Jun could replace Gcn4 function suggested that v-Jun also contained a transcriptional activation domain (TAD); progressive N-terminal deletions localised this domain to two acidic regions between amino acids 15 and 102 of v-Jun (Struhl, 1988).

1.1.5 c-Jun as a component of AP-1.

The DNA recognition sequence of Gcn4, ATGACTCAT, is similar to an element found in the promoters of genes induced by tumour-promoting phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Angel *et al.*, 1987b). These TPA response elements (TREs) have the consensus sequence TGAG/CTCA and are bound by Activator Protein-1 (AP-1), which activates transcription from TRE-containing promoters in response to TPA (Angel *et al.*, 1987b; Lee *et al.*, 1987). The similarity between the AP-1 and Gcn4 recognition sequences suggested that c-Jun may be related to AP-1.

Antibodies raised against N- and C-terminal domains of v-Jun specifically recognised human AP-1 in Western blots, and tryptic peptide fragments of AP-1 were found to correspond in amino acid sequence to regions of c-Jun (Angel *et al.*, 1988; Bohmann *et al.*, 1987). In DNA footprinting analysis, v-Jun and human c-Jun protected consensus TRE sequences, but not mutated derivatives, with a similar pattern to human AP-1 (Angel *et al.*, 1988; Bohmann *et al.*, 1987; Bos *et al.*, 1988). v-Jun was also shown to activate transcription from TRE-containing promoters (Angel *et al.*, 1988). Taken together, these data showed that c-Jun and v-Jun bind specifically to TREs, and that c-Jun comprises a major component of the cellular AP-1 transcription factor.

1.1.6 Post-translational regulation of c-Jun and v-Jun.

The expression and activity of cellular proto-oncogenes such as c-*jun* are tightly regulated to prevent inappropriate cell growth. Identification of c-Jun as a major component of AP-1 placed the protein at the end of signal transduction pathways induced by phorbol esters such as TPA (Angel *et al.*, 1987b; Lee *et al.*, 1987), and the activation of oncogenes such as Ha-*ras* and v-*src* (Schonthal *et al.*, 1988; Wasylyk *et al.*, 1988). This discovery enabled investigation of the way in which c-Jun is regulated by extra-cellular signals, and how v-Jun may escape regulation and so cause cell transformation and tumorigenesis.

Treatment with phorbol esters, or activation of Ha-Ras, increased the level of c-Jun phosphorylation at serine residues at positions 63 and 73 (Adler *et al.*, 1992; Binetruy *et al.*, 1991; Pulverer *et al.*, 1991; Smeal *et al.*, 1991). Phosphorylation of these residues, in particular serine 73, correlated with increased transcriptional activation of TPA-responsive genes such as collagenase (Binetruy *et al.*, 1991; Black *et al.*, 1994; Hibi *et al.*, 1993; May *et al.*, 1998; Pulverer *et al.*, 1991; Smeal *et al.*, 1991), and with the ability of c-Jun to increase the efficiency of cell transformation by Ha-Ras (Smeal *et al.*, 1991). In contrast, the level of phosphorylation of v-Jun was not increased by these stimuli (Adler *et al.*, 1992; Black *et al.*, 1994; Black *et al.*, 1991; May *et al.*, 1998; Smeal *et al.*, 1991), and mutation of the serine residues corresponding to positions 63 and 73 of c-Jun did not affect the transcriptional activity of v-Jun (Black *et al.*, 1994). This difference between the Jun proteins was found to be a result of the deletion of the delta region from v-Jun (Adler *et al.*, 1992; Black *et al.*, 1991; Derijard *et al.*, 1994).

c-Jun N-terminal kinase (JNK) was identified as a novel member of the mitogen activated protein kinase (MAPK) family (Derijard *et al.*, 1994; Hibi *et al.*, 1993). JNK proteins are activated by cellular stress such as ultra-violet (UV) light, phorbol esters, or activation of Ha-Ras, and phosphorylate c-Jun on serines 63 and 73 (Derijard *et al.*, 1994; Hibi *et al.*, 1993). The JNK binding domain of c-Jun encompasses the delta domain, with the result that JNK binds c-Jun, but not v-Jun (Hibi *et al.*, 1993; Kilbey *et al.*, 1996; May *et al.*, 1998). The lack of phosphorylation of v-Jun at N-terminal serine residues renders the oncoprotein refractory to signal transduction pathways induced by JNK agonists such as UV light, TPA and activated Ha-Ras (Hibi *et al.*, 1993; Kilbey *et al.*, 1996; May *et al.*, 1998; Tsang *et al.*, 1994).

The point mutations within the C-terminal half of v-Jun also alter the regulation of the protein by signal transduction cascades. The serine to phenylalanine mutation at position

222 abolishes phosphorylation of the site, which releases v-Jun from inhibition of DNA binding (Boyle *et al.*, 1991; Lin *et al.*, 1992). The cysteine to serine mutation at position 248 has also been shown to increase the affinity of DNA binding by v-Jun in oxidising conditions, due to insensitivity of the serine residue to redox regulation (Abate *et al.*, 1990). However, an equivalent mutation has been shown to decrease the DNA binding activity of c-Jun homodimers in reducing conditions (Oehler *et al.*, 1993). Regulation of the DNA binding activity of c-Jun is clearly complex, but the double point mutation in the C-terminal half of v-Jun has been shown to permit specific DNA binding by the viral protein in conditions where binding by c-Jun was abolished (Oehler *et al.*, 1993).

Interestingly, the v-Jun cysteine to serine mutation subjects the protein to an additional level of regulation. Amino acid position 248 is contained within the Jun nuclear localistion signal, and introduction of a serine residue at this site has been shown to cause cell-cycle dependent nuclear translocation; unlike c-Jun, which was constitutively nuclear, the nuclear translocation of v-Jun was specifically increased during the G2 phase of the cell-cycle (Chida and Vogt, 1992). This was due to phosphorylation of v-Jun at serine 248 during G1 and S phase (Tagawa *et al.*, 1995). The significance of this phenomenon is not known, but represents a further example of how the mutations within v-Jun alter its regulation compared to c-Jun.

c-Jun is also regulated by other pathways. For example, an increase in intracellular calcium levels stimulated the phosphorylation of the protein, at sites other than serines 63 and 73, and independently of JNK activation (Cruzalegui *et al.*, 1999). In addition, c-Jun, but not v-Jun, has been shown to be phosphorylated at tyrosine 170 by c-Abl (Barila *et al.*, 2000). Little is known about the regulation of v-Jun by these mechanisms, and how the mutations within the protein may alter the effects of such signal transduction pathways in v-Jun transformed cells. Further investigation of these pathways may reveal further examples of the phenotypic effects of the mutations within v-Jun, possibly with implications for cell transformation and tumorigenesis.

1.1.7 Interaction of Jun with other proteins.

1.1.7.1 Fos and other bZip proteins.

Early attempts to purify AP-1 revealed that the transcription factor consists of more than one protein (Angel *et al.*, 1987b; Lee *et al.*, 1987). The product of the c-*fos* proto-oncogene was known to bind DNA sequences similar to a TRE, and was indeed shown to associate

with Jun proteins as part of the AP-1 complex bound to TREs (Halazonetis *et al.*, 1988; Rauscher *et al.*, 1988a; Rauscher *et al.*, 1988c; Sassone-Corsi *et al.*, 1988).

v-Jun, c-Jun, JunB and JunD have all been shown to bind TREs in the absence of any other protein (Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988b; Ryseck and Bravo, 1991; Turner and Tjian, 1989). While c-Fos alone could not bind TREs, co-expression of c-Fos or the related proteins FosB and Fos-related antigen-1 (Fra-1) increased the affinity of DNA binding by each of the Jun proteins (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988b; Ryseck and Bravo, 1991; Turner and Tjian, 1989). This was due to stabilisation of the protein-DNA complex (Rauscher *et al.*, 1988b; Ryseck and Bravo, 1991). Co-translation of full-length and truncated forms of Jun and Fos revealed that Jun proteins could form homodimers (Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988; Turner and Tjian, 1989), and heterodimers with other Jun proteins (Nakabeppu *et al.*, 1988) or with c-Fos and related proteins (Gentz *et al.*, 1989; Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Nakabeppu *et al.*, 1988; Turner and Tjian, 1989). In contrast, c-Fos could not form stable homodimers (Turner and Tjian, 1989). The ability of proteins to bind DNA therefore correlated with the presence of stable Jun / Jun or Jun / Fos dimers. Dimerisation occurred in the absence of DNA (Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988), suggesting that dimerisation was required for DNA binding.

The Jun dimerisation domains were localised by various studies to the C-terminal halves of the proteins. Deletion and site directed mutagenesis studies with Jun and Fos proteins revealed that dimerisation was mediated by the leucine zipper motif adjacent to the basic DBD (Gentz *et al.*, 1989; Kouzarides and Ziff, 1988; Schuermann *et al.*, 1989; Turner and Tjian, 1989). This combination of a basic region and leucine zipper, responsible for DNA binding and dimerisation, is known as a bZip domain. The specific orientation of binding between two leucine zippers brings together the basic regions of the two components of the dimer (Gentz *et al.*, 1989), to form a bi-partite DNA-binding domain.

A conserved element similar to a TRE is found in the promoters of many genes activated in response to increased levels of cyclic AMP (cAMP) (Comb *et al.*, 1986). This cAMP response element (CRE) has the sequence TGACGTCA and is bound by members of the activating transcription factor (ATF) / CRE binding protein (CREB) family. These transcription factors contain bZip domains with limited sequence homology to the Jun and Fos families. This, and the similarity between the TRE and CRE sequences, led to investigation of the relationship between the AP-1 and ATF / CREB families.

Protein complexes bound to TREs were also capable of binding to oligonucleotides containing various CREs (Hoeffler *et al.*, 1989). c-Jun, JunB and JunD homodimers have been shown to directly bind certain CREs, depending on the flanking sequences, with a lower affinity than to TREs (Chatton *et al.*, 1994; Ivashkiv *et al.*, 1990; Macgregor *et al.*, 1990; Nakabeppu *et al.*, 1988; Ryseck and Bravo, 1991). In contrast, v-Jun homodimers bound preferentially to CREs (Kataoka *et al.*, 1994). Jun proteins heterodimerise via leucine zipper motifs with certain members of the ATF/CREB family, including CRE-BP2, CRE-BPa, ATF-2, -3, -4, ATF-a, B-ATF, and Jun dimerisation proteins (JDP) -1 and -2, but not with CREB or ATF-1 (Aronheim *et al.*, 1997; Benbrook and Jones, 1990; Chatton *et al.*, 1994; Hai and Curran, 1991; Ivashkiv *et al.*, 1990; Macgregor *et al.*, 1990; Nomura *et al.*, 1993).

Interestingly, while Jun / Jun or Jun / Fos dimers bind preferentially to TREs, dimers of c-Jun with members of the ATF/CREB family generally bind specifically to CREs (Benbrook and Jones, 1990; Chatton *et al.*, 1994; Hai and Curran, 1991; Ivashkiv *et al.*, 1990; Macgregor *et al.*, 1990; Nomura *et al.*, 1993). Some combinations, such as Jun / ATF-2, Jun / ATF-4, and Jun / ATFa, can also bind to TREs, but with a lower affinity than to CREs (Chatton *et al.*, 1994; Hai and Curran, 1991). JunB / B-ATF dimers are highly unusual in binding to TREs with a higher affinity than to CREs (Echlin *et al.*, 2000). The implications of this alteration in binding site preference are discussed below. Jun and Fos proteins also dimerise with members of the Maf family, bZip oncoproteins which bind to sequences containing TREs and CREs (Kataoka *et al.*, 1994), and c-Jun and c-Fos dimerise with the nuclear factor for interleukin-6 expression (NF-IL6) transcription factor in response to IL-6 (Hsu *et al.*, 1994).

The ability of Jun proteins to form dimers with such a wide range of bZip transcription factors has implications for their role as transcriptional regulators. Opposing effects of different heterodimer combinations on transcription have been demonstrated. For example, co-expression of CRE-BP2 or B-ATF interfered with the activation of TRE-containing promoters by Jun / Fos dimers (Benbrook and Jones, 1990; Echlin *et al.*, 2000), by formation of dimers with Jun which do not activate transcription from TREs. As described above, dimerisation of Jun with different partners also alters the DNA binding preference of the complex, directing Jun / AP-1 family dimers to TREs and a sub-set of CREs, and Jun / ATF/CREB family dimers predominately to CREs. This would be expected to direct different dimers to bind and regulate distinct but overlapping spectra of target promoters *in vivo*.

Convincing evidence for distinct roles of Jun / AP-1 family and Jun / ATF/CREB family heterodimers in transcriptional regulation and cell transformation has been obtained, by manipulation of the Jun leucine zipper motif to create c-Jun and v-Jun mutants which dimerise preferentially with either Fos or ATF proteins (van Dam *et al.*, 1998). Jun-m0 proteins, which preferentially dimerised with c-Fos and Fra-2, specifically bound and activated transcription from a consensus TRE, while Jun-m1 proteins, which formed dimers with ATF-2, did not. The mutant proteins induced different aspects of the v-Jun transformed phenotype in CEFs; v-Jun-m0 mediated anchorage independent growth in soft agar, while expression of v-Jun-m1 enabled growth in low serum. Co-expression of both proteins reconstituted the full v-Jun transformed phenotype. These results suggested a model of v-Jun transformation whereby v-Jun / Fos family dimers regulate TRE-containing promoters to induce anchorage independent growth, and v-Jun / ATF family dimers regulate promoters containing CREs to enable growth-factor independence.

This hypothesis is supported by data from various studies. Over-expression of Fra-2, but not ATF-2, induced some degree of anchorage independent growth (van Dam *et al.*, 1998), although with a lower efficiency than v-Jun. Co-expression of ATF-2 with v-Jun-m1, and to a lesser extent with wild-type v-Jun, enhanced tumorigenesis and growth in low serum, but decreased the efficiency of colony formation in soft agar, possibly by sequestration of v-Jun from complexes containing Fos family proteins (Huguier *et al.*, 1998). Over-expression of ATF-3 in CEFs caused the characteristic change in cell morphology seen in v-Jun transformed cells, and enhanced growth in low serum (Perez *et al.*, 2001). However, ATF-3 did not induce anchorage independent growth, and regulated (by repression) only a sub-set of v-Jun target genes, further supporting the model described above. Forced expression of v-Jun homodimers, which bind TREs and CREs (Kataoka *et al.*, 1994), caused growth factor independence, anchorage independent growth, and tumorigenesis (Jurdic *et al.*, 1995). Similarly, formation of c-Jun homodimers, which bind TREs and certain CREs (Nakabeppu *et al.*, 1988; Ryseck and Bravo, 1991), led to both growth-factor independence and anchorage independent growth (Castellazzi *et al.*, 1993).

Even within the Fos family, different dimerisation partners can modify the function of Jun proteins. This was first suggested due to the changes in composition of the protein complex bound to TREs observed in rodent fibroblasts in the 24hr following stimulation with serum or specific mitogens (Cook *et al.*, 1999; Kovary and Bravo, 1991; Kovary and Bravo, 1992; Lallemand *et al.*, 1997). The composition of the complex varied with differences in the relative levels of expression of the Jun and Fos proteins. The JunD / Fra-2 dimers predominant in quiescent cells were rapidly replaced, initially by dimers of JunB with c-

Fos and FosB, and then by c-Jun and JunB with Fra-1 and Fra-2. In contrast, TREs in asynchronous cultures of exponentially growing cells were bound predominantly by dimers of c-Jun and JunD with Fra-2 and some Fra-1 (Kovary and Bravo, 1992; Lallemand *et al.*, 1997). Distinct roles for the different Fos proteins were suggested by micro-injection of specific inhibitory antibodies. Simultaneous inhibition of c-Fos and FosB function had the greater effect on DNA synthesis during serum stimulation of cells, while inhibition of Fra-1 and Fra-2 function had the greater effect in exponentially growing cells (Kovary and Bravo, 1992).

Evidence exists that the distinct roles of different Fos proteins are due to the highly specific regulation of transcription by different dimer combinations. For example, c-Jun / c-Fos and c-Jun / Fra-2 dimers have similar DNA binding properties, but c-Jun / c-Fos dimers activated, while c-Jun / Fra-2 dimers repressed, a promoter driven by the collagenase TRE (Suzuki *et al.*, 1991). The differences between the Fos proteins were largely attributable to sequence divergence in their C-terminal halves. c-Fos and Fra-2 also have distinct roles in mouse keratinocyte differentiation in response to different stimuli (Rutberg *et al.*, 1997). c-Fos expression was induced specifically by TPA, and c-Fos activated transcription from certain TRE-containing promoters, while Fra-2 expression was induced by calcium treatment and was shown to reduce transcription from a promoter strongly activated by c-Fos. This difference between the proteins is thought to contribute to the activation of overlapping but distinct sets of target genes in response to the different stimuli.

1.1.7.2 Unrelated transcription factor families.

As described above, dimerisation of c-Jun and v-Jun with different members of the bZip transcription families can significantly alter their DNA binding preference and transcriptional effects. c-Jun is also known to interact with members of many structurally unrelated transcription factor families, providing a further level of transcriptional regulation. The recent trend towards investigation of combinatorial transcriptional regulation by multiple factors from complex enhanceosome elements has yielded much information about the interactions of the AP-1 family with other regulatory factors, and only a few well-characterised examples are discussed below; for a more comprehensive review, see (Chinenov and Kerppola, 2001).

One example involves certain members of the signal transducer and activator of transcription (Stat) family. In response to cytokines such as IL-6, c-Jun and Stat proteins

interact and synergistically activate transcription from promoters containing a Stat recognition sequence, with or without a TRE (Schaefer *et al.*, 1995; Schuringa *et al.*, 2001; Zhang *et al.*, 1999). c-Jun and c-Fos also interact with the nuclear factor of activated T cells (NFAT) family to activate transcription from promoters containing a NFAT recognition sequence adjacent to a TRE. NFAT proteins bound to DNA form a complex with Jun / Jun or Jun / Fos dimers (Jain *et al.*, 1993), which has been shown to increase the affinity of binding of a c-Jun / c-Fos dimer to a non-consensus TRE (Peterson *et al.*, 1996). Similarly, interaction between c-Jun and the retinoblastoma (Rb) tumour suppressor protein led to synergistic activation of transcription from target promoters and increased the affinity of c-Jun binding to a consensus (Nead *et al.*, 1998; Nishitani *et al.*, 1999) or non-consensus (Slack *et al.*, 2001) TRE. Interactions such as these clearly have consequences for target promoter selection as well as regulation by c-Jun.

Jun proteins also undergo a complex series of interactions with Smad proteins in response to transforming growth factor-β (TGF-β). TGF-β induced a strong interaction between c-Jun and Smad-3, and the two proteins co-regulated promoters containing a Smad binding element (SBE) and TRE (Zhang *et al.*, 1998). Promoters containing overlapping TRE / SBE sequences, as well as promoters such as c-*jun*, which contain a TRE and SBE some distance apart, were activated by TGF-β, with DNA binding by the Smad and AP-1 families required for full activation (Qing *et al.*, 2000; Wong *et al.*, 1999). However, TGF-β-induced activation of certain SBE-containing promoters by Smad-3 was inhibited by JNK activation of c-Jun and JunB (Dennler *et al.*, 2000; Verrecchia *et al.*, 2001), suggesting the existence of complex cross-talk networks linking the Smad and AP-1 family pathways. The induction of the c-*jun* promoter by TGF-β may therefore provide a negative feedback mechanism.

The examples discussed above represent only a fraction of the known interactions of c-Jun with a wide range of structurally diverse transcription factors. While this area of research is expanding, little is known about the interaction of v-Jun with these regulatory factors. It would be expected that the structural differences between v-Jun and c-Jun could alter the interaction of v-Jun with some c-Jun-binding proteins, possibly affecting the selection and regulation of a diverse range of target promoters. Any such alterations may be found to contribute to the v-Jun transformed phenotype.

1.2 Transformation as a result of altered transcriptional regulation: the search for v-Jun target genes.

As described in Chapter 1.1, v-Jun and c-Jun have been characterised as transcription factors which dimerise with a variety of proteins via the leucine zipper, bind a specific recognition sequence in promoter DNA, and regulate the transcription of a range of target genes in a highly complex manner. Structural differences exist between v-Jun and c-Jun, and these have been shown to contribute to cell transformation and tumorigenesis by the viral protein (See Chapter 1.1.3). The role of the Jun proteins as transcription factors suggests that tumorigenesis and the increased efficiency of cell transformation by v-Jun are due to the altered expression of target genes, via mis-regulation of their promoters by the viral protein. Evidence for cell transformation as a consequence of altered transcriptional regulation by v-Jun, and a review of the search for target genes relevant to this process, are presented below.

1.2.1 Transcriptional regulation and cell transformation.

In the context of over-expressed murine c-Jun, deletion of the DBD or leucine zipper abolished transcriptional regulation from TREs and cell transformation, while point mutations in these domains decreased transcriptional activation and the efficiency of cell transformation (Morgan *et al.*, 1992). Deletion of the v-Jun TAD similarly abolished transcriptional activation and cell transformation by the viral protein. These results suggest that sequence-specific DNA binding and the activation of transcription are required for cell transformation by c-Jun and v-Jun, supporting the hypothesis that the phenotypic effects of the proteins are due to their role as regulators of transcription.

Manipulation of the v-Jun DBD to alter its binding specificity from the TRE to the unrelated c/EBP recognition sequence further supported this hypothesis (Basso *et al.*, 2000). v-Jun proteins with a mutated DBD failed to alter the expression of known v-Jun target genes, and did not induce deregulated cell growth, anchorage independence or release from contact inhibition. This strongly suggests that the specific binding and regulation of promoters containing TREs is required for cell transformation by v-Jun.

v-Jun has been shown to down-regulate the endogenous c-Jun protein and thereby replace c-Jun as a major component of the complex bound to TREs and CREs in v-Jun transformed CEFs (Kilbey *et al.*, 1996). Various studies have investigated the effect of this

alteration on the transcriptional regulation of promoters containing these elements. Initial observations with a promoter driven by four copies of the TRE sequence in an *in vitro* assay suggested that v-Jun was a stronger activator of transcription than c-Jun (Bohmann and Tjian, 1989). Deletion of a region similar to the delta domain increased the transcriptional activity of c-Jun in this system, implying that the delta deletion was largely responsible for the increased activity of v-Jun. This was supported by the study in CEFs of transcriptional activation from a promoter containing five binding sites for the yeast Gal4 transcription factor. A fusion of v-Jun to the Gal4 DBD was a stronger activator of transcription than the equivalent Gal4-c-Jun protein (Black *et al.*, 1994). Again, the different activities of c-Jun and v-Jun were reported to be due to the deletion of delta from v-Jun.

In the murine F9 embryonal carcinoma cell line, which expresses low levels of the endogenous AP-1 family proteins, c-Jun has been shown to be a more potent activator than v-Jun of the natural collagenase I promoter (Morgan *et al.*, 1993), which contains a consensus TRE (Angel *et al.*, 1987b). Systematic analysis revealed that while the introduction of the serine to phenylalanine mutation into c-Jun increased transactivation of the collagenase promoter, the cysteine to serine or double mutation decreased transcription to the levels obtained with v-Jun (Morgan *et al.*, 1993). These v-Jun C-terminal point mutations also had opposing effects on the efficiency of cell transformation, further supporting the relationship between transcriptional mis-regulation and cell transformation. Deletion of the delta domain from c-Jun also decreased transcriptional activation of the collagenase I promoter, but to a lesser degree than introduction of the cysteine to serine mutation.

A decreased transcriptional activity of v-Jun compared to c-Jun has also been observed in CEFs, which have a much higher basal level of AP-1 family activity than do F9 cells. Various artificial promoters containing a TRE, as well as the natural collagenase I promoter, were specifically repressed in v-Jun transformed CEFs compared to control cells (Kilbey *et al.*, 1996), and co-expression of c-Jun or v-Jun with the collagenase I promoter confirmed that c-Jun activates this promoter more strongly than does v-Jun (Gao *et al.*, 1996). In a comparison between a minimal c-*jun* promoter driven by multiple copies of the c-*jun* TRE or of a consensus CRE, c-Jun activated the TRE-containing promoter more strongly than did v-Jun, while v-Jun was the more potent activator of transcription from the CRE-driven promoter (Gao *et al.*, 1996).

Taken together, these results suggest that v-Jun can both activate and repress transcription compared with c-Jun, depending on the promoter context, cell type, and transcription assay utilised. It has been reported that the delta deletion is largely responsible for the increased transcriptional activity of v-Jun at certain promoters (Black *et al.*, 1994; Bohmann and Tjian, 1989), while the cysteine to serine point mutation appears more important for transcriptional repression by v-Jun (Morgan *et al.*, 1993). This has not been specifically investigated by direct comparison in the same system of promoters activated or repressed by v-Jun. It therefore remains unclear whether this represents a genuine difference between the two modes of transcriptional regulation by v-Jun. However, it is clear that the replacement of c-Jun with v-Jun in transformed cells mis-regulates, by activation or repression, various target promoters containing TREs or CREs, due to the structural differences between the proteins.

1.2.2 v-Jun target genes.

1.2.2.1 Identification of v-Jun targets.

In accordance with the observations described above, methods such as subtractive hybridisation have identified examples of both activated and repressed targets of v-Jun. Examples of negative v-Jun targets include CO6, a transmembrane protein with homology to a subunit of calcium-activated potassium channels (Oberst *et al.*, 1997); apolipoprotein A-1, whose down-regulation is associated with certain cancers (Hadman *et al.*, 1998); the endogenous c-*jun* gene (Hussain *et al.*, 1998); and secreted protein, acidic and rich in cysteine (SPARC), which is associated with tissue remodelling and cell cycle control (Vial and Castellazzi, 2000; Vial *et al.*, 2000).

Many up-regulated v-Jun targets have also been identified, including β-keratin in *jun*-transformed cells (Bkj), a highly hydrophobic protein related to β–keratins (Hartl and Bister, 1995; Hartl and Bister, 1998); a putative cysteine protease related to cathepsin O (Hadman *et al.*, 1996); glutaredoxin, which is involved in DNA synthesis (Goller *et al.*, 1998); heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Fu *et al.*, 1999); homologues of cytokine-inducible SH2-containing proteins, reversion-induced LIM protein, MAPK phosphatase 2, and other targets with no homology to known genes (Bader *et al.*, 2000; Fu *et al.*, 2000).

A more recent study has utilised emerging micro-array technology to identify a large number of genes (more than 200) either activated or repressed by v-Jun, including a

putative tumour suppressor, Src-suppressed C kinase substrate (SSeCKS), which is repressed by v-Jun (Cohen *et al.*, 2001). This approach necessitates the expression of v-Jun in murine fibroblasts, as chicken micro-arrays are not yet commercially available. This may be found to limit the value of the technique for the identification of target genes associated with cell transformation by v-Jun in its natural CEF target cells. However, at present, this appears to be an extremely useful approach to large-scale identification of v-Jun target genes.

Many genes mis-regulated in v-Jun transformed cells will not be direct transcriptional targets of v-Jun, but may be activated or repressed in response to the altered expression of other, direct, targets. A small number of genes have been identified as direct v-Jun targets by cloning and analysis of their promoters. For example, the activation of Bkj and the repression of c-Jun have been shown to be due to direct transcriptional regulation by v-Jun, via TREs in the respective gene promoters (Hartl and Bister, 1998; Hussain *et al.*, 1998). However, this approach, while potentially informative about the transcriptional regulation of specific v-Jun target promoters, is not practical with the advent of technologies such as micro-array analysis. The large amount of data now being generated requires a screening approach simultaneously applicable to many putative target genes.

One such possibility is the use of a quail cell system, in which the expression of v-Jun and subsequent transformation of the cells are controlled by the addition of doxycycline (Bader *et al.*, 2000). *bkj*, a known direct target of v-Jun, was activated in this system with kinetics consistent with rapid induction by v-Jun. The expression of a novel up-regulated v-Jun target gene was also dependent on the induction of v-Jun expression, indicating that it may also be a direct transcriptional target. However, the dependence of this system on the kinetics of gene induction to discriminate between direct and indirect v-Jun targets represents a potential problem, as this approach could lead to ambiguity in certain cases.

A more reliable system involves a protein with hormone-inducible v-Jun function (Kruse *et al.*, 1997). Previous fusion of c-Jun, JunD and Fos family proteins to the hormone-binding domain of human estrogen receptor-α (ER-α) had been shown to induce the characteristic regulation of known target genes only in the presence of the agonist ligand estradiol (Fialka *et al.*, 1996; Francis *et al.*, 1995; Kim *et al.*, 1996; Schuermann *et al.*, 1993; Superti-Furga *et al.*, 1991). Fusion of the ER-α hormone-binding domain to c-Jun or JunD proteins lacking a functional TAD enabled hormone-dependent transcriptional activation (Francis *et al.*, 1995; Kim *et al.*, 1996). This suggested that the ER-α activating function 2 (AF-2) domain, a hormone-dependent TAD which overlaps the hormone-binding domain,

contributed to the activation of Jun target promoters in the presence of its activating ligand. The ER-α hormone-binding domain was therefore fused to full-length v-Jun, to create a hormone-inducible v-Jun protein (vJ1-hER), and to an amino-terminally truncated v-Jun protein lacking its TAD, to investigate the contribution of the AF-2 domain to hormone-dependent transcriptional activation by the chimaeric protein (Kruse *et al.*, 1997).

Both vJ1-hER and the truncated ΔvJ-hER protein initiated hormone-dependent activation of *bkj*, a direct transcriptional target of v-Jun. The level of activation by ΔvJ-hER was lower than that obtained with vJ1-hER, suggesting that the ER-α AF-2 domain contributed to, but was not completely responsible for, hormone-dependent transactivation (Kruse *et al.*, 1997). Both ER-α fusion proteins induced cell transformation in the presence of estradiol. ΔvJ-hER also mediated hormone-dependent cell-cycle progression in the absence of serum growth factors (Clark *et al.*, 2000). This indicated that the ER-α fusion proteins regulated at least some of the same critical transformation effector genes as v-Jun. Of the two fusion proteins, the function of ΔvJ-hER was regulated more tightly by estradiol, probably due to degradation of vJ1-hER to release full-length functional v-Jun protein even in the absence of estradiol (Kruse *et al.*, 1997).

ΔvJ-hER, with its strictly hormone-dependent v-Jun function, represents an excellent system for discrimination between direct and indirect v-Jun target genes. As the addition of hormone activates a pre-existing protein, simultaneous treatment of cells with estradiol and a protein synthesis inhibitor such as cyclohexamide leads to the activation only of direct v-Jun target genes. Indirect targets, which may depend on the increased synthesis of a regulatory factor in response to v-Jun, are less likely to be activated. Hormone-inducible v-Jun systems have been used to identify glutaredoxin, HB-EGF and MAPK phosphatase 2 (Fu *et al.*, 1999; Fu *et al.*, 2000; Goller *et al.*, 1998), but not other v-Jun target genes (Fu *et al.*, 2000), as probable direct v-Jun targets. This system is not ideal. Hormone-dependent transcriptional activation by ΔvJ-hER is thought to be due to the ER-α AF-2 domain, a transcriptional activator, and the effect on down-regulated v-Jun target genes has not been investigated. However, for genes up-regulated by v-Jun, this is an excellent system for discrimination between direct and indirect transcriptional targets.

1.2.2.2 v-Jun effector genes.

The altered expression of only a sub-set of v-Jun target genes is thought to be necessary for cell transformation and tumorigenesis. Both direct and indirect transcriptional targets of v-

Jun may be relevant for these processes. Identification of these "effector genes" is of primary importance in the investigation of the mechanisms of cell transformation and tumorigenesis by v-Jun.

One approach has been to investigate the effects of over-expression of individual up-regulated v-Jun target genes in control cells, or of re-expression of negative target genes in v-Jun transformed cells. This had yielded some extremely interesting results. For example, over-expression of HB-EGF, a gene directly up-regulated by v-Jun, induced anchorage independent growth and release from contact inhibition, albeit with a lower efficiency than v-Jun (Fu *et al.*, 1999). This marks HB-EGF as an extremely good candidate v-Jun effector gene. A further putative effector gene is SSeCKS. Co-expression of this protein with v-Jun in murine fibroblasts strongly decreased the efficiency of cell transformation, indicating that repression of SSeCKS by v-Jun is necessary for the activity of the oncoprotein (Cohen *et al.*, 2001).

A potential limitation of this approach is demonstrated by the example of SPARC. Re-expression of this protein in v-Jun transformed CEFs increased the rate of cell division and efficiency of anchorage independent growth (Vial and Castellazzi, 2000). This was unexpected, as SPARC is a negative target of v-Jun. However, co-expression with SPARC was shown to decrease the efficiency of tumorigenesis by v-Jun. The analysis of gene expression in cultured cells may therefore prove inadequate if potential v-Jun effector genes are not to be overlooked.

Analysis of the effects of individual target genes presents a further difficulty. It is likely that v-Jun induces cell transformation and tumorigenesis by the simultaneous mis-regulation of a number of effector genes. Alteration of the expression of an individual v-Jun effector gene may not therefore induce any phenotypic effect, as the activity of the protein may require the co-ordinate mis-regulation of other targets.

In summary, while analysis of individual v-Jun target genes has the potential to be highly informative, this approach is not a practical proposition for large-scale investigation of the mechanisms of cell transformation and tumorigenesis. As with the discrimination between direct and indirect transcriptional targets of v-Jun, screening procedures have been developed which may facilitate management of the large amounts of data currently being generated. These involve determination of the expression of putative v-Jun effector genes in a panel of cell lines, expressing mutated Jun proteins with varying transformation efficiencies (Basso *et al.*, 2000; Havarstein *et al.*, 1992). Such screening approaches have

correlated the degree of mis-regulation of HB-EGF (Fu *et al.*, 1999) and reversion-induced LIM protein (Fu *et al.*, 2000), but not homologues of cytokine-inducible SH2-containing proteins, MAPK phosphatase 2 (Fu *et al.*, 2000), cathepsin O, or apolipoprotein A-1 (Basso *et al.*, 2000) with transformation potential. This technique is suitable for positive and negative, direct and indirect v-Jun target genes, and is potentially highly important as a method of identifying novel targets worthy of investigation as putative v-Jun effector genes.

1.3 Mechanisms of transcriptional regulation by v-Jun.

As described in Chapter 1.2.2, many positive and negative target genes of v-Jun have been identified, with a number known to be direct transcriptional targets. However, only a few v-Jun target promoters have been studied in any detail. While such studies have provided information about the effect of v-Jun on specific TRE-containing promoters such as *bkj* (Hartl and Bister, 1998) and c-*jun* (Hussain *et al.*, 1998), little is known about the mechanisms of transcriptional regulation by v-Jun. Elucidation of these mechanisms is important to our understanding of the relationship between target gene regulation and cell transformation by v-Jun. The mechanisms responsible for the differential regulation of target promoters by v-Jun and c-Jun are of particular interest.

After discussion of general mechanisms of transcriptional regulation, a review of the literature concerning the regulation of transcription by the Jun proteins will be presented.

1.3.1 General mechanisms of transcriptional regulation.

It is commonly believed that most sequence-specific DNA binding transcription factors regulate transcription by control of the affinity of binding of the pre-initiation complex to core gene promoters. The pre-initiation complex is an extremely large complex containing RNA polymerase II, its associated general transcription factors (TFIIs) and other proteins necessary for the initiation of transcription. As discussed below, transcription factors can increase the affinity of binding of the pre-initiation complex at core promoters directly, by binding to components of the complex, or indirectly, by alteration of the chromatin environment surrounding the promoter.

1.3.1.1 Direct recruitment of the pre-initiation complex.

The pre-initiation complex binds sequences known as core promoter elements situated close to the transcriptional start site. The best characterised element is the TATA box. This element is generally located 25-30bp upstream of the transcriptional start site, and has the loose consensus sequence TATAAAA. However, various non-consensus A/T-rich sequences have been shown to enable basal levels of transcription in an *in vitro* assay (Hahn *et al.*, 1989), and considerable sequence variation between naturally occurring, functional TATA boxes has been observed.

The TATA box provides the initial binding site for TATA binding protein (TBP), a component of TFIID, and is the most important core promoter element for the transcription of many promoters. The TATA box also contributes to the selection of the transcriptional start site. It has been shown that the majority of transcriptional events initiate from sites 25-30bp downstream of the TATA box, independently of its position with respect to other core promoter elements (O'Shea-Greenfield and Smale, 1992).

The Initiator (Inr) element, with the consensus sequence PyPyANPyPyPy (Javahery *et al.*, 1994; Lo and Smale, 1996), surrounds the major transcriptional start site of many promoters. In the absence of a TATA box, an Inr is sufficient for the initiation of basal and activated transcription from a specific start site (Smale and Baltimore, 1989). In promoters containing TATA box and Inr sequences, both elements contribute to transcriptional start site selection. A distance of 25bp between the elements is optimal for the initiation of high levels of transcription from a site within the Inr (O'Shea-Greenfield and Smale, 1992). An increase in the distance between the elements decreased the overall level of transcription. The majority of the remaining transcriptional initiation events occurred from sites 25-30bp downstream of the TATA box, with additional events initiating from a site within the Inr. This suggests that, while both elements can independently influence the selection of the transcriptional start site, optimal spacing allows the elements to co-operate to activate high levels of transcription from a specific site.

More recently, a conserved sequence known as the downstream promoter element (DPE) has been characterised as a common core promoter element. A consensus DPE has the sequence A/G G A/T CGTG and is located between positions +29 and +32 relative to the transcriptional start site (Burke and Kadonaga, 1996). Like the TATA box, the DPE provides a binding site for TFIID (Burke and Kadonaga, 1996; Burke and Kadonaga, 1997) and is dependent on an optimal distance from the Inr for full activity (Burke and

Kadonaga, 1997; Kutach and Kadonaga, 2000). Analysis of 200 *Drosophila* promoters revealed that around 30% of promoters contained a TATA box, a similar number contained a DPE, and an additional 15% contained both elements (Kutach and Kadonaga, 2000). While introduction of a consensus DPE could partially rescue basal transcription from a promoter with a mutated TATA box (Burke and Kadonaga, 1996), mutational analysis revealed that the TATA box is the more important sequence in promoters containing both elements (Burke and Kadonaga, 1997).

As described above, the TATA box and DPE provide binding sites for TFIID. Components of TFIID also contact the Inr (Chalkley and Verrijzer, 1999; Kaufmann and Smale, 1994). However, most studies have investigated the role of the TATA box, and recruitment of the pre-initiation complex to TATA boxes will be discussed below. It should be noted that evidence exists for the recruitment of components of the pre-initiation complex in a step-wise manner, and also as a pre-formed complex or series of complexes. The step-wise model will be followed below for convenience.

TFIID contains TBP and a number of TBP-associated factors (TAF_{II}s). Binding of TFIID has been shown to be the first stage in the assembly of the pre-initiation complex at core promoters (Buratowski *et al.*, 1989), with the addition of TFIIA increasing the affinity of TFIID binding at some promoters (Buratowski *et al.*, 1989; Emami *et al.*, 1997; Kaufmann and Smale, 1994; Maldonado *et al.*, 1990). TBP / TFIID can bind consensus and non-consensus TATA boxes (Hahn *et al.*, 1989; Patikoglou *et al.*, 1999), in the minor groove of the DNA helix (Lee *et al.*, 1991a; Starr and Hawley, 1991). The assembly of the pre-initiation complex proceeds in the order TFIID / A, TFIIB, TFIIF / RNA polymerase II, TFII E / H (Buratowski *et al.*, 1989; Ha *et al.*, 1993; Maldonado *et al.*, 1990); see (Orphanides *et al.*, 1996) and (Pugh, 2000) for reviews.

Various studies have shown, by fusion of TBP to transcriptionally inactive sequence-specific DBDs or by a 2-hybrid approach, that recruitment of components of the pre-initiation complex by upstream binding factors enhanced transcriptional activation (Chatterjee and Struhl, 1995; Keaveney and Struhl, 1998; Klages and Strubin, 1995; Xiao *et al.*, 1995). A functional TATA box and the TBP DBD were required for full activation (Chatterjee and Struhl, 1995; Klages and Strubin, 1995). Fusion of TBP to the Gal4 DBD directed the binding of TBP to a non-consensus TATA box close to the Gal4 binding site (Xiao *et al.*, 1995), suggesting that transcriptional activation occurred by direct recruitment of the pre-initiation complex to core promoter elements. The fusion of acidic TADs to TBP or TAF_{II}s did not increase the level of transcription from a core promoter (Keaveney and

Struhl, 1998). This ruled out an effect on the transcriptional activity of the pre-initiation complex by the binding of TADs to component proteins. This supports the hypothesis that recruitment of the pre-initiation complex to core promoters is responsible for the activation of transcription by factors binding to sites within the promoter.

Various transcription factors are known to bind components of the pre-initiation complex. For example, members of the nuclear hormone receptor family, such as steroidogenic factor 1, the progesterone receptor and ER-α, bind TFIIB (Ing *et al.*, 1992; Li *et al.*, 1999). TFIIB bound the AF-2 domain of ER-α, but not the N-terminal AF-1 domain (Ing *et al.*, 1992). The AF-2 domain also bound $TAF_{II}30$ in a hormone-independent manner; depletion or inactivation of $TAF_{II}30$ specifically decreased transcriptional activation by the AF-2 domain, suggesting that this interaction had functional significance (Jacq *et al.*, 1994). Over-expression of $TAF_{II}28$ has been shown to enhance transcriptional activation by the AF-2 domains of the retinoid X receptor, retinoic acid receptor-γ, ER-α and the vitamin D receptor (May *et al.*, 1996), suggesting that interaction with TAF_{II} proteins is a common mechanism of transcriptional activation by nuclear hormone receptors.

The Herpes simplex virus VP16 protein has been shown to bind TFIID, TFIIB and TFIIH (Gupta *et al.*, 1996; Stringer *et al.*, 1990; Xiao *et al.*, 1994). Interestingly, mutations in the VP16 TAD which impaired its ability to activate transcription decreased its binding to TFIIH (Xiao *et al.*, 1994), but not to TFIIB (Gupta *et al.*, 1996). This implies that binding to some components of the pre-initiation complex may be more important than others in the activation of transcription.

p53 has been shown to bind TFIIH (Xiao *et al.*, 1994) and has also been shown to increase the affinity of TFIID binding to the TATA box, especially in the presence of TFIIA (Xing *et al.*, 2001). A transcriptionally inactive p53 protein had a much smaller effect on the affinity of TATA box binding. This further strengthens the relationship between binding to components of the pre-initiation complex and activation of transcription.

1.3.1.2 Histone modification and chromatin remodelling.

In vivo transcription occurs from DNA bound by histone proteins, and packaged into nucleosomes and higher-order chromatin structures. This has a general repressive effect on transcription by denying access to the DNA by sequence-specific binding proteins such as transcription factors and the pre-initiation complex. RNA polymerase II is associated with

proteins involved in covalent histone modification and chromatin remodelling, linking these processes to transcriptional regulation.

Covalent modification of the histone components of nucleosomes, by acetylation, phosphorylation or methylation, is associated with transcriptional regulation (see below). The best characterised mode of modification is by acetylation and deacetylation of lysine residues in the N-terminal histone tail regions, mediated by enzymes known as histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation of these residues is proposed to decrease the affinity of histone-DNA binding, and so increase the accessibility of DNA to other proteins. $TAF_{II}250$ has been identified as a HAT, preferentially acetylating histones 3 and 4 (H3 and H4) (Mizzen *et al.*, 1996). $TAF_{II}250$ HAT activity was found to be required for transcription of the promoters of cyclin A and cyclin D1, and therefore for cell cycle progression (Dunphy *et al.*, 2000). The RNA polymerase II holoenzyme has also been shown to contain various other HATs (Cho *et al.*, 1998).

The yeast (Wilson *et al.*, 1996) and human (Cho *et al.*, 1998) RNA polymerase II holoenzymes contain various mating-type switch / sucrose non-fermenter (SWI/SNF) proteins. The SWI/SNF complex is involved in ATP-dependent nucleosome structure disruption, and is implicated in transcriptional activation. The yeast and human RNA polymerase II holoenzymes could both remodel chromatin, and this function in yeast was dependent on SWI/SNF proteins and ATP (Wilson *et al.*, 1996).

As well as their presence within the RNA polymerase II holoenzyme, other evidence links histone modification and chromatin modelling proteins to transcription. For example, TFIIIA, a co-factor in the activation of 5S rRNA gene promoters, could only bind a target promoter assembled into nucleosomes containing hyper-acetylated histones (Lee *et al.*, 1993). Removal of the N-terminal histone tails also permitted binding of TFIIIA to a nucleosome template, indicating that these domains restrict DNA binding within chromatin templates. As mentioned above, N-terminal histone tails are the targets for modification by HATs and other enzymes, and modification of these domains is proposed to regulate transcription by altering the accessibility of DNA to binding factors.

A chromatin immuno-precipitation (ChIP) assay using an antibody specific for acetylated H3 revealed that estrogen treatment caused histone hyper-acetylation in a region of chromatin containing a promoter activated by ER-α, and enabled TBP to bind the promoter (Sewack *et al.*, 2001). Rapid and specific histone phosphorylation in response to heat

shock has similarly been shown to occur at *Drosophila* chromosome regions containing multiple heat-shock response genes (Nowak and Corces, 2000). Conversely, specific regions of DNA known to be transcriptionally silent in certain yeast strains were associated with hypo-acetylation of H4 proteins *in vivo* (Braunstein *et al.*, 1993). Treatment with trichostatin A (TSA), an inhibitor of HDAC enzymes, activated the transcription of various viral promoters (Dressel *et al.*, 2000), suggesting a role for HDACs in transcriptional repression. These findings strengthen further the relationship between histone modification and transcriptional regulation.

Further evidence for this relationship came with the discovery that transcriptional co-activators, which enhance transcriptional activation by a wide range of transcription factors, have intrinsic HAT activity. These co-activators include Gcn5 (Brownell *et al.*, 1996), CREB binding protein (CBP) (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996), the highly similar p300 protein (Ogryzko *et al.*, 1996), p300/CBP associated factor (PCAF) (Yang *et al.*, 1996), and steroid receptor co-activator-1 (SRC-1) (Spencer *et al.*, 1997). Binding between p300/CBP, PCAF and SRC-1 (Spencer *et al.*, 1997; Yang *et al.*, 1996) suggested the presence of co-activator complexes. A chimaeric protein consisting of the Gal4 DBD and CBP HAT domain acted as a HAT-dependent transcriptional activator at certain promoters (Martinez-Balbas *et al.*, 1998). The ability of Gcn5, p300 and PCAF to acetylate histones has been shown to correlate with their transcriptional co-activator function (Chakravarti *et al.*, 1999; Kuo *et al.*, 1998; Lau *et al.*, 2000; Wang *et al.*, 1998). The relationship between histone modification and transcriptional regulation is therefore well established.

Nucleosome disruption and chromatin remodelling by the SWI/SNF complex are also associated with transcriptional activation. Mutations in yeast SNF2, SNF5 or SNF6 reduced the accessibility of core promoter DNA to binding proteins, and decreased transcription from certain promoters. However, this phenotype was partially rescued by a further mutation which reduced the production of H2A and H2B (Hirschhorn *et al.*, 1992), suggesting a link between the function of the SNF proteins, nucleosome structure and transcription. Addition of a large SWI/SNF complex with DNA-dependent ATPase activity increased the affinity of binding of Gal4 or TBP/TFIIA to sites contained within a nucleosome (Cote *et al.*, 1994; Imbalzano *et al.*, 1994; Kwon *et al.*, 1994). This suggests that, like histone modification, ATP-dependent chromatin remodelling by SWI/SNF proteins causes transcriptional activation by increasing the accessibility of promoter DNA.

As discussed in Chapter 1.3.1.1, transcription factors can activate transcription by direct recruitment of the pre-initiation complex to core promoters. There is now a great deal of evidence that transcription factors can also regulate transcription by indirect control of the binding of the pre-initiation complex to core promoters, via recruitment of factors involved in histone modification and chromatin remodelling.

One of the better characterised examples of this kind of transcriptional regulation involves the manipulation of histone acetylation status by members of the nuclear hormone receptor family. These proteins contain a conserved AF-2 domain, which mediates activation of target promoters in the presence of agonist ligand (Danielian *et al.*, 1992), and transcriptional repression when unliganded or bound to antagonist ligand. The search for effectors of hormone-dependent transcriptional regulation by nuclear hormone receptors identified various co-activator and co-repressor proteins. Co-activators such as p300, CBP, PCAF, SRC-1 and activator of thyroid and retinoic acid receptors (ACTR) bind members of the nuclear hormone receptor family in a ligand-dependent manner (Chakravarti *et al.*, 1996; Chen *et al.*, 1997; Hanstein *et al.*, 1996; Korzus *et al.*, 1998; Yao *et al.*, 1996) and have been shown to be involved in hormone-dependent transcriptional activation (Chakravarti *et al.*, 1996; Chen *et al.*, 1997; Hanstein *et al.*, 1996; Henttu *et al.*, 1997; Korzus *et al.*, 1998; Smith *et al.*, 1996).

Nuclear hormone receptor co-repressors include N-CoR (Horlein *et al.*, 1995) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) (Chen and Evans, 1995). These proteins formed complexes with nuclear hormone receptors bound to DNA, but were released upon addition of agonist ligand (Chen and Evans, 1995; Horlein *et al.*, 1995). N-CoR has also been shown to bind ER-α specifically in the presence of a tamoxifen-derived antagonist ligand (Lavinsky *et al.*, 1998). A Gal4-N-CoR protein acted as a transcriptional repressor (Horlein *et al.*, 1995), and N-CoR and SMRT were both associated with transcriptional repression by unliganded or antagonist-bound nuclear hormone receptors (Chen and Evans, 1995; Horlein *et al.*, 1995; Lavinsky *et al.*, 1998).

Structural studies have shown that ligands bind to a deep hydrophobic pocket within the nuclear hormone receptor C-terminal half (Bourguet *et al.*, 1995; Renaud *et al.*, 1995). Comparison between unliganded and agonist-bound receptors revealed a major conformational change upon ligand binding; helix 12 folded back towards the ligand-binding pocket, and the AF-2 domain assumed a generally more compact structure (Bourguet *et al.*, 1995; Renaud *et al.*, 1995). The integrity of helix 12 and associated protein domains was found to be essential for binding to co-activators and hormone-

dependent transcriptional activation (Feng *et al.*, 1998; Henttu *et al.*, 1997; Renaud *et al.*, 1995).

It has been proposed that the conformational change induced by agonist ligand provides a hydrophobic surface for co-activator binding (Feng *et al.*, 1998). Comparison between ER-α hormone-binding domains bound to agonist or antagonist ligand confirmed that both ligands bound within the same hydrophobic pocket (Shiau *et al.*, 1998). However, different contacts formed between each ligand and its binding domain caused distinct effects. In the presence of agonist ligand, helix 12 moved to form a hydrophobic cleft with residues from helices 3, 4, and 5. This cleft was bound by a peptide derived from a co-activator protein. However, binding of antagonist ligand did not induce movement of helix 12 to form this hydrophobic cleft. Structural differences between unliganded, agonist- and antagonist-bound nuclear hormone receptors therefore appear to account for the binding of different proteins in response to different stimuli.

ChIP assays have shown that ER-α rapidly recruited co-activators, including p300, CBP, PCAF and SRC-1, to target promoters in the presence of estradiol, whereas N-CoR and SMRT were recruited upon addition of tamoxifen (Shang *et al.*, 2000). This suggests a functional role for co-activators and repressors in the regulation of transcription by nuclear hormone receptors.

As described above, many nuclear receptor co-activators have been identified as HATs, providing a link between histone modification and hormone-dependent transcriptional regulation. The dependence on p300 for enhancement of estradiol-induced transcription by ER-α was limited to chromatinised templates (Kraus and Kadonaga, 1998); this enhancement was shown to be dependent on the HAT activity of p300 (Kraus *et al.*, 1999). PCAF HAT activity has also been shown to be essential for hormone-dependent transcriptional activation by nuclear hormone receptors (Korzus *et al.*, 1998). Direct evidence for a role for histone acetylation in transcriptional regulation by nuclear hormone receptors has been obtained using ChIP assays. These have shown that thyroid hormone receptor and ER-α mediated an increase in histone acetylation at target promoters in response to agonist ligand, correlating with the activation of transcription from these promoters (Sachs and Shi, 2000; Shang *et al.*, 2000).

Similarly, the binding of co-repressors to unliganded or antagonist-bound nuclear hormone receptors has been shown to recruit HDACs such as Rpd3 and HDAC1 (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). HDAC function has been shown to be

important for transcriptional repression by nuclear hormone receptor proteins (Heinzel *et al.*, 1997; Lavinsky *et al.*, 1998; Nagy *et al.*, 1997; Sachs and Shi, 2000). Nuclear hormone receptors therefore represent an example of how the recruitment of histone modifying proteins can lead to the highly specific, tightly-controlled positive and negative regulation of transcription.

Many other transcription factors have been shown to recruit HATs and/or HDACs and so regulate transcription. Knowledge in this area is increasing rapidly, and only selected examples are discussed below. For example, the activation of a promoter driven by Sp1 and NF-κB p65 was enhanced by wild-type p300, but to a lesser extent by a p300 mutant protein with no HAT activity (Kraus *et al.*, 1999), implicating histone acetylation in transcriptional activation by these factors. Transcriptional activation by E2F was also dependent on co-expression of CBP. However, a CBP mutant protein with no HAT activity, but competent for binding to E2F and TBP, could not co-activate transcription by E2F (Ait-Si-Ali *et al.*, 2000). This suggests that CBP HAT activity, rather than any bridging role between the transcription factor and pre-initiation complex, is important in some cases. The importance of HAT activity has also been shown in a system assaying transcription from a chromatinised promoter containing a Gal4 site. Transcriptional activation by Gal4-VP16 required p300 and acetyl co-A; activation was decreased in the presence of a p300 inhibitor which specifically inhibits p300 HAT activity (Kundu *et al.*, 2000).

Examples are also known of transcription factors recruiting HDAC proteins. These include Rb, which bound HDAC1 and 2 and recruited the proteins to form a complex with E2F (Brehm *et al.*, 1998). When recruited by Rb, HDAC1 was shown to repress transcription from E2F target promoters. Repression was relieved by treatment with TSA, supporting the role of histone deacetylation in transcriptional repression. Sp1 has also been shown to bind HDAC1 and repress transcription from a chromatinised promoter via histone deacetylation (Doetzlhofer *et al.*, 1999).

Transcription factors are also known to regulate transcription by recruitment of components of the SWI/SNF chromatin remodelling complex. For example, human SNF2α and β homologues have been implicated in hormone-dependent transcriptional activation by ER-α (Ichinose *et al.*, 1997). The AF-2 domain has also been shown to bind a SWI2 homologue and recruit it to target promoters, specifically in the presence of agonist ligand (DiRenzo *et al.*, 2000). The function of the SWI2 protein was essential for

hormone-dependent transcriptional activation, and also for the co-activation function of SRC-1 and CBP.

c-Myc also activates transcription by a mechanism involving chromatin remodelling. c-Myc bound directly to a SNF5 homologue, and a mutant SNF5 protein deficient for binding to other SWI/SNF complex components decreased transcriptional activation by c-Myc (Cheng *et al.*, 1999). Similarly, Gcn4 bound SNF5 and other components of the SWI/SNF complex, and mutations in these proteins reduced transcription from Gcn4 target promoters (Natarajan *et al.*, 1999). Gal4-VP16 also bound components of the SWI/SNF complex via the VP16 TAD, and recruited these proteins to target promoters (Neely *et al.*, 1999; Yudkovsky *et al.*, 1999). This led to ATP-dependent chromatin remodelling at Gal4 target promoters (Yudkovsky *et al.*, 1999) and an increase in transcriptional activity (Neely *et al.*, 1999).

As described above, the recruitment of histone modifying or chromatin remodelling activities is a regulatory mechanism utilised by many transcription factors. Examples are given above of factors such as ER-α which recruit HATs and components of the SWI/SNF complex in response to similar stimuli. Evidence exists that these two mechanisms can co-operate in the regulation of certain promoters. CBP is known to interact with a protein related to Snf2 (Johnston *et al.*, 1999); this interaction enhanced transcriptional activation by a Gal4-CBP protein (Johnston *et al.*, 1999) and by activated CREB (Monroy *et al.*, 2001). Other factors are also thought to activate transcription by co-operative recruitment of histone modifying and chromatin remodelling proteins. For example, while the separate addition of SWI/SNF proteins or p300 each enabled some degree of hormone-dependent transcriptional activation by the retinoic acid receptor, their simultaneous addition synergistically activated transcription to a much higher level (Dilworth *et al.*, 2000).

Co-operation between histone modification and chromatin remodelling has also been reported in the activation of transcription by Gal4-VP16 (Mizuguchi *et al.*, 2001). Pre-incubation of a chromatinised template with p300 or PCAF did not in itself induce chromatin remodelling, but increased the level of transcription observed upon the addition of Gal4-VP16 and a chromatin remodelling factor (Mizuguchi *et al.*, 2001). Similarly, in the case of interferon-β enhancer-driven transcription, pre-incubation of the template with CBP or Gcn5 did not induce chromatin remodelling, but increased the ability of the enhanceosome to recruit a SWI/SNF protein (Agalioti *et al.*, 2000). This led to a greater chromatin remodelling effect on pre-acetylated templates. Recruitment of TBP and $TAF_{II}250$ to the promoter was dependent on this remodelling activity, and was enhanced

by pre-acetylation of the chromatin. These examples show that recruitment of histone modifying and chromatin remodelling activities by transcription factors can lead to transcriptional activation, by an indirect increase in the affinity of binding of the pre-initiation complex to target promoters. These are obviously extremely important mechanisms of transcriptional regulation within a chromatin context.

1.3.2 Mechanisms of transcriptional regulation by Jun proteins.

As described in Chapter 1.3.1, many transcription factors have been shown to regulate transcription via control of the affinity of binding of the pre-initiation complex to core promoters. This control can occur directly, by protein-protein interaction with components of the pre-initiation complex, or indirectly, via recruitment of proteins which alter the chromatin environment of target promoters and their accessibility to the pre-initiation complex. As discussed below, the relevance of these mechanisms for transcriptional regulation by Jun proteins has begun to be investigated.

1.3.2.1 Recruitment of the pre-initiation complex.

Jun proteins have been shown to bind components of the pre-initiation complex *in vitro*. An initial study revealed that c-Jun bound TBP via the bZip domain (Ransone *et al.*, 1993). This was later confirmed, and additional binding of TBP to the c-Jun TAD was also detected (Franklin *et al.*, 1995). Phosphorylation of c-Jun at serines 63 and 73 did not affect the interaction between c-Jun and TBP. Interestingly, TBP also bound the N-terminal TADs of v-Jun, JunD and JunB, but with different affinities. TBP bound the v-Jun TAD with a higher affinity than to c-Jun; deletion of the delta domain from c-Jun increased the affinity of TBP binding. However, TBP and TFIIB bound to the C-terminal half of c-Jun with a higher affinity than to the equivalent region of v-Jun (Franklin *et al.*, 1995). The relative affinities of binding of TBP and TFIIB to full-length c-Jun and v-Jun have not been determined. This is potentially an extremely significant result, as different affinities of binding to the pre-initiation complex could provide an explanation for the altered transcriptional regulation of certain promoters by v-Jun compared to c-Jun. The implication that the delta deletion and C-terminal point mutations within v-Jun alter the affinity of TBP binding is especially important, as these mutations are known to contribute to transcriptional mis-regulation and cell tranformation by the viral protein (see Chapter 1.1.3 and 1.2.1).

Interactions between c-Jun and other components of the pre-initiation complex have also been reported. The bZip domain bound to components of TFIIE and TFIIF, with dimerisation to c-Fos increasing the affinity of binding (Martin *et al.*, 1996). Transcriptional activation of a TRE-containing promoter was "squelched" at high levels of expression of the c-Jun / c-Fos dimer, suggesting that the over-expressed proteins sequestered factors required for transcription from the core promoter. Co-expression of TFIIE specifically relieved this squelching effect, suggesting that TFIIE was a limiting factor for transcriptional activation by c-Jun and c-Fos.

The functional significance of c-Jun binding to a component of the pre-initiation complex has been explored in one case. In an *in vitro* assay, TBP activated transcription to a higher level than TFIID (Lively *et al.*, 2001). This was found to be due to the inhibition of TBP-TATA box binding by the N-terminal domain of $TAF_{II}250$. c-Jun bound to this N-terminal region, and was found to enhance transcription by TFIID, but not TBP. Footprinting analysis revealed that the binding of c-Jun to $TAF_{II}250$ derepressed TATA box binding by TBP. Binding between c-Jun and components of the pre-initiation complex therefore appears to be highly significant, at least *in vitro*.

The binding of v-Jun to TFIIE, TFIIF or $TAF_{II}250$, and any contribution of these factors to transcriptional activation by v-Jun, have not been investigated. It may be that, as with TBP (Franklin *et al.*, 1995), v-Jun binds these proteins with altered affinity compared to c-Jun. The differential regulation of certain promoters by v-Jun and c-Jun may, in that case, be due to the differing abilities of the proteins to recruit the pre-initiation complex to target promoters. Determination of the relative *in vivo* binding affinities of c-Jun and v-Jun to TBP and other components of the pre-initiation complex, and the abilities of the transcription factors to recruit the complex to target promoters, could potentially determine whether this is the case.

1.3.2.2 Histone modification.

Like many other transcription factors, members of the Jun and Fos families bind HATs. This was first suggested by the observation that micro-injection of inhibitory CBP antibodies abolished TPA-induced transcription from a TRE-containing promoter (Arias *et al.*, 1994). c-Jun phosphorylated by JNK, but not phosphorylated at C-terminal sites by casein kinase II, bound directly to CBP *in vitro* (Arias *et al.*, 1994). The binding of CBP to un-phosphorylated c-Jun was not determined. It has since been revealed that CBP binds to the N-terminal TAD of c-Jun and v-Jun (Bannister *et al.*, 1995). Serine to alanine

substitutions at c-Jun positions 63 and 73 decreased, but did not abolish, binding to CBP. Introduction of alanine residues at the equivalent positions within the v-Jun TAD had a similar effect on CBP binding. As v-Jun is not phosphorylated at these sites by JNK, this suggested that serines 63 and 73 were involved in binding to CBP, but that N-terminal phosphorylation of c-Jun did not affect the affinity of co-activator binding. However, this has not been specifically tested by comparison of the binding of CBP to c-Jun phosphorylated by JNK or treated with phosphatases.

p300, SRC-1 and the nuclear hormone receptor co-activator ASC-2 have also been shown to bind c-Jun (Lee *et al.*, 1996; Lee *et al.*, 1998; Lee *et al.*, 2000b). However, the dependence of this binding on c-Jun phosphorylation, and the affinity of co-activator binding to v-Jun, have not been determined. In contrast, a putative transcriptional adaptor protein known as Jun-activation-domain binding protein (JAB1) has been shown to bind c-Jun, stabilise its binding to a TRE, and enhance transcription from TRE-containing promoters (Claret *et al.*, 1996). However, JAB1 did not bind or enhance transcriptional activation by v-Jun. JAB1 has been shown to bind SRC-1 (Chauchereau *et al.*, 2000) and may therefore recruit HAT activity to c-Jun, but not v-Jun. Differential recruitment of HAT proteins, whether direct or via selective binding to adaptors such as JAB1, may be found to be important for the differences in transcriptional regulation between c-Jun and v-Jun.

Co-expression of CBP, p300, SRC-1 or ASC-2 has been shown to enhance transcriptional activation by c-Jun (Bannister *et al.*, 1995; Lee *et al.*, 1996; Lee *et al.*, 1998; Lee *et al.*, 2000b). p300 and SRC-1 co-operated to increase transcriptional activation by c-Jun (Lee *et al.*, 1998), suggesting that, like nuclear receptor co-activators, Jun proteins may recruit co-activator complexes. Transcriptional activation by a Gal4-v-Jun protein was enhanced by co-expression of CBP (Bannister *et al.*, 1995), but the role of other co-activators in transcriptional activation by v-Jun has not been determined.

Interestingly, in an investigation into the interaction between ATF-2 and p300, it was discovered that ATF-2 possessed intrinsic HAT activity (Kawasaki *et al.*, 2000). ATF-2 specifically acetylated the N-terminal tails of H2B and H4. ATF-2 HAT activity was induced by JNK phosphorylation in response to UV light, and correlated with transcriptional activation of a promoter containing a CRE. As described in Chapter 1.1.7.1, ATF-2 forms heterodimers with Jun proteins and is implicated in the induction of growth factor independence by v-Jun. The contribution of ATF-2 HAT activity to this process is

not known, but may be important in transcriptional regulation and cell transormation by v-Jun.

As described in Chapter 1.3.1.2, negative, as well as positive, regulation of transcription can occur by recruitment of histone modifying proteins. In line with this, c-Jun and c-Fos have been shown to bind SMRT (Lee *et al.*, 2000a). In the case of c-Fos, co-expression with SMRT or HDAC1 decreased transcriptional activation from a TRE-containing promoter, and this repression was relieved by TSA. This suggests that c-Fos may repress transcription by recruitment of HDAC activity to target promoters. It would be interesting to determine whether c-Jun and v-Jun can repress transcription by a similar mechanism.

In summary, while c-Jun and v-Jun have been shown to bind proteins with HAT activity, the role of histone modification in the transcriptional regulation of Jun target promoters has not been established. ChIP and *in vitro* transcription assays have been used to demonstrate the involvement of HAT activity in transcriptional regulation by nuclear hormone receptors and Gal4-VP16 (Kundu *et al.*, 2000; Sachs and Shi, 2000; Shang *et al.*, 2000). Similar analyses could help to determine whether c-Jun and v-Jun activate transcription by recruitment of HAT activity.

The contribution of HAT co-activators and HDAC co-repressors, as well as proteins such as ATF-2, to the differential regulation of transcription by c-Jun and v-Jun also remains elusive. Determination of the relative *in vivo* binding affinities of these proteins to c-Jun and v-Jun may reveal differences in their ability to recruit histone modifying activity. Comparison of the acetylation status of the chromatin surrounding target promoters in control and v-Jun transformed cells may disclose whether histone modification has functional significance for the mis-regulation of target genes by v-Jun.

An interesting possibility is raised by the observation that hormone-dependent activation of certain nuclear hormone receptors repressed TPA-induced, but not basal, transcription from TRE-containing promoters. This repression is mutual, and could be relieved by expression of co-activators such as p300, CBP, SRC-1 and ASC-2 (Aarnisalo *et al.*, 1998; Kamei *et al.*, 1996; Lee *et al.*, 1998; Lee *et al.*, 2000b). One interpretation is that trans-repression occurs via competition for a limiting amount of common HAT co-activators. Alternative models, such as the specific repression of JNK activity by ligand-bound glucocorticoid receptor (Caelles *et al.*, 1997), have been proposed. However, the observation that over-expression of HAT co-activator proteins relieved repression implies that TPA-induced transcriptional activation from TREs occurs via the enhanced

recruitment of co-activators. Indeed, CBP function has been implicated in TPA-induction of transcription from a TRE-containing promoter (Arias *et al.*, 1994).

This suggests a model whereby phosphorylation of c-Jun by JNK in response to TPA and other stimuli leads to increased transcriptional activation by recruitment of HAT co-activators. This is an attractive model, as it would explain the inability of v-Jun, which is not phosphorylated by JNK, to activate transcription of TPA and UV-responsive promoters such as collagenase, stromelysin and c-*jun* (Hussain *et al.*, 1998; May *et al.*, 1998; Tsang *et al.*, 1994). While *in vitro* studies have implied that the phosphorylation of c-Jun serines 63 and 73 may not be important for binding to CBP (Bannister *et al.*, 1995), this has not been studied *in vivo*. Neither has the regulation of binding of other co-activators by c-Jun phosphorylation been investigated. Resolution of these issues may develop our understanding of the differential transcriptional responses of c-Jun and v-Jun to external stimuli.

1.3.2.3 Chromatin remodelling.

As described in Chapter 1.3.1.2, the recruitment of chromatin remodelling activity to target promoters is a mechanism employed by various transcription factors. In the case of c-Jun / c-Fos dimers, binding to a TRE can be sufficient to induce nucleosome disruption (Ng *et al.*, 1997). A promoter fragment with a TRE at its centre was assembled into a nucleosome, using acetylated histones. The c-Jun / c-Fos dimer bound to a TRE within naked DNA with a higher affinity than to the site within the nucleosome. However, binding to the nucleosome site occurred when the c-Jun / c-Fos dimer was present at higher concentrations. This completely disrupted the structure of the nucleosome and led to the dissociation of histones from the promoter DNA. The c-Jun and c-Fos TADs were not required for disruption, suggesting that TRE binding by the proteins was sufficient to disrupt DNA-histone contacts. Disruption of the nucleosome structure by c-Jun / c-Fos enabled binding of a second factor to a site previously masked by the nucleosome, illustrating that TRE binding by these proteins increased the accessibility of the promoter DNA. This model has not been tested *in vivo*, but has potential implications for transcriptional activation by indirect recruitment of the pre-initiation complex.

c-Jun and c-Fos have also been shown to bind BAF60a, a component of the SWI/SNF complex (Ito *et al.*, 2000). A c-Jun / c-Fos dimer bound BAF60a as well as other SWI/SNF proteins, including the DNA-dependent ATPase subunit, *in vivo*. While the interaction between BAF60a and v-Jun was not investigated, BAF60a did not bind JunD, or Fos

family proteins other than c-Fos. The ability of heterodimers consisting of different Jun and Fos family members to bind BAF60a correlated with their activation of a TRE-containing promoter in F9 cells. Re-constitution of a functional SWI/SNF complex in cells lacking this chromatin remodelling activity enhanced the ability of a c-Jun / c-Fos dimer to activate a TRE-containing promoter.

Taken together, this suggests a role for the SWI/SNF complex in transcriptional activation by c-Jun / c-Fos dimers. However, as with the recruitment of the pre-initiation complex and histone modifying proteins, the functional significance of chromatin remodelling activity in transcriptional activation by c-Jun has not been definitively verified, and its role in transcriptional activation by v-Jun has not been investigated.

1.3.2.4 Summary

As described above, there is evidence for recruitment of the pre-initiation complex, histone modifying proteins, and a chromatin remodelling complex in transcriptional regulation by c-Jun. However, the functional relevance of these interactions for the *in vivo* regulation of c-Jun target genes has not been established. As our understanding of these mechanisms of transcriptional regulation develops, this situation may be resolved.

Our understanding of the mechanisms of transcriptional regulation by v-Jun, and how these differ from c-Jun, is more limited still. Comparison of the ability of c-Jun and v-Jun to disrupt nucleosome structure, and to bind and recruit components of the pre-initiation complex, SWI/SNF complex and histone modifying proteins, may reveal important differences between the proteins. More detailed analyses could then follow, for example *in vitro* transcription assays and ChIP analysis to determine whether alterations in histone modification, ATP-dependent chromatin remodelling, pre-initiation complex recruitment, or a combination of mechanisms, are likely to be involved in the mis-regulation of specific target genes by v-Jun. Coupled with the identification of v-Jun effector genes, this would improve our understanding of the mechanisms of transcriptional regulation and cell transformation by v-Jun.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Amersham Pharmacia Biotech

D-*threo*-[*dichloroacetyl*-1-^{14}C]-chloramphenicol

Redivue adenosine 5'-[γ-^{32}P]-triphosphate, triethylammonium salt

Redivue deoxycytidine 5'-[α-^{32}P]-triphosphate, triethylammonium salt

BDH Laboratory Supplies

Ethyl acetate

Fisher Scientific

Acetic acid, glacial

Ammonium persulphate (APS)

Boric acid

Chloroform

Ethylene diaminetetra-acetate (EDTA), disodium salt

Glycine

Hydrochloric acid

Magnesium chloride

Methanol

Potassium chloride

Propan-2-ol

Sodium acetate

Sodium chloride

Sodium dodecyl sulphate (SDS)

Sodium hydrogen orthophosphate

Fisons

Glycerol

Gibco BRL

N-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid (Hepes) buffer

Tris

Ultra-pure agarose, electrophoresis grade

James Burrough (F.A.D.) Ltd

Ethanol

Kramel Biotech

Bovine serum albumin (BSA)

New England BioLabs

SDS sample buffer, 3x

Pierce

Coomassie protein assay reagent

Premier Beverages

Marvel skimmed milk powder

Promega

Deoxyadenosine triphosphate (dATP)

Reporter lysis buffer, 5x

Roche

Biotin-16-dUTP

Severn Biotech Ltd.

30%(w/v) acrylamide, 1.6%(w/v) bisacrylamide

30%(w/v) acrylamide, 0.8%(w/v) bisacrylamide

Sigma

Acetyl coenzyme A, sodium salt

Adenosine 5' triphosphate (ATP), disodium salt

Aprotinin

Benzamidine HCl

Bromophenol blue (BPB)

Dithiothreitol (DTT)

Ethidium bromide

Ethylene glycol-bis(β–aminoethyl ether)N,N,N',N'-tetra-acetic acid (EGTA)

Leupeptin

Okadaic acid

Phenyl methyl sulphonyl fluoride (PMSF)

Polydeoxyinosinic-deoxycytidylic acid (Poly dI.dC)

Ponceau S solution

Sodium fluoride

Sodium orthovanadate

Spermidine

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

Triton X-100 (t-Octylphenoxypoly-ethoxy ethanol)

Tween-20 (Polyoxyethylene sorbitan nonolaurate)

2.1.2 Enzymes and kits

Amersham Pharmacia Biotech

Enhanced chemiluminescence (ECL) Western detection agent

Oligolabelling kit

Applied Biosystems Inc.

GeneAmp PCR core reagents

PRISM BigDye terminator cycle sequencing ready reaction kit

Gibco BRL

All restriction enzymes with the exception of BstYI

Kramel Biotech

Klenow fragment of *E.coli* DNA polymerase I

New England BioLabs

BstYI

Qiagen

QIAGEN plasmid maxi kit

QIAprep spin miniprep kit

QIAquick gel extraction kit

QIAquick PCR purification kit

Roche

Rapid DNA ligation kit

Stratagene

QuikChange XL site-directed mutagenesis kit

Transgenomic

Alkaline phosphatase

T4 DNA ligase

T4 polynucleotide kinase (PNK)

2.1.3 Oligonucleotides

2.1.3.1 Probes

NB Where the antisense strand is an exact complement, only the sense strand is shown. TREs are shown underlined.

bkj(D)	5' GGA TGG GTG ACT CAG AGT GAG
bkj(P)	5' CCA GGC CTG ACT CAG CAG CCT
*bkj*mt(P)	5' CCA GGC CAG ACC CAG CAG GCT
bkj-777	5' CAC GTG GTG ACT CAG CCT CTC
bkj-610	5' GCA GAG ATG AGT CAT GAA GCG
bkj-477	5' ACA CAT CTG AGT CAC CTG CAG
bkj-323	5' GGA GCC ATG ACT CAT GGG ATA

bkj-169	5' CGC AGC ATG ACT CAG GAG CCA
bkj-109	5' CAG AGC TTG AGT CAC CAA AAT
bkj-65	5' TTT TTG CTG ACT CAG CTC CCT
Col TRE	5' AAG CAT GAG TCA GAC ACC TC
ColEco sense	5' AAT TAA GCA TGA GTC AGA CAC CTC
ColEco antisense	3' TT CGT ACT CAG TCT GTG GAG TTA A

2.1.3.2 Primers

Mutagenic bases shown in bold type; restriction enzyme sites underlined.

HindIII *bkj*5'	5' TCC AAG CTT GCG TCT CAG GGT GCA TGT CTG GAA AAA C
XbaI *bkj*3'	5' TGT TCT AGA ACG GCA GCA GGG AAT GGA GTG GCT GG
mt(P)TRE *bkj*5'	5' TCC AGG CC**A** GAC **C**CA GCA GC
-777TRE *bkj*5'	5' GGT GA**C** **T**CA GCC TCT CCC C
-610TRE *bkj*5'	5' GAG TC**A** TGA AGC GGC CTG AC
-477TRE *bkj*5'	5' CTG AGT C**A**C CTG CAG AGG C
-323TRE *bkj*5'	5' GAC **TCA** TGG GAT AGG GAG G
-169TRE *bkj*5'	5' ACG CAG CAT GAC **TCA** GGA GC
-109TRE *bkj*5'	5' TGA G**TC** **A**CC AAA ATC ACC C
-65TRE *bkj*5'	5' GTT TTT GC**T** **GAC** TCA GCT CC

HindIII-65TRE *bkj*5' 5'TCC AAG CTT GCG CTG ACT CAG CTC CCT CGG

bkj Col TATA 5'	5' CCA TCA GGC GCA GTA TA**T** ATA GTC GCC TCA TCT CC
bkj Col TATA 3'	3' GGT AGT CCG CGT C**A**T ATA TAT CAG CGG AGT GAG GG
bkj Col ILS 5'	5' CCT CAT CTC CCA GCC ACT **A**TA TT**G** **G**CT GCT GCA GTG C

bkj Col ILS 3'	3' GGA GTA GAG GGT CGG TGA **TAT** AA**C** **C**GA CGA CGT CAC G
Col *bkj* TATA 5'	5' GCA AGG ACT C**C**A TA**A** ATA CAG AGG GAG C
Col *bkj* TATA 3'	3' CGT TCC TGA G**GT** AT**T** TAT GTC TCC CTC G
Col *bkj* ILS 5'	5' CCT AGC TGG G**CC** ATT **C**CA GCA GCA AGA GG
Col *bkj* ILS 3'	3' GGA TCG ACC C**GG** TAA **GG**T CGT CGT TCT CC'
AF2mt5'	5' GAC CTG CTG CTG GAG **GC**G **GC**G GAC GCC CAC CGC CTA C
AF2mt3'	3' CTG GAC GAC GAC CTC **CG**C **CG**C CTG CGG GTG GCG GAT G
SalI p3005'	5' ATT GCA TCC GAG TCG ACT TTG GAG GCA CTT TAC CGT CAG G
HindIII p3003'	5' GAT CCA TAG CAA GCT TCT AGC AGC CTG CTG GTT GTT GC

2.1.4 Plasmids

ASV17 provirus with flanking chicken genomic sequences from clone 241, cloned into EMBL3, and pV, the corresponding proviral DNA without flanking sequences, cloned into M13mp19, have been described (Maki *et al.*, 1987) and were a gift from Peter Vogt.

Cla12Nco has been described (Hughes *et al.*, 1987) and was a gift from Stephen Hughes.

-73/+63 ColCAT has been described (Angel *et al.*, 1987a). –60/+63 ColCAT is a deletion mutant lacking the TRE at position –72. Both plasmids were a gift from Peter Angel.

pBlueScript KS p300 WT and pBlueScript KS p300 MutAT2 contain the p300 WT and p300 MutAT2 constructs respectively (Kraus *et al.*, 1999) and were a gift from W.Lee Kraus.

pCAT-Basic is commercially available from Promega.

pCAT-*BKJ*(WT), pCAT-*BKJ*(DP), pCAT-*BKJ*(P), pCAT-*BKJ*(D) and pCAT-*BKJ*(0) have been described (Hartl and Bister, 1998) and were a gift from Markus Hartl.

pckGAPDH contains chicken GAPDH DNA (Hussain *et al.*, 1998).

pJC6 has been described (Han *et al.*, 1992) and was a gift from Ron Prywes.

pRc/RSV is commercially available from Invitrogen.

pRc/RSV-cJ3 and pRc/RSV-vJ1 have been described (Morgan *et al.*, 1993) and were a gift from Iain Morgan.

pSPT19-J-D300 contains the chicken c-*jun* 3' UTR sequence (Hussain *et al.*, 1998).

RCAS has been described (Hughes *et al.*, 1987) and was a gift from Stephen Hughes.

RCAS AV2 has been described (Schuur *et al.*, 1993) and was a gift from Peter Vogt; see Figure 2.1.

RCAS ΔvJ-hER and RCAS hER have been described (Kruse *et al.*, 1997) and were a gift from Ulrich Kruse; see Figure 2.1.

2.1.5 Bacteriology

Beatson Institute Central Services

L-Broth

Sterile glycerol

Difco Laboratories

Bacto-agar

Gibco BRL

E.coli DH5α competent cells

Sigma

Ampicillin

Stratagene

XL10-Gold β-mercaptoethanol mix

XL10-Gold ultracompetent cells

2.1.6 Cell culture

Chicken embryo fibroblasts (CEFs) are fibroblasts derived from 10.5-11 day-old White Leghorn chicken embryos.

Beatson Institute Central Services

Sterile phosphate-buffered serum (PBS)

Sterile PBS + EDTA (PE)

BioWhittaker Molecular Applications

SeaPlaque agarose

Fisher Scientific

Dimethyl sulphoxide (DMSO)

Gibco BRL

Chicken serum

Dulbecco's modified Eagle medium (DMEM)

L-glutamine

New-born calf serum

Trypsin solution

Tryptose phosphate broth

Harlan Sera-Lab Ltd.

Foetal calf serum

Roche

N-[1-(2,3-dioleoyloxyl)propyl]-*N,N,N*-trimethylammoniummethyl sulphate (DOTAP) liposomal transfection reagent

Sigma

10 x DMEM

17β-estradiol (estradiol)

4-Hydroxy tamoxifen (tamoxifen)

Penicillin G

Sodium bicarbonate

Sodium pyruvate

Streptomycin sulphate

2.1.7 Antibodies

599-3 is an affinity-purified rabbit polyclonal antibody, raised against a peptide spanning amino acids 60-77 of chicken c-Jun.

730-5 is a rabbit polyclonal antiserum, raised against a full-length chicken c-Jun protein expressed in *E.coli.*

Cell Signalling Technology

Anti-rabbit IgG (H&L), Horseradish Peroxidase (HRP)-linked.

Anti-mouse IgG (H&L), Horseradish Peroxidase (HRP)-linked.

Santa Cruz Biotechnology, Inc.

c-Fos(4)X: rabbit polyclonal, c-Fos

FosB(102)X: rabbit polyclonal, FosB

HC20: rabbit polyclonal, estrogen receptor α

K-25: rabbit polyclonal, pan Fos

KM-1X: mouse monoclonal, c-Jun

N-17X : rabbit polyclonal, Fra-1

Q-20X : rabbit polyclonal, Fra-2

VP16(1-21): mouse monoclonal, VP16

2.1.8 Miscellaneous

Alltech

Silica gel 60 thin liquid chromatography (TLC) plates

Amersham Pharmacia Biotech

Hybond-N^+ nylon membrane

BioRad

Bio-Spin 30 chromatography columns

Fuji Film

Phosphorimager imaging plate

Super RX medical X-ray film

Gibco BRL

100bp DNA ladder

1kb DNA ladder

Osmonics

Supported nitrocellulose membrane

Pall Gelman

Nanosep MF 0.2μm filtration tubes

Promega

Streptavidin MagneSphere paramagnetic particles

Sigma

Prestained protein molecular weight markers

Whatman International Ltd.

Whatman 3MM filter paper

2.2 Methods

2.2.1 Bacteriology

2.2.1.1 Transformation of DNA into bacterial hosts

DH5α cells were thawed on ice, and a 20μl aliquot added to 2μl DNA solution in a pre-chilled polypropylene tube. A range of DNA concentrations was generally used. After incubation on ice for 45min, cells were subjected to heat-shock at 42°C for 40s. 80μl L-broth was added and the tubes were incubated for 1hr at 37°C with shaking. The mixture was spread onto plates containing 1.5%(w/v) agar and 100μg/ml ampicillin in L-broth. The plates were incubated overnight at 37°C.

For transformation of DNA into XL10-Gold ultracompetent cells, the following modifications were made to the above procedure: a 45μl aliquot of cells was pre-incubated

with 2μl XL10-Gold β-mercaptoethanol mix for 10min on ice; incubation on ice with DNA took place for 30min; heat-shock was for 30s; and 500μl L-broth, pre-warmed to 42°C, was added after heat-shock.

2.2.1.2 Bacterial culture

Single bacterial colonies were picked from agar plates, and inoculated into an appropriate volume of L-broth containing 100μg/ml ampicillin. The culture was grown overnight at 37°C with shaking.

2.2.1.3 Preparation of glycerol stocks

0.5ml of overnight bacterial culture was mixed with an equal volume of sterile glycerol and stored at –70°C.

2.2.1.4 Small-scale plasmid DNA preparation

1ml overnight bacterial culture was pelleted by centrifugation for 5min at 5000rpm. The pellet was resuspended and processed using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

2.2.1.5 Large-scale plasmid DNA preparation

500ml overnight bacterial culture was pelleted by centrifugation for 15min at 6000rpm, 4°C. The pellet was resuspended and processed using the QIAGEN Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions.

2.2.2 Cell culture

2.2.2.1 Maintenance and storage of cell lines

CEFs were grown as a monolayer at 41°C, 5% CO_2, in DMEM supplemented with 10%(v/v) new-born calf serum, 1%(v/v) heat-inactivated chicken serum, 10%(v/v) tryptose phosphate broth, 2mM glutamine, 5U/ml penicillin and 50μg/ml streptomycin. 2μM estradiol, 200nM tamoxifen or an equivalent volume of ethanol was added as required.

Cells were routinely passaged every 3-4 days. Cells were washed once in PE, exposed briefly to 0.25%(v/v) trypsin solution in PE, and harvested by agitation in normal growth

medium. For long-term storage, cells were pelleted by centrifugation for 5min at 1000rpm. The pellet was resuspended in an appropriate volume of 10%(v/v) DMSO in foetal calf serum, aliquoted, and stored in liquid nitrogen.

2.2.2.2 Transfection of cells with plasmid DNA

Plasmid DNA was transfected using DOTAP liposomal transfection reagent. For each transfection, 2.5-10μg DNA in 170μl 200mM Hepes buffer was added to 30μl DOTAP. After 5min, the mixture was added to sub-confluent cells. The cell medium was changed after 24hr.

2.2.2.3 Cell photography

Photographs were taken using a Fuji Fine pix digital camera attached to an Axiovert25 light microscope (Zeiss).

2.2.2.4 Cell growth assays

Cells were set up at either $2x10^5$ cells per 60mm plate or $4x10^5$ cells per 90mm plate. Cell numbers were determined in duplicate every 24hr by trypsinisation of the cells and scraping into 2ml growth medium. Cells were counted automatically using a Casy1 cell counter (Schärfe System).

2.2.2.5 Soft agarose growth assays

2 x complete growth medium was prepared using 10xDMEM in sterile water with the addition of 2mM sodium pyruvate, 0.375%(w/v) sodium bicarbonate, and all other growth medium components (see Chapter 2.2.2.1) at double the usual concentration. SeaPlaque agarose in sterile water was prepared at 2%(w/v) and 0.7%(w/v) and sterilised by autoclaving.

Assays were prepared in 30mm plates. The base layer, consisting of 1.5ml 1% agarose in 1 x growth medium, containing 2μM estradiol or an equivalent volume of ethanol as appropriate, was allowed to set at room temperature (RT). Growing cells were trypsinised and resuspended in 1 x growth medium as before, and counted using a haemocytometer. 10^2, 10^3 or 10^4 cells were suspended in 2ml 0.35% agarose in 1 x growth medium, containing 2μM estradiol or an equivalent volume of ethanol as appropriate, and overlaid

onto the base layer. The agarose was allowed to set and the cells incubated at 41°C, 5% CO_2. Cells were fed every 3-4 days by overlaying with 1.5ml 1% agarose in 1 x growth medium, containing 2μM estradiol or an equivalent volume of ethanol as appropriate. Colonies were counted under a light microscope after 2 weeks.

2.2.2.6 Preparation of whole cell extracts (WCEs)

Cells were grown to approximately 90% confluence on 90mm or 140mm plates. On ice, growth medium was removed and the cells washed twice with ice-cold PBS. After draining well, the cells were scraped into 1ml PBS and pelleted by centrifugation at 4000 rpm. The pellet was resuspended in a suitable volume of WCE buffer (0.4M KCl, 20mM Hepes, 10%(v/v) glycerol with 10mM EGTA, 5mM EDTA, 5mM NaF, 1mM sodium orthovanadate, 1mM DTT, 50μg/ml PMSF, 5μg/ml aprotinin, 5μg/ml leupeptin, 50ng/ml okadaic acid, 0.4%(v/v) Triton X-100). After 20min on ice, cells were subjected to 2 rounds of freeze-thaw (dry ice-37°C waterbath) and pelleted by centrifugation at 14000rpm, 4°C. The supernatant was removed and stored at –70°C.

2.2.2.7 Chloramphenicol acetyltransferase (CAT) assays

Cells were plated at $2.5x10^5$ cells per 60mm plate in 4ml growth medium, containing 2μM estradiol, 200nM tamoxifen or an equivalent volume of ethanol as appropriate. After 24hr, cells were transiently transfected with reporter plasmid, and co-expression plasmids as required (see Chapter 2.2.2.2 for details). Cells were harvested 24-48hr after transfection. Cells were washed twice with PBS, and 300μl 1 x reporter lysis buffer was added. After 10min, cells were scraped and pelleted by centrifugation at 13000rpm. 10μl supernatant was retained for estimation of the protein concentration (see Chapter 2.2.4.1). 180μl supernatant was incubated at 37°C with 0.8mg/ml acetyl coenzyme A and 2μl D-*threo*-[*dichloroacetyl*-1-^{14}C]-chloramphenicol.

After 2-3hr, the reaction was stopped on ice and 300μl ethyl acetate was added. After mixing and separation by brief centrifugation at 13000rpm, 200μl of the organic phase was removed to a clean tube and dried down by centrifugation under vacuum. The pellet was resuspended in 10μl ethyl acetate and spotted onto silica gel 60 TLC plates. TLC was performed in an air-tight glass tank in 95%(v/v) chloroform, 5%(v/v) methanol for 1hr. Plates were then dried and exposed to a phosphporimager imaging plate. The plate was read using a Personal Molecular Imager FX (BioRad), and radio-labelled products were

quantified using Molecular Analyst software (BioRad). Relative CAT activity was calculated by division of the percentage acetylation of D-*threo*-[*dichloroacetyl*-1-^{14}C]-chloramphenicol by the sample protein concentration.

2.2.3 Nucleic acid protocols

2.2.3.1 Oligonucleotide synthesis

Oligonucleotides were synthesised by Beatson Institute Technical Services staff, using Cruachem reagents in a 392 DNA/RNA oligonucleotide synthesiser according to the manufacturer's instructions. DNA pellets were dissolved in sterile TE (10mM Tris, 1mM EDTA).

2.2.3.2 Quantitation of DNA concentration

DNA concentrations were calculated by determining the UV light absorbency of a DNA solution at 260 and 280nm, using a Beckman DU650 Spectrophotometer. Samples were read in duplicate against a suitable blank. The DNA concentration was calculated using de Beer's law, which states that an optical density of 1 at 260nm corresponds to 50μg/ml double-stranded DNA or 33μg/ml single-stranded DNA.

2.2.3.3 Oligonucleotide annealing

Equal amounts of two complementary oligonucleotides in annealing buffer (67mM Tris pH8, 13mM $MgCl_2$, 6.7mM DTT, 1.3mM spermidine, 1mM EDTA) were heated to 95°C and cooled to RT.

2.2.3.4 Polyacrylamide gel electrophoresis (PAGE)

DNA samples were resolved on polyacrylamide gels, prepared by adding an appropriate volume of 30%(w/v) acrylamide, 1.6%(w/v) bisacrylamide to 1xTBE buffer (90mM Tris, 90mM boric acid, 2mM EDTA). The gel was polymerised by the addition of APS and TEMED. Samples, containing 10%(v/v) loading dye (10%(v/v) glycerol with BPB in 1xTBE), were subjected to electrophoresis at 150V in 1xTBE buffer. The gel was washed for 15min in ethidium bromide in 1xTBE, and DNA was visualised and photographed under short-wave UV illumination.

2.2.3.5 Agarose gel electrophoresis

0.7-1%(w/v) agarose gels were used, depending on the size of the DNA fragment of interest. The appropriate amount of agarose was heated in 1xTAE buffer (40mM Tris, 16mM acetic acid, 1mM EDTA) and 1μg/ml ethidium bromide was added before setting. Samples, containing 10% loading dye (see Chapter 2.2.3.4), were loaded along with appropriate size markers and subjected to electrophoresis at 80-100V in 1xTAE buffer. DNA fragments were visualised and photographed under short-wave UV illumination.

2.2.3.6 Extraction of DNA fragments from agarose gels

DNA fragments were excised from agarose gels and recovered using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.2.3.7 Restriction enzyme digests

DNA was generally digested at 37°C for 1hr with an excess of enzyme, in the buffer supplied by the manufacturer. Double digests were carried out simultaneously in the buffer most suitable for both restriction enzymes. PCR products with restriction sites close to their ends were digested overnight at 37°C. Digests with the BstYI enzyme were carried out at 60°C for 1hr with the addition of 10%(w/v) BSA.

2.2.3.8 De-phosphorylation reactions

Linearised plasmid DNA was de-phosphorylated by treatment with an excess of alkaline phosphatase at 37°C for 1hr, in the buffer recommended by the manufacturer.

2.2.3.9 DNA ligation reactions

DNA fragments were ligated either by overnight incubation at 11°C with an excess of T4 DNA ligase in the buffer supplied by the manufacturer, or at RT for 30min using the Rapid DNA Ligation Kit (Roche) according to the manufacturer's instructions. A range of insert:vector ratios was generally used.

2.2.3.10 Polymerase chain reaction (PCR)

PCR was carried out using GeneAmp PCR Core Reagents (Applied Biosystems Inc). 0.75ng plasmid DNA template was amplified by AmpliTaq DNA polymerase using

primers at a concentration of 0.2μM (p300 templates) or 0.6μM (*bkj* templates) in 4mM $MgCl_2$. Reactions were carried out using thin-walled PCR tubes in a PTC-100 PCR machine (MJ Research, Inc). Reaction conditions were as follows: initial step 95°C, 2min; followed by 25 cycles of 95°C, 1min 30; 50°C, 2min; 72°C, 2min; final step 72°C, 7min.

2.2.3.11 Purification of PCR products

10% of each PCR reaction was resolved on an agarose gel and visualised. The remainder of each reaction was processed using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

2.2.3.12 Site-directed mutagenesis by a PCR cassette method

A panel of *bkj* promoter mutants with TREs in different positions was created using a PCR cassette method. In the first stage, the flanking primer pair HindIII *bkj*5' and XbaI *bkj*3' was used to amplify the *bkj* promoter region from –929 to +13, using pCAT-*BKJ*(WT) as a template (see Chapter 2.1.3.2 for details of all primer sequences). The PCR product was digested overnight with HindIII and XbaI and ligated into the pCAT-Basic reporter vector.

This wt(P)TRE *bkj* plasmid was used as the template for the next stage, involving point mutation of the proximal TRE at position –815. The first round of PCR used the mutagenic primer mt(P)TRE *bkj*5' with the XbaI *bkj*3' flanking primer. 50% of the purified first-round product was then used as the 3' primer in the second round, along with the HindIII *bkj*5' flanking primer, to amplify the full-length mutated PCR product. This was digested and ligated into pCAT-Basic as before.

The resulting mt(P)TRE *bkj* plasmid was used as the template in the third stage. Again, this involved the use of each mutagenic 5' primer with the XbaI *bkj*3' flanking primer in the first round of PCR, and 10-50% of the purified first-round product with the HindIII *bkj*5' flanking primer in the second. All full-length mutated products were digested and ligated into pCAT-Basic as before to create the panel of reporter vectors.

2.2.3.13 Site-directed mutagenesis by a one-stage PCR method

All other site-directed mutants were made using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). PCR using 50ng template DNA and 125ng each of 2 complementary mutagenic primers was carried out according to the manufacturer's

instructions (see Chapter 2.1.3.2 for details of all primer sequences). The original template DNA was degraded by incubation at 37°C for 1hr with DpnI endonuclease, which specifically cleaves methylated and hemimethylated DNA, and the resulting mutated product was transformed into XL10-Gold ultracompetent cells (see Chapter 2.2.1.1).

2.2.3.14 Plasmid construction

RCAS ΔvJ-hER mt

Due to the large size of the RCAS vector, the ClaI fragment of RCAS ΔvJ-hER was cloned into the Cla12Nco adaptor plasmid to enable site-directed mutagenesis. The AF-2mt primer pair (see Chapter 2.1.3.2) was used to introduce the M543A / L544A mutation into Cla12Nco ΔvJ-hER (see Chapter 2.2.3.13). The ClaI fragment was then cloned into RCAS to create RCAS ΔvJ-hER mt.

RCAS ΔvJ-p300 wt and mt

The Cla12Nco ΔvJ-hER plasmid (see above) was digested with NcoI and BamHI to remove the ΔvJ-hER construct. The resulting DNA fragment was digested further with BstYI, and the NcoI-BstYI fragment containing ΔvJ was cloned into Cla12Nco digested with NcoI and BamHI, to create the Cla12Nco ΔvJ plasmid. pBlueScript KS p300 WT and p300 Mut AT2 were each used as the template in PCR reactions using the SalI p3005' and HindIII p3003' primers (see Chapter 2.1.3.2) to amplify the p300 HAT domain (amino acids 1062-1723). PCR products were digested with SalI and HindIII, and cloned into SalI-HindIII digested Cla12Nco ΔvJ, downstream of and in frame with ΔvJ. The resulting plasmids were digested with ClaI and cloned into RCAS to create RCAS ΔvJ-p300 wt and mt; see Figure 2.1.

2.2.3.15 Automated DNA sequencing

All plasmids were sequenced using the PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc.). Approximately 500ng DNA was mixed with 3.2pmol appropriate primer in a 6μl volume, and 4μl BigDye terminator ready reaction mix was added. PCR reaction conditions were as follows: initial step 95°C, 2min; followed by 25 cycles of 95°C, 15s; 50°C, 1min; 60°C, 4min, then a 4°C soak. The PCR

product was precipitated by incubation on ice with 2µl 3M sodium acetate pH4.5 and 50µl ethanol, followed by centrifugation and washing in 70%(v/v) ethanol.

Samples were loaded and run overnight on an Applied Biosystems 373A automated sequencer by Beatson Institute Technical Services staff.

2.2.3.16 Probing of Northern blots

Extraction of total RNA from cells, mRNA selection, RNA formaldehyde gel electrophoresis, and blotting onto nylon membrane were performed by Mr B. Clark of the Beatson Institute.

Nylon membranes were pre-hybridised at 65°C for at least 3hr in hybridisation buffer (0.25M Na_2HPO_4 pH 7.2, 1mM EDTA pH 8, 7%(w/v) SDS). Double-stranded DNA probes were boiled for 5min then cooled on ice. Probes were labelled by incubation at 37°C for 1hr with an excess of deoxycytidine 5'-[α-^{32}P]-triphosphate using the Oligolabelling Kit (Amersham Pharmacia Biotech). Radio-labelled probes were collected by centrifugation in a Bio-Spin 30 chromatography column. Probes were boiled for 5min and cooled on ice before adding to pre-heated hybridisation buffer. The nylon membranes were hybridised overnight at 65°C in this mixture.

Membranes were washed at 65°C for 2x10min in 20mM Na_2HPO_4 pH 7.2, 1mM EDTA pH8, 5%(w/v) SDS, then 3x10min in 20mM Na_2HPO_4 pH 7.2, 1mM EDTA pH 8, 1%(w/v) SDS. Bound probe was detected by autoradiography; membranes were exposed to X-ray film at –70°C and films developed using an X-OMAT 480 RA processor (Kodak). Membranes were stripped by washing at 65°C for 3x10min in 0.1%(w/v) SDS, and then re-probed.

2.2.4 Protein protocols

2.2.4.1 Estimation of protein concentrations

Protein concentration standards were prepared over a range of 0-4mg/ml using BSA in a suitable buffer. 10µl of each standard was added to 1ml 50%(v/v) Coomassie protein assay reagent in a plastic cuvette. The light absorbencies of the protein standards were read at 595nm, using a Beckman DU650 Spectrophotometer, and a standard curve was produced.

10µl of each protein sample was then treated in the same way, and the protein concentration estimated from the standard curve.

2.2.4.2 SDS-PAGE

10% polyacrylamide resolving gels were prepared using 30%(w/v) acrylamide, 0.8%(w/v) bisacrylamide in 375mM Tris pH8.8, 0.1%(w/v) SDS. 4.8% polyacrylamide stacking gel layers were prepared using 30%(w/v) acrylamide, 0.8%(w/v) bisacrylamide in 125mM Tris pH6.8, 0.1%(w/v) SDS. Gels were polymerised by the addition of APS and TEMED.

WCEs in 1xSDS sample buffer were boiled for 2min, cooled on ice and loaded onto the gel along with 10µl prestained protein molecular weight markers. Electrophoresis was carried out at 250V in buffer containing 25mM Tris, 192mM Glycine, 0.1%(w/v) SDS.

2.2.4.3 Western transfer of proteins

Proteins were transferred from polyacrylamide gels to supported nitrocellulose membrane using a semi-dry electroblotter (Millipore). Blot components were soaked in transfer buffer (48mM Tris pH, 39mM glycine, 1.3mM SDS, 20%(v/v) methanol) and assembled, from anode to cathode, in the order: 6 sheets Whatman 3MM filter paper, nitrocellulose membrane, polyacrylamide gel, 6 sheets Whatman 3MM filter paper. Electoblotting was performed at 20V, 200mA for 1hr.

2.2.4.4 Immunological detection of blotted proteins

Nitrocellulose membranes were blocked for 1hr at RT in 100ml Tris-buffered saline-Tween (TBST: 10mM Tris, 100mM NaCl, 0.1%(v/v) Tween-20) containing 5%(w/v) skimmed milk powder. The membrane was then incubated in 5% milk powder in TBST containing an appropriate concentration of primary antibody. Incubation was carried out overnight at 4°C (599-3 antibody) or for 1hr at RT (all other antibodies). The membrane was washed at RT for 3x10min with TBST, and then incubated for 1hr at RT with 5% milk powder in TBST containing a 1:5000 dilution of an HRP-linked secondary antibody. The membrane was washed as before, and incubated for 1min at RT with ECL Western detection agent. Proteins were visualised by autoradiography. Membranes were stripped by incubation in 0.2M glycine, 1%(w/v) SDS for 30-60min at RT.

2.2.4.5 Protein staining

Total protein was visualised by incubating membranes in Ponceau S solution for 10-20min and rinsing in dH_20.

2.2.5 DNA binding assays

2.2.5.1 Oligonucleotide labelling

200ng double-stranded oligonucleotide was end-labelled by incubation with T4 PNK in the presence of an excess of adenosine 5'-[γ-^{32}P]-triphosphate for 30min at 30°C. 10% loading dye (see Chapter 2.2.3.4) was added to the reaction, and the sample was loaded onto a 12% polyacrylamide gel. Electrophoresis was carried out at 150V in 1xTBE buffer. The gel was wrapped and exposed to X-ray film to enable visualisation and excision of the labelled probe. The gel fragment was placed in a Nanosep MF 0.2µm filtration tube (Pall Gelman), minced, and 400µl sterile TE was added. After overnight incubation at RT, the probe was recovered by centrifugation and stored at –20°C until needed.

2.2.5.2 Electrophoretic mobility shift assays (EMSAs)

10µg WCE was added to 0.5µg polydI.dC in 1 x binding buffer (10mM Hepes, 4mM DTT, 0.2mM EDTA, 100nM NaCl, 0.1mg/ml BSA, 4%(v/v) glycerol). The extract was pre-incubated for 15min on ice with antibody or unlabelled competitor oligonucleotide as required. 1ng radio-labelled probe was added, and the extract was incubated for 30min on ice.

A 4.2% polyacrylamide gel was prepared and pre-electrophoresed at 150V in 1xTBE buffer for 30min, 4°C. Samples were then loaded, along with 10% loading dye in 1 x binding buffer in a separate well. Electrophoresis was carried out at 150V in 1xTBE buffer, 4°C. The gel was then fixed by incubation in 10%(v/v) acetic acid, 10%(v/v) methanol for 15-30min at RT, and dried under vacuum onto Whatman 3MM filter paper. DNA-binding complexes were visualised by autoradiography at –70°C.

2.2.5.3 Preparation of biotinylated concatenated probes

Double-stranded ColEco oligonucleotide, with 5'-overhanging EcoRI complementary ends, was phosphorylated by incubation with T4 PNK in the presence of an excess of ATP

for 30min at 30°C. T4 DNA ligase was added, and the reaction was incubated for 2hr at RT. The concatenated probe was biotinylated by incubation with the Klenow fragment of *E.coli* DNA polymerase I, in the presence of an excess of dATP and biotin-16-dUTP. After 1hr at 37°C, the reaction was stopped on ice. The probe was collected by centrifugation in a Bio-Spin 30 chromatography column (BioRad). After each stage, a portion of the reaction was retained and checked by resolving on a 12% polyacrylamide gel.

2.2.5.4 Biotinylated oligonucleotide capture of DNA-binding complexes

WCEs were pre-cleared using 10µl streptavidin-conjugated paramagnetic particles in 1 x binding buffer (see Chapter 2.2.5.2), 1M NaCl. After incubation with mixing at 4°C for 1hr, particles were pulled down using a magnetic stand (Promega). A portion of the supernatant was removed for analysis by EMSA.

0.5µg biotinylated concatenated probe was bound to 10µl streptavidin-conjugated paramagnetic particles by incubation with mixing at 4°C for 1hr in 1 x binding buffer, 1M NaCl. DNA-bound particles were pulled down, washed twice in 400µl 1 x binding buffer, and resuspended in the remaining supernatant from the pre-clearing stage. After incubation for 1hr as before, particles were pulled down and a portion of the supernatant was removed for analysis by EMSA. The remaining supernatant was discarded. Particles were washed as before, then resuspended in 40µl 1 x SDS sample buffer. The particles were pulled down, and the supernatant, containing eluted DNA-binding complexes, was analysed by SDS-PAGE and Western blotting. The particles from the pre-clearing stage were also resuspended in 1 x SDS sample buffer, pulled down, and the supernatant removed for use as a negative control.

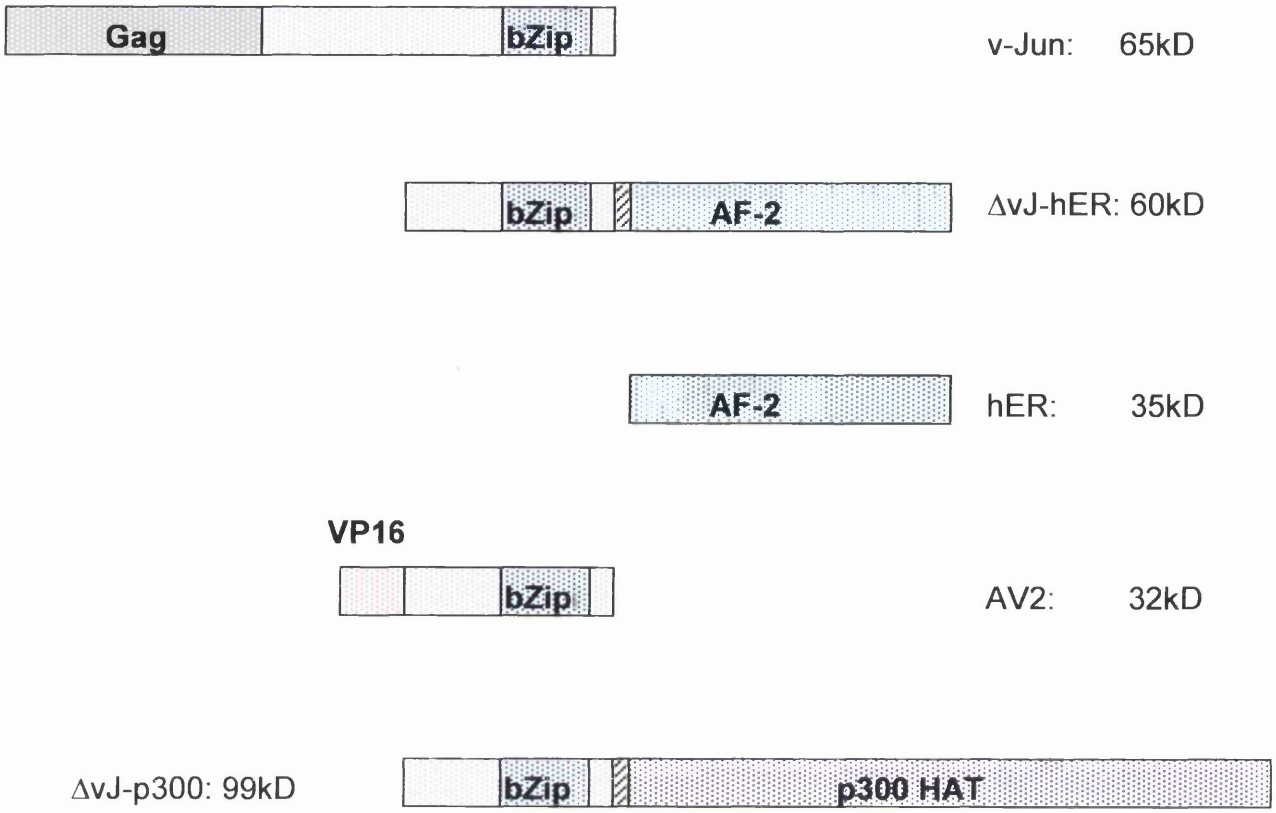


Figure 2.1

Structure of RCAS expression constructs.

ΔvJ-hER consists of the v-Jun C-terminal region fused to the hormone binding / AF-2 domain of human estrogen receptor-α (see Chapter 2.1.4)

hER consists of the hormone binding / AF-2 domain of human estrogen receptor-α (see Chapter 2.1.4)

AV-2 consists of the C-terminal region of v-Jun fused to the VP16 transcriptional activation domain (see Chapter 2.1.4).

ΔvJ-p300 consists of the v-Jun C-terminal region fused to the histone acetyl transferase (HAT) domain of human p300 (see Chapter 2.2.3.14).

Point mutation derivatives of ΔvJ-hER and ΔvJ-p300 were also constructed: see Chapter 2.2.3.14.

Linker regions are shown as hatched boxes.

3 Results: Comparative studies of gene promoters activated or repressed by v-Jun.

3.1 Introduction and aims.

While many positive and negative targets of v-Jun have been identified (see Chapter 1.2.2), little is known about the mechanisms of transcriptional regulation by v-Jun. However, the transcriptional regulation of a number of specific v-Jun target genes has been studied, with *bkj* and collagenase I representing two of the better characterised promoters.

Expression of *bkj* (β-keratin in Jun-transformed cells) was detected specifically in quail embryo fibroblasts (QEFs) transformed by v-Jun, c-Jun, or a chimaeric v-Jun / JunD protein (Hartl and Bister, 1995), CEFs transformed by v-Jun or v-Fos (Hartl and Bister, 1998), and in RCAS-ΔvJ-hER infected QEFs treated with estradiol (Kruse *et al.*, 1997). Two consensus TREs were identified at positions –1073 and –815 relative to the major transcriptional start site (Hartl and Bister, 1998). Transcription from the full-length *bkj* promoter in QEFs was activated strongly by v-Jun, and to a lesser degree by c-Jun and JunD. Deletion analysis of the promoter suggested that the proximal TRE was necessary for transcriptional activation by v-Jun (Hartl and Bister, 1998).

The collagenase I promoter contains a consensus TRE at position –72 relative to the transcriptional start site (Angel *et al.*, 1987b). Mutation of this element abolished basal and TPA-induced transcription from the promoter (Auble and Brinckerhoff, 1991; Gutman and Wasylyk, 1990; Jonat *et al.*, 1990; Westermarck *et al.*, 1997). Other sites, including a non-consensus TRE at position –186, have been shown to contribute to transcriptional regulation (Auble and Brinckerhoff, 1991; Chamberlain *et al.*, 1993; Gutman and Wasylyk, 1990; Westermarck *et al.*, 1997; White and Brinckerhoff, 1995). However, the TRE at position –72 appears to be sufficient for regulation by v-Jun, as transcription from the collagenase I promoter region –73/+63 has been shown to be repressed in v-Jun transformed CEFs compared to control cells (Kilbey *et al.*, 1996).

bkj and collagenase, then, represent examples of gene promoters which are respectively activated or repressed by v-Jun. The transcriptional mechanisms responsible for the opposing effects of v-Jun on the two promoters are not known. The aim of this work was to further characterise transcription from the *bkj* and collagenase promoters in v-Jun

transformed and control CEFs, with the emphasis on investigation of the differences between the promoters which determine the nature of their regulation by v-Jun.

3.2 *bkj* is activated by v-Jun, whereas collagenase is repressed.

3.2.1 Promoter regulation in control and v-Jun transformed CEFs.

A systematic analysis of the activity of *bkj* and collagenase promoters and deletion mutant derivatives was carried out in CEFs uniformly infected with ASV17 or the RCAS retroviral vector. Prior transfection with a construct consisting of the CAT reporter gene driven by the RSV long terminal repeat promoter established that the transfection efficiencies of the two cell types were equivalent (data not shown).

5μg each of the pCAT-*BKJ*(WT), -*BKJ*(DP), -*BKJ*(P), -*BKJ*(D) and -*BKJ*(0) reporter plasmids was transfected in triplicate into CEFs and v-Jun transformed CEFs, and relative CAT activities were determined (see Chapter 2.2.2.7 for details). The results of a representative experiment are shown in Figure 3.1A.

The full-length promoter, *BKJ*(WT), was more active in v-Jun transformed CEFs than in controls. The *BKJ*(DP) promoter, which retains both TREs but lacks 690bp of upstream sequence, was also more active in v-Jun transformed CEFs than in controls; however, the overall levels of promoter activity in both cell types were much higher than the corresponding values obtained with the *BKJ*(WT) promoter. Deletion of the distal TRE and flanking sequences from *BKJ*(DP), resulting in the *BKJ*(P) promoter, did not affect promoter activity in v-Jun transformed CEFs, but decreased transcription in control CEFs. Further deletion of the proximal TRE and flanking sequences, resulting in the *BKJ*(0) promoter, decreased promoter activity in v-Jun transformed CEFs to basal levels. Deletion of the proximal TRE and flanking sequences from *BKJ*(WT) resulted in the *BKJ*(D) promoter, which displayed low levels of activity in both v-Jun transformed and control CEFs.

These results are in line with previous observations in QEFs (Hartl and Bister, 1998) that the *BKJ*(WT) promoter was activated more strongly by v-Jun than c-Jun, the *BKJ*(DP) promoter was more active in v-Jun transformed cells than in controls, and that v-Jun activated the *BKJ*(DP) and *BKJ*(P) promoters more strongly than *BKJ*(WT), *BKJ*(D) and

BKJ(0). The fact that the *BKJ*(DP) promoter was more active than *BKJ*(WT) suggests that there may be a negative regulatory element, for example a binding site for a transcriptional repressor, within the 690bp deleted sequence. The difference in activity between the *BKJ*(WT) and *BKJ*(DP) promoters was much more pronounced in CEFs (Figure 3.1A) than in QEFs (Hartl and Bister, 1998). The two promoters have not been compared in QEFs stably infected with ASV17, or by co-transfection with c-Jun and v-Jun in CEFs; the discrepancy may be due to the different approach used in each case, or to the species difference.

Previous studies have suggested that the proximal, rather than the distal, TRE is necessary for activation of the *bkj* promoter by v-Jun; this was based on a comparison between the *BKJ*(P) and *BKJ*(D) promoters (Hartl and Bister, 1998). However, unlike *BKJ*(P), *BKJ*(D) retains the 690bp sequence upstream of the distal TRE which, as described above, mediated down-regulation of the *BKJ*(WT) promoter in v-Jun transformed and control CEFs (Figure 3.1A). Any transcriptional effect of the distal TRE may therefore be masked in this promoter context. Additionally, comparison of the *BKJ*(DP) and *BKJ*(P) promoters reveals that deletion of the distal TRE decreased promoter activity in control CEFs. The distal TRE may therefore contribute to *bkj* regulation. However, the most important conclusions for the purpose of this comparative study were that the *BKJ*(P) promoter is more active in v-Jun transformed CEFs than in controls, and that deletion of the proximal TRE from this construct decreases transcription to basal levels in v-Jun transformed CEFs.

5μg each of the –73/+63 ColCAT and –60/+63 ColCAT reporter plasmids was transfected in triplicate into CEFs and v-Jun transformed CEFs, and relative CAT activities were determined as before. The results of a representative experiment are shown in Figure 3.1B. The –73/+63 ColCAT promoter had a high level of basal activity in control CEFs, and was down-regulated approximately 10-fold in v-Jun transformed CEFs. This confirmed previous observations (Hussain *et al.*, 1998; Kilbey *et al.*, 1996). Deletion of the TRE at position –72, resulting in the –60/+63 ColCAT promoter, abolished basal promoter activity. The decreased activity of this promoter in v-Jun transformed CEFs compared with control CEFs was not reproducible, and therefore unlikely to be a transcriptional effect of v-Jun on the –60/+63 ColCAT promoter.

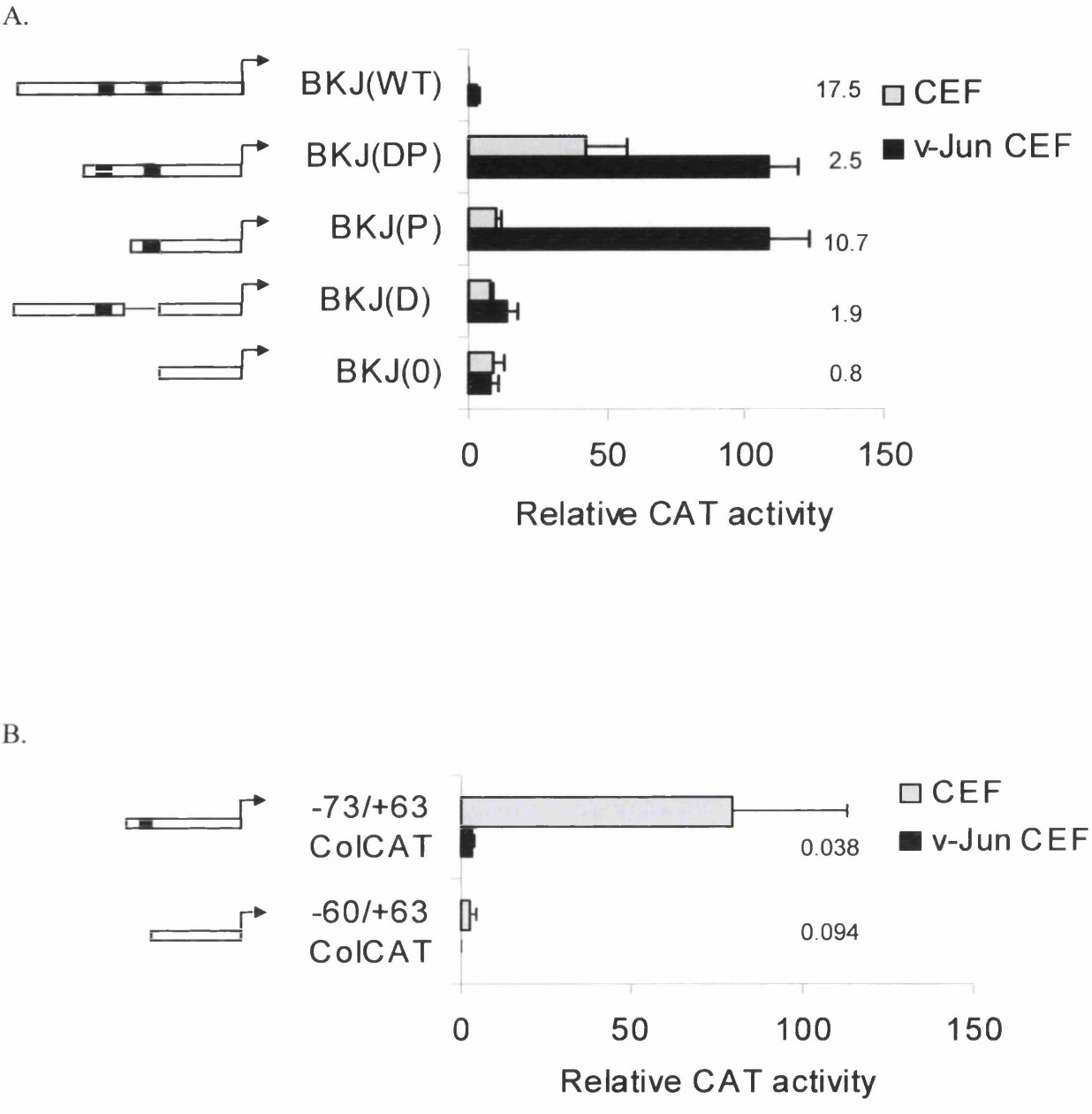


Figure 3.1

Transcription from (A) the *bkj* promoter and deletion mutants thereof and (B) the collagenase promoter and a deletion mutant.

Black boxes represent consensus TRE sequences. Promoter diagrams are not to scale.

5μg each reporter plasmid was transfected in triplicate into CEFs and v-Jun CEFs. Fold activation of transcription in v-Jun CEFs compared to control CEFs is shown in each case.

Error bars denote standard deviations.

3.2.2 Effect of ectopic v-Jun expression on the *bkj* and collagenase promoters.

As described in Chapter 1.2.1, v-Jun replaces c-Jun as the major component of the protein complexes bound to TREs and CREs in v-Jun transformed CEFs, and this is thought to account for the altered transcription of certain target promoters in these cells. However, it was possible that the activation of *bkj* and repression of collagenase in v-Jun transformed CEFs were not direct effects of v-Jun expression and binding to TREs, but were caused by some other altered property of the transformed cells. Co-expression experiments were therefore carried out to analyse the effects of c-Jun and v-Jun protein expression on the *bkj* and collagenase promoters.

0.5μg *BKJ*(P) reporter plasmid, along with 2μg pRc/RSV-cJ3, -vJ1 or empty vector, was transfected in triplicate into CEFs, and relative CAT activities were determined as before. Figure 3.2A shows that, while co-expression of c-Jun had little or no effect on promoter activity, co-expression of v-Jun strongly activated the *BKJ*(P) promoter. This confirmed previous observations in QEFs (Hartl and Bister, 1998).

Similarly, 2μg –73/+63 ColCAT reporter plasmid, along with 1μg pRc/RSV-cJ3, -vJ1 or empty vector, was transfected in triplicate into CEFs, and relative CAT activities were determined as before. Figure 3.2B shows that, while co-expression of c-Jun activated the promoter slightly, co-expression of v-Jun repressed the basal activity of the promoter. This confirmed previous reports (Gao *et al.*, 1996).

The results of *bkj* and collagenase promoter reporter assays in v-Jun transformed and control cells (Figure 3.1) therefore correlate well with experiments involving the ectopic expression of c-Jun and v-Jun (Figure 3.2; see also (Hartl and Bister, 1998)). This strongly suggests that the activation of *bkj* and repression of collagenase in v-Jun transformed CEFs are direct effects of the v-Jun protein on the two gene promoters.

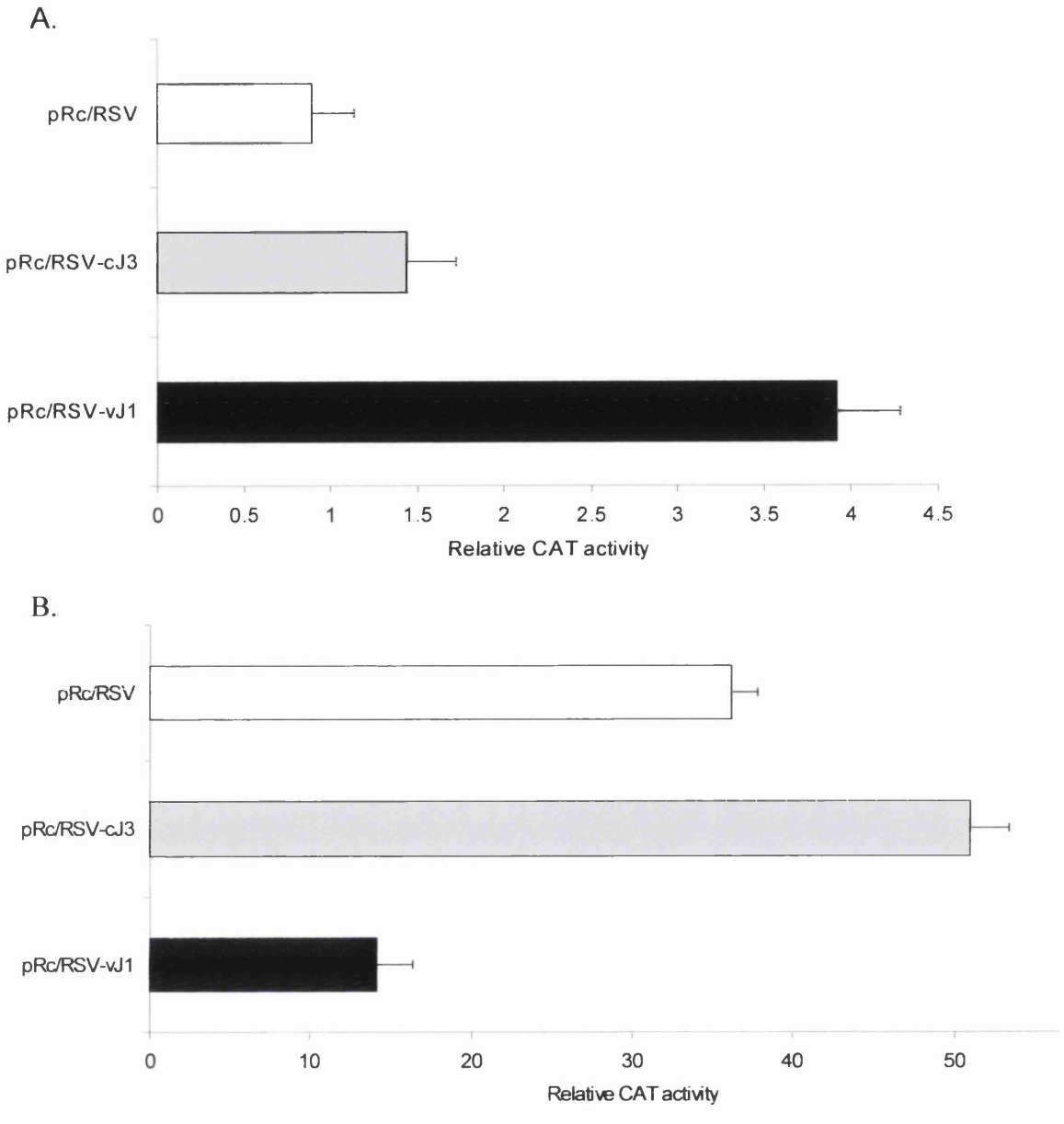


Figure 3.2

(A) Co-expression of c-Jun and v-Jun with the *BKJ*(P) promoter.

0.5μg reporter plasmid, along with 2μg pRc/RSV-cJ3, -vJ1 or empty vector, was transfected in triplicate into CEFs. Error bars denote standard deviations.

(B) Co-expression of c-Jun and v-Jun with the –73/+63 ColCAT promoter.

2μg reporter plasmid, along with 1μg pRc/RSV-cJ3, -vJ1 or empty vector, was transfected in triplicate into CEFs. Error bars denote standard deviations.

3.3 The collagenase and *bkj* TREs are bound by similar protein complexes.

It has been shown that dimers consisting of different members of the Jun and Fos families have different transcriptional effects on certain promoters (see Chapter 1.1.7.1). It was therefore possible that dimerisation with different Fos family members contributed to the different effects of v-Jun on the *bkj* and collagenase promoters.

The collagenase TRE has been shown to be specifically bound by c-Jun / Fos dimers in CEFs, and by v-Jun / Fos dimers in v-Jun transformed CEFs (Hawker *et al.*, 1993; Kilbey *et al.*, 1996). The major Fos family component was thought to be Fra-2 in both cell types (Kilbey *et al.*, 1996). The proximal *bkj* TRE has been shown to be specifically bound by recombinant chicken c-Jun protein (Hartl and Bister, 1998). Neither of the *bkj* TREs has previously been tested for binding using cell extracts.

An EMSA was carried out to visualise the protein complexes bound to the collagenase TRE, and the distal (D) and proximal (P) TREs of *bkj,* in v-Jun transformed and control CEFs (Figure 3.3A). 10μg of each WCE was incubated with radio-labelled oligonucleotide probes comprising the relevant TRE and flanking sequences (see Chapter 2.1.3.1 for details) and subjected to electrophoresis (see Chapter 2.2.5.2). The complex bound to each TRE in v-Jun transformed CEFs was of similar intensity, but lower electrophoretic mobility, than the corresponding complex in control cells. In the case of the collagenase TRE, this has been shown to be due to the greater size of the Gag-v-Jun protein (65kD) compared to c-Jun (39kD) (Kilbey *et al.*, 1996).

These results suggested that all three TREs were bound by similar protein complexes. Competition assays were carried out to determine whether this was the case. 10μg of WCE from CEFs or v-Jun transformed CEFs was pre-incubated with a 100-fold excess of unlabelled competitor oligonucleotide before addition of radio-labelled probe. Results are shown in Figure 3.3B. It should be noted that the relatively low intensity of the complex bound to the *bkj*(P) TRE in v-Jun transformed CEFs was due to the reduced efficiency of the probe end-labelling reaction in this case.

In v-Jun transformed CEFs, each TRE competed complex bound to each of the other two probes. In CEFs, there was mutual competition between the collagenase and *bkj*(P) TREs. This suggests that identical complexes bound to these TREs in each cell type. However,

the complex bound to the *bkj*(D) TRE in CEFs was not fully competed by an excess of *bkj*(P) TRE; this suggests that a component of this complex may be unique to the *bkj*(D) TRE under these conditions. However, as self-competition by *bkj*(P) was not complete, this may reflect a technical fault rather than a genuine difference between the two TREs.

The complexes bound to each probe were analysed by super-shift experiments. WCEs from CEFs were pre-incubated with the c-Jun specific KM-1X antibody, or with the K-25 antibody, which is broadly reactive to all Fos family members. WCEs from v-Jun transformed CEFs were pre-incubated with K-25, or the Jun 730-5 antibody (Black *et al.*, 1994). Results are shown in Figure 3.3C.

In CEFs, the majority of the complex bound to each probe was super-shifted by the c-Jun antibody, while in v-Jun transformed CEFs, each complex was almost completely disrupted by the Jun antibody. The K-25 pan Fos antibody completely shifted the complex bound to each probe in both cell types. This supports previous observations (Hawker *et al.*, 1993; Kilbey *et al.*, 1996) that the major components of the complex bound to the collagenase TRE are c-Jun / Fos family dimers in CEFs and v-Jun / Fos family dimers in v-Jun transformed CEFs, and additionally suggests that the *bkj*(D) and *bkj*(P) TREs are bound by similar protein complexes in each cell type.

Further analysis was carried out using antibodies specific for the individual Fos family members (Figure 3.3D). A Fra-2 specific antibody shifted each bound complex; no other specific antibody had any discernible effect. It is important to note that the Fra-2 antibody effected only a partial shift of each complex. That the residual bound proteins contained Fos family members seems clear from the fact that the K-25 antibody completely shifted the same complexes. As only the c-Fos and Fra-2 proteins have so far been identified in chickens, it is possible that the residual bound complexes contained FosB and/or Fra-1 proteins which were not recognised by the antibodies used in this experiment.

Within the limits of the materials used, however, Figure 3.3 suggests that the collagenase, *bkj*(D) and *bkj*(P) TREs are bound by highly similar protein complexes in v-Jun transformed CEFs, i.e. predominately v-Jun / Fra-2 dimers.

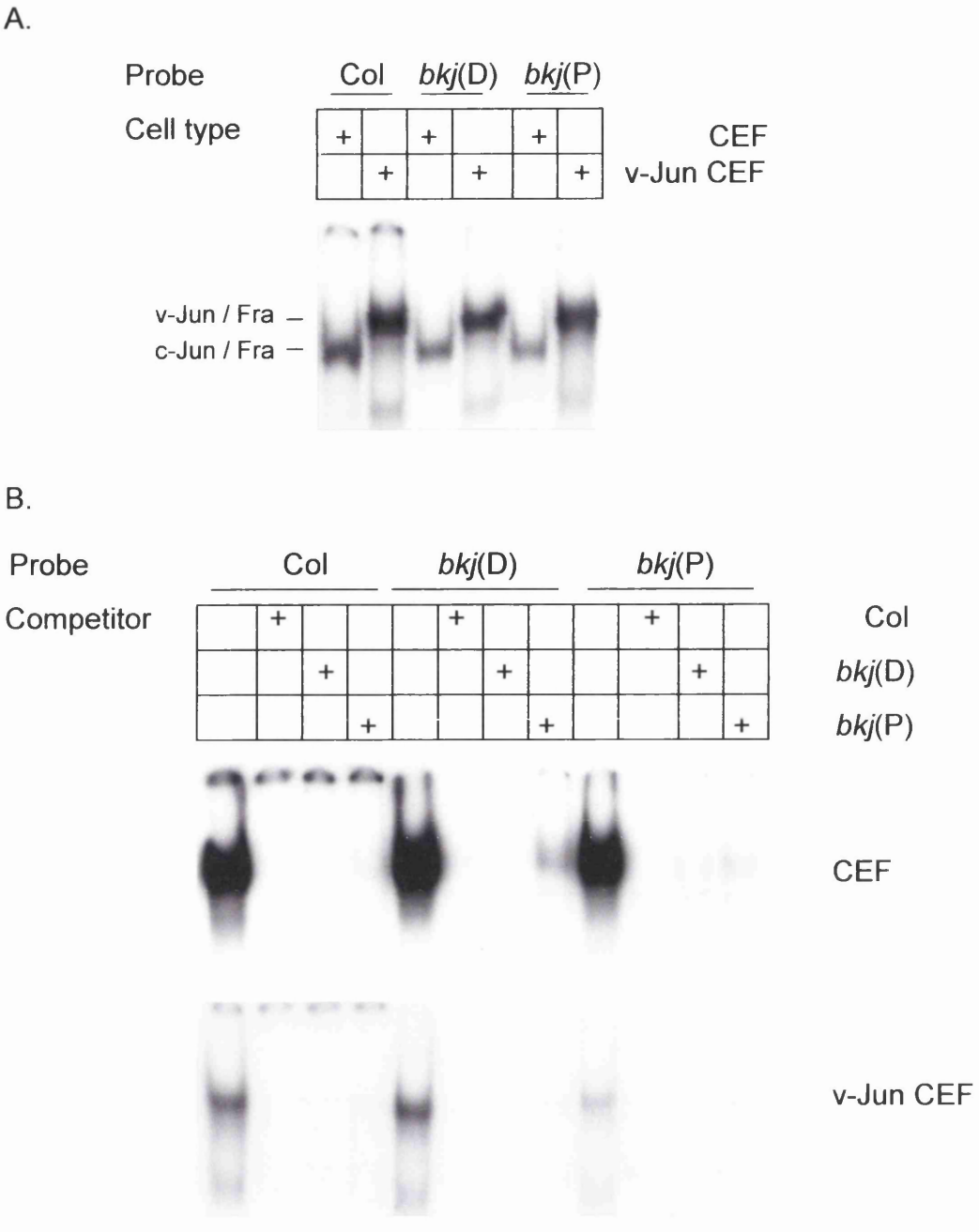


Figure 3.3

(A) EMSA showing protein complexes bound to radio-labelled probes encompassing the collagenase (Col) or *bkj* (Distal and Proximal) TREs.

(B) EMSAs showing mutual competition for binding complexes between the three probes in CEFs (upper panel) and v-Jun CEFs (lower panel). Extracts were pre-incubated with a 100-fold excess of unlabelled competitor DNA, as indicated, before addition of radio-labelled probe.

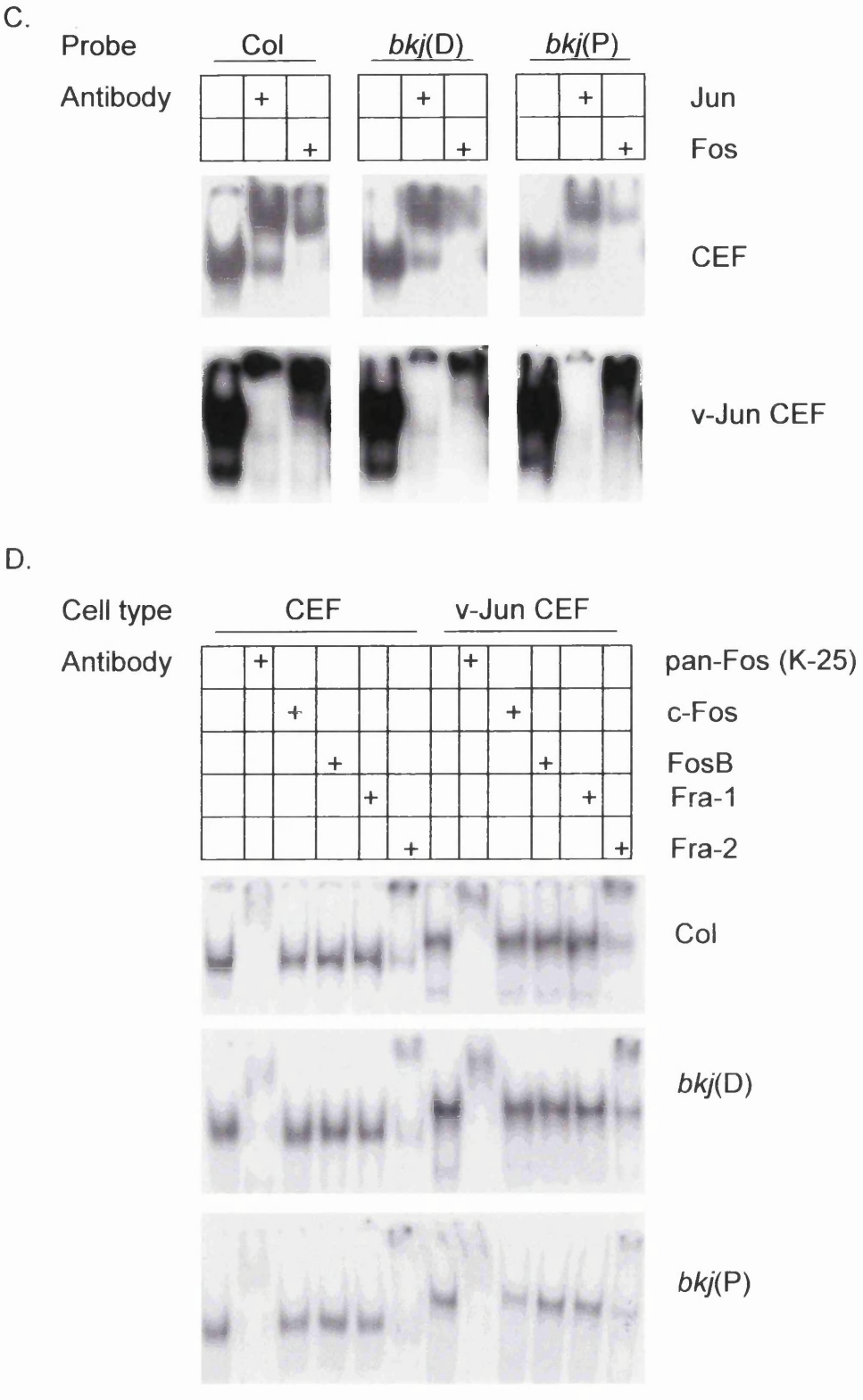


Figure 3.3

Analysis of protein complexes bound to collagenase and *bkj* TREs in CEFs and v-Jun CEFs, using antibodies directed against Jun / Fos (C) and Fos family proteins (D).

Extracts were pre-incubated with 3μl antibody, as indicated, before addition of radio-labelled probe.

3.4 The effect of TRE position on transcriptional regulation by v-Jun.

As described above, the binding of different Jun / Fos complexes to the collagenase and *bkj*(P) TREs is unlikely to account for the different effects of v-Jun on the –73/+63 ColCAT and *bkj*(P) promoters. It is possible that the effects of v-Jun may instead be mediated by the different position of the TRE relative to the transcriptional start site in the two promoters. At –72, the collagenase TRE is situated close to the transcriptional start site, whereas at position –815, the *bkj*(P) TRE is relatively distant.

It has been shown that the ability of a CRE at position -45 to mediate basal and cAMP-induced transcription from the tyrosine hydroxylase promoter was decreased upon its insertion at positions further from the transcriptional start site (Tinti *et al.*, 1997). While this site was not bound by c-Jun (Lim *et al.*, 2000), it is possible that TRE position is similarly important for the nature of target promoter regulation by v-Jun. It is interesting that the stromelysin (transin) and endogenous c-*jun* genes, which are, like collagenase, down-regulated by v-Jun (Gao *et al.*, 1996; Hussain *et al.*, 1998; Kilbey *et al.*, 1996; Tsang *et al.*, 1994), have a consensus TRE at position –71 and –72 respectively (Hattori *et al.*, 1988; Matrisian *et al.*, 1986). In the case of the c-*jun* promoter, repression by v-Jun was shown to be mediated by the non-consensus TRE at position –72 (Hussain *et al.*, 1998). Such close correlation in the positions of TREs close to the transcriptional start site in three v-Jun-repressed promoters suggests that TRE position may be critical for the nature of target promoter regulation by v-Jun.

To investigate this possibility, a panel of variant *bkj* promoters was constructed, each with its TRE in a different position relative to the transcriptional start site. The aim was to determine whether moving the TRE closer to the transcriptional start site would convert *bkj* from a v-Jun-activated to a v-Jun-repressed promoter.

3.4.1 Mutation of the proximal *bkj* TRE abolishes DNA binding and transactivation by Jun / Fra-2.

The first stage of the procedure was to mutate the *bkj*(P) TRE, to ensure that binding of Jun / Fra-2 dimers to this element did not interfere with transcription from the introduced TREs. This was achieved using a PCR cassette site-directed mutagenesis protocol (see Chapter 2.2.3.12). A mutagenic primer was used to alter two bases of the *bkj*(P) TRE, from

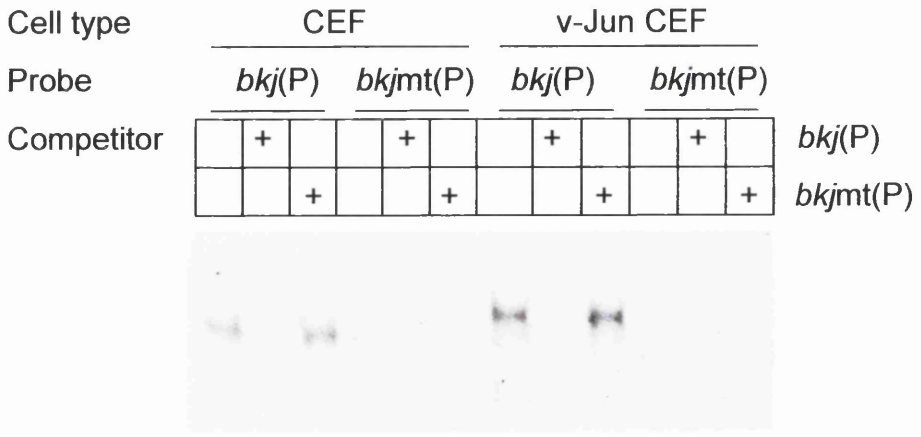
TGACTCA to AGACCCA. This mutation has been shown to abolish binding by recombinant chicken c-Jun protein (Hartl and Bister, 1998).

Oligonucleotides containing the wild-type or mutated *bkj*(P) TRE (see Chapter 2.1.3.1) were each used as radio-labelled probe and unlabelled competitor in an EMSA, to determine whether mutation of the *bkj*(P) TRE abolished binding by Jun / Fra-2 dimers. As shown in Figure 3.4A, the *bkj*mt(P) TRE failed to compete for the complex bound to the *bkj*(P) TRE in CEFs and v-Jun transformed CEFs. No complex was detected bound to radio-labelled *bkj*mt(P) TRE in either cell type. This confirmed that mutation of the *bkj*(P) TRE abolishes normal TRE binding by Jun / Fra-2 dimers.

The activities of the wt(P)TRE *bkj* and mt(P)TRE *bkj* promoters were compared in CEFs and v-Jun transformed CEFs. *BKJ*(P) and *BKJ*(0) were included as controls. 5μg of each reporter plasmid was transfected in triplicate into CEFs and v-Jun transformed CEFs, and relative CAT activities were determined as before. Results from a representative experiment are shown in Figure 3.4B.

Like *BKJ*(P), wt(P)TRE *bkj* was activated much more strongly in v-Jun transformed CEFs than in CEFs. The difference in the overall level of activation of these two promoters was probably due to the slightly different promoter region used in each case (-886/+12 for *BKJ*(P) and -929/+13 for wt(P)TRE *bkj*). mt(P)TRE *bkj* was not activated in v-Jun transformed CEFs to the high levels seen with the wt(P)TRE *bkj* promoter, although basal transcription was not affected by mutation of the TRE. It is clear, therefore, that the *bkj*(P) TRE is essential for high levels of transcription of the *bkj* promoter in v-Jun transformed CEFs. This strongly supports the hypothesis that the high level of expression of *bkj* in v-Jun transformed CEFs is due to direct binding and regulation of the promoter by v-Jun.

A.



B.

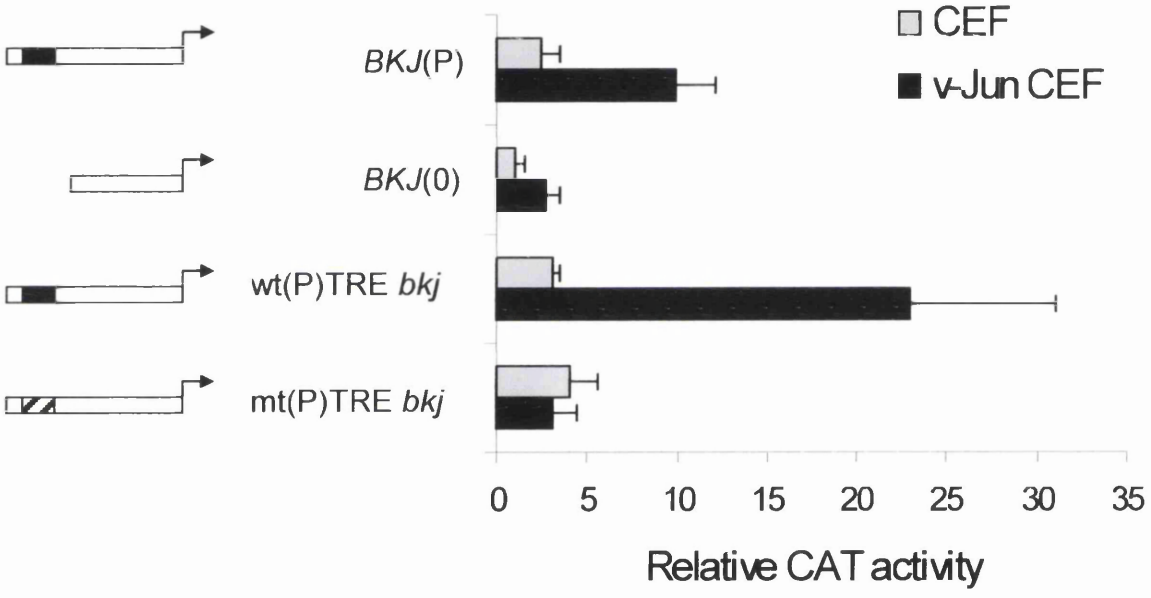


Figure 3.4

(A) EMSA showing that the mutated proximal TRE was not bound by Jun/Fos dimers in CEFs or v-Jun CEFs, and failed to compete bound complexes from the wild-type *bkj*(P) TRE. Extracts were pre-incubated with a 100-fold excess of unlabelled competitor DNA, as indicated, before addition of radio-labelled probe.

(B) Transcription from *bkj* promoters containing wild-type or mutated proximal TREs. Black boxes represent consensus TRE sequences; the hatched box represents the mutated proximal TRE at position –815. Promoter diagrams are not to scale.

5μg each reporter plasmid was transfected in triplicate into CEFs and v-Jun CEFs.

Error bars denote standard deviations.

3.4.2 Introduction of TRE sequences into the *bkj* promoter.

The mt(P)TRE *bkj* promoter was used as the template to create a panel of variant promoters with TREs at different positions relative to the transcriptional start site. Site-directed mutagenesis was used to convert sites with sequence similarity to a TRE into the consensus TRE sequence (see Chapter 2.2.3.12). In this way, only the 1-4 nucleotides which diverged from the consensus TRE sequence at each site were altered, avoiding disruption of the sequences flanking each element. To avoid disruption of functional elements which may be involved in the regulation of *bkj* by factors other than Jun, the *bkj* promoter sequence was checked for potential binding sites of other transcription factors by the RGSiteScan programme (wwwmgs.bionet.nsc.ru/mgs/programs/yura/ RecGropScanStart.html). No consensus binding sites for other cellular transcription factors were found in the mt(P)TRE *bkj* promoter. Suitable sequences for conversion into a consensus TRE were found at positions -777, -610, -477, -323, -169, -109 and -65 relative to the transcriptional start site.

Before the effect of TRE position on transcription could be analysed, it was necessary to determine whether all introduced TREs were bound by c-Jun and v-Jun with comparable affinities. EMSAs were carried out to determine the efficiency with which each introduced TRE competed for complex bound to the *bkj*(P) TRE. 10μg of WCE was pre-incubated with increasing amounts of each introduced TRE and its flanking sequences (see Chapter 2.1.3.1), before incubation with radio-labelled *bkj*(P) TRE. An example is shown in Figure 3.5A. All probes were assayed for competition in CEFs and v-Jun transformed CEFs. After autoradiography, bound complexes were quantified using PDI Gel Scan software (Precision Digital Images Inc), and the percentage competition of bound complex was calculated. Full results are shown in Figure 3.5B.

The efficiencies with which the introduced TREs competed for bound complex varied to some degree. For example, the *bkj*-610 TRE competed for bound complex more efficiently than did *bkj*-477 in CEFs and in v-Jun transformed CEFs. The relative competition efficiencies of the TREs were not conserved between the two cell types in all cases. For example, the *bkj*-777 TRE competed less effectively for bound complex than did the *bkj*(P) TRE in CEFs, but *bkj*-777 was the more effective competitor in v-Jun transformed CEFs.

It was possible that the observed variations in competition efficiencies were due to the preferential binding of different Jun / Fos proteins to different probes. The composition of the complexes bound to each TRE in CEFs and v-Jun transformed CEFs was therefore

analysed. Radio-labelled probes containing the relevant TRE were incubated with 10μg of WCE from CEFs or v-Jun transformed CEFs. The complex bound to each TRE was analysed by pre-incubation with antibodies specific for c-Jun (KM-1X) or Fra-2 in CEFs, and Jun (730-5) or Fra-2 in v-Jun transformed CEFs. Results are shown in Figure 3.5C.

Within each cell type, every probe was bound by a complex with an equivalent electrophoretic mobility. Pre-incubation with antibodies produced results similar to those seen with the *bkj*(P) TRE (see Figure 3.3C and D); the majority of the complex bound to each TRE in CEFs was super-shifted by the c-Jun specific antibody, and each complex from v-Jun transformed CEFs was almost completely disrupted by the 730-5 Jun antibody. The Fra-2 antibody super-shifted the majority of each complex in both cell types. This suggests that, like the *bkj*(P) TRE, each introduced TRE is bound predominately by c-Jun / Fra-2 dimers in CEFs and v-Jun / Fra-2 dimers in v-Jun transformed CEFs.

In conclusion, there were some minor variations in the affinity of binding of protein complexes to the panel of TREs. This was probably due to the different sequences flanking each site, as the core TRE was identical in each case. However, no major differences were detected in the composition of the complexes bound to the panel of TREs in v-Jun transformed and control CEFs.

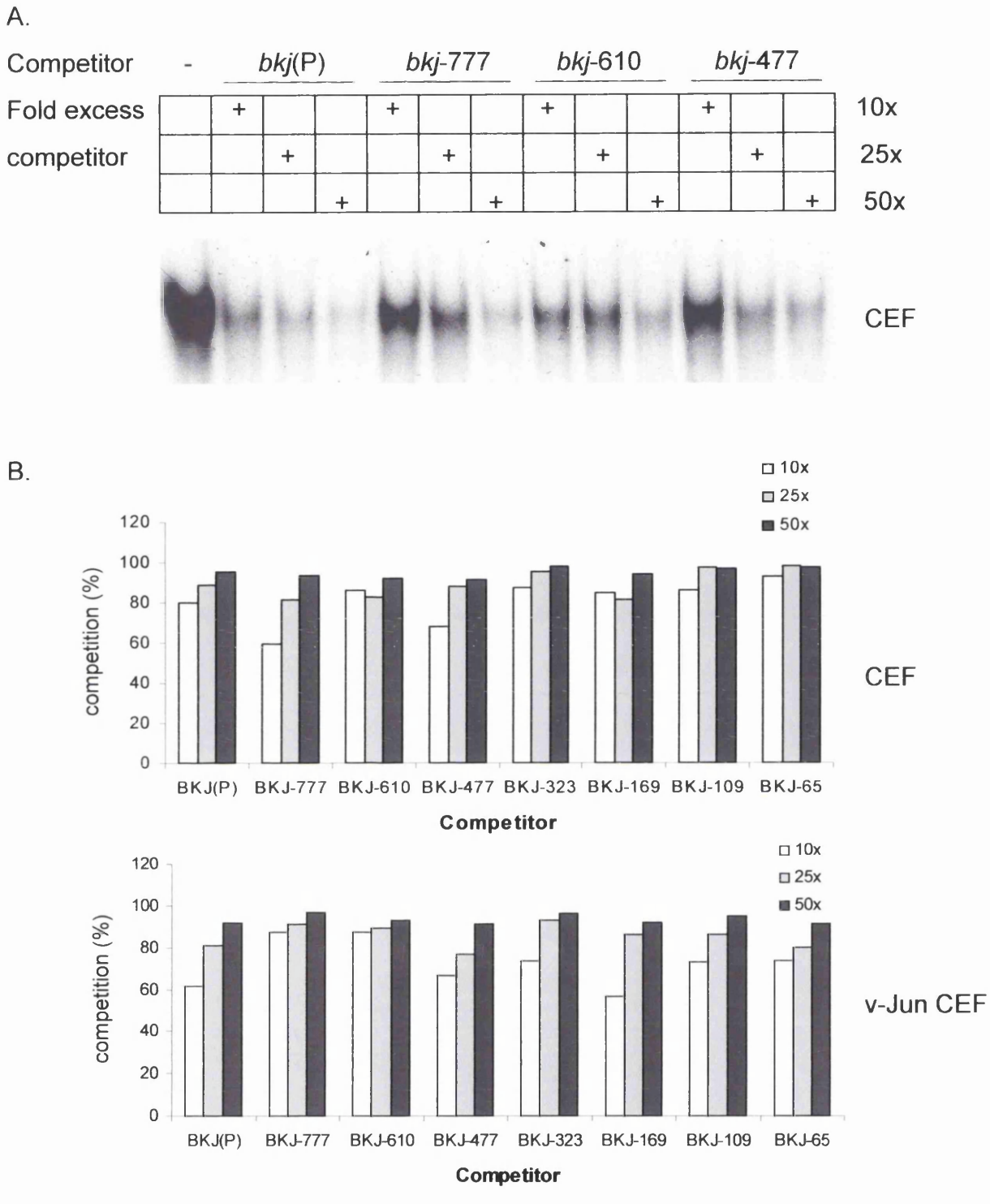


Figure 3.5

(A) Representative EMSA showing competition of bound complex from the *bkj*(P)TRE by the addition of increasing amounts of unlabelled competitor. Extracts were pre-incubated with competitor DNA, as indicated, before addition of radio-labelled probe.

(B) Graphical representation of EMSA data. All probes were tested for competition as in (A), in CEF and v-Jun CEF extracts.

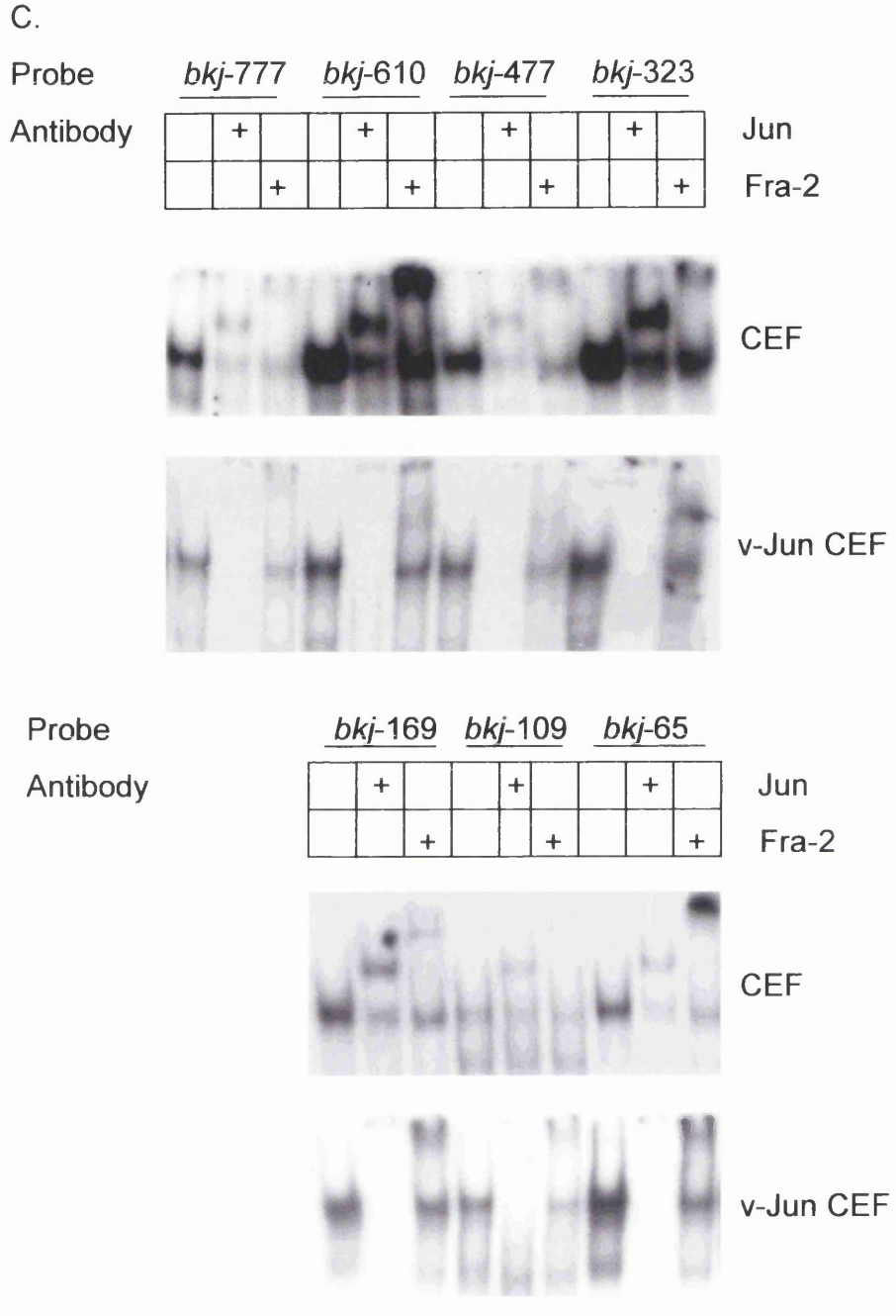


Figure 3.5

(C) Analysis of protein complexes bound to the panel of TRE position mutants in CEFs and v-Jun CEFs, using antibodies directed against Jun or Fra-2.

Extracts were pre-incubated with 3μl antibody, as indicated, before addition of radio-labelled probe.

3.4.3 The effect of TRE position on transcription of *bkj*.

5μg of each of the panel of *bkj* promoter mutants was transfected in duplicate into CEFs and v-Jun transformed CEFs, and relative CAT activities were determined as before. Results from a representative experiment are shown in Figure 3.6.

Compared to the wt(P)TRE *bkj* promoter, the activities of the –777TRE *bkj* and –610 TRE *bkj* promoters were decreased by approximately 50% in v-Jun transformed CEFs. A further, progressive, decrease was seen in the activities of –477TRE *bkj* and –323TRE *bkj*; transcription from the –323TRE *bkj* promoter was also decreased in CEFs. Indeed, –323 TRE *bkj* was active at levels similar to mt(P)TRE *bkj* in both cell types. The –169 TRE *bkj* and –109TRE *bkj* promoters were both activated in v-Jun transformed CEFs to levels similar to wt(P)TRE *bkj,* while their activity in CEFs was increased. –109TRE *bkj* was almost as active in CEFs as in v-Jun transformed CEFs. –65TRE *bkj*, in contrast, was activated over 4-fold more in v-Jun transformed CEFs than in controls, although overall levels of activation were lower than for the wt(P)TRE *bkj* promoter.

These results show that alteration of TRE position affected the activation of the *bkj* promoter in CEFs and v-Jun transformed CEFs. The initial effect of movement of the TRE from its wild-type position at –815 towards the transcriptional start site was to decrease the activity of the promoter in v-Jun transformed CEFs. This held true until a TRE position of –323, from where transactivation by both c-Jun and v-Jun was almost completely abolished. However, this trend was not continued with further reduction of the distance between the TRE and the transcriptional start site. Instead, introduction of a TRE 169 or 109bp from the transcriptional start site produced promoters which were activated strongly in both CEFs and v-Jun transformed CEFs. –65TRE *bkj*, the promoter with its TRE closest to the transcriptional start site, was activated more strongly in v-Jun transformed CEFs than in CEFs. It is important to note that the relative activities of the promoters did not correlate with the variations in the affinity of Jun / Fra-2 binding to each TRE seen in Figure 3.5B, in either cell type. The results shown in Figure 3.6 are therefore not due solely to differences in the affinity of Jun / Fra-2 binding to the different promoters.

Therefore, while TRE position influenced the transcriptional regulation of *bkj*, the introduction of a TRE close to the transcriptional start site did not result in transcriptional repression by v-Jun. In conclusion, TRE position alone does not account for the difference in regulation of the collagenase and *bkj* promoters by v-Jun.

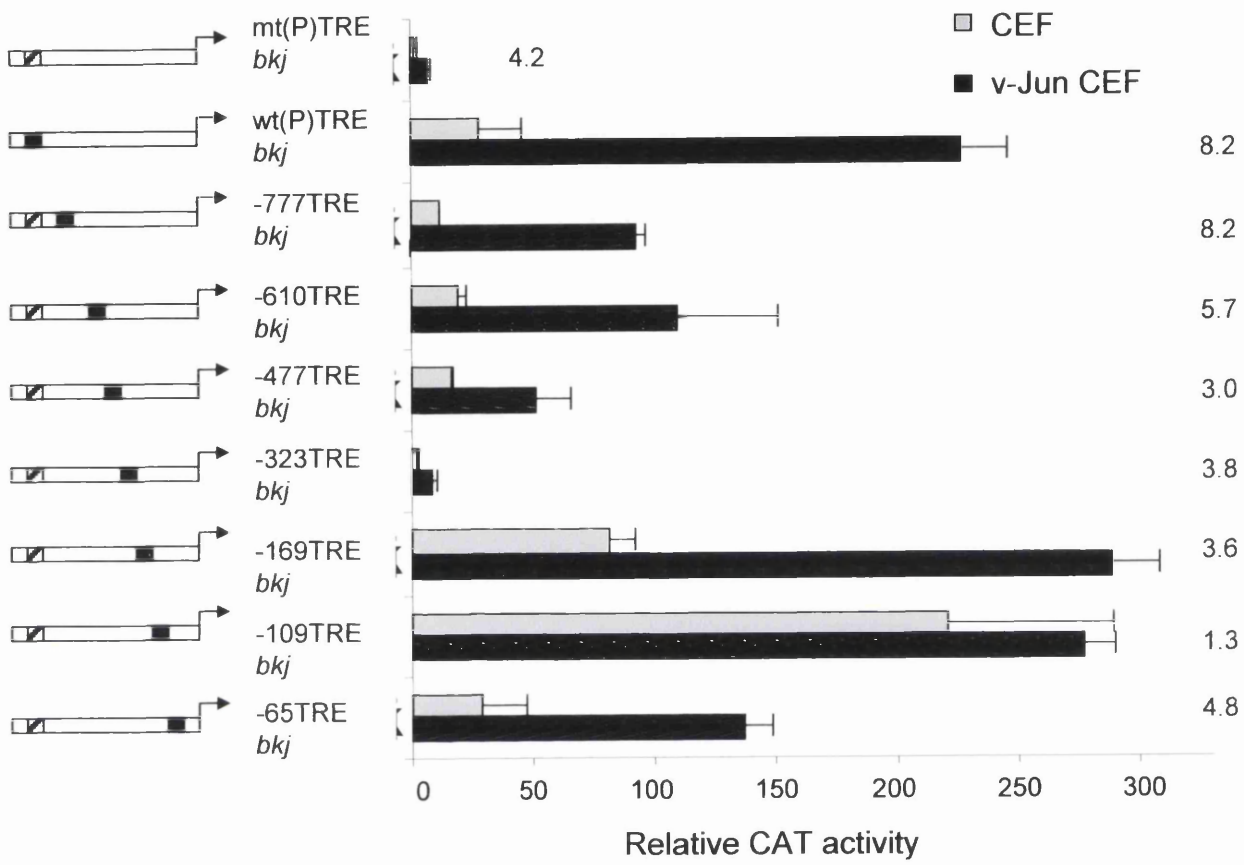


Figure 3.6

Transcription from a panel of *bkj* promoter mutants with TREs introduced at different positions.

Black boxes represent consensus TRE sequences; hatched boxes represent the mutated proximal TRE at position –815. Promoter diagrams are not to scale.

5μg each reporter plasmid was transfected in duplicate into CEFs and v-Jun CEFs. Fold activation of transcription in v-Jun CEFs compared to control CEFs is shown in each case.

Error bars denote standard deviations.

3.5 The effect of core promoter sequences on transcriptional regulation by v-Jun

As described in Chapter 1.3.2.1, c-Jun and v-Jun have both been shown to bind components of the pre-initiation complex, including TBP. The sequence of the TATA box and other core elements of target gene promoters may therefore be important for transcriptional regulation by v-Jun.

Previous studies have shown that exchange of core promoter elements between heterologous promoters affects activation by certain transcription factors. For example, the levels of transcriptional activation by ATF (Taylor and Kingston, 1990b), Varicella Zoster virus IE62 protein (Perera, 2000) and Herpes simplex virus ICP4 protein (Cook *et al.*, 1995) vary with TATA box sequence. However, other transcription factors, including CP1, Sp1 (Taylor and Kingston, 1990b), VP16 (Perera, 2000) and adenovirus E1a (Taylor and Kingston, 1990a) are not affected by TATA box sequence alterations. Variations in Inr sequence have been shown to affect the ability of Sp1 to activate transcription (Chalkley and Verrijzer, 1999).

Interestingly, ATF facilitated the recruitment of a pre-formed pre-initiation complex from one transcription template to another, while CP1, which functions regardless of TATA box sequence, did not (Taylor and Kingston, 1990b). This suggests that activators which interact with and recruit components of the pre-initiation complex are more likely to be affected by sequence variations in core promoter elements. A possible explanation for this is suggested by the observation that the highest levels of induction by ICP4 were seen from the TATA boxes with the lowest affinities for TBP and the lowest levels of basal transcription (Cook *et al.*, 1995). ICP4 is known to form a complex with TFIIB, TFIID and the TATA box (Smith *et al.*, 1993). This implies that ICP4 activates transcription by enhancing the binding of TFIID to the TATA box, with a greater effect therefore observed on promoters where the basal level of binding is naturally low.

Transcription from a promoter containing a TRE has previously been assayed with a panel of TATA box sequences (Taylor and Kingston, 1990b). No major variations were observed, but the overall levels of transcription were so low that any differences in activation may not have been detected.

A comparison of the *bkj* (Hartl and Bister, 1998) and collagenase (Angel *et al.*, 1987a) core promoter regions is shown in Figure 3.7A. Both promoters contain a non-consensus TATA box at position –25 relative to the transcriptional start site. The *bkj* promoter also contains a consensus Inr sequence surrounding the major transcriptional start site. As this sequence has not yet been functionally analysed, it has been designated as an Initiator-like sequence (ILS). The same region in the collagenase promoter differs from a consensus Inr by 3 nucleotides, and from the *bkj* ILS by 4 nucleotides. The TATA boxes of the two promoters differ at two positions. Neither of the promoters contains a DPE or similar sequence.

To investigate the possibility that the *bkj* and collagenase TATA box and ILS elements contribute to the nature of promoter regulation by v-Jun, these sequences were exchanged between the two promoters. The –65TRE *bkj* promoter was mutated as well as wt(P)TRE *bkj*, to investigate any combined effect of TRE position and core promoter sequence on transcriptional regulation by v-Jun. The *bkj* Col TATA and *bkj* Col ILS mutagenic primer pairs (see Chapter 2.1.3.2 for all primer sequences) were used to convert the *bkj* TATA box and ILS, respectively, into the corresponding collagenase sequence (see Chapter 2.2.3.13 for details). The Col *bkj* TATA and Col *bkj* ILS primer pairs were used to convert the collagenase TATA box and ILS into the corresponding *bkj* sequence. Promoters with double element exchanges were created by two sequential rounds of mutagenic PCR, plasmid selection and sequencing.

The effect of TATA box exchange on transcription in CEFs and v-Jun transformed CEFs was investigated. 5μg each of wt(P)TRE *bkj*: Col TATA, –65TRE *bkj*: Col TATA and –73/+63 ColCAT: *bkj* TATA, as well as each parent plasmid, was transfected in triplicate into CEFs and v-Jun transformed CEFs, and relative CAT activities were determined as before. Results from a representative experiment are shown in Figure 3.7B.

Introduction of the collagenase TATA box into the wt(P)TRE *bkj* promoter reduced promoter activity in CEFs and v-Jun transformed CEFs over 4-fold. A much smaller decrease in activity was observed upon introduction of the collagenase TATA box into –65 TRE *bkj*. Both wt(P)TRE *bkj*: Col TATA and –65TRE *bkj*: Col TATA were activated more strongly in v-Jun transformed CEFs than in controls. Introduction of the *bkj* TATA box into –73/+63 ColCAT had no discernible effect on transcription in CEFs or v-Jun transformed CEFs.

The effect of ILS exchange was investigated in the same way. Results from a representative experiment are shown in Figure 3.7C. Introduction of the collagenase ILS

into both *bkj*-derived promoters decreased their overall activity in CEFs and v-Jun transformed CEFs, while retaining the difference in activation between the two cell types. Again, introduction of the *bkj* ILS into –73/+63 ColCAT had no discernible effect on transcriptional regulation.

The combined effect of TATA box and ILS exchange was also investigated. Results from a representative experiment are shown in Figure 3.7D. Introduction of the collagenase TATA box and ILS into the wt(P)TRE *bkj* promoter increased transcriptional activation around 2-fold in v-Jun transformed CEFs, whereas no major effect of the double exchange was seen in the context of the –65TRE *bkj* promoter. Introduction of the *bkj* TATA box and ILS into –73/+63 ColCAT increased promoter activity in CEFs and v-Jun transformed CEFs over 3-fold; however, the promoter was still repressed in v-Jun transformed CEFs relative to controls.

Figures 3.7B and C show that the sequences of the TATA box and ILS are important for transcriptional regulation of the *bkj* promoter by v-Jun. Substitution of either element for the corresponding collagenase sequence decreased promoter activity in CEFs and v-Jun transformed CEFs. This suggests that the *bkj* ILS is a functional Initiator, as mutation to the non-consensus collagenase sequence reduced transcription from the wt(P)TRE *bkj* and –65TRE *bkj* promoters. However, a consensus Inr does not appear to be necessary or sufficient for transcriptional activation by v-Jun; wt(P)TRE *bkj*: Col ILS and –65TRE *bkj*: Col ILS remained more active in v-Jun transformed CEFs than in controls, and the introduction of the *bkj* ILS into –73/+63 ColCAT did not alleviate transcriptional repression by v-Jun. Likewise, as the effects of TATA box exchange were similar to those of ILS exchange, v-Jun can not be said to depend on a particular TATA box sequence for transcriptional activation of target promoters.

Unexpectedly, the combined exchange of the TATA box and ILS elements increased the activity of the wt(P)TRE *bkj* promoter in v-Jun transformed CEFs and of –73/+63 ColCAT in CEFs. The reason for this is not clear. It is possible that the core elements of a particular promoter evolve together to activate transcription most efficiently when combined, and that this combination may serve to increase the activity of a heterologous promoter. However, the reason why both of the reciprocal double exchanges between wt(P)TRE *bkj* and –73/+63 ColCAT should increase promoter activity is not known. It is interesting that no such effect was seen with the –65TRE *bkj* promoter.

Overall, these results suggest that the differential regulation of the *bkj* and collagenase promoters by v-Jun can not be accounted for by the particular sequence of the TATA box or the ILS of either promoter. A combined effect of TRE position and core promoter sequences was also ruled out by the results of experiments involving core element exchanges between the collagenase and –65TRE *bkj* promoters.

A.

Position	-25	+1
Consensus	**TATAAAAN**	**PyPy A NPyPyPy**
bkj	AG **CATAAATA** GTCGCCTCATCTCCCAGCCACT	**CC A TTCC** CTG
Collagenase	TC **TATATATA** CAGAGGGAGCTTCCTAGCTGGG	**AT A TTGG** AGC

B.

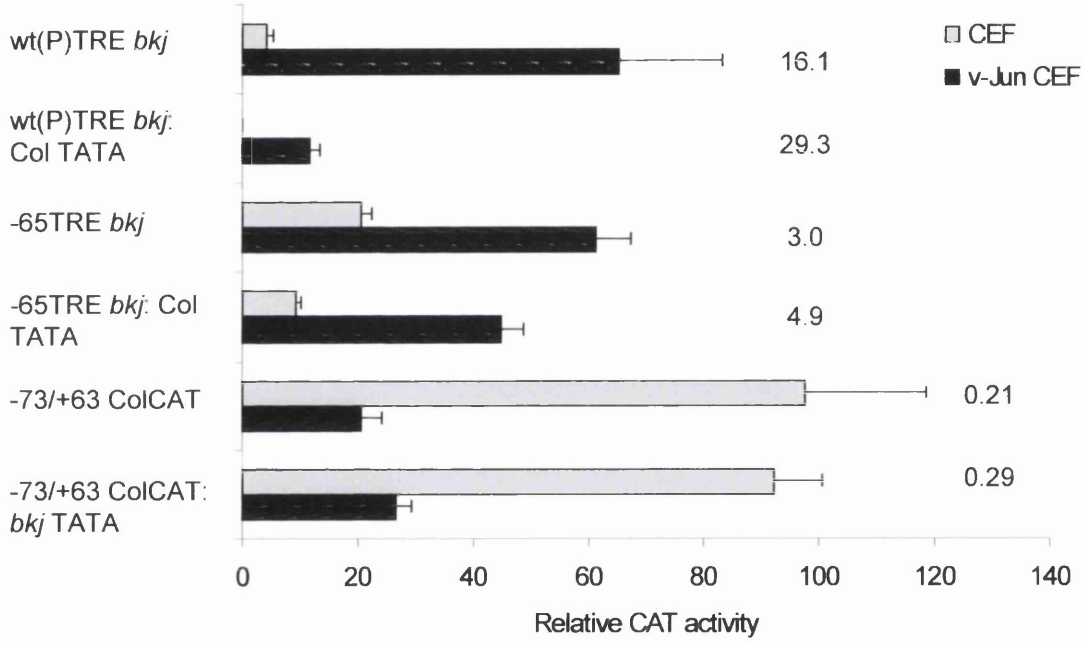


Figure 3.7

(A) Comparison of the *bkj* and collagenase core promoter sequences.

Putative core promoter elements are shown in bold type.

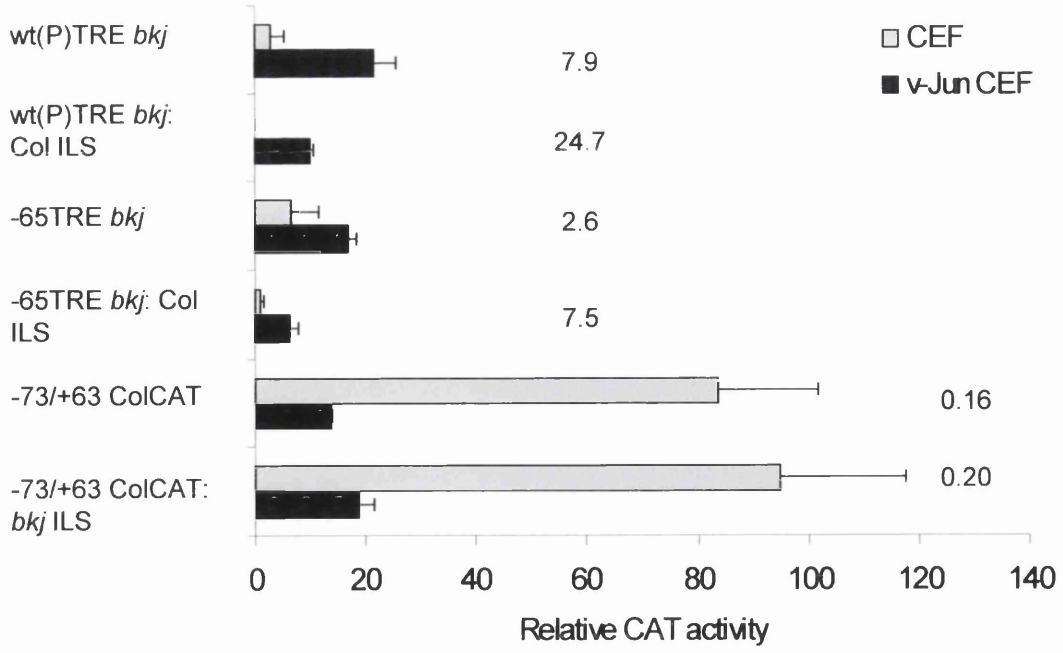
Arrows denote the major transcriptional start sites.

(B) Transcription from *bkj* and collagenase promoters with interchanged TATA box elements.

5μg each reporter plasmid was transfected in triplicate into CEFs and v-Jun CEFs. Fold activation of transcription in v-Jun CEFs compared to control CEFs is shown in each case.

Error bars denote standard deviations.

C.



D.

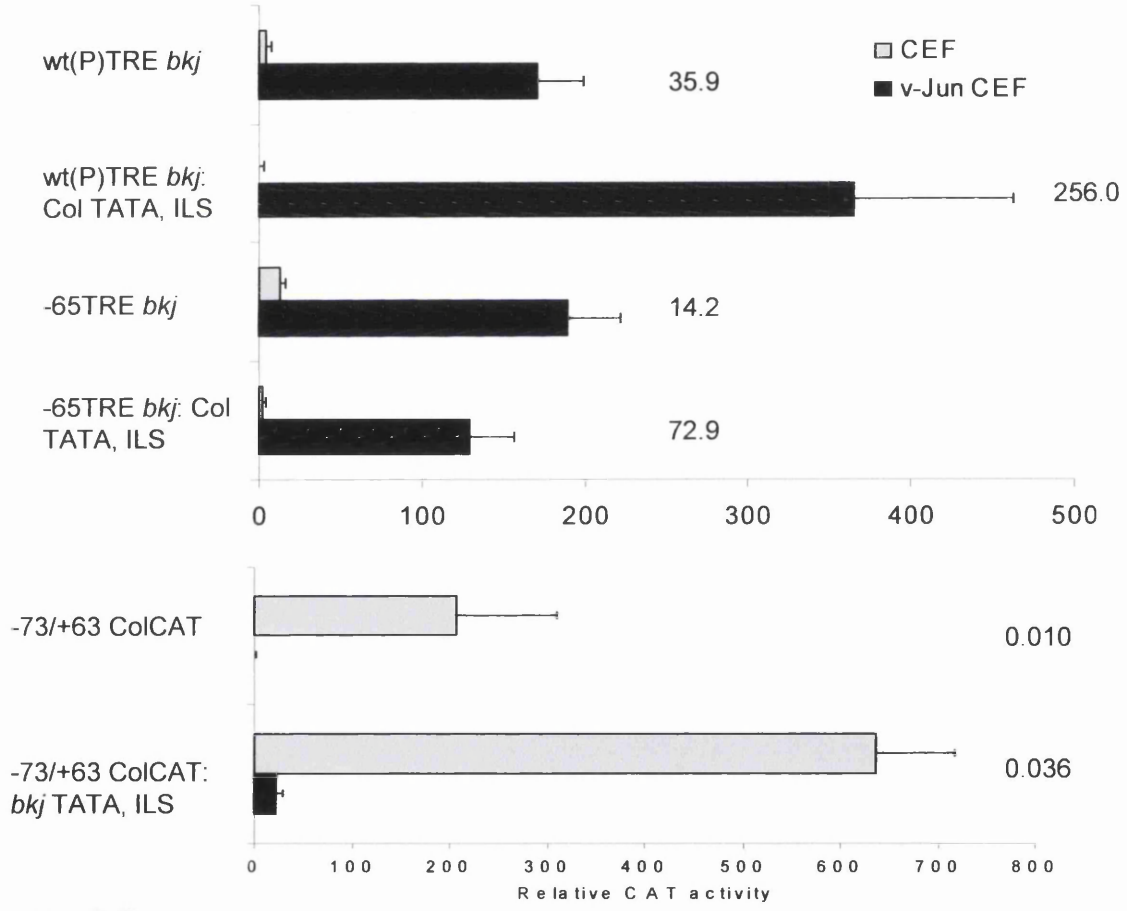


Figure 3.7

Transcription from *bkj* and collagenase promoters with interchanged ILS elements (C) or interchanged TATA box and ILS elements (D).

5μg each reporter plasmid was transfected in triplicate into CEFs and v-Jun CEFs. Fold activation of transcription in v-Jun CEFs compared to control CEFs is shown in each case.

Error bars denote standard deviations.

3.6 The contribution of upstream *bkj* promoter regions to transcriptional regulation by v-Jun.

As already described, the different effects of v-Jun on the *bkj* and collagenase promoters could not be explained by differences in the composition of the complex bound to each TRE, TRE position relative to the transcriptional start site, or core promoter sequences. To facilitate future comparisons between the two promoters, a derivative of the –65TRE *bkj* promoter was created, which lacks the *bkj* promoter regions upstream of its TRE. The HindIII –65TRE *bkj* 5' and XbaI *bkj* 3' primers (see Chapter 2.1.3.2) were used to amplify the –65TRE *bkj* promoter region from –66 to +13. The resulting PCR product was digested and ligated into pCAT-Basic as before (see Chapter 2.2.3.12).

5μg each of –66/+13 –65TRE *bkj* and full-length –65TRE *bkj* (shown as –929/+13 –65 TRE *bkj* for ease of comparison) was transfected into CEFs and v-Jun transformed CEFs, and relative CAT activities were determined as before. Results from a representative experiment are shown in Figure 3.8. –66/+13 –65TRE *bkj*, like the full-length plasmid, was activated more strongly in v-Jun transformed CEFs than in controls. However, its overall activity was much higher (more than 10-fold) than that of –929/+13 –65 TRE *bkj*. This suggests that, while the *bkj* promoter region from –929 to –67 has a general repressive effect on transcription, it is not responsible for the activation of the *bkj* promoter by v-Jun.

This result will simplify future comparative studies of gene promoters activated or repressed by v-Jun. As –66/+13 –65TRE *bkj* is, like the full-length wild-type *bkj* promoter, up-regulated in v-Jun transformed CEFs, it can be directly compared with the –73/+63 ColCAT promoter without the risk of interference from upstream sequences.

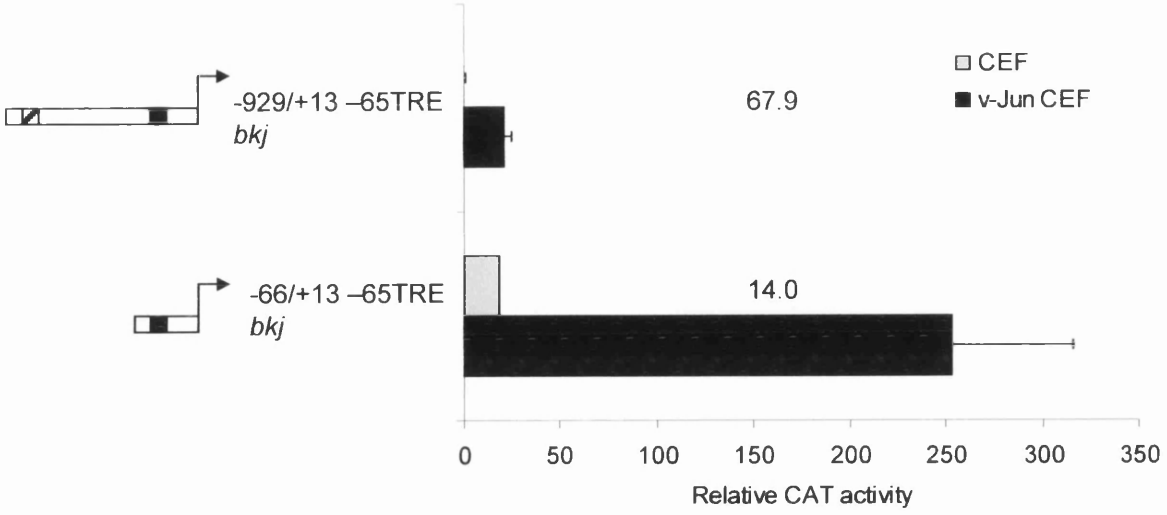


Figure 3.8

Transcription from the –65TRE *bkj* promoter and a mutant derivative lacking the upstream promoter region from –929 to –67.

Black boxes represent consensus TRE sequences; the hatched box represents the mutated TRE at position –815. Promoter diagrams are not to scale.

5μg each reporter plasmid was transfected in triplicate into CEFs and v-Jun CEFs. Fold activation of transcription in v-Jun CEFs compared to control CEFs is shown in each case.

Error bars denote standard deviations.

4 Results: Cell transformation and activation of v-Jun target genes.

4.1 Introduction and aims.

As described in Chapter 1.2.2.1, fusion of the v-Jun bZip region to the hormone-binding domain of human ER-α created a protein which exhibited estradiol-dependent transcriptional activation and cell transformation (Kruse *et al.*, 1997). This was thought to be due to activation of v-Jun target promoters by the ER-α AF-2 domain, as ΔvJ-hER lacks any other known TAD, and transcriptional activation and cell transformation by the chimaeric protein were not induced by tamoxifen, an AF-2 antagonist ligand.

While full-length v-Jun has been shown to both activate and repress target genes, the estradiol-activated ER-α AF-2 domain has not been shown to directly repress transcription. This suggests that cell transformation in the presence of estradiol is likely to be due to the activation of positive v-Jun target promoters by the AF-2 domain. This is supported by the observation that fusion of the strong transcriptional activation domain of VP16 to amino-terminally truncated v-Jun or c-Jun caused cell transformation and tumorigenesis (Schuur *et al.*, 1993).

The aim of this work was to investigate the relationship between transcriptional activation of v-Jun target genes and cell transformation, in particular the role of the ER-α AF-2 domain in hormone-dependent cell transformation by ΔvJ-hER.

4.2 Characterisation of cells expressing ΔvJ-hER.

4.2.1 Ligand-activated ΔvJ-hER down-regulates the endogenous c-Jun protein.

The first stage was to further characterise cells expressing the ΔvJ-hER protein. CEFs were transfected with 10μg RCAS-ΔvJ-hER, or RCAS-hER, expressing the ER-α hormone-binding domain (Kruse *et al.*, 1997), as a control. Transfection with replication-competent RCAS vectors results in uniform infection of the cell culture due to viral production. Cells were passaged 5 times in the presence of estradiol before plating onto 140mm tissue

culture dishes in the presence of estradiol, tamoxifen, or an equivalent volume of ethanol as a carrier control. WCEs were prepared after 48hr.

50μg of each WCE was resolved by SDS-PAGE, transferred to nitrocellulose membrane, and the membrane was probed with antibody specific for human ER-α (see Chapter 2.2.4.2 – 2.2.4.4). The membrane was also probed with 599-3 antibody to monitor expression of the endogenous c-Jun protein. Results are shown in Figure 4.1.

As described previously (Kruse *et al.*, 1997), the ΔvJ-hER protein ran with an apparent molecular weight of around 60kD, with higher mobility bands thought to represent protein degradation products also present. The hER protein ran as a single band of around 35kD. The addition of estradiol or tamoxifen did not affect the expression of either protein. Unexpectedly, the level of endogenous c-Jun protein was markedly reduced in ΔvJ-hER CEFs in the presence of estradiol, but not tamoxifen. This was a highly reproducible result, and was not due to ligand activation of the exogenous hER AF-2 domain or endogenous ER proteins, as treatment of hER CEFs with estradiol did not affect expression of c-Jun. Down-regulation of c-Jun, a negative target of v-Jun, therefore correlated with activation of the AF-2 domain of ΔvJ-hER.

Some degree of down-regulation of c-Jun has been demonstrated in CEFs expressing ΔvJ-hER or vJ1-hER (Kruse *et al.*, 1997), but this effect was not shown to be hormone-dependent. The fusion of c-Jun, JunD or Fos proteins to the ER-α ligand-binding domain has been shown to cause hormone-dependent down-regulation of natural repressed target genes (Crowe *et al.*, 2000; Fialka *et al.*, 1996; Francis *et al.*, 1995; Schuermann *et al.*, 1993; Superti-Furga *et al.*, 1991). However, this was in the context of the full-length Jun or Fos protein in each case, and hormone-dependent transcriptional repression was thought to be mediated by unmasking of the activity of the relevant Jun or Fos domain. In the case of ΔvJ-hER, the results shown in Figure 4.1 imply that the estradiol-bound AF-2 domain mediated target gene down-regulation, as the protein contains no other known TAD.

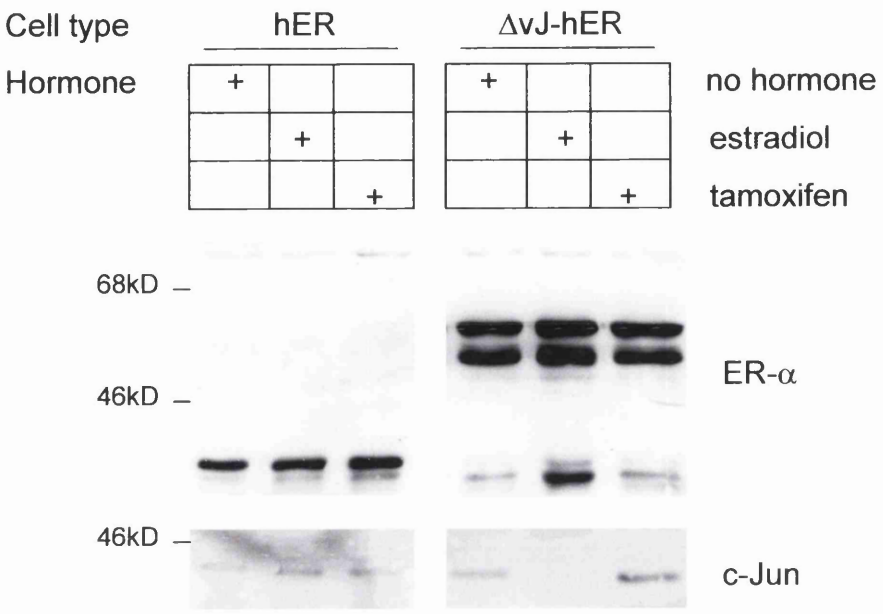


Figure 4.1

Western blot showing expression of the hER, ΔvJ-hER and endogenous c-Jun proteins in CEFs infected with RCAS-hER or -ΔvJ-hER, treated with estradiol, tamoxifen, or carrier control.

Blots were probed with antibodies specific for human ER-α (upper panel) and chicken c-Jun (lower panel).

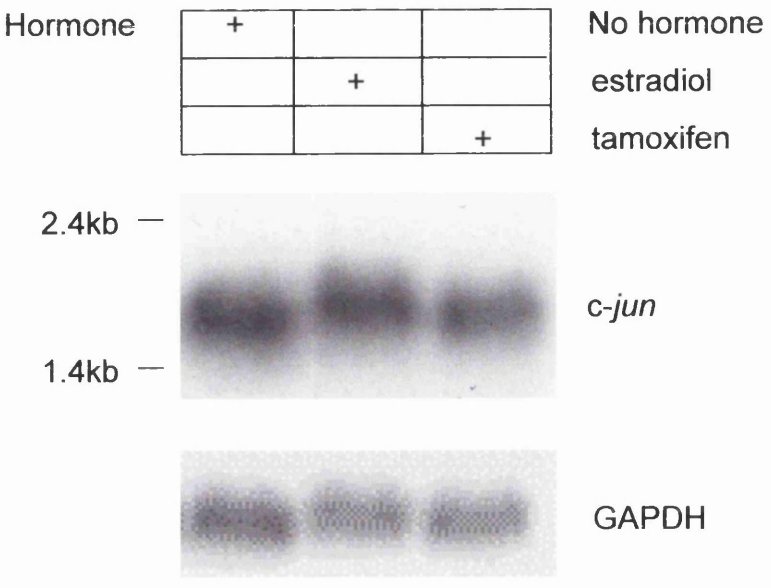
4.2.2 ΔvJ-hER does not down-regulate c-Jun at the level of transcription.

v-Jun directly represses transcription from a non-consensus TRE at position –72 in the c-*jun* promoter (Hussain *et al.*, 1998). It was important to determine whether the down-regulation of c-Jun protein in estradiol-treated ΔvJ-hER CEFs was also due to transcriptional repression, or to some other hormone-dependent mechanism. mRNA was isolated from ΔvJ-hER CEFs treated with estradiol, tamoxifen or carrier control, and a Northern blot was performed (see Chapter 2.2.3.16). The blot was probed using sequences from the c-*jun* 3' UTR, or the GAPDH coding sequence as a loading control. Figure 4.2A shows that treatment with estradiol did not decrease the level of c-*jun* mRNA in ΔvJ-hER CEFs.

Transcription from the c-*jun* promoter was assayed using the pJC6 reporter plasmid, which contains the murine c-*jun* promoter region from –225 to +150 (Han *et al.*, 1992). 5μg of the reporter vector was transfected in triplicate into ΔvJ-hER CEFs treated with estradiol, tamoxifen or carrier control, and relative CAT activities were determined as before. Figure 4.2B shows that transcription from the c-*jun* promoter was not repressed in the presence of estradiol.

These results contrast with the regulation of c-Jun by v-Jun. c-*jun* mRNA is absent in v-Jun transformed CEFs, and transcription from the c-*jun* promoter is repressed by v-Jun (Gao *et al.*, 1996; Hussain *et al.*, 1998). Figure 4.2 suggests that the down-regulation of c-Jun protein in estradiol-treated ΔvJ-hER CEFs does not occur at the level of transcription. The mechanism of c-Jun protein down-regulation is not known, but may involve alterations in processes such as protein translation or degradation.

A.



B.

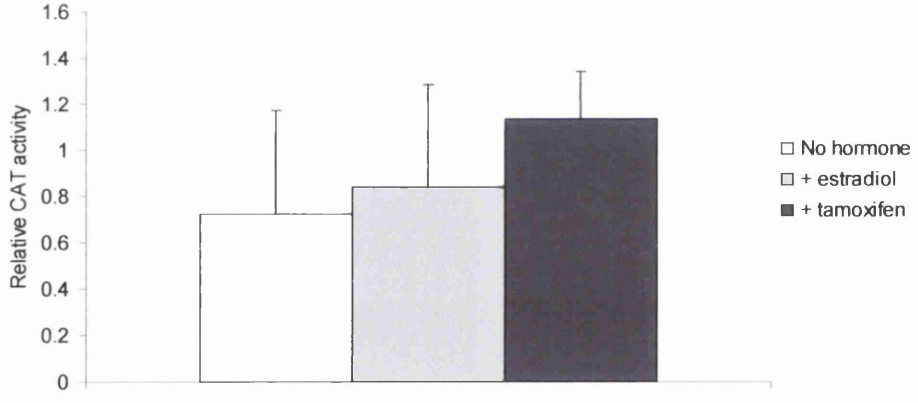


Figure 4.2

(A) Northern blot showing expression of c-*jun* and GAPDH mRNA in ΔvJ-hER CEFs treated with estradiol, tamoxifen or carrier control.

Blots were probed with radio-labelled DNA containing chicken c-*jun* 3' UTR (upper panel) or chicken GAPDH coding (lower panel) sequences.

(B) Activity of the c-*jun* promoter in ΔvJ-hER CEFS treated with estradiol, tamoxifen or carrier control.

5μg reporter vector was transfected in triplicate.

Error bars denote standard deviations.

4.2.3 ΔvJ-hER binds constitutively to TREs.

Chimaeric proteins consisting of Jun or Fos fused to the ER-α hormone-binding domain have, in some cases, been shown to be regulated in a hormone-dependent manner at the level of nuclear translocation or DNA binding (Fialka *et al.*, 1996; Francis *et al.*, 1995; Kim *et al.*, 1996). If this is also the case for ΔvJ-hER, then cell transformation in the presence of estradiol may be an indirect effect of nuclear accumulation or binding of the protein to TREs, rather than a direct effect of AF-2 domain activation at v-Jun target promoters. The sub-cellular location and DNA binding activity of ΔvJ-hER were therefore investigated in cells treated with estradiol, tamoxifen or carrier control.

Confocal immunocytochemistry performed by Dr. E. Black of the Beatson Institute revealed that the hER and ΔvJ-hER proteins were predominately nuclear in the presence of estradiol, tamoxifen and carrier control (data not shown).

An EMSA was carried out to visualise the protein complexes bound to TREs in hER and ΔvJ-hER CEFs treated with estradiol, tamoxifen or carrier control. 10μg of each WCE was incubated with radio-labelled probe containing the collagenase TRE (see Chapter 2.1.3.1). Results are shown in Figure 4.3A. Each complex ran with a similar electrophoretic mobility. A decrease was observed in the intensity of the complex bound to the collagenase TRE in ΔvJ-hER CEFs treated with estradiol. This was a highly reproducible result.

Bound complexes were analysed by antibody super-shifts. WCEs were pre-incubated with antibodies specific for c-Jun (KM-1X), human ER-α, or the broad-specificity Fos family antibody (K-25). Results are shown in Figure 4.3B. In hER CEFs, the majority of each complex was super-shifted by the c-Jun antibody, and complexes were completely shifted by the pan-Fos antibody. This is in line with previous observations in CEFs ((Hawker *et al.*, 1993; Kilbey *et al.*, 1996) and Figure 3.3C of this work), and indicates that expression of hER and treatment with estradiol or tamoxifen did not disrupt the usual c-Jun / Fos dimers bound to TREs. As expected, complexes from hER CEFs were not super-shifted by the ER-α antibody, indicating that hER can not bind to TREs in the absence of the v-Jun DBD.

Complexes from ΔvJ-hER CEFs treated with estradiol, tamoxifen or carrier control were also super-shifted by the c-Jun and pan-Fos antibodies. Pre-incubation with the ER-α antibody caused a slight decrease in the intensity of the bound complex in each case, and

appeared to cause the formation of a low-mobility complex which did not migrate into the gel (Figure 4.3B, see arrows), suggesting that ΔvJ-hER might bind TREs in a hormone-independent manner. However, this putative complex was present in an area of the gel with some background radiation, due to retention of a fraction of each complex in the wells of the polyacrylamide gel, and therefore could not be identified with any certainty.

Complexes were analysed further using antibodies specific for the individual members of the Fos family. Figure 4.3C shows that the Fra-2 specific antibody super-shifted the complexes bound to the collagenase TRE in hER and ΔvJ-hER CEFs treated with estradiol or carrier control. No other specific antibody had any effect. These results show that the protein complexes bound to TREs in hER CEFs contain predominately c-Jun / Fra-2 dimers. In ΔvJ-hER CEFs, the complex bound to TREs appears to consist of c-Jun / Fra-2, and possibly ΔvJ-hER / Fra-2 dimers, regardless of hormone treatment.

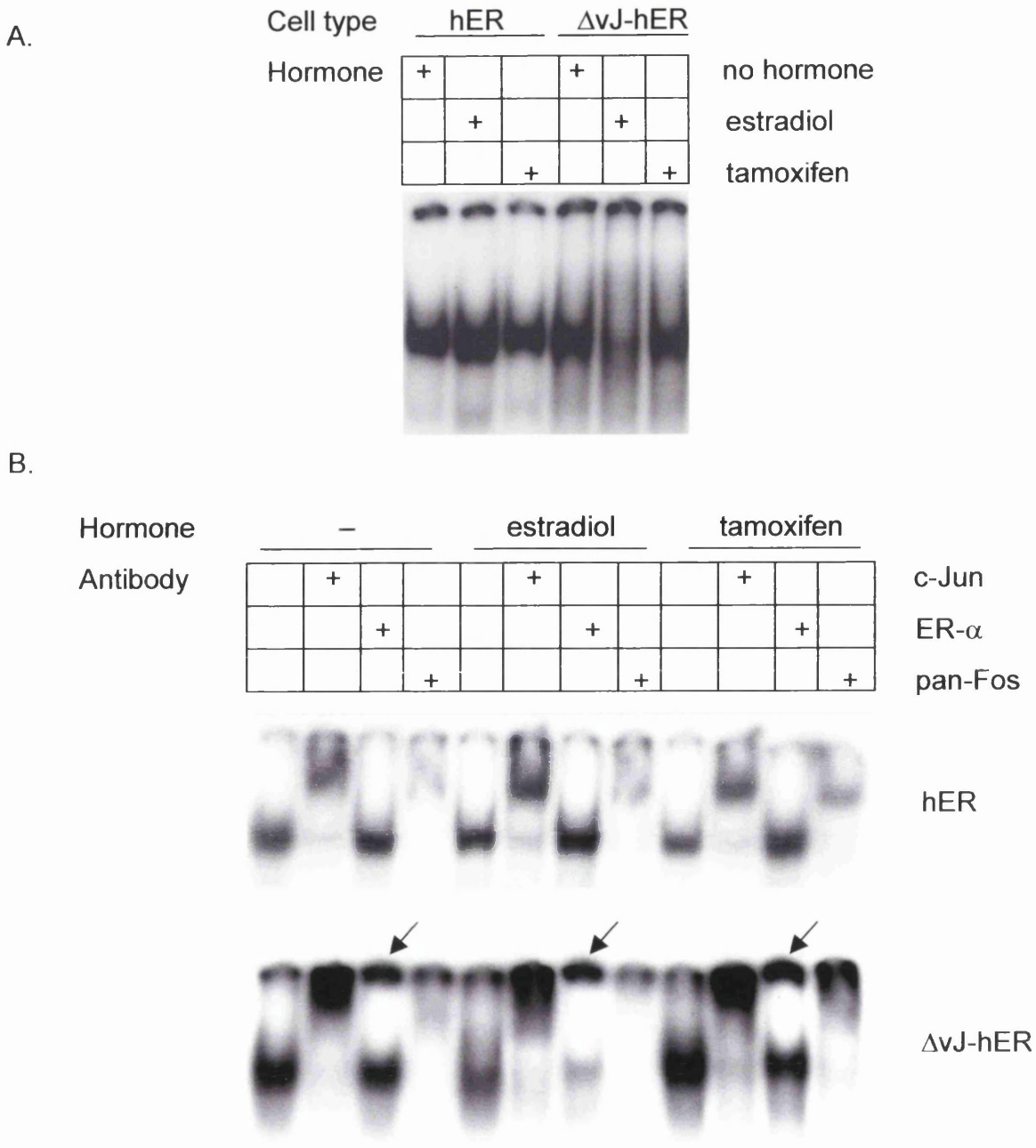


Figure 4.3

(A) EMSA showing protein complexes bound to the collagenase TRE in CEFs infected with RCAS-hER and -ΔvJ-hER, treated with estradiol, tamoxifen, or carrier control.

(B) Analysis of protein complexes bound to the collagenase TRE in hER (upper panel) and ΔvJ-hER (lower panel) CEFs treated with estradiol, tamoxifen, or carrier control, using antibodies specific for c-Jun, ER-α or the Fos family.

Extracts were pre-incubated with 3μl antibody, as indicated, before addition of radio-labelled probe.

Arrows indicate the putative complex super-shifted by the ER-α antibody.

C.

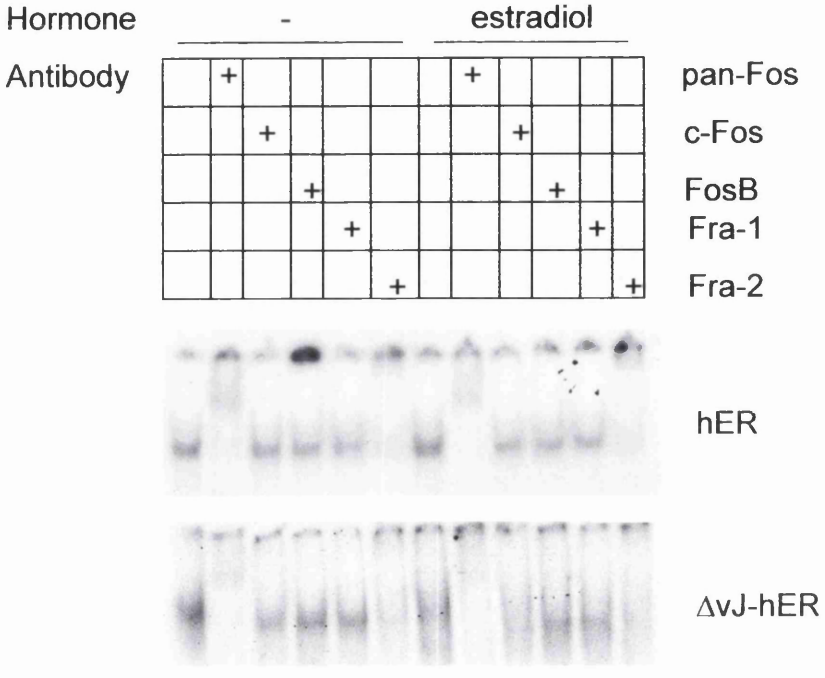


Figure 4.3

(C) Analysis of protein complexes bound to the collagenase TRE in hER and ΔvJ-hER CEFs treated with estradiol or carrier control, using antibodies specific for Fos family proteins.

Extracts were pre-incubated with 3μl antibody, as indicated, before addition of radio-labelled probe.

Biotinylated-oligonucleotide capture experiments were carried out to enable unambiguous identification of the proteins bound to TREs in hER and ΔvJ-hER CEFs treated with estradiol or carrier control. 500μg of each WCE was pre-cleared by incubation with streptavidin-conjugated paramagnetic particles. Complexes bound to TREs were captured by incubation with particles bound to a biotinylated oligonucleotide containing multiple copies of the collagenase TRE (see Chapter 2.2.5.3 and 2.2.5.4). EMSA analysis of samples removed from the supernatant after pre-clearing and oligonucleotide capture revealed depletion of TRE-binding complexes from WCEs after incubation with the biotinylated oligonucleotide (Figure 4.4A), indicating that these complexes were successfully captured.

Proteins bound to the biotinylated oligonucleotide were eluted after washing and analysed by Western blotting (Figure 4.4B). ΔvJ-hER bound to TREs in a hormone-independent manner, while the hER protein, which lacks the v-Jun DBD, did not bind TREs. c-Jun bound TREs in a hormone-independent manner in hER CEFs. In ΔvJ-hER CEFs, detectable levels of c-Jun bound TREs in the absence, but not the presence, of estradiol. Equal amounts of Fra-2 bound to TREs in each case. The binding of all proteins was specific for the biotinylated oligonucleotide, as no protein was detected in samples eluted after the pre-clearing stage.

The results from Figure 4.4 show that c-Jun / Fra-2 dimers bind TREs in hER CEFs treated with estradiol or carrier control. This is in agreement with antibody supershift experiments (Figure 4.3B, C). In ΔvJ-hER CEFs, the ΔvJ-hER protein binds TREs in a hormone-independent manner. In untreated cells, c-Jun also binds TREs; however, no c-Jun was detected bound to TREs in estradiol-treated cells. This is consistent with the down-regulation of c-Jun in these cells (Figure 4.1), and provides an explanation for the estradiol-induced decrease in the intensity of the protein complex bound to the collagenase TRE (Figure 4.3A); both c-Jun / Fra-2 and ΔvJ-hER / Fra-2 dimers bind TREs in untreated cells, whereas treatment with estradiol decreases the c-Jun / Fra-2 component of the complex.

Previous experiments in estradiol-treated ΔvJ-hER CEFs clearly showed a super-shift of the complex bound to TREs with a c-Jun specific antibody (Figure 4.3B). This would suggest that down-regulation of endogenous c-Jun in the presence of estradiol is not complete, and that residual protein binds TREs at levels too low to allow detection by

Western blot methods. However, the most important conclusion of this work is that the binding of ΔvJ-hER to TREs is hormone-independent.

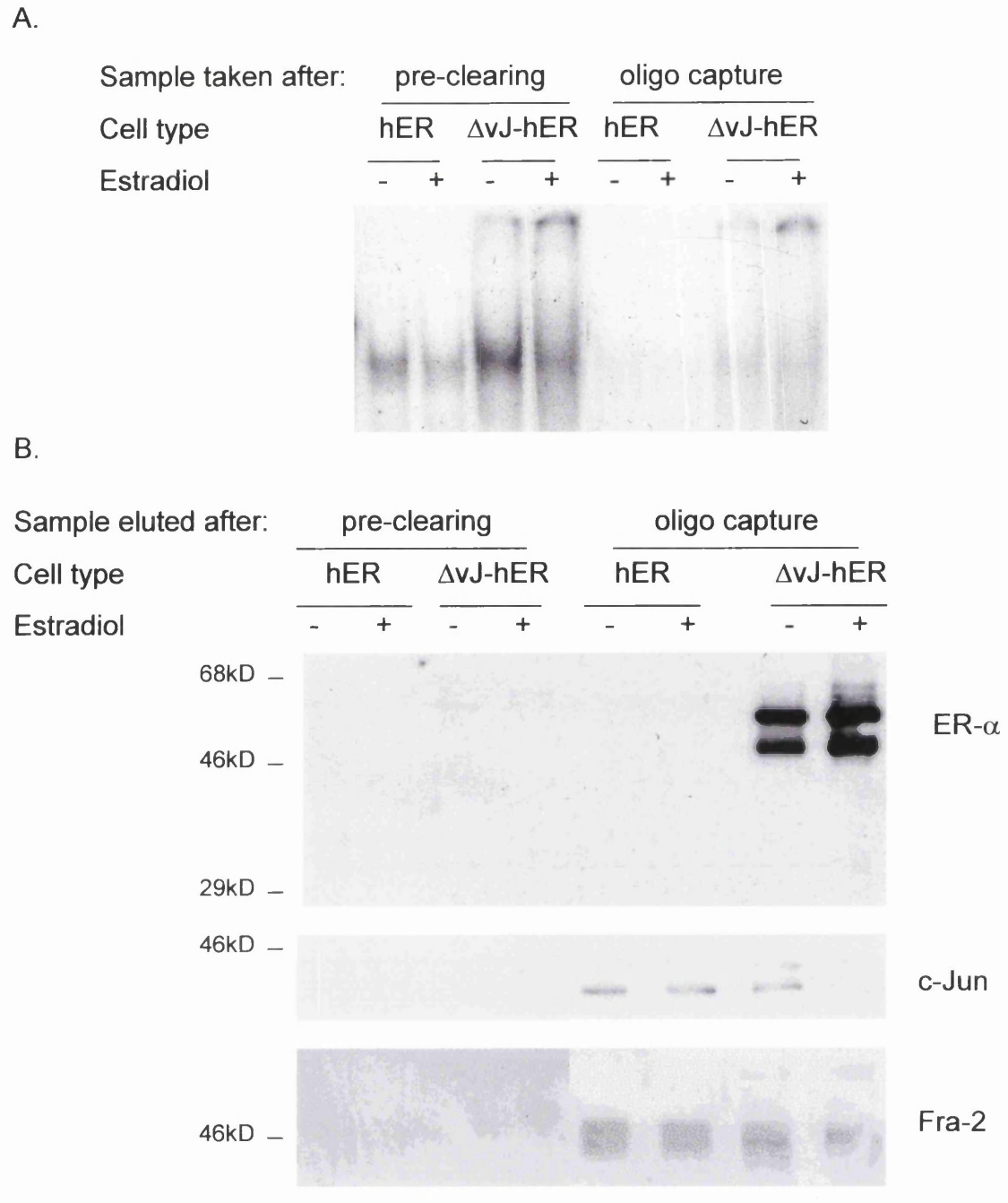


Figure 4.4

Biotinylated-oligonucleotide capture of DNA-binding complexes

(A) EMSA analysis of supernatants removed after the pre-clearing stage and after biotinylated-oligonucleotide capture of DNA-binding complexes. 4% of each supernatant was incubated with radio-labelled collagenase TRE.

(B) Western blot analysis of proteins eluted from paramagnetic particles after the pre-clearing stage and after biotinylated-oligonucleotide capture of DNA-binding complexes.

Blots were probed with antibodies specific for human ER-α (upper panel), chicken c-Jun (middle panel) or Fra-2 (lower panel).

4.3 The role of the ER-α AF-2 domain in hormone-dependent transcriptional activation and cell transformation by ΔvJ-hER.

As described in Chapter 1.2.2.1, the ER-α AF-2 domain has been proposed to account for transcriptional activation by ΔvJ-hER in the presence of estradiol, but not the antagonist ligand tamoxifen. Since ΔvJ-hER binds constitutively to TREs (see Figure 4.4), cell transformation in the presence of estradiol is likely to be a direct effect of activation of the AF-2 domain at v-Jun target promoters. This hypothesis was investigated by the introduction of an inactivating mutation into the AF-2 domain of ΔvJ-hER. The M543A / L544A double amino acid substitution within helix 12 of the ER-α AF-2 domain has previously been shown to specifically abolish hormone-dependent transcriptional activation in the context of the full-length ER-α (Danielian *et al.*, 1992) and a Gal4·ER·VP16 fusion protein (Stafford and Morse, 1998), without affecting hormone binding. This mutation was introduced into RCAS-ΔvJ-hER to create RCAS-ΔvJ-hER mt; see Chapter 2.2.3.14.

4.3.1 The mutant protein is expressed at a lower level than wild-type ΔvJ-hER.

CEFs were transfected with 10μg RCAS-ΔvJ-hER wt; -ΔvJ-hER mt; -AV2, expressing a fusion of the v-Jun C-terminal domains to the VP16 TAD (Schuur *et al.*, 1993); pV and RCAS, to reconstitute ASV17; or empty RCAS vector as control. ΔvJ-hER wt and mt CEFs were each maintained in the presence or the absence of estradiol. After 3-4 passages, cells were plated onto 140mm plastic tissue culture dishes, in the presence of estradiol or carrier control as appropriate. WCEs were prepared after 48hr.

40μg of each WCE was resolved by SDS-PAGE and subjected to Western blotting as before. The ΔvJ-hER mt protein ran as a major band of around 60kD, with small amounts of a higher mobility product also detected (Figure 4.5, upper panel). The expression of the protein was not affected by passaging in the presence of estradiol. This is in contrast to ΔvJ-hER wt, where expression of the protein was higher in cells passaged in the presence of estradiol. As previously reported (Schuur *et al.*, 1993), the AV2 protein ran as a single band of around 32kD (Figure 4.5, middle panel).

As described previously ((Gao *et al.*, 1996; Hussain *et al.*, 1998; Kilbey *et al.*, 1996), Figure 4.1 of this work), the c-Jun protein was down-regulated in v-Jun transformed CEFs, and in ΔvJ-hER wt CEFs treated with estradiol (Figure 4.5, lower panel). Down-regulation of c-Jun was also observed in CEFs infected with RCAS-AV2. However, little if any decrease in c-Jun expression was observed in ΔvJ-hER mt CEFs treated with estradiol or carrier control. This suggests that hormone-dependent down-regulation of c-Jun by ΔvJ-hER wt requires a functional AF-2 domain.

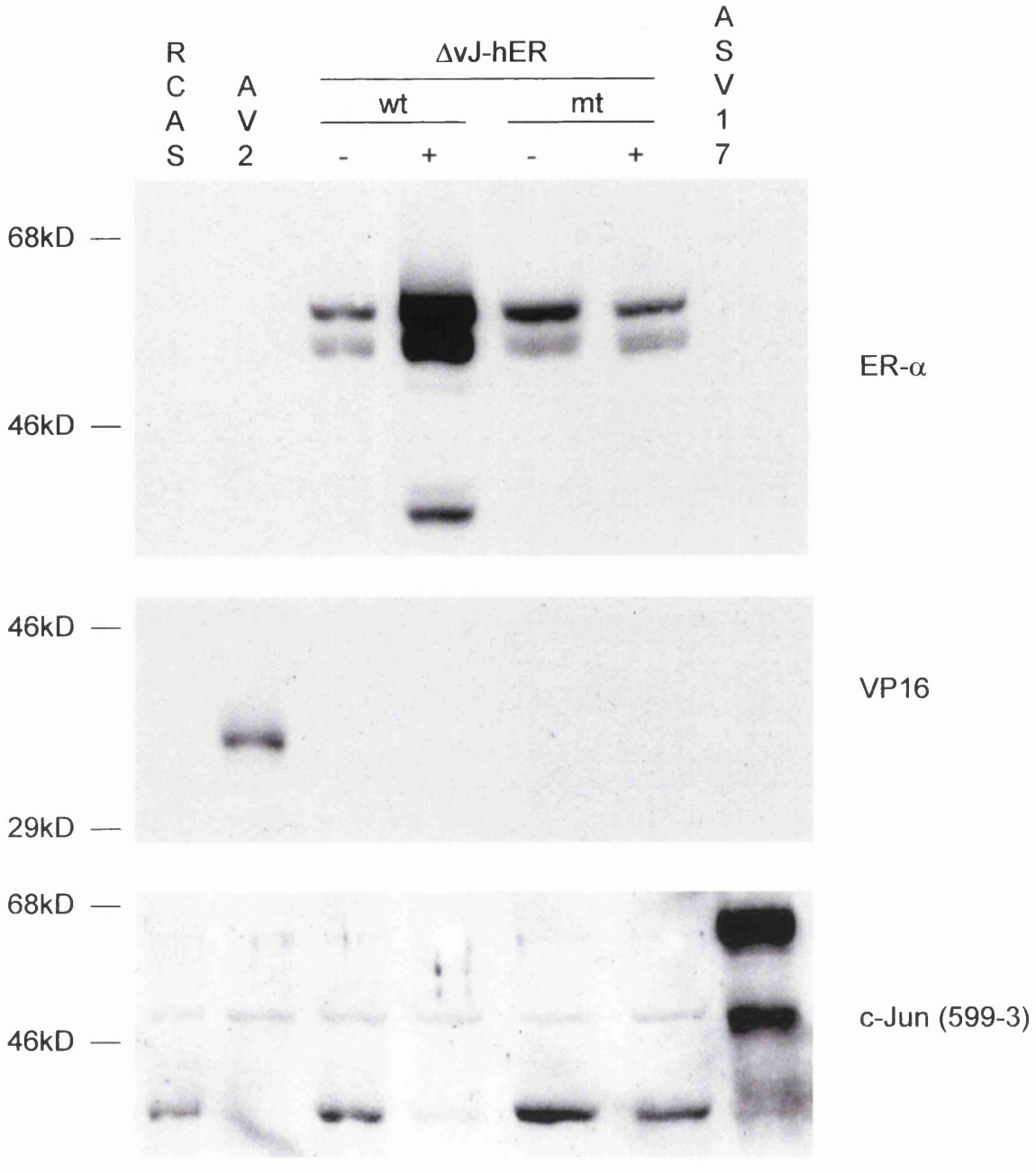


Figure 4.5

Western blot analysis of protein expression in CEFs infected with RCAS, RCAS-AV2, ASV17, or RCAS-ΔvJ-hER wt and mt, grown in the presence of estradiol (+) or carrier control (-).

Blots were probed with antibodies specific for human ER-α (upper panel), VP16 (middle panel), or chicken c-Jun (lower panel).

It is interesting to note that the expression levels of ΔvJ-hER mt were lower than those of ΔvJ-hER wt in cells passaged in the presence of estradiol (Figure 4.5, upper panel). This was a highly reproducible result, observed in four independent rounds of transfection with RCAS-ΔvJ-hER wt and mt. The expression of the two proteins during the infection process was determined. CEFs were transfected with 10μg RCAS-ΔvJ-hER wt or mt and passaged in the presence or absence of estradiol. A portion of the trypsinised cells was retained at each passage, and WCEs were prepared.

20μg of WCEs prepared from cells at passage numbers 3 to 5 were resolved by SDS-PAGE, subjected to Western blotting, and probed with antibody specific for human ER-α. Figure 4.6 shows that the expression level of ΔvJ-hER wt was consistently higher in cells passaged in the presence of estradiol, with a greater increase compared to controls observed at the later stages of transfection. This was a reproducible result.

As expression of ΔvJ-hER wt has been reported to increase the rate of cell proliferation in the presence of estradiol, but inhibit cell proliferation in the absence of ligand (Kruse *et al.*, 1997), it may be that high levels of expression of this protein in the presence of estradiol confer a selective advantage on RCAS-ΔvJ-hER wt infected cells. This could lead to an increased rate of spread of the RCAS-ΔvJ-hER wt virus in the presence of estradiol. The increased expression of the ΔvJ-hER wt protein seen in Figure 4.5 may therefore reflect a higher proportion of cells in the population expressing this protein, rather than higher levels of expression in each cell. The fact that passaging in the presence of estradiol did not increase the expression of ΔvJ-hER mt suggests that mutation of the AF-2 domain abolished the hormone-dependent increase in the rate of cell proliferation observed with ΔvJ-hER wt.

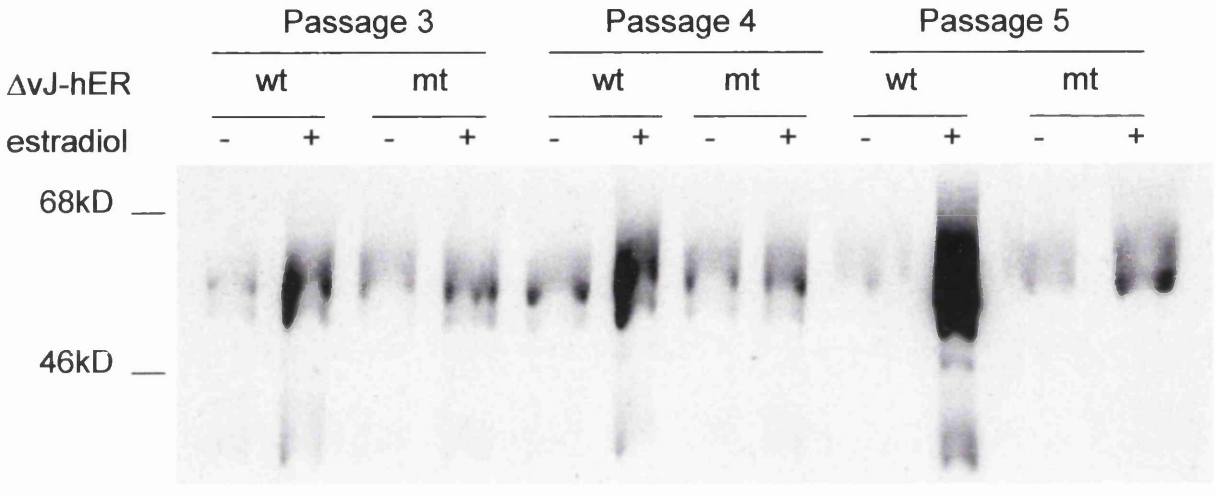


Figure 4.6

Western blot analysis of ΔvJ-hER wt and mt expression in CEFs 3 to 5 passages post-transfection in the presence of estradiol (+) or carrier control (-).

Blots were probed with antibody specific for human ER-α.

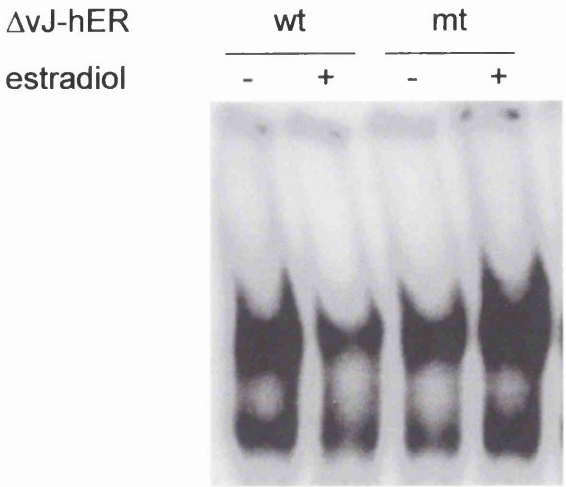
4.3.2 ΔvJ-hER mt binds constitutively to TREs.

The introduction of AF-2 domain mutations into full-length ER-α did not affect binding of the protein to DNA (Danielian *et al.*, 1992). However, the effect of such mutations on a heterologous DBD in the context of a fusion protein has not previously been determined. An EMSA was carried out to visualise the complexes bound to TREs in ΔvJ-hER mt CEFs treated with estradiol or carrier control. 10μg of each WCE was incubated with radio-labelled probe containing the collagenase TRE (Figure 4.7A). In contrast to the results of similar experiments with ΔvJ-hER wt CEFs, no decrease in the intensity of the bound complex was observed in the presence of estradiol.

Biotinylated-oligonucleotide capture experiments were carried out to identify the proteins bound to the collagenase TRE in ΔvJ-hER CEFs treated with estradiol or carrier control. 265μg of each WCE was pre-cleared and then incubated with paramagnetic particles bound to a biotinylated collagenase TRE oligonucleotide, as before. Proteins were eluted and analysed by Western blotting (Figure 4.7B).

The ΔvJ-hER mt and c-Jun proteins bound specifically to the collagenase TRE in a hormone-independent manner. In contrast to ΔvJ-hER wt, the higher mobility groups thought to represent proteolytic degradation products did not appear to bind DNA (compare Figure 4.7B and Figure 4.4B). The reason for this difference is not known. However, it is clear that the full-length ΔvJ-hER mt protein bound DNA in a hormone-independent manner. The different appearance of the complexes bound to TREs in ΔvJ-hER wt and mt CEFs treated with estradiol (Figure 4.7A) is therefore likely to be due to down-regulation of c-Jun by ligand-activated ΔvJ-hER wt, but not its mutant derivative. These results show that introduction of the M543A / L544A mutation into the AF-2 domain of ΔvJ-hER does not affect the ability of the protein to bind TREs.

A.



B.

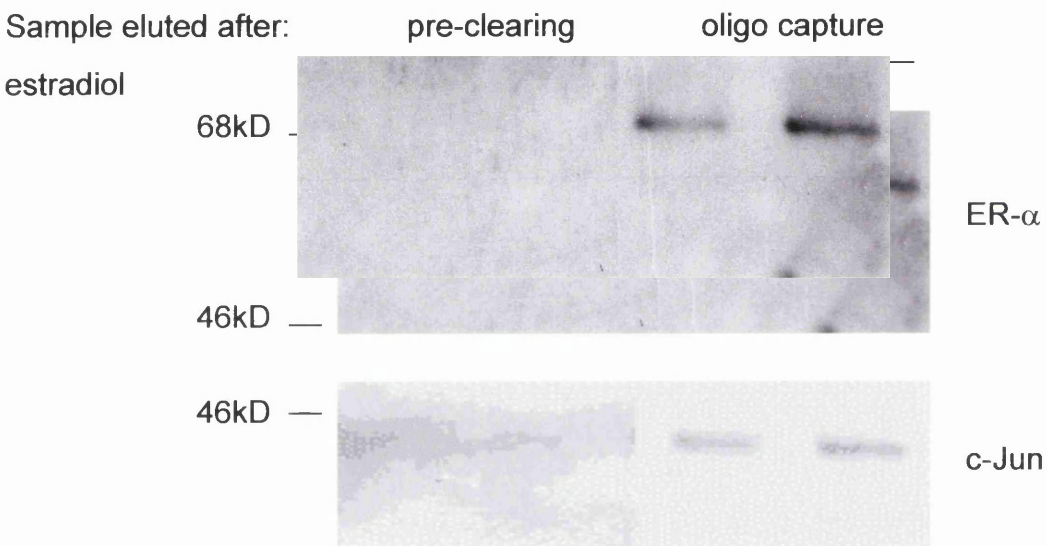


Figure 4.7

(A) EMSA showing protein complexes bound to the collagenase TRE in ΔvJ-hER wt and mt CEFs, treated with estradiol (+) or carrier control (-).

(B) Western blot analysis of proteins eluted from paramagnetic particles after pre-clearing and after biotinylated-oligonucleotide capture of DNA-binding complexes from ΔvJ-hER mt CEFs treated with estradiol (+) or carrier control (-).

Blots were probed with antibodies specific for human ER-α (upper panel) or chicken c-Jun (lower panel).

4.3.3 ΔvJ-hER mt does not transactivate the *bkj* promoter in the presence of estradiol.

The role of the AF-2 domain in hormone-dependent transcriptional activation of v-Jun target promoters by ΔvJ-hER was investigated. 5μg of the wt(P)TRE *bkj* reporter vector (see Chapter 2.2.3.12) was transfected in triplicate into ΔvJ-hER wt and mt CEFs, grown in the presence of estradiol or carrier control; AV2 CEFs; and RCAS and ASV17 CEFs as controls. Relative CAT activities were determined as before. Results from a representative experiment are shown in Figure 4.8.

As shown previously (Figure 3.4), the wt(P)TRE *bkj* promoter was activated more strongly in ASV17 CEFs than in RCAS controls. The *bkj* promoter was also strongly activated in AV2 CEFs, to a level around 60% of that observed in ASV17 CEFs. This contrasts with previous reports, in which AV2 only weakly activated transcription from v-Jun target promoters (Schuur *et al.*, 1993). The discrepancy may be due to the different promoters studied in each case.

As previously reported (Kruse *et al.*, 1997), transcriptional activation of *bkj* by ΔvJ-hER wt was hormone-dependent, with expression from the promoter induced around 8-fold in estradiol-treated cells. The overall level of transcriptional activation by ligand-activated ΔvJ-hER wt was around 30% of that induced in ASV17 CEFs; this is consistent with previous reports (Kruse *et al.*, 1997). In contrast, treatment with estradiol did not induce activation of the *bkj* promoter by ΔvJ-hER mt, indeed the promoter was reproducibly down-regulated in hormone treated cells. These results strongly support the hypothesis that the ER-α AF-2 domain is responsible for hormone-dependent activation of v-Jun target genes by ΔvJ-hER.

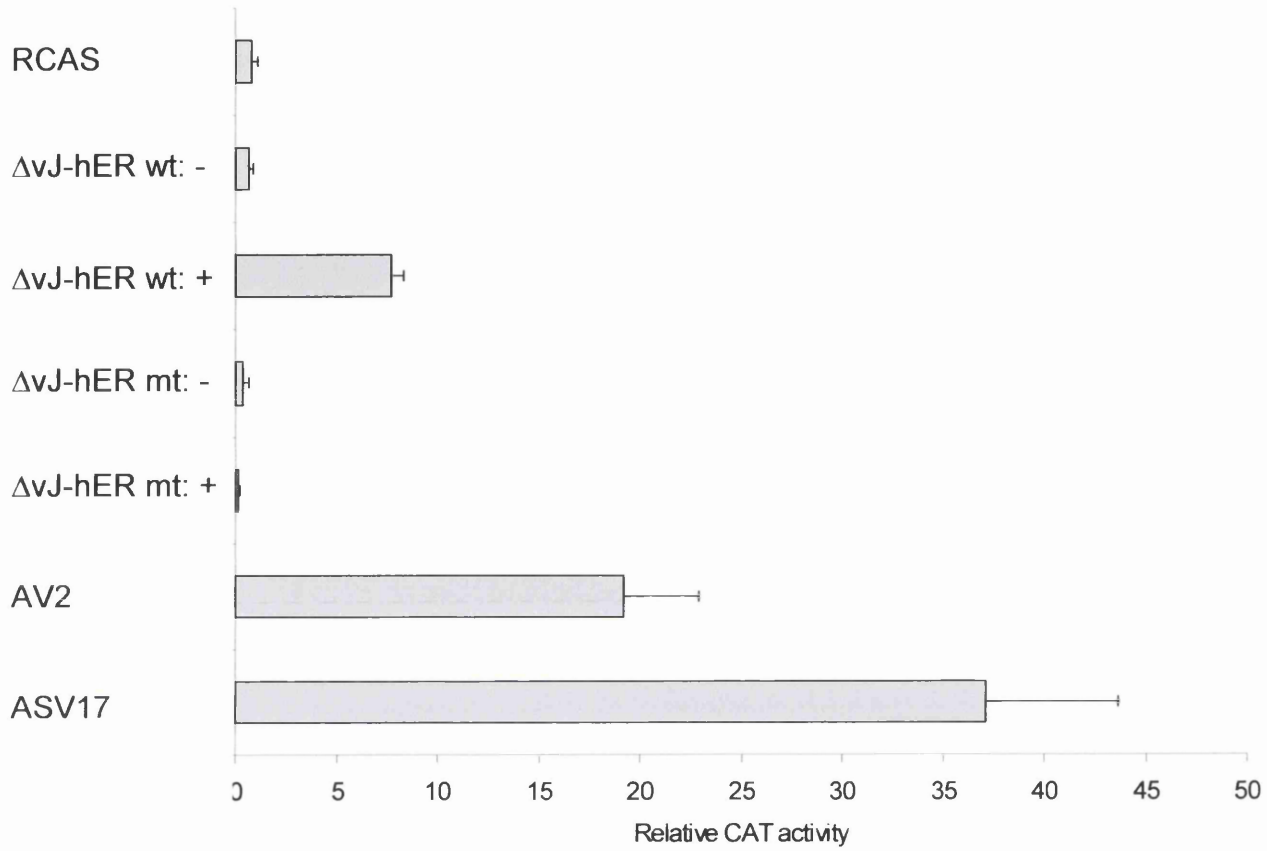


Figure 4.8

Activity of the wt(P)TRE *bkj* promoter in CEFs infected with RCAS, RCAS-ΔvJ-hER wt in the presence (+) and absence (-) of estradiol, -ΔvJ-hER mt in the presence (+) and absence (-) of estradiol, -AV2 or ASV17.

5μg reporter plasmid was transfected in triplicate into each cell type.

Error bars denote standard deviations.

4.3.4 ΔvJ-hER mt does not induce hormone-dependent cell transformation.

4.3.4.1 Cell morphology.

Cells infected with the ASV17 virus adopt a characteristic elongated morphology compared to control CEFs, are more refractile, and grow in parallel arrays ((Cavalieri *et al.*, 1985; Maki *et al.*, 1987), Figure 4.9A and B of this work). CEFs expressing AV2 adopt a similar morphology to ASV17 transformed cells (Figure 4.9C).

In the absence of estradiol, the morphology of ΔvJ-hER wt and mt CEFs resembles that of control cells (Figure 4.9D and F). Estradiol-treated ΔvJ-hER wt CEFs adopt a morphology similar to ASV17 infected cells (Figure 4.9E). However, CEFs expressing ΔvJ-hER mt do not undergo a change in morphology in the presence of estradiol; cells remain similar in appearance to control CEFs (Figure 4.9G). This suggests that a functional AF-2 domain is required for the change in morphology of cells expressing ΔvJ-hER wt in the presence of estradiol.

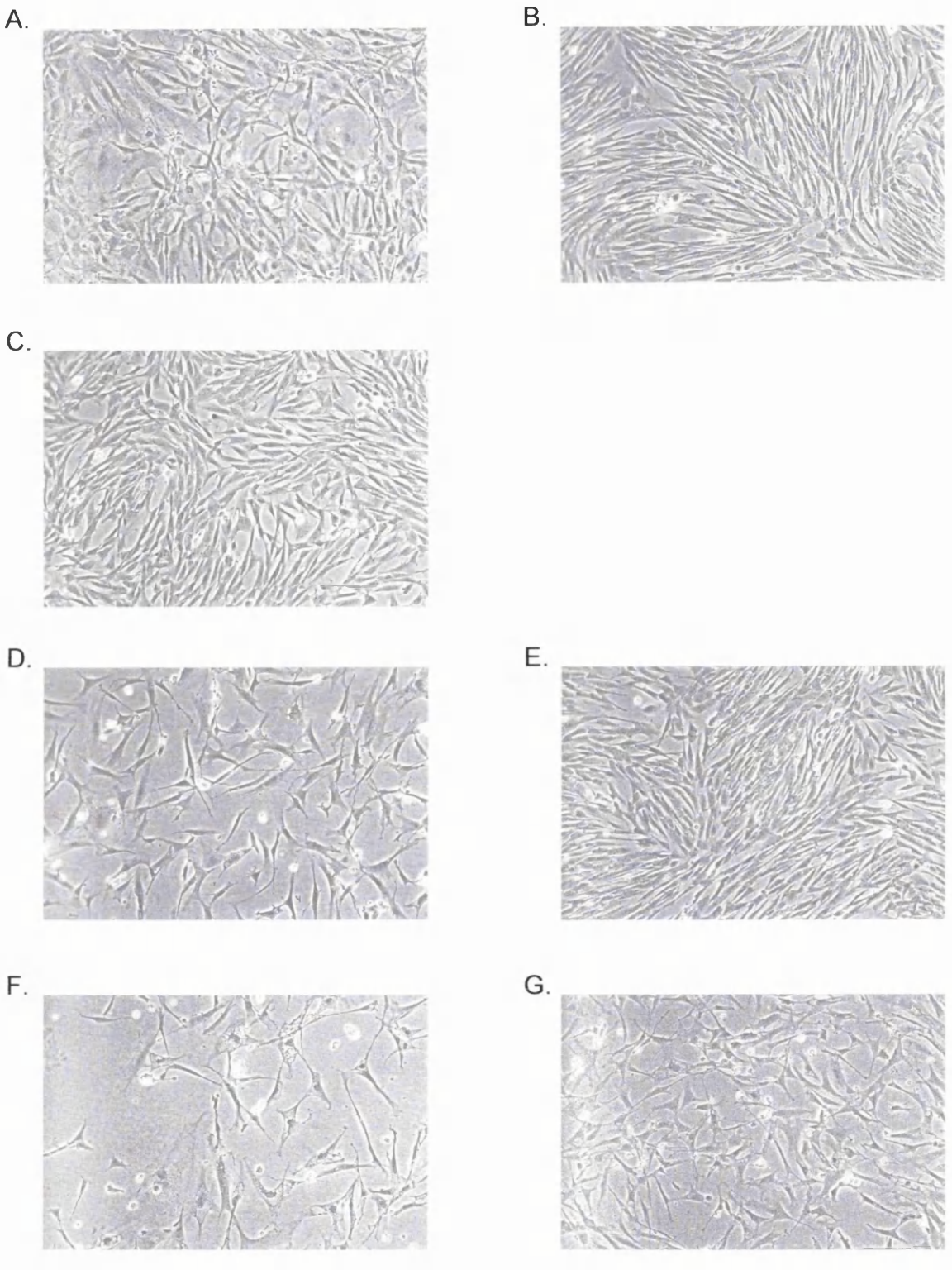


Figure 4.9

CEFs infected with RCAS (A); ASV17 (B); RCAS-AV2 (C); -ΔvJ-hER wt in the presence of carrier control (D) or estradiol (E); -ΔvJ-hER mt in the presence of carrier control (F) or estradiol (G).

4.3.4.2 Rate of cell proliferation.

It has been reported that the ΔvJ-hER wt protein mediates a hormone-dependent increase in the rate of cell proliferation (Kruse *et al.*, 1997). Cell growth assays were carried out to investigate the role of the ER-α AF-2 domain in this process. Equal numbers of CEFs infected with RCAS-ΔvJ-hER wt or mt, in the presence of estradiol; RCAS-AV2; ASV17 or RCAS were plated onto 60mm plastic tissue culture dishes, and cell numbers determined every 24hr (see Chapter 2.2.2.4). Results of a representative experiment are shown in Figure 4.10.

As previously reported (Clark and Gillespie, 1997), ASV17 CEFs grew at a greater rate and reached a higher saturation density than control cells. AV2 CEFs also grew at an increased rate. ΔvJ-hER wt CEFs in the presence of estradiol grew at a similar rate to ASV17 CEFs, while estradiol-treated ΔvJ-hER mt CEFs proliferated at a rate similar to control CEFs.

These results suggest that a functional AF-2 domain is necessary for the increased rate of proliferation of estradiol-treated ΔvJ-hER CEFs, and may provide an explanation for the increase in expression of ΔvJ-hER wt in cells passaged in the presence of estradiol (see Chapter 4.3.1).

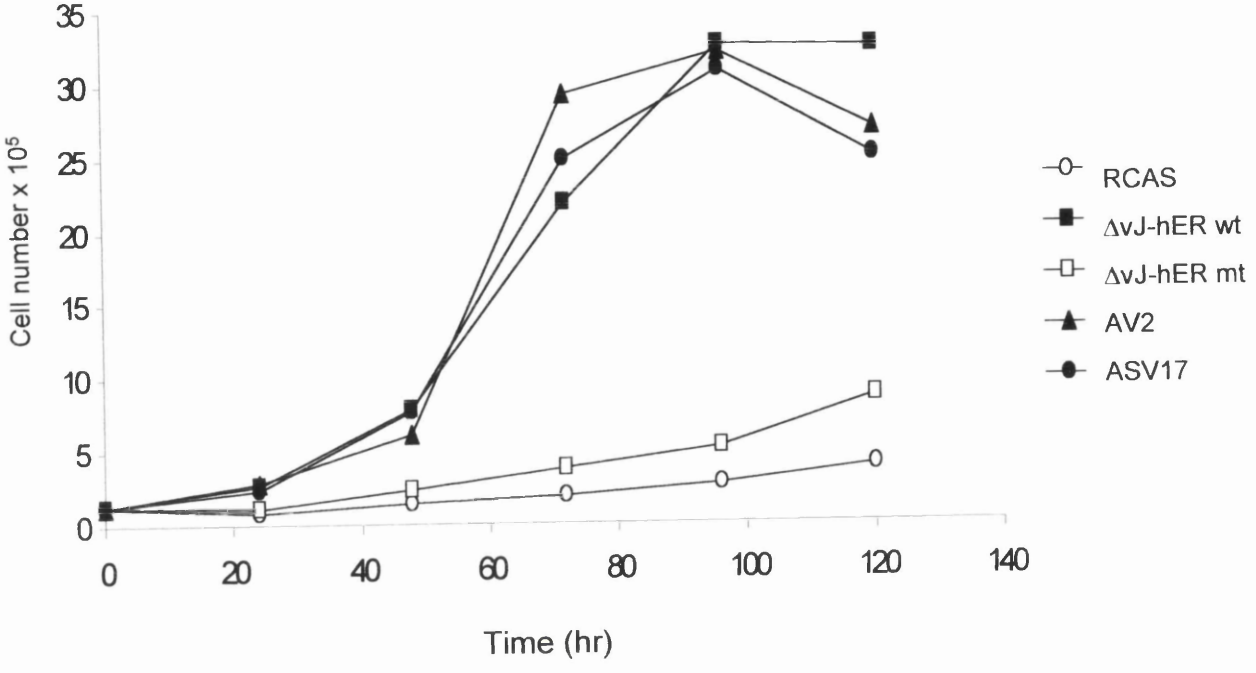


Figure 4.10

Rate of growth of CEFs infected with RCAS, RCAS-ΔvJ-hER wt (+ estradiol), -ΔvJ-hER mt (+ estradiol), -AV2 or ASV17.

Cells were counted in duplicate every 24 hours.

4.3.4.3 Anchorage-independent growth.

ΔvJ-hER wt has been shown to induce anchorage-independent growth in the presence of estradiol, with an efficiency equivalent to v-Jun (Kruse *et al.*, 1997). It was proposed that the transforming ability of the chimaeric protein was due to the function of the ER-α AF-2 domain. To investigate this hypothesis, the ability of ΔvJ-hER wt and mt CEFs to form colonies in agarose was determined. Cells were suspended in agarose containing estradiol or carrier control as appropriate, at 10^4, 10^3 and 10^2 cells per 30mm plastic tissue culture dish (see Chapter 2.2.2.5 for details). Colonies were counted and photographed after 2 weeks. Results from a representative experiment are shown in Table 4.1 and Figure 4.11.

As previously described (Bos *et al.*, 1990), ASV17 CEFs formed colonies in agarose with high efficiency (Table 4.1, Figure 4.11B), while control CEFs did not form colonies. AV2 CEFs formed colonies with an approximately 2-fold greater efficiency than ASV17 CEFs. This contradicts a previous study reporting a relatively low efficiency of cell transformation by AV2 (Schuur *et al.*, 1993). While ASV17 CEFs formed predominately large dispersed colonies, as well as compact colonies (Figure 4.11B), the colonies formed by AV2 CEFs were generally small and compact (Figure 4.11C).

ΔvJ-hER wt and mt CEFs did not form colonies in the absence of estradiol. However, estradiol-treated ΔvJ-hER wt CEFs formed colonies with an approximately 3-fold higher efficiency than ASV17 CEFs. The colonies resembled those formed by ASV17 CEFs, with many large dispersed colonies present as well as smaller, compact colonies (Figure 4.11E). In contrast, ΔvJ-hER mt CEFs did not form colonies in agar in the presence of estradiol. These results support the hypothesis that hormone-dependent cell transformation by ΔvJ-hER wt is dependent on a functional AF-2 domain.

Infecting construct	Cells plated		
	10^4	10^3	10^2
RCAS vector	0	0	0
ASV17	131 / 159	14 / 25	5 / 3
RCAS-AV2	301 / 265	36 / 14	3 / 10
RCAS-ΔvJ-hER wt: no hormone	0	0	0
RCAS-ΔvJ-hER wt: + estradiol	423 / 397	37 / 59	11 / 7
RCAS-ΔvJ-hER mt: no hormone	0	0	0
RCAS-ΔvJ-hER: + estradiol	0	0	0

Table 4.1

Agarose colony formation by CEFs infected with RCAS v-Jun constructs. The number of colonies at each cell concentration is shown in duplicate.

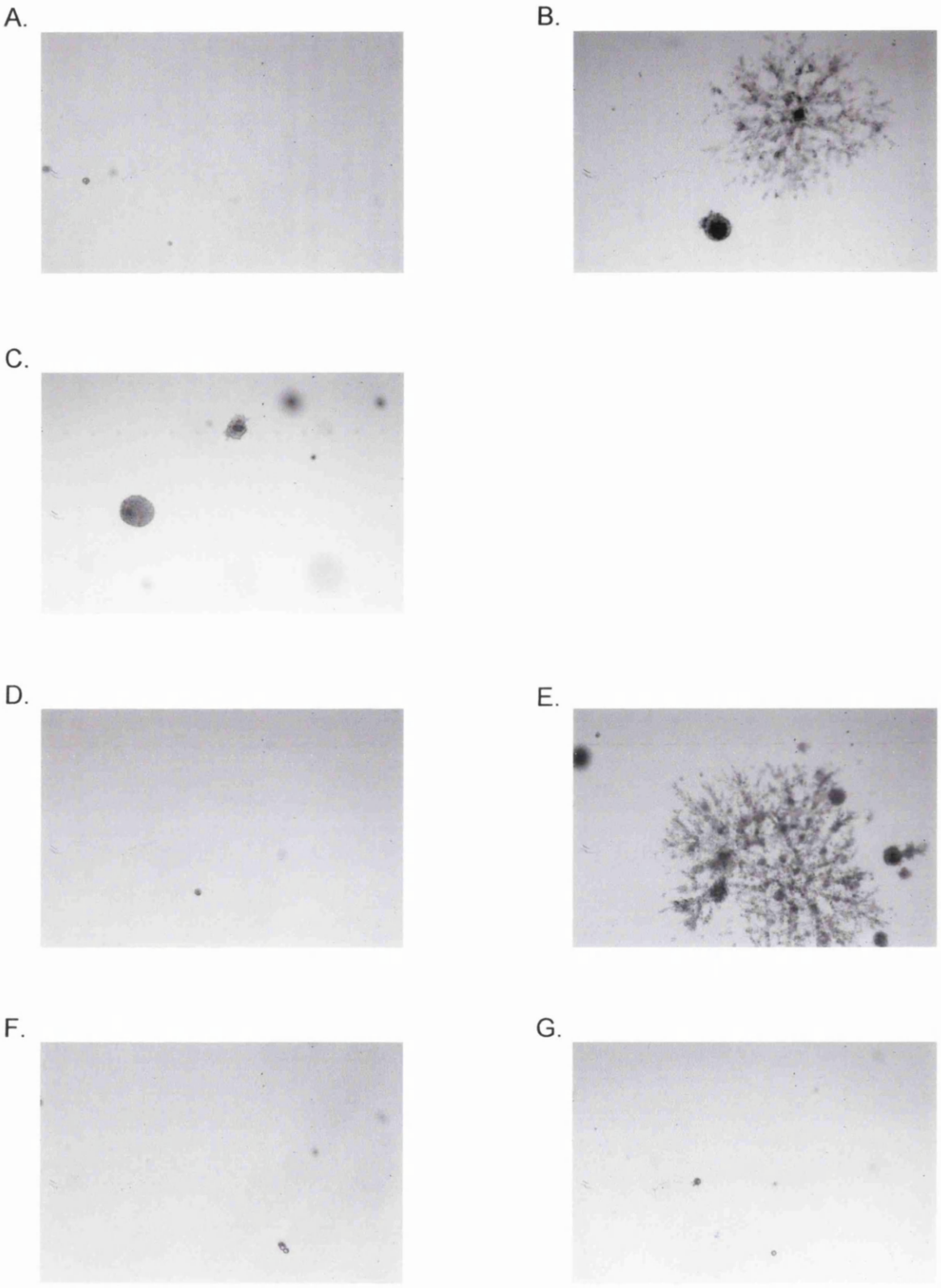


Figure 4.11

Agarose suspension cultures of CEFs infected with RCAS (A); ASV17 (B); RCAS-AV2 (C); -ΔvJ-hER wt in the presence of carrier control (D) or estradiol (E); -ΔvJ-hER mt in the presence of carrier control (F) or estradiol (G).

4.4 The role of a HAT co-activator in transcriptional activation of v-Jun target promoters and cell transformation.

The results described in Chapter 4.3 show that introduction of an inactivating mutation into helix 12 of the AF-2 domain abolishes hormone-dependent transcriptional activation and cell transformation by ΔvJ-hER. As helix 12 provides a binding surface for HAT co-activator proteins in the presence of agonist ligand (see Chapter 1.3.1.2), the deficiency of the ΔvJ-hER mt protein may be due to the disruption of co-activator binding.

It was decided to investigate whether direct recruitment of a HAT domain to v-Jun target promoters could substitute for the function of the v-Jun TAD or ER-α AF-2 domain in transcriptional activation and cell transformation. The p300 co-activator was selected for analysis, as there is evidence for a role of the protein in transcriptional activation by Jun proteins (Lee *et al.*, 1996), and mutated derivatives of the protein exist which enable analysis of the role of the HAT domain in p300 function (Kraus *et al.*, 1999)

Fusions were created of the v-Jun DBD and dimerisation domain to the HAT domain of wild-type p300, and of p300 Mut AT2, which lacks HAT function and has a reduced ability to co-activate transcription by ligand-activated ER-α (Kraus *et al.*, 1999). A chimaeric protein consisting of the homologous region of CBP fused to the Gal4 DBD has been shown to retain HAT activity and to activate transcription from certain promoters containing Gal4 binding sites (Martinez-Balbas *et al.*, 1998). It was expected that recruitment of the wild-type, but not the mutated, p300 HAT domain to TREs and CREs by the v-Jun DBD would activate transcription from v-Jun target promoters and induce cell transformation, as with ligand-activated ΔvJ-hER wt. The construction of RCAS-ΔvJ-p300 wt and mt is described in Chapter 2.2.3.14.

4.4.1 Expression of the chimaeric proteins.

CEFs were transfected with 10μg RCAS-ΔvJ-p300 wt; -ΔvJ-p300 mt; or the empty RCAS vector as control. After 3 passages, cells were plated onto 140mm plastic tissue culture dishes. WCEs were prepared after 48hr.

40μg of each WCE was resolved by SDS-PAGE and subjected to Western blotting as before. The ΔvJ-p300 wt and mt proteins ran as a single band with an apparent molecular

weight of around 99kD (Figure 4.12, upper panel). The predicted molecular weight of the 840 amino-acid proteins was 93.3kD. The level of c-Jun protein was not affected by expression of ΔvJ-p300 wt or mt (Figure 4.12, lower panel).

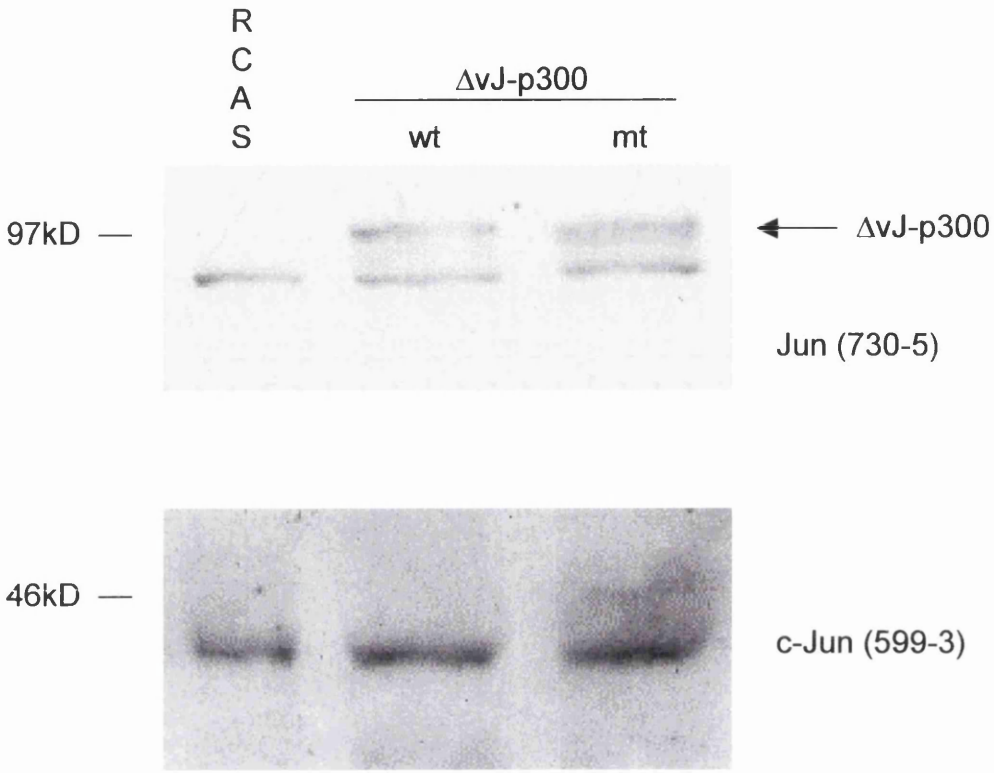


Figure 4.12

Western blot analysis of protein expression in CEFs infected with RCAS, RCAS-ΔvJ-p300 wt or –ΔvJ-p300 mt.

Blots were probed with chicken Jun antibodies 730-5 or 599-3.

4.4.2 The ΔvJ-p300 proteins bind to TREs.

Biotinylated-oligonucleotide capture experiments were carried out to determine whether the ΔvJ-p300 proteins bound to TREs. 125μg of each WCE was pre-cleared before incubation with paramagnetic particles bound to a biotinylated collagenase TRE oligonucleotide, as before. Proteins were eluted and analysed by Western blotting (Figure 4.13).

The ΔvJ-p300 wt and mt proteins both appeared to bind specifically to the collagenase TRE, showing that the p300 HAT domains were recruited to TREs by the v-Jun DBD. c-Jun bound to the collagenase TRE in both cell types. However, due to the poor technical quality of the Western blot, binding of the ΔvJ-p300 proteins to TREs can not be identified with absolute certainty.

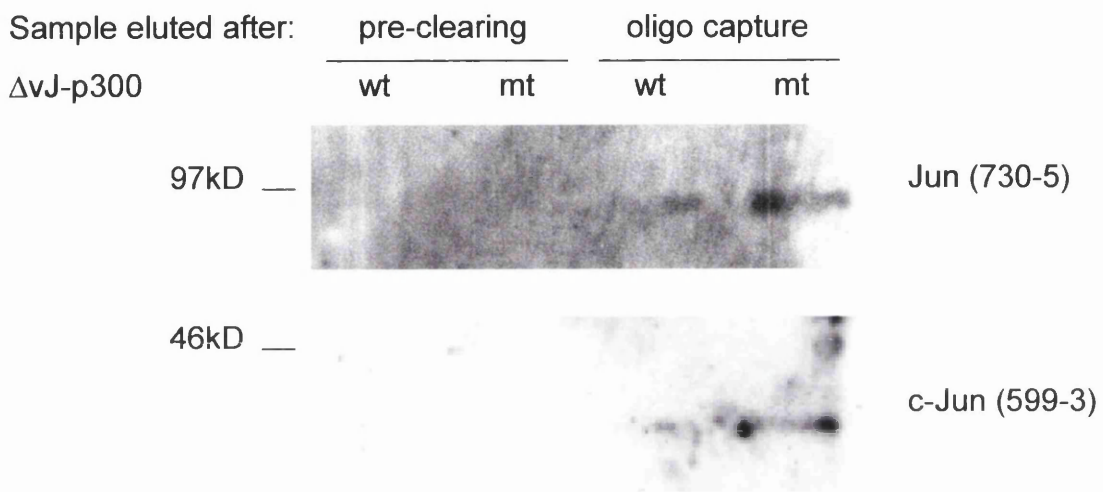


Figure 4.13

Western blot analysis of proteins eluted from paramagnetic particles after pre-clearing and after biotinylated-oligonucleotide capture of DNA-binding complexes from ΔvJ-p300 wt or mt CEFs.

Blots were probed with chicken Jun antibodies 730-5 or 599-3.

4.4.3 The ΔvJ-p300 proteins do not transactivate the *bkj* promoter.

The ΔvJ-hER mt protein does not activate transcription from the *bkj* promoter in the presence of estradiol, suggesting that the ER-α AF-2 domain is necessary for hormone-dependent transcriptional activation by ΔvJ-hER (see Chapter 4.3.3). Having determined that ΔvJ-p300 wt and mt bind to TREs, the contribution of the p300 HAT domain to the transcriptional activation of *bkj* was investigated. 5μg of the wt(P)TRE *bkj* promoter was transfected in triplicate into CEFs infected with RCAS ΔvJ-p300 wt and mt, ASV17, or RCAS, and relative CAT activities were determined as before. The results of a representative experiment are shown in Figure 4.14.

The *bkj* promoter was not activated above the basal level by ΔvJ-p300 wt or mt. This suggests that recruitment of the p300 HAT domain to a TRE-containing promoter could not substitute for the v-Jun TAD or ER-α AF-2 domain in transcriptional activation.

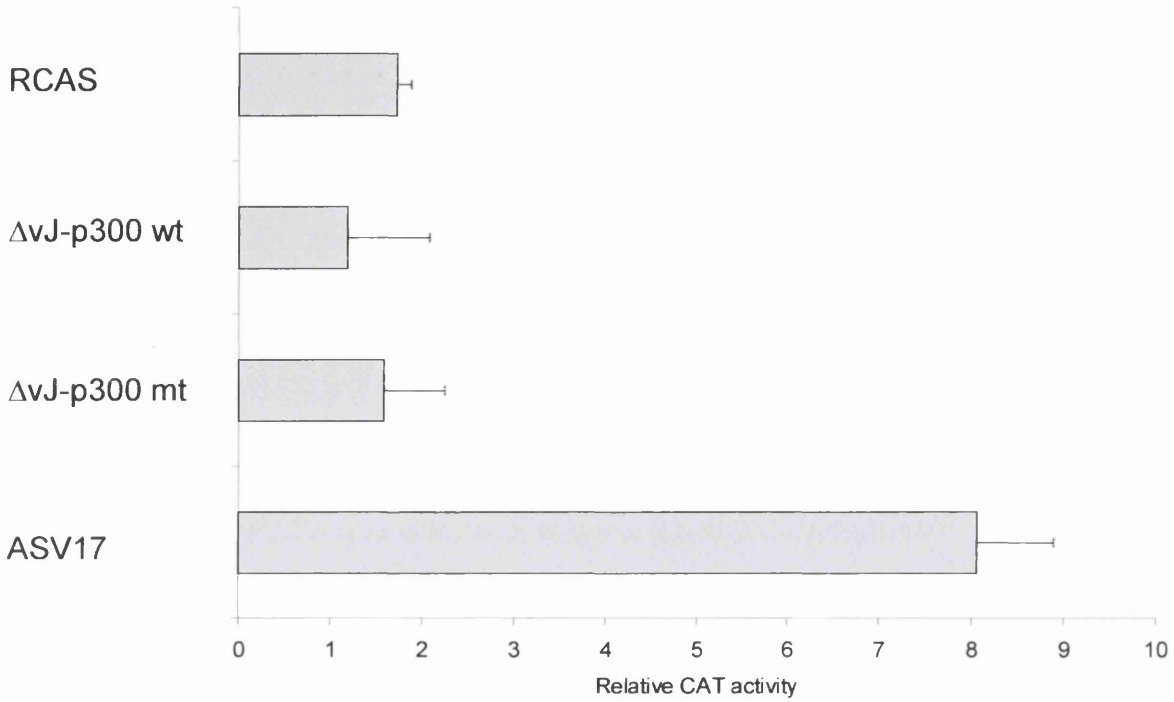


Figure 4.14

Activity of the wt(P)TRE *BKJ* promoter in CEFs infected with RCAS, RCAS-ΔvJ-p300 wt, -ΔvJ-p300 mt, or ASV17.

5μg reporter plasmid was transfected in triplicate into each cell type.

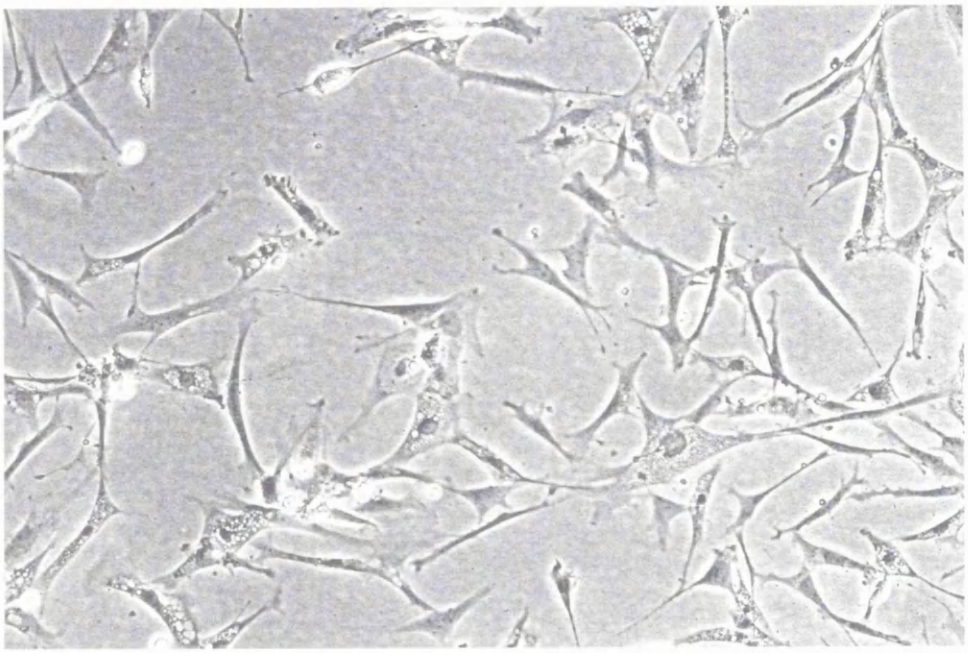
Error bars denote standard deviations.

4.4.4 The ΔvJ-p300 proteins do not induce cell transformation.

4.4.4.1 Cell morphology.

The morphology of CEFs infected with ΔvJ-p300 wt and mt is shown in Figure 4.15. These cells do not adopt the characteristic morphology of v-Jun transformed CEFs, or CEFs expressing AV2 or ligand-activated ΔvJ-hER wt, but appear similar in appearance to control CEFs (compare with Figure 4.9). The p300 HAT domain, therefore, can not substitute for the function of the v-Jun TAD or ER-α AF-2 domain in the induction of an altered cell morphology.

A.



B.

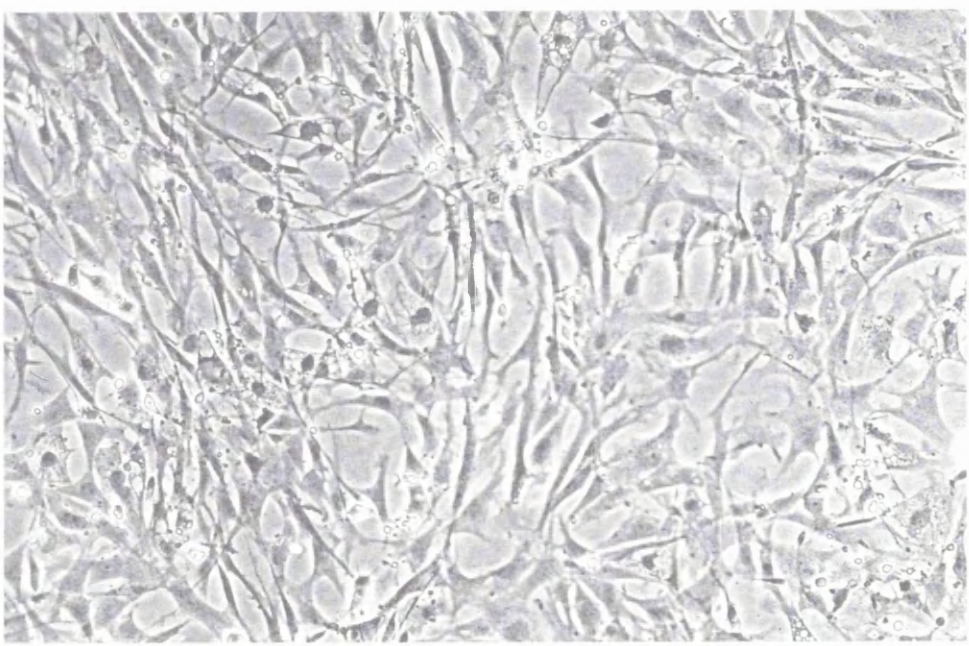


Figure 4.15

CEFs infected with RCAS-ΔvJ-p300 wt (A) or -ΔvJ-p300 mt (B).

4.4.4.2 Rate of cell proliferation.

The ligand-activated AF-2 domain of ΔvJ-hER mediates an increase in the rate of cell proliferation (Figure 4.10). The contribution of the p300 HAT domain to this process was investigated. CEFs infected with ΔvJ-p300 wt and mt, or the empty RCAS vector, were plated onto 90mm plastic tissue culture dishes, and cell numbers were determined every 24hr (see Chapter 2.2.2.4). The results of a representative experiment are shown in Figure 4.16.

Fusion of the p300 HAT domain to the v-Jun DBD did not induce an increase in the rate of cell growth; indeed, ΔvJ-p300 wt CEFs proliferated at a slightly lower rate than controls. ΔvJ-p300 mt CEFs grew at a similar rate to control CEFs. Again, therefore, the recruitment of a HAT domain to TRE-containing promoters was not sufficient to cause the increased rate of cell growth mediated by the v-Jun TAD and ligand-activated AF-2.

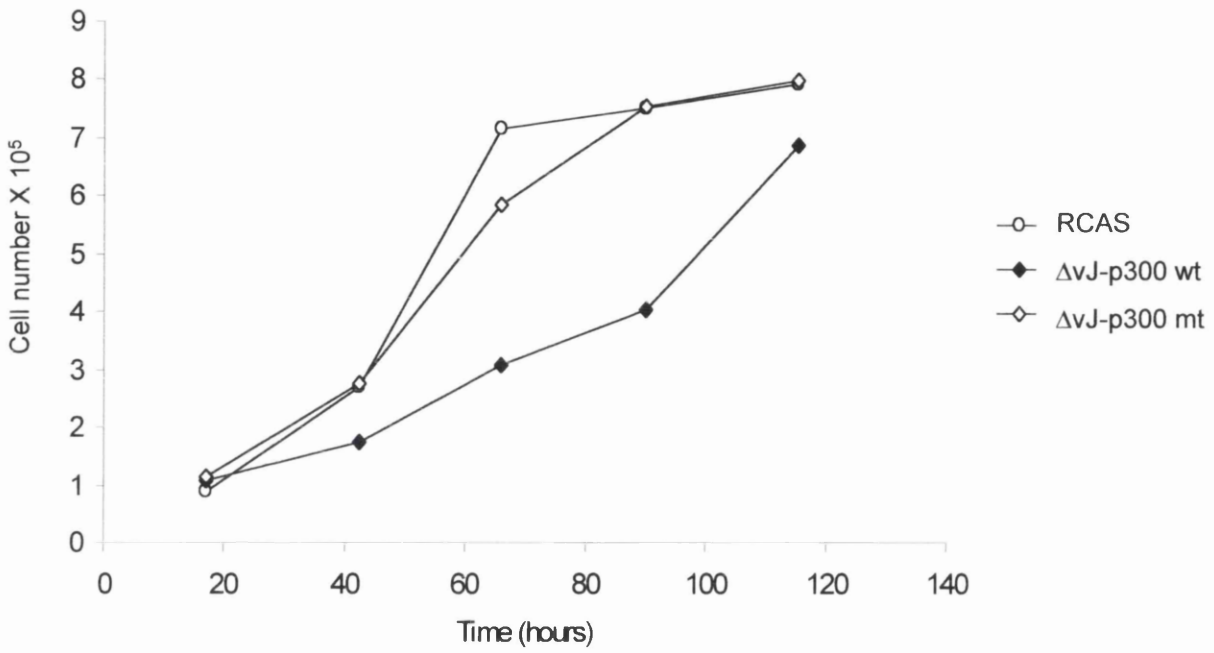


Figure 4.16

Rate of growth of CEFs infected with RCAS, RCAS-ΔvJ-p300 wt or -ΔvJ-p300 mt.

Cells were counted in duplicate every 24 hours.

4.4.4.3 Anchorage-independent growth.

The AF-2 domain of ΔvJ-hER wt mediates hormone-dependent cell transformation (see Chapter 4.3.4). The ability of the p300 HAT domains to substitute for this function was investigated. ΔvJ-p300 wt and mt CEFs were suspended in agarose at 10^4, 10^3 and 10^2 cells per 30mm plastic tissue culture dish, as before. This experiment was performed alongside the analysis described in Chapter 4.3.4.3, to provide negative and positive controls.

Table 4.2 shows that ΔvJ-p300 wt and mt CEFs were unable to form colonies in agarose suspension. Recruitment of the p300 HAT domains to v-Jun target promoters was therefore not able to substitute for the function of the v-Jun TAD or ligand-activated AF-2 domain in cell transformation.

Infecting construct	Cells plated		
	10^4	10^3	10^2
RCAS vector	0	0	0
ASV17	131 / 159	14 / 25	5 / 3
RCAS-ΔvJ-p300 wt	0	0	0
RCAS-ΔvJ-p300 mt	0	0	0

Table 4.2

Agarose colony formation by CEFs infected with RCAS-ΔvJ-p300 constructs. The number of colonies at each cell concentration is shown in duplicate.

5 Discussion

5.1 General introduction

Identification of the v-Jun oncoprotein as a member of a family of cellular transcription factors suggested that cell transformation by v-Jun was a result of the mis-regulation of specific target genes. Many positive and negative targets of v-Jun have since been identified, with the altered expression of some having been shown to contribute to the transformed phenotype (see Chapter 1.2.2). However, much work remains to be done to elucidate the mechanisms by which v-Jun mis-regulates transcription, and to determine which of its many target genes are specifically involved in the transformation process. The work described in Chapters 3 and 4 aimed to better characterise the mechanisms of transcriptional activation and repression by v-Jun, and to relate these different processes to cell transformation.

5.2 Transcriptional regulation by v-Jun

5.2.1 Introduction

While v-Jun is known to activate some target genes and repress others, its transcriptional mechanisms are not well understood. This work sought to better understand these mechanisms by comparison of gene promoters activated or repressed by v-Jun. *bkj*, which is activated in v-Jun transformed cells, and collagenase, which is repressed, were chosen, as the promoters of these genes have been relatively well characterised.

5.2.2 Regulation of the *bkj* and collagenase promoters by v-Jun.

A systematic analysis of transcription from the *bkj* and collagenase promoters was carried out, to verify that the regulation of the two promoters in control and v-Jun transformed CEFs occurred as previously reported.

Transcriptional regulation of the human collagenase I promoter is known to be complex. A non-consensus TRE at position –186 (Chamberlain *et al.*, 1993; White and Brinckerhoff, 1995) and a PEA3 binding site at position –81 (Gutman and Wasylyk, 1990) contribute to TPA induction of transcription, in co-operation with the TRE at position –72. Transcription factors such as ETS-1 and PU.1 also regulate the collagenase promoter, with

transcriptional repression by PU.1 dependent on an intact TRE at position –72 (Westermarck *et al.*, 1997). Other sequences in the promoter region between –180 and +60 have been implicated in TPA induction of transcription (Auble and Brinckerhoff, 1991). However, various studies have shown that the TRE at position –72 is the most important regulatory element in the collagenase promoter (Auble and Brinckerhoff, 1991; Gutman and Wasylyk, 1990; Jonat *et al.*, 1990; Westermarck *et al.*, 1997), and a promoter containing this site is repressed in v-Jun transformed CEFs (Kilbey *et al.*, 1996). The importance of the TRE at position –72 for transcriptional repression by v-Jun was confirmed by transfection of –73/+63 ColCAT and –60/+63 into control and v-Jun transformed CEFs (Figure 3.1B), and also by co-transfection of c-Jun and v-Jun with –73/+63 ColCAT in CEFs (Figure 3.2B).

The quail *bkj* promoter was previously shown to be up-regulated by v-Jun through the more proximal of two TREs (Hartl and Bister, 1998). This was confirmed by transfection of *BKJ*(P) and its deletion mutant *BKJ*(0) into control and v-Jun transformed CEFs (Figure 3.1A), and also by co-transfection of c-Jun and v-Jun with *BKJ*(P) in CEFs (Figure 3.2A).

However, throughout this study, it became clear that the regulation of *bkj* is more complex than previously thought. As described in Chapter 3.2.1, comparison of *BKJ*(WT) and *BKJ*(DP) indicated that the sequence upstream of the distal TRE contains negative regulatory elements, possibly with a stronger effect in chicken cells than in quail cells. Also, the function of the distal *bkj* TRE has not been adequately investigated. Figure 3.1A indicates that this element contributes to *bkj* regulation in control CEFs, and may have a role in transcriptional regulation by v-Jun that had been masked in previous studies.

Later studies revealed further complexities in the regulation of *bkj*. Comparison of the *BKJ*(P) and wt(P)TRE *bkj* promoters (Figure 3.4B) suggests that the region between –929 and –886 may mediate an increase in the level of transcription (See Chapter 3.4.1). Figure 3.8 indicates that the promoter region from –929 to –67 negatively regulates transcription from the –65TRE *bkj* promoter. Whether these effects are mediated by the binding of specific regulatory factors to the promoter, or by some other process, is not known.

Further characterisation of the *bkj* promoter, for example by a comprehensive search for transcription factor binding sites, finer deletion analysis, footprinting etc., would facilitate a better understanding of the transcriptional regulation of this gene, and the interaction of c-Jun and v-Jun with any other regulatory factors. The role of the distal TRE is of particular interest. The construction of reporter vectors containing point mutations of the

two TREs within the same promoter context would enable analysis of the contribution of each site to regulation by c-Jun and v-Jun. Further characterisation of the protein complexes bound to each TRE would also be beneficial, as any differences between them may contribute to the different roles of the two elements in transcriptional regulation.

However, the important conclusions for the purposes of the comparative study were that, as reported, the *bkj* promoters used are up-regulated by v-Jun through the proximal TRE, and that the –73/+63 ColCAT promoter is repressed by v-Jun through its TRE at position –72.

5.2.3 The effect of the protein complexes bound to different TREs.

The first stage in the comparison of the *bkj* and collagenase promoters was identification of the proteins bound to their TREs. EMSAs (Figure 3.3A) and antibody super-shift experiments (Figure 3.3C, D) showed the binding of similar protein complexes to each TRE within each cell type. As previously seen with the collagenase TRE in chicken cells (Hawker *et al.*, 1993; Kilbey *et al.*, 1996), the complex contained v-Jun in v-Jun transformed CEFs and predominately c-Jun in CEFs, with Fra-2 as the major dimerisation partner in each case.

The complex bound to each TRE in CEFs was not completely shifted by a c-Jun specific antibody (Figure 3.3C). The collagenase TRE has been shown to be bound by JunD in rabbit fibroblasts (White and Brinckerhoff, 1995), but not in CEFs (Kilbey *et al.*, 1996). Further antibody super-shifts would determine whether JunD binds the *bkj* TREs in CEFs. In both cell types, a broad-specificity Fos family antibody completely shifted each complex, while a Fra-2 specific antibody effected only a partial shift. As discussed in Chapter 3.3, the residual complex may contain the chicken homologues of the FosB and Fra-1 proteins. Any differences between the TREs in the composition of this residual complex may be important for their function in transcriptional regulation.

However, competition assays showed that all three TREs were bound by an identical complex in v-Jun transformed CEFs (Figure 3.3B). Differences in the composition of the protein complex bound to each TRE are therefore unlikely to account for the different effects of v-Jun on the two promoters.

5.2.4 The effect of TRE position.

Comparisons between the collagenase, stromelysin and c-*jun* promoters suggested that the position of the TRE relative to the transcriptional start site may have an effect on target promoter regulation by v-Jun (see Chapter 3.4). This was investigated by the creation of a panel of variant *bkj* promoters with TREs introduced at different positions relative to the transcriptional start site.

As shown in Figure 3.6, alteration of TRE position modified regulation of the *bkj* promoter by v-Jun. This was not due to different levels of co-operation of each introduced TRE with the wild-type proximal TRE at position –815, as prior point mutation had eliminated this element (Figure 3.4). Nor was the binding of different proteins likely to be responsible for the variations in transcriptional activity, as each TRE was, like the wt(P)TRE, bound predominately by c-Jun / Fra-2 dimers in CEFs and v-Jun / Fra-2 dimers in v-Jun transformed CEFs (Figure 3.5C). These proteins bound to each TRE with slightly different affinities (Figure 3.5B), probably due to the different sequences flanking the TRE in each case, which have been shown to contribute to the affinity of binding by Jun / Fos dimers (Ryseck and Bravo, 1991). However, TRE binding affinity did not correlate with the level of activation of the *bkj* promoters in either cell type. The variations in promoter activity seen in Figure 3.6 were therefore due primarily to the different TRE position in each case.

The reasons for these variations are unclear. There appear to be three phases: a progressive decrease in transcription in v-Jun transformed CEFs from TREs at positions from –815 to –323; high levels of transcription in both cell types from TREs at positions –169 and –109; and a pattern of activation resembling that of the wild-type promoter from TRE position –65. It would be interesting to investigate whether these three phases represent different transcriptional mechanisms of v-Jun from different TRE positions, for example by recruitment of different components of the pre-initiation complex, different HAT co-activators or chromatin remodelling factors. The use of specific inhibitors could potentially determine which, if any, of the mutant promoters are activated by a mechanism involving HATs.

The principal conclusion from these experiments was that the introduction of a TRE close to the transcriptional start site did not convert a v-Jun activated to a v-Jun repressed promoter. However, an effect of TRE position on the nature of transcriptional regulation by v-Jun can not be ruled out. A major limitation of this study was the requirement for sites with sequence similarity to a TRE, to enable conversion into a consensus TRE using

mutagenic PCR primers. In the attempt to create a TRE in a position closely corresponding to that of the site in the collagenase promoter, the nearest available site for conversion was at position –65. Repression of other promoters by v-Jun occurs from TREs at position –72 (collagenase and c-*jun*) or –71 (stromelysin: see Chapter 3.4). Binding of v-Jun / Fra-2 to the –65TRE *bkj* promoter would therefore occur on the opposite face of the DNA helix (relative to the transcriptional start site) than on the three natural v-Jun repressed promoters. This difference may be crucial for the nature of transcriptional regulation by v-Jun, for example by affecting binding and assembly of the pre-initiation complex.

Certain transcription factors have been shown to depend on the stereospecific alignment between their binding site and the TATA box for their transcriptional activity at certain promoters. Examples include myoD and myogenin, which bind to the proximal E-box of the murine desmin gene promoter (Li and Capetanaki, 1994), and Sp1 within the SV40 early promoter (Takahashi *et al.*, 1986). Within the tyrosine hydroxylase promoter, insertions of full or half DNA helical turns between the CRE and the TATA box caused variations in the level of basal transcription, but did not affect the induction of transcription by cAMP (Tinti *et al.*, 1997).

A possible role for a precise stereospecific alignment between the TRE and the TATA box is suggested by comparison of the –169TRE and –109TRE *bkj* promoters (Figure 3.6). These promoters contain TREs on the same face of the DNA helix relative to the transcriptional start site, and are regulated in a similar way, with high levels of activation in CEFs and v-Jun transformed CEFs. While other factors may contribute to the regulation of these promoters, their similarity suggests that the stereospecific alignment between the TRE and the TATA box may be important.

Introduction of a TRE at position –71 or –72 in the *bkj* promoter would be likely to alter the overall levels of transcription in CEFs and v-Jun transformed CEFs, but it is less clear whether the TRE would mediate repression by v-Jun. Mutation of the six bases at this site which differ from a consensus TRE would require two rounds of mutagenic PCR. Alternatively, a TRE could be introduced at this site by insertion, but this would cause greater overall sequence disruption than would site-directed mutagenesis. Construction of such a promoter would provide a better comparison with the collagenase promoter, and may yet provide evidence for TRE position as a determinant of transcriptional regulation by v-Jun.

5.2.5 The effect of core promoter sequence.

As described in Chapter 3.5, regulation by certain transcription factors has been shown to be influenced by the sequences of core promoter elements, such as the TATA box and Inr. These sequences were therefore exchanged between the *bkj* and collagenase core promoters, to investigate their possible role in determining the nature of target promoter regulation by v-Jun.

Figures 3.7B-D show the effect of TATA box and / or ILS exchange between the collagenase and *bkj* promoters. It is clear that the TATA box and ILS do not determine whether these target genes are activated or repressed by v-Jun. However, the overall levels of transcription from the *bkj* promoter, but not collagenase, were altered by interaction of c-Jun and v-Jun with different core promoter element sequences.

Figure 3.7C indicates a role for a consensus initiator in transcription from the *bkj* promoter. It would be interesting to analyse this element functionally, for example by analysis of the effect of point mutations on the choice of transcriptional start site. The *bkj* promoter contains two transcriptional start sites (Hartl and Bister, 1998). The minor site has no consensus Inr sequence and is not active in QEFs expressing c-Jun or v-Jun (Hartl and Bister, 1995; Hartl and Bister, 1998). However, this alternative site is used to initiate a small number of transcriptional events in ASV17-transformed CEFs (Hartl and Bister, 1995; Hartl and Bister, 1998). It would be interesting to analyse the contribution of the ILS at the major transcriptional start site to this species difference in transcription initiation.

Another intriguing result is the difference between the wt(P)TRE *bkj* and –65TRE *bkj* promoters. Introduction of the collagenase ILS decreased transcription from both promoters (Figure 3.7C), implying that v-Jun activates transcription by a mechanism involving an Inr in both cases. However, introduction of the collagenase TATA box, alone or in combination with the collagenase ILS, affected transcriptional regulation from wt(P)TRE *bkj*, but had little or no effect in the context of the –65TRE *bkj* promoter (Figure 3.7B, D). This suggests that, while alteration of TRE position does not convert *bkj* into a v-Jun repressed promoter, it may alter the mechanism whereby v-Jun activates the promoter. Figure 3.7B suggests that this may be by removing v-Jun's dependence on TATA box sequence. This could provide an explanation for the three distinct phases of the variations in *bkj* promoter activity seen in Figure 3.6 (see Chapter 5.2.4).

As described in Chapter 3.5, some studies have shown a link between a transcription factor's dependence on TATA box sequence within a particular promoter, and its binding to components of the pre-initiation complex (Cook *et al.*, 1995; Taylor and Kingston, 1990b). This relationship could suggest distinct mechanisms for the regulation of different promoters by v-Jun, i.e. by direct recruitment of the pre-initiation complex to the core promoter of wt(P)TRE *bkj*, but not –65TRE *bkj* or collagenase. However, the correlation between TATA box sequence dependence and binding to the pre-initiation complex is not absolute. E1a and VP16 each bind components of the pre-initiation complex (Gupta *et al.*, 1996; Horikoshi *et al.*, 1991; Lee *et al.*, 1991b; Stringer *et al.*, 1990; Xiao *et al.*, 1994), but have been shown to activate transcription independently of TATA box sequence (Perera, 2000; Taylor and Kingston, 1990a). Further work, for example footprinting or template commitment assays with the wt(P)TRE *bkj*, –65TRE *bkj* and collagenase core promoters, may help to resolve this issue.

5.2.6 Future work.

A *bkj*-derived promoter with a TRE at position –65 and none of the upstream sequence is, like wt(P)TRE *bkj* and –65TRE *bkj*, activated more strongly in v-Jun transformed CEFS than controls (Figure 3.8). This construct may provide a better comparison with –73/+63 ColCAT, as both promoters contain a TRE at the extreme 5' end of the promoter. While activation of –66/+13 –65TRE *bkj* by v-Jun may be found to differ in some respects from the regulation of the natural *bkj* promoter, comparisons between –66/+13 –65TRE *bkj* and –73/+63 ColCAT will serve as a useful model in further investigations of the transcriptional mechanisms responsible for the opposing effects of v-Jun on different promoters.

The first stage would be to determine whether the different effects of v-Jun on the two promoters are mediated through sequence variations in the TREs, or in the core promoters. Differences in the sequences of TREs, and flanking DNA up to 10bp from the central base of the TRE, have been shown to affect the affinity and orientation of Jun / Fos heterodimer binding (Leonard *et al.*, 1997; Rajaram and Kerppola, 1997; Ramirez-Carrozzi and Kerppola, 2001a; Ramirez-Carrozzi and Kerppola, 2001c; Ryseck and Bravo, 1991). Different orientations of heterodimer binding to a TRE affected DNA bending at the site and transcriptional synergy with NFAT proteins bound to a site adjacent to the TRE (Ramirez-Carrozzi and Kerppola, 2001a; Ramirez-Carrozzi and Kerppola, 2001b).

The collagenase TRE, *bkj* proximal TRE and the TRE introduced at *bkj* position –65 are not predicted to show a strong preference for the orientation of Jun / Fos heterodimer binding (V.R. Ramirez-Carrozzi, personal communication), suggesting that the *bkj* and collagenase TRE flanking sequences are unlikely to determine the nature of transcriptional regulation by v-Jun. Exchange of the TRE and flanking sequences between the two promoters would verify this.

A more likely explanation for the different effects of v-Jun on the collagenase and *bkj* promoters is variation in core promoter sequences other than the TATA box and ILS. Exchange of whole core promoters between *bkj* and collagenase, followed by the exchange of smaller sequence blocks, would determine whether this is the case, and may identify the core promoter regions which are likely to determine the transcriptional effect of v-Jun. Possible candidates are the sequence blocks immediately flanking the TATA box, as exchange of these sequences between promoters has been shown to affect the level of transcriptional induction by a chimaeric Gal4-VP16 transcription factor (Wolner and Gralla, 2000).

However, it may be that this kind of dissection of the *bkj* and collagenase gene promoters will not identify the determinants of transcriptional regulation by v-Jun. The whole sequence of such natural promoters has evolved together to determine how the gene is regulated by transcription factors, and it may be that individual sequence elements can not function correctly outside the natural core promoter context. However, as with the experiments described in Chapter 3, further comparative studies between genes activated or repressed by v-Jun are likely to provide insights into the transcriptional mechanisms of v-Jun at specific target promoters. Such findings may be found to be applicable to other v-Jun target genes, and may lead indirectly to a better understanding of the transcriptional mechanisms responsible for the opposing effects of v-Jun on different target promoters.

5.3 Activation of v-Jun target genes and cell transformation.

5.3.1 Introduction

Fusion to the ligand-binding domain of ER-α is a widely used method to confer hormone-inducible function upon a heterologous protein. In the case of ER-α fusions to transcription factors such as Jun, activation of the ER-α AF-2 domain is thought to contribute to

hormone-dependent transcriptional regulation of target promoters by the chimaeric protein (Francis *et al.*, 1995; Kim *et al.*, 1996). Therefore, when constructing a hormone-regulatable v-Jun-ER fusion protein, an amino-terminally truncated v-Jun protein was also fused to the ER-α hormone-binding domain, to determine the contribution of the AF-2 domain to the hormone-dependent functions of the v-Jun-ER protein (Kruse *et al.*, 1997).

It was found that the ligand-activated AF-2 domain could substitute for the function of the v-Jun TAD, in transcriptional activation of v-Jun target promoters and induction of some of the characteristics of cell transformation. This observation suggested a common mechanism of cell transformation by v-Jun and ΔvJ-hER, with activation, rather than repression, of v-Jun target promoters thought to be important for the transformation process. The work described in Chapter 4 sought to better characterise the relationship between activation of v-Jun target genes and cell transformation.

5.3.2 Characterisation of cells expressing ΔvJ-hER.

The premise that hormone-dependent cell transformation by ΔvJ-hER is due to the activation of v-Jun target promoters by the ER-α AF-2 domain assumes that the chimaeric protein is recruited to TRE and CRE-containing promoters by the v-Jun DBD. It was not clear whether the addition of estradiol directly activated the AF-2 domain of ΔvJ-hER bound constitutively to target promoters, or caused transcriptional activation and cell transformation by regulation of the sub-cellular location or DNA binding activity of the protein.

Previous studies with Jun and Fos-ER proteins suggest that the level at which hormone regulates the function of such chimaeric proteins varies. For example, proteins consisting of JunD or Fos proteins fused to the ER-α ligand-binding domain were constitutively nuclear in location (Francis *et al.*, 1995; Schuermann *et al.*, 1993), while c-Jun-ER was expressed throughout the cytoplasm and nucleus in untreated cells, with the addition of estradiol stimulating nuclear accumulation of the protein (Fialka *et al.*, 1996). Similarly, JunD-ER was strictly hormone-dependent for DNA binding (Francis *et al.*, 1995), while c-Fos-ER has been shown to bind TREs in the absence of estradiol (Crowe *et al.*, 2000), and DNA binding by a c-Jun bZip-ER protein was increased by estradiol treatment (Kim *et al.*, 1996). The regulation of ΔvJ-hER at these levels had not previously been studied.

The results described in Chapter 4.2.3 show that ΔvJ-hER is not regulated by hormone at the level of sub-cellular location or DNA binding, but binds constitutively to TREs. The hormone-dependent effects of ΔvJ-hER are therefore likely to be a direct result of activation of the AF-2 domain at v-Jun target promoters.

One unexpected effect of AF-2 domain activation was down-regulation of the endogenous c-Jun protein (Figure 4.1). Antibody super-shift experiments suggested that this down-regulation was not complete, and that residual c-Jun protein bound TREs (see Chapter 4.2.3). However, the level of residual c-Jun protein was below the limits of detection by Western blotting techniques. As described in Chapter 4.2.2, the levels of c-*jun* mRNA and transcription from the c-*jun* promoter were not decreased in ΔvJ-hER CEFs treated with estradiol. This suggested that, unlike the v-Jun TAD, the ligand-activated AF-2 domain of ΔvJ-hER does not directly repress c-*jun* at the level of transcription. It would be interesting to determine whether other negative targets of v-Jun, such as collagenase and SSeCKS, are also repressed in estradiol-treated ΔvJ-hER CEFs. The results described in Chapter 4.2.2 suggest that direct transcriptional targets of v-Jun may not be repressed. The down-regulation of c-Jun at the translational or post-translational level is likely to be specific, possibly due to its homology with the v-Jun domains of ΔvJ-hER.

The mechanism of specific c-Jun protein down-regulation by ligand-activated ΔvJ-hER is unknown. One possibility is that the activated protein specifically decreases the rate of c-Jun translation. Evidence exists that chicken c-*jun* mRNA is translated by a mechanism involving internal initiation, due to a putative internal ribosome entry segment(s) within the 5' UTR (Sehgal *et al.*, 2000). Translation by internal initiation is not well understood, and it is not clear how ligand-activation of a protein such as ΔvJ-hER could specifically disrupt this process.

Perhaps a more likely explanation for the down-regulation of c-Jun in estradiol-treated ΔvJ-hER CEFs is specific protein degradation. In the presence of activating ligand, c-Jun was down-regulated by ΔvJ-hER wt, but not by the transcriptionally inactive ΔvJ-hER mt protein (Figure 4.5). c-Jun was also down-regulated in cells expressing AV2, a fusion of the v-Jun bZip domain to the VP16 TAD. Fusion of a functional TAD to the v-Jun bZip domain is therefore associated with down-regulation of the endogenous c-Jun protein.

Recently, there has been much interest in the link between transcriptional activation and protein degradation by the ubiquitin / proteasome pathway, initiated by the observation that

the Myc "degron", the region of the protein which signals its degradation, overlaps its TAD (Salghetti *et al.*, 1999). This is now known to be a common feature of many transcription factors with acidic TADs, including Jun, Fos, and Gcn4 (Salghetti *et al.*, 2000). Studies with various wild-type and mutated TADs have shown a correlation between ubiquitin / proteasome-mediated protein degradation and transcriptional activation (Molinari *et al.*, 1999; Salghetti *et al.*, 1999; Salghetti *et al.*, 2000).

Phosphorylation of yeast Gcn4 by Srb10, a component of the RNA polymerase II holoenzyme, signalled the ubiquitination and degradation of the transcription factor (Chi *et al.*, 2001). Similarly, cdk7, a component of TFIIH, phosphorylated the E2F-1 TAD; this was thought to stimulate degradation of the protein (Vandel and Kouzarides, 1999). These observations have suggested the "Black Widow" model of transcription-linked protein degradation, whereby recruitment of the pre-initiation complex and activation of transcription by certain transcription factors signals their destruction, mediated by components of the transcription machinery (Tansey, 2001). This is proposed to provide an additional level of transcriptional regulation.

While recent studies suggest that, in some cases, ubiquitination regulates the activity of a transcription factor independently of its function in protein degradation (Salghetti *et al.*, 2001), the proteasome itself has been shown to be essential for transcriptional activation in the case of ER-α (Lonard *et al.*, 2000). However, both mechanisms result in the degradation of the ubiquitinated transcription factor. Various studies have shown that proteins non-covalently bound to a ubiquitinated transcription factor are also targeted for degradation. For example, activation of the retinoid X receptor with specific ligand signalled the destruction of the receptor and its heterodimerisation partner, even when the partner protein was itself transcriptionally inactive (Osburn *et al.*, 2001).

ER-α was ubiquitinated and degraded by the proteasome in the presence of estradiol, but not tamoxifen, while a transcriptionally inactive helix 12 mutant was not degraded (Nirmala and Thampan, 1995; Wijayaratne and McDonnell, 2001). The VP16 TAD was also ubiquitinated and degraded by the proteasome (Salghetti *et al.*, 2001). This suggests a mechanism for the repression of c-Jun by AV2 and ligand-activated ΔvJ-hER. c-Jun could theoretically dimerise with these proteins via the v-Jun leucine zipper, and become degraded by the proteasome upon transcription-linked ubiquitination of its heterodimerisation partner. This is an attractive model, as it provides an explanation for the down-regulation of c-Jun by transcriptionally active AV2 and agonist-bound ΔvJ-hER wt,

but not by transcriptionally inactive ΔvJ-hER mt, ΔvJ-p300 proteins, and unliganded or antagonist-bound ΔvJ-hER wt.

There are possible problems with this model. Figure 4.1 shows that the expression of ΔvJ-hER was not decreased by the addition of estradiol. However, any transcription-linked degradation of this protein may be masked by its high level of expression from the RCAS retroviral vector. Indeed, the level of a high-mobility degradation product of ΔvJ-hER was increased in the presence of estradiol (Figure 4.1), indicating some degree of protein degradation in the presence of activating ligand. This was not an effect of ligand binding, as the ΔvJ-hER mt protein was not degraded in estradiol-treated cells (Figure 4.5 and 4.6). Additionally, super-shift and biotinylated-oligonucleotide capture experiments show that, while ΔvJ-hER wt and c-Jun bound to TREs in untreated cells, both proteins appeared to dimerise with Fra-2, not with each other (Figure 4.3 and 4.4). However, this may not reflect the situation *in vivo*. Alternatively, ΔvJ-hER and c-Jun may dimerise in response to estradiol, promoting degradation of c-Jun.

Further investigation of this model is required. Firstly, it is important to determine whether c-Jun binds to AV2, and to ΔvJ-hER wt in the presence and absence of estradiol. Determination of the half-lives of ΔvJ-hER wt and mt in the presence and absence of estradiol would establish whether any degradation of these proteins correlates with their transcriptional activity. Time-course experiments would reveal the rate of c-Jun degradation in ΔvJ-hER CEFs upon addition of estradiol, and whether this process can be blocked by the inhibition of ubiquitination or proteasome function. These experiments may suggest a mechanism for the down-regulation of c-Jun by AV2 and ligand-activated ΔvJ-hER.

5.3.3 The role of the ER-α AF-2 domain in hormone-dependent transcriptional activation and cell transformation by ΔvJ-hER.

As described above, the ER-α AF-2 domain is proposed to contribute to hormone-dependent transcriptional activation by ΔvJ-hER and other Jun-ER fusion proteins. Changes in the position of helix 12 within this domain are thought to regulate the binding of transcriptional co-activators and co-repressors in response to agonist or antagonist ligands (see Chapter 1.3.1.2). This motif is therefore critical for ligand-specific

transcriptional activation and repression by nuclear hormone receptors. A double amino acid substitution was introduced into helix 12 of ΔvJ-hER, to determine whether hormone-dependent transcriptional activation and cell transformation by the chimaeric protein were mediated by the ER-α AF-2 domain.

Figure 4.5 and 4.6 show that, while passaging cells in the presence of estradiol increased the expression of the ΔvJ-hER wt protein, no such effect was observed with ΔvJ-hER mt. Cell growth assays confirmed a previous report (Kruse *et al.*, 1997) that expression of ΔvJ-hER wt increased the rate of cell proliferation in the presence of estradiol, and showed that a functional AF-2 domain was required for this effect (Figure 4.10). This result suggests that in the presence of estradiol, expression of ΔvJ-hER wt, but not ΔvJ-hER mt, conferred a selective advantage on infected cells and specifically increased the rate of spread of the RCAS-ΔvJ-hER wt virus.

A JunD-ER fusion protein was reported to delay G1-S transition, and therefore decrease the rate of cell proliferation, in the absence of hormone (Francis *et al.*, 1995). In the attempt to isolate clonal JunD-ER cell lines by antibiotic selection, it was reported that expression of the fusion protein could only be detected if cells were selected in the presence of agonist ligand as well as antibiotic (Francis *et al.*, 1995). Similarly, the efficiency of cell transformation induced by a panel of mouse c-Jun mutant proteins correlated with their level of expression from RCAS vectors in CEFs (Morgan *et al.*, 1992). This suggests that correlation between the level of expression of a protein and its ability to increase the rate of cell proliferation may be a common effect.

With this kind of correlation, it is difficult to distinguish between cause and effect. However, in the case of ΔvJ-hER, direct comparison between the wild-type and mutated proteins suggests that their levels of expression are a result of their effects on the rate of cell proliferation. Immunocytochemistry experiments performed at various stages throughout the infection process would determine the proportion of cells expressing ΔvJ-hER wt or mt in the presence of estradiol or carrier control. The relative levels of expression of the proteins in individual infected cells could also be compared. It may be possible by this method to begin to quantitate the rate of spread of the RCAS-ΔvJ-hER wt and mt viruses.

Introduction of the M543A / L544A mutation into helix 12 of full-length ER-α or a Gal4·ER·VP16 fusion protein abolished hormone-dependent activation of target promoters

(Danielian *et al.*, 1992; Stafford and Morse, 1998). It was expected that the equivalent mutation within ΔvJ-hER would have a similar effect on the transcription of v-Jun target promoters. Having determined that full-length ΔvJ-hER mt bound constitutively to TREs (Figure 4.7B), its ability to activate transcription from a v-Jun target promoter was determined. Transcription from the *bkj* promoter was assayed, as *bkj* is a direct transcriptional target of v-Jun ((Hartl and Bister, 1995; Hartl and Bister, 1998), Chapter 3 of this work) and estradiol-induced ΔvJ-hER wt (Kruse *et al.*, 1997).

As expected, ΔvJ-hER mt did not activate the *bkj* promoter in the presence of estradiol (Figure 4.8). In fact, ΔvJ-hER mt reproducibly repressed the basal level of *bkj* transcription in a hormone-dependent manner. A similar effect has been observed in the context of an ER-α protein with a deletion of the AF-2 domain. This protein repressed basal transcription from positive ER-α target promoters in the presence of estradiol (Jung *et al.*, 2001). Unlike wild-type ER-α, which interacts with co-repressor proteins only in the presence of antagonist ligands (Lavinsky *et al.*, 1998; Shang *et al.*, 2000), AF-2-deleted ER-α bound constitutively to SMRT, and estradiol-dependent transcriptional repression was relieved by TSA treatment (Jung *et al.*, 2001). This suggests that the AF-2-deleted protein repressed basal transcription via histone deacetylation.

In contrast to ΔvJ-hER mt, the AF-2-deleted ER-α protein bound DNA in a hormone-dependent manner. This could account for hormone-dependent transcriptional repression by recruitment of HDAC activity to target promoters only in the presence of estradiol. As ΔvJ-hER mt binds constitutively to TREs, its mechanism of hormone-dependent transcriptional repression may differ from that of AF-2-deleted ER-α in some respects. One possibility is that repression by ΔvJ-hER mt is an effect of the increased availability of co-repressor proteins upon estradiol treatment, due to their release by endogenous ER proteins. It would be interesting to determine whether TSA treatment relieves repression of the *bkj* promoter by estradiol-bound ΔvJ-hER mt, and whether the mutated protein binds co-repressors such as SMRT in the presence and absence of estradiol.

As full-length ΔvJ-hER mt bound constitutively to TREs, its failure to activate the *bkj* promoter is thought to be a direct transcriptional effect of AF-2 domain mutation. This supports the hypothesis that hormone-dependent transcriptional activation of v-Jun target promoters by ΔvJ-hER is mediated by the ER-α AF-2 domain. It would be expected that ΔvJ-hER mt is also deficient in the activation of other v-Jun target genes such as HB-EGF. However this has not yet been investigated.

The ability of the ΔvJ-hER proteins to induce hormone-dependent cell transformation was determined. As described in Chapter 4.3.4, ΔvJ-hER wt induced hormone-dependent cell transformation based on three criteria: altered cell morphology, an increased rate of cell proliferation, and the induction of anchorage-independent growth. This confirms previous reports (Kruse *et al.*, 1997). Unexpectedly, estradiol-bound ΔvJ-hER wt induced anchorage-independent growth approximately 3-fold more efficiently than v-Jun (Table 4.1).

In contrast, the transcriptionally inactive ΔvJ-hER mt protein did not induce cell transformation in the presence of estradiol. This supports the hypothesis that hormone-dependent cell transformation by ΔvJ-hER wt is mediated by the ER-α AF-2 domain, and provides evidence for a relationship between activation of v-Jun target promoters and cell transformation (see Chapter 5.3.5).

5.3.4 The role of a HAT co-activator protein in transcriptional activation of v-Jun target promoters and cell transformation.

As described in Chapter 1.3.1.2, there is a great deal of evidence that hormone-dependent transcriptional activation by the AF-2 domain of nuclear hormone receptors is mediated by the recruitment of HAT co-activator proteins. Thyroid hormone receptor proteins containing point mutations within the AF-2 domain have been shown to be defective for binding to SRC-1 and other co-activators (Liu *et al.*, 1998; Tagami *et al.*, 1998). The failure of ΔvJ-hER mt to mediate hormone-dependent transcriptional activation of a v-Jun target promoter may therefore be due to a similar disruption of co-activator binding.

While various co-activators bind nuclear hormone receptors, the nature of their contribution to hormone-dependent transcriptional activation has not, in many cases, been directly determined. However, the HAT activity of p300 has specifically been shown to mediate hormone-dependent transcriptional activation by ER-α (Kraus *et al.*, 1999; Shang *et al.*, 2000). There is also evidence for a role of p300 in transcriptional activation by c-Jun (Lee *et al.*, 1996). The ΔvJ-p300 wt and mt fusion proteins were therefore created, to determine whether direct recruitment of HAT activity to v-Jun target promoters could substitute for the function of the v-Jun TAD or ligand-activated AF-2 domain in transcriptional activation and cell transformation.

Although both ΔvJ-p300 wt and mt were expressed in CEFs and bound TREs (Figure 4.12, 4.13), neither protein induced transcriptional activation of the *bkj* promoter or cell transformation (Figure 4.14, Chapter 4.4.4). The inactivity of the ΔvJ-p300 mt protein was expected, as its HAT domain was derived from p300 Mut AT2, which had negligible HAT activity *in vitro* and was deficient in the hormone-dependent co-activation of ER-α target promoters (Kraus *et al.*, 1999). However, ΔvJ-p300 wt contains a HAT domain which was active in these assays in the context of the full-length p300 protein (Kraus *et al.*, 1999).

The failure of ΔvJ-p300 wt to activate v-Jun target promoters could have several causes. One possibility is that the chimaeric protein has no HAT activity. This would not be expected, as DNA sequencing of RCAS-ΔvJ-p300 wt showed that the p300 HAT domain was fused in-frame with the v-Jun bZip region, and was not mutated during the PCR and cloning process. Additionally, fusion of the equivalent domain of the highly similar CBP protein to the Gal4 DBD created a protein with *in vitro* HAT activity (Martinez-Balbas *et al.*, 1998).

However, differences between p300 and CBP may render a p300 HAT domain fusion such as ΔvJ-p300 wt transcriptionally inactive. The p300 domain used lacks protein regions which interact with other co-activators such as PCAF and SRC-1. These interactions may be necessary for transcriptional activation of v-Jun target promoters by p300. This would not be unprecedented. It has been shown that, while p300 and PCAF both co-activated transcription by MyoD, the HAT activity of PCAF, but not p300, was essential for co-activation (Puri *et al.*, 1997). This suggests that, at some promoters, p300 may activate transcription by the recruitment of PCAF and other HAT proteins. The HAT activity of ΔvJ-p300 wt and mt could be determined by an in-gel HAT assay or similar method. Any HAT deficiency of ΔvJ-p300 wt would provide a probable explanation for the failure of this protein to activate the transcription of a v-Jun target promoter.

However, it may be found that ΔvJ-p300 wt has a functional HAT domain but does not activate transcription. A Gal4-CBP HAT domain fusion protein activated transcription from five Gal4 binding sites located upstream of the adenovirus AdML and E4 promoters, but not the E1B promoter (Martinez-Balbas *et al.*, 1998). It may be that ΔvJ-p300 wt could similarly activate only a sub-set of TRE-containing promoters. Assessment of the effect of ΔvJ-p300 wt expression on other known v-Jun target genes may determine whether this is the case. However, given the failure of ΔvJ-p300 wt to induce cell transformation, it seems

unlikely that the chimaeric protein would activate the transcription of putative v-Jun effector genes such as HB-EGF.

A further possibility is that transcriptional activation of v-Jun target promoters is mediated not by p300, but by other co-activator proteins such as CBP, PCAF or SRC-1. As described in Chapter 1.3.1.2 and 1.3.2.2, various HAT proteins are known to bind c-Jun and nuclear hormone receptors, possibly as co-activator complexes. However, the specific roles of these proteins in transcriptional activation of c-Jun and v-Jun target genes are not known.

Despite the high degree of sequence conservation between p300 and CBP, examples are known where the two proteins have distinct functions. The use of ribozymes which specifically cleave p300 or CBP mRNA showed that p300, but not CBP, was required for transcriptional activation and cell differentiation in response to retinoic acid (Kawasaki *et al.*, 1998; Kawasaki *et al.*, 1999). However, both proteins contributed to retinoic acid-induced growth arrest and apoptosis. It was shown that p300 was required for activation of the p21 promoter, while CBP was specifically involved in activation of the p27 promoter (Kawasaki *et al.*, 1998). A similar study in human cells indicated a role for p300, but not CBP, in ionising radiation-induced apoptosis (Yuan *et al.*, 1999).

These results suggest that, despite the similarity of p300 to CBP, the two proteins are involved in different processes and co-activate different sets of promoters. It may be that v-Jun and ligand-activated ΔvJ-hER wt activate transcription via the recruitment of CBP or other HAT proteins, rather than p300. Fusions of the v-Jun bZip domain to other HAT co-activator proteins may therefore be able to activate transcription from v-Jun target promoters, possibly leading to cell transformation.

Alternatively, the deficiency of ΔvJ-hER mt in hormone-dependent transcriptional activation may be due to a failure to recruit not HAT co-activators, but other factors such as TAF_{II} or SWI/SNF proteins. Determination of the histone acetylation status of the *bkj* promoter in estradiol-treated ΔvJ-hER wt and mt CEFs, for example by ChIP assays, could potentially reveal whether transcriptional activation by ΔvJ-hER wt is dependent on the activities of HAT co-activators, or other proteins.

5.3.5 Cell transformation as a result of v-Jun target gene activation.

As described in Chapter 5.3.3, hormone-dependent transcriptional activation and cell transformation by ΔvJ-hER wt are mediated by the ER-α AF-2 domain. The failure of ΔvJ-hER mt and the ΔvJ-p300 proteins to induce cell transformation is proposed to be due to their inability to activate v-Jun target promoters. The relationship between transcriptional activation of v-Jun target genes and cell transformation is further supported by parallel observations with AV2. This protein consists of the v-Jun bZip domain fused to the VP16 TAD, and has been shown to bind TREs and CREs (Schuur *et al.*, 1993).

The AV2 protein strongly activated the *bkj* promoter (Figure 4.8). AV2 had previously been shown to activate transcription only very weakly compared with v-Jun (Schuur *et al.*, 1993). However, this comparison was performed using the –73/+63 ColCAT promoter. While Schuur *et al* reported some degree of activation of this promoter in CEFs by v-Jun and AV2, other reports have shown the collagenase promoter to be a negative transcriptional target of v-Jun ((Hussain *et al.*, 1998; Kilbey *et al.*, 1996), Chapter 3 of this work). While the reason for this discrepancy is not clear, Figure 4.8 clearly shows that recruitment of the VP16 TAD to the *bkj* promoter induced a high level of transcription. The expression of other v-Jun target genes in AV2 CEFs, such as collagenase or HB-EGF, has not been investigated. As described in Chapter 4.3.4, expression of AV2 induced cell transformation, as assayed by cell morphology, rate of cell proliferation, and anchorage-independent growth. This supports the relationship between activation of v-Jun target genes and cell transformation.

Comparison between Figure 4.8 and Table 4.1 reveals a negative correlation between the efficiency of *bkj* promoter activation and of cell transformation by v-Jun, AV2 and ligand-activated ΔvJ-hER wt. The significance of this relationship is not known. A previous report assayed the efficiency of transcriptional activation and cell transformation by a panel of c-Jun mutant proteins containing various deletions within the delta domain (Havarstein *et al.*, 1992). A negative correlation between transcriptional activation and cell transformation was observed. However, the –73/+63 ColCAT promoter was again used to assay transcription. As this is a negative target of v-Jun, a higher level of activation of this promoter by a mutated c-Jun protein could indicate a lesser degree of transcriptional mis-regulation compared to v-Jun. Indeed, the use of some of these mutant c-Jun proteins in a screening panel revealed a positive correlation between their activation of HB-EGF and

reversion-induced LIM protein, and their efficiency of cell transformation (Fu *et al.*, 1999; Fu *et al.*, 2000).

Others have reported that estradiol-bound ΔvJ-hER wt induced anchorage-independent growth with a similar efficiency to v-Jun (Kruse *et al.*, 1997), and that AV2 induced cell transformation with a significantly decreased efficiency compared to v-Jun (Schuur *et al.*, 1993). While the relative abilities of these proteins to induce cell transformation may vary between studies, it is clear from Figure 4.8 and Table 4.1 that all three constructs induced the activation of a v-Jun target gene and anchorage-independent growth with a high efficiency compared to negative controls. It may be that a threshold level of activation of v-Jun target genes is sufficient to induce cell transformation. It is equally possible that *bkj* is not a v-Jun effector gene, and that the level of activation of genuine effector genes correlates more closely with the efficiency of anchorage-independent growth. Determination of the expression of putative effector genes such as HB-EGF in these cells may help to resolve this issue.

It is also possible that, like v-Jun, AV2 and ligand-activated ΔvJ-hER wt repress certain target genes. While ligand-activated ΔvJ-hER wt did not repress transcription from the c-*jun* promoter, this negative target of v-Jun was nevertheless down-regulated by another mechanism, possibly specific protein degradation (see Chapter 5.3.2). While it would be expected that negative transcriptional targets of v-Jun, such as collagenase, would not be repressed by ligand-activated ΔvJ-hER wt and AV2, other targets may be down-regulated by indirect mechanisms. It is not known whether putative v-Jun effector genes such as SSeCKS and SPARC are down-regulated by v-Jun at the level of transcription, or whether they are indirect targets. Proteins such as these may be found to be down-regulated in AV2 and estradiol-treated ΔvJ-hER wt CEFs. However, this would not necessarily contradict the hypothesis that activation, rather than repression, of direct transcriptional v-Jun target genes is the primary cause of cell transformation.

Cell transformation by v-Jun, AV2 and ligand-activated ΔvJ-hER wt may be a result of altered target promoter selection, as well as increased transcriptional activation, compared to c-Jun. As described in Chapter 1.1.7.1, c-Jun bound preferentially to TREs and a sub-set of CREs, depending on the flanking sequence. However, analysis of DNA sequences bound by the v-Jun bZip domain revealed that v-Jun bound preferentially to CREs, rather than TREs (Kataoka *et al.*, 1994). Also, interaction of c-Jun with other transcription factors can alter its affinity of binding to non-consensus TREs (see Chapter 1.1.7.2). The binding

and activity of v-Jun at such promoters have not been investigated. However, based on the relative preferences of the proteins for binding to TREs and CREs, there are likely to be differences between the spectra of target promoters bound and regulated by c-Jun and v-Jun.

The v-Jun bZip domain would be expected to recruit the ER-α AF-2 domain or VP16 TAD to the same set of target promoters as the v-Jun TAD. This may contribute to cell transformation by AV2 and ligand-activated ΔvJ-hER wt. Indeed, while fusion of the ER-α hormone-binding domain to the v-Jun bZip domain created ΔvJ-hER, which induced hormone-dependent cell transformation (Kruse *et al.*, 1997), fusion of the same ER-α region to the bZip domain of c-Jun created a protein with an altered activity. This TAM67-ER protein induced transcriptional activation from a TRE-containing promoter, but not cell transformation, in the presence of estradiol (Kim *et al.*, 1996). Indeed, expression of TAM67-ER inhibited transformation by Ras and Raf in a hormone-independent manner. This suggests that target promoter selection, as well as activation, is important for the induction of cell transformation by v-Jun.

Emerging technologies such as micro-array analysis may determine whether AV2 and ligand-activated ΔvJ-hER wt alter the expression of the same set of target genes as v-Jun. Analysis of common mis-regulated targets may facilitate the identification of v-Jun effector genes. Identification of these genes, and study of the effect of v-Jun on their regulation, would improve our understanding of the relationship between transcriptional regulation and cell transformation by v-Jun.

References

Aarnisalo, P., Palvimo, J. J., and Janne, O. A. (1998). CREB-binding protein in androgen receptor-mediated signaling, Proc Natl Acad Sci U S A *95*, 2122-7.

Abate, C., Patel, L., Rauscher, F. J., 3rd, and Curran, T. (1990). Redox regulation of fos and jun DNA-binding activity in vitro, Science *249*, 1157-61.

Adler, V., Franklin, C. C., and Kraft, A. S. (1992). Phorbol esters stimulate the phosphorylation of c-Jun but not v-Jun: regulation by the N-terminal delta domain, Proc Natl Acad Sci U S A *89*, 5341-5.

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000). Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter, Cell *103*, 667-78.

Ait-Si-Ali, S., Polesskaya, A., Filleur, S., Ferreira, R., Duquet, A., Robin, P., Vervish, A., Trouche, D., Cabon, F., and Harel-Bellan, A. (2000). CBP/p300 histone acetyl-transferase activity is important for the G1/S transition, Oncogene *19*, 2430-7.

Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R. A. (1997). Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression, Nature *387*, 49-55.

Angel, P., Allegretto, E. A., Okino, S. T., Hattori, K., Boyle, W. J., Hunter, T., and Karin, M. (1988). Oncogene jun encodes a sequence-specific trans-activator similar to AP- 1, Nature *332*, 166-71.

Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H. J., and Herrlich, P. (1987a). 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region, Mol Cell Biol *7*, 2256-66.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987b). Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor, Cell *49*, 729-39.

Arias, J., Alberts, A. S., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J., and Montminy, M. (1994). Activation of cAMP and mitogen responsive genes relies on a common nuclear factor, Nature *370*, 226-9.

Aronheim, A., Zandi, E., Hennemann, H., Elledge, S. J., and Karin, M. (1997). Isolation of an AP-1 repressor by a novel method for detecting protein- protein interactions, Mol Cell Biol *17*, 3094-102.

Auble, D. T., and Brinckerhoff, C. E. (1991). The AP-1 sequence is necessary but not sufficient for phorbol induction of collagenase in fibroblasts, Biochemistry *30*, 4629-35.

Bader, A. G., Hartl, M., and Bister, K. (2000). Conditional cell transformation by doxycycline-controlled expression of the ASV17 v-jun allele, Virology *270*, 98-110.

Ball, A. R., Jr., Bos, T. J., Loliger, C., Nagata, L. P., Nishimura, T., Su, H., Tsuchie, H., and Vogt, P. K. (1988). Jun: oncogene and transcriptional regulator, Cold Spring Harb Symp Quant Biol *53*, 687-93.

Bannister, A. J., and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase, Nature *384*, 641-3.

Bannister, A. J., Oehler, T., Wilhelm, D., Angel, P., and Kouzarides, T. (1995). Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro, Oncogene *11*, 2509-14.

Barila, D., Mangano, R., Gonfloni, S., Kretzschmar, J., Moro, M., Bohmann, D., and Superti-Furga, G. (2000). A nuclear tyrosine phosphorylation circuit: c-Jun as an activator and substrate of c-Abl and JNK, EMBO J *19*, 273-281.

Basso, J., Briggs, J., Findlay, C., and Bos, T. (2000). Directed mutation of the basic domain of v-Jun alters DNA binding specificity and abolishes its oncogenic activity in chicken embryo fibroblasts, Oncogene *19*, 4876-85.

Benbrook, D. M., and Jones, N. C. (1990). Heterodimer formation between CREB and JUN proteins, Oncogene *5*, 295-302.

Binetruy, B., Smeal, T., and Karin, M. (1991). Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain, Nature *351*, 122-7.

Black, E. J., Catling, A. D., Woodgett, J. R., Kilbey, A., and Gillespie, D. A. (1994). Transcriptional activation by the v-Jun oncoprotein is independent of positive regulatory phosphorylation, Oncogene *9*, 2363-8.

Black, E. J., Street, A. J., and Gillespie, D. A. (1991). Protein phosphatase 2A reverses phosphorylation of c-Jun specified by the delta domain in vitro: correlation with oncogenic activation and deregulated transactivation activity of v-Jun, Oncogene *6*, 1949-58.

Bohmann, D., Bos, T. J., Admon, A., Nishimura, T., Vogt, P. K., and Tjian, R. (1987). Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1, Science *238*, 1386-92.

Bohmann, D., and Tjian, R. (1989). Biochemical analysis of transcriptional activation by Jun: differential activity of c- and v-Jun, Cell *59*, 709-17.

Bos, T. J., Bohmann, D., Tsuchie, H., Tjian, R., and Vogt, P. K. (1988). v-jun encodes a nuclear protein with enhancer binding properties of AP- 1, Cell *52*, 705-12.

Bos, T. J., Monteclaro, F. S., Mitsunobu, F., Ball, A. R., Jr., Chang, C. H., Nishimura, T., and Vogt, P. K. (1990). Efficient transformation of chicken embryo fibroblasts by c-Jun requires structural modification in coding and noncoding sequences, Genes Dev *4*, 1677-87.

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995). Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha, Nature *375*, 377-82.

Boyle, W. J., Smeal, T., Defize, L. H., Angel, P., Woodgett, J. R., Karin, M., and Hunter, T. (1991). Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity, Cell *64*, 573-84.

Braunstein, M., Rose, A. B., Holmes, S. G., Allis, C. D., and Broach, J. R. (1993). Transcriptional silencing in yeast is associated with reduced nucleosome acetylation, Genes Dev *7*, 592-604.

Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription, Nature *391*, 597-601.

Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation, Cell *84*, 843-51.

Buratowski, S., Hahn, S., Guarente, L., and Sharp, P. A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II, Cell *56*, 549-61.

Burke, T. W., and Kadonaga, J. T. (1996). Drosophila TFIID binds to a conserved downstream basal promoter element that is present in many TATA-box-deficient promoters, Genes Dev *10*, 711-24.

Burke, T. W., and Kadonaga, J. T. (1997). The downstream core promoter element, DPE, is conserved from Drosophila to humans and is recognized by TAFII60 of Drosophila, Genes Dev *11*, 3020-31.

Caelles, C., Gonzalez-Sancho, J. M., and Munoz, A. (1997). Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway, Genes Dev *11*, 3351-64.

Castellazzi, M., Loiseau, L., Piu, F., and Sergeant, A. (1993). Chimeric c-Jun containing an heterologous homodimerization domain transforms primary chick embryo fibroblasts, Oncogene *8*, 1149-60.

Cavalieri, F., Ruscio, T., Tinoco, R., Benedict, S., Davis, C., and Vogt, P. K. (1985). Isolation of three new avian sarcoma viruses: ASV 9, ASV 17, and ASV 25, Virology *143*, 680-3.

Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M., and Evans, R. M. (1996). Role of CBP/P300 in nuclear receptor signalling, Nature *383*, 99-103.

Chakravarti, D., Ogryzko, V., Kao, H. Y., Nash, A., Chen, H., Nakatani, Y., and Evans, R. M. (1999). A viral mechanism for inhibition of p300 and PCAF acetyltransferase activity, Cell *96*, 393-403.

Chalkley, G. E., and Verrijzer, C. P. (1999). DNA binding site selection by RNA polymerase II TAFs: a TAF(II)250- TAF(II)150 complex recognizes the initiator, EMBO J *18*, 4835-45.

Chamberlain, S. H., Hemmer, R. M., and Brinckerhoff, C. E. (1993). Novel phorbol ester response region in the collagenase promoter binds Fos and Jun, J Cell Biochem *52*, 337-51.

Chatterjee, S., and Struhl, K. (1995). Connecting a promoter-bound protein to TBP bypasses the need for a transcriptional activation domain, Nature *374*, 820-2.

Chatton, B., Bocco, J. L., Goetz, J., Gaire, M., Lutz, Y., and Kedinger, C. (1994). Jun and Fos heterodimerize with ATFa, a member of the ATF/CREB family and modulate its transcriptional activity, Oncogene *9*, 375-85.

Chauchereau, A., Georgiakaki, M., Perrin-Wolff, M., Milgrom, E., and Loosfelt, H. (2000). JAB1 interacts with both the progesterone receptor and SRC-1, J Biol Chem *275*, 8540-8.

Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300, Cell *90*, 569-80.

Chen, J. D., and Evans, R. M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors, Nature *377*, 454-7.

Cheng, S. W., Davies, K. P., Yung, E., Beltran, R. J., Yu, J., and Kalpana, G. V. (1999). c-MYC interacts with INI1/hSNF5 and requires the SWI/SNF complex for transactivation function, Nat Genet *22*, 102-5.

Chi, Y., Huddleston, M. J., Zhang, X., Young, R. A., Annan, R. S., Carr, S. A., and Deshaies, R. J. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase, Genes Dev *15*, 1078-92.

Chida, K., and Vogt, P. K. (1992). Nuclear translocation of viral Jun but not of cellular Jun is cell cycle dependent, Proc Natl Acad Sci U S A *89*, 4290-4.

Chinenov, Y., and Kerppola, T. K. (2001). Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity, Oncogene *20*, 2438-52.

Cho, H., Orphanides, G., Sun, X., Yang, X. J., Ogryzko, V., Lees, E., Nakatani, Y., and Reinberg, D. (1998). A human RNA polymerase II complex containing factors that modify chromatin structure, Mol Cell Biol *18*, 5355-63.

Claret, F. X., Hibi, M., Dhut, S., Toda, T., and Karin, M. (1996). A new group of conserved coactivators that increase the specificity of AP-1 transcription factors, Nature *383*, 453-7.

Clark, W., Black, E. J., MacLaren, A., Kruse, U., LaThangue, N., Vogt, P. K., and Gillespie, D. A. (2000). v-Jun overrides the mitogen dependence of S-phase entry by deregulating retinoblastoma protein phosphorylation and E2F-pocket protein interactions as a consequence of enhanced cyclin E-cdk2 catalytic activity, Mol Cell Biol *20*, 2529-42.

Clark, W., and Gillespie, D. A. (1997). Transformation by v-Jun prevents cell cycle exit and promotes apoptosis in the absence of serum growth factors, Cell Growth Differ *8*, 371-80.

Cohen, S. B., Waha, A., Gelman, I. H., and Vogt, P. K. (2001). Expression of a down-regulated target, SSeCKS, reverses v-Jun-induced transformation of 10T1/2 murine fibroblasts, Oncogene *20*, 141-6.

Comb, M., Birnberg, N. C., Seasholtz, A., Herbert, E., and Goodman, H. M. (1986). A cyclic AMP- and phorbol ester-inducible DNA element, Nature *323*, 353-6.

Cook, S. J., Aziz, N., and McMahon, M. (1999). The repertoire of fos and jun proteins expressed during the G1 phase of the cell cycle is determined by the duration of mitogen-activated protein kinase activation, Mol Cell Biol *19*, 330-41.

Cook, W. J., Gu, B., DeLuca, N. A., Moynihan, E. B., and Coen, D. M. (1995). Induction of transcription by a viral regulatory protein depends on the relative strengths of functional TATA boxes, Mol Cell Biol *15*, 4998-5006.

Cote, J., Quinn, J., Workman, J. L., and Peterson, C. L. (1994). Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex, Science *265*, 53-60.

Crowe, D. L., Brown, T. N., Kim, R., Smith, S. M., and Lee, M. K. (2000). A c-fos/Estrogen receptor fusion protein promotes cell cycle progression and proliferation of human cancer cell lines, Mol Cell Biol Res Commun *3*, 243-8.

Cruzalegui, F. H., Hardingham, G. E., and Bading, H. (1999). c-Jun functions as a calcium-regulated transcriptional activator in the absence of JNK/SAPK1 activation, EMBO J *18*, 1335-44.

Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors, EMBO J *11*, 1025-33.

Dennler, S., Prunier, C., Ferrand, N., Gauthier, J. M., and Atfi, A. (2000). c-Jun Inhibits Transforming Growth Factor beta -mediated Transcription by Repressing Smad3 Transcriptional Activity, J Biol Chem *275*, 28858-28865.

Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain, Cell *76*, 1025-37.

Dilworth, F. J., Fromental-Ramain, C., Yamamoto, K., and Chambon, P. (2000). ATP-Driven chromatin remodeling activity and histone acetyltransferases act sequentially during transactivation by RAR/RXR In vitro, Mol Cell *6*, 1049-58.

DiRenzo, J., Shang, Y., Phelan, M., Sif, S., Myers, M., Kingston, R., and Brown, M. (2000). BRG-1 is recruited to estrogen-responsive promoters and cooperates with factors involved in histone acetylation, Mol Cell Biol *20*, 7541-9.

Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E., and Seiser, C. (1999). Histone deacetylase 1 can repress transcription by binding to Sp1, Mol Cell Biol *19*, 5504-11.

Dressel, U., Renkawitz, R., and Baniahmad, A. (2000). Promoter specific sensitivity to inhibition of histone deacetylases: implications for hormonal gene control, cellular differentiation and cancer, Anticancer Res *20*, 1017-22.

Dunphy, E. L., Johnson, T., Auerbach, S. S., and Wang, E. H. (2000). Requirement for TAF(II)250 acetyltransferase activity in cell cycle progression, Mol Cell Biol *20*, 1134-9.

Echlin, D. R., Tae, H. J., Mitin, N., and Taparowsky, E. J. (2000). B-ATF functions as a negative regulator of AP-1 mediated transcription and blocks cellular transformation by Ras and Fos, Oncogene *19*, 1752-63.

Emami, K. H., Jain, A., and Smale, S. T. (1997). Mechanism of synergy between TATA and initiator: synergistic binding of TFIID following a putative TFIIA-induced isomerization, Genes Dev *11*, 3007-19.

Feng, W., Ribeiro, R. C., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushner, P. J., and West, B. L. (1998). Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors, Science *280*, 1747-9.

Fialka, I., Schwarz, H., Reichmann, E., Oft, M., Busslinger, M., and Beug, H. (1996). The estrogen-dependent c-JunER protein causes a reversible loss of mammary epithelial cell polarity involving a destabilization of adherens junctions, J Cell Biol *132*, 1115-32.

Francis, M. K., Phinney, D. G., and Ryder, K. (1995). Analysis of the hormone-dependent regulation of a JunD-estrogen receptor chimera, J Biol Chem *270*, 11502-13.

Franklin, C. C., McCulloch, A. V., and Kraft, A. S. (1995). In vitro association between the Jun protein family and the general transcription factors, TBP and TFIIB, Biochem J *305*, 967-74.

Fu, S., Bottoli, I., Goller, M., and Vogt, P. K. (1999). Heparin-binding epidermal growth factor-like growth factor, a v-Jun target gene, induces oncogenic transformation, Proc Natl Acad Sci U S A *96*, 5716-21.

Fu, S. L., Waha, A., and Vogt, P. K. (2000). Identification and characterization of genes upregulated in cells transformed by v-Jun, Oncogene *19*, 3537-45.

Gao, M., Morgan, I., and Vogt, P. K. (1996). Differential and antagonistic effects of v-Jun and c-Jun, Cancer Res *56*, 4229-35.

Gentz, R., Rauscher, F. J., 3rd, Abate, C., and Curran, T. (1989). Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains, Science *243*, 1695-9.

Goller, M. E., Iacovoni, J. S., Vogt, P. K., and Kruse, U. (1998). Glutaredoxin is a direct target of oncogenic jun, Oncogene *16*, 2945-8.

Gupta, R., Emili, A., Pan, G., Xiao, H., Shales, M., Greenblatt, J., and Ingles, C. J. (1996). Characterization of the interaction between the acidic activation domain of VP16 and the RNA polymerase II initiation factor TFIIB, Nucleic Acids Res *24*, 2324-30.

Gutman, A., and Wasylyk, B. (1990). The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites, EMBO J *9*, 2241-6.

Ha, I., Roberts, S., Maldonado, E., Sun, X., Kim, L. U., Green, M., and Reinberg, D. (1993). Multiple functional domains of human transcription factor IIB: distinct interactions with two general transcription factors and RNA polymerase II, Genes Dev *7*, 1021-32.

Hadman, M., Gabos, L., Loo, M., Sehgal, A., and Bos, T. J. (1996). Isolation and cloning of JTAP-1: a cathepsin like gene upregulated in response to V-Jun induced cell transformation, Oncogene *12*, 135-42.

Hadman, M., Lin, W., Bush, L., and Bos, T. J. (1998). Apolipoprotein A-1 is a negative target of v-Jun overexpression, Oncogene *16*, 655-60.

Hahn, S., Buratowski, S., Sharp, P. A., and Guarente, L. (1989). Yeast TATA-binding protein TFIID binds to TATA elements with both consensus and nonconsensus DNA sequences, Proc Natl Acad Sci U S A *86*, 5718-22.

Hai, T., and Curran, T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity, Proc Natl Acad Sci U S A *88*, 3720-4.

Halazonetis, T. D., Georgopoulos, K., Greenberg, M. E., and Leder, P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities, Cell *55*, 917-24.

Han, T. H., Lamph, W. W., and Prywes, R. (1992). Mapping of epidermal growth factor-, serum-, and phorbol ester- responsive sequence elements in the c-jun promoter, Mol Cell Biol *12*, 4472-7.

Hanstein, B., Eckner, R., DiRenzo, J., Halachmi, S., Liu, H., Searcy, B., Kurokawa, R., and Brown, M. (1996). p300 is a component of an estrogen receptor coactivator complex, Proc Natl Acad Sci U S A *93*, 11540-5.

Hartl, M., and Bister, K. (1995). Specific activation in jun-transformed avian fibroblasts of a gene (bkj) related to the avian beta-keratin gene family, Proc Natl Acad Sci U S A *92*, 11731-5.

Hartl, M., and Bister, K. (1998). Structure and transcriptional regulation of BKJ, a novel AP-1 target gene activated during jun- or fos-induced fibroblast transformation, Oncogene *17*, 2901-13.

Hattori, K., Angel, P., Le Beau, M. M., and Karin, M. (1988). Structure and chromosomal localization of the functional intronless human JUN protooncogene, Proc Natl Acad Sci U S A *85*, 9148-52.

Havarstein, L. S., Morgan, I. M., Wong, W. Y., and Vogt, P. K. (1992). Mutations in the Jun delta region suggest an inverse correlation between transformation and transcriptional activation, Proc Natl Acad Sci U S A *89*, 618-22.

Hawker, K. L., Pintzas, A., Hennigan, R. F., Gillespie, D. A., and Ozanne, B. W. (1993). Transformation by the fos or jun oncogene does not increase AP-1 DNA- binding activity, J Virol *67*, 5487-95.

Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., *et al.* (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression, Nature *387*, 43-8.

Henttu, P. M., Kalkhoven, E., and Parker, M. G. (1997). AF-2 activity and recruitment of steroid receptor coactivator 1 to the estrogen receptor depend on a lysine residue conserved in nuclear receptors, Mol Cell Biol *17*, 1832-9.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain, Genes Dev *7*, 2135-48.

Hirai, S. I., Ryseck, R. P., Mechta, F., Bravo, R., and Yaniv, M. (1989). Characterization of junD: a new member of the jun proto-oncogene family, EMBO J *8*, 1433-9.

Hirschhorn, J. N., Brown, S. A., Clark, C. D., and Winston, F. (1992). Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure, Genes Dev *6*, 2288-98.

Hoeffler, J. P., Deutsch, P. J., Lin, J., and Habener, J. F. (1989). Distinct adenosine 3',5'-monophosphate and phorbol ester-responsive signal transduction pathways converge at the level of transcriptional activation by the interactions of DNA-binding proteins, Mol Endocrinol *3*, 868-80.

Horikoshi, N., Maguire, K., Kralli, A., Maldonado, E., Reinberg, D., and Weinmann, R. (1991). Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID, Proc Natl Acad Sci U S A *88*, 5124-8.

Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and et al. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor, Nature *377*, 397-404.

Hsu, W., Kerppola, T. K., Chen, P. L., Curran, T., and Chen-Kiang, S. (1994). Fos and Jun repress transcription activation by NF-IL6 through association at the basic zipper region, Mol Cell Biol *14*, 268-76.

Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J., and Sutrave, P. (1987). Adaptor plasmids simplify the insertion of foreign DNA into helper- independent retroviral vectors, J Virol *61*, 3004-12.

Huguier, S., Baguet, J., Perez, S., van Dam, H., and Castellazzi, M. (1998). Transcription factor ATF2 cooperates with v-Jun to promote growth factor-independent proliferation in vitro and tumor formation in vivo, Mol Cell Biol *18*, 7020-9.

Hussain, S., Kilbey, A., and Gillespie, D. A. (1998). v-Jun represses c-jun proto-oncogene expression in vivo through a 12-O- tetradecanoylphorbol-13-acetate-responsive element in the proximal gene promoter, Cell Growth Differ *9*, 677-86.

Ichinose, H., Garnier, J. M., Chambon, P., and Losson, R. (1997). Ligand-dependent interaction between the estrogen receptor and the human homologues of SWI2/SNF2, Gene *188*, 95-100.

Imbalzano, A. N., Kwon, H., Green, M. R., and Kingston, R. E. (1994). Facilitated binding of TATA-binding protein to nucleosomal DNA, Nature *370*, 481-5.

Ing, N. H., Beekman, J. M., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1992). Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II), J Biol Chem *267*, 17617-23.

Ito, T., Yamauchi, M., Nishina, M., Yamamichi, N., Mizutani, T., Ui, M., Murakami, M., and Iba, H. (2000). Identification of SWI/SNF complex subunit BAF60a as a determinant of transactivation potential of Fos/Jun dimers, J Biol Chem *26*, 26.

Ivashkiv, L. B., Liou, H. C., Kara, C. J., Lamph, W. W., Verma, I. M., and Glimcher, L. H. (1990). mXBP/CRE-BP2 and c-Jun form a complex which binds to the cyclic AMP, but not to the 12-O-tetradecanoylphorbol-13-acetate, response element, Mol Cell Biol *10*, 1609-21.

Jacq, X., Brou, C., Lutz, Y., Davidson, I., Chambon, P., and Tora, L. (1994). Human TAFII30 is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor, Cell *79*, 107-17.

Jain, J., McCaffrey, P. G., Miner, Z., Kerppola, T. K., Lambert, J. N., Verdine, G. L., Curran, T., and Rao, A. (1993). The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun, Nature *365*, 352-5.

Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B., and Smale, S. T. (1994). DNA sequence requirements for transcriptional initiator activity in mammalian cells, Mol Cell Biol *14*, 116-27.

Johnson, R. S., van Lingen, B., Papaioannou, V. E., and Spiegelman, B. M. (1993). A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture, Genes Dev *7*, 1309-17.

Johnston, H., Kneer, J., Chackalaparampil, I., Yaciuk, P., and Chrivia, J. (1999). Identification of a novel SNF2/SWI2 protein family member, SRCAP, which interacts with CREB-binding protein, J Biol Chem *274*, 16370-6.

Jonat, C., Rahmsdorf, H. J., Park, K. K., Cato, A. C., Gebel, S., Ponta, H., and Herrlich, P. (1990). Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone, Cell *62*, 1189-204.

Jung, D. J., Lee, S. K., and Lee, J. W. (2001). Agonist-dependent Repression Mediated by Mutant Estrogen Receptor alpha That Lacks the Activation Function 2 Core Domain, J Biol Chem *276*, 37280-3.

Jurdic, P., Treilleux, I., Vandel, L., Tabone, E., Huguier, S., Sergeant, A., and Castellazzi, M. (1995). Tumor induction by v-Jun homodimers in chickens, Oncogene *11*, 1699-709.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors, Cell *85*, 403-14.

Kataoka, K., Noda, M., and Nishizawa, M. (1994). Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun, Mol Cell Biol *14*, 700-12.

Kaufmann, J., and Smale, S. T. (1994). Direct recognition of initiator elements by a component of the transcription factor IID complex, Genes Dev *8*, 821-9.

Kawasaki, H., Eckner, R., Yao, T. P., Taira, K., Chiu, R., Livingston, D. M., and Yokoyama, K. K. (1998). Distinct roles of the co-activators p300 and CBP in retinoic-acid-induced F9-cell differentiation, Nature *393*, 284-9.

Kawasaki, H., Schiltz, L., Chiu, R., Itakura, K., Taira, K., Nakatani, Y., and Yokoyama, K. K. (2000). ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation, Nature *405*, 195-200.

Kawasaki, H., Taira, K., and Yokoyama, K. K. (1999). Functional analysis of the transcriptional coactivators p300 and CBP using ribozyme, Nucleic Acids Symp Ser *42*, 263-4.

Keaveney, M., and Struhl, K. (1998). Activator-mediated recruitment of the RNA polymerase II machinery is the predominant mechanism for transcriptional activation in yeast, Mol Cell *1*, 917-24.

Kilbey, A., Black, E. J., Unlu, M., and Gillespie, D. A. (1996). The v-Jun oncoprotein replaces p39 c-Jun as the predominant AP-1 constituent in ASV17-transformed fibroblasts: implications for SAPK/JNK- mediated signal transduction, Oncogene *12*, 2409-18.

Kim, S., Brown, P. H., and Birrer, M. J. (1996). The inhibitory activity of a transdominant c-jun mutant fused to the ligand binding domain of the estrogen receptor, Oncogene *12*, 1043-53.

Klages, N., and Strubin, M. (1995). Stimulation of RNA polymerase II transcription initiation by recruitment of TBP in vivo, Nature *374*, 822-3.

Korzus, E., Torchia, J., Rose, D. W., Xu, L., Kurokawa, R., McInerney, E. M., Mullen, T. M., Glass, C. K., and Rosenfeld, M. G. (1998). Transcription factor-specific requirements for coactivators and their acetyltransferase functions, Science *279*, 703-7.

Kouzarides, T., and Ziff, E. (1988). The role of the leucine zipper in the fos-jun interaction, Nature *336*, 646-51.

Kovary, K., and Bravo, R. (1991). Expression of different Jun and Fos proteins during the G0-to-G1 transition in mouse fibroblasts: in vitro and in vivo associations, Mol Cell Biol *11*, 2451-9.

Kovary, K., and Bravo, R. (1992). Existence of different Fos/Jun complexes during the G0-to-G1 transition and during exponential growth in mouse fibroblasts: differential role of Fos proteins, Mol Cell Biol *12*, 5015-23.

Kraus, W. L., and Kadonaga, J. T. (1998). p300 and estrogen receptor cooperatively activate transcription via differential enhancement of initiation and reinitiation, Genes Dev *12*, 331-42.

Kraus, W. L., Manning, E. T., and Kadonaga, J. T. (1999). Biochemical analysis of distinct activation functions in p300 that enhance transcription initiation with chromatin templates, Mol Cell Biol *19*, 8123-35.

Kruse, U., Iacovoni, J. S., Goller, M. E., and Vogt, P. K. (1997). Hormone-regulatable neoplastic transformation induced by a Jun-estrogen receptor chimera, Proc Natl Acad Sci U S A *94*, 12396-400.

Kundu, T. K., Palhan, V. B., Wang, Z., An, W., Cole, P. A., and Roeder, R. G. (2000). Activator-dependent transcription from chromatin in vitro involving targeted histone acetylation by p300, Mol Cell *6*, 551-61.

Kuo, M. H., Zhou, J., Jambeck, P., Churchill, M. E., and Allis, C. D. (1998). Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo, Genes Dev *12*, 627-39.

Kutach, A. K., and Kadonaga, J. T. (2000). The downstream promoter element DPE appears to be as widely used as the TATA box in Drosophila core promoters, Mol Cell Biol *20*, 4754-64.

Kwon, H., Imbalzano, A. N., Khavari, P. A., Kingston, R. E., and Green, M. R. (1994). Nucleosome disruption and enhancement of activator binding by a human SW1/SNF complex, Nature *370*, 477-81.

Lallemand, D., Spyrou, G., Yaniv, M., and Pfarr, C. M. (1997). Variations in Jun and Fos protein expression and AP-1 activity in cycling, resting and stimulated fibroblasts, Oncogene *14*, 819-30.

Lau, O. D., Kundu, T. K., Soccio, R. E., Ait-Si-Ali, S., Khalil, E. M., Vassilev, A., Wolffe, A. P., Nakatani, Y., Roeder, R. G., and Cole, P. A. (2000). HATs off: selective synthetic inhibitors of the histone acetyltransferases p300 and PCAF, Mol Cell *5*, 589-95.

Lavinsky, R. M., Jepsen, K., Heinzel, T., Torchia, J., Mullen, T. M., Schiff, R., Del-Rio, A. L., Ricote, M., Ngo, S., Gemsch, J., *et al.* (1998). Diverse signaling pathways modulate nuclear receptor recruitment of N- CoR and SMRT complexes, Proc Natl Acad Sci U S A *95*, 2920-5.

Lee, D. K., Horikoshi, M., and Roeder, R. G. (1991a). Interaction of TFIID in the minor groove of the TATA element, Cell *67*, 1241-50.

Lee, D. Y., Hayes, J. J., Pruss, D., and Wolffe, A. P. (1993). A positive role for histone acetylation in transcription factor access to nucleosomal DNA, Cell *72*, 73-84.

Lee, J. S., See, R. H., Deng, T., and Shi, Y. (1996). Adenovirus E1A downregulates cJun- and JunB-mediated transcription by targeting their coactivator p300, Mol Cell Biol *16*, 4312-26.

Lee, S. K., Kim, H. J., Na, S. Y., Kim, T. S., Choi, H. S., Im, S. Y., and Lee, J. W. (1998). Steroid receptor coactivator-1 coactivates activating protein-1- mediated transactivations through interaction with the c-Jun and c-Fos subunits, J Biol Chem *273*, 16651-4.

Lee, S. K., Kim, J. H., Lee, Y. C., Cheong, J., and Lee, J. W. (2000a). Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-kappaB, and serum response factor, J Biol Chem *275*, 12470-4.

Lee, S. K., Na, S. Y., Jung, S. Y., Choi, J. E., Jhun, B. H., Cheong, J., Meltzer, P. S., Lee, Y. C., and Lee, J. W. (2000b). Activating protein-1, nuclear factor-kappaB, and serum response factor as novel target molecules of the cancer-amplified transcription coactivator ASC-2, Mol Endocrinol *14*, 915-25.

Lee, W., Mitchell, P., and Tjian, R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements, Cell *49*, 741-52.

Lee, W. S., Kao, C. C., Bryant, G. O., Liu, X., and Berk, A. J. (1991b). Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor, Cell *67*, 365-76.

Leonard, D. A., Rajaram, N., and Kerppola, T. K. (1997). Structural basis of DNA bending and oriented heterodimer binding by the basic leucine zipper domains of Fos and Jun, Proc Natl Acad Sci U S A *94*, 4913-8.

Li, H., and Capetanaki, Y. (1994). An E box in the desmin promoter cooperates with the E box and MEF-2 sites of a distal enhancer to direct muscle-specific transcription, EMBO J *13*, 3580-9.

Li, L. A., Chiang, E. F., Chen, J. C., Hsu, N. C., Chen, Y. J., and Chung, B. C. (1999). Function of steroidogenic factor 1 domains in nuclear localization, transactivation, and interaction with transcription factor TFIIB and c- Jun, Mol Endocrinol *13*, 1588-98.

Lim, J., Yang, C., Hong, S. J., and Kim, K. S. (2000). Regulation of tyrosine hydroxylase gene transcription by the cAMP- signaling pathway: involvement of multiple transcription factors, Mol Cell Biochem *212*, 51-60.

Lin, A., Frost, J., Deng, T., Smeal, T., al-Alawi, N., Kikkawa, U., Hunter, T., Brenner, D., and Karin, M. (1992). Casein kinase II is a negative regulator of c-Jun DNA binding and AP-1 activity, Cell *70*, 777-89.

Liu, Y., Takeshita, A., Misiti, S., Chin, W. W., and Yen, P. M. (1998). Lack of coactivator interaction can be a mechanism for dominant negative activity by mutant thyroid hormone receptors, Endocrinology *139*, 4197-204.

Lively, T. N., Ferguson, H. A., Galasinski, S. K., Seto, A. G., and Goodrich, J. A. (2001). c-Jun binds the N terminus of human TAFII250 to derepress RNA polymerase II transcription in vitro, J Biol Chem *276*, 25582-8.

Lo, K., and Smale, S. T. (1996). Generality of a functional initiator consensus sequence, Gene *182*, 13-22.

Lonard, D. M., Nawaz, Z., Smith, C. L., and O'Malley, B. W. (2000). The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation, Mol Cell *5*, 939-48.

Macgregor, P. F., Abate, C., and Curran, T. (1990). Direct cloning of leucine zipper proteins: Jun binds cooperatively to the CRE with CRE-BP1, Oncogene *5*, 451-8.

Maki, Y., Bos, T. J., Davis, C., Starbuck, M., and Vogt, P. K. (1987). Avian sarcoma virus 17 carries the jun oncogene, Proc Natl Acad Sci U S A *84*, 2848-52.

Maldonado, E., Ha, I., Cortes, P., Weis, L., and Reinberg, D. (1990). Factors involved in specific transcription by mammalian RNA polymerase II: role of transcription factors IIA, IID, and IIB during formation of a transcription-competent complex, Mol Cell Biol *10*, 6335-47.

Martin, M. L., Lieberman, P. M., and Curran, T. (1996). Fos-Jun dimerization promotes interaction of the basic region with TFIIE-34 and TFIIF, Mol Cell Biol *16*, 2110-8.

Martinez-Balbas, M. A., Bannister, A. J., Martin, K., Haus-Seuffert, P., Meisterernst, M., and Kouzarides, T. (1998). The acetyltransferase activity of CBP stimulates transcription, EMBO J *17*, 2886-93.

Matrisian, L. M., Leroy, P., Ruhlmann, C., Gesnel, M. C., and Breathnach, R. (1986). Isolation of the oncogene and epidermal growth factor-induced transin gene: complex control in rat fibroblasts, Mol Cell Biol *6*, 1679-86.

May, G. H., Funk, M., Black, E. J., Clark, W., Hussain, S., Woodgett, J. R., and Gillespie, D. A. (1998). An oncogenic mutation uncouples the v-Jun oncoprotein from positive regulation by the SAPK/JNK pathway in vivo, Curr Biol *8*, 117-20.

May, M., Mengus, G., Lavigne, A. C., Chambon, P., and Davidson, I. (1996). Human TAF(II28) promotes transcriptional stimulation by activation function 2 of the retinoid X receptors, EMBO J *15*, 3093-104.

Mizuguchi, G., Vassilev, A., Tsukiyama, T., Nakatani, Y., and Wu, C. (2001). ATP-dependent nucleosome remodeling and histone hyperacetylation synergistically facilitate transcription of chromatin, J Biol Chem *276*, 14773-83.

Mizzen, C. A., Yang, X. J., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S. L., Kouzarides, T., *et al.* (1996). The TAF(II)250 subunit of TFIID has histone acetyltransferase activity, Cell *87*, 1261-70.

Molinari, E., Gilman, M., and Natesan, S. (1999). Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency in vivo, EMBO J *18*, 6439-47.

Monroy, M. A., Ruhl, D. D., Xu, X., Granner, D. K., Yaciuk, P., and Chrivia, J. C. (2001). Regulation of cAMP-responsive Element-binding Protein-mediated Transcription by the SNF2/SWI-related Protein, SRCAP, J Biol Chem *276*, 40721-40726.

Morgan, I. M., Asano, M., Havarstein, L. S., Ishikawa, H., Hiiragi, T., Ito, Y., and Vogt, P. K. (1993). Amino acid substitutions modulate the effect of Jun on transformation, transcriptional activation and DNA replication, Oncogene *8*, 1135-40.

Morgan, I. M., Havarstein, L. S., Wong, W. Y., Luu, P., and Vogt, P. K. (1994). Efficient induction of fibrosarcomas by v-jun requires mutations in the DNA binding region and the transactivation domain, Oncogene *9*, 2793-7.

Morgan, I. M., Ransone, L. J., Bos, T. J., Verma, I. M., and Vogt, P. K. (1992). Transformation by Jun: requirement for leucine zipper, basic region and transactivation domain and enhancement by Fos, Oncogene *7*, 1119-25.

Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997). Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase, Cell *89*, 373-80.

Nakabeppu, Y., Ryder, K., and Nathans, D. (1988). DNA binding activities of three murine Jun proteins: stimulation by Fos, Cell *55*, 907-15.

Natarajan, K., Jackson, B. M., Zhou, H., Winston, F., and Hinnebusch, A. G. (1999). Transcriptional activation by Gcn4p involves independent interactions with the SWI/SNF complex and the SRB/mediator, Mol Cell *4*, 657-64.

Nead, M. A., Baglia, L. A., Antinore, M. J., Ludlow, J. W., and McCance, D. J. (1998). Rb binds c-Jun and activates transcription, EMBO J *17*, 2342-52.

Neely, K. E., Hassan, A. H., Wallberg, A. E., Steger, D. J., Cairns, B. R., Wright, A. P., and Workman, J. L. (1999). Activation domain-mediated targeting of the SWI/SNF complex to promoters stimulates transcription from nucleosome arrays, Mol Cell *4*, 649-55.

Ng, K. W., Ridgway, P., Cohen, D. R., and Tremethick, D. J. (1997). The binding of a Fos/Jun heterodimer can completely disrupt the structure of a nucleosome, EMBO J *16*, 2072-85.

Nirmala, P. B., and Thampan, R. V. (1995). Ubiquitination of the rat uterine estrogen receptor: dependence on estradiol, Biochem Biophys Res Commun *213*, 24-31.

Nishimura, T., and Vogt, P. K. (1988). The avian cellular homolog of the oncogene jun, Oncogene *3*, 659-63.

Nishitani, J., Nishinaka, T., Cheng, C. H., Rong, W., Yokoyama, K. K., and Chiu, R. (1999). Recruitment of the retinoblastoma protein to c-Jun enhances transcription activity mediated through the AP-1 binding site, J Biol Chem *274*, 5454-61.

Nomura, N., Zu, Y. L., Maekawa, T., Tabata, S., Akiyama, T., and Ishii, S. (1993). Isolation and characterization of a novel member of the gene family encoding the cAMP response element-binding protein CRE-BP1, J Biol Chem *268*, 4259-66.

Nowak, S. J., and Corces, V. G. (2000). Phosphorylation of histone H3 correlates with transcriptionally active loci, Genes Dev *14*, 3003-13.

Oberst, C., Weiskirchen, R., Hartl, M., and Bister, K. (1997). Suppression in transformed avian fibroblasts of a gene (CO6) encoding a membrane protein related to mammalian potassium channel regulatory subunits, Oncogene *14*, 1109-16.

Oehler, T., Pintzas, A., Stumm, S., Darling, A., Gillespie, D., and Angel, P. (1993). Mutation of a phosphorylation site in the DNA-binding domain is required for redox-independent transactivation of AP1-dependent genes by v-Jun, Oncogene *8*, 1141-7.

Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases, Cell *87*, 953-9.

Orphanides, G., Lagrange, T., and Reinberg, D. (1996). The general transcription factors of RNA polymerase II, Genes Dev *10*, 2657-83.

Osburn, D. L., Shao, G., Seidel, H. M., and Schulman, I. G. (2001). Ligand-dependent degradation of retinoid X receptors does not require transcriptional activity or coactivator interactions, Mol Cell Biol *21*, 4909-18.

O'Shea-Greenfield, A., and Smale, S. T. (1992). Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription, J Biol Chem *267*, 1391-402.

Patikoglou, G. A., Kim, J. L., Sun, L., Yang, S. H., Kodadek, T., and Burley, S. K. (1999). TATA element recognition by the TATA box-binding protein has been conserved throughout evolution, Genes Dev *13*, 3217-30.

Perera, L. P. (2000). The TATA motif specifies the differential activation of minimal promoters by varicella zoster virus immediate-early regulatory protein IE62, J Biol Chem *275*, 487-96.

Perez, S., Vial, E., van Dam, H., and Castellazzi, M. (2001). Transcription factor ATF3 partially transforms chick embryo fibroblasts by promoting growth factor-independent proliferation, Oncogene *20*, 1135-41.

Peterson, B. R., Sun, L. J., and Verdine, G. L. (1996). A critical arginine residue mediates cooperativity in the contact interface between transcription factors NFAT and AP-1, Proc Natl Acad Sci U S A *93*, 13671-6.

Pugh, B. F. (2000). Control of gene expression through regulation of the TATA-binding protein, Gene *255*, 1-14.

Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991). Phosphorylation of c-jun mediated by MAP kinases, Nature *353*, 670-4.

Puri, P. L., Sartorelli, V., Yang, X. J., Hamamori, Y., Ogryzko, V. V., Howard, B. H., Kedes, L., Wang, J. Y., Graessmann, A., Nakatani, Y., and Levrero, M. (1997). Differential roles of p300 and PCAF acetyltransferases in muscle differentiation, Mol Cell *1*, 35-45.

Qing, J., Zhang, Y., and Derynck, R. (2000). Structural and Functional Characterization of the Transforming Growth Factor-beta -induced Smad3/c-Jun Transcriptional Cooperativity, J Biol Chem *275*, 38802-38812.

Rajaram, N., and Kerppola, T. K. (1997). DNA bending by Fos-Jun and the orientation of heterodimer binding depend on the sequence of the AP-1 site, EMBO J *16*, 2917-25.

Ramirez-Carrozzi, V. R., and Kerppola, T. K. (2001a). Control of the orientation of Fos-Jun binding and the transcriptional cooperativity of Fos-Jun-NFAT1 complexes, J Biol Chem *276*, 21797-808.

Ramirez-Carrozzi, V. R., and Kerppola, T. K. (2001b). Dynamics of Fos-Jun-NFAT1 complexes, Proc Natl Acad Sci U S A *98*, 4893-8.

Ramirez-Carrozzi, V. R., and Kerppola, T. K. (2001c). Long-range electrostatic interactions influence the orientation of Fos- Jun binding at AP-1 sites, J Mol Biol *305*, 411-27.

Ransone, L. J., Kerr, L. D., Schmitt, M. J., Wamsley, P., and Verma, I. M. (1993). The bZIP domains of Fos and Jun mediate a physical association with the TATA box-binding protein, Gene Expr *3*, 37-48.

Rauscher, F. J., 3rd, Sambucetti, L. C., Curran, T., Distel, R. J., and Spiegelman, B. M. (1988a). Common DNA binding site for Fos protein complexes and transcription factor AP-1, Cell *52*, 471-80.

Rauscher, F. J., 3rd, Voulalas, P. J., Franza, B. R., Jr., and Curran, T. (1988b). Fos and Jun bind cooperatively to the AP-1 site: reconstitution in vitro, Genes Dev *2*, 1687-99.

Rauscher, F. J. d., Cohen, D. R., Curran, T., Bos, T. J., Vogt, P. K., Bohmann, D., Tjian, R., and Franza, B. R., Jr. (1988c). Fos-associated protein p39 is the product of the jun proto-oncogene, Science *240*, 1010-6.

Renaud, J. P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1995). Crystal structure of the RAR-gamma ligand-binding domain bound to all- trans retinoic acid, Nature *378*, 681-9.

Rutberg, S. E., Saez, E., Lo, S., Jang, S. I., Markova, N., Spiegelman, B. M., and Yuspa, S. H. (1997). Opposing activities of c-Fos and Fra-2 on AP-1 regulated transcriptional activity in mouse keratinocytes induced to differentiate by calcium and phorbol esters, Oncogene *15*, 1337-46.

Ryder, K., Lau, L. F., and Nathans, D. (1988). A gene activated by growth factors is related to the oncogene v-jun, Proc Natl Acad Sci U S A *85*, 1487-91.

Ryseck, R. P., and Bravo, R. (1991). c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins, Oncogene *6*, 533-42.

Sachs, L. M., and Shi, Y. B. (2000). Targeted chromatin binding and histone acetylation in vivo by thyroid hormone receptor during amphibian development, Proc Natl Acad Sci U S A *97*, 13138-43.

Salghetti, S. E., Caudy, A. A., Chenoweth, J. G., and Tansey, W. P. (2001). Regulation of transcriptional activation domain function by ubiquitin, Science *293*, 1651-3.

Salghetti, S. E., Kim, S. Y., and Tansey, W. P. (1999). Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc, EMBO J *18*, 717-26.

Salghetti, S. E., Muratani, M., Wijnen, H., Futcher, B., and Tansey, W. P. (2000). Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis, Proc Natl Acad Sci U S A *97*, 3118-23.

Sassone-Corsi, P., Lamph, W. W., Kamps, M., and Verma, I. M. (1988). fos-associated cellular p39 is related to nuclear transcription factor AP-1, Cell *54*, 553-60.

Schaefer, T. S., Sanders, L. K., and Nathans, D. (1995). Cooperative transcriptional activity of Jun and Stat3 beta, a short form of Stat3, Proc Natl Acad Sci U S A *92*, 9097-101.

Schonthal, A., Herrlich, P., Rahmsdorf, H. J., and Ponta, H. (1988). Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters, Cell *54*, 325-34.

Schuermann, M., Hennig, G., and Muller, R. (1993). Transcriptional activation and transformation by chimaeric Fos-estrogen receptor proteins: altered properties as a consequence of gene fusion, Oncogene *8*, 2781-90.

Schuermann, M., Neuberg, M., Hunter, J. B., Jenuwein, T., Ryseck, R. P., Bravo, R., and Muller, R. (1989). The leucine repeat motif in Fos protein mediates complex formation with Jun/AP-1 and is required for transformation, Cell *56*, 507-16.

Schuringa, J. J., Timmer, H., Luttickhuizen, D., Vellenga, E., and Kruijer, W. (2001). c-Jun and c-Fos cooperate with STAT3 in IL-6-induced transactivation of the IL-6 respone element (IRE), Cytokine *14*, 78-87.

Schuur, E. R., Parker, E. J., and Vogt, P. K. (1993). Chimeras of herpes simplex viral VP16 and jun are oncogenic, Cell Growth Differ *4*, 761-8.

Sehgal, A., Briggs, J., Rinehart-Kim, J., Basso, J., and Bos, T. J. (2000). The chicken c-Jun 5' untranslated region directs translation by internal initiation, Oncogene *19*, 2836-45.

Sewack, G. F., Ellis, T. W., and Hansen, U. (2001). Binding of TATA binding protein to a naturally positioned nucleosome is facilitated by histone acetylation, Mol Cell Biol *21*, 1404-15.

Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. (2000). Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription, Cell *103*, 843-52.

Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen, Cell *95*, 927-37.

Slack, A., Pinard, M., Araujo, F. D., and Szyf, M. (2001). A novel regulatory element in the dnmt1 gene that responds to co- activation by Rb and c-Jun, Gene *268*, 87-96.

Smale, S. T., and Baltimore, D. (1989). The "initiator" as a transcription control element, Cell *57*, 103-13.

Smeal, T., Binetruy, B., Mercola, D. A., Birrer, M., and Karin, M. (1991). Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73, Nature *354*, 494-6.

Smith, C. A., Bates, P., Rivera-Gonzalez, R., Gu, B., and DeLuca, N. A. (1993). ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB, J Virol *67*, 4676-87.

Smith, C. L., Onate, S. A., Tsai, M. J., and O'Malley, B. W. (1996). CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription, Proc Natl Acad Sci U S A *93*, 8884-8.

Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997). Steroid receptor coactivator-1 is a histone acetyltransferase, Nature *389*, 194-8.

Stafford, G. A., and Morse, R. H. (1998). Mutations in the AF-2/Hormone-binding domain of the chimeric activator GAL4.Estrogen Receptor.VP16 inhibit hormone-dependent transcriptional activation and chromatin remodeling in yeast, J Biol Chem *273*, 34240-6.

Starr, D. B., and Hawley, D. K. (1991). TFIID binds in the minor groove of the TATA box, Cell *67*, 1231-40.

Stehelin, D., Varmus, H. E., Bishop, J. M., and Vogt, P. K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA, Nature *260*, 170-3.

Stringer, K. F., Ingles, C. J., and Greenblatt, J. (1990). Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID, Nature *345*, 783-6.

Struhl, K. (1987). The DNA-binding domains of the jun oncoprotein and the yeast GCN4 transcriptional activator protein are functionally homologous, Cell *50*, 841-6.

Struhl, K. (1988). The JUN oncoprotein, a vertebrate transcription factor, activates transcription in yeast, Nature *332*, 649-50.

Superti-Furga, G., Bergers, G., Picard, D., and Busslinger, M. (1991). Hormone-dependent transcriptional regulation and cellular transformation by Fos-steroid receptor fusion proteins, Proc Natl Acad Sci U S A *88*, 5114-8.

Suzuki, T., Okuno, H., Yoshida, T., Endo, T., Nishina, H., and Iba, H. (1991). Difference in transcriptional regulatory function between c-Fos and Fra- 2, Nucleic Acids Res *19*, 5537-42.

Tagami, T., Gu, W. X., Peairs, P. T., West, B. L., and Jameson, J. L. (1998). A novel natural mutation in the thyroid hormone receptor defines a dual functional domain that exchanges nuclear receptor corepressors and coactivators, Mol Endocrinol *12*, 1888-902.

Tagawa, T., Kuroki, T., Vogt, P. K., and Chida, K. (1995). The cell cycle-dependent nuclear import of v-Jun is regulated by phosphorylation of a serine adjacent to the nuclear localization signal, J Cell Biol *130*, 255-63.

Takahashi, K., Vigneron, M., Matthes, H., Wildeman, A., Zenke, M., and Chambon, P. (1986). Requirement of stereospecific alignments for initiation from the simian virus 40 early promoter, Nature *319*, 121-6.

Tansey, W. P. (2001). Transcriptional activation: risky business, Genes Dev *15*, 1045-50.

Taylor, I. C., and Kingston, R. E. (1990a). E1a transactivation of human HSP70 gene promoter substitution mutants is independent of the composition of upstream and TATA elements, Mol Cell Biol *10*, 176-83.

Taylor, I. C., and Kingston, R. E. (1990b). Factor substitution in a human HSP70 gene promoter: TATA-dependent and TATA-independent interactions, Mol Cell Biol *10*, 165-75.

Tinti, C., Yang, C., Seo, H., Conti, B., Kim, C., Joh, T. H., and Kim, K. S. (1997). Structure/function relationship of the cAMP response element in tyrosine hydroxylase gene transcription, J Biol Chem *272*, 19158-64.

Tsang, T. C., Chu, Y. W., Powell, M. B., Kittelson, J., Meade-Tollin, L., Hendrix, M. J., and Bowden, G. T. (1994). v-jun oncogene suppresses both phorbol ester-induced cell invasion and stromelysin gene expression in a mouse papilloma cell line, Cancer Res *54*, 882-6.

Turner, R., and Tjian, R. (1989). Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers, Science *243*, 1689-94.

van Dam, H., Huguier, S., Kooistra, K., Baguet, J., Vial, E., van der Eb, A. J., Herrlich, P., Angel, P., and Castellazzi, M. (1998). Autocrine growth and anchorage independence: two complementing Jun- controlled genetic programs of cellular transformation, Genes Dev *12*, 1227-39.

Vandel, L., and Kouzarides, T. (1999). Residues phosphorylated by TFIIH are required for E2F-1 degradation during S-phase, EMBO J *18*, 4280-91.

Verrecchia, F., Tacheau, C., Schorpp-Kistner, M., Angel, P., and Mauviel, A. (2001). Induction of the AP-1 members c-Jun and JunB by TGF-beta/Smad suppresses early Smad-driven gene activation, Oncogene *20*, 2205-11.

Vial, E., and Castellazzi, M. (2000). Down-regulation of the extracellular matrix protein SPARC in vSrc- and vJun-transformed chick embryo fibroblasts contributes to tumor formation in vivo, Oncogene *19*, 1772-82.

Vial, E., Perez, S., and Castellazzi, M. (2000). Transcriptional control of SPARC by v-Jun and other members of the AP1 family of transcription factors, Oncogene *19*, 5020-9.

Vogt, P. K., Bos, T. J., and Doolittle, R. F. (1987). Homology between the DNA-binding domain of the GCN4 regulatory protein of yeast and the carboxyl-terminal region of a protein coded for by the oncogene jun, Proc Natl Acad Sci U S A *84*, 3316-9.

Wang, L., Liu, L., and Berger, S. L. (1998). Critical residues for histone acetylation by Gcn5, functioning in Ada and SAGA complexes, are also required for transcriptional function in vivo, Genes Dev *12*, 640-53.

Wasylyk, C., Imler, J. L., and Wasylyk, B. (1988). Transforming but not immortalizing oncogenes activate the transcription factor PEA1, EMBO J *7*, 2475-83.

Westermarck, J., Seth, A., and Kahari, V. M. (1997). Differential regulation of interstitial collagenase (MMP-1) gene expression by ETS transcription factors, Oncogene *14*, 2651-60.

White, L. A., and Brinckerhoff, C. E. (1995). Two activator protein-1 elements in the matrix metalloproteinase-1 promoter have different effects on transcription and bind Jun D, c-Fos, and Fra-2, Matrix Biol *14*, 715-25.

Wijayaratne, A. L., and McDonnell, D. P. (2001). The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators, J Biol Chem *276*, 35684-92.

Wilson, C. J., Chao, D. M., Imbalzano, A. N., Schnitzler, G. R., Kingston, R. E., and Young, R. A. (1996). RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling, Cell *84*, 235-44.

Wolner, B. S., and Gralla, J. D. (2000). Roles for non-TATA core promoter sequences in transcription and factor binding, Mol Cell Biol *20*, 3608-15.

Wong, C., Rougier-Chapman, E. M., Frederick, J. P., Datto, M. B., Liberati, N. T., Li, J. M., and Wang, X. F. (1999). Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta, Mol Cell Biol *19*, 1821-30.

Wong, W. Y., Havarstein, L. S., Morgan, I. M., and Vogt, P. K. (1992). c-Jun causes focus formation and anchorage-independent growth in culture but is non-tumorigenic, Oncogene *7*, 2077-80.

Xiao, H., Friesen, J. D., and Lis, J. T. (1995). Recruiting TATA-binding protein to a promoter: transcriptional activation without an upstream activator, Mol Cell Biol *15*, 5757-61.

Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, J. L., Triezenberg, S. J., Reinberg, D., Flores, O., Ingles, C. J., and et al. (1994). Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53, Mol Cell Biol *14*, 7013-24.

Xing, J., Sheppard, H. M., Corneillie, S. I., and Liu, X. (2001). p53 Stimulates TFIID-TFIIA-promoter complex assembly, and p53-T antigen complex inhibits TATA binding protein-TATA interaction, Mol Cell Biol *21*, 3652-61.

Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996). A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A, Nature *382*, 319-24.

Yao, T. P., Ku, G., Zhou, N., Scully, R., and Livingston, D. M. (1996). The nuclear hormone receptor coactivator SRC-1 is a specific target of p300, Proc Natl Acad Sci U S A *93*, 10626-31.

Yuan, Z. M., Huang, Y., Ishiko, T., Nakada, S., Utsugisawa, T., Shioya, H., Utsugisawa, Y., Shi, Y., Weichselbaum, R., and Kufe, D. (1999). Function for p300 and not CBP in the apoptotic response to DNA damage, Oncogene *18*, 5714-7.

Yudkovsky, N., Logie, C., Hahn, S., and Peterson, C. L. (1999). Recruitment of the SWI/SNF chromatin remodeling complex by transcriptional activators, Genes Dev *13*, 2369-74.

Zhang, X., Wrzeszczynska, M. H., Horvath, C. M., and Darnell, J. E., Jr. (1999). Interacting Regions in Stat3 and c-Jun That Participate in Cooperative Transcriptional Activation, Mol Cell Biol *19*, 7138-7146.

Zhang, Y., Feng, X. H., and Derynck, R. (1998). Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription, Nature *394*, 909-13.