The Biological Consequences and Molecular Basis of Jun-Mediated Autorepression in Avian Fibroblasts.

by
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For Malcolm and Mairi.

Abstract

c-Jun expression is down-regulated in v-Jun- and c-Jun-transformed chicken embryo fibroblasts. The down-regulation is a specific consequence of high Jun expression suggesting that *c-jun*, like several other immediate early genes, is subject to negative autoregulation.

The work in this thesis has attempted to define the consequences of and the molecular basis for Jun-mediated autorepression in vivo. c-Jun is the sole or predominant Jun family protein expressed in primary CEFs. Repression of endogenous p39 c-Jun in ASV17-transformed cultures results in the replacement of c-Jun-containing AP-1/TRE binding complexes with v-Jun-containing alternatives. The absence of auxiliary Jun family proteins facilitates the displacement and may contribute to the unique transforming activites of v-Jun (and c-Jun) in avian cells. v-Jun-mediated auto-repression is primarily directed at the level of transcription and correlates with specific changes in occupancy at the proximal junTRE and adjacent junRSRE binding sites in the *c-jun* promoter. In normal asynchronous cultures specific binding factors compete for the adjacent junTRE and junRSRE regulatory elements. In ASV17-transformed cells the junTRE is exclusively occupied by v-Jun-containing complexes and endogenous *c-jun* expression is down-regulated. The absence of junRSRE occupancy in ASV17-transformed cells is associated with high levels of the v-Jun oncoprotein which physically disrupt or inhibit the binding activity of junRSRE-specific complexes. Mutually exclusive binding at the junTRE and junRSRE or a Jun-dependent sequestration of specific accessory factors have been proposed to direct the single pattern of occupancy in ASV17-transformed cells and to, thereby, contribute to the down-regulation of endogenous *c-jun* expression.

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Abbreviations.

A	: Adenine.
aa	: Amino Acid.
A_X	: Absorbance wavelength(nm).
AMP	: Adenosine monophosphate.
AP-1	: Activator protein 1.
ASV17	: Avian sarcoma virus 17.
ATF	: Activating transcription factor.
B-gal	: B galactosidase.
bp	: Base Pair.
BR	: Basic Region.
C	: Cytosine.
^oC	: Degrees centigrade.
cDNA	: Complementary deoxyribonucleic acid.
c/EBP	: CCAAT/ enhancer-binding protein.
CEF	: Chicken Embryo Fibroblasts.
Ci	: Curie.
cpm	: Counts per minute.
CRE	: Cyclic AMP response element.
CREB	: CRE binding protein.
CREM	: CRE modulator
CTF	: CCAAT binding transcription factor.
DABCO	: 1,4, Diazabicyclo (2,2,2) octane.
DMEM	: Dulbecco's Modified Eagles Medium.
DMSO	: Dimethylsulphoxide.
DNA	: Deoxyribonucleic acid.
DNAse	: Deoxyribonuclease.
dNTP	: 3' deoxyribonucleoside 5' triphosphate.
DTT	: Dithiothreitol.
E1A	: Adenovirus early region 1A.
EDTA	: Ethylenediaminetetra-acetic acid, disodium salt.
EGF	: Epidermal growth factor.
EGTA	: Ethylenediguaninetetra-acetic acid, disodium salt
ERK	: Extracellular signal regulatory protein kinase 1 & 2.
ERT	: EGF receptor threonine protein kinase.
g	: Gram.

G	: Guanine.
gag	: Group antigen.
GAPDH	: Glyceraldehyde phosphate dehydrogenase.
GAR FITC	: Goat-anti-rabbit Fluoresceine Isothiocyanate.
GM-CSF	: Granulocyte-macrophage colony-stimulating factor.
Hepes	: N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid).
HI	: Heat inactivated.
HLH	: Helic-Loop-Helix.
hr	: Hour.
HS	: DNase I Hypersensitivity site.
hsp	: Heat shock protein.
HSV-IE5	: Herpes Simplex virus - Immediate Early gene 5 promoter
IL-1	: Interleukin-1.
IL-2	: Interleukin-2.
IPTG	: Isopropylthiogalactoside.
k	: Kilo.
l	: Litre.
LTR	: Long terminal repeat.
LZ	: Leucine zipper.
μ	: Micro.
m	: Milli.
M	: Molar.
mA	: Milliamps.
MAP	: Mitogen activated protein kinase.
MHC	: Major histocompatability complex.
min	: Minute.
mm	: Millimetre.
MADS	: MCM1-Arg80-agamous-deficiens-SRF).
MEM	: Minimal essential medium.
mRNA	: Messenger ribonucleic acid.
n	: nano.
NF-AT	: Nuclear factor of activated T cells.
NF-E2	: Nuclear factor of erythroid cells.
NP40	: Nonyl phenoxy polyethoxy ethanol.
ONPG	: o-Nitrophenyl-B-D Galactopyranoside.
phage	: Bacteriophage.
PBS	: Phosphate-buffered saline.
PCR	: Polymerase chain reaction.

PKC	: Protein kinase C.
PIPES	: Piperazine-N, N'-bis (2-ethanesulphonic acid).
PMSF	: Phenylmethylsulfonyl flouride.
poly(dI-dC): dI-dC)	: Polydeoxyinosinic deoxycytidylic acid.
RCAN	: Replication Competent, Avian leukaemia virus LTR, No splice acceptor.
RCAS	: As above with splice acceptor.
REF	: Rat embryo fibroblast.
Ref-1	: Redox factor 1.
RERE	: Retinoic acid response element.
RNA	: Ribonucleic acid.
RNAse	: Ribonuclease.
RNAsin	: Ribonuclease inhibitor.
rNTP	: 3' ribonucleoside 5' triphosphate.
rpm	: Revolutions per minute.
RSRE	: Related-serum-response element.
RSRF	: Related-serum-response factor.
s.c	: Subcutaneous.
sec	: Second.
SDS	: Sodium dodecyl sulphate.
SV40	: Simian virus 40.
Sp-1	: Promoter-specific transcription factor 1.
SRE	: Serum response element.
SRF	: Serum response factor.
T	: Thymine.
TAFs	: TBP-associated factors.
TCA	: Tri-chloroacetic acid.
TBP	: TATA-box binding protein
TEMED	: Tetramethylenediamine.
TGF-B	: Transforming growth factor-B
TNF-α	: Tumour necrosis factor-alpha.
TPA	: 12-0-tetradecanoyl phorbol 13-acetate.
TRE	: TPA response element.
Tris	: 2-amino-2-(hydroxymethyl) propane-1,3-diol.
tRNA	: Transfer ribonucleic acid.
Tween 20	: Polyoxyethylene sorbitan monolaurate.
U	: Units.

UV	: Ultra Violet.
V	: Volts.
v-	: Viral.
v/v	: Volume for volume.
YY-1	: Ying-Yang 1.

Throughout this thesis genes are indicated by italics e.g. *c-jun*, whilst proteins remain unitalicised e.g. c-Jun.

CHAPTER I - INTRODUCTION.

CHAPTER I.A. - INTRODUCTION

c-JUN - MODULAR DOMAINS AND KINETICS OF EXPRESSION

I.A.1 HISTORICAL BACKGROUND

c-jun was isolated as a retroviral transforming gene from Avian Sarcoma Virus 17 (ASV17), and derived its name from the Japanese translation of seventeen : Ju-nana (Maki *et al*, 87). v-Jun is expressed as a 65kD gag-Jun fusion protein. Homology between human c-Jun and v-Jun is approximately 80% but increases to 98% at the C-terminus (Bohmann *et al*, 87). This region also exhibits a 44% homology with the DNA binding domain of the yeast transactivator, GCN4 (Vogt *et al*, 87). Domain swap experiments between v-Jun and GCN4 revealed that v-Jun was able to functionally substitute for GCN4 in DNA binding assays, suggesting a role for v-Jun in transactivation (Struhl *et al*, 87).

The consensus binding site for GCN4 closely ressembles that of the mammalian transcription factor AP-1 (Activator Protein 1). AP-1 was originally identified as a DNA binding activity in HeLa cell extracts that recognized a specific enhancer element in the SV40 viral promoter (Angel *et al*, 87). The sequence, TGACTCA, is found in the upstream regions of many genes, and imparts sensitivity to phorbol esters such as 12-o-tetradecanoyl phorbol 13-acetate (TPA). Consequently, it has been named the TPA Response Element or the TRE (Lee *et al*, 87). The similarity between the DNA binding sites of GCN4 and AP-1 prompted an investigation into their relationship, which demonstrated that c-Jun was

capable of binding to a synthetic TRE and was contained within the AP-1 transcription factor complex (Angel *et al*, 88(i)). Other proteins within the complex had not been identified, but the product of the cellular proto-oncogene, *c-fos*, was a strong candidiate. *c-fos*, like *c-jun*, was isolated as retroviral oncogene from two independent murine osteosarcoma viruses : Finkel Biskis Jinkins and Finkel Biskis Reilly (Reviewed in Curran, 91). c-Fos and v-Fos are nuclear phosphoproteins. c-Fos is more highly phosphorylated than v-Fos and is transiently induced in response to mitogenic, differentiation, and neural cell depolarizing stimuli. Both c-Fos and v-Fos form part of a nuclear protein complex of which the major Fos associated protein in fibroblasts is p39 (Curran *et al*, 85). From in vitro DNA binding and transactivation analyses it was proposed that the Fos/p39 complex played a role in gene regulation (Sambucetti *et al*, 86; Setoyama *et al*, 86). Specifically, the Fos/p39 complex was shown to interact with a DNA binding element within the promoter region of an adipocyte gene, aP2, that was subsequently identified as the recognition sequence for AP-1 (Distel *et al*, 87).

The availability of Jun-specific antisera facilitated immunological comparisons between p39 and c-Jun, and revealed that they were identical. In addition, p39 c-Jun was shown to form a stable complex with c-Fos and v-Fos in the nucleus, and to bind DNA directly and specifically (Franza *et al*, 88; Rauscher *et al*, 88(i)). This provided conclusive evidence that the products of the proto-oncogenes *c-fos* and *c-jun* formed the major components of the mammalian transcription factor AP-1 (Rauscher *et al*, 88(ii)). It has subsequently been shown that c-Jun and c-Fos belong to multigene families. Under different conditions individual members from both families contribute to the overall AP-1 activity in the cell (Nakabeppu *et al*, 88).

I.A.2. JUN FUNCTIONAL DOMAINS

c-Jun is structurally arranged in modular domains that direct its hierarchical functions of dimerization, DNA binding, and transactivation.

I.A.2a Dimerization

c-Jun dimerization is mediated through a leucine zipper domain (Ransome *et al*, 89). The leucine zipper was initially described in the transcription factor C/EBP, as a heptad repeat of leucine residues capable of forming an alpha helix (Landshultz *et al*, 88). Leucine zippers have subsequently been identified in many other transcription factors, including the Fos and Jun multigene families, and are an absolute requirement for dimerization (Kouzarides *et al*, 89). The Coiled Coil model for dimerization describes an alignment of two parallel alpha helices (Kouzarides *et al*, 89). Hydrophobic interactions between extending leucine residues are proposed to stabilize the dimer. Additional interactions between two minor hydrophobic ridges and between charged nucleotide bases contribute to the overall stability and increase the specificity of dimerization (Kouzarides *et al*, 89; Smeal *et al*, 89).

In vitro analysis has shown that c-Jun is capable of homo and heterodimerization. c-Jun/c-Fos heterodimers are relatively more stable than c-Jun homodimers and exhibit a higher affinity for the TRE (Kouzarides *et al*, 88; Rauscher *et al*, 88(iii)). This has been partially attributed to ionic interactions between c-Fos and c-Jun which are not permitted within the c-Jun homodimer (O'Shea *et al*, 92, Smeal 89).

In vitro dimerization analysis has now been extended to include other members of the Fos and Jun gene families : FosB, Fra1, Fra2, FosB-

short form, JunB, and JunD. This analysis has demonstrated that homo and heterodimerization between Jun and Fos family proteins is always permitted, but that dimerization between Fos family members is physically inhibited (Nakabeppu *et al*, 88). Interfamily dimerization generates a wide variety of different AP-1 transcription factors that specifically modulate gene expression through distinct but related AP-1 regulatory elements, thereby expanding the AP-1 mediated transcriptional response (Abate *et al*, 90(ii); Lamb *et al*, 91).

I.A.2b DNA Binding

AP1 DNA binding is absolutely dependent on the leucine zipper and an adjacent domain described as the basic region. The basic region consists of a cluster of basic amino acids which, upon dimerization, align to form the DNA contact surface (Rauscher *et al*, 88(ii)). In the presence of sequence-specific DNA, the basic regions adopt an alpha helical configuration (Patel *et al*, 90). The palindromic TRE sequence was initially described as the consensus AP-1 DNA binding site (Abate *et al*, 90(iii)), but subsequent analysis has shown that AP-1 dimers are capable of binding to a wide variety of TRE and TRE-like sites. Binding affinities vary for different AP-1 dimers, and include both the core and the flanking DNA sequences (Ryseck *et al*, 91)

From crystallographic analysis, sequence-specific AP-1 DNA binding is best described by the "Scissor Grip" model (Ellenberger *et al*, 92). In this model a continuous alpha helix is proposed to track the major groove of the dyad symmetrical binding site, contacting it at specific bases and phosphate oxygens as it wraps the DNA (Ellenberger *et al*, 92; Sauer *et al*, 90). The dimerization interphase is oriented almost perpendicular to the

DNA axis in the shape of the letter T. Contacts between the palindromic half sites and the basic regions of each contributing monomer have been confirmed by UV cross linking experiments (Risse *et al*, 89).

I.A.2c Transactivation

Dimerization and DNA binding are essential for c-Jun transactivation. Transactivation activity has been mapped to several independent transactivation domains depending on the assay system used. Their functional integration is proposed to contribute to the overall complexity of the transcriptional response (Abate *et al*, 91). The major transactivation domains are discussed below.

- N-Terminal Domain

A cell-type-specific transactivation domain has been described at the extreme amino terminus of c-Jun (Alani *et al*, 91; Angel *et al*, 89). Similar assays in different cell types have failed to confirm this region as a transactivation domain (Baichwal *et al*, 90; Bohman *et al*, 89; Bos *et al*, 90) and, consequently, its overall contribution to transactivation is not known.

- A2 Transactivation Domain

A2 was identified using an in vitro transcription assay as a constitutively active transactivation domain adjacent to the basic region (Bohman *et al*, 89). Subsequent analysis failed to confirm A2 as a bonafide transactivation domain (Abate *et al*, 91; Alani *et al*, 91; Baichwal *et al*, 91;

Hirai *et al*, 90), but did show that this region plays an important role in the regulation of AP-1 : DNA binding activity (Boyle *et al*, 91).

- delta (δ) Domain

The δ domain has been identified as a negative transactivation domain in vitro (Bohman *et al*, 89) and in vivo (Abate *et al*, 91; Bos *et al*, 90). From initial observations, it was proposed that the δ domain facilitated or stabilized an interaction between c-Jun and a cell-type-specific inhibitor (Baichwal *et al*, 90). Subsequent experiments have shown that the δ domain overlaps with the binding site for a c-Jun amino terminal kinase, JNK, essential for the regulation of c-Jun transactivation activity (Hibi *et al*, 93, section IV.A.2).

- A1 Transactivation Domain

A1 is a composite transactivation domain composed of two subdomains : a1 and E (Baichwal *et al*, 92). E is proposed to interact directly with a cell-type-specific inhibitor, thereby regulating the activity of the a1 transactivation domain (section I.C.2a). The a1 transactivation domain is highly conserved within the Jun family (Abate *et al*, 91; Alani *et al*, 91; Baichwal *et al*, 91; Hirai *et al*, 90) but does not encompass serines 63 and 73, whose phosphorylation has been positively associated with c-Jun transactivation in vitro and in vivo (Binetruy *et al*, 91; Hibi *et al*, 93). a1 does contain multiple threonie residues that occur in a sequence known to be phosphorylated by proline-dependent protein kinases in vitro (Baichwal *et al*, 92). Two of these threonines, positioned directly adjacent to a proline residue, are phosphorylated by kinases isolated from Ha-ras transformed FR3T3 and UV irradiated HeLa cells. The same kinases have

been associated with the phosphorylation of serines 63 and 73, which are also followed by a highly conserved proline residue in all characterized c-Jun amino acid sequences (Hibi *et al*, 93).

An independent transactivation domain, Homology Domain 2, has been identified that overlaps with the E region of the A1 transactivation domain (Sutherland *et al*, 92). HOB2 synnergizes with a second domain, Homology Domain 1, that encompasses serine 73. The functional activities of HOB domains 1 & 2 are discussed in section I.B.3a.

I.A.3. c-JUN EXPRESSION

I.A.3a Expression

c-jun was initially described as an immediate early gene as the mRNA was rapidly and transiently induced in response to serum growth factors and tumour promoters (Lamph *et al*, 88; Quantin *et al*, 88; Ryder *et al*, 88; Ryseck *et al*, 88) and superinduced in the presence of protein synthesis inhibitors (Greenberg *et al*, 86; Ryder *et al*, 88; Ryseck *et al*, 88).

Subsequent analysis has revealed that *c-jun* expression is not exclusively characterized by immediate early gene kinetics. c-Jun mRNA has been observed at G1/S transitions (Carter *et al*, 91), and during differentiation as cells exit from the cell cycle. In embryonal carcinoma and embryonal stem cells, for example, differentiation is associated with a concomitant and prolonged increase in *c-jun* mRNA expression. Likewise, overexpression of c-Jun in these cells induces differentiation and the loss of the tumourigenic phenotype (de Groot *et al*, 90(i); de Groot *et al*, 90(ii); Yang-Yen *et al*, 90(ii)). Differentiation along the monocyte macrophage

lineage is similarly associated with an increase in c-Jun expression. The pattern of expression reflects the nature of the inducing stimulus and is mediated through both transcriptional and post-transcriptional levels of control (Kharbanda *et al*, 92; Nakamura *et al*, 91; Sherman *et al*, 90).

I.C.3b Induction

Serum growth factors and tumour promoters were traditionally associated with the induction of *c-jun* and other immediate early genes (Lamph *et al*, 88; Quantin *et al*, 88; Ryder *et al*, 88; Ryseck *et al*, 88). Subsequent observations have extended the range of *c-jun*-inducing stimuli to include transforming oncogenes, DNA damaging agents, and steroid hormones (de Groot *et al*, 90(ii); Stein *et al*, 92; Wasylyk *et al*, 90). One consequence of multiple inducing stimuli is to expand the c-Jun transcriptional response. Many transforming oncogenes, for example, cooperate on a single signal transduction pathway that results in the activation of Jun/AP-1 (Reviewed in Gutman *et al*, 91; Karin *et al*, 92). c-Jun is similarly activated by DNA damaging agents such as oxidative stress and UV irradiation, but under these conditions alternative activation pathways are favoured (Hallahan *et al*, 91; Stein *et al*, 92; Xanthoudakis *et al*, 92(ii)), suggesting that c-Jun is the effector of multiple signal transduction pathways in vivo.

A wide range of independent inducing stimuli may also be important for the fine regulation of AP-1-dependent transactivation in vivo. Independent AP-1 dimers exhibit distinct transcriptional activities (Chiu *et al*, 89) and are induced in response to specific patterns of external stimuli. Depolarization of PC12 cells, a pheochromocytoma cell line, for example, rapidly induces *c-fos* and *JunB* expression. *c-jun*, in contrast, is refractory to

depolarization but is induced with *c-fos* and *junB* in response to serum growth factors (Bartel *et al*, 89). These observations suggest that subtle changes in AP-1-dependent gene expression may be mediated through the activation of distinct AP-1 effector molecules, that are specifically induced in response to different inducing stimuli.

I.A.4. c-JUN PROMOTER

c-jun expression is regulated by a number of cooperating and independent enhancer elements within the *c-jun* promoter. It is presumed that the integration of these activities is responsible for the specific patterns of *c-jun* expression observed in response to different inducing stimuli.

I.A.4a Post-Translational Modifications and Factor Exchange

In vivo DNA footprinting analysis has revealed that UV- and TPA-induced expression of *c-jun* is not accompanied by crude changes in promoter site occupancy (Herr *et al*, 94; Rozek *et al*, 93). Similar results were reported at the *c-fos* promoter in response to serum stimulation, suggesting that post-translational modifications to pre existing factors and/or subtle factor exchanges may be responsible for specific alterations in immediate early gene expression (Herrera *et al*, 89). Serum-induced expression of *c-fos* is regulated through the Serum Response Element (SRE) in the *c-fos* promoter (Rivera *et al*, 90). The SRE is recognized by the Serum Response Factor (SRF) which binds as a ternary complex with the Elk-1 transcription factor (Dalton *et al*, 93; Marais *et al*, 93). Elk-1 is multiply

phosphorylated in the conserved "C Box" in response to mitogenic stimuli (Marais *et al*, 93). Phosphorylation of Elk-1 is both rapid and transient and contributes to the subsequent transactivation activity of the SRF. *c-jun*, like *c-fos*, is also induced in response to serum stimulation although serum associated changes in *c-jun* promoter site occupancy have not been investigated. *c-jun* does not posess an SRE in the gene promoter but does contain a related element that shares many characteristics with the SRE. The Related-to-Serum Response Element (RSRE) is found in the promoter regions of several growth factor responsive and muscle specific genes, and binds a family of transcription factors known as the Related-to-Serum Response Factors (RSRF). The RSRFs share sequence homology with the SRF over a conserved MADS BOX. Homology is highest over the DNA binding and dimerization domains, but cross dimerization and DNA binding is not exhibited by the SRF and RSRFs (Pollock *et al*, 91). Sequence homology between the SRF and the RSRFs completely diverges at the carboxy terminus of the MADS BOX. This region is rich in proline and glutamine residues and functions as the Elk-1 binding domain in the SRF (Dalton *et al*, 93). The divergent region is also an absolute requirement for SRF and RSRF DNA binding activity suggesting, that additional protein:protein interactions may also be important for RSRF DNA binding and the subsequent regulation of RSRF transactivation activity (Pollock *et al*, 91). The functional significance of the junRSRE in vivo is complicated by the refractibility of this element to DNase 1 digestion (Flemington *et al*, 90; Rozek *et al*, 93). However, it has been associated with serum and EGF-induced expression of *c-jun*, and is specifically recognized by HeLa cell RSRFs in vitro (Han *et al*, 92).

I.A.4b Cooperation and Redundancy

- Cooperation

Cooperative regulatory elements have been demonstrated to mediate the wide ranging patterns of *c-jun* expression in response to different inducing stimuli. Maximal activation of the *c-jun* promoter by adenovirus E1A proteins, for example, requires cooperativity between two TRE-like sites and an additional CTF element within the *c-jun* promoter (van Dam *et al*, 93). Retinoic acid-induced expression of *c-jun* in differentiating embryonal carcinoma cells is similarly mediated through multiple cooperating elements that have been described as retinoic acid responsive elements or REREs. Induction is restricted to differentiating conditions and depends on the exact number and spacing of REREs. Two junREREs overlap with the proximal and distal TRE-like sites in the *c-jun* promoter (Kitabayashi *et al* 92). This may account for the potentiation of retinoic acid-induced *c-jun* expression by E1A, and suggests that E1A cooperates with independent factors to regulate mitogenic- and differentiation-induced patterns of *c-jun* expression (van Dam *et al*, 93).

- Redundancy

Functional analyses of full length and minimal gene promoters has demonstrated that single mutations within the context of a complete promoter often have little effect on subsequent gene expression. This has led to the proposal that cooperative sites compensate for lost activity and that individual sites are redundant for the functional activity of full length gene promoters.

Redundant binding sites are characteristic of several immediate early gene promoters, including *c-jun*. Multiple independent regulatory elements have been identified in the *c-jun* promoter that specifically activate *c-jun* expression in response to a wide range of different inducing stimuli. Mutation of these individual binding motifs has no effect on *c-jun*-induced expression in the context of a full length *c-jun* promoter fragment but can completely abolish a response when minimal *c-jun* promoter fragments are assembled in a reporter construct (Angel *et al*, 88(ii); Han *et al*, 92; Unlap *et al*, 92). From these observations, it has been proposed that *c-jun* expression is regulated through a complex integration of multiple but cooperative enhancer elements. Each is presumed to respond to distinct signal transduction pathways and thereby contribute to the multiple patterns of *c-jun* expression observed in response to different inducing stimuli.

I.A.5. c-JUN AUTOREGULATION

Autoregulation is a characteristic shared by many independent transcription factors (Serfling *et al*, 89). c-Fos and c-Myc, for example, negatively regulate their own gene promoters in a concentration-dependent manner (Grignani *et al*, 90; Lucibello *et al*, 89; Sassoni-Corsi *et al*, 88). Multiple homeobox-containing genes in drosophila and certain eukaryotic transcription factors, including MyoD, Myogenin, and c-ets1, in contrast, exhibit positive autogenous transactivation (Serfling *et al*, 89; Seth *et al*, 90; Weintraub *et al*, 91(i)). c-Jun has been associated with both positive and negative autoregulation in vivo, and this has been proposed to contribute to its complex patterns of gene expression.

I.A.5a Positive Autogenous Transactivation

Two TRE-like sites within the *c-jun* promoter have been shown to mediate *c-jun* expression in response to a wide range of inducing stimuli, including steroid hormones, UV irradiation, and phorbol esters (Kitabayashi *et al*, 92; Stein *et al*, 92; Unlap *et al*, 92). Both junTREs differ from the canonical TRE by the inclusion of single base pair (Angel *et al* 88(ii); Unlap *et al*, 92). The proximal junTRE has retained the ability to bind c-Jun homodimers in vitro and has been reported to activate CAT expression in response to c-Jun overexpression in two independent cell lines (Angel *et al*, 88(ii)). A number of other in vitro observations support a positive autoregulatory role for c-Jun in vivo. Collagenase gene expression in primary human fibroblasts, for example, is prolonged in response to TNF-α. The response has been attributed to a persistent overexpression of c-Jun/AP-1, which is proposed to result from a putative positive autoregulatory loop (Brenner *et al*, 89). A similar enhancement and extension of *c-jun* expression has been observed during the in vitro differentiation of P19 embryonal carcinoma cells (de Groot *et al*, 90(i); de Groot *et al*, 90(ii)). The mechanism responsible for this has not been investigated, but it has been suggested that the negative feedback loop associated with transient *c-jun* expression is, in some way, delayed or overridden during differentiation.

Subsequent investigations have failed to confirm a positive role for c-Jun homodimers in vivo (Kovary *et al*, 92; Kovary *et al*, 91). In addition, characterization of the proximal and distal junTRE binding complexes has identified contributions by CREB/ATF factors rather than c-Jun homodimers (van Dam *et al*, 93). This supports the observation that the junTREs more closely resemble CREs than TREs and that Jun/ATF heterodimers exhibit a higher affinity for CRE- than TRE-containing

oligonucleotides (Benbrook *et al*, 90). Distinct junTRE-specific protein complexes may provide an explanation for the differences reported between the junTRE and collagenase TRE binding sites in vitro. The collagenase TRE, for example, binds c-Jun/c-Fos heterodimers and c-Jun homodimers, but has no affinity for CREB/ATF-containing complexes and does not compete for junTRE-specific complexes in electrophoretic mobility shift assays (Hai *et al*, 91; Stein *et al*, 92). Similarly, adenovirus E1A proteins and retinoic acid have been reported to repress gene expression through a canonical TRE but to activate *c-jun* expression through the proximal and distal junTRE-like sites (de Groot *et al*, 91; Kitabayashi *et al*, 91; Offringa *et al*, 90; van Dam *et al*, 90). E1A-induced transactivation of the *c-jun* promoter is proposed to depend on CREB/ATF2 proteins which both bind to the junTREs and are widely recognized transactivation targets of the 289R E1A protein (van Dam *et al*, 93).

From these observations it is hypothesized that auto-induction of *c-jun* may not represent true autoregulation, and that alternative c-Jun-containing or c-Jun-lacking heterodimeric complexes positively regulate *c-jun* expression in vivo.

I.A.5b Auto-repression

Overexpression of c-Jun or v-Jun in primary CEFs induces a repression of endogenous *c-jun* expression that is dependent on high levels of exogenous Jun proteins (Castellazzi *et al*, 90; Castellazzi *et al*, 91; Hughes *et al*, 92). Elements within the *c-jun* promoter responsible for auto-repression have not been identified but observations suggest that, in common with positive autogenous transactivation, Jun homodimers may

not be the true in vivo effector. GCN4/v-Jun fusion proteins, for example, exclusively homodimerize and, in contrast to wild type p65 gag-v-Jun, have been shown to up regulate *c-jun* expression in primary CEFs (Hughes *et al*, 92). In addition, no correlation has been reported between exogenous v-Jun expression and the levels of TRE or TRE-like DNA binding activity in ASV17-transformed CEFs (Hadman *et al*, 93; Hawker *et al*, 93). Consequently, Jun-dependent auto-repression may be mediated through a squelching mechanism that requires high levels of c-Jun or v-Jun protein but is independent of Jun/AP-1 DNA binding activity (Oehler *et al*, 92). Conventional squelching mechanisms are directed by productive protein:protein interactions mediated through intact transactivation domains (section I.B.6b). Mutational analysis of c-Jun has identified the singular importance of the Jun leucine zipper domain for endogenous c-Jun repression in primary CEFs (Castellazzi *et al*, 91). Consequently, an alternative type of squelching mechanism may be essential for Jun auto-repression in vivo, that may or may not require functional Jun\AP-1 DNA binding activity but absolutely depends on intact Jun dimerization domains. The kinetics of *c-jun* expression clearly include a period of gene repression in response to a wide range of different inducing stimuli (Lamph *et al*, 88; Quantin *et al*, 88; Ryder *et al*, 88; Ryseck *et al*, 88). This, together with the down-regulation of c-Jun expression in c-Jun and v-Jun transformed primary CEFs, suggests that auto-repression plays an important role in the regulation of *c-jun* expression during both normal and transformed conditions of growth (Castellazzi *et al*, 90; Castellazzi *et al* 91; Hartle *et al*, 92; Havarstein *et al*, 92).

CHAPTER 1.B - INTRODUCTION

TRANSCRIPTIONAL ACTIVATION AND REPRESSION BY CELLULAR TRANSCRIPTION FACTORS

Factors involved in the accurate initiation of eukaryotic transcription can be classified into two groups : general or basal transcription factors, and sequence-specific activator proteins. (Reviewed in Saltzman *et al*, 89; Ptashne *et al*, 90). The general transcription factors assemble on the promoter in an ordered fashion to form a preinitiation complex. Gene expression is then modulated through subsequent interactions with DNA-bound activator proteins. Activator proteins or sequence-specific cellular transcription factors can transactivate gene expression through direct interactions with components of the basal transcriptional machinary that may (Lin *et al*, 91) or may not (Kerr *et al*, 93; Liu *et al*, 93) facilitate the assembly of the pre-initiation complex on the DNA. Alternatively, co-activators have been isolated that function as bridging proteins (Chrivia *et al*, 93; Gill *et al*, 94) or DNA structural re-organizers (Natesan *et al*, 93), and thereby mediate indirect protein:protein interactions that enhance the transactivation of specific genes.

Transcriptional repression has similarly been associated with several cellular and oncogenic transcription factors in vivo and is mediated through direct and indirect interactions with components of the basal transcriptional machinary. c-Myc, for example, has been reported to repress transcriptional initiation through a direct interaction with the initiator binding protein, TFII-I (Lucas *et al*, 93; Roy *et al*, 93), and a direct and non-productive interaction between v-Rel and the TATA-box binding protein

contributes to the dominant negative phenotype exhibited by the v-Rel oncoprotein (Kerr *et al*, 93).

Indirect transcriptional repression is generally mediated through protein:protein interactions outwith the DNA. The interaction between c-Myc and Yin-Yang-1 (YY1), a zinc-finger eukaryotic transcription factor, for example, (Shrivastava *et al*, 93), inhibits the dual transactivator and repressor functions of YY1 (Riggs *et al*, 93; Seto *et al*, 91) and has been proposed to mediate *c-myc* transcriptional auto-repression in vivo (Grignani *et al*, 90)

Transactivation and transrepression have been associated with several cellular transcription factors in vivo. Dual functionality is directly and indirectly modulated by a variety of complex mechanisms; some of which are described below.

I.B.1. FUNCTIONAL MODIFICATION BY PHOSPHORYLATION

Phosphorylation of pre-existing and newly synthesized transcription factors provides a rapid and highly specific mode of transcriptional control. Phosphorylation has been reported to regulate the transactivation, DNA binding and nuclear localization of cellular transcription factors depending on the precise domain targeted for modification.

I.B.1a Transactivation

c-Jun transactivation is regulated by the phosphorylation of two amino-terminal sites : serines 63 and 73 (Pulverer *et al*, 91; Smeal *et al*, 91; Smeal *et al*, 92). A c-Jun amino-terminal kinase (JNK) has now been

identified (Hibi *et al*, 93) that is upregulated under conditions associated with the phosphorylation of serines 63 and 73 in c-Jun (Devary *et al*, 92; Pulverer *et al*, 91; Pulverer *et al*, 92; Smeal *et al*, 92). A series of threonine residues in the c-Jun A1 transactivation domain are also phosphorylated under these conditions and may present additional targets for JNK phosphorylation (Devary *et al*, 92). A major 46kD isoform of JNK has been identified that binds to a specific site within the c-Jun transactivation domain and phosphorylates c-Jun on serines 63 and 73 in vitro. Phosphorylation is proposed to induce a conformational change in the c-Jun protein that is responsible for c-Jun transactivation and the concomitant dissociation of JNK (Hibi *et al*, 93).

JNK is distantly related to the Mitogen Activated Protein Kinases (Derijard *et al*, 94). Mitogen Activated protein kinases have been shown to phosphorylate numerous transcription factors in vitro including c-Jun (Pulverer *et al*, 91; Pulverer *et al*, 92) and the serum response factor (SRF) associated protein Elk-1 (Marais *et al*, 93). DNA binding by monomeric Elk-1 is potentiated by the formation of a ternary complex with the SRF at the *c-fos* serum response element (SRE) (Hipskind *et al*, 91; Shaw *et al*, 89). Two conserved domains are required to mediate the Elk-1/SRF-interaction. A third conserved domain is phosphorylated at multiple sites in vitro by MAP kinases. Like c-Jun, phosphorylation is associated with increased Elk-1 transactivation, thereby linking growth factor regulated phosphorylation to transactivation through the SRE (Marais *et al*, 93)

For certain transcription factors phosphorylation-induced transcriptional activation is potentiated by more than one kinase. CREB is phosphorylated at serine 133 in response to at least two independent signal transduction pathways. Activation of protein kinase A, through the adenyl cyclase pathway, leads to a dramatic increase in the

transactivating potential of CREB (Gonzalez *et al*, 91). A similar increase is observed upon membrane depolarization of PC12 cells but, in this case, the calmodulin-dependent protein kinases I and II have been proposed as the in vivo effectors (Dash *et al*, 91; Sheng *et al*, 91).

The mechanisms responsible for phosphorylation-induced transactivation are poorly understood but phosphorylation is frequently associated with conformational changes that may be significant for subsequent transcriptional regulation. Immunoprecipitated, phosphorylated c-Jun, for example, migrates as a ladder of 4-5 specific bands that correlate with amino terminal phosphorylations (Pulverer *et al*, 92) and an increase in the activity of JNK (Hibi *et al*, 93). A similar increase in the electrophoretic mobility of the SRE ternary complex has been observed following the post-translational phosphorylation of Elk-1 (Marais *et al*, 93).

Conformational changes may be significant for subsequent protein:protein interactions necessary for transactivation and transrepression. CREB phosphorylation at serine 133, for example, induces a conformational change that exposes a glutamine rich transactivation domain essential for transactivation (Gonzalez *et al*, 91). Direct interactions between transcription factors and components of the basal transcriptional machinary have similarly been associated with phosphorylation-induced conformational changes. Phosphorylated c-Jun, for example, binds purified TATA-binding factors TFIIB and TBP in vitro (Kraft; *in press*), while SRF transactivation is enhanced through an interaction with the TATA-binding factor TFIID that facilitates formation of an active preinitiation complex (Zhu *et al*, 91).

Post-translational modifications to transactivation domains have also been associated with a stimulation of DNA binding activity that indirectly activates transactivation. Phosphorylation of Elk-1, for example, has

been reported to increase ternary complex formation over the SRE (Gille *et al*, 92). The affinity of CREB for low affinity CRE binding sites is similarly increased following serine 133 phosphorylation by protein kinase A (Nichols *et al*, 92).

I.B.1b DNA Binding

c-Jun DNA binding activity is directly regulated through phosphorylation. c-Jun is phosphorylated at three carboxy-terminal sites : threonine 231, serine 243 and serine 249, located immediately adjacent to the DNA binding domain in the A2 region (Bohman *et al*, 89; Boyle *et al*, 91). An additional site lies within this cluster: threonine 239, but does not appear to be phosphorylated in vivo (Lin *et al*, 92). All three sites are phosphorylated in non-stimulated cells but upon exposure to TPA or certain transforming oncogenes, including activated Ha-ras, v-Sis and v-Src, two sites are rapidly dephosphorylated (Binetruy *et al*, 91; Smeal *et al*, 92), and there is a subsequent increase in DNA binding activity (Boyle *et al*, 91). Casein kinase II has been shown to phosphorylate threonine 231 and serine 249 in vivo. Serine 243 is not phosphorylated by Casein kinase II but has been implicated as a potential substrate for the MAP 2 kinases and MAP Related kinases : ERT and ERK 1&2. Phosphorylation of serine 243 increases the reactivity of the remaining sites. Conversly, mutation of serine 243 to an alanine, as in v-Jun, has been shown to interfere with subsequent phosphorylation events and is believed to contribute to the increased oncogenicity of v-Jun relative to c-Jun (Lin *et al*, 92). Casein kinase II is a constitutively active kinase which is predominantly located in the nucleus (Boyle *et al*, 91). Consequently, it has been proposed that activation of c-Jun DNA binding may be controlled

at the level of a specific phosphatase rather than a specific kinase (Lin *et al*, 92).

In contrast to c-Jun, SRF phosphorylation at serine 103, enhances the affinity of the SRF for the SRE (Rivera *et al*, 93). Transcriptional induction of *c-fos* correlates with a transient phosphorylation of the SRF and an enhancement of DNA binding activity. Phosphorylation does not affect the ability of the SRF to interact with Elk-1 but may influence other protein:protein interactions necessary for the SRF:SRE binding interaction. Mechanisms responsible for phosphorylation-induced changes in DNA binding activity are poorly understood. It has been suggested that allosteric and/or electrostatic changes influence the integrity of the DNA binding pocket and thereby alter the affinity of a transcription factor for a specific DNA recognition sequence. Alternatively, phosphorylation-induced changes may direct specific protein:protein interactions that indrectly regulate subsequent DNA binding activity. In vivo footprinting data from several immediate early gene promoters has revealed that promoter site occupancy is unchanged in response to certain inducing stimuli (Herr *et al*, 94; Herrera *et al*, 89; Rozek *et al*, 93). These observations would appear to directly contradict the in vitro studies described above unless stimulation induces a rapid exchange of factors on the gene promoter leaving the overall crude DNA footprint unchanged. Alternatively, post-translational modifications affecting DNA binding activity may only be significant for the recognition of low affinity DNA binding sites and, consequently, not be detected by footprinting analysis (Nichols *et al*, 92).

I.B.1c Sequestration

Regulated cytoplasmic sequestration controls the activity of certain transcriptional activators and repressors in eukaryotic cells. Regulation is rapid and obviates the need for a mechanism to propagate signals across the nuclear membrane. Phosphorylation of the transcription factor itself or a cytoplasmic anchor protein can facilitate nuclear translocation and subsequent activation. A well documented example of phosphorylation-regulated nuclear translocation is provided by the Rel-related family of transcription factors which includes NF-κB p50, NF-κB p65, p49 c-Rel, RelB and Dorsal (Reviewed in Gilmore, 91). The Rel proteins transactivate as homo and heterodimers through sequences related to the NF-κB binding site. Inactive proteins are sequestered in the cytoplasm as dimeric complexes or as single monomers (Ganchi *et al*, 93) by a common inhibitory protein, IκB (Baeuerle *et al*, 88). The IκB anchor protein is unphosphorylated in the cytoplasm but becomes rapidly phosphorylated in response to a variety of stimuli, including phorbol esters and DNA damaging agents. Phosphorylation triggers the degradation of IκB and the subsequnt nuclear translocation of NF-κB-related proteins. The mechanism is likely to be more complex in vivo but phosphorylation is clearly integral to the overall pattern of regulation (Baeuerle *et al*, 1988; Ghosh *et al*, 90; Sun *et al*, 93).

I.B.2. FUNCTIONAL MODIFICATION BY CELLULAR REGULATORY FACTORS

I.B.2a Transactivation

Transactivation activity can be modulated by cellular regulatory proteins. Co-activators have been identified that bind to specific transcription factors and enhance their transactivation function. A TBP associated factor, $TAF_{II}110$, for example, has been shown to form a bridge between the RNA polymerase II preinitiation complex and a glutamine rich transactivation domain within the cellular transcription factor Sp1 (Gill *et al*, 94). A nuclear protein, CBP (CREB Binding Protein) has similarly been demonstrated to enhance the transactivation activity of CREB. CPB exclusively binds to phosphorylated CREB which forms a scaffold for CBP enhanced, CRE-dependent gene expression (Chrivia *et al*, 93).

Protein:protein interactions have also been reported that mask the activity of transactivation domains. In yeast, the transcription of galactose-inducible genes is regulated by the interaction between an activator protein, GAL4, and a negative regulatory protein, GAL80. Under non-inducing conditions GAL80 binds to and masks the GAL4 transactivation domain. Following induction, GAL80 remains bound to GAL4 but the transactivation domain is unmasked to promote transactivation (Leuther *et al*, 92). A similar pattern of regulation has been reported for the cellular oncogene MDM2 which binds to an acidic transactivation domain in p53. Binding masks the activity of p53 and contributes to the transforming function of MDM2 in human soft tissue sarcomas (Oliner *et al*, 93).

Protein:protein interactions can also indirectly inhibit the activity of adjacent transactivation domains. The activity of PHO4, a basic-helix-loop-helix

yeast transactivator, for example, is modulated by the PHO80 repressor. PHO80 physically interacts with two independent sites on PHO4 to mediate transcriptional repression. Neither site overlaps with the PHO4 transactivation domain and repression is presumed to result from an indirect masking effect (Jayaraman *et al*, 94). The PHO4/PHO80 system exhibits strong parallels with repression of the c-Jun a1 transactivation domain. a1 is flanked by two independent repressor domains: δ and E, both of which are required for full transcriptional repression. The δ domain has been reported to facilitate or stabilize the interaction between a cell-type-specific inhibitor and the E domain (Baichwal *et al*, 92). In addition the δ domain binds to the c-Jun amino-terminal kinase prior to c-Jun phosphorylation and subsequent activation (Hibi *et al*, 93). It remains to be seen whether a1 repression is a consequence of masking induced by a δ/E /I interaction.

I.B.2b DNA Binding

The DNA binding activity of cellular transcription factors is modulated by a wide variety of regulatory proteins.

Post-translational modifications involving the reduction/oxidation (redox) of DNA binding sensitive residues have been reported to regulate the DNA binding activity of the AP-1, Myb and NF-κB transcription factor families (Abate *et al*, 90(i); Abate *et al*, 90(iv); Frame *et al*, 91; Toledano *et al*, 91). The AP-1 transcription factors are reversibly reduced on a conserved cysteine residue in the basic region. Reduction is mediated by a cellular redox/DNA repair enzyme, Ref-1 (Xanthoudakis *et al*, 94), and is required for optimal DNA binding activity of all the Fos and Jun family proteins (Xanthoudakis *et al*, 92(i); Xanthoudakis *et al*, 92(ii)). The importance of

redox regulation in vivo is illustrated by the v-Jun oncoprotein, which retains a cysteine to serine mutation at the redox sensitive residue and is refractory to DNA binding regulation (Maki *et al*, 87; Nishimura *et al*, 88). The same substitution in c-Fos augments both its DNA binding and transforming potential and has consequently been proposed to represent a gain of function mutation (Okuno *et al*, 93).

Direct protein:protein interactions have similarly been reported to alter the DNA binding activity of several transcription factors. Inhibitory proteins have been identified that recognize specific transcription factors, and bind to form complexes with altered or reduced DNA-binding activity. Several c-Jun dimerization partners have been reported that alter its DNA binding activity in vitro. Jun-interacting factor 1 (Jif-1) binds specifically to the c-Jun leucine zipper and down regulates c-Jun DNA binding and transactivation through the canonical collagenase TRE (Monteclaro *et al*, 93). Jif-1 does not show any structural resemblance to known transcription factor motifs but is closely related to the product of a putative human tumour suppressor gene, QM. JunB, in contrast, is highly related to c-Jun but attenuates c-Jun-mediated transactivation of the collagenase gene through a small number of amino acid changes within its DNA binding and dimerization domains (Deng *et al*, 93). c-Jun/JunB heterodimers form in preference to either homodimer, but have a relatively low affinity for the collagenase TRE. It has been proposed that the c-Jun/JunB heterodimer binds to a distinct subset of TRE-dependent genes, and thereby regulates an alternative pattern of c-Jun-dependent transactivation (Deng *et al*, 93; Ryseck *et al*, 91).

Other transcription factors whose DNA binding activity is modulated by protein:protein interactions include MyoD and the CAAT/enhancer binding protein, C/EBP. MyoD and C/EBP dimerize with dominant repressors proteins that lack functional DNA binding domains. MyoD is sequestered

as an inactive heterodimer with Id, a relatively ubiquitous negative regulator of helix-loop-helix proteins (Benezra *et al*, 90), whereas C/EBP interacts specifically with a highly homologous repressor, CHOP (Ron *et al*, 92).

I.B.2c Sequestration

The activity of certain transcription factors is regulated through specific interactions with cellular regulatory proteins that mediate cytoplasmic sequestration in vivo. The glucocorticoid receptor, for example, is sequestered in the cytoplasm as an inactive complex with heat shock protein 90, hsp90 (Pratt *et al*, 88). Binding of hsp90 to the hormone binding domain is believed to induce an unfolding of the receptor which inactiviates its nuclear localization, DNA binding and transactivation functions. The conformational change is reversed through hormone binding which triggers the release of hsp90 and the concomitant nuclear localization of the glucocorticoid receptor (Picard *et al*, 88).

I.B.3. FUNCTIONAL MODIFICATION THROUGH DOMAIN INTERACTIONS

I.B.3a Intermolecular Interactions

Synnergism between independent transactivation domains has been reported to modulate the transcriptional activity of several transcription factors in vivo. c-Jun, for example, posesses two transactivation

domains, Homology Domains (HOB) 1 & 2, that cooperate and are functionally interchangeable with HOB-like domains found in other transcription factors such as c-Fos and C/EBP (Sutherland *et al*, 92). Synnergistic transactivation domains have also been identified in Sp-1, a transcription factor essential for basal and enhancer mediated transactivation of certain gene promoters (Dusing *et al*, 94). Synnergism between the glutamine rich transactivation domains of proximally and distally bound Sp-1 molecules superactivates Sp-1-dependent transcription in vitro (Courey *et al*, 89).

Antagonistic transactivation domains can similarly modulate transactivation activities. CREB transactivation, for example, is repressed by different isoforms of the CREB Modulator protein, CREM. CREM proteins bind to the CRE as homodimers or as heterodimers with CREB, and thereby directly down-regulate CREB transactivation (Foulkes *et al*, 91(i); Foulkes *et al*, 91(ii)).

I.B.3b Intramolecular Interactions

Intramolecular interactions regulate both the transactivation and the DNA binding activity of cellular transcription factors. Certain ATF-2-like proteins, for example, contain an inhibitory domain in the carboxy-terminus that weakens transactivation through a mechanism of intramolecular masking. Repression is alleviated through heterodimerization with Fos or Jun proteins that provide alternative transactivation domains, or by the Adenovirus E1a protein that binds and directly unmasks the ATF-2 zinc finger transactivation domain (Chatton *et al*, 94). In vitro transactivation by p53 is similarly regulated by intramolecular interactions. Transactivation is mediated through a direct interaction with the TATA-box binding protein,

TBP, but is modulated by additional activator and repressor domains located outwith the TBP binding domain (Liu *et al*, 93).

The DNA binding activity of the c-Ets1 transcription factor is regulated through an intramolecular interaction involving the full length c-Ets1 protein. c-Ets1 DNA binding activity is directed by the conserved "ets domain" but repressed in the context of full length c-Ets1. The importance of this regulatory mechanism is demonstrated by the v-Ets1 oncoprotein whose DNA binding activity is refractory to intramolecular constraints (Lim *et al*, 92). Intramolecular interactions have an opposing effect on the DNA binding activity of JunD. Amino terminal sequences modulate the DNA binding activity of JunD but, unlike c-Ets1, increase the affinity of JunD for its specific DNA binding sites (Hirai *et al*, 90).

I.B.4. FUNCTIONAL MODIFICATION THROUGH DNA ORGANIZATION

Structural re-organization of the DNA has been associated with the DNA binding activity of several transcription factors in vitro (Kerppola *et al*, 93; Van der Vliet *et al*, 93). c-Jun homodimers and c-Jun/c-Fos heterodimers, for example, bind to the same DNA consensus site but bend the DNA in opposite orientations (Kerppola *et al*, 91). Bending introduces the potential for cooperativity between widely spaced DNA bound factors and can contribute to transcriptional repression and activation. YY1, for example, is a ubiquitously expressed protein that exhibits functional flexibility as a transactivator. Flexibility is dependent on a relatively degenerate DNA binding specificity coupled with an ability to induce DNA bending (Reviewed in Hahn, 92). In vitro experiments have shown that alterations in promoter topology are sufficient for YY1-dependent transactivation and

repression, and suggest that under certain conditions direct interactions with the basal transcriptional machinary are not required (Natesan *et al*, 93).

I.B.5. FUNCTIONAL MODIFICATION THROUGH DISPLACEMENT AND REPLACEMENT

Activation and repression from a single gene promoter can be mediated in vitro through the direct replacement of activating factors with repressors. A direct antagonism between YY1 and the SRF, for example, has been described at the *c-fos* SRE. YY1 competes with the SRF for binding to the SRE and induces a concomitant repression of *c-fos* transcription in vitro (Gualberto *et al*, 92). A similar pattern of regulation has been reported at the proximal TRE-like site in the *c-jun* promoter. Overexpression of under-phosphorylated, inactive CREB displaces endogenous TRE binding factors from the proximal junTRE and represses serum- and TPA-induced expression of *c-jun* (Lamph *et al*, 90).

The contribution made by displacement and replacement to the regulation of transcription in vivo is less well defined. Direct factor replacement is difficult to monitor and may account for the apparently identical in vivo footprints observed for some induced and non-induced immediate early gene promoters (Herr *et al*, 94; Herrera *et al*, 89; Rozek *et al*, 93). The best evidence to support qualitative differences in promoter site occupancy in vivo is provided by differentiation model systems. Two independent nuclear factors, for example, have been shown to compete for a conserved 28-base pair element in the skeletal actin gene promoter (Walsh *et al*, 87). Muscle actin promoter factor 1 (MAPF1) is the

predominant binding activity in nuclear extracts from non-muscle cell types, whereas a distinct binding activity, MAPF2, is detected in differentiated skeletal and cardiac muscle cells (Walsh *et al*, 88). MAPF1- or MAPF2-containing nuclear extracts produce identical footprints over the 28-base pair conserved element but skeletal actin is restricted to MAPF2-expressing cells, suggesting a functional association between MAPF2 DNA binding and skeletal actin expression in vivo..

I.B.6. QUANTITATIVE MECHANISMS TO MODIFY FUNCTION

I.B.6a Dimerization

Heterodimerization expands the DNA binding potential and transactivation activity of individual transcription factors. Dimers between c-Jun and members of the Jun, Fos and CREB/ATF gene families, for example, exhibit independent DNA binding and transactivation activities (Hai *et al*, 91; Kerrpola *et al*, 93; Macgregor *et al*, 90; Ryseck *et al*, 91). The proportion of any one dimer has been proposed to reflect the relative abundance of each constituent protein present within the cell (Kovary *et al*, 92). Consequently, dimerization provides a regulatory mechanism that is sensitive to changing cellular environments. The Rel-related family of transcription factors, for example, dimerize and transactivate through NF-κB-related elements in vitro. Strong transactivation is mediated through NF-κB p50/NF-κB p65 heterodimers and NF-κB p65 homodimers, whereas weak transactivation is mediated through NF-κB p50 homodimers

(Ganchi *et al*, 93). The relative proportions of each dimer is presumed to contribute to the overall transactivation of NF-κB-dependent genes in vivo. Evidence to support a role for dimerization in the regulation of transcription in vivo is provided by erythropoiesis. NF-E2 is an erythroid-specific transcription factor which binds as an NF-E2 / Maf heterodimer to AP-1-like sites in the promoters of erythroid-specific genes (Andrews *et al*, 93; Kataoka *et al* 94). The small Maf proteins lack canonical transactivation domains but are capable of dimerization and DNA binding (Kataoka *et al*, 94). Consequently, Maf homodimers supress transcription through NF-E2 binding sites. Unlike NF-E2, the Maf proteins are ubiquitously expressed, albeit at markedly different levels, in specific cell types (Andrews *et al*, 93). From this observation it has been proposed that the relative concentration of Maf proteins acts as a sensitive switch by which erythroid-specific gene expression is controlled (Igarashi *et al*, 94)

I.B.6b Squelching

The transactivation of sequence-specific transcription factors is enhanced through direct interactions with components of the basal transcriptional machinary and/or specific accessory factors (section I.B.2). Over expression of transactivation domains can repress this effect through non-productive protein:protein interactions (Angel *et al*, 89; Gill *et al*, 88; Oehler *et al*, 92). This phenomenon is described as Squelching and is predominantly dependent on functional transactivation domains. Transcription factors lacking a DNA binding domain (Angel *et al*, 89) or possessing an unrelated DNA binding domain (Gill *et al*, 88) are not compromised in their ability to titrate factors required for productive transcription.

The specificity of squelching interactions is challenged by domain swap experiments whereby one transactivation domain is replaced by an unrelated domain (Oehler *et al*, 92). c-Jun mutants, lacking an intact DNA binding domain, for example, have been shown to repress c-Jun transactivation in yeast (Angel *et al*, 89). Repression is mimicked by other acidic transactivation domains such as JunB, GAL4 and VP16, but not by unrelated transactivation domains present in the steroid receptor family of transcription factors (Angel *et al*, 89; Oehler *et al*, 92). These observations have prompted a classification of transactivation domains according to their ability to interact with similar components of the basal transcriptional machinary (Lin *et al*, 91; Tasset *et al*, 90). Acidic transactivation domains, for example, are functionally interchangeable and, consequently, have been shown to repress several non-specific genes in vitro that are recognized targets of independent acidic transactivators in vivo (Gill *et al*, 88).

The ability to repress transactivation when overexpressed may be an important regulatory mechanism in vivo. The rapid and transient expression of immediate early genes, for example, could be regulated by a concentration-dependent switch in gene expression. One example of concentration-dependent transregulation in vivo is provided by the yeast transactivator, GAL4. At high concentrations Gal4 exhibits a repressor function that, in contrast to its ability to transactivate, is independent of DNA binding activity. This, together with the requirement for intact acidic transactivation domains, suggests that Gal4-mediated repression is directed by a squelching mechanism that is essential for the activity of Gal4 in vivo (Gill *et al*, 88).

I.B.7. FUNCTIONAL MODIFICATION THROUGH TRANSCRIPTIONAL CROSS TALK

Interactions between transcription factors from unrelated gene families facilitate the integration of independent signal transduction pathways and expand and refine the overall transcriptional response. This process has been described as "transcriptional cross talk". Some of the consequences of transcriptional cross talk for gene regulation are described below.

I.B.7a Consequences of Transcription Cross Talk For Gene Regulation In Vivo

1. Expansion of DNA Binding Potential

Heterodimerization alters the sequence specificity of DNA binding transcription factors in vitro and in vivo. c-Jun/c-Fos heterodimers , for example, bind to the canonical TRE whereas heterodimers between c-Jun and ATF2, exhibit a greater affinity for the ATF/CREB (CRE) consensus sequence (Hai *et al*, 91).

More detailed analyses have demonstrated the importance of flanking residues for the optimum binding activity of sequence-specific transcription factors (Ryseck *et al*, 91). The Maf-related proteins, for example, were isolated on the basis of their homology to the product of the v-Maf oncogene, but also exhibit a weak homology over the DNA binding domain with members of the AP-1 and ATF/CREB transcription factor families (Kataoka *et al*, 93). MAF proteins homodimerize through a leucine zipper domain and bind 13- or 14-bp palindromic sequences containing the TRE or CRE consensus site. Maf heterodimers between c-Fos and c-Jun

exhibit alternative binding specificities that are distinct from both Maf homodimers and Jun/AP-1. The differences reside in unique flanking sequences surrounding the TRE and CRE consensus sites and serve to expand the repertoire of DNA binding sites available to the Maf transcription factor family (Kataoka *et al*, 94).

2. Integration of Independent Signal Transduction Pathways

Transcriptional "cross talk" facilitates the integration of independent signal transduction pathways in vivo. Well characterized examples of this type of regulation are provided by tissue-specific and differentiation-dependent patterns of gene expression.

- Tissue-Specific Gene Expression

Antigen-dependent activation of T-lymphocytes leads to the immediate early expression and secretion of the cytokine, IL-2. Expression of IL-2 is regulated through the integration of at least two independent signalling pathways that have been proposed to cooperatively activate the Rel and AP-1-like components of NF-AT, the Nuclear Factor of activated T cells (Jain *et al*, 92; Nolan *et al*, 94). Antigen-dependent activation can be mimicked in vitro by treatment of T cells with a PKC agonist and a calcium ionophore. Calcium is proposed to stimulate the nuclear translocation of pre-existinig cytoplasmic NF-AT (Rel) which then combines with a PKC- and calcium-induced nuclear subunit representing newly synthesized AP-1 (Jain *et al*, 92; Su *et al*, 94). Nuclear NF-AT binds to two regulatory elements in a 300bp enhancer region upstream of the transcriptional initiation site of IL-2. The AP-1 component is believed to stabilize the NF-AT:DNA interaction and thereby enhance subsequent IL-2 transactivation.

Functional synnergy has similarly been reported between IL-6 and IL-1 in association with several physiological responses in vivo, including immunoglobulin secretion and the acute-phase response in T-cell activation (Brasier *et al*, 90; Hirano *et al*, 90). IL-1 and IL-6 activate the expression of NF-$\varkappa$B p65 (Osborn *et al*, 89) and C/EBPb (Poli *et al*, 89) repectively. NF-$\varkappa$B and C/EBP synnergistically regulate gene expression through C/EBP DNA binding sites in vivo (Stein *et al*, 93(i)). Synnergism is dependent on a direct physical interaction between NF-$\varkappa$B and C/EBPb that stimulates the DNA binding activity of C/EBPb and enhances transactivation through an additional NF-$\varkappa$B transactivation domain. It has not been determined whether the cross coupling of NF-$\varkappa$B and C/EBP observed in vitro is relevent to the signal integration of IL-1 and IL-6 reported in vivo.

- Differentiation-Specific Gene Expression.

The integration of two independent signalling pathways is proposed to regulate myogenesis in vivo. MyoD, a member of the helix-loop-helix group of transcription factors, is a key regulator in the process of myogenic differentiation, and is capable of activating many muscle-specific genes (Weintraub *et al*, 91(ii)). Overexpression of c-Jun and v-Jun in immature myoblasts has been reported to suppress muscle-specific gene expression and inhibit terminal differentiation (Bengal *et al*, 92; Grossi *et al*, 91; Su *et al*, 91). Inhibition is a consequence of a direct physical interaction between the helix-loop-helix domain of MyoD and the c-Jun leucine zipper. c-Jun inhibition of myogenesis is mediated through the repression of MyoD responsive promoters and is refractory to differentiating-inducing stimuli . Similarly, induction of AP-1 responsive genes by activated c-Jun and c-Fos is inhibited by MyoD expression

(Bengal *et al*, 92). These observations suggest that the interaction between MyoD and c-Jun regulates the integration of two antagonistic signalling pathways important for myoblast proliferation and myogenic differentiation in vivo.

An alternative mechanism of transcriptional cross coupling has been reported to regulate the switch between osteoblast proliferation and differentiation. The osteocalcin gene is repressed in proliferating osteoblasts through an AP-1 binding site within the gene promoter. Differentiation stimuli, such as Vitamin D3, bind to an overlapping hormone response element (HRE) thereby inducing a concomitant displacement of Jun/AP-1 binding factors. Competitive binding to the HRE has been proposed to regulate the osteoblast response to independent proliferation and differentiation signalling pathways (Owen *et al*, 90; Schule *et al* 90(i)).

3. Integration Through Common Signal Transduction Pathways

Several independent families of transcription factors are activated by common inducing stimuli. Cross talk between co-activated factors can result in functional synnergism in vivo. The NF-κB and AP-1 transcription factor families, for example, are related to structurally distinct oncogenes but are co-induced in response to growth factors, mitogens, tumour promoters, and DNA damaging agents (Reviewed in Angel *et al*, 91; Baeuerle *et al*, 91). NF-κB and AP-1 independently recognize distinct enhancer motifs but have recently been reported to cooperatively transactivate gene expression through NF-κB and AP-1 DNA binding sites. The functional synnergism is dependent on intact NF-κB and AP-1 transactivation domains, and is proposed to result from a physical

interaction between the bzip domains of c-Jun and c-Fos and the rel homology domain of NF-κB (Stein *et al*, 93(ii)).

I.B.7b Mechanisms of Transcriptional Cross Talk In Vivo

- DNA Involvement

Transcriptional cross talk is mediated through protein:protein interactions between unrelated transcription factor families. Auxiliary protein:DNA interactions may or may not contribute to the functional activity of different protein complexes in vivo. Transcriptional cross talk between Jun/AP-1 and members of the steroid hormone receptor gene family, for example, results in a mutual repression of their respective transactivaiton activities (Desbois *et al*, 91; Jonat *et al*, 90; Schule *et al*, 90(ii); Yang-Yen *et al*, 91). Auxiliary protein:DNA interactions have not been detected and repression is proposed to result from a direct protein: protein interaction that inhibits the DNA binding activity of both components (Schule *et al*, 90(ii); Yang-Yen *et al*, 91). Jun/AP-1-mediated expression of the collagenase gene, for example, is repressed by glucocorticoids through a direct interaction between the DNA binding domain of the glucocorticoid receptor and the c-Jun leucine zipper (Jonat *et al*, 90; Schule *et al*, 90(ii)). The DNA binding domain of the Retinoic Acid Receptor or the Thyroid Hormone Receptor can physically substitute for the Glucocorticoid Receptor in AP-1-associated collagenase gene repression, suggesting that the important feature of mutual repression is not to recognize a specific DNA sequence but to achieve a specific overall structure (Desbois *et al*, 91; Yang-Yen *et al*, 91).

For other transcription factors, the effects of transcriptional cross talk are absolutely dependent on auxiliary protein:DNA interactions. The

interaction between monomers of Elk-1 and SRF dimers for example precipitates Elk-1 DNA binding activity and thereby enhances transactivation of the SRF ternary complex through phosphorylatable residues on Elk-1 (Hipskind *et al*, 91; Marais *et al*, 93). The DNA binding activity of NF-$\varkappa$B is similarly enhanced in electrophoretic mobility shift assays in the presence of c-Fos or c-Jun proteins. The bzip region of c-Fos and c-Jun is required for the enhanced DNA binding activity in vivo, and for the physical interaction between NF-$\varkappa$B and AP-1 in vitro (Stein *et al*, 93(ii)). A similar mechanism of transcriptional cross talk has been reported between NF-$\varkappa$B p65 and C/EBP. NF-$\varkappa$B physically interacts with the C/EBP protein in vitro and thereby enhances the affinity of C/EBP for its cognate DNA binding site (Stein *et al*, 93(i)). The electrophoretic mobility shift of the C/EBP:DNA complex is unaltered in the presence of NF-$\varkappa$B but this may simply reflect the constraints of the electrophoretic mobility shift assay which does not account for DNA bending, transient interactions or dissociations during electrophoresis (Stein *et al*, 93(i)).

- Auxiliary Factors

The functional domains directing the effects of transcriptional cross talk are commonly identical to those required to mediate the physical interaction (Stein *et al*, 93(i); Stein *et al* 93(ii)). Consequently, it has proved difficult to establish the structure of many functional protein complexes in vivo. The nature of the physical interaction may be further complicated by contributions from auxiliary proteins. The hormone bound glucocorticoid receptor, for example, has been shown to interact with c-Jun and c-Fos monomers (Yang-Yen *et al*, 90(i)), and with specific AP-1 dimers (Diamond *et al*, 90) in vitro, but cannot be detected in either complex in vivo (Diamond *et al*, 90). This may be the consequence of an intrinsic

dimer instability or reflect a requirement for auxiliary factors that are essential for the functional activity of the protein complex but preclude its detection in vivo .

Auxiliary factors may also provide an explanation for the cell-type-specific effects and antigenic profiles of certain protein complexes in vivo. AP-1-mediated collagenase expression, for example, is repressed in HeLa cells in the presence of dexamethasone. A parallel effect is not observed in NIH3T3 cells which are presumed to lack essential cell-type-specific auxiliary proteins (Jonat *et al*, 90). Expression of the IL-2 gene is induced in activated T cells by a multiprotein complex containing NF-AT and an AP-1-related component. c-Fos and c-Jun directly enhance the DNA binding activity of NF-AT through an adjacent TRE binding site within the IL-2 promoter (Jain *et al*, 92). AP-1-containing oligonucleotides specifically inhibit the binding of NF-AT to its cognate motif, but neither c-Fos nor c-Jun-specific antisera completely disrupt the protein complex in vitro, suggesting that alternative AP-1 dimers or auxiliary factors contribute to the NF-AT complex in vivo (Boise *et al*, 93; Jain *et al*, 92).

CHAPTER 1.C. - INTRODUCTION

c-JUN TRANSACTIVATION AND TRANSFORMATION.

I.C.1 c-JUN - TRANSACTIVATION AND CELL GROWTH

Cell growth is regulated through the transactivation and transrepression of growth promoting and growth attenuating genes. In different cell types, c-Jun expression has been associated with both the onset and the cessation of cell growth. This, together with the ability of c-Jun to transactivate gene expression, has led to the proposal that it functions as a dual regulator of growth in vivo.

- c-Jun Transactivating Function

c-Jun transactivation has been associated with the expression of growth promoting and growth attenuating genes.

Early reports describing the kinetics of *c-jun* expression demonstrate a direct correlation between growth promotion and the onset of *c-jun* expression: *c-jun* mRNA is rapidly and transiently induced in response to serum growth factors and tumour promoters (Lamph *et al*, 88; Quantin *et al*, 88; Ryder *et al*, 88; Ryseck *et al*, 88); the mRNA and protein levels are markedly repressed in quiescent NIH3T3 fibroblasts (Pfarr *et al*, 94); and elevated *c-jun* expression is associated with Go/G1 transitions (Kovary *et al*, 91; Ryseck *et al*, 88; Zwiller *et al*, 91). The relationship between *c-jun* expression and growth promotion has been further substantiated by the

increase in AP-1-dependent transactivation commonly observed in growth stimulated cells. De-regulated expression of human c-Jun, for example, induces cellular transformation of primary REFs in association with activated Ha-ras. Transformation correlates with an up-regulation of AP-1-dependent gene expression and is reversed by a dominant negative mutant of c-Jun lacking a functional amino terminal transactivation domain (Schutte *et al*, 89). The same mutant has been shown to repress the transactivation of several oncoproteins including c-Fos and c-Jun (Lloyd *et al*, 91), and to repress AP-1-dependent transactivation in two malignant epidermal cell lines, thereby inhibiting their sub-cutaneous tumour formation in nude mice (Domann *et al*, 94).

In other cell types c-Jun expression has been associated with growth cessation. Stimulation of Balb/c keratinocytes with TGF-B1, for example, induces a repression of cell growth and a concomitant activation of *c-jun* expression (Ginsberg *et al*, 91). A similar reversal of *c-jun* expression has been associated with certain in vivo differentiation pathways. F9 and P19 embryonal carcinoma cells exhibit a high proliferative potential in the absence of *c-jun* expression, but exit from the cell cycle and rapidly express *c-jun* in response to differentiation stimuli (de Groot *et al*, 90(i); de Groot *et al*, 90(ii)). In addition, an inverse correlation has been observed between the cellular transformation of F9 embryonal carcinoma cells and AP-1-dependent transactivation (Havarstein *et al*, 92). This correlation suggests that, in specific cell types, *c-jun* expression is associated with the regulation of growth attenuating genes. In support of this hypothesis, overexpression of c-Jun (or c-Fos) has been shown to reduce the tumourigenic and metastatic potential of high metastatic cell lines through an induction of MHC class 1 gene expression (Yamit-Hezi *et al*, 94). An inverse correlation between AP-1-dependent transactivation and cellular transformation has similarly been reported in c-Jun- and v-Jun-expressing

primary CEF cultures (Hartle *et al*, 92; Havarstein *et al*, 92; Wong *et al*, 92), leading to the proposal that c-Jun functions as a tumour suppressor gene in specific cell types, activating the expression of growth attenuating genes under normal growth conditions.

Transactivation of growth attenuating and growth promoting genes would clearly enable c-Jun to function as a dual regulator of cell growth in vivo. The evidence described above supports this proposal and suggests that each regulatory function is restricted to particular cell types, presumably due to the presence or absence of additional AP-1 transcription factors and cell-type-specific accessory proteins.

- c-Jun Transrepression Function

Transrepression provides an alternative interpretation of the results decribed above. Evidence associating c-Jun with a transrepressor activity, however, has not been widely reported, and is mainly confined to analyses of the v-Jun oncoprotein. v-Jun, for example is a poor transactivator in primary CEFs but a strong inducer of in vitro transformation and in vivo tumourigenesis (Bos *et al*, 90; Wong *et al*, 92). This has led to the proposal that v-Jun mediates transformation of primary CEFs through a passive inhibition of growth attenuating genes (Hartle *et al*, 92; Havarstein *et al*, 92; Wong *et al*, 92; section I.C 2.). Active inhibition of growth promoting genes has similarly been associated with the v-Jun oncoprotein. The metastatic potential of a mouse papilloma cell line, for example, was reduced through an overexpression of v-Jun. Metastatic suppression correlated with a specific repression of the AP-1-dependent stromalysin gene required for the invasion of the basement membrane by malignant epithelial tumours (Tsang *et al*, 94).

These observations suggest that transrepression is a specific function of the v-Jun oncoprotein that might contribute to the differential transforming activities of v-Jun and c-Jun in vitro and in vivo. This interpretation is not favoured by the results presented in one report. In this investigation overexpression of murine c-Jun was associated with a reversal of the transformed phenotype in primary REFs co-transfected with c-Myc and activated Ha-ras (Ginsberg *et al*, 91). Repression was dependent on a domain immediately ajacent to the basic region and did not require the leucine zipper or amino-terminal transactivation domains. A novel mechanism of transrepression was proposed. Curiously, this domain is essential for c-Jun-induced growth promotion in CEFs (Hartl *et al*, 92), which exhibit an inverse correlation between Jun transactivation and transformation (Hartl *et al* 92; Havarstein *et al*, 92; Wong *et al*, 92). Consequently this previously uncharacterized domain may be involved in c-Jun-dependent transrepression of both growth promoting (REFs) and growth attenuating (CEFs) genes, and thereby contribute to the specific growth regulatory functions of c-Jun in different cell types.

I.C.2. MECHANISMS OF JUN-INDUCED CELLULAR TRANSFORMATION

I.C.2a Qualitative and Quantitative Changes in Gene Expression

Overexpression of c-Jun and v-Jun has been proposed to induce cellular transformation through aberrant gene expression (Castellazzi *et al*, 90; Castellazzi *et al*, 91; Schutte *et al*, 89). c-Jun and v-Jun transactivate

gene expression through AP-1/TRE regulatory elements (Angel *et al*, 88(i); Bohmann *et al*, 89; Chiu *et al*, 89). In accordance with this, a direct correlation has been observed between AP-1-dependent transactivation and the co-transformation of primary REFs by c-Jun and activated Ha-ras (Alani *et al*, 91). Transformation of primary REFs was not abolished in the presence of c-Jun/GCN4 or c-Fos/GCN4, which exclusively homodimerize to transactivate AP-1-dependent genes (Oliviero *et al*, 92). Consequently, quantitative changes in AP-1-regulated gene expression may be sufficient to mediate the cellular transformation of specific cell types in vitro (Oliviero *et al*, 92). In vivo tumourigenesis, however, is exclusively associated with high levels of the v-Jun oncoprotein and presumably requires additional qualitative mutations not present in the c-Jun protein. Overexpression of c-Jun and JunB, for example, is associated with third stage dermal fibrosarcomas but is not sufficient to induce a fully transformed phenotype (Bossy-Wetzel *et al*, 92). Similarly, primary CEFs exhibit a partially transformed phenotype in the presence of high c-Jun expression, but are incapable of forming tumours in nude mice (Wong *et al*, 92).

The structural mutations present in v-Jun are located in the carboxy and amino-terminal regions of the protein and are described below. They have been proposed to relieve v-Jun from DNA binding and transcriptional regulation, thereby facilitating v-Jun-mediated tumourigenesis in vivo (Figure 1.1).

- δ deletion

The δ domain is essential for the transcriptional regulation of c-Jun. It is recognized by the Jun-amino-terminal kinase, JNK, which phosphorylates c-Jun at serines 63 and 73 in response to various inducing stimuli (Hibi *et al*, 93). The v-Jun oncoprotein lacks an intact δ domain (Maki *et al*, 87;

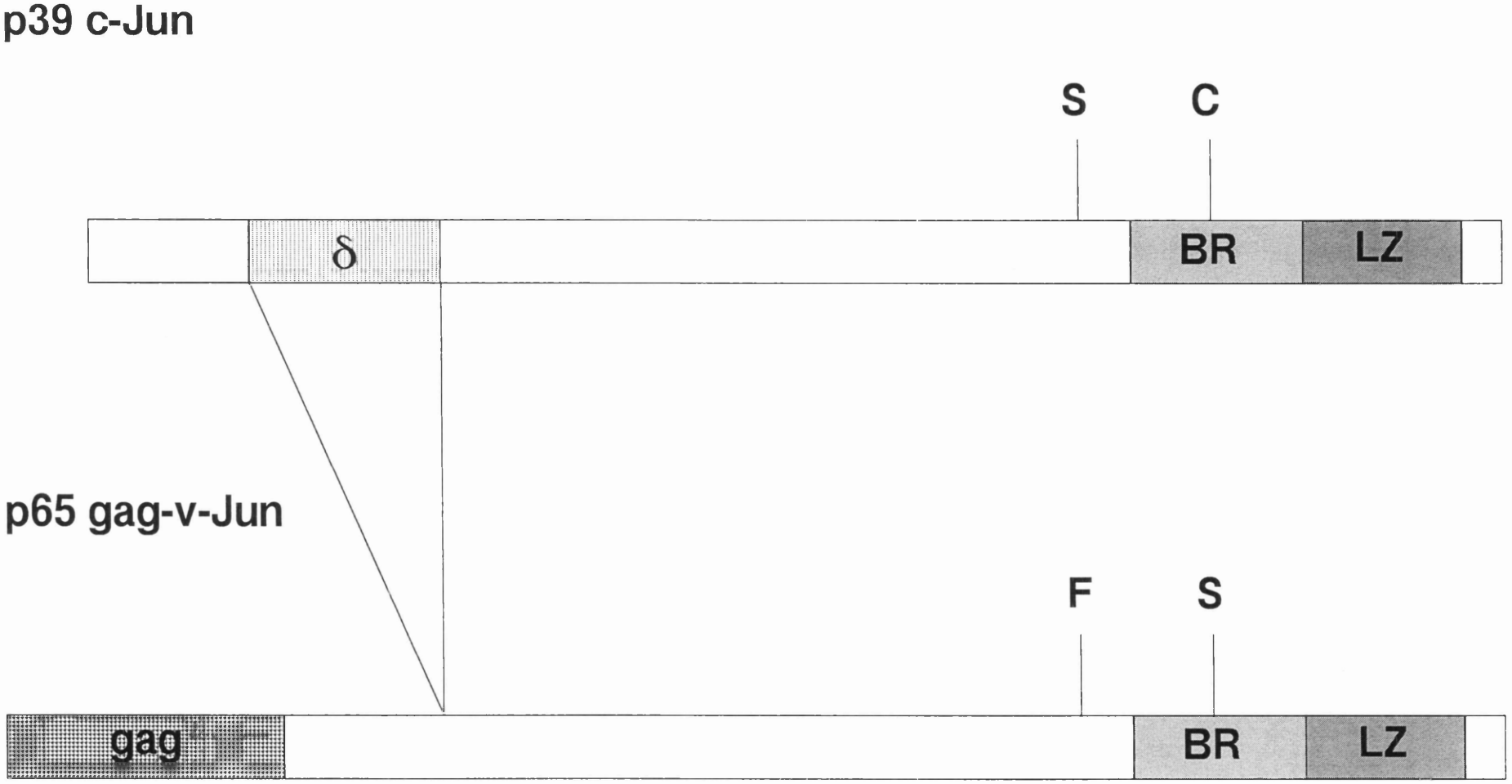


FIGURE 1.1

Nishimura *et al*, 88; Figure 1.1). Consequently, v-Jun does not present a binding site for JNK and has been proposed to express an intrinsically weaker transactivation activity than the c-Jun protein (Hibi *et al*, 93). The consequences of this are complex and cell-type dependent. In avian cells, for example, c-Jun is believed to transactivate growth attenuating genes, thereby effecting an an inverse correlation between transactivation and transformation (Havarstein *et al*, 92; Lloyd *et al*, 91); whereas in REFs, the reverse is true (Alani *et al*, 91; Lloyd *et al*, 91). If v-Jun transactivation is intrinsically weaker than c-Jun, owing to the lack of JNK regulation, transformation of both cell types would ensue through the poor transactivation of growth attenuating (CEFs) or growth promoting (REFs) genes. In support of this hypothesis, it has been reported that c-Jun is a more potent oncogene than v-Jun in the REF co-transformation assay (M.Birrer - *personal commun.*), and that v-Jun is a poor transactivator in CEFs (Havarstein *et al*, 92).

In other cell types v-Jun has been reported to transactivate gene expression more strongly than c-Jun (Baichwal *et al*, 90; Bohmann *et al*, 89). Enhanced transactivation is directly associated with the absence of the δ domain (Bohmann *et al*, 89), which is proposed to act as a transcriptional repressor in c-Jun by mediating an interaction between a cell-type-specific inhibitor (I) and the c-Jun E domain (Baichwal *et al*, 92). In support of this hypothesis, competition analysis has demonstrated super-transactivation in the presence of exogenous full length c-Jun protein, that is presumed to result from a Jun-mediated titration of a cell-type-specific inhibitor (Baichwal *et al*, 90). Enzymatic or allosteric mechanisms could mediate an E/I/δ interaction in vivo. JNK clearly binds to a domain that overlaps with the δ domain (Hibi *et al*, 93) and consequently may be masked by the E/I interaction. However, v-Jun is slightly super-transactivated in the presence of c-Jun, supporting the

proposal that the E/I interaction exerts a negative transcriptional effect even in the absence of a productive JNK/Jun interaction (Baichwal *et al*, 90).

The relevence of these observations in vivo is not clear. The experiments described above were exclusively performed with c-Jun and v-Jun homodimers, although studies to detect c-Jun homodimers in vivo have not been successful (Kovary *et al*, 91; Kovary *et al*, 92). In addition, when c-Jun/c-Fos heterodimers were used in the titration analysis no super-transactivation was observed, suggesting that the heterodimer is not subject to this additional level of control (Baichwal *et al*, 90). Thus, cell-type-specific inhibition of c-Jun transactivation may be confined to c-Jun homodimers and provide a regulatory mechanism by which c-Jun homodimeric transactivation is suppressed and transformation of specific cell types, such as primary CEFs, mediated (Castellazzi *et al*, 90; Castellazzi *et al*, 91; Wong *et al*, 92). By this analogy, additional qualitative changes in gene expression, associated with v-Jun-mediated tumourigenesis in vivo, would presumably depend on the weaker, but constitutively active, v-Jun-specific transactivation domain.

- C-Terminal Point Mutations

Two point mutations in the carboxy terminus of v-Jun have been reported to directly affect its DNA binding activity in vitro. The serine to phenylalanine mutation at serine 243 converts v-Jun into a constitutively active DNA binding protein, no longer inhibited by casein kinase II phosphorylation (Lin *et al*, 92). The cysteine to serine mutation in the v-Jun DNA binding domain, represents a gain of function mutation that relieves v-Jun from Redox regulation (Abate *et al*, 90(iv)). Both mutations have been proposed to activate v-Jun DNA binding and are presumed to

contribute to its transforming activity in vitro and in vivo (Abate *et al*, 90(i); Lin *et al*, 92).

An additional effect of the carboxy-terminal mutations may be to alter the spectrum of DNA binding sequences recognized by v-Jun-containing homo and heterodimers. Preferential recognition could be mediated directly through differential binding affinies, or indirectly through a unique subset of v-Jun dimerization partners. In support of this proposal, in vivo viral and cellular Jun complexes have been reported to interact differentially with a variety of AP-1 and CREB-like target sequences (Hadman *et al*, 93) and in vivo tumourigenesis requires both carboxy-terminal mutations in the v-Jun oncoprotein (Wong *et al*, 92). The δ domain, in contrast, contributes to the latency of tumour development but is otherwise dispensible for tumourigenesis. These observations suggest that the carboxy-terminal mutations direct the expression of an overlapping but distinct subset of target genes, essential for v-Jun-dependent tumourigenesis in vivo (Wong *et al*, 92). Alternative subsets of c-Jun and v-Jun-specific target genes could mediate their differential transforming effects in vivo and in vitro (Castellazzi *et al*, 90; Castellazzi *et al*, 91; Wong *et al*, 92), and provide an explanation for the distinct morphologies exhibited by c-Jun and v-Jun transformed fibroblasts (Pfarr *et al*, 94; section III.B.2b).

I.C.2b. Concentration-Dependent Changes in AP-1 Regulated Gene Expression

Overexpression of c-Jun (or v-Jun) is sufficient for the induction of cellular transformation in primary CEFs (Bos *et al*, 90; Hartle *et al*, 92; Havarstein *et al*, 92). This contrasts with the proposed tumour suppressor effects of c-

Jun in non-transformed CEF cultures (Hartle *et al*, 92; Havarstein *et al*, 92), and suggests that c-Jun mediates growth promotion and growth attenuation in a concentration-dependent manner. Concentration-dependent gene expression has been observed for many cellular transcription factors, including c-Jun (Schneikert *et al*, 91), and is often associated with the phenomenon of squelching (Gill *et al*, 88; section I.B.6b). c-Jun has been reported to interact with components of the basal transcriptional machinary (Kraft; *in press*), and with transcriptional co-activators (Oehler *et al*, 92), suggesting that it may be capable of mediating squelching effects at high concentrations in vivo. Alternatively, concentration-dependent changes in gene expression may be mediated through the formation of different AP-1 dimers. The spectrum of AP-1 dimers detected in vivo is thought to be determined by the relative concentrations of each constituent protein (Kovary *et al*, 92). c-Jun/Fra2 heterodimers, for example, are the predominant AP-1 DNA binding activity detected in exponential and quiescent CEFs (Nishina *et al*, 90; Suzuki *et al*, 94). In the presence of high exogenous c-Jun or v-Jun, the dimerization eqilibrium is proposed to shift in favour of c-Jun or v-Jun homodimers (Hughes *et al*, 92). The consequences of this for Jun-mediated cellular transformation are not known, but it may be significant that different AP-1 dimers exhibit independent transactivation activities and preferentially bind distinct subsets of target regulatory sequences (Deng *et al*, 93; Ryseck *et al*, 91; Suzuki *et al*, 91). If preferential recognition includes c-Jun- and v-Jun-specific non-consensus AP-1-regulated target sequences, then this may provide an explanation for the apparent inverse correlation between Jun-mediated cellular transformation and AP-1-dependent transactivation in primary CEFs (Hartle *et al*, 92; Havarstein *et al*, 92), and the differential effects of c-Jun and v-Jun on cellular transformation in vitro.

CHAPTER II - METHODS and MATERIALS.

CHAPTER II - METHODS

II.A. CELL CULTURE

II.A.1. CELL GROWTH AND PREPARATION

Primary cultures of CEF cells were prepared as described by Tato *et al.* Cells were maintained in DMEM supplemented with 10% tryptose phosphate broth, 10% newborn calf serum, 2% heat inactivated chicken serum (56^{o}C for 2hrs), and 2mM glutamine, in humidified 37^{o}C incubators containing 5% (v/v) CO_2. Confluent cultures were passaged approximately 1:5 every 2-3 days. For a T25 tissue cultue flask, the growth medium was aspirated and the cells washed with 5ml PBS. 5ml of 0.25% trypsin in CT buffer (see Materials) was slowly added and then aspirated off to leave a thin film. The culture flasks were left at room temperature until the monolayer could be detatched by gentle agitation. The cells were resuspended in growth medium and reseeded at an appropriate density. Cultures were maintained in this manner for no longer than 4 weeks or 7 subcultures.

Quiesced cultures were prepared as follows. Growth medium was aspirated from subconfluent flasks and the cells washed twice in DMEM. For a T25 flask, 5ml of DMEM supplemented with 0.2% newborn calf serum and 2mM glutamine was added, and the culture maintained at 37^{o}C for 48hrs. Under these conditions the cultures reached confluence but then underwent a complete cessation of growth, as measured by uptake of tritiated thymidine (Dr A. Catling; *personal commun.*).

II.A.2. STORAGE

Confluent cultures of CEFs were trypsinized as described, and resuspended in DMEM supplemented with 40% fetal calf serum at 1-2 $x10^7$ cells/ml. An equal volume of DMEM containing 20% DMSO was added dropwise to the cells over approximately 2min. The cells were divided into 1ml aliquots in 1-2ml Nunc cryotubes and frozen well insulated at -70°C. After 1-2 days individual tubes were transferred to liquid nitrogen for long term storage.

II.A.3. TRANSFECTION AND G418 SELECTION

II.A.3a Collagen

All CEF transfections and clonal expansions were performed on collagenated tissue culture dishes. Dishes were coated in 1mg/ml collagen in 1% acetic acid, for 2-5min, and then drained and air dried.

II.A.3b Transfection and Selection

Secondary CEFs were seeded at $3x10^5$/T25 flask or $8x10^5$/90mm dish and incubated at 37°C overnight. A Calcium Phosphate precipitate was prepared as follows:

1. 10ug of SFCV based plasmid (Figure 3.6) in 10ul TE buffer (10mM Tris HCl, 1mM EDTA, pH 8.0), was mixed with 2ug RCAN (1mg/ml in TE) as a helper virus, and 240ul 0.2x SSC

(20x stock is 175.3g NaCl, 88.2g tri sodium citrate per litre) in a sterile plastic bijoux.

2. 35ul of 2M $CaCl_2$ was added and the contents gently mixed.

3. 250ul of 2x HEBS (2x HEBS is 274mM NaCl, 10mM KCl, 1.4mM $Na_2HPO_4.7H_20$, 11mM glucose, 38mM HEPES, pH 7.1) was added and the mixture allowed to stand at room temperature for 30min.

The overnight cultures were washed in 5ml PBS and refed with 5ml of growth medium. The resulting precipitate was added dropwise with gentle mixing to the medium, and the cultures incubated at 37^oC for 4hrs. After 4hrs the medium was aspirated and the cells washed in 3ml growth medium. The cultures were shocked in 1ml HEBS-glycerol (1x HEBS with 15% glycerol) for 4min at room temperature and then washed as before. 5ml of growth medium was added and the cells incubated at 37^oC for 4-6 days until confluent. The cultures were split 1:2 and G418 added to a final concentration of 1mg/ml. Selection continued in this way for 14-21 days. This resulted in the death of mock transfected cultures and uniform infection, as judged by western blot analysis and cell morphology.

Virus particles were harvested from 90mm dishes of uniformly-infected cultures. The growth medium was aspirated and the cells washed twice with DMEM. The cultures were fed with complete growth medium supplemented with 1% DMSO and reincubated for 4hrs (5ml medium) or overnight (10ml medium) at 37^oC. The media was collected and filtered through a 0.22um sterile filter to remove any detatched cells. Virus particles were stored for long term use at -70^oC.

II.A.4. VIRAL INFECTION AND SOFT AGAR CLONING ASSAYS

II.A.4a Viral Infection

Soft Agar Assays were used as an indicator of anchorage indepent growth. Primary CEFs were subcultured 1:6 on 60mm dishes and incubated overnight at 37°C until approximately 70% confluent. The cells were washed in sterile PBS and fed with 4ml of growth medium supplemented with 2ug/ml polybrene. 1ml of overnight virus collection was added to the medium and the contents gently mixed. The cultures were incubated at 37°C until confluent and then expanded into two T75 flasks for seeding into soft agar.

II.A.4b Soft Agar Assay

0.72% base agar plates were prepared by mixing 43ml of sterile 2.5% Difco bacto-agar with 81ml of hard agar mix (described below) at 44°C. The base agar was poured into 60mm dishes at 4ml/dish and left to set at room temperature. The dishes were equilibrated in a 37°C incubator before overlaying.

Virally-infected cells were diluted to the desired concentration (10^3, 10^4, 10^5/ml) in growth medium and diluted with an equal volume of 0.72% base agar mix, maintained at 44°C. The base plates were overlayed with 2ml of the soft agar/cell mix and allowed to set at 4°C. The dishes were incubated in a humidified 37°C incubator containing 5% CO_2 (v/v), and fed every 3-4 days with 2ml of soft agar. The assays were typically performed in triplicate with colonies appearing 14 - 21 days after seeding.

Hard Base Agar (0.72%)

DMEM x2	50ml
Tryptose phosphate	10ml
Foetal calf serum	10ml
Chicken serum (HI)	2ml
Penicillin (50U/ml)	0.5ml
Streptomycin (50ug/ml)	0.5ml
Glutamine (200mM)	1ml
Folic acid (0.8%)	1ml
MEM Vitamins 100x	1ml
Sodium bicarbonate	5ml
2.5% agar	43ml

DMEM x2

10x DMEM	40ml
Sodium bicarbonate	20ml
Sodium pyruvate	4ml
distilled water	160ml

Soft Agar Colonies were picked for screening and a second round of cloning approximately 21 days after seeding. Individual colonies were picked with a one way valve mouth pipette into 500ul of growth medium in a 24 well dish, and disseminated with repeated pipetting. After overnight incubation any remaining non-adherent cells were further disseminated through a 23G needle. The cultures were fed and expanded into 90mm dishes over 1-2 weeks. Virus was collected for a second round of soft agar cloning and lysates prepared for western blot analysis (section II.D.3).

II.A.5. IMMUNOFLUORESCENCE

II.A.5a Indirect Immunoflourescence

Sterile 13mm diammeter coverslips were coated in poly-L-lysine (13.2ug/ml distilled H_2O) and incubated at 37°C overnight. Dried coverslips were transferred into 24 well dishes and seeded with $2x10^4$ cells in 500ul growth medium at 37°C overnight. The cells were fixed in 100ul of 3.3% paraformaldehyde (see below) for 15min, and then permeablized three times in 100ul of PBS/0.5% Triton X-100 for 10min. Permeabilized cells were incubated with 50ul of anti c-Jun specific antisera (see section II.E.2), diluted 1:100 or 1:500 in PBS/0.5% Triton X-100, in a humidified dark environment to prevent cellular dessication. The cells were washed three times in 50ul PBS/0.5% Triton X-100 for 5min and incubated as described above with 50ul of GAR FITC, diluted 1:100 in PBS/0.5% Triton X-100. After three washes in PBS/0.5% Triton X-100 and three rinses in sterile PBS, the coverslips were mounted in antifade (2.5% DABCO in 50:50 glycerol/PBS), and sealed with nail varnish on microscope slides. The slides were stored in the dark at 4°C for up to 6 months.

All manipulations were performed at room temperature and followed by two washes in sterile PBS unless otherwise stated.

3.3% Paraformaldehyde

Heat 3.3g of paraformaldehyde and 100ml sterile PBS to 70°C in a fume cupboard.

Dissolve with drops of 1.0M NaOH.

Cool on ice and pH to 7.3 with 1.0M NaOH.

II.B. RECOMBINANT DNA TECHNIQUES

II.B.1. BACTERIAL HOST CELLS FOR TRANSFORMATION

II.B.1a Growth Conditions

E. coli strain DH1 was obtained from D. Crouch. Strain DH5α was obtained as competent cells from Life Technology Industries. Host cells and derivatives were grown at 37^{o}C with good aeration in L-broth supplemented with 100ug/ml ampicillin.

II.B.1b Competent Cells

2.5ml of an overnight culture of DH1 was diluted with L-broth to 250ml in a 2L flask, and grown until the absobance at 600nm reached 0.3 (1cm path length, L-broth blank). The cell suspension was cooled on ice and centifuged at 8,000 rpm for 8min at 4^{o}C in a Sorvall GS3 rotor. The log phase cells were collected as a pellet and washed twice in 100ml 0.1M NaCl, 5mM $MgCl_2$. The cell pellet was collected by centrifugation as described above and carefully resuspended in 50ml 100mM $CaCl_2$, 5mM $MgCl_2$. The suspension was incubated on ice for 20min and then centrifuged as before. The supernatant was decanted and the cell pellet resupended in 10ml 100mM $CaCl_2$, 5mM $MgCl_2$, 15% glycerol. The suspension of competant cells was divided into 0.1-1.0ml aliquots on dry ice and stored at -70^{o}C.

All solutions were sterilized and maintained at 4^{o}C before use.

II.B.1c Transformation of Bacterial Hosts DH1 and DH5α

E. coli strains DH1 and DH5α were routinely used to propagate cloned and commercially obtained plasmids. 25-50% of ligation mix or 10-50ng of plasmid DNA was added to a precooled eppendorf tube and stored on ice. 100ul or 200ul of competent DH5α or DH1 cells respectively were thawed on ice and then mixed gently with the DNA of interest. The mixture was incubated on ice for a further 30-45min and then heat shocked at 42°C for 2min to allow DNA uptake. 800ul of prewarmed L-broth was added and the cell suspension incubated at 37°C for 60min to induce expression of the antibiotic resistance marker. 100ul was plated on agar plates (1.5% agar in L-broth) supplemented with the appropriate antibiotic. The remaining 900ul of cell suspension was gently centrifuged for 10sec and all but 100ul of the supernatant drawn off. The cell pellet was resuspended in the remaining 100ul and plated out as before. The agar plates were incubated inverted at 37°C overnight to allow colony formation.

II.B.1d Glycerol Stocks

E. coli transformants of interest were stored as glycerol stocks for future use. 500ul of a stationary phase L-broth culture was mixed gently with an equal volume of glycerol in a sterile plastic 1-2ml nunc cryotube and stored at -70°C. A sterile plastic loop was used to retrieve cells as and when required.

II.B.2. BACTERIAL HOST CELLS FOR PHAGE PROPAGATION

II.B.2a Growth conditions

E Coli strain WL87 was obtained from V. Fincham. Host cells were grown at 37°C with good aeration in L-broth supplemented with 10mM $MgSO_4$ and 0.4% maltose. Stationary phase plating bacteria were recovered by bench top centrifugation at 3000rpm for 5min, and resuspended at approximately 10^8 cells/ml (1 OD_{600} = $8x10^8$ cell/ml) in sterile 10mM $MgSO_4$. Plating bacteria were stored at 4°C for up to 48hrs. Glycerol stocks were prepared as described in section II.B.1d and stored at -70°C.

II.B.2b Phage Quantitation

Caesium chloride preparations of bacteriophage λ (section II.B.6bi) were titred onto stationary phase WL87 cultures to estimate the concentration of intact phage particles present. Serial dilutions of bacteriophage λ were made into TMN buffer (10mM Tris-HCl, pH 7.5, 0.1M NaCl, 10mM $MgCl_2$) and 10ul of each dilution adsorbed to 0.2ml plating bacteria at 37°C for 20min. Sterile top agar (7.5% agar in L-broth supplemented with 10mM $MgSO_4$) was melted in a boiling water bath and cooled to 47°C. 3.0ml was added to each adsorption mix and the contents plated out onto 100mm agar plates (15% agar in L-broth supplemented with 10mM $MgSO_4$). After overnight incubation plates were scored for individual plaque forming units.

II.B.2c Packaging and Adsorption

An appropriate amount of phage lambda ligation mix was packaged using the Gigapack II Plus Packaging Extract Kit according to the manufacturer's instructions. The packaging mix was serially diluted and plated out as described above. An optimum dilution was replated in proportionally bigger volumes on 20x20cm dishes for screening.

II.B.2d Screening Bacteriophage λ Libraries

Nitrocellulose filter lifts were made in triplicate from 20x20cm dishes using a series of needle marks to align the membranes. Filters were washed in denaturation buffer (section II.B.8) for 30-60sec and then in neutralization buffer (section II.B.8) for 5min. They were rinsed in 5x SSC (20x SSC is 175.3g NaCl, 88.2g NaOH in 1l distilled water, pH 7.0) for 5min and blot dried. The membranes were fixed at 80^{o}C for 2hrs and screened as described in section II.B.8. Autorads were aligned by the original needle marks and used to identify positive plaques. These were picked as soft agar plugs with a blunt ended pasteur pipette. Plugs were transferred into 1.0ml TMN buffer supplemented with a drop of chloroform in a sterile universal. After elution at room temperature for 1-2hrs, the phage particles were titrated as before and plated onto 100mm agar dishes for secondary screening. Minipreparation phage DNA was prepared from positive secondary plaques (section II.B.6bii) and digested with an appropriate enzyme for Southern Blotting analysis (section II.B.8)

II.B.3. RESTRICTION DIGESTS AND SIZE SEPARATION OF DIGESTED FRAGMENTS

II.B.3a Restriction Digests

Restriction digests were performed under buffered conditions using commercially obtained buffers specific for the enzyme of choice. 1-10 U of restriction enzyme per ug of DNA was added, depending on the enzyme used and the number of restriction sites present. The total volume of enzyme did not exceed one tenth of the final digestion volume. Typically, 1-2ug plasmid DNA was digested in a volume of 20ul for 1-2hrs at 37°C. Larger quantities of DNA were digested in proportionally greater volumes. Genomic DNA was digested overnight in the presence of maximal concentrations of enzyme and 1.0mM spermidine. Occasionally a second aliquot of enzyme was required for complete digestion of genomic DNA. Double digests were performed sequentially or as a single reaction depending on the buffering conditions specified by the manufacturer. Digests were terminated by the addition of one tenth gel loading buffer (50% sucrose w/v, 1.5% bromophenol blue w/v).

II.B.3b Size Separation of Digested Fragments

(i) Agarose Gel Electrophoresis

DNA Restriction fragments were resolved on non-denaturing agarose gels. Resolution was dependent upon the DNA fragments of interest and the agarose concentration used, but for most purposes a 0.7% agarose gel was sufficient. Gels were prepared by heating powdered agarose in an appropriate volume of TAE buffer (10x TAE is g/l 48.44g tris, 27.22g sodium acetate, 7.5g EDTA ; pH 7.8 with glacial acetic acid) until all the

particles had dissolved. The solution was cooled to approximately 50°C and ethidium bromide added to a final concentration of 0.5ug/ml. The contents of the flask were poured into a gel cast and allowed to set at room temperature. The well former was removed and the gel installed in an electrophoresis tank containing TAE buffer. Samples for electrophoresis were mixed with one tenth volume of gel loading buffer and pipetted through the TAE buffer into the wells. 1ug of molecular weight standards (materials) were treated in the same manner. The samples were resolved by electrophoresis towards the anode and visualized by ethidium bromide staining. A UV sourse was shone from below to illuminate the gel and a permanent record made onto polaroid film.

(ii) Sucrose Gradient Centrifugation

Sucrose gradient centrifugation was used to enrich for a specific population of restriction fragments within a total digest pool. Breifly, 250-500ug of genomic DNA was digested according to conditions established for a previous pilot reaction, and an aliquot analysed on a 0.4% agarose gel for correct size distribution. The DNA was extracted and resuspended in 500-1000ul of 1xTE buffer. A 10-40% sucrose gradient was mixed in a gradient former according to Manniatis and decanted into a 13.2ml Beckman polyallomer tube. The DNA sample was heated to 68°C for 10min, cooled to 20°C and loaded gently onto the gradient. The tubes were spun in a Sorval TH641 rotor at 25,000rpm at 20°C for 16hrs. 0.5ml fractions were recovered from the top of the gradient downwards using a slow speed peristaltic pump. 10ul of each sample was diluted with 20ul of distilled water and electrophoresed on a 1.0% agarose gel. Fractions containing the fragment of interest were identified by southern blotting (section II.B.8) and the DNA extracted for bacteriophage λ cloning.

II.B.4. ELUTION, EXTRACTION AND QUANTITATION OF DNA

II.B.4a Elution

(i) Agarose Gel

DNA fragments were excised from agarose gels under longwave uv illumination (300-360nm) to minimize DNA damage, and eluted using geneclean. This was carried out according to the manufactures instructions (Materials).

(ii) Low Melting Point Agarose Gel

DNA fragments for oligolabeling (section II.B 8) were excised from 1% low melting point agarose gels as described above. The restriction digest was designed such that the band of interest contained at least 250ng of DNA. The gel slice was transferred to a sterile eppendorf tube and distilled water added to a final concentration of 3.0ml/gram gel slice. The eppendorf was heated to dissolve the agarose and the contents divided into aliquots for storage at -20^{o}C.

(iii) Acrylamide Gel.

DNA fragments of less than 500bp in length were resolved on 10% polyacrylamide gels (section II.D.7a). Polyacrylamide gels were stained in 0.5ug/ml ethidium bromide for 30min at room temperature and the band excised as described above. The gel slice was cut up and the DNA eluted overnight at 37^{o}C in no more than 400ul of polyacrylamide gel elution buffer (0.5M NH4OAc, 0.01M MgOAc, 0.1mM EDTA, 0.1% SDS). The acrylamide was removed by centrifugation and the DNA ethanol precipitated for further analysis.

II.B.4b Extraction with Organic Solvents and Ethanol Precipitation

DNA samples were extracted with chloroform and/or phenol to remove contaminants such as restriction enzymes, nucleases and detergents, that can interfere with subsequent DNA manipulations. Typically the initial extraction was performed in an equal volume of phenol/chloroform. Phenol/Chloroform was prepared by mixing equal volumes of 1M Tris-HCl, pH 8.0 equilibrated phenol and chloroform/isoamyl alcohol (24:1) immediately before addition to the DNA sample. After addition the aqueous and organic phases were mixed thoroughly by vortexing and then separated by centrifugation in a bench top microfuge for 2min at 14,000rpm. This extraction was repeated if necessary. The aqueous phase was transferred into a fresh tube with an equal volume of chloroform (chloroform/isoamyl alcohol, 24:1) and the sample vortexed and centrifuged as before. This was repeated one more time to remove any traces of phenol from the upper aqueous phase. After the final extraction the aqueous phase was collected for ethanol precipitation.

Ethanol precipitation was used to collect and concentrate DNA samples, and to separate them from traces of salt, sucrose, and other solute contaminants. The aqueous solution of DNA was gently mixed with one tenth volume of 3M Sodium Acetate, pH5.2, and 2-2.5 volumes of ice cold ethanol. The sample was stored overnight at -20^{o}C or alternatively placed on dry ice for 10-20min, to precipitate the DNA. Yeast tRNA was added to facilitate precipitation if the sample contained concentrations of less than 10ug/ml DNA. Precipitated DNA was recovered by centrifugation in a bench top microfuge at 14,000rpm for 10-20min at room temperature or 4^{o}C. Precipitates were washed in a large volume of 70% ice cold ethanol to remove any traces of salt, air dried, and resuspended in an appropriate volume of TE buffer.

II.B.4c Quantitation of DNA by Spectrophotometric Analysis

The concentration of DNA preparations was calculated from their absorbance at 260nm. Typically an appropriate dilution of a DNA solution was made into TE buffer and the absorbance of the sample read at 260nm and 280nm against a blank of TE. The readings were made in a quartz cuvette with a path length of 1cm. An A_{260} of 1.0 was taken as being equivalent to a concentration of 50ug/ml plasmid or genomic DNA and 30ug/ml oligonucleotide. The chemical purity of the DNA sample was determined by the ratio of absorbance at 260nm and 280nm. A ratio of 1.8-2.0 indicated that the DNA solution was essentially pure. Preparations with a ratio of significantly less than 1.8 were purified further by extraction and precipitation.

II.B.5. ENZYME MODIFICATION

II.B.5a Dephosphorylation of 5'termini

Calf Intestinal Alkaline Phosphatase was used to dephosphorylate 5'termini after restriction endonuclease digestion. Phosphatase reactions were performed under buffered conditions with 1U of enzyme essentially according to the method outlined in Maniatis. Phosphatased DNA was extracted twice with phenol/chloroform and ethanol precipitated as described in section II.B.4b.

II.B.5b 5' Phosphorylation of 5' Termini

5' phosphorylation of 5' termini was used to label DNA probes for electrophoretic mobility shift assays. Briefly, 200ng of double stranded oligonucleotides was phoshorylated in a final volume of 20ul kinase buffer (100mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 7mM DTT) with 2 units of polynucleotide kinase and 25-50 uCi (γ-^{32}P) ATP (3000Ci / mmol). The reaction was allowed to proceed at 37^{o}C for 30min.

II.B.5c Ligation of DNA Fragments

DNA fragments were ligated over a range of molar concentrations to determine the optimal conditions for ligation. Reactions were performed in small volumes, typically 10-20ul. For a 20ul reaction the following components were assembled on ice:

10-20ng linearized phosphatased vector DNA
2-10 fold molar excess of purified DNA insert
4ul 5x ligation buffer (250mM Tris-HCl,pH7.8, 50mM $MgCl_2$, 100mM DTT, 5mM ATP, 250ug/ml BSA)
Distilled water to 18ul
2.0ul T4 DNA Ligase

The ligation was mixed gently and allowed to stand at room temperature for 30min before overnight incubation at 4^{o}C. Optimum ligation conditions were determined by visual inspection of pre and post ligation samples on agarose gels. Successful ligations were used to transform bacterial hosts immediately or stored at -20^{o}C for future use.

II.B.6. DNA PREPARATION

II.B.6a Preparation of Plasmid DNA

(i) Plasmid Minipreparations

Small-scale preparations of plasmid DNA were prepared according to an unpublished method of Dr D.Crouch.

Solutions

Lysis Solution	25mM Tris-HCl, pH 8.0
	10mM EDTA, pH 8.0
	50mM glucose
Alkaline/SDS	2M NaOH
	1.0% SDS

Single bacterial colonies were picked into 2.0ml L-broth supplemented with the appropriate antibiotic and grown up overnight. 1.5ml was transferred to an eppendorf tube and the bacteria collected by centrifugation at 6000rpm for 1min in a bench top microfuge. The bacteria were resuspended in 200ul lysis solution and lysed with 400ul alkaline / SDS on ice for 5min. 300ul of 3.0M sodium acetate pH 4.8 was added and the solution mixed thoroughly by vortexing. Chromosomal DNA was precipitated on ice for 5-10min and recovered by centrifugation at high speed for 5min in a bench top microfuge. 500ul of supernatant was transferred into a fresh eppendorf tube and the plasmid DNA precipitated

on dry ice in the presence of an equal volume of isopropanol. The precipitate was centrifuged as before and reprecipitated from 0.3M sodium acetate pH 5.2 and 2.5 volumes of ethanol. The pellet was resuspended in 75ul 1x TE and used at 5.0ul per restriction endonuclease digestion. Digestions also included a final concentration of 1.0mg/ml RNAse A.

(ii) Large Scale Preparation of Plasmid DNA

Large scale preparations of plasmid DNA essentially followed a modification of the method outlined above. A 500ml overnight culture was harvested by centrifugation in a Sorvall GS3 rotor at 4,000rpm for 10min at 4°C. The bacterial pellet was resuspended in 10ml of lysis solution and allowed to stand at room temperature for 5min before the addition of 20ml alkaline/SDS. The solution was mixed well and incubated on ice for 10min to ensure complete bacterial cell lysis. 15ml of 3M potassium acetate pH4.8 was added and the lysates incubated for a further 5min on ice. The chromosomal DNA precipitate was recovered by centrifugation at 8,000rpm for 10min at 4°C. The supernatant was filtered through a double thickness of fine gauze and precipitated in 0.6 volumes of iso-propanol at room temperature for 15min. The bacterial DNA pellet was collected by centrifugation in a Sorvall SS34 rotor at 10,000rpm for 15min at room temperature. Precipitates were drained thoroughly and resuspended in 8.0ml TE in a sterile plastic universal. Exactly 7.0g caesium chloride was added and the universal placed at 37°C until all the particulate matter had dissolved. 500ul of ethidium bromide was added and the solution transferred to a polycarbonate ultracentrifuge tube. Tubes were balanced to within 0.1g and centrifuged at 40,000rpm for 40-48hrs at 20°C in a Sorvall T1270 rotor. Plasmid DNA was carefully syringed from the gradient with a 19G needle and extracted twice with an equal volume of butanol to remove the ethidium bromide. The DNA was dialysed overnight against a

large volume of TE buffer and then ethanol precipitated and resuspended in TE at 0.5-1mg\ml. Large scale plasmid preparations were stored at 4°C or -20°C.

II.B.6b Preparation of Bacteriophage λ DNA

(i) Large Scale Preparation

Step gradient solutions

CsCl/ TMN
ϱ 1.3: 20.3g CsCl dissolved in 44.7ml TMN
ϱ 1.5: 34.0g CsCl dissolved in 41.0ml TMN

Bacteriophage λ DNA was prepared essentially according to the protocol outlined in Manniatis (Maniatis *et al*, 78; Yamamoto *et al*, 70). 2-3ml of phage particles were purified on a caesium chloride step gradient prepared from 5ml of ϱ1.5 CsCl and 4.5ml of ϱ1.3 CsCl in 14ml Beckman cellulose nitrate centrifuge tubes. The gradients were balenced to within 0.1g with TMN buffer and spun at 20,000rpm for 2hrs at 4°C in a Sorvall TH641 rotor. Phage particles were syringed from the gradient with a 21G needle and stored at 4°C in caesium chloride or dialysed against 2l of TMN buffer overnight. Dialysed bacteriophage particles were treated with 50ug\ml proteinase K for 60min at 37°C in the presence of 20mM EDTA, pH 8.0 and 0.5% SDS. The DNA was extracted in phenol/chloroform and ethanol precipitated. Precipitates were collected by spooling and washed in 70% ethanol. Phage particles were resuspended in TE buffer at 0.5mg\ml and stored at -20°C.

(ii) Bacteriophage λ Minipreparation

Single, well isolated plaques were picked into 50ul of TMN buffer and adsorbed to 50ul of WL87 plating bacteria for 20min at 37°C. 10ml of L-broth supplemented with 10mM $MgSO_4$ was added and the cultures incubated at 37°C with agitation for 6-8hrs until cell lysis was evident. Phage particles were extracted from the cultures as outlined above (Maniatis *et al*, 78; Yamamoto *et al*, 70) but in proportionally smaller volumes and excepting the dialysis step. Precipitated DNA was resuspended in 100ul of TE and digested at 20ul per reaction with 1.0mg/ml RNAse A.

II.B.6c Preparation of Genomic DNA

(i) Preparation of undigested Genomic DNA

Solutions

TNE buffer	10mM Tris-HCl
	400mM NaCl
	2mM EDTA, pH 8.2
Lysis Buffer	40ug/ml proteinase K
	0.5% SDs
	TNE buffer

Cells (~$5x10^7$) in T175 flasks were washed twice in PBS and lysed in 5.0ml lysis buffer at 37°C for 30min. The viscous contents were scraped into 15ml falcon 2059 tubes and incubated at 37°C overnight. The solution

was extracted twice in phenol/chloroform and once in chloroform. Each extraction was centrifuged at 5000rpm for 5min in a Sorvall HB4 rotor. The aqueous phase was transferred into a fresh tube using a wide bore pipette. Genomic DNA was then ethanol precipitated with 0.3M sodium acetate at room temperature. Precipitates were collected at the end of a sealed pasteur pipette and rinsed thoroughly in 70% ethanol. Excess ethanol was removed by air drying and the DNA dissolved in 1-2ml TE/T175 flask at room temperature overnight. Genomic DNA was stored at 0.5-1mg/ml in TE at 4^{o}C.

(ii) Preparation of DNase 1 Treated Genomic DNA

Preparation of DNase 1 treated genomic DNA was essentially according to Stewart *et al*, with modifications made for CEF cell culture.

Solutions

Permeabilization solution	15mM Tris-HCl, pH 7.5
(pH 7.5 with NaOH)	15mM NaCl
	5mM MgCl2
	300mM Sucrose
	60mM KCl
	0.5mM EGTA
Lysis solution	50mM Tris-HCl, pH 8.0
	20mM EDTA, pH 8.0
	1.0% SDS
DNase 1 Storage Buffer	20mM sodium acetate, pH 6.5
	5mM CaCl2

0.1mM PMSF

50% (v/v) glycerol

Secondary CEFs were plated at approximately 5 x10^6 cells per 90mm dish and incubated overnight at 37°C. Cells were washed twice in PBS and drained thoroughly. DNase 1 (2mg/ml in storage buffer) was diluted over a range of concentrations (10-100ug/ml) in permeabilization buffer supplemented with 0.2% NP40 and 0.5mM B-Mercaptoethanol. 2.0ml of each dilution was layered onto a single monolayer and the cells incubated at room temperature for 3.5 min. The solution was aspirated off and replaced with 1.0ml of lysis solution. Proteinase K was added to 200ug/ml and the contents scraped into a sterile capped eppendorf tube and incubated overnight at 37°C. After a single extraction in 1.5ml phenol, nucleic acids were precipitated twice from 0.25M ammonium acetate and 2 volumes ethanol. The pellet was rinsed in 70% ethanol, air dried, and resuspended in TE at 0.5-1mg/ml.

II.B.6d Preparation of Oligonucletides

Tritylated oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer. The oligonucleotide was eluted from the column in 2.0ml of concentrated ammonia (29%) for 1.5hrs at room temperature. The solution was transferred to a sealed glass vial and incubated overnight at 55°C. The deprotected oligonucleotide was passed through an oligonucleotide purification cartridge according to the manufacturers instructions (see materials). Full length oligonucleotides were dried down under vacuum and resuspended in TE at 1.0mg/ml.

II.B.7. DOUBLE STRANDED DNA SEQUENCING

Double stranded sequencing was used to sequence avian *c-jun* genomic DNA and cloned derivatives thereof. The DNA was prepared and sequenced according to an unpublished method of Dr D. Crouch. 50ul of RNase A treated minipreparation plasmid DNA or 2.5ug of plasmid DNA (in 50ul TE) was mixed with 30ul of 2.5M NaCl / 20% PEG and incubated on ice for 1hr. DNA precipitates were recovered by centrifugation at 14,000rpm for 15min in a bench top microfuge and washed in 70% ethanol. Air dried pellets were resuspended in 16.0ul TE and alkali denatured in 0.2M NaOH for 30min at 37°C. The DNA was precipitated on dry ice from 0.3M sodium acetate, and 2 volumes of ethanol. The pellet was washed as before and resuspended in 20ul TE. 7.0ul of this preparation was mixed with 2.0ul sequencing reaction buffer (USB Sequenase Kit) and 1.0ul primer (25ng/ul) and annealed for 30min at 37°C. Sequencing reactions were then carried out using the USB Sequenase Kit according to the manufacturers instructions. The reactions were resolved as described in section II.C.3.

II.B.8. SOUTHERN BLOT ANALYSIS

10-20ug of genomic DNA was digested with an appropriate enzyme and resolved by agarose gel electrophoresis. Gels were washed with gentle agitation for 1hr at room temperature in 200ml denaturation buffer (1.5M NaCl, 0.5M NaOH). The buffer was changed twice over this period. After rinsing briefly in water to remove traces of alkali, gels were gently agitated in 200ml of neutralisation buffer (1.5M NaCl, 0.5M Tris-HCl, pH7.5) for 40min at room temperature.

Gel transfer to Hybond N(fp) membrane was effected by capillary action in 20x SSC buffer. Transfer and subsequent fixation by UV cross linking, were performed according to the manufacturer's instructions. Membranes were sealed in 100ml of prehybridization buffer (6x SSC, 0.5% SDS, 5X Denhardts, 100ug/ml denatured salmon sperm DNA), and incubated at 65^{o}C in a shaking water bath for 2-4hrs. DNA probes were oligolabelled using commercially available kits from Pharmacia and Boehringer. Radiolabelled probes were separated from unincorporated nucleotides through Biorad Biospin 30 columns according to the manufacturer's instructions. Probes were denatured at 95-100^{o}C for 2-5min and coooled on ice immediately prior to hybridization. Hybridization was carried out in sealed boxes in a minimal volume of prehybridization fluid supplemented with 10mM EDTA and the denatured oligolabelled probe. Boxes were incubated overnight in a shaking water bath at 65^{o}C. Membranes were washed to high stringency according to the manufacturer's instructions, blot dried to remove excess fluid, and subjected to autoradiography at -70^{o}C.

II.C. RNA ANALYSIS

II.C.1. TOTAL RNA EXTRACTION AND QUANTITATION

Total RNA was harvested from confluent CEF cell monolayers grown in the presence and absence of serum growth factors. A T175 flask was lysed in 10ml RNAzol solution and the contents transferred to a 15ml polypropylene falcon 2059 tube. RNA extraction was then carried out according to the manufacturer's instructions. Washed precipitates were air dried and resuspended in sterile distilled water. The concentration of total RNA preparations was measured spectrophotometrically as described in section II.B.4c. The spectrophotometer was calibrated using a blank of sterile distilled water. An A260 of 1.0 was taken as being equivalent to 40ug/ml of RNA. RNA preparations were stored at 1.0mg/ml at -20^{o}C and at -70^{o}C.

II.C.2. PREPARATION OF ANTISENSE RNA PROBES

The transcription vector pSPT19 was used to generate antisense cjun riboprobes for RNase protection analysis. pSPT19 contains diametrically opposed SP6 and T7 transcription promoter sequences, separated by a multiple cloning site. JF4 *c-jun* cDNA, was subcloned into pSPT19 in the SP6 orientation by Dr E. Black (Figure 2.1). pSPT19/JF4(SP6) was linearized in the absence of RNase A, at a single Bgl II site prior to transcription. The linearized template was extracted sequentially with phenol/chloroform, and chloroform, and precipitated in 2 volumes of ethanol and 0.3M sodium acetate. The DNA pellet was air dried and

Figure 2.1

A plasmid map to illustrate the structure of pSPT19/JF4. pSPT19 /JF4 contains an EcoRI fragment spanning an avian c-jun cDNA cloned into the transcription vector, pSPT19 (Figure 5.3). The cDNA contains ~ 25bp of 5' untranslated leader sequence and ~300bp of 3' untranslated downstream sequences.

Figure 2.2

A plasmid map to illustrate the structure of pHSV-Bgal. pHSV-Bgal, used as an internal control for transfection efficiency, is derived from the lacZ-containing plasmid pCH110 (Hall *et al*, 83). Transcription of the lacZ gene is driven by the HSV-2 IE-5 promoter obtained from pLW2 (Gaffney *et al*, 85).

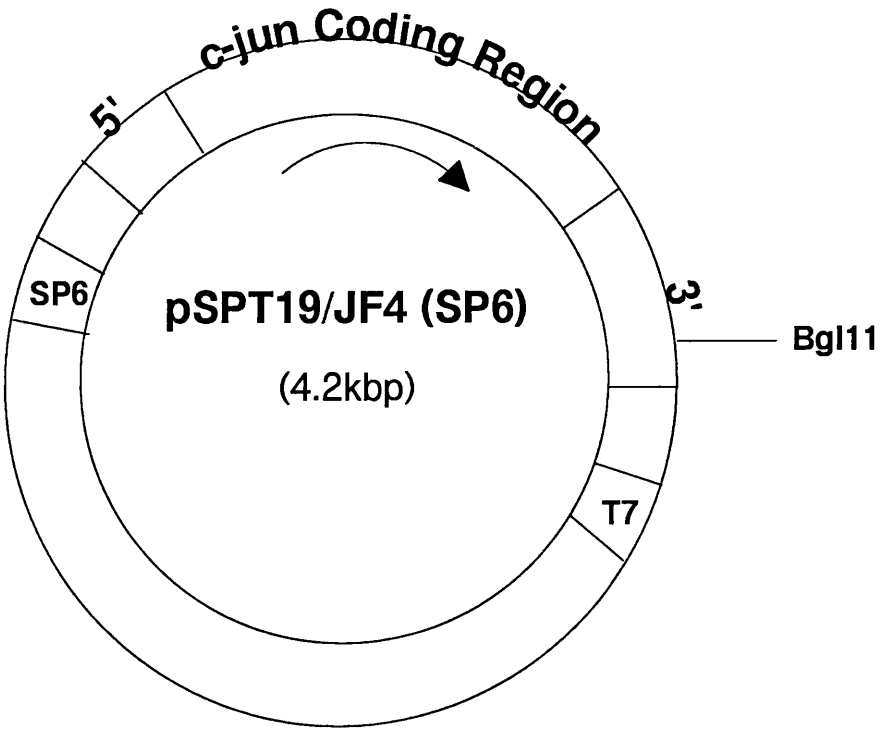


FIGURE 2.1

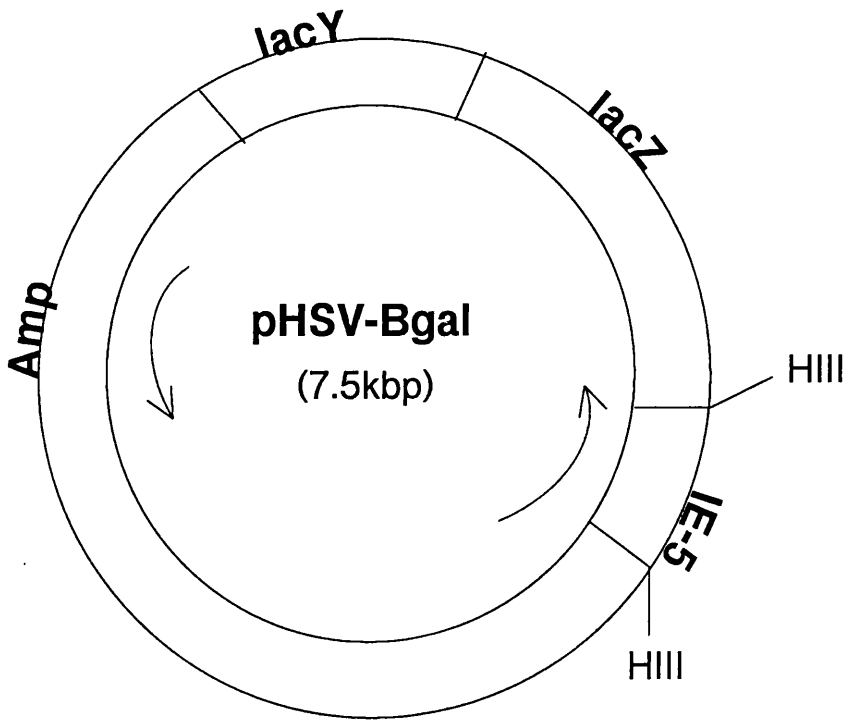


FIGURE 2.2

resuspended at 1.0mg/ml in sterile distilled water. Stock solutions were stored at -20^{o}C.

For a single in vitro transcription reaction the following components were mixed on ice in a final volume of 20ul:

1-2ug linearized DNA template

4ul 5x transcription buffer (200mM Tris-HCl, pH 7.5, 30mM MgCl2, 10mM Spermidine, 50mM NaCl)

1ul RNasin

5mM GTP:ATP:UTP

100uCi (α-^{32}P) CTP (800Ci/mmol)

2ul T7 RNA polymerase

The reaction was incubated at 37^{o}C for 30-60min. 0.5mg/ml RNase free DNase 1 was added and the incubation continued for a further 10-15min. RNA was extracted sequentially in phenol, phenol/chloroform, and chloroform. The aqueous phase was passed through a Pharmacia Nick Column according to the manufacturer's instructions. 100ul fractions were collected into STE buffer (10x STE is 100mM Tris-HCl, pH 8.0, 1.0M NaCl, 10mM EDTA, pH 8.0) and 1.0ul of each counted in 5.0ml Ecoscint. The hottest fraction was retained as a probe for subsequent RNase protection analysis. A second smaller peak represented unincorporated radioactive nucleotides.

II.C.3. RNase PROTECTION ANALYSIS

Solutions

80% FAB	80% deionized formamide
	400mM NaCl
	40 mM PIPES, pH 6.4
	1mM EDTA
Digest Buffer	10mM Tris-HCl, pH 7.6
	5mM EDTA, pH 8.0
	0.3M NaOAc, pH 7.0
	40ug/ml RNase A
	150 U/ml RNase T1

30ug of total cellular RNA was mixed with approximately 10^6 cpm of antisense riboprobe in a sterile eppendorf, and dried down under vacuum spinning at room temperature. The pellets were resuspended in 20ul FAB and incubated in a water bath at 85°C for 5min. The temperature of the bath was slowly reduced to 50°C and the hybridization continued overnight. 300ul of digestion buffer was added and the samples incubated at 30°C for 60min to digest single stranded RNA. 2.5ul proteinase K (10mg/ml) and 3.2ul 20% SDS was then added for 15min at 37°C to terminate the digestion. Double stranded RNA was extracted sequentially with 5-20ug of carrier yeast tRNA, in phenol/chloroform (x1) and chloroform (x2). Two volumes of ethanol was added and the RNA precipitated on dry ice for 30min. The pellet was washed in 70% ethanol, air dried, and resuspended in 5ul loading buffer (95% formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF). Controls

containing the digested or the non-digested riboprobe alone were included in each experiment to illustrate the extent of digestion and to compare the migration of the full length riboprobe relative to the hybridized counterpart. A 1kb DNA ladder was 5' end labelled (section II.B.5b) to size the protected fragments. Reactions were resolved on 6% denaturing acrylamide gels (see below) at a constant 40W for 2hrs. Gels were fixed for 10-20min in 10% methanol, 10% acetic acid in water and dried down before autoradiography at room temperature.

Gel Mix - 75ml in distilled water

30% Acrylamide-bis (19:1 ; seq. grade)	15ml
Urea	31.5g
10X TBE buffer	7.5ml
(g/l; 108 tris base, 9.3 EDTA, 55 boric acid)	
10% ammonium persulphate	450ul
TEMED	75ul

II.C.4. NUCLEAR RUN-OFF ANALYSIS

II.C.4a Nuclei Extraction

Solutions

Hypotonic Buffer	10mM Tris-HCl, pH 7.5
	10mM KCl
	1.5mM $MgCl_2$

Glutamate Buffer	125mM Potassium glutamate
(stored at -20°C)	100mM Hepes, pH 8.0
	5mM $MgCl_2$
	2mMM DTT
	1mM EGTA
	40% Glycerol

Confluent CEF cells in T175 flasks were washed in ice cold PBS and trypsinized in 12.5ml 0.25% trypsin in CT buffer. 2x T175 flasks were harvested into 12.5ml newborn calf serum in a 50ml falcon tube. Any remaining cells were collected in a further 12.5ml ice cold PBS. The cells were recovered by bench top centrifugation at 1000rpm for 5min and washed in 50ml ice cold PBS. Cells were pelleted as before and resuspended in 5.0ml Hypotonic Buffer in a dounce homogenizer. The cells were dounced 2-3x to obtain an even suspension and a further 15-20x in the presence of 5.0ul Triton X-100 to promote cell lysis. Nuclei were harvested in a Sorvall HB4 rotor at 2000rpm for 10min at 4°C. Opaque pellets were resuspended in 0.5ml ice cold glutamate buffer in a sterile eppendorf tube. Nuclei were harvested for a second time at 3000rpm for 30-60sec in a bench top microfuge, and the supernatant carefully aspirated to approximately 40ul. Pellets were vortexed briefly to resuspend the nuclei and stored at -70°C for several months.

II.C.4b Immobilization of DNA to Nitrocellulose Filters

Plasmid DNA was immobilized on nitrocellulose membranes using a Biorad Bio-Slot Microfiltration Apparatus. 10ug of DNA was diluted to 80ul in TE buffer, and alkali denatured in 0.4M NaOH, 10mM EDTA, pH 8.0 at 100°C for 10min. The samples were neutralized in an equal volume of 2.0M

ammonium acetate, pH 7.0, and transferred to nitrocellulose according to the manufacturer's instructions. The membrane was fixed under vacuum at 80°C for 2hrs and prehybridized as described in section II.B.8. Individual filters were transferred to sterile plastic bijouxs containing a minimal volume of hybridization fluid (section II.B.8) and hybridized as described below.

II.C.4c Run-Off Assay

Nuclei from 4 T175 flasks were thawed on ice and mixed with the following components in a sterile eppendorf tube:

12ul ^{32}P-UTP (240 uCi, 800 Ci/mmol)
0.5ul 1.0M creatine phosphate in 10mM Hepes, pH 8.0
(stored in aliquots at -70°C)
1.2ul 2.0mg/ml creatine kinase in glutamate buffer
1.0ul nucleotide triphosphate solution
(50mM ATP, 25mM GTP, 25mM CTP)

Nuclear runoff reactions were incubated in a 37°C water bath for 15min. RNA was extracted according to the manufacturer's instructions, in 800ul of RNAzol and 400ul of chloroform. Precipitates were washed in 70% ethanol, air dried, and resuspended in 90ul 0.5% SDS, 1.0mM EDTA, pH 8.0. 10ul of 3M NaOH was added to degrade the RNA to approximately 100bp fragments, and the tubes incubated on ice for 10min. 100ul of 0.48M HEPES was added to neutralize the sodium hydroxide and the samples assayed for TCA precipitable radioactivity. Typically 2.0ul of labelled extract was mixed with 5.0ul of a 10mg/ml solution of yeast tRNA, and 83ul of sterile distilled water in a precooled eppendorf tube. Ice cold

TCA was added to a final concentration of 10% and the tube incubated on ice for 1hr. Precipitates were collected on Whatman GF-C filters and washed extensively in ice cold 10% TCA. Filters were washed with 95% ethanol, dried thoroughly and counted in 5.0ml Ecoscint. An eqivalent amount of TCA precipitable radioactivity was added directly to each parallel nitrocellulose filter in hybridization fluid. The filters were hybridized in a shaking water bath at 65^{o}C for 48-72hrs and then washed to high stringency according to the manufacturer's instructions. Filters were rinsed twice in 1x SSC and incubated with 10ug/ml RNAse A in 20ml 10mM Tris-HCl, pH 7.5, 5mM EDTA, pH 8.0, 0.3M NaCl, in a 37^{o}C shaking water bath for 30min. After a final wash in 1x SSC at 65^{o}C for 15min, the filters were blot dried and subjected to autoradiography at -70^{o}C.

II.D. PROTEIN ANALYSIS

II.D.1. PROTEIN EXTRACTION

II.D.1a Preparation of Non-Denatured Nuclear Protein Extracts

Nuclear protein extracts for footprinting analysis (section II.D.6a) were prepared from normal and ASV 17-infected CEF stocks essentially according to O'Prey *et al.*

Group A Inhibitors - 100x stocks (-20°C).

50mM PMSF in isopropanol

50mM Benzamidine

1.0M sodium butyrate

1.0M B-glycerophosphate

1.0mM sodium orthovanadate, pH 8.0 (20x stock)

Group B Inhibitors - 1000x stocks (-20°C).

1.0mg/ml Leupeptin

1.0mg/ml Aprotinin in PBS

1.0mg/ml Bestatin in PBS

1.0mg/ml Pepstatin A in methanol

E50

50mM $(NH_4)2SO_4$

20mM Hepes, pH 7.9

5mM $MgCl_2$

0.1mM EDTA, pH 8.0

0.1% (v/v) Brij 35

20% (v/v) glycerol

1mM DTT (add fresh)

Storage Buffer 50mM NaCl

20mM Hepes, pH 7.9

5mM $MgCl_2$

20% (v/v) glycerol

1mM DTT (add fresh)

CEF cells were grown to confluence in 10 roller bottles (~10^{10} cells) and detatched with sterile glass beads. The nuclei were harvested according to the published protocol and resuspended in 7.0ml TMS (5mM Tris-HCl pH 7.5, 2.5mM $MgCl_2$, 125mM sucrose) in a sterile 30ml Corex tube. 0.1 volumes of 4M NaCl was added dropwise, with stirring, and the solution centrifuged in a Sorvall SS34 rotor at 17,000rpm for 15min at 4°C. The supernatant was ultracentrifuged in a Sorvall T1270 rotor at 35,000rpm for 60min at 4°C. Solid ammonium sulphate was added to 3.4g/7.5ml, and the supernatant left on ice for 30min. The precipitate was pelleted in a Sorvall SS34 rotor at 15,000rpm for 20min, and redissolved in 3.0ml E50 buffer. Nuclear protein preparations were dialysed overnight against 1l of storage buffer in ready prepared BRL dialysis tubing washed sequentially in distilled water and TMS. The crude protein extract was cleared by ultracentrifugation in a Beckman TL-100 rotor at 35,000rpm for 60min at 4°C. The supernatant was divided into aliquots on dry ice and stored at -70°C.

All manipulations were carried out at 4°C in solutions containing Group A + B inhibitors.

II.D.1b Preparation of Non-Denatured Whole Cell Extracts

Whole cell extracts were prepared for Electrophoretic Mobility Shift assays essentially according to the method of Marais *et al.* Typically, CEF cells were grown to confluence in 150mm dishes and lysed in 300ul lysis buffer (20mM Hepes, pH 7.9, 5mM EDTA, 10mM EGTA, 5mM NaF, 0.1ug/ml okadaic acid, 10% glycerol, 1mM DTT), containing 0.4M KCl, 0.4% Triton X-100 and protease inhibitors as follows : 5ug/ml pepstatin A, 1mM benzamidine, 50ug/ml PMSF. The crude extract was cleared in a bench top microfuge at 14,000rpm for 15min and the protein concentration estimated essentially according to the method of Bradford *et al.* 1ul of extract was diluted to 500ul or 1ml in distilled water and mixed with an equal volume of Coomassie protein reagent. The absorbance reading at 595nm was taken in a plastic cuvette with a path length of 1cm. The spectrophotometer was calibrated using a lysis buffer blank. Protein concentrations were read off a standard curve constructed from dilutions of bovine serum albumin under the same assay conditions. Extracts were divided into aliquots and stored at -70^{o}C.
All manipulations were carried out at 4^{o}C sterile eppendorf tubes.

II.D.1c Preparation of Denatured Whole Cell Extracts

Denatured whole cell extracts were routinely prepared from confluent T25 flasks for western blot analysis (section II.D.3). Monolayers were washed in ice cold PBS, drained thoroughly, and lysed in 500ul sample buffer (50mM Tris-HCl, pH 6.8, 30% glycerol, 2.5% SDS, 2% B-mercaptoethanol). Lysates were sonnicated to shear any genomic DNA present and cleared in a bench top microfuge at 14,000rpm for 10min at 4^{o}C. The extracts were stored at -70^{o}C.

II.D.2. PREPARATION OF POLYCLONAL c-JUN-SPECIFIC ANTISERA

II.D.2a Expression and Purification of T7 expressed c-Jun

A T7 RNA polymerase expression system was adopted to prepare sufficient quantities of c-Jun protein for rabbit immunizations and subsequent antibody production. The system was developed by Dr E. J. Black essentially according to Studier *et al.* Plasmid pRK 171 and pLys S cells were kindly provoded by Dr F.W. Studier for this purpose. *c-jun* was expressed in pLys S cells from a T7 promoter within the pRK 171 plasmid, after induction of T7 RNA polymerase by IPTG. Briefly a 2l culture of cells grown to an OD_{600} of 0.3-0.5 in L-broth supplemented with 100ug/ml ampicillin and 25ug/ml chloramphenicol, was induced with 0.4mM IPTG at 37^{o}C for 3-5hrs. The cells were harvested in a Sorvall GS3 rotor at 4000rpm for 10min at 4^{o}C and resuspended in 20ml 50mM Tris-HCl, pH 7.5, 5mM EDTA. The suspension was quick frozen in dry ice and then rapidly thawed at 37^{o}C to lyse the cells. 1.4ml 5M NaCl was added dropwise with stirring at 4^{o}C. 1.5ml Triton X-100 was added and the solution sonnicated for 5sec three times to shear any genomic DNA. Proteins were recovered in a Sorvall SS34 rotor at 15,000rpm for 10min at 4^{o}C. The pellet was washed in 10mM Tris-HCl, pH 7.5 and resuspended in 2.0ml 20mM Tris-HCl, pH 7.5, 1mM EDTA, 2mM DTT, 0.1% Triton X-100 for long term storage at -20^{o}C.

II.D.2b Immunization

1ml of full length c-Jun protein suspension was emulsified with 3ml of Freunds Complete Adjuvant for immunization of two New Zealand White rabbits at 4 independent subcutaneous sites. Secondary immunizations

were performed at day 14 and day 21, using Freunds Incomplete Adjuvant. The rabbits were test bled before (pre-immune) and after each successive immunization. 5ml of blood was transferred into a sterile universal and left to clot at 4°C. The serum was decanted and spun in a bench top centrifuge at 1000rpm for 5min to remove any remaining intact red blood cells. Aliquots were stored at -20°C and then tested over a range of concentrations against control western blot lysates as described below. After two successive specific positive reactivities the animal was sacrificed and the serum divided into aliquots for long term storage at -70°C. Thawed aliquots were stored at 4°C in the presence of 0.025% azide.

II.D.3. WESTERN BLOTTING

II.D.3a Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was adopted to separate proteins according to their apparent molecular weight. Gels were resolved by electrophoresis essentially according to Laemlli *et al*, using a Biorad Protean II apperatus. For western blots gels were typically 9% with respect to acrylamide. A stock solution containing 29.2% (w/v) acrylamide, 0.8% bis-acrylamide was used to prepare the resolving gels as follows:

	ml
30:0.8 Acrylamide-bis	12.0
1M Tris-HCl, pH 8.8	15.0
distilled water	12.3

10% SDS	0.4
10% ammonium persulphate	0.305
TEMED	0.02

The gel mix was pipetted between two glass plates and overlayed with water saturated butan-2-ol. After polymerization the overlay was poured off and the gel surface washed extensively with distilled water. Excess water was removed with a sheet of 3mm Whatman filter paper, and the comb inserted before the stacking gel was poured. The stacking gel contained the following components:

	ml
30:0.8 Acrylamide-bis	3.2
1M Tris-HCl, pH 6.8	2.5
distilled water	14.0
10% SDS	0.2
ammonium persulphate	0.2
TEMED	0.02

After polymerization the comb was removed and the gel transferred to a gel tank. Both reservoirs were filled with 1x running buffer (10x stock, g/l tris base, 30, SDS, 10, glycine, 144, in distilled water, pH8.3). The wells were flushed through with running buffer and bubbles removed from the lower surface of the plate before the samples were loaded.

50ul of lysate (section II.D.1c) from a confluent T25 flask was resolved with an aqual amount of protein (by Coomassie brilliant blue staining) from the same culture maintained under different growth conditions or from an alternative culture of interest. The samples were denatured at 100^{o}C for

3-7min prior to loading, and resolved at a 200-250V for 2hrs or 30V overnight with pre-stained protein molecular weight standards.

II.D.3b Electroblotting

Proteins were transferred from acrylamide resolving gels to Hybond ECL nitrocellulose membranes using a CAM LAB Semi Dry Blotter according to the manufacturer's instructions. Briefly, the resolving gel was equilibrated in 1x transfer buffer (60mM tris, 50mM glycine, 1.6mM SDS, 20% (v/v) methanol) for 15min at room temperature. Six pieces of Whatman 3MM paper and one piece of membrane were rinsed in transfer buffer and placed on the blotter, membrane uppermost. The gel was placed on top of the membrane and covered with six more pieces of rinsed Whatman 3MM paper. Air bubbles were removed and transfer effected at full power for 45-60min. Molecular weight marker positions were highlighted and the membrane stored at room temperature in a sealed box.

II.D.3c ECL detection

Enhanced Chemiluminescence (ECL) is a light-emitting-non-radioactive method for detecting immobilized specific primary antibodies conjugated with horse radish peroxidase (HRP)-linked secondary antibodies. Light emission is generated by the HRP/hydrogen peroxide catalysis of luminol to an excited oxidated state. This reaction is dramatically enhanced in the ECL system by chemical enhancers such as phenols, and can be detected by a short exposure to blue-light sensitve autoradiography film. Hybond-ECL nitrocellulose membranes were blocked overnight at 4^{o}C in Tris-buffered-saline-Tween (10mM Tris-HCl, 150mM NaCl, 0.05% Tween-20 - pH 8.0) containing 5% Marvel. ECL detection reactions were

then carried out according to the manufacturer's instructions. The primary antibody was diluted as required in TBS-T / 5% Marvel. The HRP-linked secondary antibody was diluted 1:5000 as above, and washes were performed in TBS-T alone.

Membranes were stripped for subsequent analysis in 100ml of 62.5mM Tris-HCl, pH 6.8, 2% SDS, 100mM B-mercaptoethanol, at 55°C for 30min. The blots were washed repeatedly at room temperature in TBS-T and stored as before.

II.D.4. IN VITRO TRANSLATION AND IMMUNOPRECIPITATION

II.D.4a In Vitro Translation

Sense RNA templates for in vitro translation reactions were prepared as described in section II.C.2. 1-2ul of invitro transcription reaction was translated in the presence of ^{35}S labelled methionine and 35ul of Promega rabbit reticulocyte lysate according to the manufacturer's instructions. The products were divided, and 5ul diluted in 20ul SDS sample buffer for Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (section II.D.3a). Samples were resolved on a 9% acrylamide/SDS gel with prestained molecular weight markers and detected by flourography of the dried gel. Co-translations were performed as above with 1-2ul of each input RNA template added per reaction.

II.D.4b Immunoprecipitations

RIPA buffer	10mM Tris-HCl, pH 7.4
	0.15M NaCl

1% NP40

1% Sodiun deoxycholate

0.1% SDS

0.5% aprotinin

20-25ul of invitro translation reaction was diluted, under non-denaturing conditions, to 500ul in RIPA buffer and gently shaken with 5ul of primary antibody at 4^{o}C for 1hr. 40ul of PAS (50% Protein A Sepharose/sterile PBS slurry) was added to bind immunoglobulin, and the samples incubated for 1hr as before. Precipitates were washed twice in RIPA buffer and resuspended in 50ul SDS sample buffer. The samples were denatured at 100^{o}C for 3-7min and spun briefly to remove the PAS before resolving on a 9% acrylamide SDS gel as above.

II.D.5. DNA TRANSIENT TRANSFECTION AND CAT EXPRESSION ASSAYS

II.D.5a Transient Transfection

Virally-infected or control CEF stocks were plated 16hr prior to transfection at 3x10^{5} cells per 60mm dish or 8x10^{5} cells per 90mm dish. Cells were transiently transfected by liposome mediated transfer using DOTAP according to the manufacturer's instructions. The quantities of DNA included in each transfection are outlined in Table 5A. After 4-5hrs incubation the transfection mix was aspirated and the cells washed twice with DMEM. Dishes were refed with growth medium (60mm dish) or quiescing medium (90mm dish), and incubated for a further 40-44hrs. Quiesced cell cultures were washed twice in DMEM and serum stimulated

for 60min in complete growth media before all the cultures were harvested for CAT analysis.

II.D.5b CAT assay

Transient transfections were washed in ice cold PBS and scraped into 1ml PBS in a precooled eppendorf tube. Cells were harvested in a bench top microfuge at 6000rpm for 2min. The pellets were resuspended by vortexing in 200ul 0.25M Tris-HCl, pH 7.8, and the cells lysed by repeated freezing and thawings. Nuclei were harvested in a bench top microfuge at 14,000rpm for 2min and collected for Hirts analysis (section II.D.4c). The supernatant was assayed for protein concentration by Bradford analysis (section II.D.1b). 20ug of protein was diluted to 180ul with 0.25M Tris-HCl, pH 7.8 and incubated at 60°C for 7min to inactivate endogenous proteases (Fromm *et al*, 85). 20ul of a 1:10 C^{14}-chloramphenicol : acetyl CoA mix was added and the tubes incubated at 37°C for 30-60min. Chloramphenicol and the mono and diacetoxy products were solubilized in 300ul of ethyl acetate, by vortexing at 4°C for 10-15sec. The products were extracted in a bench top microfuge at 14,000rpm for 2min, and 250-280ul of the organic phase dried down under vaccum by centrifugation at room temperature. Samples were taken up in 20ul of ethyl acetate and spotted, by repeated applications, at the origin of a thin layer chromatography sheet. Chromatograms were transferred to an equilibrated tank and run in chloroform/methanol (95:5 v/v) until the front was approximately 5cm from the top of the sheets. After removal from the tank chromatograms were air dried and subjected to autoradiography.

The percentage conversion of chloramphenicol was calculated directly by aligning the exposed X-ray film with the translucent chromatogram over a light source. Spots corresponding to chloramphenicol or to the aceylated

products were excised and transferred into separate scintillation vials for counting in 5ml Ecoscint.

II.D.5c B- Galactosidase Assay

pHSV-Bgal was kindly provided by J. O'Prey (Figure 2.2) and was used as an internal control to standardize for transfection efficiency in CEF cells. The levels of B-galactosidase activity were quantified by the catalytic conversion of colourless ONPG to yellow o-nitrophenol. Typically, 30-60ul of cell lysate was incubated with 0.5ml solution 1 and 0.1ml solution 2 (see below) at 37°C for 30-90min or until a yellow colour change was apparent. The reaction was stopped with 0.25ml 1M sodium carbonate and an absorbance reading taken at 420nm in a plastic cuvette with a path length of 1cm

Solution 1	Solution 2
60mM Na_2HPO_4	60mM Na_2HPO_4
40mM NaH_2PO_4	40mM NaH_2PO_4
10mM KCl	2mg/ml ONPG
1mM $MgCl_2$	
50mM B-mercaptoethanol	

II.D.5d Hirts Supernatants

Hirts Supernatants measure the concentration of plasmid DNA within a cell population and were used as a control to standardize for transfection efficiency between different cell types. Briefly, cell pellets were resuspended in 200ul TE buffer and mixed on ice for 5min with 200ul TSE (1% SDS, 5mM Tris-HCl, pH 8.0, 10mM EDTA). 100ul of 3M potassium

acetate, pH 4.8 was mixed in gently and the tubes incubated for a further 10min on ice. Chromosomal DNA precipitates were collected in a bench top microfuge at 14,000rpm for 10min. The supernatants were extracted 3 times in phenol/chloroform and precipitated with 0.3M sodium acetate and 2.5 volumes of ethanol. Precipitates were recovered by centrifugation and resuspended in 36ul TE buffer. The DNA was digested with an approprate enzyme and analysed by Southern Blotting (section II.B.8).

II.D.6. PROTEIN:DNA INTERACTIONS

II.D.6a Footprinting Analysis

(i) Labelling Plasmid for Footprinting

Footprinting probes were prepared by 5' end labelling of DNA restriction fragments with T4 polynucleotide kinase and (γ-^{32}P) ATP, and isolated by secondary restriction according to Plumb *et al.*

Labelled fragments were resolved on a non-denaturing 8% acrylamide/TBE gel in sample buffer (10x TBE, 20% glycerol). Gels were exposed wet and the released fragment located by aligning the exposed X-ray film. Bands were excised and eluted as described in section II.B.4a. Precipitates were resuspended in 100ul storage buffer (50mM NaCl, 20mM Hepes, pH 7.9, 5mM $MgCl_2$, 0.1mM EDTA, 20% glycerol, 1mM DTT, 0.1mM EGTA, 0.1mM $CaCl_2$), and assayed for TCA precipitable activity as outlined above in section II.C.4c.

(ii) DNase 1 Footprint Protection Assays

DNase 1 footprint protection assays were performed in 100ul storage buffer (see above), in the presence of 6ug poly(dI-dC):(dI-dC), 1ul end-

labelled restriction fragment and up to 80ul (30ug) nuclear protein extract (section II.D.1a). DNA binding reactions were incubated on ice for 60-90min and then partially digested with DNAse 1 (20-200ug/ml in storage buffer) at room temperature for 15-30sec. Digestions were terminated in 100ul STOP buffer for 30min at 37^{o}C and proteinase K then inactivated at 90^{o}C for 2min. Nucleic acid was extracted sequentially in phenol (x1) and chloroform (x2) and precipitated in 10ul 5M LiCl and 600ul ethanol. Precipitates were washed in 95% ice cold ethanol, air dried, and resuspended in 10ul loading buffer (95% Formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF). 2-5ul of each sample was denatured at 100^{o}C for 3-5min and resolved on a 6% denaturing acrylamide gel (section II.C.3) with markers prepared from double stranded sequencing reactions of the same DNA.

STOP buffer - 1.5ml

1.3ml 100mM Tris-HCl, pH 8.0 / 10mM EDTA

70ul 10% SDS

60ul Proteinase K (10mg/ml)

15ul yeast tRNA (10mg/ml)

38ul 4M NaCl

II.D.6b Electrophoretic Mobility Shift Assay

(i) Preparation of Double Stranded Oligonucleotide Probes

5ug of paired single stranded oligonucleotides were diluted in annealing buffer (67mM Tris-HCl, pH 7.3, 13mM $MgCl_2$, 6.7mM DTT, 1.3mM Spermidine, 1.3mM EDTA) to a final volume of 100ul. The annealing reaction was heated for 2min at 100^{o}C and then cooled slowly to room temperature. 200ng of double stranded oligonucleotide was end-labelled

as described in section II.B.5b, and separated from unincorporated nucleotides using a Biorad Biospin 30 column according to the manufacturer's instructions. 2ul was assayed for TCA precipitable activity as outlined in section II.C.3.

(ii) Gel Mobility Shift Assay

Solutions

10x Binding Buffer	4% Acrylamide Gel
100mM Hepes, pH 8.0	8ml 30% Acrylamide/bis
2mM EDTA	(19:1 seq. grade)
0.94M NaCl	6ml 10X TBE buffer
1mM PMSF	46ul distilled water
1mg/ml BSA	500ulAmmonium persulphate
40% Glycerol	50ul TEMED

Binding reactions were performed in a volume of 30ul distilled water in the presence of 5-10ug whole cell extract (section II.D.1b), 1x binding buffer, 0.5ug poly(dI-dC):(dI-dC), 5mM DTT, and 2ul end labelled oligonucleotide ($2x10^4$ cpm). Reactions were incubated on ice for 30min and resolved at 4^oC on a 4% non-denaturing acrylamide gel in TBE running buffer for 2-3hrs at 150V. Samples were loaded between two marker tracks (0.05% bromophenol blue and Xylene Cyanol FF in 2% glycerol/TBE) to monitor the electrophoresis. Gels were fixed for 15-30 min in 10% methanol, 10% acetic acid in water, and dried, before autoradiography at -70^oC.
Modifications to this procedure were made for competition and supershift analysis. Cold binding reactions were assembled with 1-2ul of antibody or 50ng of a specific cold competitor in a final volume of 28ul. After

incubation on ice for 30min, 2ul end labelled oligonucleotide ($2x10^4$ cpm) was added and the samples incubated for a further 30min before electrophoresis as described above.

CHAPTER II.E. - MATERIALS

II.E.1. ANTISERUM

Rabbit anti-Fra-2 (PEP-2)	NBS Biologicals Hatfielf, UK.
Donkey anti-rabbit IgG, alkaline phosphatase conjugate	Promega Southampton, UK.
Rabbit anti-murine Jun B Rabbit anti-murine Jun D	A gift of Dr. R Bravo
Rabbit anti-human RSRF-C4	A gift of Dr. R. Treisman
Goat anti-rabbit FITC	Europath Ltd. Bude, Cornwall, UK.
Rabbit anti-TrpE-v-Jun (CASTOR)	A gift of Dr T Oehler Inst.fur Genetik und Tox. Postfach 3640 7500 Karlsruhe, Germany.

Rabbit anti-c-Fos	Laboratory stocks (Dr. D.A.F. Gillespie and Dr. A. Darling)
Rabbit anti-gag 5202	A gift of Dr R.N Eisenman FHCRC, Seattle Wa. 98104, USA
Rabbit anti-bzip c-Jun 948/4	Laboratory stocks (Dr. D.A.F. Gillespie)
Rabbit anti-avian c-Jun 730/5	Laboratory stocks (A. Kilbey)
Normal rabbit serum	Laboratory stocks

II.E.2. BACTERIAL HOSTS

E. coli host strains DH1 and WL87 were obtained from laboratory stocks held by Dr D. Crouch (DH1) and V. Fincham (WL87). E. coli DH5α was obtained as a competant strain from Life Technology Industries, Paisley, Scotland.

II.E.3. CELLS

CEF were prepared as described from White cross Brown Leghorn chicken embryos supplied by Wickham Laboratories, Hants., UK.

pLys S cells, used for the expression preparation of T7 expressed c-Jun (section II.D.2a), were a gift from F.W. Studier and held as laboratory stocks by Dr E. Black.

II.E.4. RABBITS

New Zealand White Rabbits supplied by Harlin Olac UK. Ltd., were used for immunization and subsequent polyclonal antisera preparation.

II.E.5. CHEMICALS

Supplier - Amersham International PLC., Aylesbury, UK.

Amplify
(α-^{32}P)dCTP 3000Ci/mmlo
(α-^{32}P)CTP 800Ci/mmol
(α-^{32}P)UTP 800Ci/mmol
(γ-^{32}P)ATP 3000Ci/mmol
^{14}C Chloramphenicol 50-62 mCi/mmol
^{14}C protein molecular weight markers
ECL Western Blot immunodetection reagents

Supplier- BDH Chemicals Ltd., Poole, Dorset, UK.

All organic solvents (AnalaR grade) were obtained from BDH unless otherwise stated. The following compounds were also obtained from BDH (AnalaR, Biochemical or Electran grades):

Acrylamide
Ammonium acetate
Ammonium persulphate
Ammonium sulphate
Aqueous ammonia
bis-Acrylamide
B-mercaptoethanol
Calcium chloride
D-glucose
Disodium $HP0_4.7H_20$
Disodium $HP0_4.2H_20$
DMSO
EDTA
Glycerol
Glycine
Hydrochloric acid
Lithium chloride
Magnesium acetate
Magnesium chloride
Magnesium sulphate
Maltose
$NaH_2P0_4.2H_20$
PEG 6000
Potassium acetate
Potassium chloride
Sodium acetate
Sodium carbonate-anhydrous
Sodium chloride
Sodium citrate
SDS
Sodium fluoride
Sodium hydroxide
Sodium orthvanadate
Tris
Xylene cyanol

Supplier - BioRad Laboratories, Hemel Hempstead, UK.

TEMED

Supplier - Boehringer Mannheim UK Ltd., Lewes, East Sussex.

Caesium chloride
Calf intestinal alkaline phosohatase
DOTAP transfection reagent
Klenow fragment E.coli DNA polymerase
100mM Nucleotide triphoshates (ATP, CTP, GTP, UTP)
Proteinase K

RNase A

Supplier - James Burrough Ltd., Witham, Essex, UK.

Ethanol

Supplier - Flow Laboratories, UK.

Vitrogen 100 purified collagen

Supplier - CP Laboratories, Bishop's Stortford, Herts, UK.

Sheared salmon sperm DNA

Supplier - Difco Laboratories.

BactoAgar

Supplier - Imperial Laboratories (Eur.) Ltd., West Portway, Hants, UK.

Vitrogen 100-Collagen

Supplier - Koch Light Ltd., Haverhill, Suffolk, UK.

Isoamyl alcohol

Supplier - Life Technology Industries, Paisley Scotland.

All DNA modifying enzymes and appropriate buffer concentrates were obtained from Life Technology Industries unless otherwise stated. The following reagents were also obtained from Life Technology Industries.

Agarose (ultrapure grade)

Bacteriophage λ (HIII digested)

Bacteriophage

10x DMEM concentrate

DNA ladder (1kb)

DNase 1 (RNase free)

Foetal calf serum

Folic acid (USP grade)

G418 (powder)

200mM Glutamine

1M HEPES

Low melting point agarose

MEM 100x vitamins

Newborn calf serum

Okadaic acid

2x MEM concentrate, phenol red free

Prestained protein molecular weight markers

7.5% sodium bicarbonate

100mM sodium pyruvate

Sucrose (enzyme grade)

Trypsin

Tryptose phosphate broth

Urea

Supplier - National Diagnostics, Manville, New Jersey, USA.

Ecoscint A

Supplier - New England Nuclear, Dupont UK Ltd., Stevenage.

^{35}S-methionine

Supplier - Pharmacia Ltd., Milton Keynes, Bucks, UK.

T4 polynucleotide kinase

Yeast tRNA

Supplier - Pierce and Warriner UK Ltd., Chester, UK.

2mg/ml albumin standard

Coomassie protein reagent

Supplier - Promega, Southhampton UK.

RNasin (40,000U/ml)

SP6 RNA polymerase (20,000U/ml)

T7 RNA Polymerase (20,000U/ml)

Rabbit reticulocyte lysate - methionine

Supplier - Rathburn Chemicals Ltd., Walkerburn, Scotland.

Phenol (water saturated)

Supplier - Safeway supermarket - Glasgow.

Non-fat milk powder ("Marvel")

Supplier - Severn Biotech. Ltd., Kidderminster, UK.

30% w/v Acrylamide - bis acrylamide (19:1)

RNAzol

Supplier - Sigma Chemical Co., Poole, Dorset, UK.

Acetyl co-A

Activated charcoal

Ampicillin

Aprotinin

Freunds incomplete adjuvant

HEPES

IPTG

Leupeptin

B-glycerophosphate

B-oestradiol

Benzamidine

Bestatin

Brij 35 (30% w/v)

Bromophenol Blue

BSA (fraction V)

Calf Thymus DNA

Coomassie Brilliant Blue

Creatine phosphokinase

Cycloheximide

DABCO

Denhardt's

DTT

EGTA

Emetine

Ethidium bromide

Formamide

Freunds complete adjuvant

ONPG

Paraformaldehyde

Pepstatin A

Phosphocreatine

PIPES

PMSF

Poly(dI-dC):(dI-dC)

Polybrene

Poly-L-Lysine

Potassium glutamate

Protein A Sepharose

Potassium glutamate

RNase T1

Sodium butyrate

Spermidine

Triton X-100

Tween 20

II.E.6. KITS

Supplier - Bio 101 Inc., Stratech Scientific, Luton UK.

Geneclean kit

Supplier - Boehringer Mannheim, Lewes UK.

Random Primed DNA Labelling kit

Supplier - Cruachem Ltd., West of Scotland Science Park, Glasgow.

Oligonucleotide Purification Cartridges and reagents

Supplier - Pharmacia LKB Biotechnologies, Milton Keynes, UK.

Oligolabelling kit

Supplier - Stratagene, La Jolla, CA, USA.

Gigapack II Plus Packaging Extract

United States Biochemical, Cleveland, Ohio, USA.

Sequenase version 2.0 kit

II.E.7. COLUMNS, MEMBRANES, PAPER, TLC PLATES AND X-RAY FILM

Supplier - Ammersham International PLC, Aylesbury, UK.

Hybond ECL nitrocellulose membrane

Hybond N(fp) nylon membrane

Hyper film - MP

Supplier - Anderman Co. Ltd., Kingston-upon-Thames, England.

Schleicher and Schuell nitrocellulose membrane

Supplier - Biorad Laboratories, Hemel Hempstead, UK.

Biospin 30 columns

Supplier - CAMLAB., Cambridge, UK.

20x20 Polygram Silica Gel TLC plates

Supplier - Eastman Kodak Co., Rochester, NY, USA.

Duplicating film (DUP-1)

X-ray film (XAR-5)

Supplier - Fuji Photo Film Co. Ltd., Japan.

X-ray film (RX)

Supplier - Life Technology Industries, Paisley, Scotland.

Dialysis tubing

Supplier - Pharmacia LKB Biotechnologies, Milton Keynes UK.

Nick Columns

Supplier - Sartorius GmbH., Epsom, Surrey, UK.

Dialysis bags

Supplier - Whatman International Ltd., Maidstone, UK.

GF-C filters

3MM chromatography paper

II.D.8. PLASMIDS AND BACTERIOPHAGES

Vector	Reference	Source
pSPT19		Boehringer Mannheim UK Ltd.Lewes, East Sussex.
pSPT19/ c-fos		Dr. D.A.F. Gillespie Laboratory stocks

pSPT19/		Dr. M. Grove
JF4 (SP6)		Laboratory stocks
pSPT19/		Dr. D.A.F. Gillespie
JF11 (SP6)		Laboratory stocks
pSPT19/		Dr D.A.F. Gillespie
vJun (T7)		Laboratory stocks
pSPT19/	Dugaiczyk *et al*,	Dr. D.A.F. Gillespie
GAPDH (SP6)	1983. Biochem.	Laboratory stocks
	22(7): 1605-1613	
pSPT19/		A. Kilbey
1.5cJ (T7)-clone 5		Laboratory stocks
pSPT19/		A. Kilbey
cJ400 (T7)		Laboratory stocks
pSPT19/		A. Kilbey
cJfp (T7)		Laboratory stocks
pSPT19/		A. Kilbey
c-jun (SP6)-clone A		Laboratory stocks
pSPT19/		A. Kilbey
cjun (T7)-clone K		Laboratory stocks
pSPT19/		A. Kilbey
cJ3' (T7)-clone I		Laboratory stocks

pSPT19/ Δ69 cJun (T7)		Dr D.A.F. Gillespie Laboratory stocks
pSPT19/ Δ69 vJun (T7)		Dr D.A.F. Gillespie Laboratory stocks
PAT	Twig & Sherratt (1980). Nature 283: 216-218	Dr E. Black Laboratory stocks
PAT / pB1		Dr D. Crouch Laboratory stocks
pCAT-Basic		Promega Southampton, UK.
pCATb-cJ-400		A. Kilbey Laboratory stocks
pUCBM21		Boehringer Mannheim UK Ltd. Lewes, E. Sussex.
pUCBM21/ cJ-400		A. Kilbey Laboratory stocks
pSFCV-LE (sa+)	Fuerstenberg *et al,* (1990). J. Virol 64(12): 5891-5902	Dr M. Zenke Inst. for Mol. Pathol. A-1030 Wien.

Δ69 PC/SFCV		Dr D.A.F. Gillespie Laboratory stocks
Δ69 PV/SFCV		Dr. D.A.F. Gillespie Laboratory stocks
RCAN **(subgroup A)**	Hughes *et al,* (1987). J. Virol 61(10): 3004-3012	Dr D. Crouch Laboratory stocks
pG5BCAT	Lillie & Green (1989). Nature 338: 33-44	Dr C. Goding Mairie Curie Res. Inst. London, UK.
pSG424	Sadowski & Ptashne (1989). NAR 17(18): 7537	Dr. Colin Goding (as above)
pSG424/ **Vmw65**	Cousens *et al* , (1989). EMBO 8(8): 2337-2342	Dr Colin Goding (as above)
pRc/RSV		Invitrogen Corp. San Diego, CA. USA.
pRC/RSV / **CJ-3**		Dr I. Morgan Cell Tech, Slough, UK.
pRc/RSV / **VJ-0**		Dr I. Morgan (as above)

pRc/RSV /		Dr. I. Morgan
VJ-1		(as above)
pHSV-B Gal	Hall *et al* ,(1983)	Dr D.J. Chester
	J-Mol-Appl-Genet 2(1)	Laboratory stocks
	101-1 09	
$\phi\lambda$1174		V.Fincham
		Laboratory stocks

II.E.9. RESEARCH SUPPLIES, MISCELLANEOUS

Supplier - Beatson Institute Central Services.

L-broth

Penicillin

Sterile CT buffer	NaCl	6.0g
	trisodium citrate	2.96g
	tricine	1.79g
	phenol red	0.005g
	distilled water	700ml
	pH	7.8

Sterile distilled water

Sterile PBS

Streptomycin

II.E.10. TISSUE CULTURE AND BACTERIOLOGICAL PLASTICWARE

Supplier - Alpha Labs. Eastleigh. Hamps.. UK.

eppendorf tubes

Supplier - A/S Nunc. Roskilde. Denmark.

T25, 75, and 175 tissue culture flasks

1 and 2ml Nunc cryotubes

Supplier - Becton Dickinson Labware. Oxford. UK.

60 and 90mm tissue culture dishes

Tissue culture roller bottles

50ml falcon tubes

12ml Polypropylene falcon 2059 tubes

19G and 21G sterile syringe needles

200ml sterile filtration unit

Supplier - Bibby Sterilin Ltd.. Stone. Staffs.. UK.

60 and 90mm bacteriological dishes

Sterile plastic bijoux containers

Sterile plastic universal containers

Supplier - Costar Corporation. High Wycombe. Bucks.. UK.

24 well tissue culture dish

Supplier - Dupont UK Ltd.. Stevenage Herts.. UK.

Polycarbonate ultracentrifuge tubes

Supplier - Gelman Sciences. Northampton. UK.

Sterile 0.2um filter Acrodisc

II.E.11. Water

Distilled water for the preparation of buffer stocks was obtained from a Millipore MilliRO 15 system. Water for protein, enzyme, and recombinantDNA procedures was further purified on a Millipore MilliQ system to18M/cm

CHAPTER III - RESULTS (1).

CHAPTER III - RESULTS

ANALYSIS OF c-JUN EXPRESSION AND AUTO-REPRESSOR ACTIVITY

III.A. *c-jun* EXPRESSION

III.A.1. TRANSCRIPTIONAL REGULATION

- Total Cellular *c-jun* mRNA

c-jun has been described as an immediate early gene. To investigate whether the kinetics of avian *c-jun* expression conform to that of other immediate early genes, subconfluent cultures of primary CEFs were serum deprived for 48hrs, and subsequently fed with serum-containing medium over an 8hr period (section II.A.1.). Total cellular RNA was prepared at different time intervals and the amount of *jun*-specific transcript determined by RNase protection analysis using a *jun*-specific riboprobe (section II.C.3.). A GAPDH-specific riboprobe was used in parallel RNase protection assays to control for loading (Figure 3.1B).

As shown in Figure 3.1A, *c-jun* mRNA was rapidly and transiently induced in response to serum growth factors. *jun*-specific transcripts were barely detectable in serum deprived cultures, but increased significantly after 30mins serum stimulation (lanes 3-4). The levels remained elevated for up to 60mins serum stimulation (lane 5) and decreased thereafter (lanes 6, 8 & 9). The decline was gradual relative to the rapid induction, with *c-jun*-

FIGURE 3.1A

RNase Protection analysis of total mRNA prepared from serum stimulated and exponentially growing CEFs. 0.1mM Emetine was included in the growth medium of cultures represented by tracks 6, 12 & 13. 20ug of total RNA was hybridized to 10^6 cpm of labelled antisense riboprobe. The riboprobe was synthesiszed from Bgl11 linearized pSPT19/JF4 (SP6) (Figure 2.1), and includes approximately 150bp of c-jun specific 3' untranslated sequence. The broken arrow denotes the undigested 150bp antisense c-jun riboprobe. Complete digestion of the non-hybridized probe is illustrated in lane 2. The solid arrow indicates the protected 75bp c-jun mRNA fragment.

	3	4	5	6	7	8	9	10	11	12	13
Serum (hrs)	-	0.5	1	2	2	4	8	+	+	+	2.0
Emetine (0.1mM)	-	-	-	-	+	-	-	-	1hr	3hr	-
Confluent	+	+	+	+	+	+	+	-	-	-	+
Exponential	-	-	-	-	-	-	-	+	+	+	-

FIGURE 3.1B

RNase Protection analysis of 2ug of RNA samples 3-11 hybridized against a 481bp chicken GAPDH anti-sense riboprobe. The riboprobe was prepared from Apa1 linearized pSPT19/GAPDH (SP6) (Figure 5.3). The 431bp protected fragment is indicated by a solid arrow. A 0.01x dilution of the input riboprobe is denoted by a broken arrow.

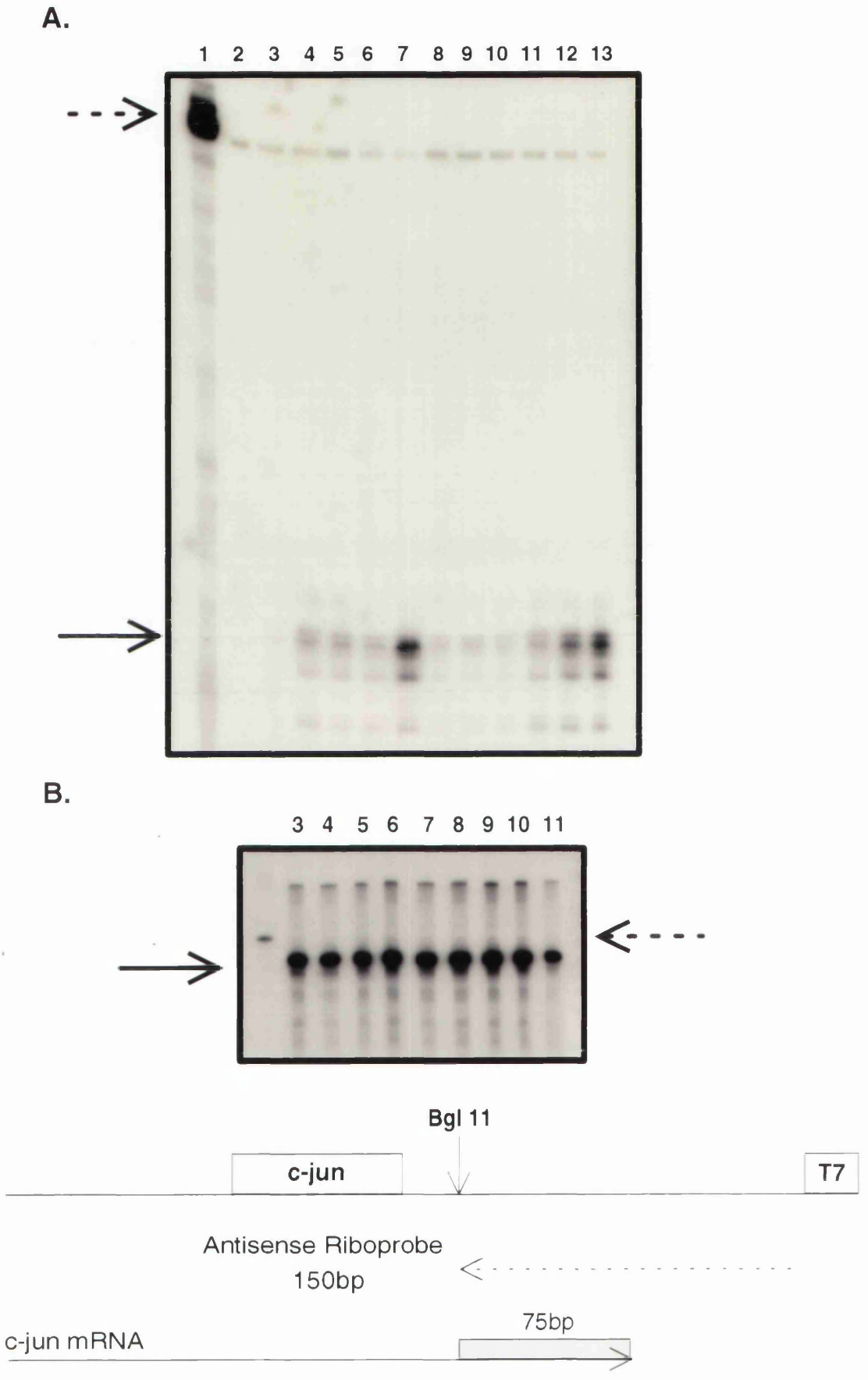

A.
1 2 3 4 5 6 7 8 9 10 11 12 13
B.
3 4 5 6 7 8 9 10 11
Bgl 11
c-jun
T7
Antisense Riboprobe
150bp
75bp
c-jun mRNA

FIGURE 3.1

specific transcripts persisting above background for at least 8hrs (compare lane 9 with lanes 3 & 10). This pattern of expression was disrupted in the presence of a protein synthesis inhibitor. Addition of 0.1mM Emetine to the 2hr serum-induced cultures dramatically increased the levels of *c-jun* mRNA (compare lanes 6 & 7). Superinduction was similarly observed in exponential CEF cultures, grown in the presence of 0.1mM Emetine. Under these conditions, *jun*-specific transcript levels increased proportionally with the period of Emetine incubation (compare lanes 10-12).

Rapid and transient kinetics of expression, together with superinduction in the presence of protein synthesis inhibitors, are typical of other immediate early genes and clearly characterize *c-jun* as a member of this class of transciption factors.

- de-novo Transcription

The levels of total cellular mRNA are determined by transcriptional and post-transcriptional regulatory mechanisms. To examine the contribution of de novo transcription to the serum induction of *c-jun* mRNA, transcription of the *c-jun* gene was measured in treated and untreated cells by the nuclear run off assay (section II.C.4.). *jun*-specific transcripts were detected using a plasmid encoding the *v-jun* gene; positive and negative controls were provided by GAPDH and pSPT19 sequences respectively (Figure 5.3).

As shown in Figure 3.2, *c-jun* transcription was detected at low levels in serum deprived CEFs. A rapid, but modest, increase in transcription was observed in response to serum stimulation. The increase was insufficient to account for the corresponding induction of *c-jun* mRNA, and peaked at an earlier time point following serum stimulation (compare Figure 3.2 &

FIGURE 3.2.

Runoff Analysis of nuclei prepared from serum deprived and serum stimulated CEF cultures. Approximately equal amounts of TCA precipitable radioactivity were included in each hybridization reaction. Plasmid DNAs were alkali denatured and immobilized onto nitrocellulose filters using a Bio-Slot Microfiltration apperatus. 5ug of plasmid DNA was immobilized per slot, and the filters fixed under vacuum for 2hrs at 80^{o}C. Hybridization proceeded for 72hrs in a final volume of 1.8ml. The filters were washed under high stringency and exposed overnight at -70^{o}C.
Negative and positive controls were provided by pSPT19 and pGAPDH respectively. pSPT19/v-Jun and pSPT19/c-Fos were included to compare the rate of c-jun and c-fos transcription at the point of cell lysis.
Details of each plasmid are outlined in Figure 5.3

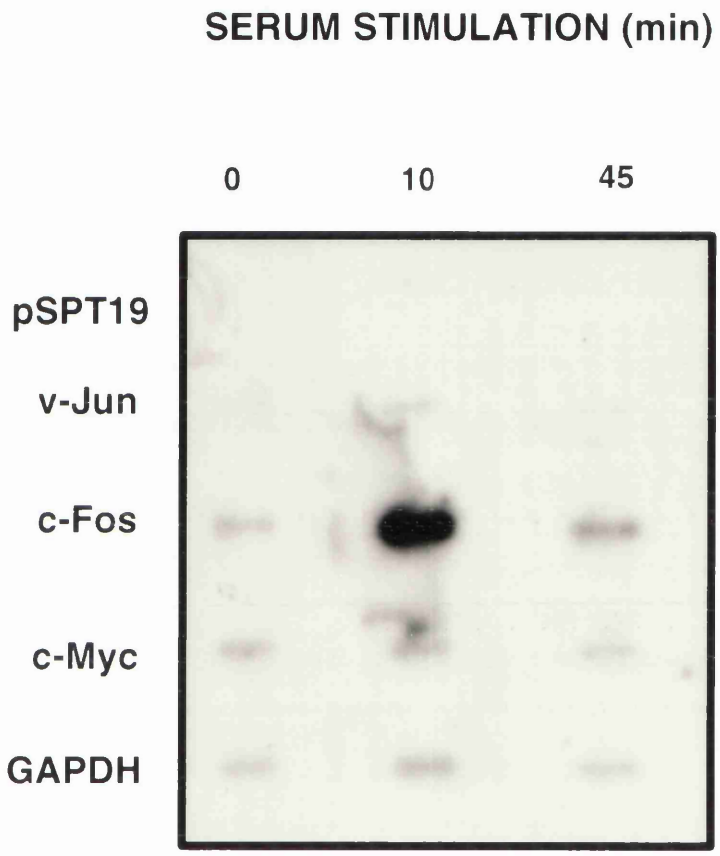


FIGURE 3.2

3.1). The decrease in *c-jun* transcription was similarly rapid. Basal levels of transcription were observed just 45mins after serum stimulation, when *c-jun* mRNA was persisting at maximum levels.

The kinetics of *c-jun* expression paralleled those of another immediate early gene, *c-myc*, but were quite distinct from the rapid and dramatic kinetics observed during the serum induction of *c-fos* (Figure 3.2).

III.A.2. TRANSLATIONAL AND POST-TRANSLATIONAL REGULATION

- Translational Regulation

To examine the relationship between *c-jun* transcription and c-Jun translation, serum deprived cultures of primary CEFs were serum stimulated over a 6hr period, and lysed under denaturing conditions for western blot analysis. Whole cell protein extracts were resolved on 9% SDS-acrylamide gels, and p39 c-Jun visualized using two independent c-Jun-specific polyclonal antisera. 730/5 was raised against bacterially expressed c-Jun protein (section II.D.2); CASTOR was raised against a 75kD trpE-vJun fusion protein and recognizes epitopes within both v-Jun and human and avian c-Jun (Angel *et al*, 88(i); Dr. T. Oehler; *personal commun.*).

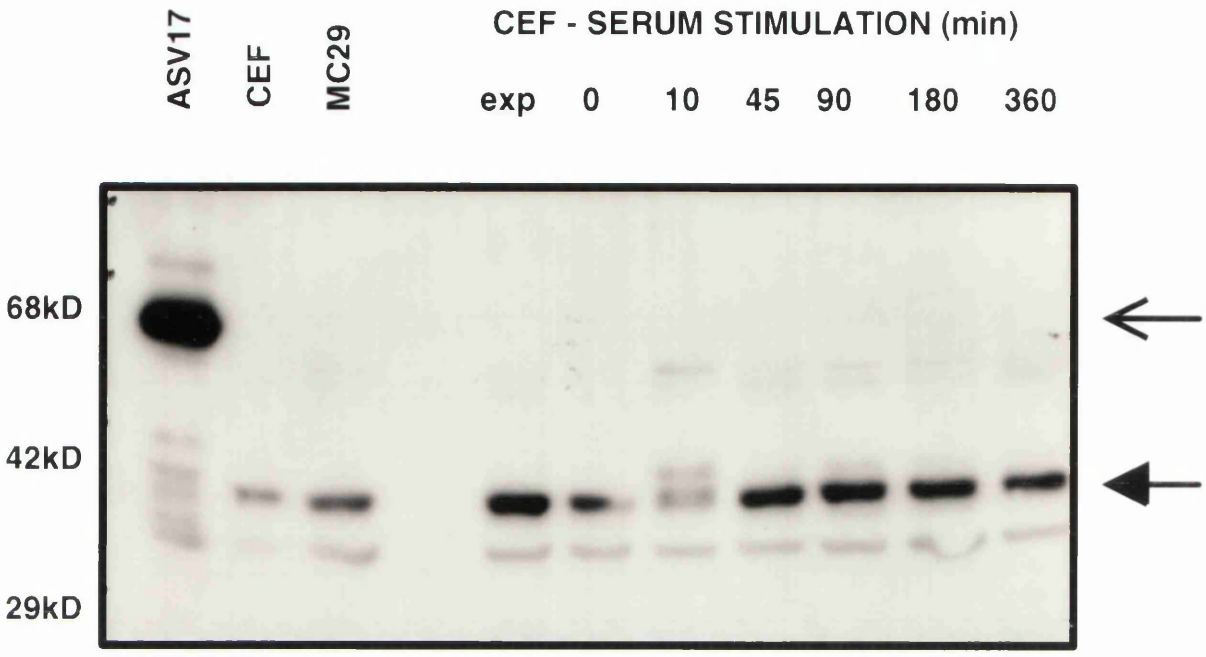
Figure 3.3A and 3.3B represent parallel western blots probed for p39 c-Jun expression with CASTOR (3A) or 730/5 (3B). As shown in Figure 3.3A, p39 c-Jun was expressed at high levels in serum deprived CEF cultures, exhibiting virtually no increase in expression in response to serum stimulation. The CASTOR antibody expresses a higher affinity for p39 c-Jun than 730/5 (compare "exp" lanes in Figure 3.3A and 3.3B).

FIGURE 3.3

Western Blot analysis of denatured whole cell protein extracts, prepared from serum deprived and serum stimulated CEF cultures. Approximately equivalent concentrations of protein were resolved on a 9% SDS/acrylamide gel and electroblotted onto ECL nylon membrane using a CAM LAB Semi-Dry Blotter. The proteins were visualized by ECL detection, with a 1:2000 dilution of CASTOR (3.3A) or a 1:4000 dilution of 730/5 (3.3B). Normal CEF extracts and ASV17 (v-Jun) and MC29 (v-Myc) transformed CEF extracts were included as controls. p65 gag v-Jun is denoted by a light arrowhead; and p39 c-Jun by a solid arrowhead.

Antibody specificities are outlined in figure legend 3.16.

A.


ASV17
CEF
MC29
CEF - SERUM STIMULATION (min)
exp
0
10
45
90
180
360
68kD
42kD
29kD

B.

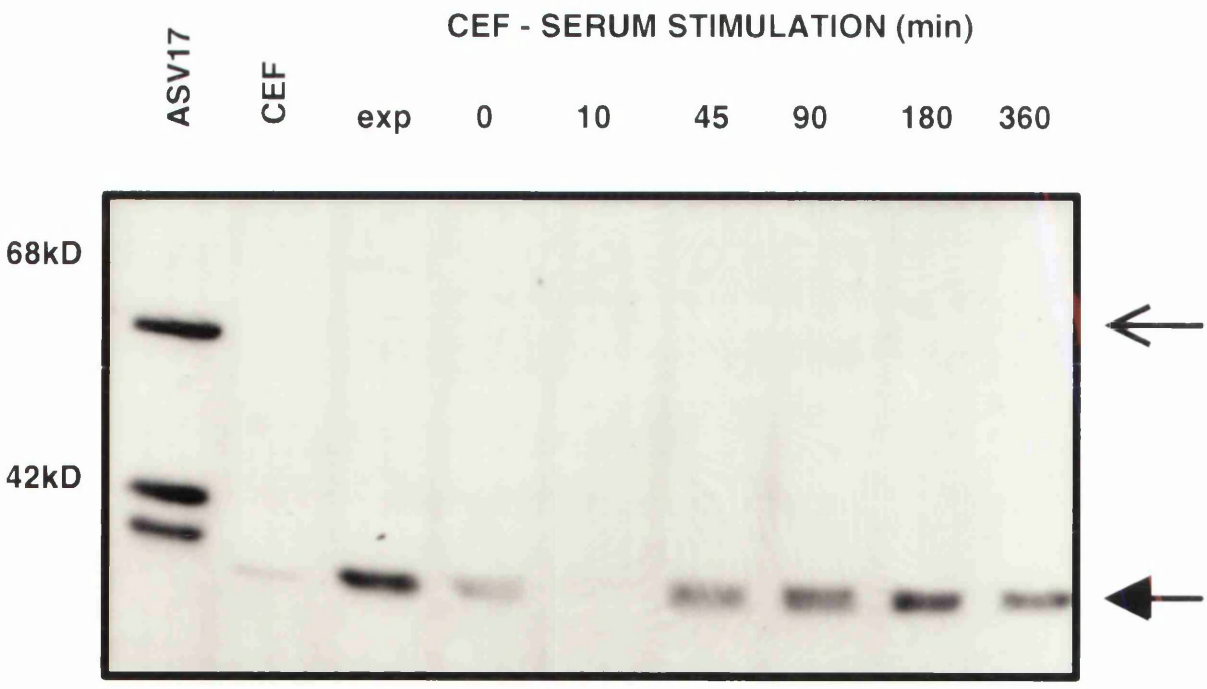

ASV17
CEF
CEF - SERUM STIMULATION (min)
exp
0
10
45
90
180
360
68kD
42kD

FIGURE 3.3

Consequently, saturating concentrations of CASTOR may mask subtle changes in c-Jun expression. In support of this hypothesis, modest increases in p39 c-Jun were detected using 730/5, with maximal expression detected approximately 3hrs after serum stimulation (Figure 3.3B).

The serum-induced expression of p39 c-Jun never exceeded the levels detected in normal exponentially growing cultures, and peaked at late time points after serum stimulation (Figure 3.3A and 3.3B). The magnitude and kinetics of the translational response contrasted with the corresponding induction of *c-jun* mRNA (Figure 3.1A). *c-jun* mRNA exhibited the most dramatic changes in expression in response to serum stimulation, with maximum levels detected within just 30mins (compare lanes 3 & 4 with lanes 3 & 10). At late time points after serum stimulation, when c-Jun protein levels were at their peak, *c-jun*-specific transcripts were in decline, owing to the rapid and transient nature of the transcriptional reponse (compare Figure 3.3B & 3.1A).

- Post-translational Modification

At very early time points after serum stimulation, a ladder of additional bands was rapidly and transiently detected by the CASTOR antibody (Figure 3:3A). The ladder was not detected by 730/5, suggesting that it represented alternative c-Jun-reactive proteins that transiently appeared at the expense of endogenous p39 c-Jun (compare Figure 3.3A & 3.3B).

It has been proposed that post-translational modifications to pre-existing proteins initiate the rapid transcriptional response of immediate early genes (Reviewed in Angel *et al*, 91). To investigate whether the additional c-Jun-reactive proteins were the products of post-translational modifications, the analysis was repeated in the presence of a protein

synthesis inhibitor. As shown in Figure 3.4, the c-Jun-reactive ladder was not abolished in the presence of 10ug/ml cycloheximide, and therefore, did not represent newly synthesized Jun proteins. Infact addition of cycloheximide accentuated the appearance of the ladder, shifting it in favour of the higher molecular weight species. This was particularly apparent at later time points after serum stimulation when the c-Jun-reactive ladder had collapsed (lane 5). Under these conditions, addition of cycloheximide induced a reappearance of the c-Jun-specific bands that mimicked their initial rapid detection in response to serum stimulation (compare lanes 6 & 3).

III.B. AUTO-REPRESSION

III.B.1. v-JUN-DEPENDENT AUTO-REPRESSION

Expression studies identified *c-jun* as an immediate early gene (section III.A.). One characteristic of several immediate early transcription factors is an ability to negatively regulate their own gene promoters (Grignani *et al*, 90; Sassoni-Corsi *et al*, 88). To investigate whether Jun exhibits an equivalent auto-repressor function, endogenous c-Jun protein levels were examined in primary CEFs infected with the ASV17 transforming retrovirus. ASV17 expresses high levels of the retrovirally transduced v-Jun oncoprotein (Bos *et al*, 90; Maki *et al*, 87). v-Jun is expressed as a 65kD gag-Jun fusion protein and is, therefore, readily distinguishable from p39 c-Jun by molecular weight (Figure 1.1).

FIGURE 3.4

Western Blot analysis of denatured whole cell protein extracts, prepared from serum deprived and serum stimulated CEF cultures. Approximately equal concentrations of protein were resolved on a 9% SDS/acrylamide gel and transferred to ECL nylon membrane using a CAM LAB Semi-Dry Blotter. The proteins were visualized, by ECL detection, with a 1:2000 dilution of CASTOR. Parallel dishes were serum stimulated in the presence of 10ug/ml cycloheximide (Linial 86) and analysed as above. Normal and ASV17 transformed cell extracts were included as controls. p65 gag-v-Jun is denoted by a solid arrowhead and p39 c-Jun by a light arrowhead.

LANE	1	2	3	4	5	6	7
SERUM (mins)	0	5	10	10	30	30	45
CHX (10ug/ml)	-	-	-	+	-	+	-

Antibody specificities are outlined in figure legend 3.16.

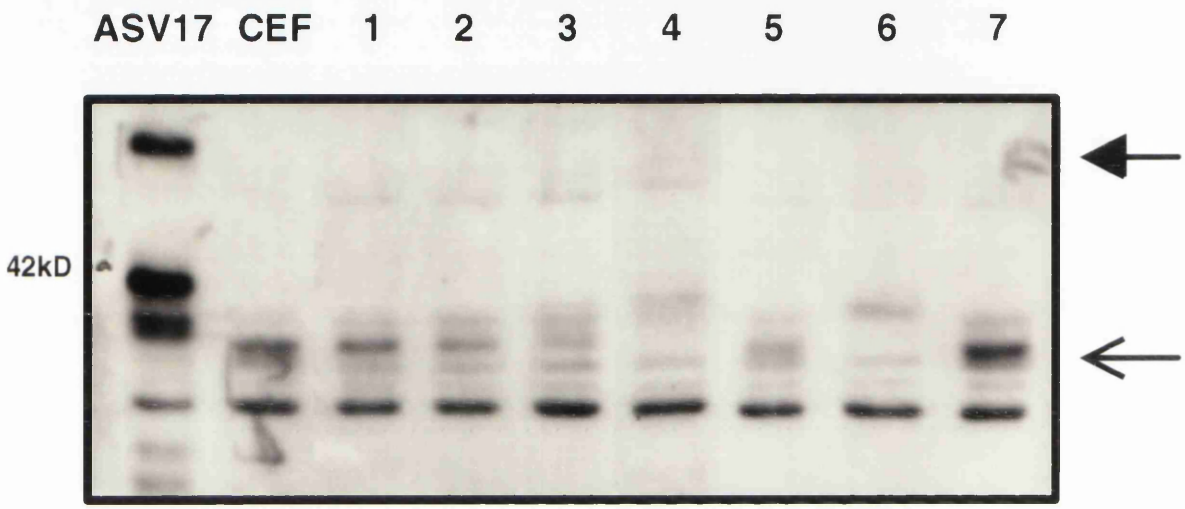


FIGURE 3.4

As shown in Figure 3:5, endogenous c-Jun protein was virtually undetectable in the presence of p65 gag-v-Jun. Wild type levels of endogenous c-Jun were expressed by equivalent v-Myc transformed cultures, suggesting that the down-regulation represented a specific effect of the v-Jun oncoprotein rather than a secondary consequence of cellular transformation.

III.B.2. c-JUN-DEPENDENT AUTO-REPRESSION

p65 gag-v-Jun was created through a non-homologous recombination event between retroviral gag sequences and the endogenous *c-jun* gene. The resulting gene is expressed at high levels from the retroviral LTR, and contains a number of structural mutations in the coding and non-coding sequences (Figure 1.1). To investigate whether repression of endogenous c-Jun represents a direct effect of the qualitative mutations in v-Jun, retroviral vectors were constructed to facilitate equivalent overexpression of c-Jun and v-Jun proteins (Figure 3.6). Specifically, gag-*c-jun* and gag-*v-jun* fusion sequences were created by PCR and inserted into the replication defective, drug-selectable, avian retrovirus SFCV-LE (Fuerstenberg *et al*, 90). The partial gag sequences were included to induce a size difference between exogenous and endogenous Jun proteins that would be evident on SDS/acrylamide gels.

III.B.2a Physical Properties of gag-c-Jun and gag-v-Jun Fusion Proteins

To confirm the predicted molecular weights of the gag-c-Jun and gag-v-Jun fusion proteins the DNA segments created by PCR were subcloned

FIGURE 3.5

Western Blot analysis of denatured whole cell protein extracts, prepared from normal and transformed CEF cultures. Transformed cell extracts were prepared from v-Jun (ASV17) and c-Myc (RCAN-c-Myc) infected chicken fibroblasts. Approximately equal concentrations of protein were resolved on a 9% SDS/acrylamide gel and transferred onto ECL nylon membrane using a CAM LAB Semi-Dry Blotter. The proteins were visualized by ECL detection, with a 1:4000 dilution of 730/5. p65 gag-v-Jun is denoted by a solid arrowhead and p39 c-Jun by a light arrowhead.

Antibody specificities are outlined in figure legend 3.16

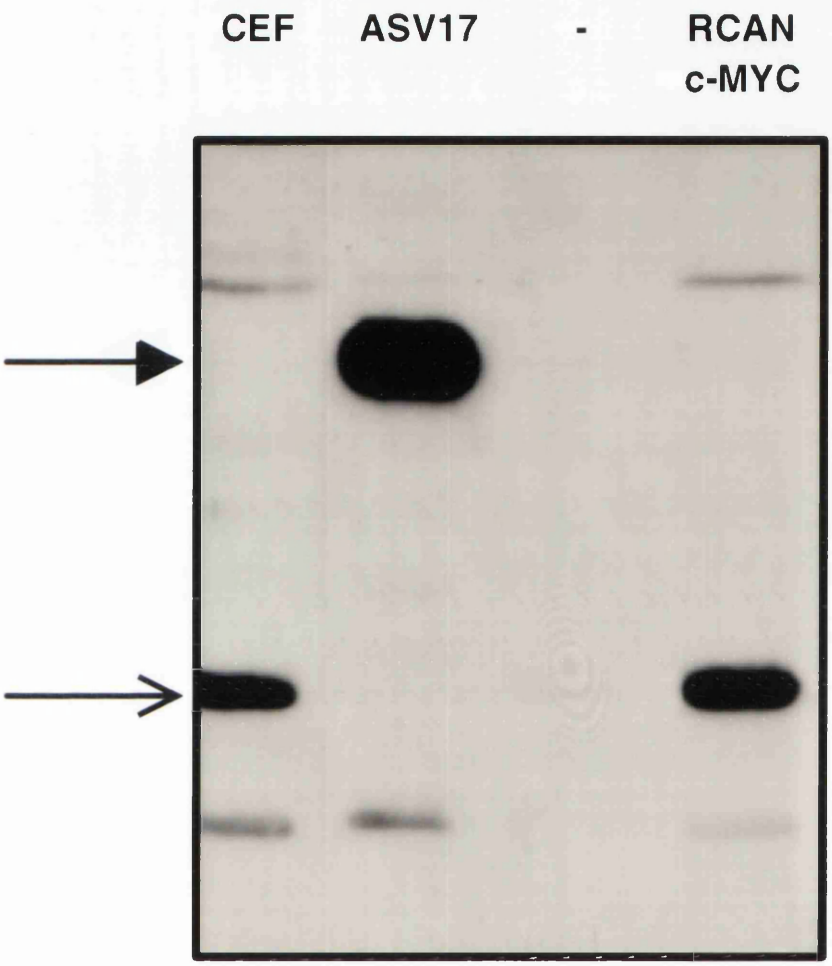


FIGURE 3.5

FIGURE 3.6

PC and PV/SFCV contain partial gag-c-jun (PC) or gag-v-Jun (PV) sequences amplified from the PC and PV retroviral vectors (constructed by D.A.F. Gillespie). PCR primer "a" was designed against a specific retroviral gag sequence, and tagged with a 5' HindIII restriction endonuclease site and an adjacent ATG initiation codon contained within a Kozak consensus sequence. The antisense PCR primer "b" was specific for c-jun 3' coding sequences, and was tagged with an antisense EcoRI restriction endonuclease site. PC and PV HindIII - EcoRI fragments encompass 67 amino acids of partial gag p19 and p10 sequences, fused in frame to the full length c-jun or v-jun coding regions respectively. The amplified product was cloned into the avian replication defective retroviral vector, pSFCV-LE (Fuerstenberg et al, 1990), at the appropriate restriction endonuclease sites. c-Jun or v-Jun sequences are denoted by a shaded box; and partial retroviral gag sequences by a hatched box. The splice donor (SD) and splice acceptor (SA) sites are identified as shown and the spliced transcript indicated by a dotted arrowhead. Translation of the gag-c-Jun and v-Jun sequences is directed from the internal ATG inserted in PCR primer "a".

For invitro transcription and sequencing analysis, the HindIII - EcoRI gag-c/v-jun fragment was subcloned directly from pSFCV-LE into the polylinker of the invitro transcription vector, pSPT19. The orientation of the HindIII and EcoRI restriction sites, within the pSPT19 polylinker, directs sense transcription from the T7 RNA polymerase promoter (Figure 5.3).

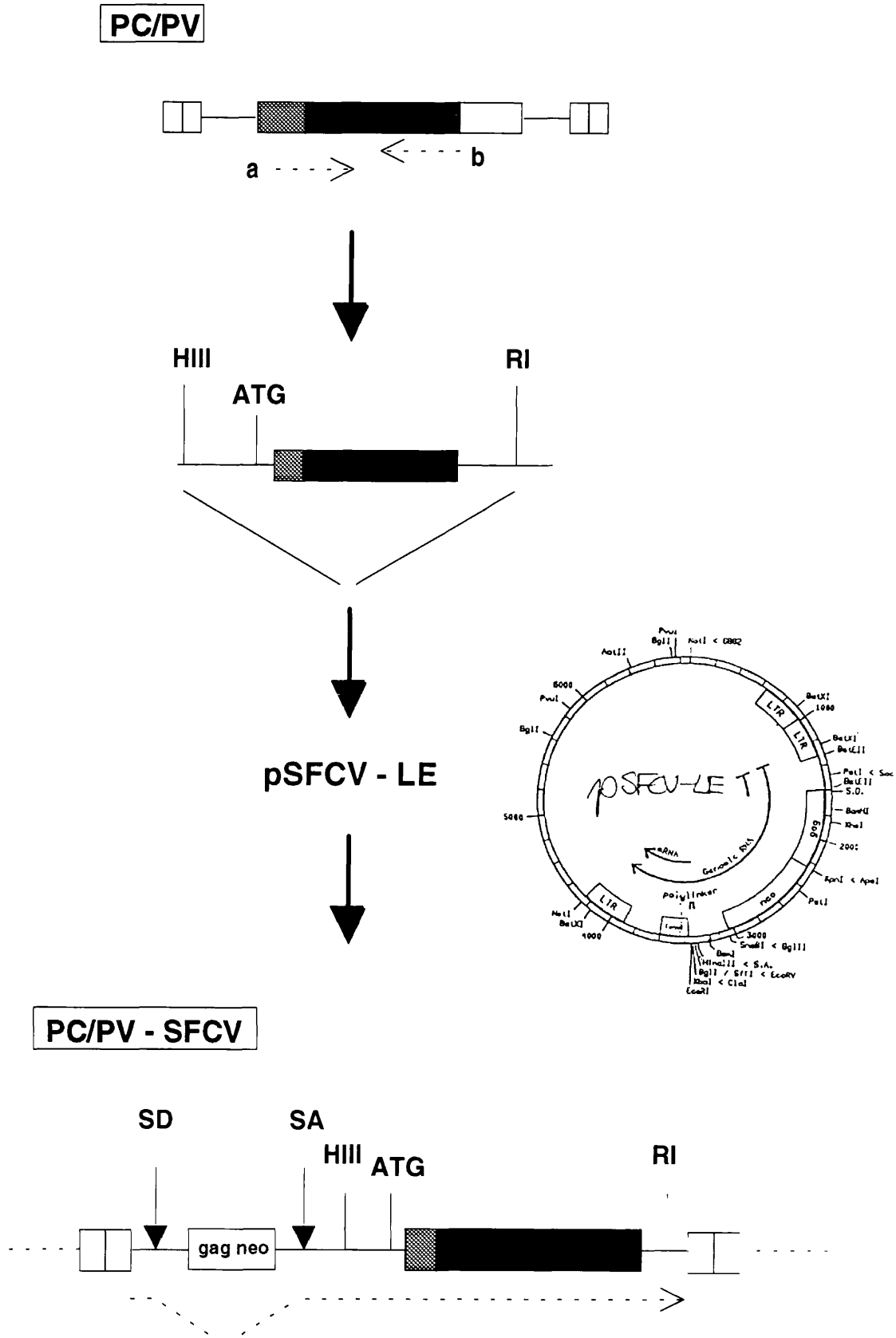


FIGURE 3.6

from SFCV into plasmid pSPT19 for in vitro translation analysis (see Figure 3.6). As shown in Figure 3.7A, c-Jun and v-Jun were expressed as 47kD and 44kD proteins respectively, in the presence of reticulocyte lysate. The additional 8kD corresponds to the predicted molecular weight of the 67aa gag sequence fused in frame to both full length proteins (Figure 3.6). A ladder of retarded bands was observed for the gag-c-Jun fusion protein. This was reminiscent of the electrophoretic mobility shift reported for in vitro translated c-Jun (but not v-Jun), that was shown to result from phosphorylations of c-Jun in reticulocyte extract (Black *et al*, 91).

To determine whether the gag-Jun fusion proteins behaved normally with respect to dimerization, the DNA segments were cotranslated in the presence of c-Fos, and the products immunoprecipitated with anti-Jun- (730/5) or anti-Fos- (388/4) specific antisera. 730/5 reacts specifically with c-Jun and v-Jun proteins, whereas 388/4 recognizes epitopes specific to c-Fos and Fos-Related Antigens. In accordance with this 703/5 immunoprecipitated the gag-Jun fusion proteins (Figure 3.7B: compare lanes 2 & 3 and lane 1), whereas 388/4 specifically immunoprecipitated c-Fos (Figure 3.7C: compare lane 1 and lanes 2 & 3). 388/4 also precipitated co-translated gag-c-Jun (lane 5) and gag-v-Jun (lane 4), indicating that heterodimerization had occured under these conditions. The extended ladder of c-Jun-specific in vitro translated proteins was not immunoprecipitated by 388/4, suggesting that c-Fos heterodimerizes with only a proportion of modified c-Jun proteins (compare lanes 5 in Figure 3.7A and 3.7C). The converse immunoprecipitation, in the presence of 730/5, was less conclusive as p55 c-Fos migrates as a weak heterogenous band and is, consequently, not readily detectable in c-Jun/c-Fos co-translations (Figure 3.7A: lanes 1, 4 & 5).

FIGURE 3.7

In vitro translations and cotranslations of p47 gag-c-Jun,(blue arrowhead) p44 gag-v-Jun (black arrowhead) and p55 c-Fos (green arrowhead).
Full length jun and fos sequences were subcloned, in the antisense orientation, into plasmid pSPT19 for in vitro transcription and subsequent in vitro translation (Figures 3.6 & 5.3). In vitro transcripts were synthesized from the T7 RNA polymerase promoter encoded by pSPT19.

	1	**2**	**3**	**4**	**5**
Plasmid sequences:					
c-Fos (p55)	+	-	-	+	+
gag-cJ (p47)	-	-	+	-	+
gag-vJ (p44)	-	+	-	+	-

In vitro translations and cotranslations were performed in a final volume of 50ul. 5ul of each reaction was removed prior to immunoprecipitation and the translated products resolved on a 9% acrylamide/SDS gel (3.7A). The remaining volume was equally divided, and immunoprecipitated against 5ul 730/5 (3.7B) or 5ul 388/4 (3.7C). p47 gag-c-Jun is denoted by a blue arrowhead; p44 gag-v-Jun by a black arrowhead and p55 c-Fos by a green arrowhead.

Antibody specificities are outlined in figure legend 3.16.

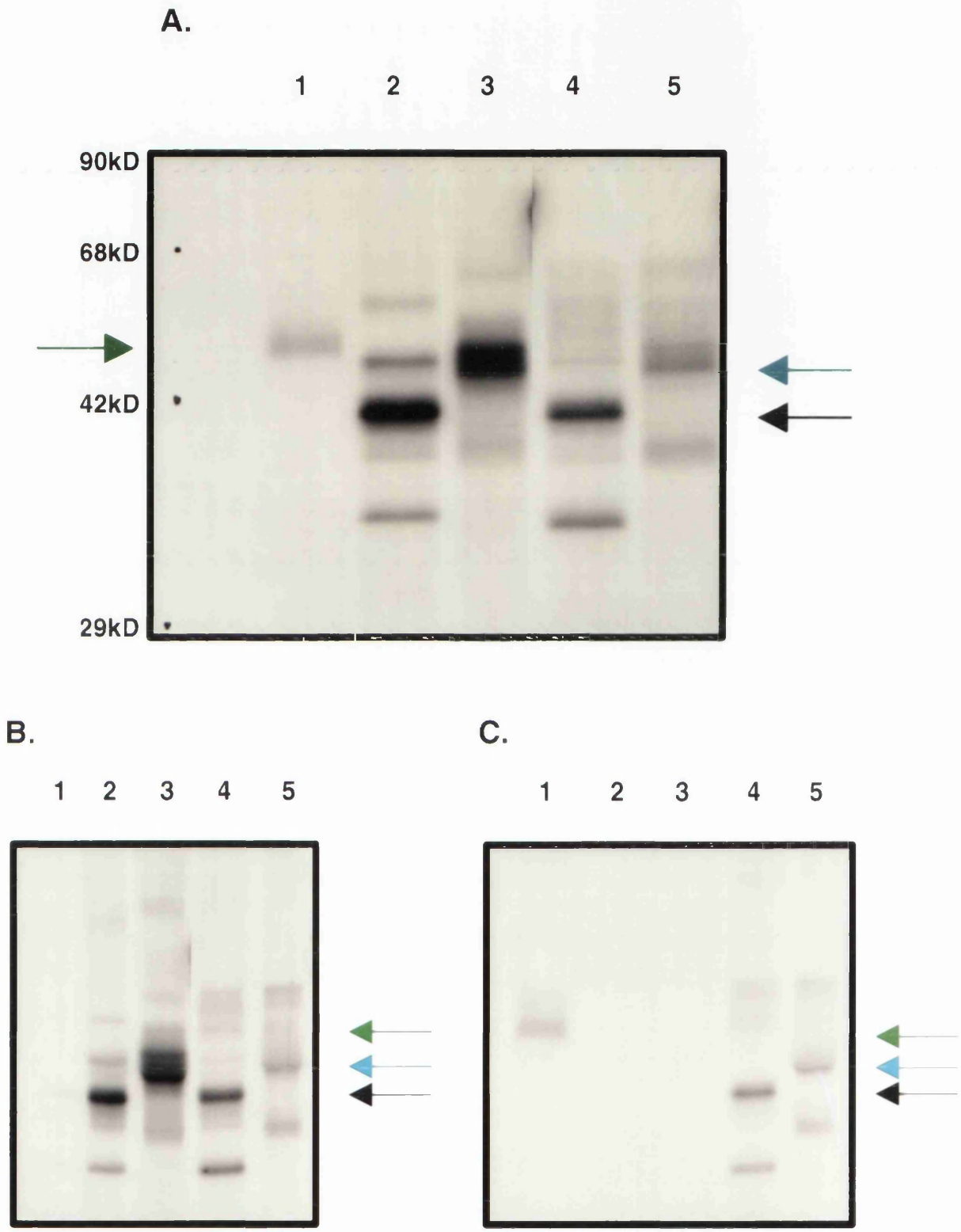


FIGURE 3.7

These results confirmed that proteins of the predicted molecular weight were encoded by the gag-jun fusion genes, and that both fusion proteins behaved normally with respect to in vitro heterodimerization with c-Fos.

III.B.2b Biological Effects of c-Jun and v-Jun Fusion Proteins

To investigate and compare the biological consequences of high c-Jun and v-Jun expression, primary CEFs were co-transfected with PC or PV/SFCV and RCAN DNA, and incubated in the presence of G418 to select for uniformly-infected cultures (section II.A.3). G418 resistant cultures were maintained in normal growth medium to examine their growth rate and cellular morphology; and lysed under denaturing conditions to quantifiy the levels of exogenous c-Jun or v-Jun expression.

- Cellular Growth Rate

The growth rate of PC and PV/SFCV transfectants was measured under normal growth conditions, over six consecutive days. As shown in Figure 3.8, PV/SFCV-transfected and ASV17-transformed cells grew more rapidly than non-transfected or vector-transfected (SFCV) CEFs. PC/SFCV-transfected CEFs grew at an intermediate rate between the fully transformed ASV17-infected cultures and the normal CEF controls.

- Cellular Morphology

The cellular morphology of PV and PC/SFCV transfected CEFs was quite distinct (Figure 3.9). PV/SFCV transfected cultures exhibited a transformed morphology typical of ASV17-transformed CEFs. The cells were elongated and refractile, and at high densities piled up in swirling

FIGURE 3.8

Growth curves of PC and PV/SFCV CEF transfectants relative to normal, vector transfected and ASV17 transformed CEF controls. Growth was measured in normal growth medium and cell numbers correspond to the average value of two T25 tissue culture flasks.

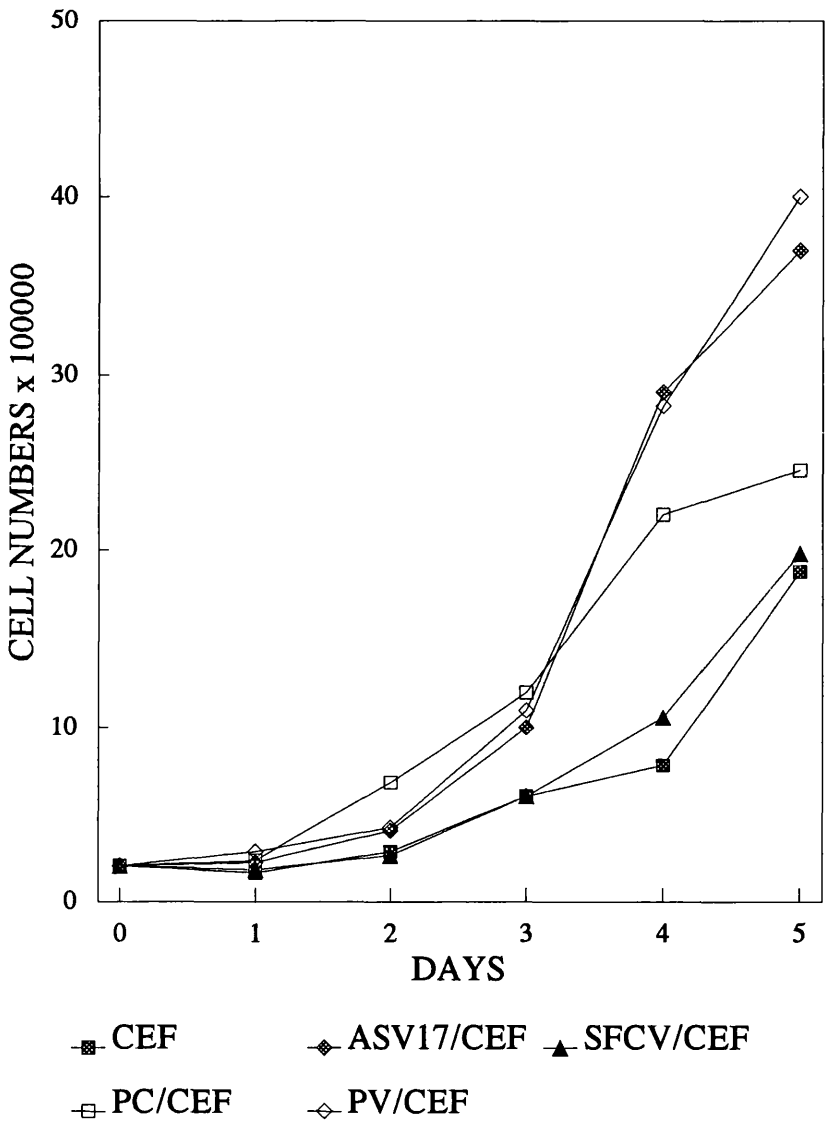
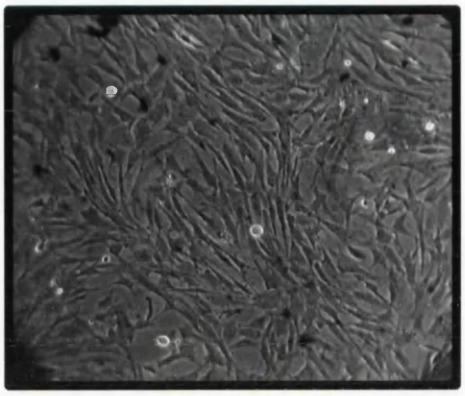

50
40
30
20
10
0
CELL NUMBERS x 100000
0
1
2
3
4
5
DAYS
CEF
ASV17/CEF
SFCV/CEF
PC/CEF
PV/CEF

FIGURE 3.8

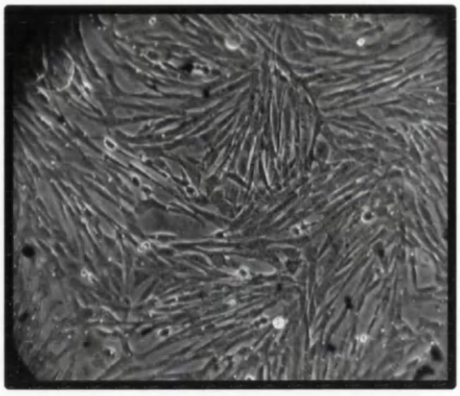
FIGURE 3.9

Morphology of PC and PV/SFCV transfected primary CEFs. A bold arrowhead denotes the epithelioid component of PC/SFCV transfected cultures. Normal and ASV17 transformed primary CEFs were included as controls. All cultures were examined at an equivalent passage.

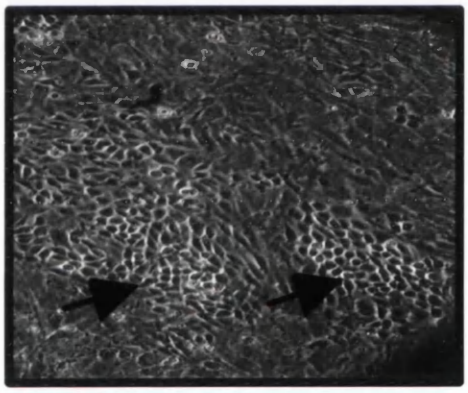
CEF / SFCV - CEF



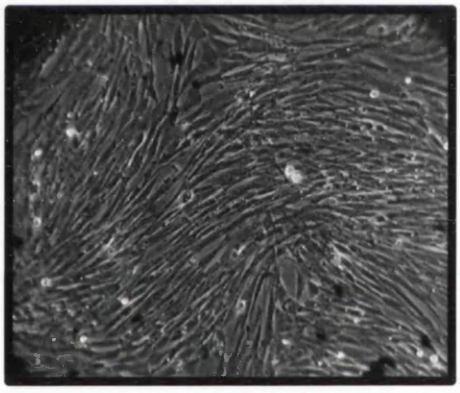
ASV17 - CEF



PC/SFCV - CEF



PV/SFCV - CEF



PC/SFCV - CEF

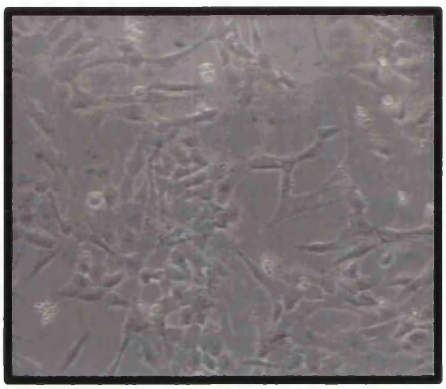


FIGURE 3.9

patterns. PC/SFCV transfected cultures exhibited a unique morphology that was distinct from normal (CEF/SFCV) and p65 gag-v-Jun-transformed (ASV17-CEF) CEFs. The newly selected cultures contained a mixture of fibroblastic and epithelioid-like-cells. With continued passage, the epithelioid-like-cells assumed a greater proportion of the culture and exhibited a tendency to slough off into the growth medium at high densities. Floating cells could be transferred into low serum-containing medium, and sustained under these conditions for several passages (Figure 3.9 - lower panel).

- Exogenous Jun Protein Expression

The expression and subcellular localization of the exogenous Jun proteins was examined by indirect immunofluorescence staining, using an antibody specific for c-Jun and v-Jun proteins (730/5). The staining pattern of PC and PV/SFCV transfected CEFs was compared to that of normal and ASV17-transformed CEF cultures.

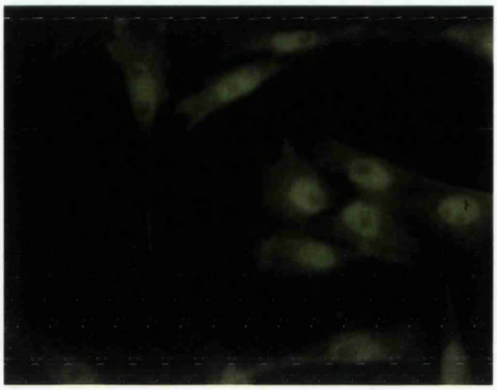
As shown in Figure 3.10, exogenous and endogenous Jun proteins were predominantly localized to the nucleus. This confirmed their function as nuclear transcription factors, and demonstrated that nuclear translocation of c-Jun and v-Jun was not disrupted by retroviral gag sequences. The intensity of nuclear staining was approximately comparable between PC and PV/SFCV transfected cultures, and a culture of ASV17-transformed CEFs. A control culture of normal CEFs exhibited a markedly reduced intensity of nuclear staining. The pattern of immunological staining was specific to c-Jun and v-Jun proteins as parallel reactions using the flouresceine-linked secondary antibody alone exhibited little or no background fluorescence (Figure 3.10 - lower panel).

FIGURE 3.10

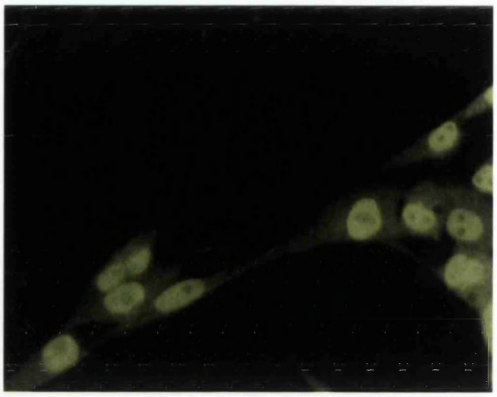
Indirect Immunfluorescence staining of PC and PV/SFCV transfected CEFs. Transfected monolayers were fixed in paraformaldehyde and incubated at room temperature with a 1:100 dilution of 730/5. c-Jun and v-Jun specific staining was visualized using a 1:100 dilution of GARFITC. Normal and ASV17 transformed CEFs were included in the analysis as controls. Non-transfected CEFs were incubated with GARFITC alone to control for non-specific immunofluorescence staining.

Antibody specificities are outlined in figure legend 3.16.

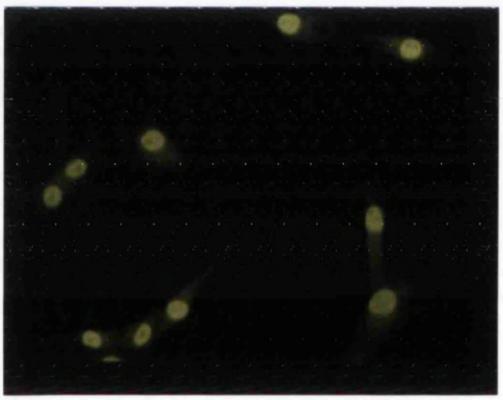
CEF



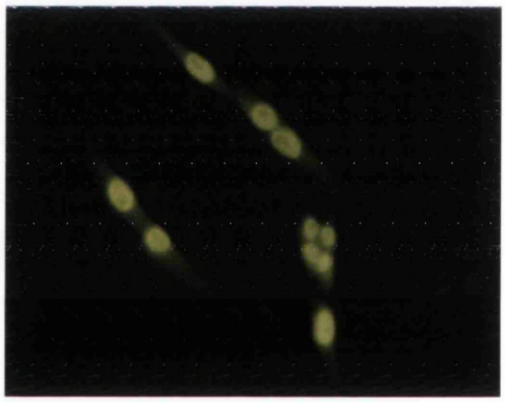
ASV17/CEF



PC/SFCV



PV/SFCV



CEF - GAR/FITC

FIGURE 3.10

These results demonstrated that exogenous c-Jun and v-Jun were expressed to equivalent levels in PC and PV/SFCV transfected CEFs, and that the levels of expression paralleled that of p65 gag-v-Jun in ASV17-transformed CEFs and exceeded that of endogenous c-Jun in normal CEFs.

To quantitate the levels of gag-c-Jun and gag-v-Jun expression more accurately, PC and PV/SFCV transfected cultures were lysed under denaturing conditions, and the whole cell protein extracts examined by western blot analysis. As shown in Figure 3.11A, endogenous c-Jun was expressed in normal and SFCV transfected cultures but was completely absent from ASV17-transformed CEFs. This confirmed previous observations (Figure 3.5), and demonstrated that the SFCV vector sequences alone did not affect endogenous c-Jun protein expression.

PC and PV/SFCV CEF extracts exhibited low, but detectable, levels of endogenous c-Jun. The levels may reflect the persistence of normal CEFs within the low passage PC and PV/SFCV transfected cultures, and/or demonstrate an inability of the gag-Jun fusion proteins to down regulate endogenous c-Jun expression. High levels of exogenous Jun-reactive proteins were also detected in the PC and PV/SFCV transfected cultures. Candidate gag-Jun fusion proteins of the predicted molecular weight (Figure 3.7) represented a small proportion of the total pool of Jun-reactive proteins (Figure 3.11A). The p44 and p47 Jun-reactive species were not recognized by a gag-specific antisera (Figure 3.11B), suggesting that they represented bona-fide protein products of the retrovirally inserted partial gag-*v-jun* and gag-*c-jun* sequences.

The vast majority of Jun-reactive proteins detected in PC and PV/SFCV transfected CEF extracts migrated with molecular weights of approximately 68kD and 65kD respectively. p68 and p65 were specific to PC and

FIGURE 3.11

Western Blot analysis of denatured whole cell protein extracts, prepared from PC and PV/SFCV transfected primary CEFs. Approximately equal concentrations of protein were resolved on a 9% SDS/acrylamide gel and transferred to ECL nylon membrane using a CAM LAB Semi-Dry Blotter. The proteins were visualized by ECL detection with a 1:4000 dilution of 730/5 (3.11A), or a 1:10000 dilution of 5202 (3.11B). Normal and ASV17 transformed cultures were included as controls, together with an extract prepared from SCFV-vector transfected CEFs alone. p68 gag-c-Jun is denoted by a broken arrowhead; p65 gag-v-Jun by a solid arrowhead; and p39 c-Jun by a black arrowhead. Candidate proteins corresponding to p44 gag-v-Jun and p47 gag-c-Jun are denoted by respective green and blue arrowheads, in Figure 3.11A. Gag reactive proteins pr76, p27 and p30 gag neo are denoted, in Figure 3.11B, by red, blue and green arrowheads respectively (11B). The ~ p42 gag-v-Jun proteolytic fragment is denoted by a **"b"** on Figure 3.11A.

Antibody specificities are outlined in figure legend 3.16

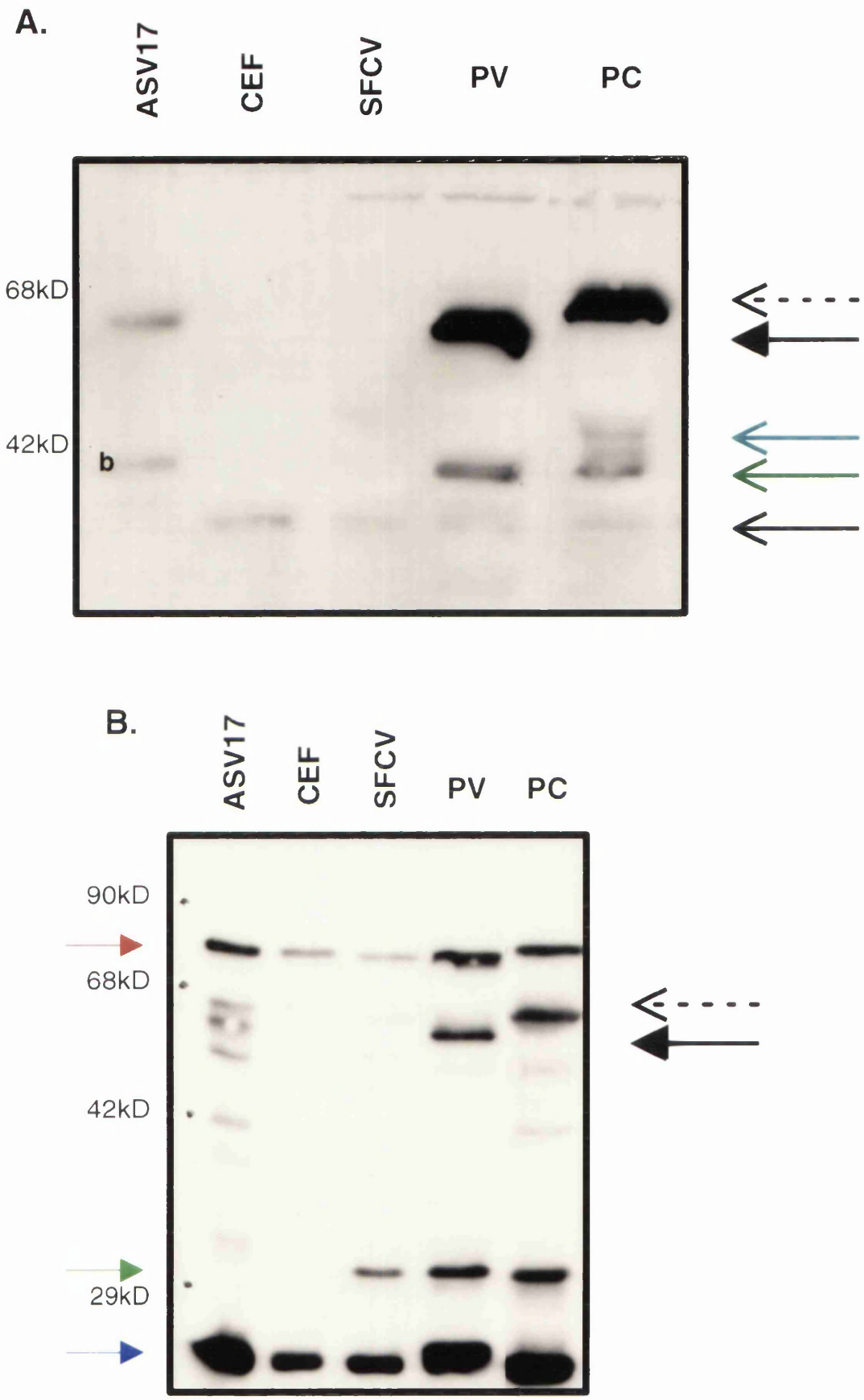

A.
ASV17
CEF
SFCV
PV
PC
68kD
42kD
b
B.
ASV17
CEF
SFCV
PV
PC
90kD
68kD
42kD
29kD

FIGURE 3.11

PV/SFCV transfected cultures (Figure 3.11A) and were recognized by anti-Jun (730/5) and anti-gag (5202) specific antisera (Figure 3.11A & 3.11B). This, together with their similarity in size to the v-Jun oncoprotein, suggested that p65 and p68 were the products of a homologous gag recombination that mimicked the non-homologous gag-jun recombination responsible for the generation of p65 gag-v-Jun (Maki *et al*, 87). c-Jun and v-Jun differ in size by 3kD due to a 27 amino acid deletion in the *v-jun* coding region (Figure 1.1). The 3kD size difference was maintained in the recombined gag-Jun fusion proteins, supporting the hypothesis that both were the consequence of an identical gene rearrangement. The gag sequences involved could have originated from endogenous retroviruses, the RCAN helper virus or gag sequences upstream of the neomycin resistance gene in SFCV (Figure 3.6). Evidence for endogenous retroviruses was provided by the identification of retroviral gag proteins in non-transfected normal CEF extracts (Figure 3.11B). The gag antiserum also recognized a 30kD protein, specific to SFCV transfected CEFs. p30 corresponds to the predicted molecular weight of the gag-neo protein responsible for conveying G418 resistance in SFCV transfected cells (Figure 3.6). The G418 resistance of PC and PV/SFCV transfected CEFs suggested that recombination within the PC and PV/SFCV constructs was unlikely to represent the sole gene rearrangement, and emphasized the importance of alternative retroviral gag sequences in the overall generation of the gag-Jun recombinant proteins.

III.B.2c Transforming Effects of gag-c-Jun and gag-v-Jun Fusion Proteins

- Soft Agar Cloning Assays

The cellular morphology and growth rate of PV/SFCV transfected CEFs were indistinguishable from ASV17-transformed cell cultures. PC/SFCV transfectants, in contrast, exhibited an intermediate phenotype between normal and fully transformed CEFs (section III.B.2b). An alternative parameter of cellular transformation is the ability to grow in the absence of anchorage to a cellular or non-cellular substrate. Anchorage independent growth is measured by soft agar cloning analysis and provides a useful tool to separate the growth of normal and ASV17-transformed CEFs (Bos *et al*, 90). At suitable densities each ASV17-derived soft agar colony represents the descendants of a single p65 gag-v-Jun-infected cell. The absence of contaminating normal CEFs has made this an ideal system for examining the auto-repressor activity of the v-Jun oncoprotein. To investigate whether the soft agar cloning assay could be similarly used to study the gag-Jun fusion proteins, PC and PV/SFCV transfected CEFs were seeded into soft agar and their anchorage independent growth monitored over 2-3 weeks (section II.A.4b). As shown in Figure 3.12, PC and PV/SFCV transfected CEFs formed multiple rapidly growing colonies in soft agar. PV/SFCV derived colonies were indistinguishable from ASV17-transformed CEF colonies in both their morphology and frequency of appearance. PC/SFCV derived colonies, in contrast, appeared at a lower frequency, but otherwise resembled their PV/SFCV transfected counterparts. In accordance with previous observations, normal and vector-transfected CEFs failed to grow in soft agar (Bos *et al*, 90).

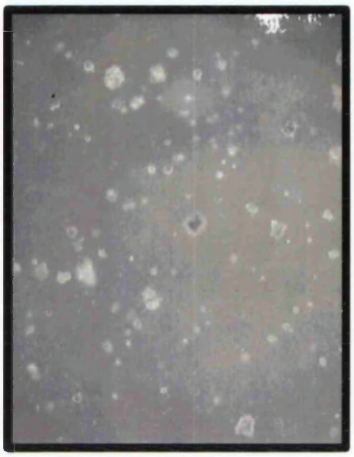
FIGURE 3.12

Soft Agar Cloning analysis of PC and PV/SFCV transfected primary CEFs. Control assays are illustrated in the upper panel. The cells were seeded into 0.36% top agar, at 10^3, 10^4 and 10^5 per 60mm dish. Cloning assays were supported on 0.76% base agar plates and fed every 2-3 days with 0.36% top agar. The dishes were incubated in a humidified 37°C incubator containing 5% CO_2 (v/v).
The colony numbers correspond to the average value of three 60mm dishes after 14 days incubation. The experiment was performed on three separate occasions. Individual soft agar colonies were picked and expanded 14 days after seeding.

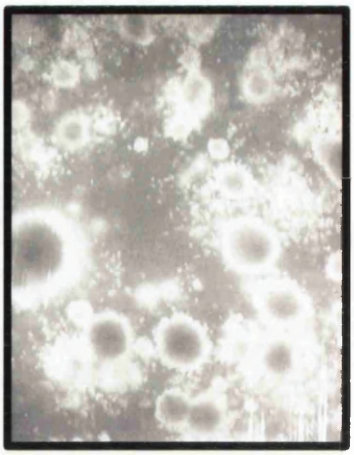
Cell Numbers x10^5	**Colonies per 60mm dish**		**Mean Colony Numbers**
CEF	-	-	-
ASV17 / CEF	207	205	230	214
SFCV/CEF	-	-	-	-
PC/SFCV	70	82	89	80
PV/SFCV	218	262	380	286

CEF

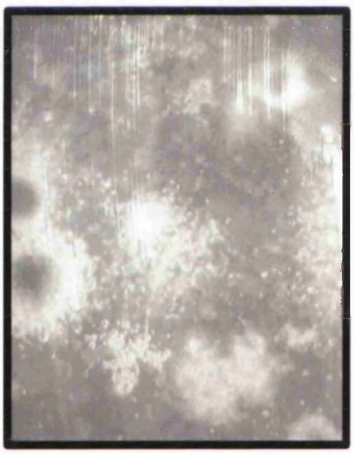
SFCV/CEF



ASV17/CEF



PC/CEF



PV/CEF

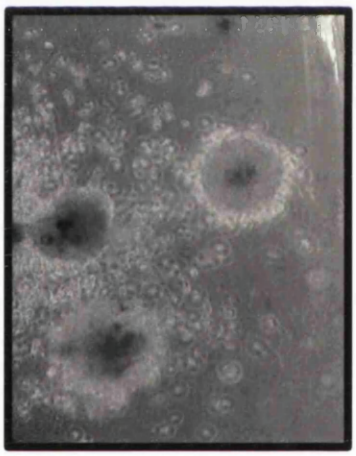


FIGURE 3.12

- Exogenous Jun Protein Expression in Primary Soft Agar Colonies

To investigate the levels of exogenous c-Jun and v-Jun expressed in PC and PV/SFCV derived soft agar colonies, individual clones were isolated and expanded for western blot analysis. As shown in Figure 3.13A, p68 gag-c-Jun and p65 gag-v-Jun were expressed at high and approximately equivalent levels in colonies derived from PC or PV/SFCV transfected CEFs. The levels of expression were comparable to the levels of p65 gag-v-Jun detected in equivalent ASV17 derived cultures (Figure 3:13A - right hand lane). Gag-Jun fusion proteins of the predicted molecular weight, were not detected, supporting the hypothesis that the homologous recombination was both stable and advantageous for cellular transformation.

Endogenous p39 c-Jun was absent in ASV17, PC/SFCV and PV/SFCV derived colonies, irrespective of the growth conditions analysed (Figure 3.13A). This observation led to the proposal that c-Jun down-regulation was a direct consequence of Jun overexpression and not dependent on qualitative mutations present within the v-Jun oncoprotein. To test this hypothesis further, virus particles were harvested from expanded primary soft agar colonies and titred onto exponential cultures of non-infected primary CEFs (section II.A.3b). The infected cultures were incubated in normal growth medium for 48hrs to permit retroviral integration and exogenous gag-Jun protein expression, and then lysed under denaturing conditions for western blot analysis. As shown in Figure 3.13B, the levels of exogenous and endogenous Jun proteins, in PC- and PV/SFCV-infected CEFs, were inversely proportional. Cells infected with the highest virus titre (1.0ml of an overnight harvest) expressed the lowest levels of endogenous p39 c-Jun, thereby supporting the conclusion that auto-

FIGURE 3.13A

Western Blot analysis of denatured whole cell protein extracts, prepared from expanded PC/SFCV, PV/SFCV and ASV17 infected soft agar colonies. Expanded colonies were grown under normal (exp) conditions, or serum stimulated for 45mins (45+), prior to cell lysis. Approximately equal concentrations of protein extract were resolved on a 9% SDS/acrylamide gel and transferred to ECL nylon membrane using a CAM LAB Semi-Dry blotter. The proteins were visualized by ECL detection with a 1:4000 dilution of 730/5. Normal and ASV17 transformed CEF extracts were included as controls. The p68 gag-c-Jun fusion protein is denoted by a broken arrowhead; the p65 gag-v-Jun fusion protein by a solid arrowhead; and p39 c-Jun by a light arrowhead. The protein extract corresponding to an expanded ASV17 infected soft agar colony is shown in the far right hand lane.

Antibody specificities are outlined in figure legend 3.16

FIGURE 3.13B

Western Blot analysis of denatured whole cell extracts, prepared from primary CEFs 48hrs after infection with titred PC/SFCV or PV/SFCV virus particles.

	1	2	3
~ Virus Titre:			
1.0ml O/N culture	+	-	-
0.1ml O/N culture	-	+	-
1.0ml 4hr culture	-	-	+

Approximately equal concentrations of protein extract were resolved on a 9% SDS/acrylamide gel and transferred to ECL nylon membrane using a CAM LAB Semi-Dry Blotter. The proteins were visualized by ECL detection with a 1:4000 dilution of 730/5. p68 gag-c-Jun is denoted by a broken arrowhead; p65 gag-v-Jun by a solid arrowhead; and p39 c-Jun by a light arrowhead.

Antibody specificities are outlined in figure legend 3.16.

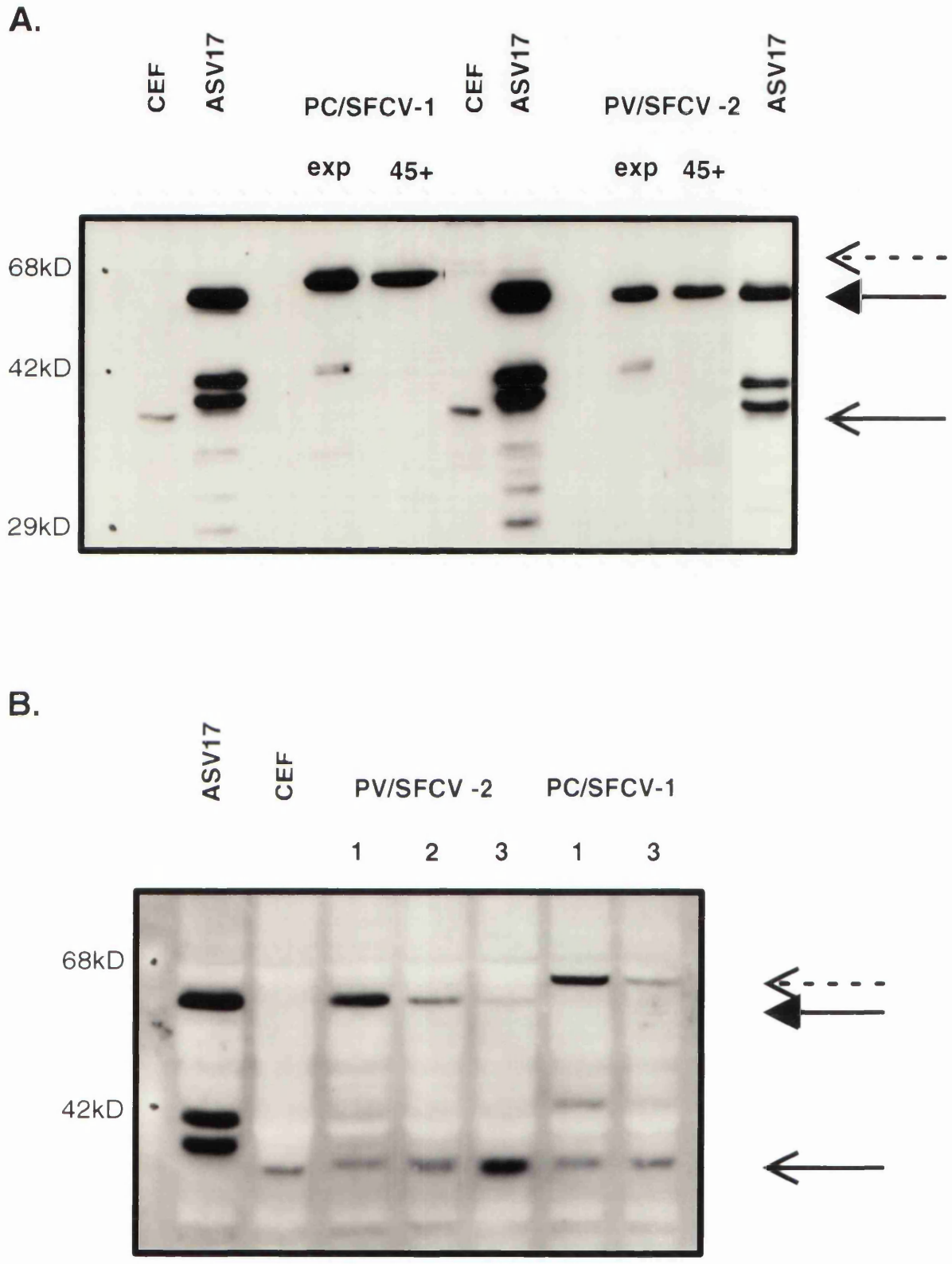

A.
CEF
ASV17
PC/SFCV-1
exp
45+
CEF
ASV17
PV/SFCV -2
exp
45+
ASV17
68kD
42kD
29kD
B.
ASV17
CEF
PV/SFCV -2
1
2
3
PC/SFCV-1
1
3
68kD
42kD

FIGURE 3.13

repression is dose-dependent, and mediated by high levels of c-Jun or v-Jun proteins.

- Exogenous Jun Protein Expression in Secondary Soft Agar colonies

To examine the stability and reproducibility of c-Jun and v-Jun-mediated auto-repression, soft agar cloning assays were repeated using PC- and PV/SFCV-infected primary CEFs. PC and PV/SFCV retroviral particles were harvested from expanded primary soft agar colonies, as described above (section II.A.4a). Second round PV and PC/SFCV derived colonies appeared at the same rate and frequency as before, and were morphologically identical to their first round counterparts (data not shown). The levels of p68 gag-c-Jun and p65 gag-v-Jun expression were similarly unchanged. Candidate exogenous proteins of the predicted molecular weight and endogenous p39 c-Jun, were consistently absent from the expanded second round colonies (Figure 3.14A & 3.14B).
From these observations it was concluded that the recombined c-Jun and v-Jun proteins were stable and that their overexpression was sufficient to down regulate endogenous c-Jun expression in avian cells.

III.C. CONSEQUENCES OF c-JUN DOWN-REGULATION FOR AP-1 DNA BINDING ACTIVITY IN ASV17-TRANSFORMED CEFS

The functional consequences of c-Jun down-regulation, in ASV17-transformed CEFs were investigated by DNA mobility shift assays. An oligonucleotide containing the collagenase TRE binding site, was used as

FIGURE 3.14

Western Blot analysis of denatured whole cell protein extracts, prepared from expanded secondary soft agar colonies. Virus particles were harvested form primary soft agar colonies, PC-1 and PV-2 (Figure 3.13A), and titred onto cultures of non-infected primary CEFs. 48hrs after infection the cells were seeded into soft agar as described in Figure 3.12. Individual secondary colonies were picked and expanded for Western Blot analysis on day 14. Approximately equal concentrations of protein extract were resolved on a 9% acrylamide gel and transferred to ECL nylon membrane using a CAM LAB Semi-Dry Blotter. The proteins were visualized by ECL detection with a 1:4000 dilution of 730/5. Figure 3.14A and 3.14B illustrate the expression of exogenous c-Jun and v-Jun proteins in respective, independent, PC and PV/SFCV derived secondary colonies. p68 gag-c-Jun is denoted by a broken arrowhead; p65 gag-v-Jun by a solid arrowhead, and p39 c-Jun by a light arrowhead.

Antibody specificities are outlined in figure legend 3.16.

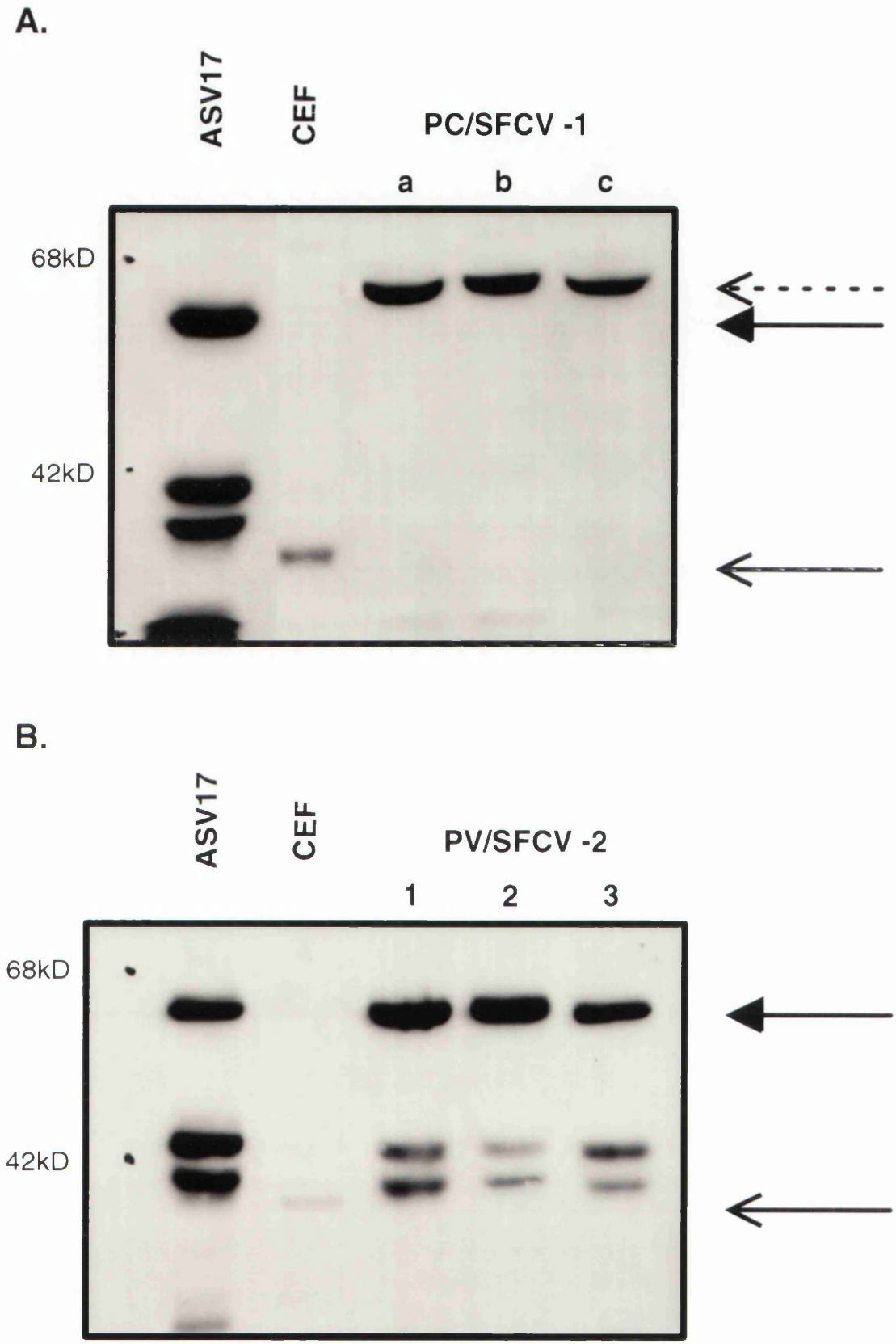

A.
ASV17
CEF
PC/SFCV -1
a
b
c
68kD
42kD
B.
ASV17
CEF
PV/SFCV -2
1
2
3
68kD
42kD

FIGURE 3.14

a probe to compare the AP-1 DNA binding activity in normal and ASV17-transformed CEF extracts. As shown in Figure 3.15, normal and ASV17-transformed CEFs were characterized by qualitatively and quantitatively distinct patterns of collagenase TRE binding activity. A single retarded band in normal CEF extracts (band I) was replaced by a weaker doublet in ASV17-transformed cells (bands II & III). The doublet was not detected in equivalent vector-infected (RCAS) or Src-(CK29) transformed CEF extracts, and therefore represented a specific consequence of v-Jun overexpression rather than a secondary effect of cellular transformation.

III.C.1. JUN COMPOSITION OF TRE BINDING ACTIVITIES I, II & III

To investigate the Jun composition of Bands I, II, and III, a panel of different polyclonal antisera was included in the in vitro AP-1/TRE binding reactions. The reactivity of each antibody is outlined in figure legend 3.16. The antibody specificities were confirmed in a parallel series of binding reactions using extracts prepared from Swiss 3T3 cells (A kind gift from M. Unlu). JunB represents the predominant Jun family protein expressed in Swiss 3T3 cells (Kovary *et al*, 91; Kovary *et al*, 92) and, in accordance with this, comprised the major AP-1/TRE binding protein detected in vitro (Figure 3.16A).

The AP-1/TRE binding activity detected in normal and ASV17-transformed CEF extracts was virtually completely disrupted by two independent c-Jun-specific antisera: 730/5 and 948/4 (Figure 3.16A). Parallel reactions in the presence of anti-JunB or anti-JunD, had no effect on the migration of Bands I, II and III. This pattern of reactivity confirmed the c-Jun-specificity of 730/5 and 948/4, and led to the proposal that c-Jun-containing

FIGURE 3.15

Electrophoretic Mobility Shift analysis of collagenase TRE binding complexes present within non-denatured whole cell protein extracts, prepared from normal and ASV17 transformed CEFs. 10ug of protein extract was incubated for 30min on ice, in the presence (+) or absence (-) of a 100 fold excess (200ng) of specific cold competitor. 2ul (~ 2x 105 cpm) of a 5' end labelled, double stranded collagenase TRE probe, was added and the contents incubated for a further 30 mins at 4^{o}C. The binding reactions were resolved on a 4% non-denaturing acrylamide gel. The right hand lanes contain control cell extracts prepared from LA29 Src (CK29) and RCAS (vector alone) infected CEFs. DNA binding complexes I, II and III are illustrated on the figure. The canonical collagenase TRE binding sequence, contained within the synthetic oligonucleotide probe, is described above the main figure.

Antibody specificities are outlined in figure legeng 3.16.

TGACTCA

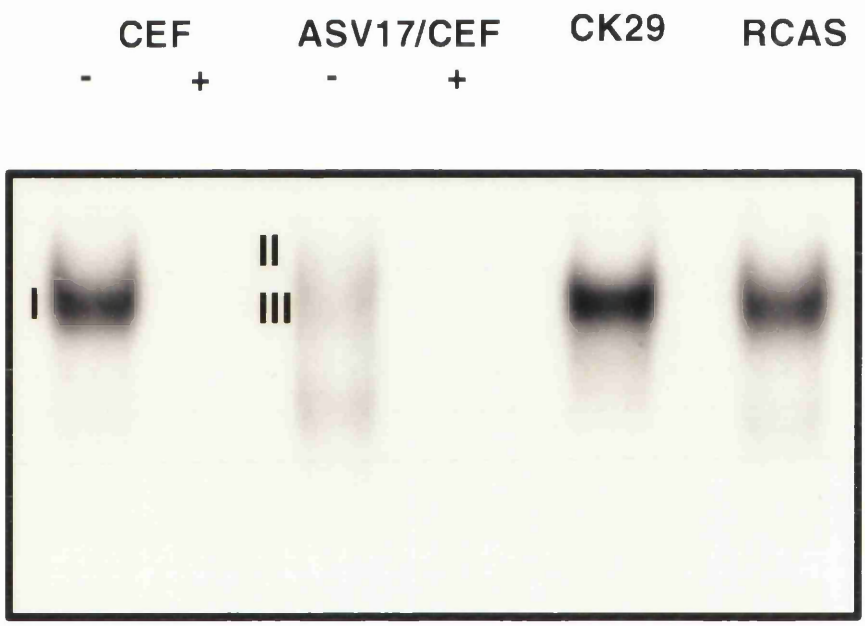


FIGURE 3.15

FIGURE 3.16A

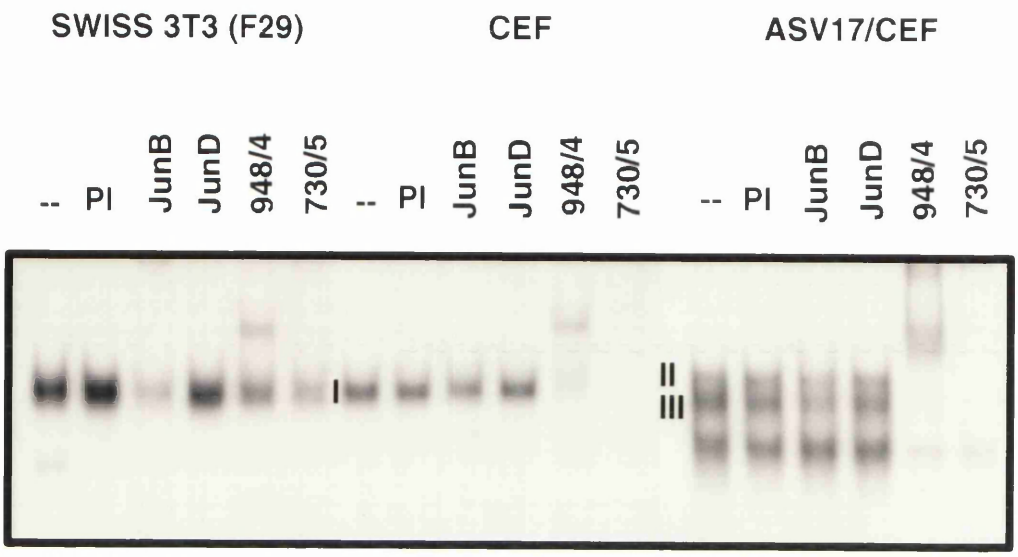
Electrophoretic Mobility Shift analysis to compare the composition of collagenase TRE binding complexes present within three independent whole cell protein extracts. 10ug of non-denatured whole cell extract, prepared from Swiss 3T3 cells, normal CEFs and ASV17 transformed CEFs, was incubated on ice for 30mins with 2ul of rabbit polyclonal antisera. 2ul (~2x 105 cpm) of a 5' end labelled, double stranded collagenase TRE probe was added, and the contents incubated for a further 30mins at 4°C. The binding reactions were resolved on a 4% non-denaturing acrylamide gel. The left hand tracks of each set of six additions represent no addition (--) or a 2ul addition of preimmune rabbit antisera (PI). Complexes I, II and III are denoted on the gel. The Swiss 3T3 and normal CEF autoradiographs were exposed for 7hrs and the ASV17/CEF autoradiograph for 24hrs.

FIGURE 3.16B

Electrophoretic Mobility Shift analysis to compare the composition of collagenase TRE binding complexes present within normal and ASV17 transformed CEF extracts. 10ug of non-denatured whole cell protein extract was incubated in the presence of 2ul of sterile water (--), 2ul preimmune rabbit sera (PI), or 2ul of specific polyclonal rabbit antisera, for 30mins at 4°C. 2ul (~2x 105 cpm) of a 5' end labelled double stranded collagenase TRE probe was added, and the contents incubated at 4°C for a further 30mins. The binding reactions were resolved on a 4% non denaturing acrylamide gel. Collagenase TRE binding complexes I, II and III are denoted on the gel.

	Immuno -gen	c-Jun	JunB	JunD	Fos/. FRA	gag	v-Jun
a 948/4	c-Jun	+					+
a 730/5	c-Jun	+					+
a JunB	JunB		+				
a JunD	JunD			+			
a 388/4	v-Fos				+		
CASTOR	v-Jun	+					+
a 5202	gag					+	

A.



B.

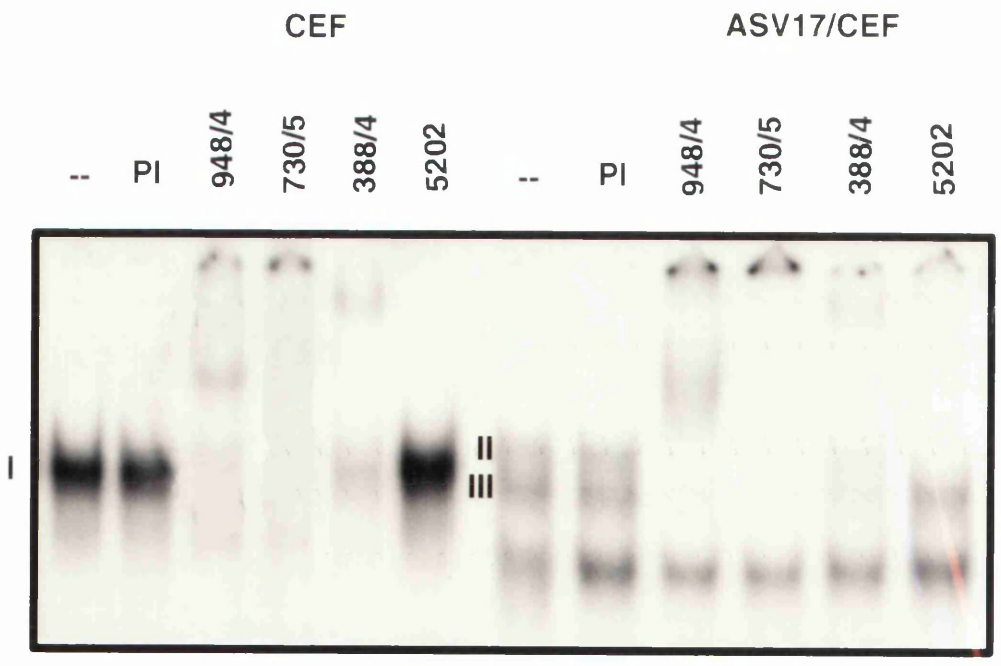


FIGURE 3.16

complexes represent the majority, or all, of the AP-1/TRE binding activity detected in normal CEFs (Band I).

The nature of the c-Jun-reactive component detected in Bands II and III, was complicated by the virtual absence of endogenous p39 c-Jun in ASV17-transformed CEFs (Figure 3.5). 730/5 and 948/4 recognize epitopes that are common to both c-Jun and p65 gag-v-Jun. To investigate the specific contribution made by p65 gag-v-Jun, a gag-specific antisera (5202) was included in the binding reactions. As shown in Figure 3.16B, anti-5202 completely disrupted band II but had no effect on the integrity of Band III (or Band I). The parallel antibody reactivities of Bands I and III, together with their similar patterns of migration in non-denaturing acrylamide gels, suggested that both might contain endogenous p39 c-Jun. To exclude the possibility of low p39 c-Jun expression in a minority of ASV17-transformed CEFs, non-denatured protein extracts were denatured in 1x SDS sample buffer and analysed for the presence or absence of endogenous c-Jun (section II.D.3). As shown in Figure 3.17A, endogenous p39 c-Jun was only detectable in normal CEF extracts. Its absence in equivalent ASV17-transformed extracts indicated that residual p39 c-Jun was unlikely to represent the c-Jun-reactive component of Band III.

III.C.2. Fos/FRA COMPOSITION OF TRE BINDING ACTIVIITIES I, II and III

c-Jun transactivates gene expression as a homo or a heterodimer (Ransone *et al*, 89). A major family of Jun dimerization partners is represented by the Fos or Fos-related proteins. To investigate their contribution to the collagenase TRE binding activity in normal and ASV17-transformed CEFs, a Fos/FRA-specific antibody (388/4) was included in

FIGURE 3.17

Western Blot analysis of denatured whole cell protein extracts, prepared from normal and ASV17 transformed CEFs. The extracts were originally prepared as non-denatured extracts for gel mobility shift analysis and were subsequently denatured in SDS sample buffer for western blotting analysis. Approximately equal concentrations of protein extract were resolved on a 9% SDS/acrylamide gel and transferred to ECL nylon membrane using a CAM LAB Semi-Dry Blotter. The proteins were visualized by ECL detection with a 1:4000 dilution of 730/5 (Figure 3.17A) and a 1:10000 dilution of 5202 (Figure 3.17B). Autorads A and B represent the same gel probed sequentially with 730/5 and 5202 after stripping in β-mercaptoethanol (Section II.D.3c). Control cell extracts are illustrated on the right hand side of the gel. They represent normal and ASV17 transformed CEF western blot extracts, and an equivalent extract prepared from c-Myc infected primary CEFs (RCAN c-MYC). p65 gag-v-Jun is denoted by a solid arrowhead; p39 c-Jun by a light arrowhead; and the p42 gag-v-Jun proteolytic fragment by a broken arrowhead.

Antibody specificities are outlined in figure legeng 3.16.

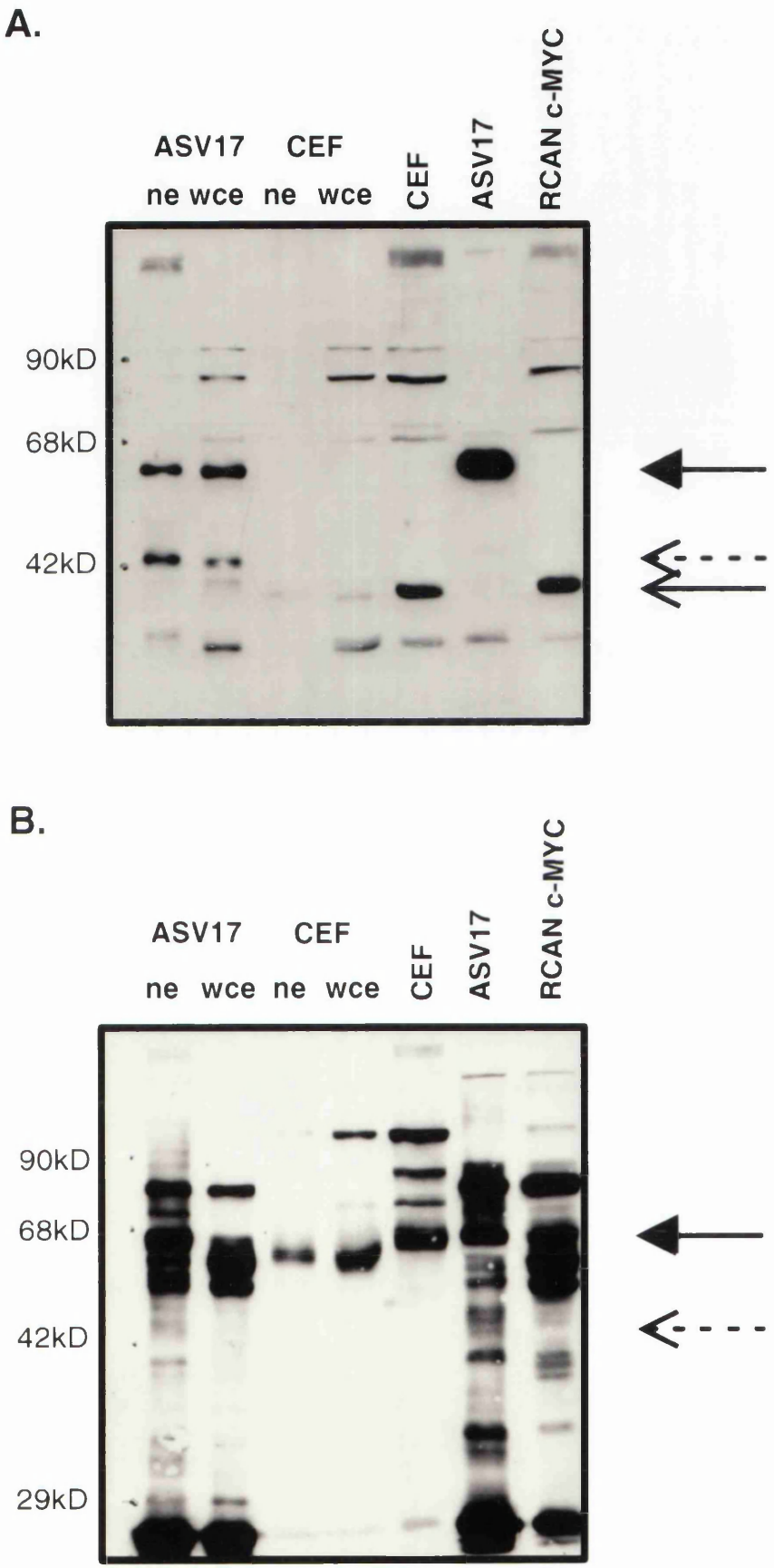

A.
ASV17
CEF
ne wce ne wce
CEF
ASV17
RCAN c-MYC
90kD
68kD
42kD
B.
ASV17
CEF
ne wce ne wce
CEF
ASV17
RCAN c-MYC
90kD
68kD
42kD
29kD

FIGURE 3.17

the in vitro DNA binding reactions. 388/4 was raised against a peptide in FBJ-MuSV provirus that spans part of the p55 v-Fos basic region and recognizes multiple members of the Fos/FRA gene family (Vosatka *et al*, 89). As shown in Figure 3.16B, Bands I, II and III were severely disrupted by a 2ul addition of 388/4. The pattern of disruption indicated a high, and approximately equivalent, presence of Fos/FRA-containing TRE binding complexes in both cell types. Complete disruption was never observed under these experimental conditions. The residual collagenase TRE binding activities was presumed to represent alternative Jun-containing complexes with identical mobilities in non-denaturing acrylamide gels to their Fos/FRA-containing counterparts.

CHAPTER IV - DISCUSSION (1).

CHAPTER IV - DISCUSSION

c-JUN EXPRESSION AND AUTO-REPRESSION

IV.A. *c-jun* EXPRESSION AND REGULATION

IV.A.1. TRANSCRIPTIONAL REGULATION

RNase protection analysis demonstrated that *c-jun* mRNA was rapidly and transiently induced in response to serum growth factors, and superinduced in the presence of protein synthesis inhibitors (Figure 3.1A). The kinetics of *c-jun* expression were characteristic of a class of transcription factors described as the immediate early genes. Immediate early genes derive their name from viral counterparts which exhibit a coordinate and transient pattern of regulation in the absence of ongoing protein synthesis (Lau *et al*, 87). The cellular immediate early genes encode transcription factors, such as c-Myc and c-Fos, which have been proposed to mediate the conversion of transient cell surface signals into long term changes in gene expression (Angel *et al*, 88(ii)).

Regulation of immediate early gene expression is both complex and specialized. As shown in Figure 3.2, the serum induction of *c-jun* transcription was modest and transient relative to the corresponding induction of *c-jun* mRNA. The pattern of transcriptional induction was insufficient to account for either the large fluctuations in *c-jun* mRNA, or the persistence of *jun*-specific transcripts at late time points after serum stimulation (compare Figure 3.1A & 3.2). The discrepancies suggest a role

for post-transcriptional regulatory mechanisms, which have been associated with the differential expression patterns of several immediate early genes in vivo (Greenberg *et al*, 84; Lau *et al*, 87; Mitchell *et al*, 86). The relative contribution made by post-transcriptional regulation depends on the identity of the immediate early gene and the nature of the inducing stimulus. Transcriptional repression of *c-jun*, for example, is delayed in the presence of inducing stimuli such as TGF-B (Kim *et al*, 90) and TNF-α (Brenner *et al*, 89). An equivalent pattern of expression is not associated with the *c-fos* gene, which is predominantly regulated at the level of transcription (Greenberg *et al*, 84; Muller *et al*, 84; Rahmsdorf *et al*, 87). In accordance with this, *c-fos* transcription was rapidly and transiently induced in response to serum stimulation (Figure 3.2). The kinetics of *c-fos* expression paralleled the reported induction of *c-fos* mRNA following serum stimulation (Greenberg *et al*, 84) but were distinct from those of *c-jun* (Figure 3.2), suggesting that the *c-jun* gene is subject to additional levels of post-transcriptional regulation under these conditions.

c-jun-specific post-transcriptional regulatory mechanisms were not investigated in this analysis, but the superinduction of *c-jun* mRNA in the presence of protein synthesis inhibitors suggested that productive translation was required for the post-transcriptional regulation of *c-jun* mRNA in response to serum stimulation (Figure 3.1A). In support of this hypothesis, ongoing protein synthesis has been associated with mRNA stability and transcriptional repression of several immediate early mRNAs in vivo, including *c-jun* (Ryseck *et al*, 88; Sherman *et al*, 90). Mechanisms responsible for regulating immediate early mRNA stability include endonuclease directed degradation through 3' untranslated target sequences (Shyu *et al*, 89; Wisdom *et al*, 91), and translation-dependent degradation through specific instability elements in the coding sequences of several immediate early genes (Bernstein *et al*, 92; Shyu *et al*, 89;

Wisdom *et al*, 91). Repression of immediate early transcription is directed through the synthesis of specific repressor proteins. For immediate early genes such as *c-myc* and *c-fos*, the protein products negatively regulate their own promoters thereby mediating exquisitely sensitive mechanisms of auto-repression (Grignani *et al*, 90; Rivera *et al*, 90).

The translation-dependent mechanisms controlling the levels of *c-jun* mRNA in response to serum growth factors, may include a regulation of *c-jun* mRNA stability and *c-jun* transcriptional repression. The differential contribution of post-transcriptional mechanisms to the serum induction of *c-jun* and *c-fos* (Figure 3.2), together with the persistence of *jun*-specific transcripts at late time points after serum stimulation (Figure 3.1A), suggests that transcriptional and post-transcriptional regulatory mechanisms induce overlapping but sequential patterns of *c-jun* and *c-fos* expression in vivo. Sequential patterns of gene expression are similarly associated with the viral immediate early genes (Lau *et al*, 87), and are presumed to regulate complex and ordered patterns of gene expression, analagous to the developmental programme of a complex virus.

IV.A.2. TRANSLATIONAL AND POST-TRANSLATIONAL REGULATION

The rapid and dramatic changes in *c-jun* mRNA levels, observed in response to serum stimulation, did not correlate with the corresponding changes in c-Jun protein expression (compare Figure 3.1A & 3.3). c-Jun protein was modestly induced under these conditions, attaining maximum levels long after the onset of decline in *jun*-specific transcripts. The discrepancies in *c-jun* mRNA and protein expression levels strongly supported a role for translational regulation. The relatively invariant levels of c-Jun protein detected in response to serum stimulation (Figure 3.3A &

3.3B), suggested that one function of translational regulation may be to maintain relatively consistent levels of c-Jun protein against widely fluctuating levels of *c-jun* mRNA.

The mechanisms governing Jun-specific translational regulation have not been elucidated, although an extended GC rich sequence in the 5' untranslated region of *c-jun* mRNA has recently been identified that represses c-Jun translation through the formation of highly stable secondary structures (A. Sehgal; *personal commun.*). Further regulatory mechanisms are likely, and presumably contribute to the high and relatively invarient levels of endogenous p39 c-Jun observed after serum stimulation (Figure 3.3). Maintaining a pool of endogenous c-Jun protein may facilitate the initial rapid transcriptional response characteristic of immediate early transcription factors. Primary immediate early transcription is independent of protein synthesis and has been proposed to depend on post-translational modifications to pre-existing proteins (Angel *et al*, 88(ii); Reviewed in Angel *et al*, 91). In support of this hypothesis, a ladder of Jun-reactive proteins was transiently detected at very early time points after serum stimulation (Figure 3.3A). The ladder comprised 4-5 individual bands and was not ablated in the presence of a protein synthesis inhibitor (Figure 3.4).

Electrophoretic retardations of the c-Jun protein have been reported in vitro (Black *et al*, 91) and in vivo (Pulverer *et al*, 92), and correlate with amino-terminal phosphorylation of c-Jun at serines 63 and 73 (Black *et al*, 94; Boyle *et al*, 91; Lin *et al*, 92). It has been proposed that phosphorylation induces a conformational change that directly or indirectly alters the electrophoretic mobility of the c-Jun protein (Black *et al*, 94; Pulverer *et al*, 92). This proposal is supported by the recently identified c-Jun amino-terminal kinase, JNK, that specifically binds to a region in the c-Jun transactivation domain and phosphorylates serines 63 and 73.

Phosphorylation results in the dissociation of the JNK-c-Jun complex, presumably through a conformational change (Hibi *et al*, 93). Stimuli that enhance c-Jun amino-terminal phosphorylation also potentiate c-Jun transcriptional activation (Binetruy *et al*, 91; Pulverer *et al*, 91; Smeal *et al*, 91; Smeal *et al*, 92). Conversely, substitution of serines 63 and 73 with non-phosphorylatable leucine or alanine residues severely impairs c-Jun transactivation, suggesting that phosphorylation at these sites is either necessary for, or potentiates c-Jun transactivation in vivo (Black *et al*, 94; Pulverer *et al*, 91; Smeal *et al*, 91). The equivalent mutations in v-Jun have no effect on v-Jun transactivation (Black *et al*, 94). v-Jun is not phosphorylated in vitro by kinases that phosphorylate c-Jun (Black *et al*, 94; Smeal *et al*, 91), and does not produce an electrophoretic mobility shift when translated in vitro in the presence of reticulocyte lysate (Black *et al*, 91). More recently it has been reported that v-Jun does not contain a binding site for JNK, suggesting that it exists in a distinct and potentially active conformation (Hibi *et al*, 93).

The conformational differences between c-Jun and v-Jun may account for the differential reactivities of CASTOR and 730/5 against the Jun-specific ladder (compare Figure 3.3A & 3.3B). 730/5 was raised against bacterial and, presumably, unmodified c-Jun; whereas CASTOR was raised against a trpE-v-Jun fusion protein (Angel *et al*, 88(ii)). If post-translational activation of c-Jun is regulated by a conformational change that permenently exists in the v-Jun protein, then recognition of the active conformation(s) would, predictably, be restricted to the CASTOR antibody. The proposed c-Jun-specific conformational change is mediated, at least in part, through the recently identified Jun amino-terminal kinase, JNK (Hibi *et al*, 93). JNK was originally identified as a cycloheximide stimulated kinase (Edwards *et al*, 92; Kyriakis *et al*, 94; Mahadevan *et al*, 91), and, consequently, may account for the enhanced

electrophoretic mobility shift of the c-Jun ladder in the presence of cycloheximide (Figure 3.4). The rapid and transient appearance of the ladder (Figure 3.3B) suggests that post-translational modifications to pre-existing Jun proteins may mediate some aspects of the initial Jun-specific transcriptional response, but that ongoing protein synthesis may be necessary for more prolonged changes in gene expression.

IV.B. JUN-MEDIATED AUTO-REPRESSION

A subset of immediate early proteins express the ability to negatively regulate their own gene promoters (Grignani *et al*, 90; Sassoni-Corsi *et al*, 88). To investigate whether Jun exhibits an equivalent auto-repressor function, expression of endogenous p39 c-Jun was analysed in ASV17-transformed CEFs. As shown in Figure 3.5, detection of endogenous p39 c-Jun was specifically and completely abolished in the presence of the v-Jun oncoprotein.

p65 gag-v-Jun contains a number of structural mutations in the coding and non-coding sequences that are not present in the non-transforming proto-oncogene (Figure 1.1). To investigate their contribution to v-Jun auto-repression, gag-*c-jun* and gag-*v-jun* fusion sequences were inserted into the SFCV-LE retroviral vector to facilitate equivalent overexpression of c-Jun and v-Jun in primary CEFs (Figure 3.6). The predicted molecular weights and dimerization properties of the gag-Jun fusion proteins were confirmed by in vitro translation and immunoprecipitation analysis (Figure 3.7)

IV.B.1. CHARACTERIZATION OF c-JUN- AND v-JUN-EXPRESSING PRIMARY CEFs

PC and PV/SFCV transfected CEFs were characterized in relation to normal and ASV17-transformed CEFs, by their growth rate and cellular morphology in normal growth medium. As shown in Figure 3.8 and 3.9, PV/SFCV transfectants exhibited an indistinguishable phenotype from ASV17-transformed CEFs. Early passage cultures expressed low levels of a candidate 44kD gag-Jun fusion protein (Figure 3.7A & 3.11A). With continued passage the 44kD protein was replaced by a larger 65kD gag-Jun fusion (Figure 3.13A & 3.13B). The molecular weight, nuclear distribution and cellular expression of p65 was identical to ASV17 p65 gag-v-Jun (Figure 3.13A & 3.10), and may provide an explanation for the ASV17-like phenotype of PV/SFCV transfected CEFs.

In contrast to their PV/SFCV transfected counterparts, PC/SFCV transfected CEFs expressed a 68kD gag-Jun fusion protein (Figure 3.13A & 3.13B). Like p65, p68 rapidly replaced the candidate 47kD gag-c-Jun fusion (compare Figure 3.11A & 3.13A), and was predominantly localized to the nucleus (Figure 3.10). The size and expression levels of p65 and p68 were consistent with a single recombination event linking either *v-jun* (PV/SFCV) or *c-jun* (PC/SFCV) coding sequences to a common portion of the retroviral gag gene (section IV.B.2). Overexpression of p68 gag-c-Jun-induced a phenotype that was intermediate between normal and fully transformed CEFs (Figure 3.8 & 3.9). The cultures expressed a dual morphology of fibroblastic and epithelioid-like cells (Figure 3.9). The small, rounded epithelioid-like cells assumed a greater proportion of the culture with continued passage, and exhibited sustained growth under low serum conditions (Figure 3.9 - lower panel). A similar morphology has been reported for high c-Jun-expressing NIH3T3 cells, which also exhibit a

slightly elevated growth rate under normal growth conditions (Pfarr *et al*, 94). Flow cytometry analysis of NIH3T3 c-Jun overexpressors, demonstrated a larger than predicted percentage of abnormally small cells in S phase. The overall growth rate of these cells was only slightly enhanced, suggesting that c-Jun overexpression accelerates entry into S phase but deccelerates exit, due to rate limiting components or negative signals.

The c-Jun-specific alterations to growth rate and cellular morphology presented in this report, favour a similar Jun-dependent pertubation of the cell cycle in primary CEFs. The precise cell cycle target was not investigated, but the changes presumably contribute to the partially transformed phenotype exhibited by c-Jun-expressing CEFs. To further investigate the transformed phenotype of PC and PV/SFCV transfected CEFs, cells were examined for their ability to grow in the absence of anchorage to a non-cellular substrate. As shown in Figure 3.12, both PC and PV/SFCV transfected CEFs were capable of anchorage independent growth. PC/SFCV derived soft agar colonies were more diffuse and approximately 3 times less frequent than their PV/SFCV and ASV17 derived counterparts. This correlated with the partially transformed phenotype of PC/SFCV transfected CEFs and confirmed the close similarity between PV/SFCV transfected and ASV17-transformed CEFs.

Similar conclusions have been drawn from independent investigations analysing the consequences of high c-Jun and v-Jun expression in primary CEFs. Overexpression of c-Jun, for example, has been associated with an enhanced growth rate, modest anchorage independent growth and a lower requirement for exogenous mitogenic stimuli (Bos *et al*, 90; Castellazzi *et al*, 90). c-Jun overexpressors, however, are not fully transformed according to other parameters of cellular transformation such as morphology and contact inhibited growth. (Castellazzi *et al*, 90).

These observations support the proposal that overexpression of c-Jun is sufficient to induce a partially transformed phenotype in primary CEFs. The requirement for high levels of c-Jun expression suggests that partial transformation is mediated through quantitative changes in c-Jun/AP-1-dependent gene expression (Wong *et al*, 92). Induction and maintenance of the fully transformed phenotype is presumed to require additional qualitative changes in gene expression that are specific to the v-Jun oncoprotein. In support of this hypothesis, v-Jun-mediated cellular transformation is dependent on high levels of v-Jun expression, and the presence of two amino acid substitutions in the carboxy-terminal region of the v-Jun oncoprotein (Figure 1.1). The structural mutations are specific to p65 gag-v-Jun and have been proposed to mediate the qualitative changes in gene expression necessary for full in vitro cellular transformation and in vivo tumourigenesis (Wong *et al*, 92).

IV.B.2. CONSEQUENCES OF c-JUN AND v-JUN OVEREXPRESSION ON ENDOGENOUS c-JUN EXPRESSION

Overexpression of c-Jun and v-Jun was sufficent to induce the cellular transformation of primary CEFs (section IV.B.1.). Transformation was accompanied by a dramatic and reproducible down-regulation of endogenous c-Jun expression that was specific to the expression of exogenous Jun proteins and not a secondary consequence of cellular transformation (Figure 3.13A & 3.14). The magnitude of the response was apparently equivalent in v-Jun- and c-Jun-expressing CEFs (Figure 3.13A), suggesting that v-Jun-specific qualitative mutations are dispensible for auto-repression in vitro. This interpretation was complicated by the appearance of the novel PC and PV gag-Jun fusion proteins which, from

their antibody reactivities and molecular weights, resembled the products of a homologous gag recombination mimicking the final structure of ASV17. The conservation and rapidity of the proposed rearrangement suggested that specific gag-Jun recombinant proteins present primary CEFs with a strong selective advantage over their normal counterparts. This proposal is supported by the observation that c-Jun, unlike c-Fos, has been captured on only one known occasion through recombination with chicken retroviral gag sequences, in spite of a propensity for gag rearrangements in vitro (Bos *et al*, 90; Reviewed in Curran, 91; Maki *et al*, 87).

The precise nature of the PC and PV/SFCV gene rearrangement was not investigated. Without direct sequencing data it remained a possibility that qualitative mutations had accumulated in c-Jun that were more significant for Jun-mediated auto-repression than any additional recombined gag sequences. Evidence from the literature does not favour this interpretation. Exogenous c-Jun proteins have consistently been detected as discrete bands in SDS gels, migrating according to their predicted molecular weight in the absence of endogenous c-Jun expression (Castellazzi *et al*, 90; Castellazzi *et al*, 91). These observations suggest that major structural alterations, such as the 27 amino acid deletion in v-Jun, are not required for Jun-dependent auto-repression in vitro. Similar conclusions have been drawn using replication defective and drug selective retroviral vectors to express high levels of murine c-Jun in primary CEFs (Castellazzi *et al*, 90). Replication defective vectors eliminate a contribution from minor, undetectable structural mutations, that may accumulate in *c-jun* during early viral replicative cycles and contribute to the auto-repressor function.

These reports, together with the appearance of the PC and PV gag-Jun fusion proteins, suggested that the principal function of recombination was

to juxtapose *c-jun* or *v-jun* genes to specific retroviral gag sequences. A requirement for specific gag rearrangements was not supported by an independent analysis of v-Jun, which identified intact *v-jun* coding sequences and high levels of v-Jun expression as the sole pre-requisites for v-Jun-mediated cellular transformation in vitro and in vivo (Bos *et al*, 90). Consequently, the principle function of homologous or non-homologous recombination, may simply be to facilitate or maximize an overexpression of exogenous Jun proteins from the retroviral LTR. The precise relationship between cellular transformation and auto-repression is not known, but the dose-dependent effects of gag-c-Jun and gag-v-Jun on endogenous c-Jun expression (Figure 3.13B), favour this interpretation, and correlate with the auto-repressor activities of other immediate early genes such as *c-myc* and *c-fos* (Grignani *et al*, 90; Sassoni-Corsi *et al*, 88).

IV.C. CONSEQUENCES OF JUN-MEDIATED AUTO-REPRESSION FOR AP-1 DNA BINDING ACTIVITY IN ASV17-TRANSFORMED CELLS

Normal and ASV17-transformed CEF extracts displayed quantitatively and qualitatively different patterns of in vitro AP-1 DNA binding activity (Figure 3.15). An oligonucleotide containing the collagenase TRE binding site was recognized by a single binding activity (Band I) in normal CEFs and a weaker doublet (Band II & III) in their ASV17-transformed counterparts. All three binding activities were disrupted by c-Jun-specific antisera (Figure 3.16A). A contrasting pattern of reactivity was observed using an extract prepared from Swiss 3T3 cells. The collagenase TRE binding activity in

Swiss 3T3 cells was identical to Band I by electrophoretic mobility but only partially disrupted by c-Jun-specific antisera. In accordance with the published data of Jun family proteins in these cells (Kovary *et al*, 92), JunB represented the major component of AP-1/TRE binding activity detected in Swiss 3T3 cell extracts. The differential composition of AP-1 DNA binding complexes in Swiss 3T3 and CEF extracts confirmed the affinity of Jun proteins for the collagenase TRE binding site (Nakabeppu *et al*, 88; Ryseck *et al*, 91) and suggested that, in contrast to Swiss 3T3 cells, c-Jun represents the sole or predominant Jun family protein expressed in primary CEFs.

The c-Jun-reactive component of Bands II and III was complicated by the absence of detectable endogenous c-Jun in ASV17-transformed CEFs (Figure 3.5). To distinguish between c-Jun- and v-Jun-specific epitopes, a gag-specific antibody (5202) was included in the DNA binding reactions. As shown in Figure 3.16B anti-5202 completely disrupted the binding activity represented by Band II. The integrity of Band III, in contrast, was unaffected by anti-5202. This, together with the absence of endogenous p39 c-Jun in parallel denatured extracts (Figure 3.17A), suggested that full length p65 gag-v-Jun and residual endogenous p39 c-Jun were unlikely to represent the Jun-reactive component of Band III. As an alternative explanation, a proteolytic or truncated varient of p65 gag-v-Jun, that lacks gag, but retains Jun-specific epitopes may represent the Jun-reactive component of Band III. In support of this hypothesis, ASV17-transformed CEF extracts contained a 42kD Jun-reactive protein that was not recognized in parallel experiments using a gag-specific antibody (compare Figure 3.17A & 3.17B). p42 was exclusively detected in ASV17-transformed CEF extracts and, consequently, may represent a highly reproducible fragment of the v-Jun oncoprotein (Figure 3.17A; Bos *et al*,

90). If p42 also retains the v-Jun DNA binding domain, then it is concievable that it contributes to AP-1 DNA binding activity in vitro.

The antibody reactivities of p42 and p65 (Figure 3.17A & 3.17B) suggested that full length and truncated variants of p65 gag-v-Jun represent the Jun-reactive components of Bands II and III respectively. The intensity of Bands I, II and III was at variance with the relative levels of c-Jun and v-Jun expression in normal and ASV17-transformed CEFs (Figure 3.5 & 3.15). p39 c-Jun and p65 gag-v-Jun, however, have been reported to express differential affinities for multiple TRE- and CRE-like target sequences in vitro (Hadman *et al*, 93; Hawker *et al*, 93). Therefore, the reduced intensity of Bands II and III may be explained by a replacement of high affinity c-Jun-containing complexes with low affinity v-Jun-containing alternatives. This explanation would also account for the indistinguishable Fos/FRA reactivities of Bands I, II and III (Figure 3.16B), and suggests that one functional consequence of endogenous c-Jun repression is the complete replacement of c-Jun TRE binding complexes with v-Jun-containg equivalents.

IV.D. CONSEQUENCES OF c-JUN DOWN-REGULATION IN PRIMARY CEFs

v-Jun- and c-Jun-dependent cellular transformation is apparently restricted to avian cells, and is associated with a concomitant down-regulation of endogenous c-Jun expression (Castellazzi *et al*, 90; Castellazzi *et al*, 91; Hughes *et al*, 92). An equivalent down-regulation in murine fibroblasts (M. Unlu; *personal commun.*) failed to induce a transformed phenotype, suggesting that the consequences of c-Jun repression are cell-type-

dependent. Alternative Jun family members may be responsible for the differential effects of c-Jun repression in vivo. It has been reported that the Jun family of proteins posess specific but overlapping DNA binding affinities, suggesting that, under certain conditions, they are functionally interchangeble (Nakabeppu *et al*, 88; Ryseck *et al*, 91). A comparison of the Jun reactivity of complex I in avian and murine cell extracts, revealed that c-Jun was the predominant or only Jun family member expressed in primary CEFs (Figure 3.16A). Swiss 3T3 fibroblasts, in contrast, expressed additional high levels of JunB, which contributed to the endogenous AP-1 DNA binding activity (Figure 3.16A; Kovary *et al*, 92). The differential expression of Jun family proteins in Swiss 3T3 and chicken fibroblasts may be critical for the activity of p65 gag-v-Jun. From the results described above in section IV.C it is proposed that v-Jun-dependent transformation of avian cells is mediated by a complete displacement of endogenous AP-1 activity with v-Jun-containing complexes. An analagous displacement is presumably prevented in murine cells by additional Jun family members, which functionally compensate for the lack of endogenous c-Jun activity, thereby inhibiting the transforming potential of the v-Jun oncoprotein.

If this interpretation is correct, it would suggest that c-Jun repression is critical for v-Jun- (and c-Jun-) dependent transformation of avian cells. Mutational analysis supports the proposal that auto-repression is necessary but not sufficient for CEF transformation. Overexpression of a bzip mutant of c-Jun, lacking the amino terminal transactivation domain, has been reported to dissociate c-Jun down-regulation from cellular transformation (Castellazzi *et al*, 91). This has led to the proposal that Jun-induced cellular transformation is an active rather than a passive process, dependent on the complete replacement of endogenous AP-1 DNA binding activity with transcriptionally active v-Jun-containing

complexes. c-Jun-dependent transformation of avian cells presumably occurs by the same mechanism but through a qualitatively and quantitatively different pattern of gene expression.

CHAPTER V - RESULTS (2).

CHAPTER V. - RESULTS

FUNCTIONAL ANALYSIS OF THE c-JUN PROMOTER

V.A. c-JUN AUTO-REPRESSION

V.A.1. TRANSCRIPTIONAL AUTO-REPRESSION

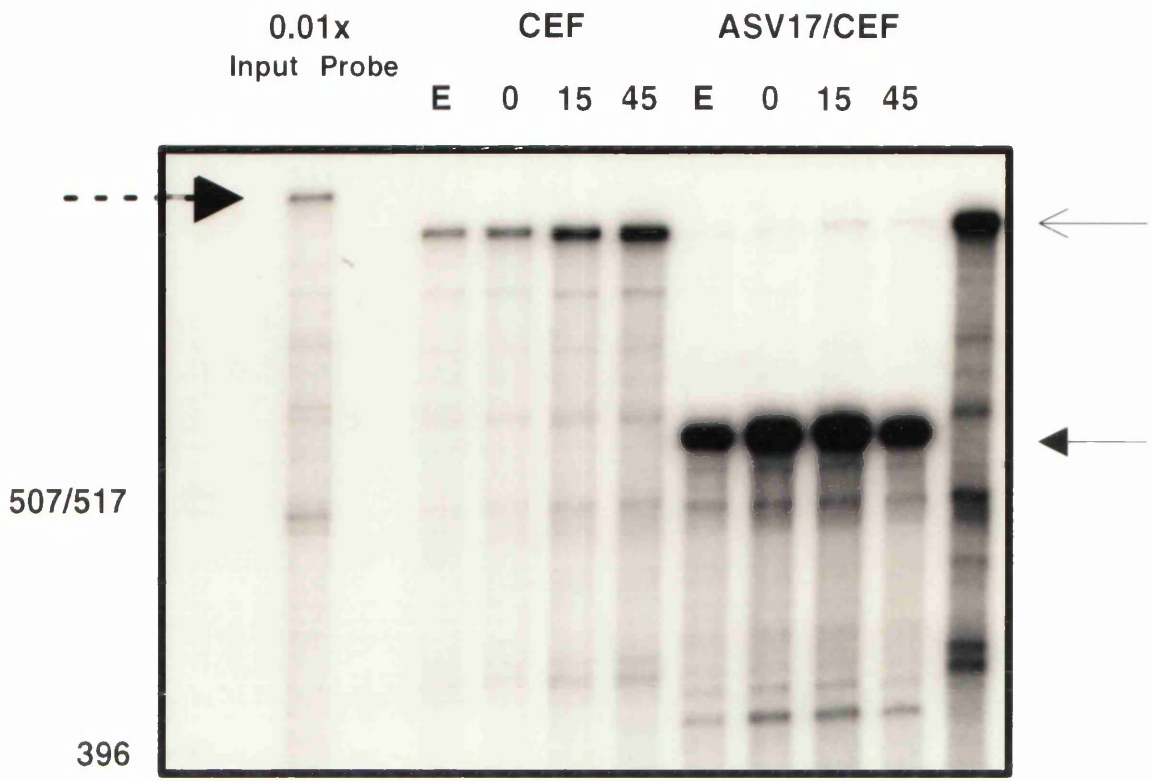
Since overexpression of c-Jun or v-Jun induces a profound down-regulation of endogenous c-Jun protein expression (Figure 3.13), it was of interest to determine at what level repression had occured. To this end RNase protection analysis was performed on mRNA samples extracted from normal and ASV17-transformed CEFs, using an antisense RNA probe designed to distinguish between *c-jun*- and *v-jun*-specific transcripts (Figure 5.1).

As shown in Figure 5.1, the levels of *c-jun* mRNA were dramatically reduced in ASV17-transformed CEFs. Repression was not absolute as low, but detectable, levels of *c-jun*-specific transcripts were maintained in exponential, serum deprived and serum stimulated cultures. *v-jun* mRNA, in contrast, was expressed from the retroviral enhancer at constitutively high levels. The quantitation of *c-jun* mRNA was considered accurate as the input probe was in excess during the hybridization (lane 1).

From these results it was clear that the reduction in c-Jun protein in ASV17-transformed cells was due to the down-regulation of endogenous *c-jun* mRNA through transcriptional and/or post-transcriptional regulatory mechanisms

FIGURE 5.1

RNase Protection anaysis of total mRNA samples prepared from normal and ASV17 transformed CEFs. The cells were in growth phase (E), serum deprived (0) or serum-stimulated (15', 45'). 20ug of RNA was hybridized to 10^6 cpm labelled antisense riboprobe. The riboprobe was prepared from Nco1 linearized pSPT19/JF4 (Dr E.J. Black - Figure 2.1) and included partial c-jun coding sequences and the 3' untranslated region (hatched box) which is specific to c-jun mRNA. The 925bp full length riboprobe is indicated by a broken arrowhead in the left hand lane. The band corresponds to a 0.01x dilution of the riboprobe concentration included in the hybridzation. 850bp c-jun-specific transcripts are denoted by a light arrowhead and 560bp v-jun-specific transcripts by a bold arrowhead. The right hand lane illustrates a positive control of normal CEFs serum-stimulated for 2hrs in the presence of 0.1mM Emetine.


0.01x
Input Probe
CEF
ASV17/CEF
E 0 15 45 E 0 15 45
507/517
396

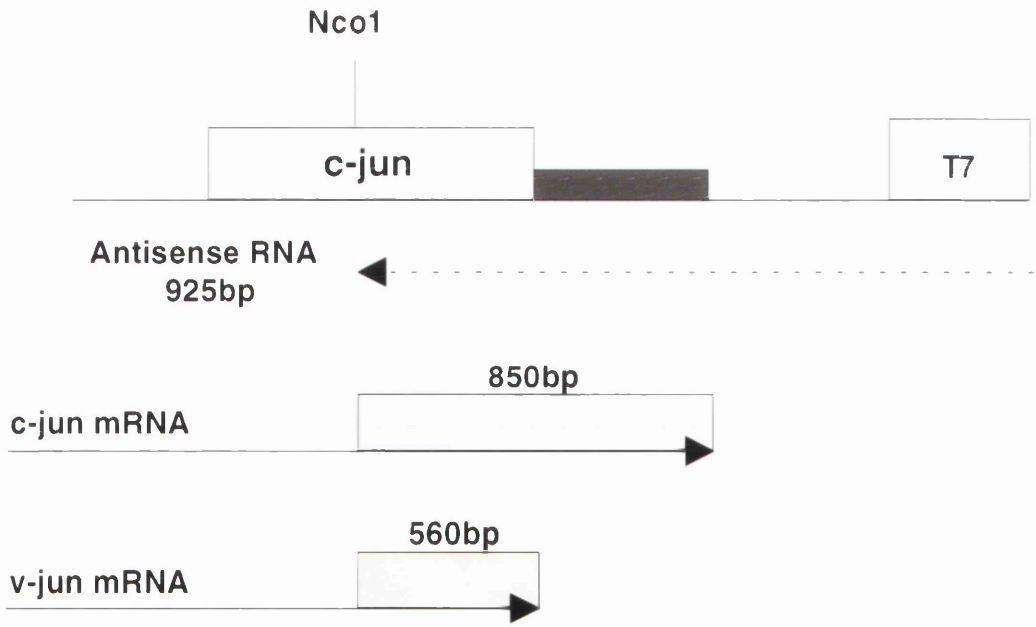

Nco1
c-jun
T7
Antisense RNA
925bp
850bp
c-jun mRNA
560bp
v-jun mRNA

FIGURE 5.1

To determine whether the down-regulation of *c-jun* mRNA was due to reduced transcription of *c-jun*, the rate of *c-jun* transcription was measured in normal and ASV17-transformed cells by the Run-Off transcription assay. Nascent *c-jun* transcripts were detected by hybridization to a plasmid encoding *c-jun* 3' untranslated sequences (c-Jun(1)). Additional jun-containing plasmids were included in the assay which were anticipated to hybridize to both *c-jun*- and *v-jun*-specific transcripts. c-Jun(5) and v-Jun encode the full length *c-jun* and *v-jun* coding sequences respectively. A *c-fos*-specific plasmid was also included in the analysis to determine whether v-Jun-mediated transformation perturbed the expression of a major c-Jun dimerization partner. Positive and negative controls were provided by chicken GAPDH and pSPT19 sequences respectively. Details of each plasmid are outlined in Figure 5.3.

As shown in Figure 5.2, the rate of *c-jun* transcription was reduced in ASV17-transformed cells, indicating that v-Jun-dependent repression of c-Jun expression was, at least partially, directed at the level of transcription. Residual *c-jun* transcription, however, was detected in ASV17-transformed CEFs, which correlated with the low levels of *c-jun* mRNA detected in ASV17-transformed cells by RNase Protection analysis (Figure 5.1). Since c-Jun protein was undetectable under these conditions (Figure 3.5), this suggests that either the residual *c-jun* mRNA is not efficiently translated, or that alternative mechanisms operate to reduce the steady state level of c-Jun protein in addition to transcriptional repression.

FIGURE 5.2

Nuclear Run Off analysis of normal and ASV17 transformed CEF nuclei, prepared from cells in exponential growth. Approximately 3.2 $x10^7$ cpm of TCA precipitable radioactivity was included in each hybridization. Plasmid DNAs were alkali denatured and immobilized onto a nitrocellulose filter using a Bio-Slot Microfiltration apparatus. 5ug of plasmid DNA was immobilized per slot and the filters fixed under vacuum at 80°C for 2hrs. Hybridization proceeded for 72hrs in a final volume of 1.8ml. The filters were wahed under high stringency and exposed overnight at -70°C.
Details of each plasmid included in the hybridization are outlined in figure 5.3. c-Jun(1) represents the c-jun specific plasmid. The arrowhead indicates the v-Jun dependent repression of c-jun transcription in ASV17 transformed CEFs.

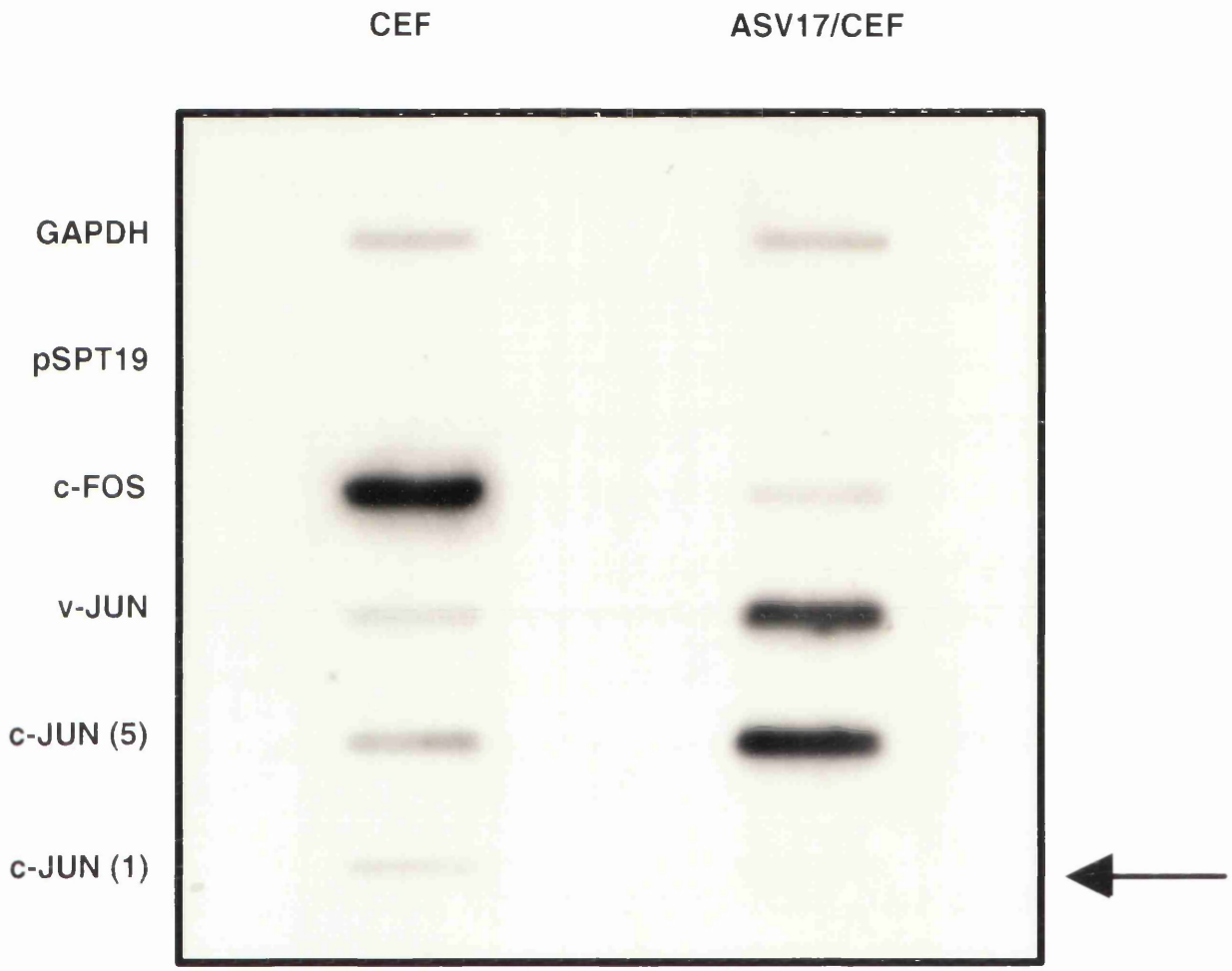


FIGURE 5.2

FIGURE 5.3

VECTOR

- pSPT19

The pSPT19 plasmid contains diametrically opposed SP6 and T7 transcription promoter sequences, separated by the multiple cloning site of PUC19. DNA fragments cloned into pSPT19 can be transcribed, with high efficiency, from the sense or the antisense strand, by SP6 and T7 RNA polymerases.

INSERTS

- pSPT19 ckGAPDH

1260bp amplified sequence of the avian GAPDH gene (Dugaiczyk 83), cloned into pSPT19, in the sense orientation, at the EcoRI restriction endonuclease site.

- pSPT19 c-Fos

Approximately 1300bp cDNA of avian c-fos cloned into pSPT19 at the EcoRI restriction endonuclease site. The orientation and sequence of pSPT19 c-Fos have not been determined.

- pSPT19 v-Jun

Approximately 1000bp BamH1/EcoRI restriction fragment spanning the entire v-Jun coding sequence. The 1000bp fragment was subcloned into pSPT19 in the antisense orientation between the Bgl11 and EcoRI restriction sites.

- pSPT19 cJun(5)

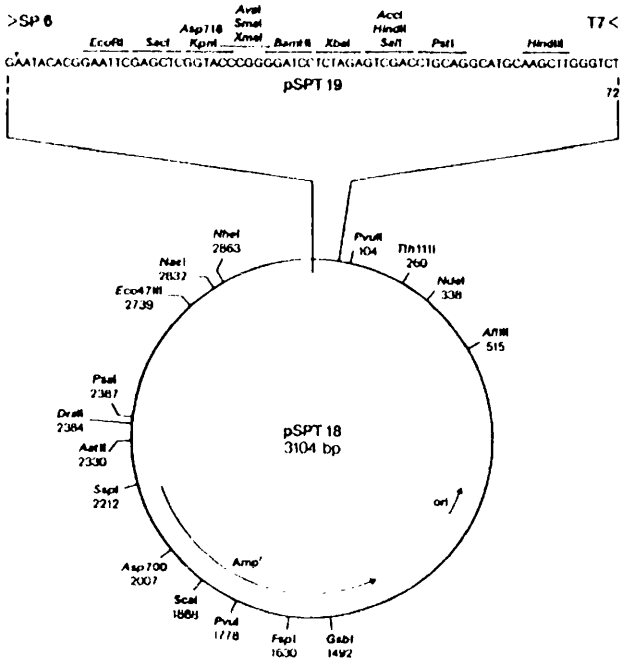
1500bp HindIII/Bgl11 restriction fragment, spanning the c-jun coding region and adjacent 3' and 5' untranslated sequences. The fragment was obtained from avian genomic c-jun (pSPT19-c-jun Clone A) and subcloned into pSPT19 between the HindIII and Bgl11 cloning sites.

- pSPT19 cJun(1)

500 - 600bp amplified sequence spanning the c-jun specific untranslated region from a cloned c-jun cDNA, pSPT19/JF11 (Dr E.J. Black). The amplified fragment was generated using an EcoRI tagged 5' primer and an SP6 specific 3' antisense primer, and subcloned into pSPT19 at the EcoRI cloning site.

Sequences corresponding to the approximate sizes of c-jun (green) and v-jun (blue) mRNAs are illustrated, to identify regions of homology with the Jun specific plasmid DNAs.

pSPT19 Vector Sequences



Avian c-jun

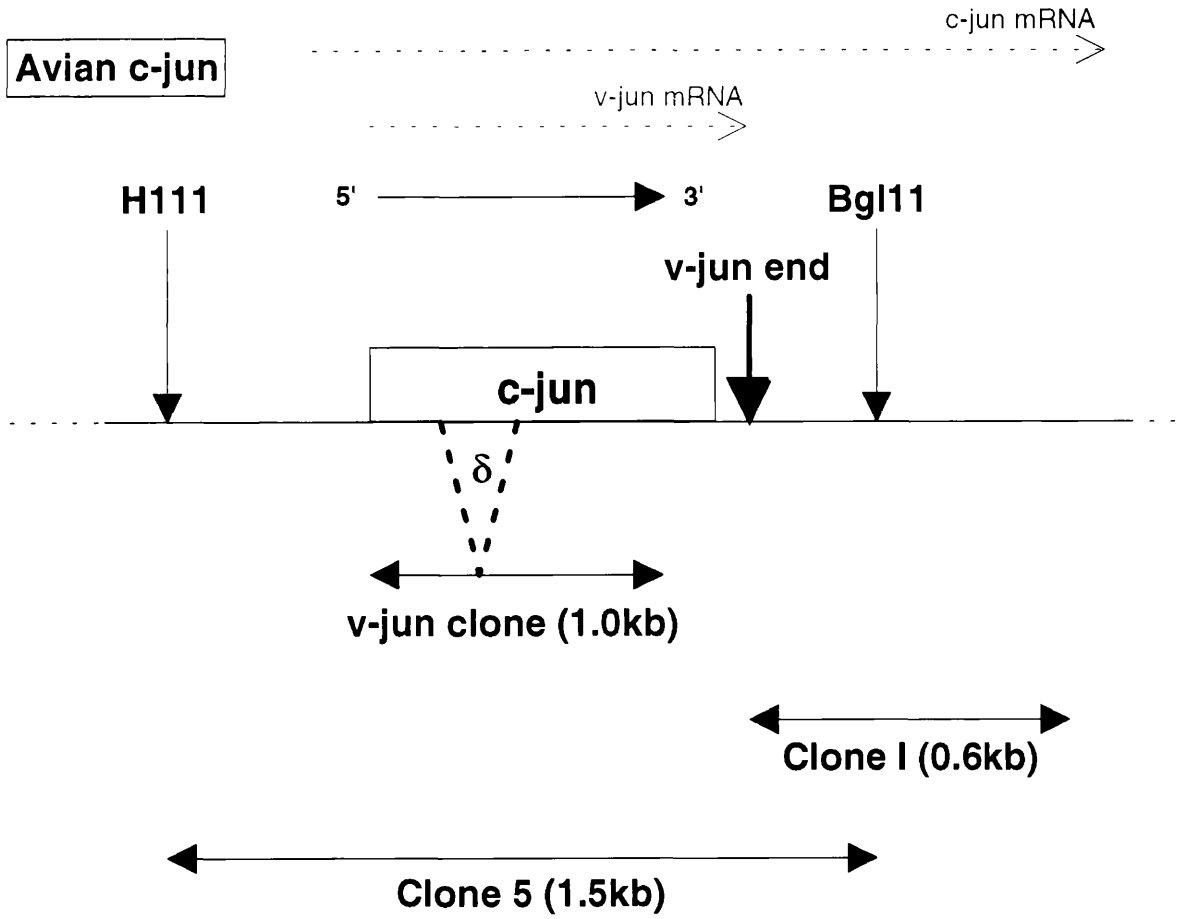


FIGURE 5.3

V.B. CHARACTERIZATION OF THE AVIAN c-JUN GENE PROMOTER

V.B.1. ISOLATION AND SEQUENCE ANALYSIS OF A GENOMIC AVIAN c-JUN CLONE

To investigate the mechanism of *c-jun* transcriptional auto-repression, it was necessary to clone the genomic gene and adjacent promoter sequences. *c-jun* had previously been reported to reside on a 5kb HindIII restriction endonuclease fragment in genomic DNA derived from primary CEFs (Dr D.A.F. Gillespie; *personal commun.*). Using this information, genomic DNA was digested with HindIII and size fractionated by sucrose gradient centrifugation. An enriched pool of approximately 5kb HindIII fragments was purified and inserted into bacteriophage λ1174 at the HindIII cloning site, and screened with a labelled v-Jun coding sequence, derived from pSPT19-v-Jun (Figure 5.3). Positive phage clones were purified by secondary screening and the inserts subcloned into pSPT19 at the HindIII site. Individual clones were orientated using a panel of different restriction endonucleases as described in Figure 5.4.

The primary sequence of this genomic *c-jun* clone, including the coding region and adjacent 5' untranslated promoter regions, was obtained and is presented in Figure 5.5A. This sequence differs from an independently derived *c-jun* sequence (Nishimura *et al*, 88) at several positions. Within the coding region, these mutations introduce five amino acid substitutions but have no effect on the final length or reading frame of the encoded protein. These alterations include: an alanine to proline substitution in the *c-jun*-specific δ domain; a triplet amino acid substitution in the previously identified E regulatory region (Baichwal *et al*, 92); and a further glycine to

FIGURE 5.4

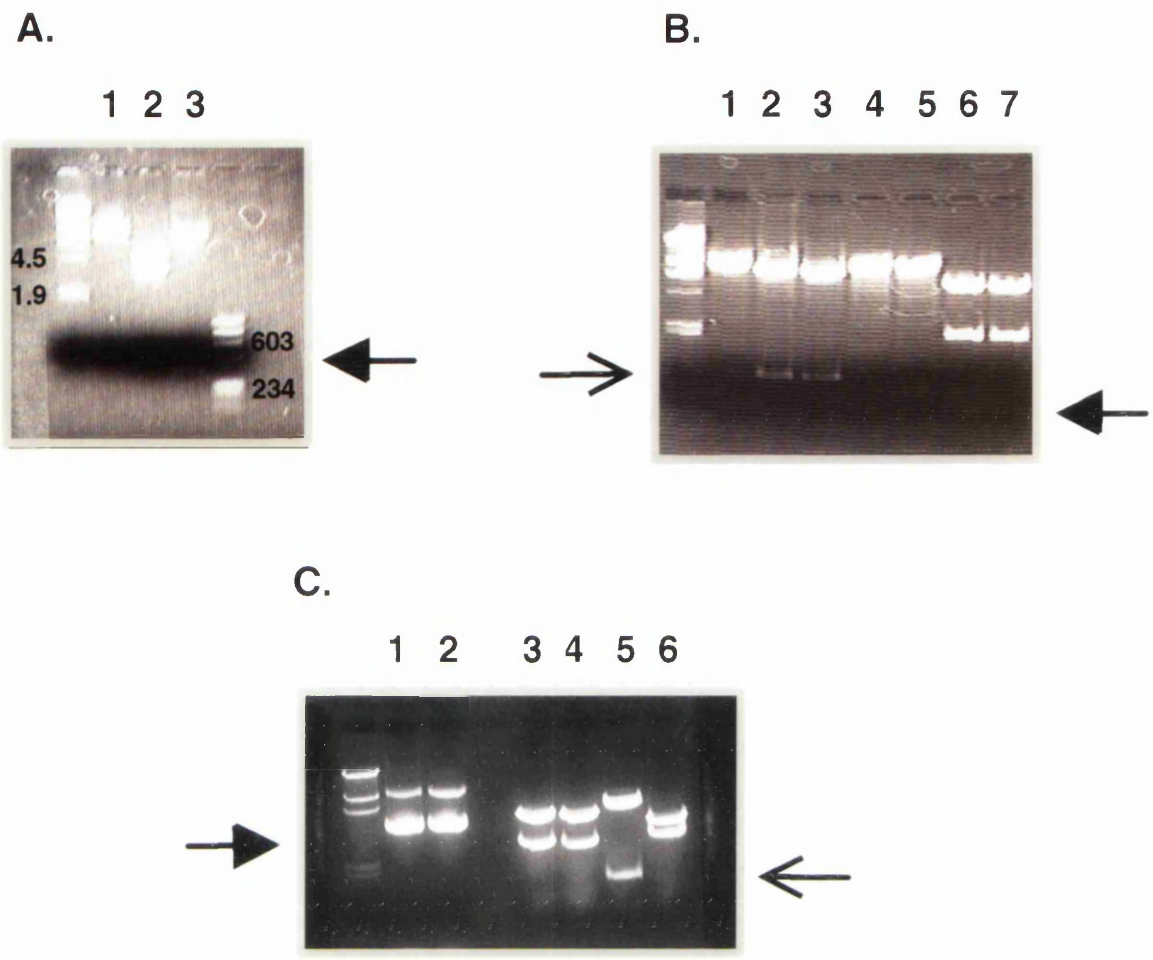
Restriction endonuclease map of the avian c-jun gene cloned into pSPT19 at the HindIII restriction site (pSPT19-c-jun/Clone A). Avian c-jun sequences are denoted in black; and pSPT19 sequences and polylinker restriction sites in green.

Panel A.
pSPT19-c-jun/Clone A undigested (1), or digested with SacI(3). Lane 2 illustrates a SacI digestion of avian c-jun subcloned into pSPT19 in the antisense orientation. The 400bp SacI fragment is denoted by a solid arrowhead. Bacteriophage λ (HindIII digested) and bacteriophage ϕ X1174 (HaeIII digested) marker tracks are illustrated in the left and right hand lanes respectively.

Panel B.
pSPT19 / Clone A digested with EcoRI (1), NcoI (2), NcoI & EcoRI (3), AccI (4), AccI & EcoRI (5), XbaI (6) and XbaI & EcoRI (7). The internal 1000bp NcoI fragment is denoted by a light arrowhead; and the internal 400bp XbaI fragment by a bold arrowhead. A bacteriophage λ (HindIII digested) marker track is illustrated in the left hand lane.

Panel C.
pSPT19 / Clone A undigested (1), and digested with HindIII (3) or EcoRI & Bgl11 (5). Even numbered lanes denote the parallel digestions of avian c-jun subcloned into pSPT19 in the antisense orientation. The 5kb HindIII avian c-jun cloning fragment is denoted by a bold arrowhead; and the 1.5kb EcoR1/Bgl11 fragment by a light arrowhead. A bacteriophage λ (HindIII digested) marker track is illustrated in the right hand lane.

The sizes of the restriction fragments are estimations approximated from the bacteriophage λ and ϕX174 marker tracks. The restriction enzymes are denoted by their first letters, except for EcoRI which is denoted by an R.


A.
1 2 3
4.5
1.9
603
234
B.
1 2 3 4 5 6 7
C.
1 2 3 4 5 6

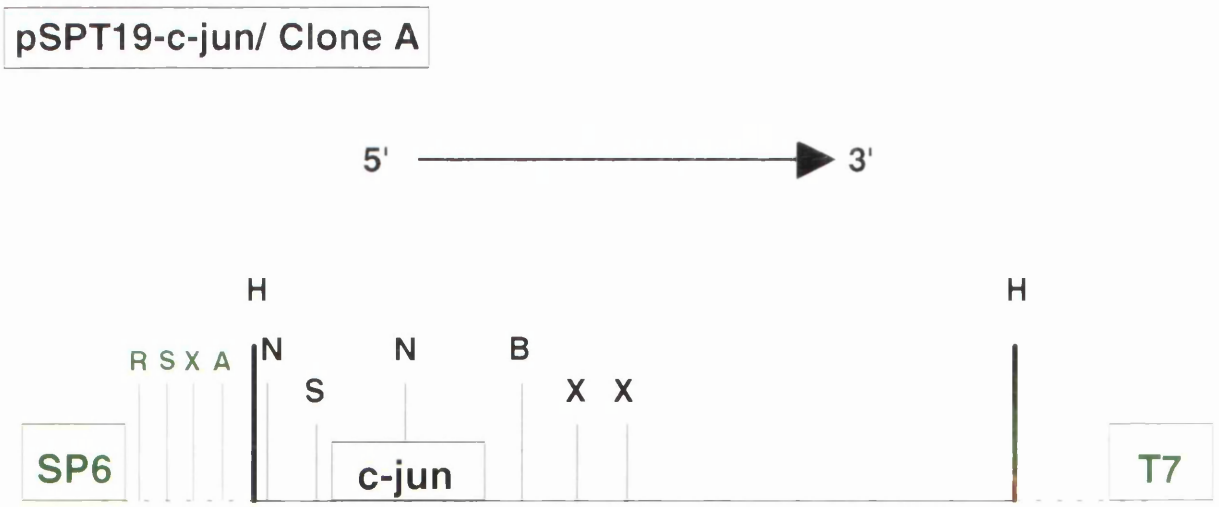

pSPT19-c-jun/ Clone A
5'
3'
H
H
R S X A
N
N
B
S
X X
SP6
c-jun
T7

FIGURE 5.4

FIGURE 5.5A

Primary sequence of avian c-jun obtained from genomic clone pSPT19/Clone A. The top panel contains partial 5' flanking sequences, including the downstream c-jun TRE-like site and adjacent RSRE (boxed in black and red respectively). Sequence variations from an independently derived c-jun sequence (Nishimura *et al*, 88) are underlined in red.
The c-jun coding sequence is illustrated in the lower panel. The initiation and termination codons are boxed in green. Sequence variations from the published sequence are underlined in red. The corresponding amino acid alterations are depicted by numbered blue codons and are presented in full in Figure 5.5B.

-202

ctaggggcgg ggtggccatg gcgaccggct gctatgcacc gggccgggcc

agcggccgct cccccgggag aggcggcggt acgaatgggg agcccgcggg

gtgacatcat ggctatttt tagcgggctc ccggtacgct gataagtgaa -8

gggctgcacg ccgcgagcgg gctcagagcg cggggcgggc gggcggcagt

+290

gggctctgtt ctatgagtgc aaaatggag cctactttct acgaggatgc

cctgaacgcc agcttcgcgc cgccggagag cggcggctat ggatataata

acgccaaggt gctgaagcag agcatgacgc tgaacctgtc cgacccggcc

agcagcctga agccgcacct gaggaacaag aacgccgaca tcctcacctc

ccccgacgtg gggctgctga agctggcctc cccggagctg gaacggctca

tcatccagtc cagcaacggg ttaatcacca ccacgccgac cccgacgcag

ttcctctgcc ccaagaacgt taccgacgag caagaggggt tcgccgacat

ggtcgtgaga gcgctggcgg aactgcacaa ccagaacacg ctgcccagcg

tcacctcagc cgcacaacct gttagcggcg gcatggcacc tgtgtcctcc

atggccggcg gcggcagctt caacacgagt ttgcacagcg agcccccggt

gtatgccaat ctcagcaact tcaaccccaa cgcgctcaac tccgcaccca

actacaacgc caacgcgatg ggctacgcgc cgcagcatca cataaacccc

cagatgcccg tgcagcatcc caggcttcag gctctgaaag aagagcctca

gactgtacct gaaatgccgg gggaaacccc tcccctgtcc cctattgaca

tggagtcgca ggagagaatc aaagccgaga gaaaacgcat gagaaacaga

attgcggcgt ccaaatgccg gaaaaggaag ttggaaagga ttgccaggtt

ggaagaaaaa gtgaaaactt tgaaagccca gaactcagag ctggcatcca

cggccaacat gctcagagaa caggttgcac agcttaagca gaaggtcatg

aaccatgtca acagcgggtg ccagctaatg ctaacacaac agttgcaaac

gtttgaaaga gacggactta aataggaact gtgatgttgt ggtataacca

aac +1292

FIGURE 5.5A

alanine substitution in a downstream region not associated with any previously recognized c-Jun functional domains. A comparison of the amino acid sequence of this avian *c-jun* clone with other animal species revealed that four out of the five amino acid alterations were not conserved (Figure 5.5B). This was surprizing as identical sequence alterations were also found in two independent *c-jun* cDNAs sequenced by Dr E.J. Black (Dr E.J. Black; *personal commun.*).

Sequence alterations found in the 5' untranslated region included three insertions, each duplicating the previous base pair; one deletion, and five G - C transversions. The changes do not affect the sequence of the conserved downstream junTRE-like site and adjacent junRSRE (Figure 5.5A - top panel).

V.B.2. DNase I HYPERSENSITIVE SITE ANALYSIS OF THE *c-jun* PROMOTER

As an initial step towards identifying potential regulatory sites within the *c-jun* gene, genomic DNA was prepared from CEF nuclei which had been digested with increasing concentrations of DNase I. Avian *c-jun* resides on a 5kb HindIII fragment and a 20kb EcoRI fragment (Dr D.A.F. Gillespie; *personal commun.*). To locate the position of potential DNase I hypersensitivity sites within these fragments, equal quantities of DNase I treated genomic DNA were digested with HindIII or EcoRI and resolved on 1% agarose gels for southern blotting analysis. The presence and location of DNase I hypersensitivity sites were mapped using *c-jun*-specific probes, derived from the 5' and the 3' region of the avian *c-jun* genomic clone (Figure 5.7B). As shown in Figure 5.6, increasing concentrations of DNase I did not reveal any hypersensitivity sites in HindIII digested

FIGURE 5.5B

A comparison of the amino acid sequence of pSPT19 c-jun/Clone A (ckjun) with the published sequences of avian, human, and murine c-Jun. The blue box represents a discrepancy between the sequence of c-jun-Clone A and the c-Jun sequence established by Nishimura *et al.* Proline is present at this position in all other known species of c-Jun. The red boxes represent other sequence discrepancies in c-jun/Clone A that introduce alterations into the amino acid sequence of avian c-Jun. The sequence discrepancies were confirmed independently through sequencing of c-jun cDNAs by Dr E.J. Black.

Codon	Nishimura *et al*	pSPT19-c-Jun/ CLONE A	Domain
1	Alanine	Proline	δ domain
2	Glutamic acid	Aspartic acid	ε domain
3	Glycine	Methionine	ε domain
4	Phenylalanine	Valine	ε domain
5	Glycine	Alanine	-

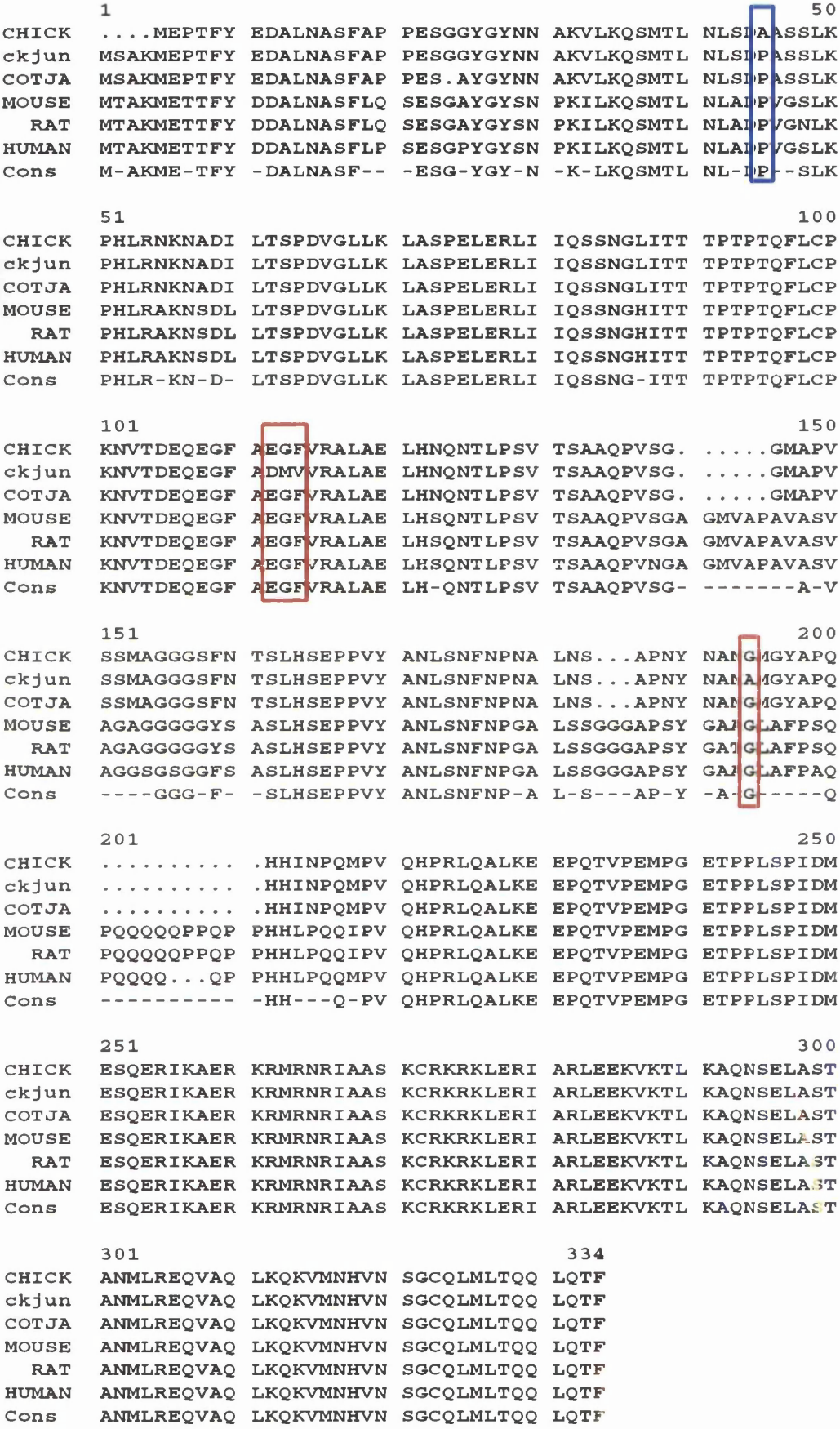

1 50
CHICK ....MEPTFY EDALNASFAP PESGGYGYNN AKVLKQSMTL NLSDAASSLK
ckjun MSAKMEPTFY EDALNASFAP PESGGYGYNN AKVLKQSMTL NLSDPASSLK
COTJA MSAKMEPTFY EDALNASFAP PES.AYGYNN AKVLKQSMTL NLSDPASSLK
MOUSE MTAKMETTFY DDALNASFLQ SESGAYGYSN PKILKQSMTL NLADPVGSLK
RAT MTAKMETTFY DDALNASFLQ SESGAYGYSN PKILKQSMTL NLADPVGNLK
HUMAN MTAKMETTFY DDALNASFLP SESGPYGYSN PKILKQSMTL NLADPVGSLK
Cons M-AKME-TFY -DALNASF-- -ESG-YGY-N -K-LKQSMTL NL-DP--SLK
51 100
CHICK PHLRNKNADI LTSPDVGLLK LASPELERLI IQSSNGLITT TPTPTQFLCP
ckjun PHLRNKNADI LTSPDVGLLK LASPELERLI IQSSNGLITT TPTPTQFLCP
COTJA PHLRNKNADI LTSPDVGLLK LASPELERLI IQSSNGLITT TPTPTQFLCP
MOUSE PHLRAKNSDL LTSPDVGLLK LASPELERLI IQSSNGHITT TPTPTQFLCP
RAT PHLRAKNSDL LTSPDVGLLK LASPELERLI IQSSNGHITT TPTPTQFLCP
HUMAN PHLRAKNSDL LTSPDVGLLK LASPELERLI IQSSNGHITT TPTPTQFLCP
Cons PHLR-KN-D- LTSPDVGLLK LASPELERLI IQSSNG-ITT TPTPTQFLCP
101 150
CHICK KNVTDEQEGF AEGFVRALAE LHNQNTLPSV TSAAQPVSG. .....GMAPV
ckjun KNVTDEQEGF ADMVVRALAE LHNQNTLPSV TSAAQPVSG. .....GMAPV
COTJA KNVTDEQEGF AEGFVRALAE LHNQNTLPSV TSAAQPVSG. .....GMAPV
MOUSE KNVTDEQEGF AEGFVRALAE LHSQNTLPSV TSAAQPVSGA GMVAPAVASV
RAT KNVTDEQEGF AEGFVRALAE LHSQNTLPSV TSAAQPVSGA GMVAPAVASV
HUMAN KNVTDEQEGF AEGFVRALAE LHSQNTLPSV TSAAQPVNGA GMVAPAVASV
Cons KNVTDEQEGF AEGFVRALAE LH-QNTLPSV TSAAQPVSG- -------A-V
151 200
CHICK SSMAGGGSFN TSLHSEPPVY ANLSNFNPNA LNS...APNY NANGMGYAPQ
ckjun SSMAGGGSFN TSLHSEPPVY ANLSNFNPNA LNS...APNY NANAMGYAPQ
COTJA SSMAGGGSFN TSLHSEPPVY ANLSNFNPNA LNS...APNY NANGMGYAPQ
MOUSE AGAGGGGGYS ASLHSEPPVY ANLSNFNPGA LSSGGGAPSY GAAGLAFPSQ
RAT AGAGGGGGYS ASLHSEPPVY ANLSNFNPGA LSSGGGAPSY GAAGLAFPSQ
HUMAN AGGSGSGGFS ASLHSEPPVY ANLSNFNPGA LSSGGGAPSY GAAGLAFPAQ
Cons ----GGG-F- -SLHSEPPVY ANLSNFNP-A L-S---AP-Y -A-G-----Q
201 250
CHICK .... .HHINPQMPV QHPRLQALKE EPQTVPEMPG ETPPLSPIDM
ckjun .... .HHINPQMPV QHPRLQALKE EPQTVPEMPG ETPPLSPIDM
COTJA .... .HHINPQMPV QHPRLQALKE EPQTVPEMPG ETPPLSPIDM
MOUSE PQQQQQPPQP PHHLPQQIPV QHPRLQALKE EPQTVPEMPG ETPPLSPIDM
RAT PQQQQQPPQP PHHLPQQIPV QHPRLQALKE EPQTVPEMPG ETPPLSPIDM
HUMAN PQQQQ...QP PHHLPQQMPV QHPRLQALKE EPQTVPEMPG ETPPLSPIDM
Cons ---------- -HH---Q-PV QHPRLQALKE EPQTVPEMPG ETPPLSPIDM
251 300
CHICK ESQERIKAER KRMRNRIAAS KCRKRKLERI ARLEEKVKTL KAQNSELAST
ckjun ESQERIKAER KRMRNRIAAS KCRKRKLERI ARLEEKVKTL KAQNSELAST
COTJA ESQERIKAER KRMRNRIAAS KCRKRKLERI ARLEEKVKTL KAQNSELAST
MOUSE ESQERIKAER KRMRNRIAAS KCRKRKLERI ARLEEKVKTL KAQNSELAST
RAT ESQERIKAER KRMRNRIAAS KCRKRKLERI ARLEEKVKTL KAQNSELAST
HUMAN ESQERIKAER KRMRNRIAAS KCRKRKLERI ARLEEKVKTL KAQNSELAST
Cons ESQERIKAER KRMRNRIAAS KCRKRKLERI ARLEEKVKTL KAQNSELAST
301 334
CHICK ANMLREQVAQ LKQKVMNHVN SGCQLMLTQQ LQTF
ckjun ANMLREQVAQ LKQKVMNHVN SGCQLMLTQQ LQTF
COTJA ANMLREQVAQ LKQKVMNHVN SGCQLMLTQQ LQTF
MOUSE ANMLREQVAQ LKQKVMNHVN SGCQLMLTQQ LQTF
RAT ANMLREQVAQ LKQKVMNHVN SGCQLMLTQQ LQTF
HUMAN ANMLREQVAQ LKQKVMNHVN SGCQLMLTQQ LQTF
Cons ANMLREQVAQ LKQKVMNHVN SGCQLMLTQQ LQTF

FIGURE 5.5B

FIGURE 5.6

DNase I Hypersite analysis of partial DNase I digested genomic DNA prepared from primary CEFs in exponential growth. The DNA was digested with HindIII (panels A & B) or EcoRI (panel C), and resolved on a 1% agarose gel. The Southern Blots were hybridized with labelled DNA probes obtained from avian c-jun Clone A (Figure 5.7B). Southern Blot A was hybridized with a 1.5kb HindIII/Bgl11 probe; Blot B with a 1.9kb Nsi1 probe and Blot C with a 3.5kb HindIII/Bgl11 probe. The 5kb HindIII and the 20kb EcoRI c-jun containing fragments are illustrated by labelled arrowheads. Bacteriophage λ (HindIII digested) marker tracks correspond to the left hand lanes of each blot. Hypersites 1 & 2 (HS-1, HS-2) are labelled on Southern Blot C.

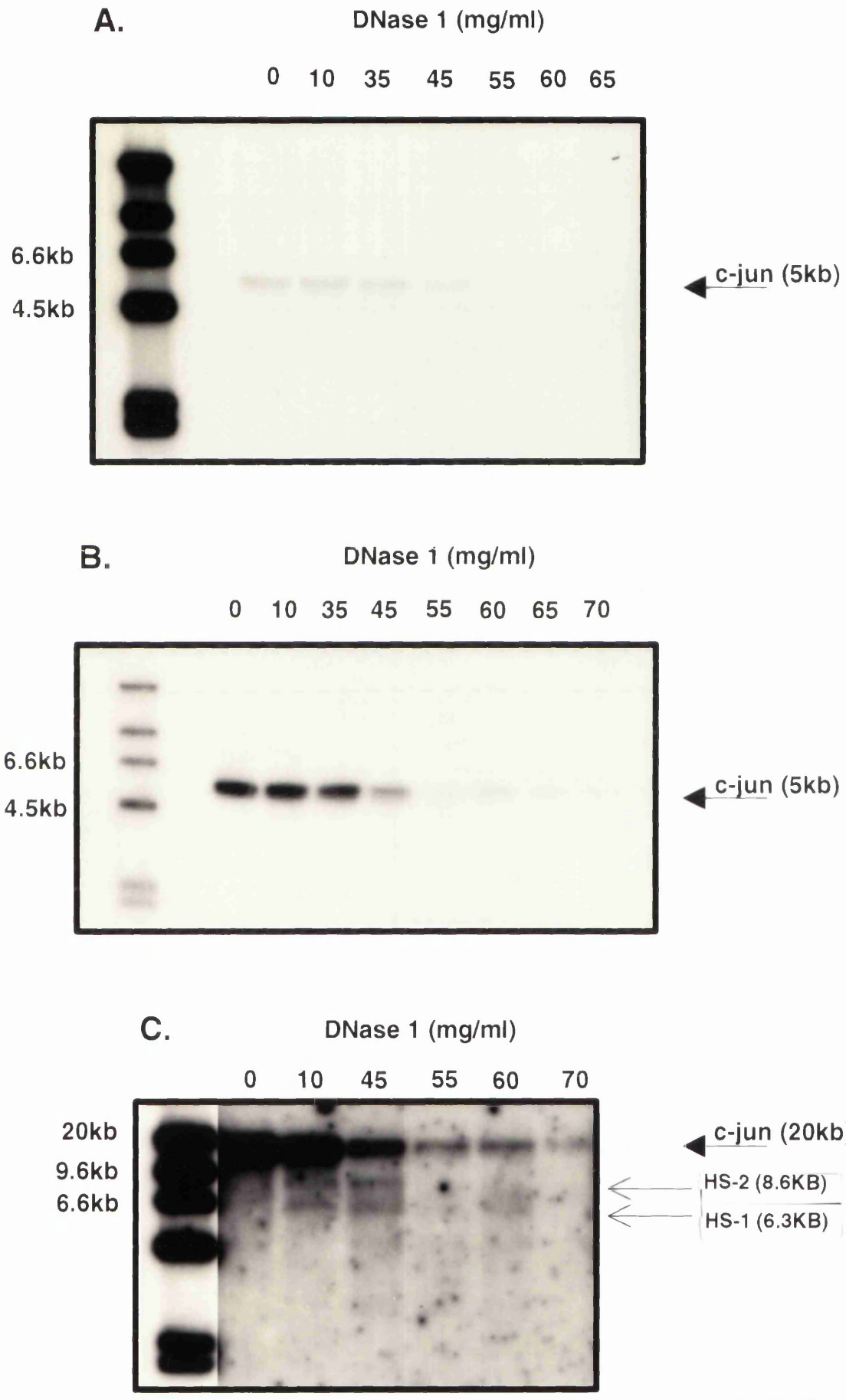

A.
DNase 1 (mg/ml)
0 10 35 45 55 60 65
6.6kb
4.5kb
c-jun (5kb)
B.
DNase 1 (mg/ml)
0 10 35 45 55 60 65 70
6.6kb
4.5kb
c-jun (5kb)
C.
DNase 1 (mg/ml)
0 10 45 55 60 70
20kb
9.6kb
6.6kb
c-jun (20kb)
HS-2 (8.6KB)
HS-1 (6.3KB)

FIGURE 5.6

genomic DNA. The gradual disappearance of the 5kb HindIII fragment indicated that successful digestion had occured, but that no specific sites within the DNA were preferentially digested under these conditions. A different pattern was observed for the 20kb EcoRI fragment. EcoRI digested DNA exhibited two DNase I hypersensitivity sites, positioned approximately 6.4kb and 8.6kb from an adjacent EcoRI site. The appearance of the bands was transient which suggests that both fragments presented highly accessible target sequences for further DNase I digestion.

The precise location of each DNase I hypersensitivity site relative to avian *c-jun* was determined by orientating *c-jun* within the 20kb EcoRI restriction fragment. To this end genomic DNA was digested with the same panel of restriction enzymes previously used to map the position and orientation of *c-jun* within the 5kb HindIII cloning fragment (Figure 5.4). Each restriction enzyme was combined with EcoRI or HindIII to locate the internal restriction sites relative to an undefined and a defined flanking site. The position of avian *c-jun* relative to the EcoRI sites was defined when the spacing between two internal restriction sites was maintained with either flanking enzyme. As shown in Figure 5.4 and 5.7A, a spacing of approximately 1.2kb was maintained between the internal Bgl11 and SacI sites. The relative position of avian *c-jun* and the two independent DNase I hypersensitivity sites was subsequently obtained, and is presented in Figure 5.7B.

As shown in Figure 5.7B, DNase I hypersensitivity sites 1 and 2 were both located to the 5' side of the *c-jun* coding region. The close proximity of DNase I hypersensitivity site 1 to the 5' HindIII restriction endonuclease cloning site positioned it within a well defined regulatory region of the *c-jun* promoter (Figure 5.8A), and may provide an explanation for its apparent absence in HindIII digested genomic DNA (Figure 5.6). The position of

FIGURE 5.7A

Southern blot analysis of double Restriction Enzyme digests of avian genomic DNA. 10ug of DNA was digested with EcoRI in combination with a panel of restriction enzymes, previously used to map the HindIII fragment containing avian c-jun (Figure 5.4). The Southern Blot was hybridized with a random primed oligolabelled DNA probe corresponding to the 3.5kb HindIII/Bgl11 fragment of avian c-jun Clone A (Figure 5.7B). Bacteriophage λ (HindIII digested) markers are illustrated in the left hand lane.

FIGURE 5.7B

The orientation and position of the c-jun coding region with respect to the EcoRI and HindIII flanking restriction sites. Sequences to the 3' side of the 5' Hind111 site are drawn to scale. The positions of the proposed DNase I Hypersites are denoted by HS-1 and HS-2. The DNA probes used in the hypersite analysis are represented by broken double arrowheads and drawn to scale.

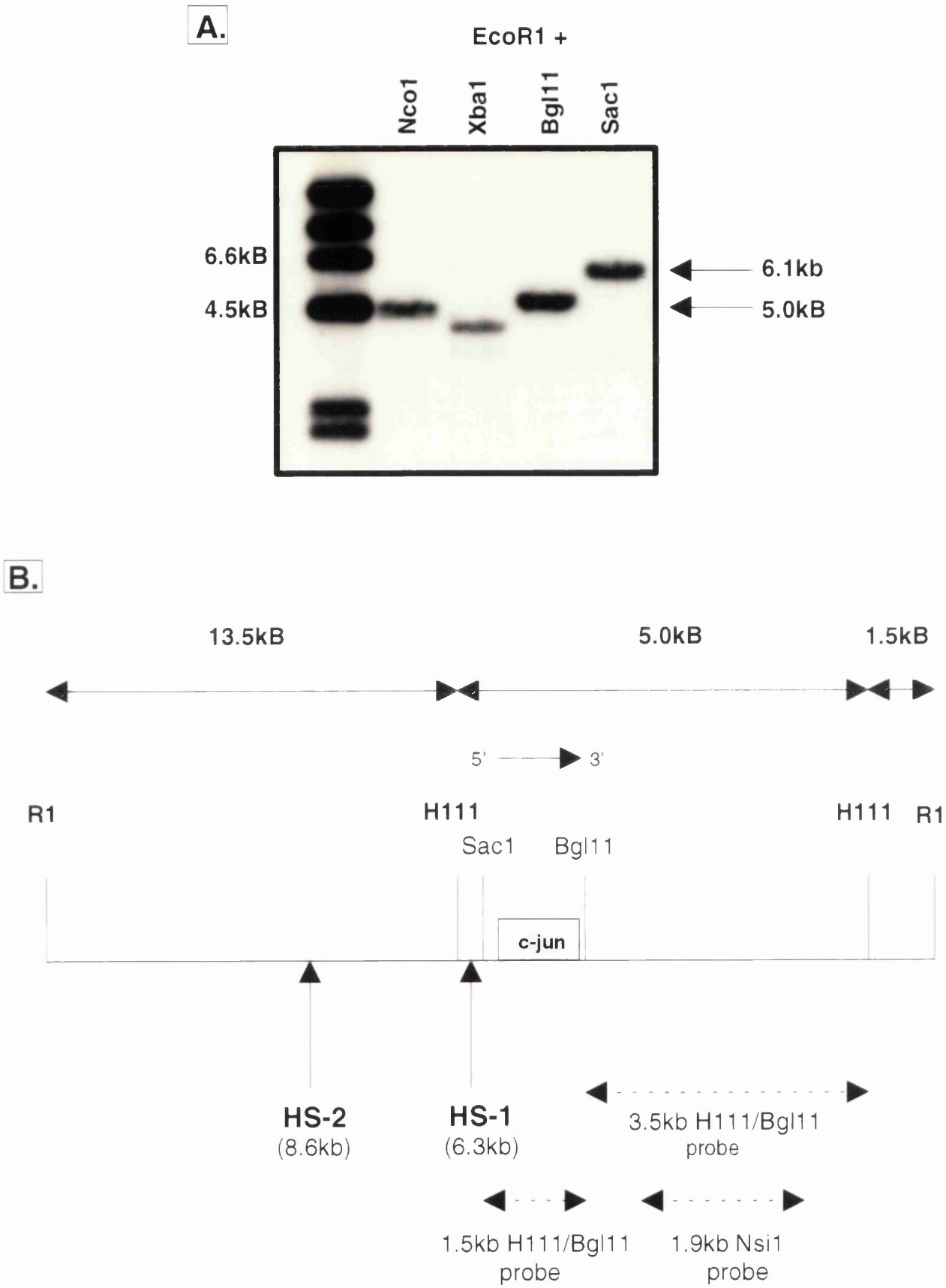

A.
EcoR1 +
Nco1
Xba1
Bgl11
Sac1
6.6kB
4.5kB
6.1kb
5.0kB
B.
13.5kB
5.0kB
1.5kB
5'
3'
R1
H111
H111
R1
Sac1
Bgl11
c-jun
HS-2
(8.6kb)
HS-1
(6.3kb)
3.5kb H111/Bgl11
probe
1.5kb H111/Bgl11
probe
1.9kb Nsi1
probe

FIGURE 5.7

DNase I hypersensitivity site 2 was less informative as it was located within a region of uncharacterized DNA at least 2.4kb upstream of the *c-jun* transcription start site.

V.C. *c-jun* PROMOTER SITE OCCUPANCY IN c-JUN-EXPRESSING (NORMAL) AND c-JUN-LACKING (ASV17-TRANSFORMED) CEFS

V.C.1. In Vitro DNA binding Analysis

Sequencing and mapping analyses identified the position of avian *c-jun* gene within the 5kb HindIII cloning fragment. As shown in Figure 5.4, the 5' HindIII site was located approximately 200bp upstream of the start site of transcription. The intervening sequence represents a region of the *c-jun* promoter that contains several highly conserved, potential regulatory sites (Figure 5.8A). Their likely importance was underlined by the presence of a DNase I hypersensitivity site within close proximity of the 5' HindIII cloning site (HS-1; Figure 5.7B).

To investigate the level of *c-jun* promoter site occupancy in *c-jun*-expressing (normal) cells, a panel of oligonucleotide probes corresponding to the conserved regions of the *c-jun* promoter were synthesized and incubated in a series of in vitro DNA binding reactions with normal CEF extracts. The pattern of binding was compared to the pattern obtained with ASV17-transformed CEF extracts to establish whether there were any changes in DNA binding activity which correlated with auto-repression.

FIGURE 5.8A

Sequence comparison of the published c-jun promoter sequences from avian, rodent and human DNA. The bottom line of each panel illustrates the regions of complete homology between the different species. The downstream TRE-like site and adjacent RSRE are boxed in blue and green respectively. The extended L oligonucleotide is denoted by a black arrowhead; the TRE containing M oligonucleotide by a blue arrowhead; and the RSRE containing R olignucleotide by a green arrowhead. Oligonucleotide probes corresponding to additional regions of homology in the cloned c-jun promoter sequences are boxed in black and labelled on the figure.

con-1	Consensus 1			
con-2	Consensus 2	-	NF-jun site	(Brach *et al*, 92)
Sp-1	Sp-1 site	-	Sp-1 site	(Angel *et al*, 88)
TATA	TATA-like	-	TATA-like	(Angel *et al*, 88)
INR	INR-like	-	Initiator-like site	(Seto *et al*, 91)

The avian promoter sequences subcloned for DNase I footprinting analysis are contained within the two bold red arrowheads. The 5' arrowhead corresponds to the extreme 5' HindIII cloning site of pSPT19/Clone A (Figure 5.4). The sequence was amplified from avian c-jun Clone A, tagged with 5' HindIII and 3' XbaI restriction sites, and subcloned into pSPT19 at the HindIII and XbaI cloning sites, in the antisense orientation (pSPT19/cJ-fp).

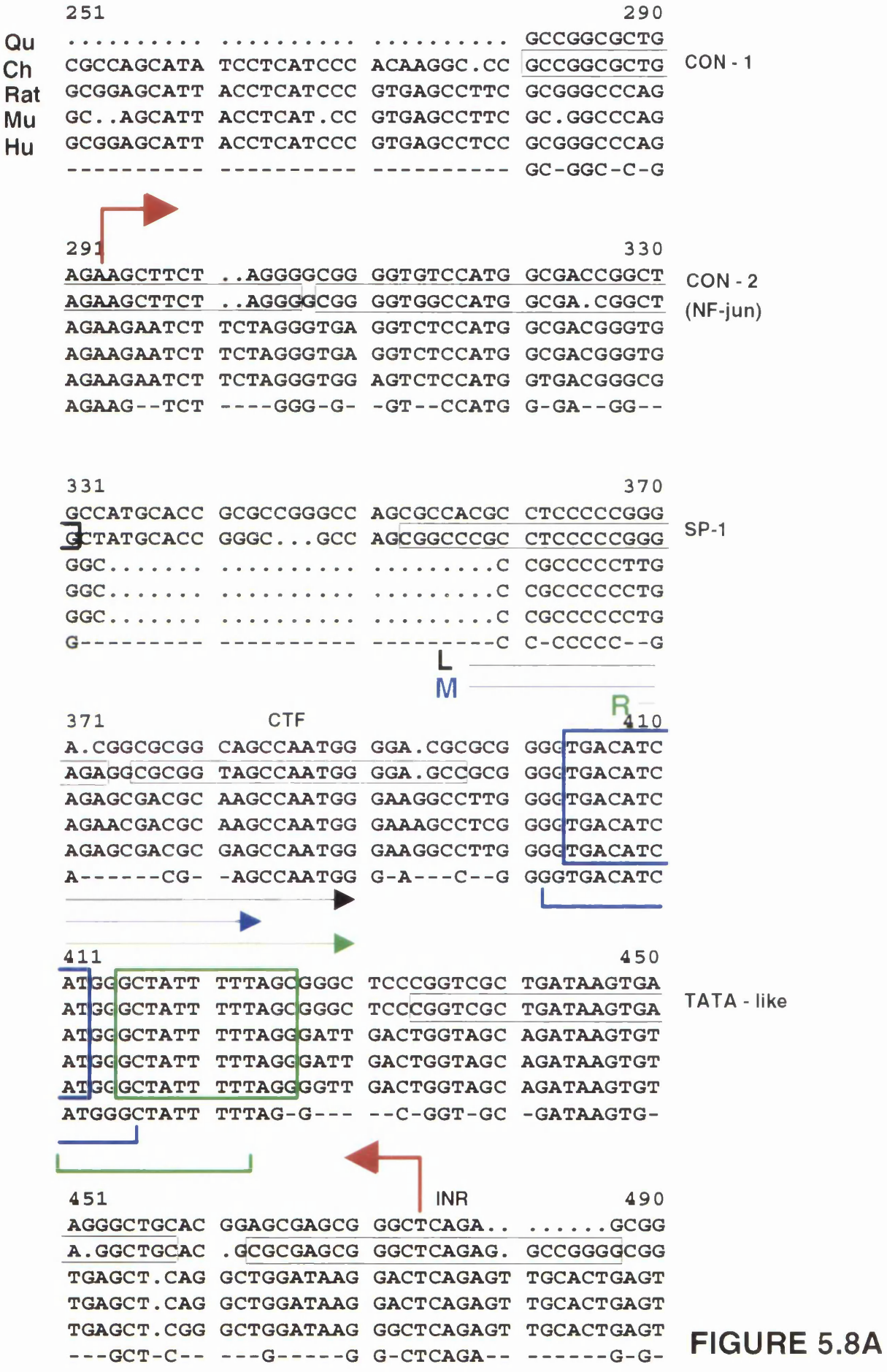

251
290
Qu .... GCCGGCGCTG
Ch CGCCAGCATA TCCTCATCCC ACAAGGC.CC GCCGGCGCTG
Rat GCGGAGCATT ACCTCATCCC GTGAGCCTTC GCGGGCCCAG
Mu GC..AGCATT ACCTCAT.CC GTGAGCCTTC GC.GGCCCAG
Hu GCGGAGCATT ACCTCATCCC GTGAGCCTCC GCGGGCCCAG
---------- ---------- ---------- GC-GGC-C-G
CON - 1
291
330
AGAAGCTTCT ..AGGGGCGG GGTGTCCATG GCGACCGGCT
AGAAGCTTCT ..AGGGGCGG GGTGGCCATG GCGA.CGGCT
AGAAGAATCT TCTAGGGTGA GGTCTCCATG GCGACGGGTG
AGAAGAATCT TCTAGGGTGA GGTCTCCATG GCGACGGGTG
AGAAGAATCT TCTAGGGTGG AGTCTCCATG GTGACGGGCG
AGAAG--TCT ----GGG-G- -GT--CCATG G-GA--GG--
CON - 2
(NF-jun)
331
370
GCCATGCACC GCGCCGGGCC AGCGCCACGC CTCCCCCGGG
GCTATGCACC GGGC...GCC AGCGGCCCGC CTCCCCCGGG
GGC..... ....C CGCCCCCTTG
GGC..... ....C CGCCCCCCTG
GGC..... ....C CGCCCCCCTG
G--------- ---------- ---------C C-CCCCC--G
SP-1
L
M
371
CTF
410
R
A.CGGCGCGG CAGCCAATGG GGA.CGCGCG GGGTGACATC
AGAGGCGCGG TAGCCAATGG GGA.GCCGCG GGGTGACATC
AGAGCGACGC AAGCCAATGG GAAGGCCTTG GGGTGACATC
AGAACGACGC AAGCCAATGG GAAAGCCTCG GGGTGACATC
AGAGCGACGC GAGCCAATGG GAAGGCCTTG GGGTGACATC
A------CG- -AGCCAATGG G-A---C--G GGGTGACATC
411
450
ATGGGCTATT TTTAGCGGGC TCCCGGTCGC TGATAAGTGA
ATGGGCTATT TTTAGCGGGC TCCCGGTCGC TGATAAGTGA
ATGGGCTATT TTTAGGGATT GACTGGTAGC AGATAAGTGT
ATGGGCTATT TTTAGGGATT GACTGGTAGC AGATAAGTGT
ATGGGCTATT TTTAGGGGTT GACTGGTAGC AGATAAGTGT
ATGGGCTATT TTTAG-G--- --C-GGT-GC -GATAAGTG-
TATA - like
451
INR
490
AGGGCTGCAC GGAGCGAGCG GGCTCAGA.. ......GCGG
A.GGCTGCAC .GCGCGAGCG GGCTCAGAG. GCCGGGGCGG
TGAGCT.CAG GCTGGATAAG GACTCAGAGT TGCACTGAGT
TGAGCT.CAG GCTGGATAAG GACTCAGAGT TGCACTGAGT
TGAGCT.CGG GCTGGATAAG GGCTCAGAGT TGCACTGAGT
---GCT-C-- ---G-----G G-CTCAGA-- ------G-G-

FIGURE 5.8A

The sequence of each oligonucleotide probe is presented in Figure 5.8A. Sequences were selected on the basis of their overall degree of homology to other animal species. Some, like the junTRE and junRSRE, represent previously recognized regulatory sites; whereas others, such as the CCAAT box and the Sp-1 element remain largely uncharacterized. Figure 5.8B is typical of the binding pattern obtained for several of these oligonucleotides which were consistently recognized by specific binding complexes in normal, *c-jun*-expressing CEF extracts. Similar complexes were also detected in v-Myc-transformed and ASV17- transformed CEF extracts, indicating that gross changes in *c-jun* promoter occupancy at these sites were unlikely to be responsible for auto-repression.

Two exceptions to this overall pattern of binding were provided by the junTRE and adjacent junRSRE regulatory elements. Qualitative and quantitative differences in junTRE and junRSRE binding activities were observed between normal and ASV17-transformed CEF extracts that were specific to ASV17-transformed CEFs and correlated with auto-repression (Figure 5.9 & 5.10).

- junTRE Binding Activity

A single junTRE binding complex was detected in normal CEF extracts, that was replaced by a weaker doublet in their ASV17-transformed counterparts (Figure 5.9A). The doublet was not observed in an equivalent extract prepared from v-Src transformed CEFs (CK29). The specificity of each binding activity was confirmed by competition analysis using unlabelled junTRE as a specific cold competitor (+ lanes). The qualitative and quantitative changes in junTRE binding activity detected in ASV17-transformed CEFs were also observed by DNase I footprinting analysis (Figure 5.9b). Footprinting was performed over a region of the *c-jun*

FIGURE 5.8B

Electrophoretic Mobility Shift analysis to compare the jun/CAAT and whole cell extracts. 10ug of extract was incubated in the presence(+) or absence (-) of a 100 fold excess of specific cold competitor, for 30min at $4^{o}C$. 2ul (~2 $x10^{5}$ cpm) of 5' end labelled, double stranded olignucleotide probe was added, and the contents incubated for a further 30min at $4^{o}C$. The binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at $4^{o}C$. An additional transformed CEF extract (MC29) derived from v-Myc) infected CEFs was included to control for v-Jun specific and transformation specific effects. The MC29 binding patterns are illustrated in the right hand lanes of both gels.

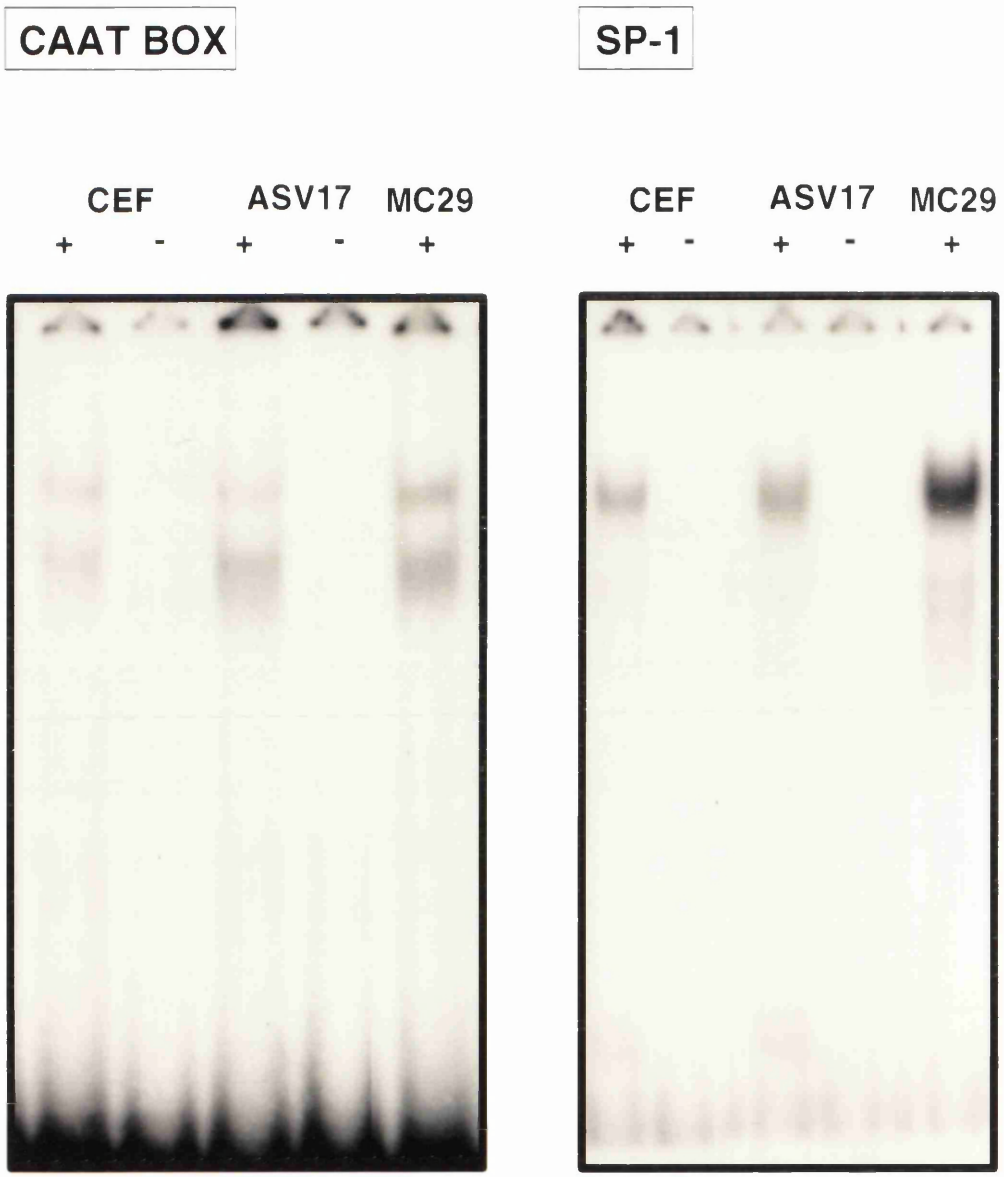


FIGURE 5.8B

FIGURE 5.9A

Electrophoretic Mobility Shift analysis to compare the junTRE binding activity in normal and ASV17 transformed whole cell extracts. 10ug of extract was incubated in the presence (+) or absence (-) of a 100 fold excess of specific cold competitor, for 30min at $4^{o}C$. 2ul (~2 $x10^{5}$ cpm) of, 5' end labelled, double stranded olignucleotide M (junTRE) was added, and the contents incubated for a further 30min at $4^{o}C$. The binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at $4^{o}C$. An additional transformed CEF extract (CK29), derived from v-Src infected CEFs, was included to control for v-Jun specific, and transformation specific effects. The CK29 TRE binding pattern is illustrated in the right hand lanes.

FIGURE 5.9B

DNase I footprinting analysis over the 171bp c-jun promoter fragment depicted in figure 5.8. The fragment was 5' end labelled on the sense strand and incubated for 60-90min on ice, in the absence (-) or presence of approximately 30ug of nuclear protein extract, prepared from normal and ASV17 transformed CEFs. The binding reactions were partially digested with DNase I, over a range of concentrations, and resolved on a 6% denaturing polyacrylamide gel. A double stranded sequencing reaction of the same strand was included to locate the positions of potential DNase1 footprints. The junTRE and adjacent RSRE are illustrated on the sequencing reaction. The dideoxy Thymidine base analogue included in the sequencing reaction is denoted by a "T". The junTRE, DNase1 protected footprint is indicated by a bracket on the right hand side of the autoradiograph.

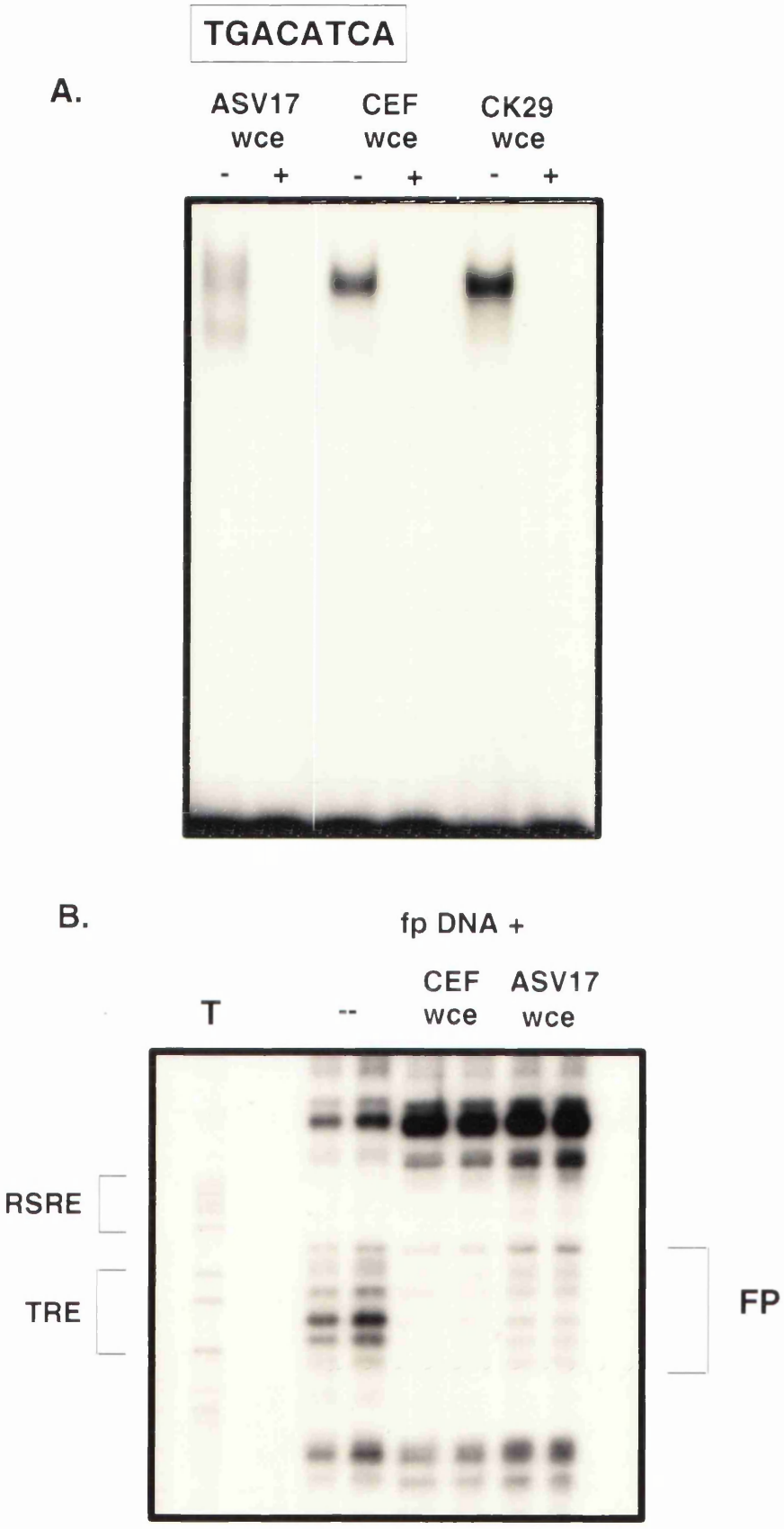

TGACATCA
A.
ASV17 wce
CEF wce
CK29 wce
- + - + - +
B.
fp DNA +
T
--
CEF wce
ASV17 wce
RSRE
TRE
FP

FIGURE 5.9

promoter identified in Figure 5.8A, which extends for 174bp downstream of the 5' HindIII cloning site and spans the jun TRE and adjacent junRSRE. As shown in Figure 5.9b, the intensity of the DNase I protected footprint over the junTRE correlated with the relative intensities of the retarded protein:DNA complexes found in normal and ASV17-transformed CEF extracts (compare Figure 5.9A & 5.9B). The protected sequence was conserved between normal and ASV17-transformed extracts, suggesting that each protein complex recognized the same junTRE binding motif in vitro.

- junRSRE Binding Activity

A junRSRE-specific binding complex was detected in normal CEF extracts that was absent in equivalent ASV17-transformed cultures (Figure 5.10A). The pattern of binding was specific to ASV17-transformed CEFs and not observed in a parallel c-Myc transformed extract.

DNase I footprinting analysis over the antisense strand of the *c-jun* promoter supported the in vitro DNA binding analysis. A DNase I protected footprint was detected using extracts prepared from normal CEFS, that encompassed the junRSRE and half of the adjacent junTRE. An equivalent pattern of protection was observed using ASV17-transformed CEF extracts. The similarity is presumed to result from an additional junRSRE-specific binding activity that was detected in both normal and ASV17-transformed CEFs (Figure 5.10A). Parallel digestions over the sense strand of the *c-jun* promoter were uninformative, as the junRSRE is refractory to DNase I digestion on this strand (Figure 5.9B; Flemington *et al*, 90; Rozek *et al*, 93; Herr *et al*, 94).

FIGURE 5.10A

Electrophoretic Mobility Shift analysis to compare the binding pattern of normal and ASV17 transformed cell extracts to the RSRE binding site in the c-jun promoter. 10ug of whole cell extract was incubated in the presence(+) or absence (-) of a 100 fold excess of specific cold competitor, for 30min at 4^{o}C. 2ul (~2 x 105 cpm) of 5' end labelled double stranded oligonucleotide R was added, and the contents incubated for a further 30min at 4^{o}C. The binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at 4^{o}C. c-Myc (wt Myc) and v-Myc (MC29) transformed CEF extracts were included in the binding reactions to control for v-Jun-specific and transformation-specific effects. A junRSRE binding complex specific to normal CEF extracts is indicated by a bold arrowhead. An additional junRSRE-specific complex detected in both cell types is indicated by a broken arrowhead

Figure 5.10B

DNase I footprinting analysis over the antisense strand of the 171bp c-jun promoter fragment illustrated in figure 5.8. The antisense strand was 5' end labelled and incubated in the absence (-) or presence (+) of approximately 30ug of nuclear protein extract, prepared from normal and ASV17 transformed CEFs. The binding reactions were partially digested with DNase I over a range of concentrations, and resolved on a 6% denaturing polyacrylamide gel. A double stranded sequencing reaction of the same strand was included as before (Figure 5.9B). The dideoxy base analogues included in each reaction are denoted by their first letters: Adenine, Cytosine, Guanine, Thymidine. The junTRE and RSRE binding sites are marked on the sequencing reaction. The junRSRE DNase I protected footprint is indicated by a bracket on the right hand side of the autoradiograph.

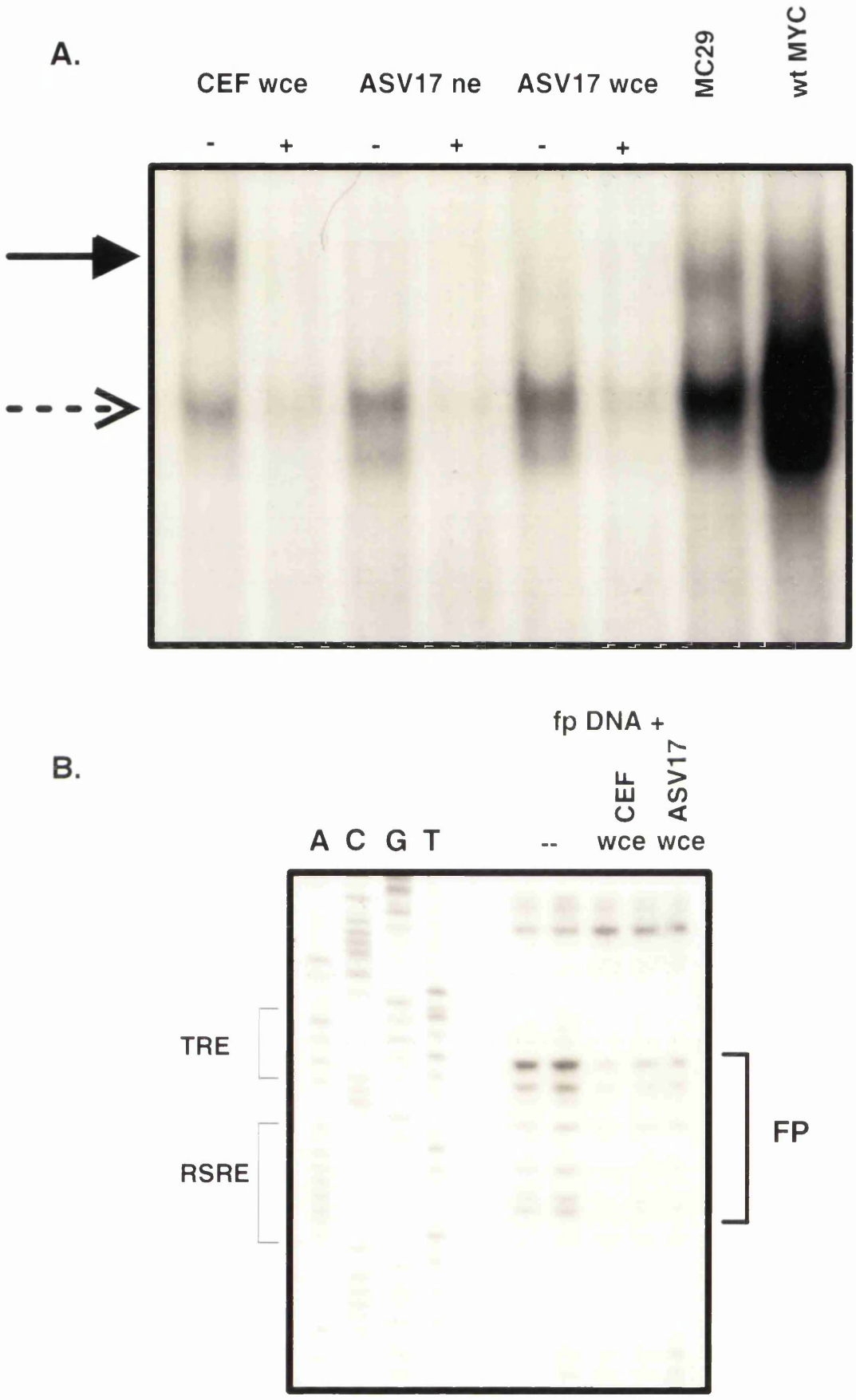

A.
CEF wce
ASV17 ne
ASV17 wce
MC29
wt MYC
-
+
-
+
-
+
B.
fp DNA +
CEF
ASV17
A C G T
--
wce
wce
TRE
RSRE
FP

FIGURE 5.10

IV.C.2. ANALYSIS OF THE RELATIONSHIP BETWEEN junTRE AND junRSRE BINDING ACTIVITIES IN NORMAL CEF EXTRACTS

The close proximity (Figure 5.8A) and overlapping DNase I protected footprints (Figure 5.9A & 5.10A) of the junTRE and junRSRE binding sites, prompted an investigation into whether both sites could be occupied simultaneously. To this end an extended oligonucleotide was synthesized that spanned both potential regulatory elements (Figure 5.8A). Oligonucleotide L was incubated in an in vitro DNA binding reaction with normal CEF extracts, and the pattern of binding compared to that obtained for the two individual sites. In support of previous analyses, the junTRE (M) and junRSRE (R) were recognized by specific DNA binding complexes in normal CEF extracts (Figure 5.11A). The junTRE was bound more avidly than the junRSRE, but both complexes were competed equally by their respective unlabelled oligonucleotides. The extended L oligonucleotide was recognized by two independent binding complexes which exhibited virtually equivalent mobilities in non-denaturing acrylamide gels as the individual junTRE- and junRSRE-specific binding activities. Since cooperative binding between adjacently bound factors would appear as a single retarded complex of an altered mobility, this pattern suggests that the junTRE and junRSRE operate as mutually exclusive binding sites when the L-oligonucleotide is in probe excess. The relative intensities of the junTRE and junRSRE binding complexes did not completely support this hypothesis. As shown in Figure 5.11A, the affinity of the junRSRE binding complex was increased relative to the junTRE binding complex, in the context of the L oligonucleotide, suggesting that an element of cooperativity may exist between the junTRE and junRSRE binding sites in vivo.

FIGURE 5.11A

Electrophoretic Mobility Shift Analysis to compare the binding pattern of normal CEF extracts to the junTRE (M), junRSRE (R), and junTRE & junRSRE (L) containing double stranded oligonucleotides (Figure 5.8). 10ug of whole cell extract was incubated in the presence (+) or absence (-) of a 100 fold excess of specific cold competitor for 30min at 4°C. 2ul (~2 x 105 cpm) of 5' end labelled oligonucleotide probe was added to the reaction and the contents incubated for a further 30min at 4°C. The binding reactions were resolved on a 4% non denaturing polyacrylamide gel at 4°C. The junRSRE binding complex is denoted by a bold arrowhead; and the junTRE binding complex by a light arrowhead.

FIGURE 5.11B

DNase1 footprinting analysis over the sense strand of the 171bp c-jun promoter fragment described in figure 5.8. 30ug of CEF nuclear protein extract was incubated with 1ug of a specific cold competitor oligonucleotide for 30min at 4°C. 1ul of 5' end labelled footprinting probe was added to the reaction and the contents incubated for a further 60min at 4°C. The binding reactions were partially digested with a range of DNase1 concentrations, and resolved on a 6% denaturing polyacrylamide gel. The junTRE and RSRE binding sites are indicated on the sequencing reaction at the left hand side of the autoradiograph and the junTRE DNase1 protected footprint by a bracket on the right hand side of the autoradiograph.

			---------- COLD COMPETITOR ----------			
Lane	**Probe**	**CEF Nuclear Extract**	**RSRE (R)**	**TRE (M)**	**RSRE + TRE (L)**	**CM1 (Myc "E" Box sequence)**
1	+	-	-	-	-	-
2	+	+	-	-	-	-
3	+	+	+	-	-	-
4	+	+	-	-	-	-
5	+	+	-	+	-	-
6	+	+	-	-	+	-
7	+	+	-	-	-	+

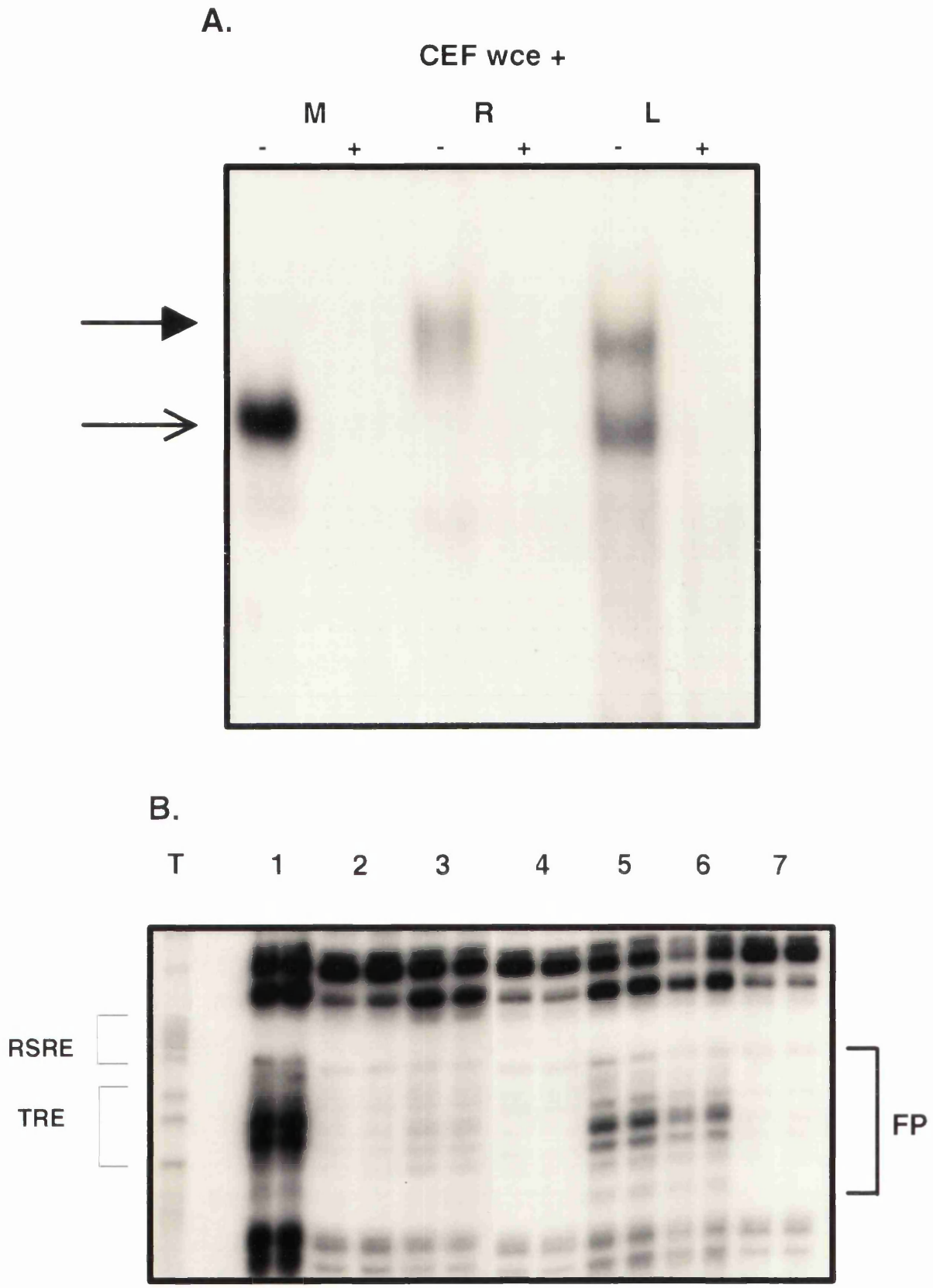


FIGURE 5.11

A pattern of competitive binding to the junTRE and junRSRE regulatory elements was supported by DNase I footprinting analysis using extracts prepared from normal CEFs (Figure 5.11B). The DNase I protected footprint corresponding to the junTRE binding site was competed by cold oligonucleotides spanning the junTRE (M) or the junTRE and adjacent junRSRE (L), but was virtually unaffected by equivalent concentrations of oligonucleotides containing the junRSRE (R), or an unrelated, c-Myc binding "E" Box sequence. As shown in lane 3, the junTRE binding complex was weakly competed by the junRSRE-containing oligonucleotide. The level of competition was reduced relative to the junTRE-containing oligonucleotide but did support the proposal that an element of cooperativity may exist between the two sites.

Mutually exclusive binding of the junTRE- and junRSRE-specific binding activities was further supported by gel retardation and cross-competition analysis. Cold oligonucleotides representing the junTRE (M), the junRSRE (R) and the combined sites (L), were used as competitors in binding reactions between labelled "M", "R" or "L" oligonucleotides and normal CEF extracts. As shown in Figure 5.12, the junTRE and junRSRE binding complexes were specifically competed by their respective cold oligonucleotide probes, and by the extended "L" oligonucleotide spanning both sites. An identical pattern of competition was also observed on the extended "L" oligonucleotide thereby confirming the identity of the junTRE- and junRSRE-specific protein complexes and their pattern of mutually exclusive binding.

FIGURE 5.12

Cross-competition analysis to investigate the binding specificity of junTRE and junRSRE binding activities in normal CEF extracts. 10ug of whole cell extract was incubated in the presence or absence (-) of a 100 fold excess of specific or non-specific cold competitor oligonucleotides for 30min at 4°C. 2ul (~2 x 105 cpm) of 5' end labelled, double stranded, oligonucleotide probe was added, and the contents incubated for a further 30min at 4°C. Panel A represents a binding reaction using olignucleotide M as probe (junTRE); panel B a binding reaction using oligonucleotide R as probe (junRSRE); and panel C, a binding reaction using the combined oligonucleotide L as probe (junTRE/RSRE). The nature of the cold competitors included in each binding reaction are indicated on the figure. Cold competitors, CM1 and SRE, were gifted by W. Clark and Dr. A. Lang, and correspond to the Myc "E" Box consensus sequence and the Serum Response Element respectively.

The binding reactions were resolved on a 4% non-denaturing poly-acrylamide gel at 4°C. The junTRE specific binding complex is denoted by a bold arrowhead and the junRSRE specific binding complex by a light arrowhead.

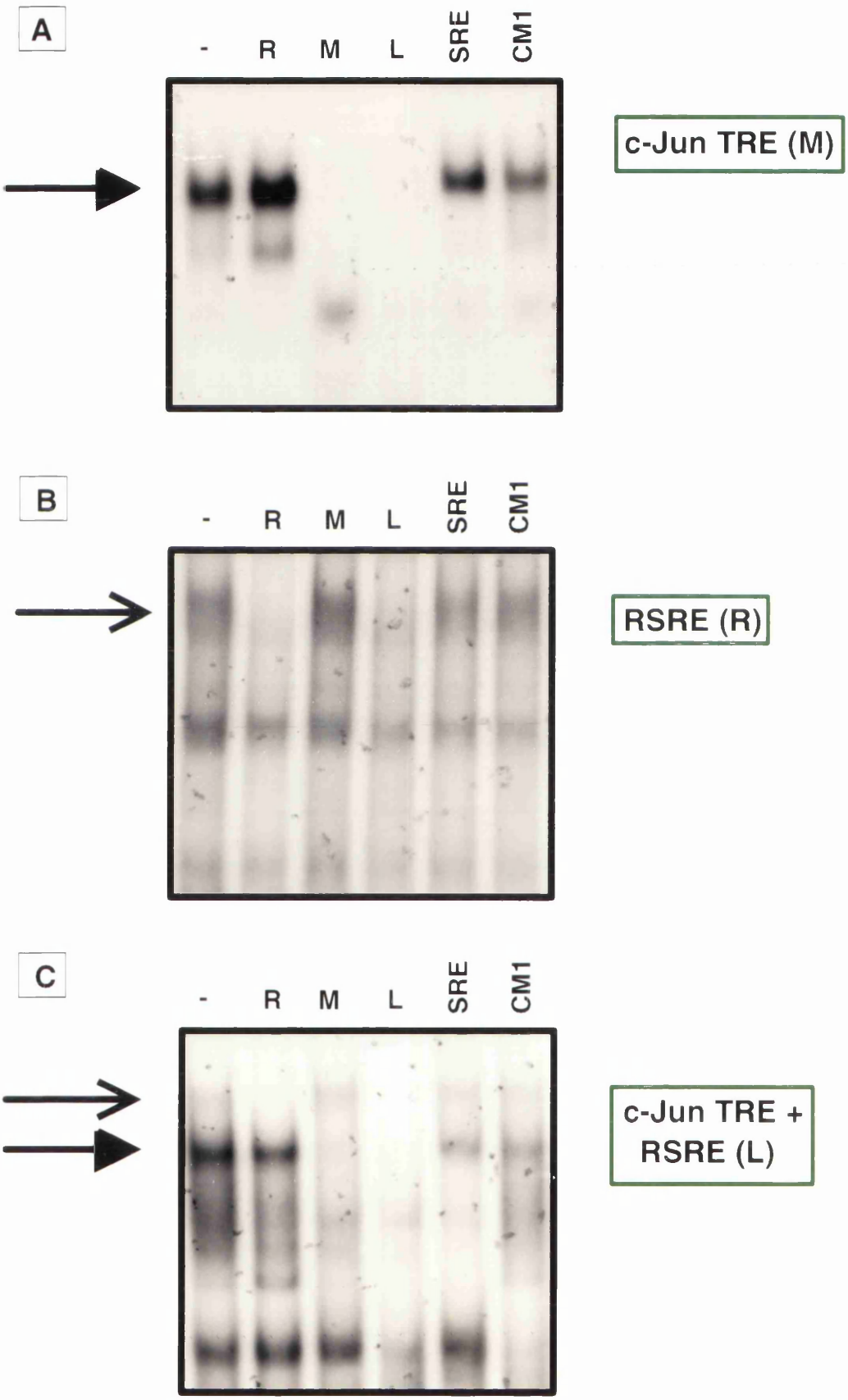

A
-
R
M
L
SRE
CM1
c-Jun TRE (M)
B
-
R
M
L
SRE
CM1
RSRE (R)
C
-
R
M
L
SRE
CM1
c-Jun TRE +
RSRE (L)

FIGURE 5.12

IV.C.3. CHARACTERIZATION OF THE junTRE AND junRSRE BINDING COMPLEXES

- junTRE Binding Complexes

To investigate the nature of the junTRE binding complexes present in normal and ASV17-transformed CEF extracts, a panel of different polyclonal antisera was included in the in vitro DNA binding reactions. As shown in Figure 5.13, the junTRE binding complexes in ASV17-transformed cell extracts, appeared identical to complexes II and III formed using the collagenase TRE (compare Figure 3.16B with 5.13). Like colTRE complexes II and III, the junTRE binding complexes were completely disrupted by c-Jun-specific antisera, partially disrupted by a Fos/FRA-specific antiserum and the upper complex disrupted by a gag-specific antiserum. The comparable antibody reactivity and electrophoretic mobility of the junTRE- and colTRE-specific protein complexes present in ASV17-transformed extracts, suggested that both contained the same composition of binding proteins. A contrasting pattern was observed for the junTRE binding activity detected in normal CEF extracts. The appearance of the junTRE binding complex closely resembled that of the collagenase TRE binding complex 1 in terms of electrophoretic mobility, but the antibody reactivity profile was quite distinct. The junTRE binding complex was partially disrupted by the c-Jun-specific antisera and only very weakly disrupted by the Fos/FRAspecific antiserum (Figure 5.13). This indicated that non-c-Jun-containing complexes constituted at least part of the junTRE binding activity in normal CEF extracts. ColTRE complex I, in contrast, was shown to consist predominantly of c-Jun-containing complexes (Figure 3.16A & 3.16B). These observations suggest that normal, but not ASV17-transformed, CEFs express a variety

FIGURE 5.13

Electrophoretic Mobility Shift analysis to compare the composition of junTRE binding activities in normal and ASV17 transformed extracts. 10ug of whole cell extract was incubated in the presence of 2ul of rabbit polyclonal antisera for 30min at 4^{o}C. 2ul (~2x10^{5} cpm) of 5' end labelled, double stranded oligonucleotide M (junTRE) was added and the contents incubated for a further 30min at 4^{o}C. The binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at 4^{o}C.

	LANE					
Antisera	**1**	**2**	**3**	**4**	**5**	**6**
-	+					
PI		+				
α-cJun(948-4)			+			
α-cJun(730/5)				+		
α-Fos(388/4)					+	
α-gag(5202)						+

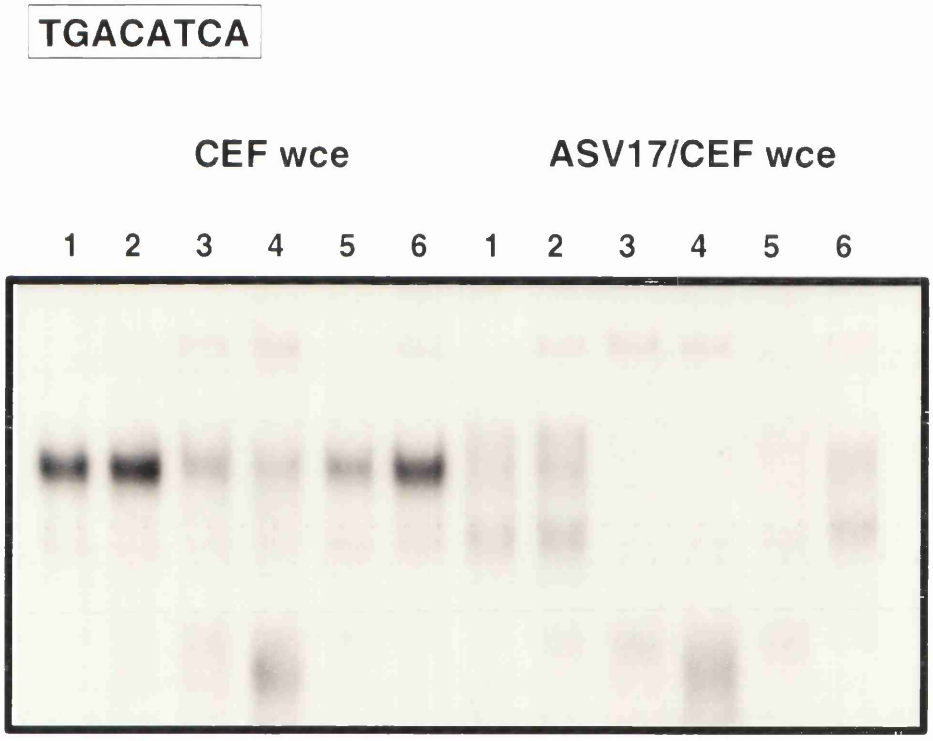


FIGURE 5.13

of different TRE binding proteins that preferentially combine to form complexes with alternative specificities for individual TRE binding sites.

- junRSRE Binding Complexes

To investigate the nature of the junRSRE binding complex in normal CEF extracts, an extended panel of different polyclonal antisera was included in the in vitro DNA binding reactions. The panel was also incubated in a parallel series of junTRE binding reactions as it included antisera not previously tested. As shown in Figure 5.14A, the junRSRE binding complex was completely disrupted by an antiserum raised against the human Related Serum Response Factor C4 (RSRF-C4). This showed that the junRSRE binding activity in normal CEFs was predominantly comprised of RSRF-related protein complexes. A weaker disruption of the junRSRE binding complex was observed with the Fos/FRA-specific antibody (lane 6). This was unexpected as the Fos and FRA proteins are not recognized dimerization partners of the RSRF transcription factor family and have not been reported to bind to the junRSRE in vitro. The remaining antisera, including a FRA2-specific antibody, had no effect on the junRSRE binding activity.

Parallel binding reactions using the junTRE, confirmed previous analyses (Figure 5.13), and demonstrated that non-c-Jun-containing complexes predominate in the normal junTRE binding activity (Figure 5.14B). Two additional antisera, anti FRA2 and anti RSRF, had no effect, indicating that neither protein contributed to the junTRE binding activity present in normal CEF extracts.

FIGURE 5.14A

Electrophoretic Mobility Shift analysis to investigate the composition of junRSRE binding activities in normal CEF extracts. 10ug of whole cell extract was incubated in the presence of 2ul of rabbit polyclonal antisera for 30min at 4°C. 2ul (~2x10^5 cpm) of 5' end labelled, double stranded oligonucleotide R (junRSRE) was added and the contents incubated for a further 30min at 4°C. The binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at 4°C.

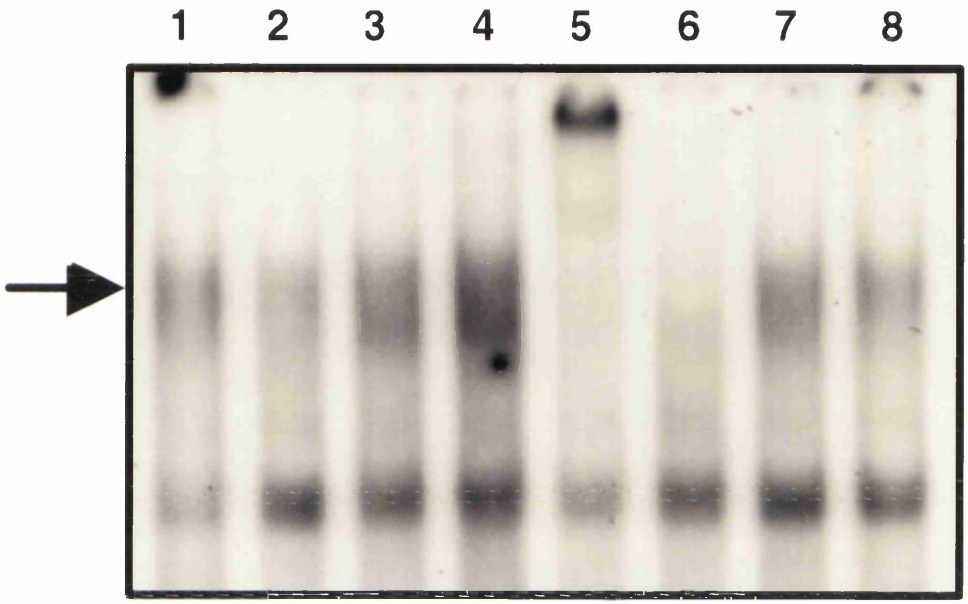
FIGURE 5.14B

Electrophoretic Mobility Shift analysis to investigate the composition of junTRE binding activities in normal CEF extracts. 10ug of whole cell extract was incubated in the presence of 2ul of rabbit polyclonal antisera for 30min at 4°C. 2ul (~2x10^5 cpm) of 5' end labelled, double stranded oligonucleotide M (junTRE) was added, and the contents incubated for a further 30min at 4°C. The binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at 4°C.

	LANE							
Anti-sera	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
-	+							
PI		+						
α-cJun(948-4)			+					
α-cJun(730/5)				+				
α-RSRF					+			
α-Fos(388/4)						+		
α-gag(5202)							+	
α-FRA2								+

A.

junRSRE (R)



B.

junTRE (M)

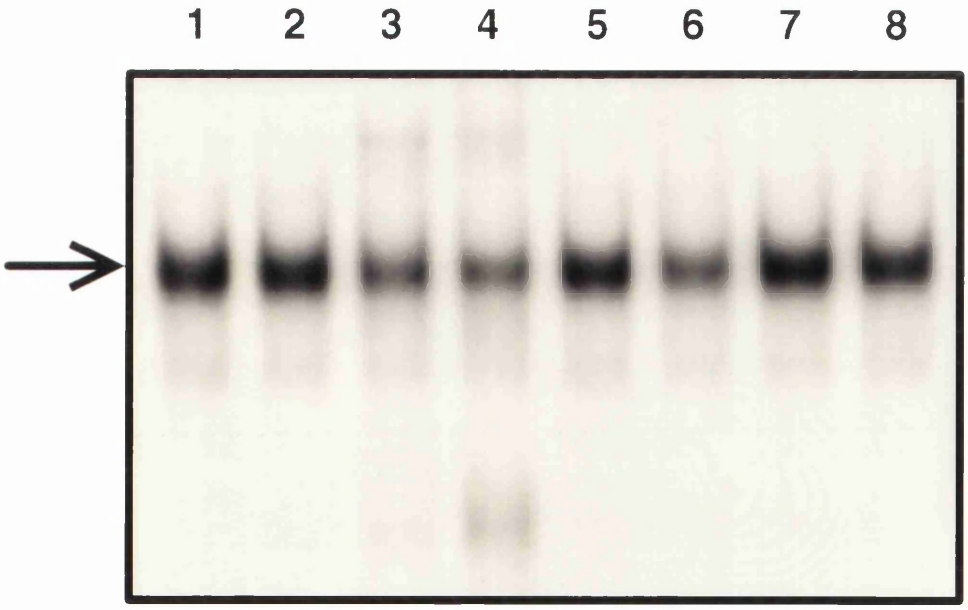


FIGURE 5.14

IV.C.4. MECHANISM OF v-JUN SPECIFIC REPRESSION OF junRSRE BINDING ACTIVITY

- RSRF Expression in Primary CEFs

To determine whether the absence of detectable junRSRE binding activity in ASV17-transformed CEFs was caused by a v-Jun-specific down-regulation of RSRF protein expression, western blot analysis was performed on normal and ASV17-transformed cell extracts. A HeLa cell extract was included in the analysis as a positive control as these cells have been reported to express high levels of RSRF-C4 (Dr. R. Treisman; *personal commun.*). RSRF protein expression was detected using a polyclonal RSRF-specific antiserum kindly provided by Dr R. Treisman. Anti RSRF-C4 was raised against the carboxy-terminal domain of human RSRF-C4. The peptide sequence is shared by an alternatively spliced varient of RSRF-C4: RSRF-C9, but not by an another member of the RSRF gene family, RSRF-C2, which is transcribed from an independent gene (Pollock *et al*, 91; Yu *et al*, 92). As shown in Figure 5.15, RSRF protein was detected in normal and ASV17-transformed extracts, and in a parallel culture of c-Myc transformed CEFs. The migration of the avian RSRF protein was distinct from the protein detected in HeLa cells, and did not conform exactly to the predicted molecular weight of RSRF-C4. RSRF-C4 has a calculated molecular mass of 52kD but undergoes extensive post-translational modification in vivo that alter the mobility of the protein in SDS/acrylamide gels (Dr. R. Treisman; *personal commun.*). Consequently it is not possible to determine whether the RSRF expressed in avian cells represents RSRF-C4 or an alternatively spliced varient such as RSRF-C9 (Pollock *et al*, 91; Yu *et al*, 92). It was clear, however, that the level of RSRF expression did not vary between normal and ASV17-transformed

FIGURE 5.15

Western Blot Analysis of denatured whole cell protein extracts. The left hand lanes (x3) represent denatured extracts prepared directly from a lysed T25 tissue culture flask (section II.D.1c). Approximately equal concentrations of each extract were loaded per lane. Protein concentrations were estimated from a Coomassie Blue stained protein gel. The right hand lanes (x5) represent whole cell protein extracts prepared for electrophoretic mobility shift analysis and subsequently denatured in SDS sample buffer. 20ug of non denatured extract was diluted in a final volume of 50ul of 1x SDS sample buffer, and sonnicated as described in section II.D.1c. The extract was cleared in a bench top microfuge and the contents loaded into a single gel lane. The HeLa cell extract was kindly provided by T Jamison and the c-Myc infected CEF extract from Dr D. Crouch. PC1 and PV2 represent extracts prepared from first round soft agar clones derived from PC/SFCV and PV/SCFV transfected CEFs (Figure 3.13A).

The samples were resolved on a 9% acrylamide gel and transferred to ECL nylon membrane using a CAM LAB Semi-Dry Blotter. The proteins were visualized by ECL detection with a 1:2000 dilution of anti-RSRF. The presumed human RSRFC4 is denoted by a bold arrowhead and the putative avian RSRF by a light arrowhead.

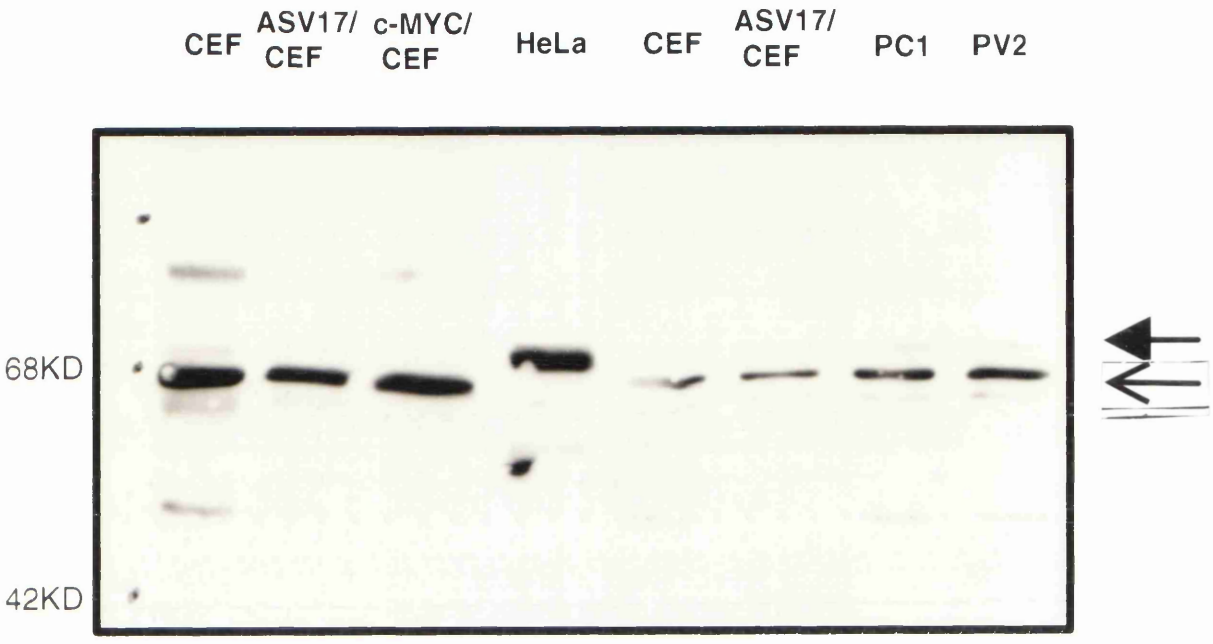


FIGURE 5.15

cell extracts (compare lanes 1 & 2 and lanes 5 & 6), indicating that repression of RSRF protein expression was unlikely to be responsible for the absence of junRSRE binding activity in ASV17-transformed CEFS.

- junRSRF Inhibitory Factor(s)

To investigate whether a repressor of RSRF binding activity was present in ASV17-transformed CEFs, normal extracts were mixed with transformed cell extracts and incubated in a standard binding reaction with labelled junRSRE-containing oligonucleotide (R). Parallel binding reactions were assembled in the presence or absence of binding buffer and incubated at 30°C for 15min prior to the addition of labelled probe. These conditions mimic those of an in vitro dimerization reaction (sectionII.D.4a), and were included to ensure the detection of potential inhibitory factors that might otherwise have been masked by standard gel mobility shift assay conditions (section II.D.6b).

As shown in Figure 5.16, the junRSRE binding activity in normal CEF extracts was consistently unaffected by the presence of ASV17-transformed cell extract, even though the transformed cell extracts exhibited no junRSRE binding activity. These results demonstrated that the absence of junRSRE binding activity in transformed cell extracts was not due to the presence of an inhibitory factor which was active in vitro.

- junRSRF Accessory Factors

Immunological analysis of the RSRE binding activity in normal CEF extracts suggested the presence of an RSRF-related transcription factor and a Fos/FRA related protein (Figure 5.14A). To investigate the function of the Fos/FRA component, bacterially expressed c-Jun and/or bzipFos,

FIGURE 5.16

Electrophoretic Mobility Shift analysis to investigate whether an inhibitor of junRSRE binding activity is present in ASV17 transformed CEF extracts. 5ug of CEF extract (1), ASV17 transformed CEF extract (2), or both cell extracts (3) were incubated in the presence of 2ul (~2.0 x10^5 cpm) 5' end labelled, double stranded oligonucleotide R (junRSRE). Three independent incubation protocols were performed to optimize the conditions of binding.

Left Hand Lanes (x3)- Incubate extracts for 30min at 4^{o}C

Middle Lanes (x3)- Incubate extracts for 15min at 30^{o}C; add binding reagents and probe, and incubate for a further 30min at 4^{o}C.

Right Hand Lanes (x3) - Incubate binding reagents and extract for 15min at 30^{o}C; add probe and incubate for a further 30min at 4^{o}C.

The binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at 4^{o}C. The RSRE specific binding activity is denoted by a solid arrowhead.

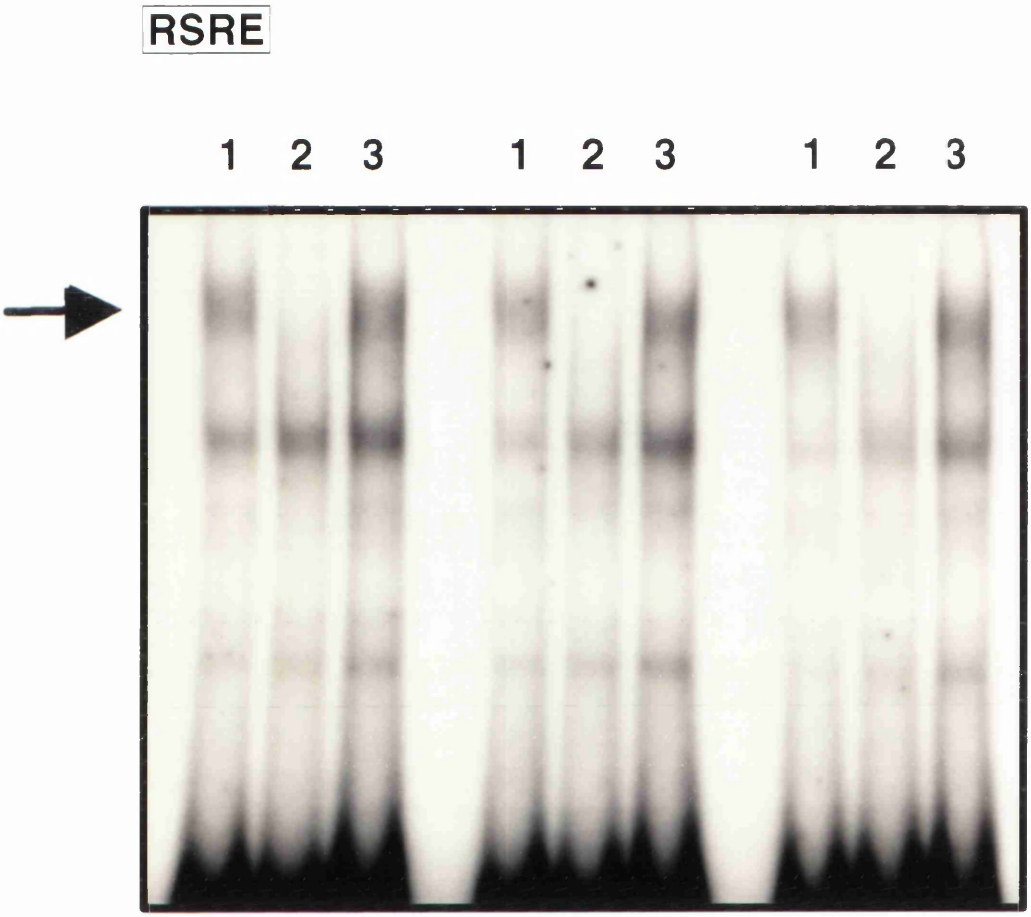


FIGURE 5.16

containing the basic region and adjacent leucine zipper, were included in the binding reactions to determine whether occupancy of the adjacent junTRE or titration of the Fos/FRA-related protein would effect the integrity of the junRSRE-bound protein complex.

As shown in Figure 5.17A, junRSRE binding activity in normal and ASV17-transformed cell extracts, was unaffected by the addition of exogenous c-Jun or bzipFos in the context of the minimal junRSRE-containing oligonucleitide (R). This was not a consequence of inefficient exogenous protein dimerization as c-Jun alone, or in combination with bzipFos, bound strongly to the collagenase TRE (lanes 11-13). The TRE binding activity of bzipFos was undetectable in the absence of full length c-Jun, consistent with the inability of c-Fos to homodimerize in vitro (Reviewed in Curran, 91).

Exogenous c-Jun and bzipFos had a contrasting effect in the context of the extended oligonucleotide, L (Figure 5.17B). Using this oligonucleotide, the junRSRE binding complex in normal CEFs was completely disrupted by the addition of c-Jun (compare lanes 3 & 7) or the co-addition of c-Jun and bzipFos (compare lanes 3 & 9). bzipFos alone had no effect on the integrity of the junRSRE-specific complex (compare lanes 3 & 5) but did markedly disrupt the junTRE binding activity in ASV17-transformed CEF extracts, presumably due to the formation of v-Jun/bzipFos heterodimers (compare lanes 4 & 6). Perhaps significantly, bzipFos did not disrupt the junTRE binding activity in normal CEFs (compare lanes 3 & 5), thereby supporting the proposal that normal and ASV17-transformed CEFs express distinct junTRE binding activities in vitro (section IV.C.3). The junTRE binding activities detected in normal and ASV17-transformed CEFs were similarly distinguished by the addition of exogenous c-Jun. Full length c-Jun had virtually no effect on the junTRE binding activity in ASV17-transformed CEFs, suggesting that c-Jun homodimers or c-Jun/v-

FIGURE 5.17A

Electrophoretic Mobility Shift analysis to investigate the effects of exogenous Jun and Fos proteins on the junRSRE binding activity in normal (odd numbers) and ASV17 transformed (even numbers) CEF extracts. 10ug of extract was incubated with approximately 2.5ug bacterially expressed protein for 15min at 30^{o}C. 2ul (~2.0 x10^{5} cpm) 5' end labelled, double stranded oligonucleotide R (junRSRE) was added and the binding reaction incubated for a further 30min at 4^{o}C. Lanes 11-13 were included as a positive control to monitor the intrinsic DNA binding activities of bacterially expressed Jun and bzip-c-Fos to an end labelled collagenase TRE probe.

The binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at 4^{o}C. The RSRE specific binding activity is denoted by a red arrowhead; the bzipFos/c-Jun colTRE binding activity by a green arrowhead; and the c-Jun colTRE binding activity by a black arrowhead.

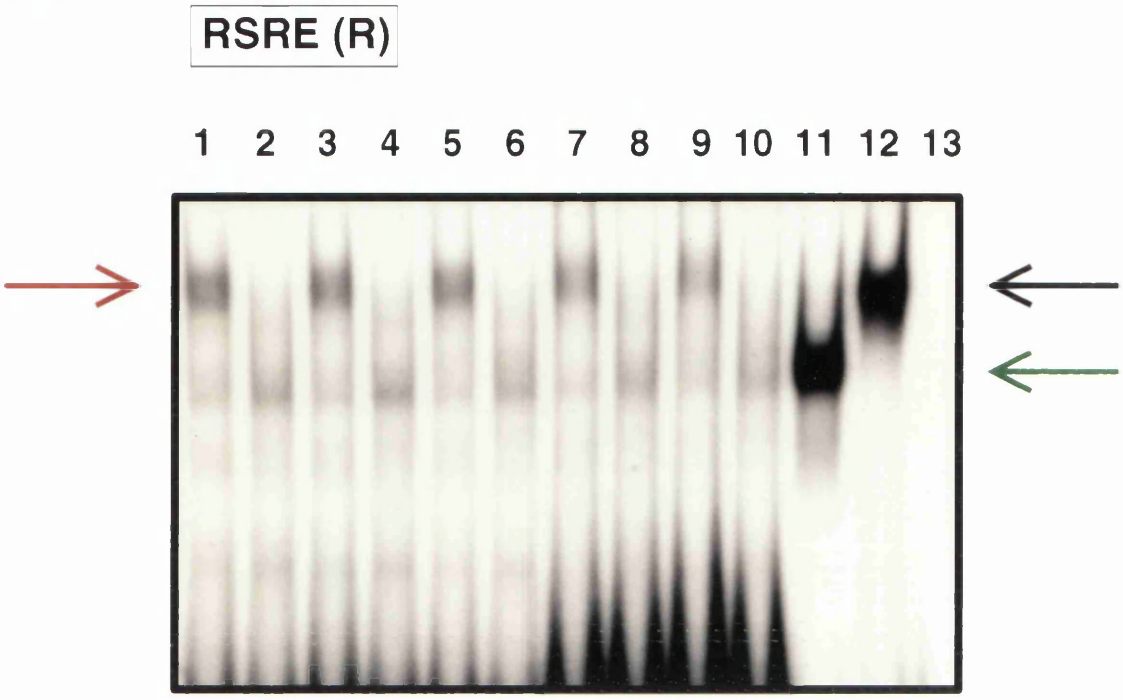
A table identifying the exogenous proteins added per lane is located at the end of Figure Legend 5.17B

FIGURE 5.17B

Electrophoretic Mobility Shift analysis to investigate the effects of exogenous Jun and Fos proteins on the junTRE and junRSRE binding activities in normal (odd numbers) and ASV17 transformed (even numbers) CEF extracts. 10ug of extract was incubated with approximately 2.5ug bacterially expressed protein for 15min at 30^{o}C. 2ul (~2.0 x10^{5} cpm) of 5' end labelled, double stranded oligonucleotide L (junTRE + junRSRE) was added and the binding reaction incubated for a further 30min at 4^{o}C. Lanes 11-13 were included as a positive control to confirm the DNA binding activities of bacterially expressed c-Jun and bzipFos on the collagenase TRE. The binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at 4^{o}C. The junTRE specific binding activity is denoted by a blue arrowhead; the RSRE specific binding activity by a red arrowhead; the c-Jun/bzipFos binding activity by a green arrowhead; the c-Jun binding activity by a black arrowhead; and the putative bzip c-Fos/v-Jun binding activity by a brown arrowhead.

	1/2	3/4	5/6	7/8	9/10	11	12	13
Extract	+	+	+	+	+	-	-	-
Exog. Protein								
TG-1		+						
bzip-c-Fos			+		+	+		+
c-Jun				+	+	+	+	

A.



B.

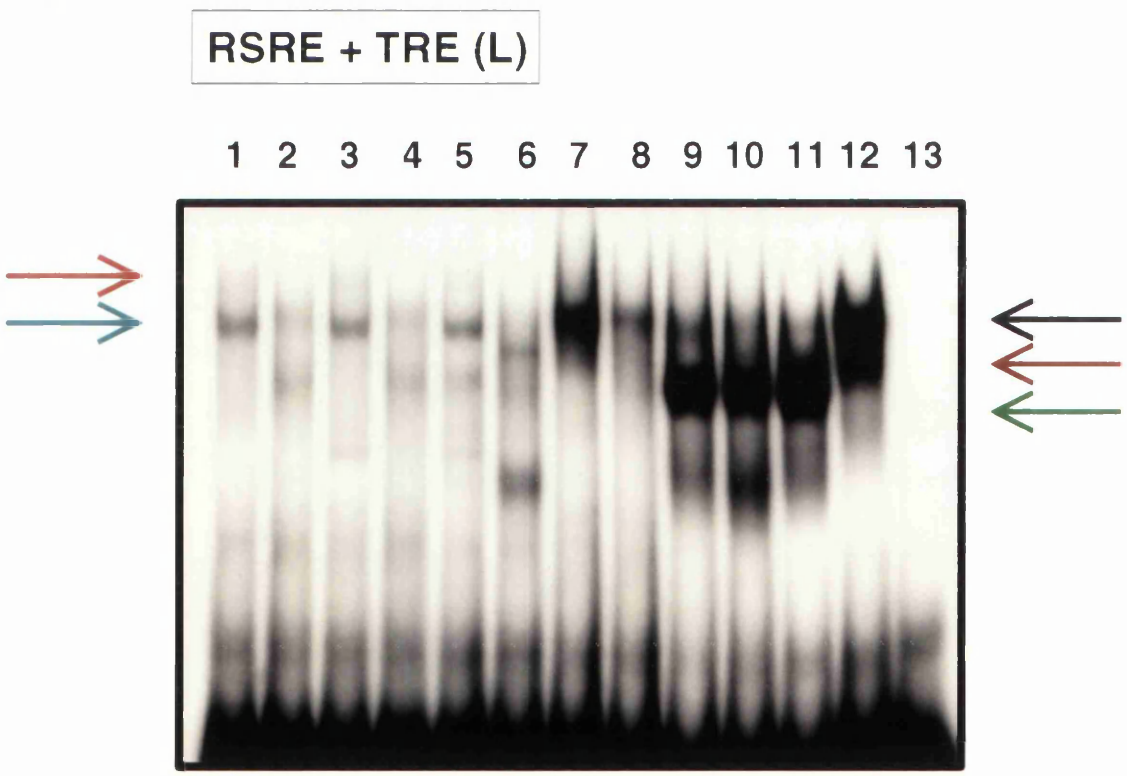


FIGURE 5.17

Jun heterodimers have little or no affinity for the junTRE binding site in vitro (compare lanes 4 & 8). The corresponding activity in normal CEFs was enhanced in the presence of exogenous c-Jun protein, at the expense of the junRSRE binding complex (compare lanes 3 & 7). This, together with the differential affinities of Jun homodimers and Jun/Fos heterodimers for the junTRE, supported the hypothesis that titration of a Fos/FRA-accessory protein was both disrupting the integrity of the junRSRE protein complex and inducing a concomitant increase in junTRE binding activity through the formation of c-Jun/Fos-FRA heterodimers.

Co-additions of c-Jun and bzipFos confirmed these observations. The exogenous proteins induced a disruption of junTRE binding activity in normal and ASV17-transformed extracts, and the appearance of junTRE-bound c-Jun/bzipFos heterodimers (compare lanes 9 & 10 with lane 11). The normal junTRE binding activity was less affected by the exogenous c-Jun and bzipFos proteins, presumably due to the presence of non-c-Jun-containing complexes with a strong affinity for the junTRE binding site (Figure 5.14B). The appearance of the c-Jun/bzipFos heterodimer also induced a concomitant displacement of the junRSRE binding complex in normal CEFs. The displacement could be the consequence of mutually exclusive binding on the combined junTRE/junRSRE-containing oligonucleotide and/or the titration of a Fos/FRA junRSRF accessory protein by excess full length c-Jun. The absence of c-Jun/Fos-FRA heterodimers at the junTRE (compare lanes 7 & 9) presumably indicates their lower affinity for the junTRE relative to c-Jun/bzipFos heterodimers, but does not distinguish between the displacement and titration hypotheses.

These results demonstrated the dual effects of exogenous c-Jun and bzipFos on the adjacent junTRE and junRSRE binding activities in normal CEFs. The relative contribution made by the displacement of junTRE-

bound endogenous factors and the Jun-mediated titration of Fos/FRA junRSRF accessory factors to the integrity of the junRSRE protein complex was not addressed. The enhanced junTRE binding activity in the presence of full length c-Jun strongly supported the formation of c-Jun/Fos-FRA heterodimers through Jun-mediated titration of junRSRF accessory proteins. However, the negative results obtained using a minimal junRSRE-containing oligonucleotide did not favour this interpretation, and suggested that Jun-mediated titration was ineffective in the absence of mutually exclusive binding.

V.D. FUNCTIONAL ANALYSIS OF THE *c-jun* PROMOTER

To further investigate the role of the *c-jun* promoter in the down-regulation of *c-jun* expression, *c-jun* promoter sequences were linked to the chloramphenicol acetyl transferase (CAT) gene and analysed for functional activity using a transient CAT expression assay (Figure 5.18B).

V.D.1. VARIABILITY IN TRANSIENT CAT EXPRESSION ASSAYS

The variability of results obtained in transient CAT expression assays is mainly due to the efficiency with which plasmid DNA is introduced into the cell. To minimize the internal variation of transient transfection assays and to maximize the transfection efficiency, various approaches were taken.

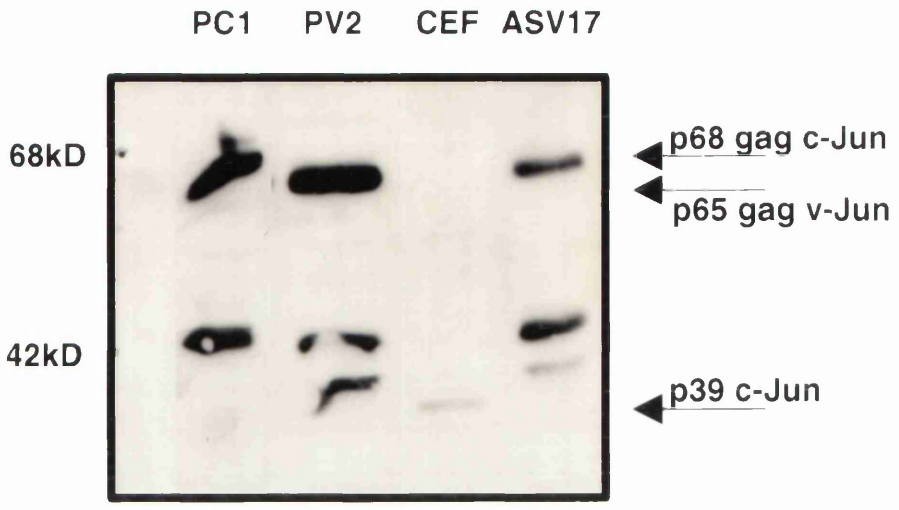
FIGURE 5.18A

Western Blot analysis of c-Jun and v-Jun expressing cells to establish the status of exogenous and endogenous Jun expression. PC-1 and PV-2 were derived from first round soft agar colonies of PC/SFCV and PV/SFCV transfected CEFs (Figure 3.13A). Virus particles were collected from PC and PV single cell colonies and used to re-infect primary CEFs. The cells were expanded and selected in G418 over five successive passages. CEFs and ASV17 transformed CEFs were harvested at an equivalent passage for the analysis. Remaining cells were frozen in separate vials at -70^{o}C for use in subsequent CAT transfections (Figure 5.19 & 5.20).
Approximately equal concentrations of whole cell protein extract were resolved on a 9% acrylamide gel and transferrred to ECL nylon membrane using a CAM LAB Semi-Dry Blotter. The proteins were visualized by ECL detection with a 1:4000 dilution of 730/5. The relative positions of exogenous and endogenous Jun proteins are illustrated on the figure.

FIGURE 5.18B

A plasmid map to illustrate the derivation of pCATb - cJ400 which was used in all subsequent functional analyses of the c-jun promoter (Figures 5.19 - 5.21). An approximately 400bp HindIII/SstI (Sac1) c-jun promoter fragment was excised from pSPT19 / Clone A (Figure 5.4), and subcloned into the pUCBM21 shuttle vector at the HindIII and SacI restriction sites in the poly linker (Boehringer Mannheim). The c-jun promoter fragment was re-excised as an ~400bp HindIII/XbaI fragment, and inserted into pCAT - Basic at the HindIII and XbaI poly linker restriction sites, in the sense orientation.

A.



B.

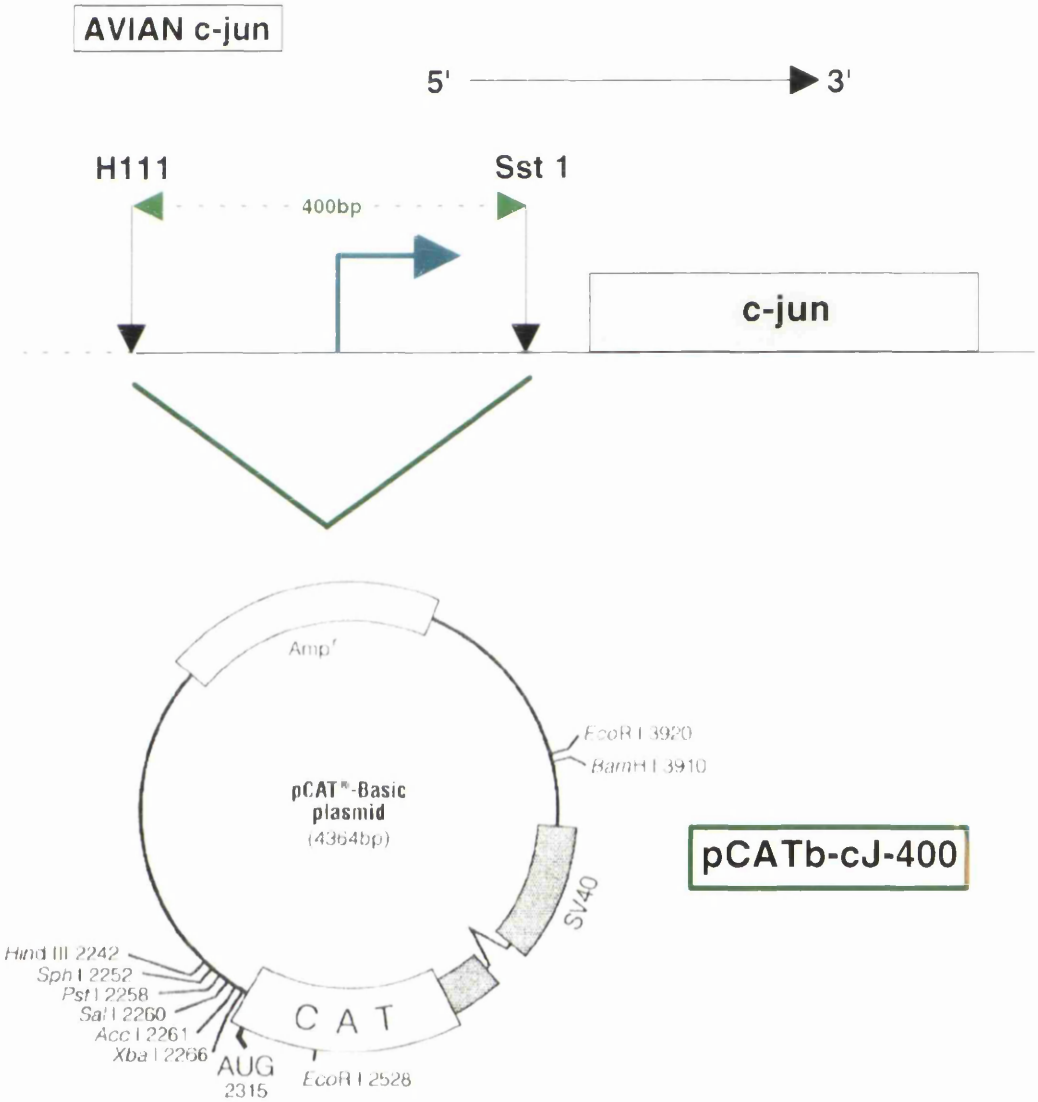


FIGURE 5.18

- Cell Culture and Transfection Conditions

Experience has shown that cell culture and transfection conditions are major contributers to variability in transient expression experiments. To reduce the former source of variability between independent transfections, primary CEFs were plated at low density on 60mm dishes to ensure healthy growth and optimum expression of introduced plasmid sequences. Normal cells were derived from a single pooled batch of chicken embryos. c-Jun- and v-Jun-transformed cultures were derived from primary CEFs infected with virus particles harvested from expanded PC/SFCV or PV/SFCV soft agar colonies (section II.A.3b). Virus spread was monitored by western blot analysis and G418 selection. Each culture was expanded through the same number of passages, and frozen down in multiple aliquots for subsequent transfection. These cell cultures were plated out at the same density as normal CEFs and transfected under identical culture conditions (section II.D 5a).

To maximize transfection efficiency, DOTAP, a commercial transfection reagent was used which delivers DNA into the cell by liposome-mediated fusion. DOTAP forms cationic liposomes in aqueous solution that interact spontaneously with DNA and fuse with the cell membrane. This method of DNA transfer is very gentle. It avoids cytotoxic effects and enables cells to be transfected with high efficiency. Variability between different DNA/DOTAP preparations was reduced by performing each transfection in triplicate and repeating each set of transfections on three separate occasions. Triplicates were transfected with DNA/DOTAP solutions, made up using the same reagents but mixed separately. Each dish was treated as an independent experimental observation. To compare the expression of a reporter plasmid in different cell backgrounds, DNA/DOTAP preparations were expanded appropriately and divided equally between

cultures of different cell types. Each preparation was assumed to represent, as far as possible, an identical transfection carried out on non-identical cell types.

Different plasmid DNA preparations were obtained by alkali lysis and caesium gradient centrifugation (section II.B.6). The DNA was dialysed and the concentration checked by OD_{260} and OD_{280} measurements. A constant total amount of DNA (Table 5.A) was added to a fixed number of plated cells in each transfection. The *c-jun* promoter-CAT construct represented 3ug of the final concentration of DNA. When required, the amount of DNA per transfection was equilibrated to 6ug (or 7ug) per 200ul using PAT plasmid DNA, as PAT vector sequences are contained within the *c-jun* promoter-CAT reporter construct (Figure 5.18B). The quantities of plasmid DNAs in micrograms added per transfection are outlined in Table 5.A

- Hirts Supernatants

Hirts supernatants provide a simple assay to quantify the uptake of plasmid DNA in different cell types. Chromosomal and plasmid DNA is extracted from nuclear cell pellets and digested with an appropriate restriction enzyme for Southern Blotting analysis. The blots are probed for the presence of plasmid sequences and autoradiographs quantified by densitometric scanning. Hirts supernatants were used to control for DNA uptake in a series of transient transfection assays designed to compare the activity of a single reporter plasmid in different cell backgrounds (section V.D.2a). Each supernatant represented the contents of a single 60mm dish. To compare the transfection efficiency between different cell types, it was necessary to assume the same number of cells in each dish. This was not achievable as each cell type exhibited an independent growth rate,

TABLE 5.A

Details of each transient transfection assay are outlined in tables 1 and 2. Single transfections (Table 1) were performed in different c-Jun and v-Jun expressing cell backgrounds (Figure 5.18 & 5.19). Assay 1 represents the test tranfection; assay 2, the negative control; and assay 3, the positive control.
Double transfections (Table 2) were performed in normal CEFs. The test reporter (pCATb-cJ-400) or the negative control (pCAT-Basic) were transfected in the absence (assays 1 & 4) or presence of the B-gal control reporter, pHSV-B-gal, (assays 2 & 5) and one of a panel of c-Jun or v-Jun expressor plasmids, pRc/RSV VJ-0/ VJ-1/ CJ-3 (assay 3 & 6). The B-galactosidase activity was unaffected by the presence of the pCAT-Basic plasmid sequences (data not shown). Consequently, the results outlined in Figure 5.21 and Table 5.C represent double transfections corresponding to assay types 4, 5 & 6.
All transfections were performed as described in section II.D.5.

TABLE 5.B

Results of the statistical "t test". Test results were expressed as a proportion of the control (CEF) mean for each assay. Single transient transfection assays were performed in different cell backgrounds to compare the functional activity of a c-jun promoter fragment (pCATb-cJ-400) in normal CEFs and in CEFs expressing high levels of c-Jun (PC-1) or v-Jun (PV-2 & ASV17). proteins. The assays were repeated three times in exponential (Table 1) and serum stimulated (Table 2) cells. Each transfection was performed in triplicate. Results from triplicate assays were pooled for each cell background and the 95% Confidence Intervals calculated.

TABLE 5.C

Mean (μ) and Standard Deviations (σ) for CAT (Bold Figures) and B-galactosidase (Light Figures) activities in CEFs co-transfected with pCATb-cJ400 and pHSV-B-gal, and a panel of c-Jun and v-Jun expressing plasmids (Table 5.A). The values represent the mean and Standard Deviation of three dishes within the same experiment. The experiment was repeated three times with different concentrations of c-Jun and v-Jun expressing plasmids. The results in Table 5.C are illustrated in Figure 5.21. Figures 5.21A and 5.21B illustrate the mean enzyme activites displayed in tables 1 and 2 respectively. The numbers on the bar charts correspond to the dish numbers shown in each table.

TABLE 5.A

(1)

	SINGLE TRANSFECTIONS		
	1	2	3
DNA (ug)			
pCATb-cJ-400	3.0		
pCAT Basic		3.0	
PAT (carrier)	3.0	3.0	2.0
GAL4 CAT			2.0
GAL4 VP16			2.0

(2)

	DOUBLE TRANSFECTIONS					
	1	2	3	4	5	6
DNA (ug)						
pCATb-cJ-400				3.0	3.0	3.0
pCAT Basic	3.0	3.0	3.0			
PAT (carrier)	4.0	2.0		4.0	2.0	
pHSV - βgal		2.0	2.0		2.0	2.0
pRc/RSV plasmids			2.0			2.0

but the transient nature of the assay, together with the inefficiency of DNA uptake, made large fluctuations in cell numbers unlikely and small fluctuations relatively insignificant.

- Internal Standards

CAT expression assays enable the activity of any given promoter to be quantified under different conditions. To investigate whether fluctuations in CAT activity are a direct consequence of a specific gene promoter or due to a more general effect, an unrelated promoter is commonly included in the transient expression assay as an internal standard. pHSV-Bgal was chosen as the internal standard for the transient expression assays described in section V.D.2b. pHSV-Bgal expresses the lacZ gene, encoding B-galactosidase, from the HSV-2 IE-5 gene promoter (Figure 2.2) and was kindly provided by J. O'Prey.

V.D.2. EXPERIMENTAL PROCEDURE

Two alternative approaches to the CAT expression assay were used to determine whether overexpression of c-Jun of v-Jun would perurb the function of the *c-jun* promoter.

V.D.2a Transfection into Different Cell Backgrounds

In the first approach a *c-jun* promoter-CAT construct (pCATb-cJ-400) was transfected into different c-Jun- and v-Jun-overexpressing cells, to examine their effects on the functional activity of a *c-jun* promoter fragment. The reporter plasmid was constructed from the avian genomic

c-jun clone and included those sequences approximately 400bp downstream of the 5' HindIII cloning site. The fragment was subcloned into the commercially available pCAT-Basic plasmid as described in Figure 5.18B. The c-Jun- and v-Jun-overexpressing cells were derived from ASV17-transformed CEFs, or from PC/SFCV and PV/SFCV virally-infected CEFs. Viral infections were monitored by G418 selection, and the levels of exogenous and endogenous Jun protein expression quantified by western blot analysis. In accordance with previous results, c-Jun- and v-Jun-overexpressing CEFs expressed undetectable levels of endogenous p39 c-Jun (Figure 5.18A).

Jun-overexpressing and normal CEFs were transfected with pCATb-cJ-400, and grown under normal growth conditions for 48hrs as described in section II.D.5a. A parallel series of dishes was serum deprived and then serum stimulated for 30mins to investigate the effect of high c-Jun and v-Jun expression on a serum-induced *c-jun* promoter fragment. Negative and positive controls were provided by an empty pCAT-Basic vector and a co-transfection with Gal4/CAT and Gal4/VP16, respectively. Gal4/VP16 encodes a strong transactivator domain that activates CAT expression through the Gal4 DNA binding site (Cousens *et al*, 89; Lillie *et al*, 89; Sadowski *et al*, 89). Details of each transfection are presented in Table 5A.

Hirts supernatants were performed on the pCATb-cJ-400 transfections to control for DNA uptake. The CAT activities were adjusted accordingly, and expressed as a proportion of the mean of the normal CEF activity in each transfection. Expressing the results in this way enabled the level of CAT activity in c-Jun- and v-Jun-overexpressing CEFs and normal CEFs to be compared directly over three independent transient expression assays. Nine observations were obtained for each cell background as each transfection was performed in triplicate. The observations were analysed

by a statisitical "t-test" to determine their significance in relation to the normal mean.

V.D.2b Co-Transfections into Primary CEFs

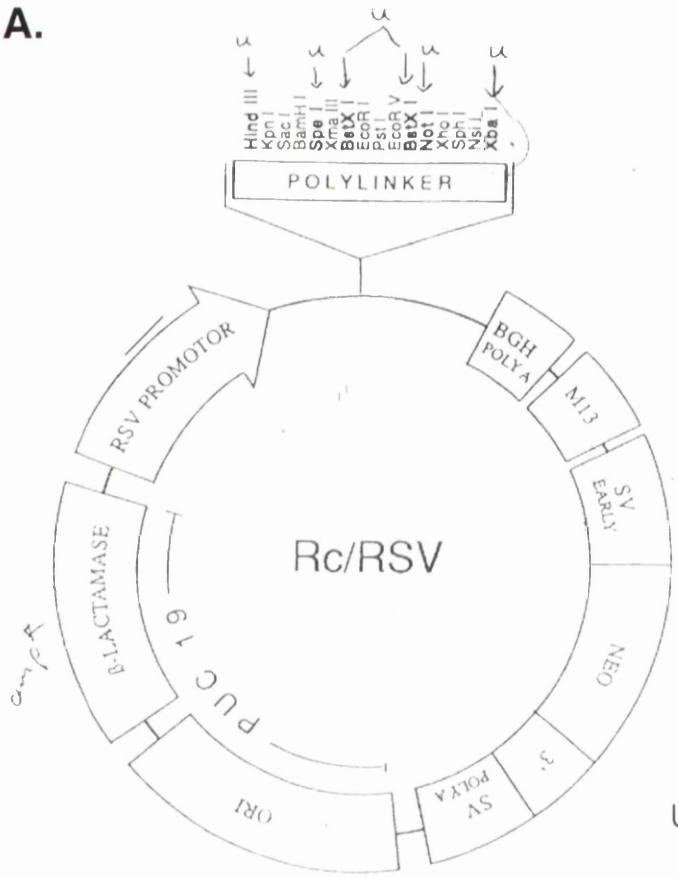
As an alternative approach to investigating the effects of c-Jun and v-Jun on the functional activity of the *c-jun* promoter, primary CEFs were co-transfected with equal molar ratios of pCATb-cJ-400 and c-Jun- or v-Jun-overexpressing plasmids. The expression plasmids were kindly provided by Dr I. Morgan and are described in detail in Figure 5.20 The advantage of this system is that it provides a direct correlation between c-Jun and v-Jun overexpression and the activity of the *c-jun* promoter, in the absence of any contributing cell-type-specific factors. Its main disadvantage is an inability to curb exogenous protein expression to within physiological levels. Consequently, transient expression results can be distorted by non-specific effects such as squelching.

Co-transfections were performed with pHSV-Bgal as an internal comparison for *c-jun* promoter activity. The assay for B-galactosidase activity relies on the enzymatic cleavage of o-nitrophenyl-B-galactopyranoside to o-nitrophenol which is yellow in colour and can be quantified spectrophotometrically at 420nm. Interpretation of these results assumes that the molar ratio of lac Z and pCATb-cJ-400 available for expression is the same in parallel transfections of normal CEFs. On the basis of this assumption, trends in CAT and B-galactosidase activity were compared in each transfection and used to determine the specificity of the c-Jun- and v-Jun-dependent effects on the functional activity of the *c-jun* promoter.

FIGURE 5.20

A plasmid map to illustrate the structure of the pRc/RSV - c-Jun and v-Jun expressing plasmids (Dr I.M. Morgan). pRc/RSV CJ-3 was obtained by cloning the Xba1 insert of RCAS-CJ-3 (Bos 90) into pRc/RSV at the XbaI cloning site; pRc/RSV VJ-0 by cloning the HindIII/XbaI insert of RCAS-VJ-0 (Bos *et al*, 90) into pRc/RSV at the HindIII and XbaI sites; and pRc/RSV CJ-1 by excising the ClaI insert of RCAS-VJ-1 (Bos *et al*, 90), converting the ClaI sites to XbaI sites and cloning the XbaI fragment into pRc/RSV at the XbaI cloning site. Details of the RCAS adaptor plasmid are outlined according to Bos *et al.*

A.



U = Unique Site

B.

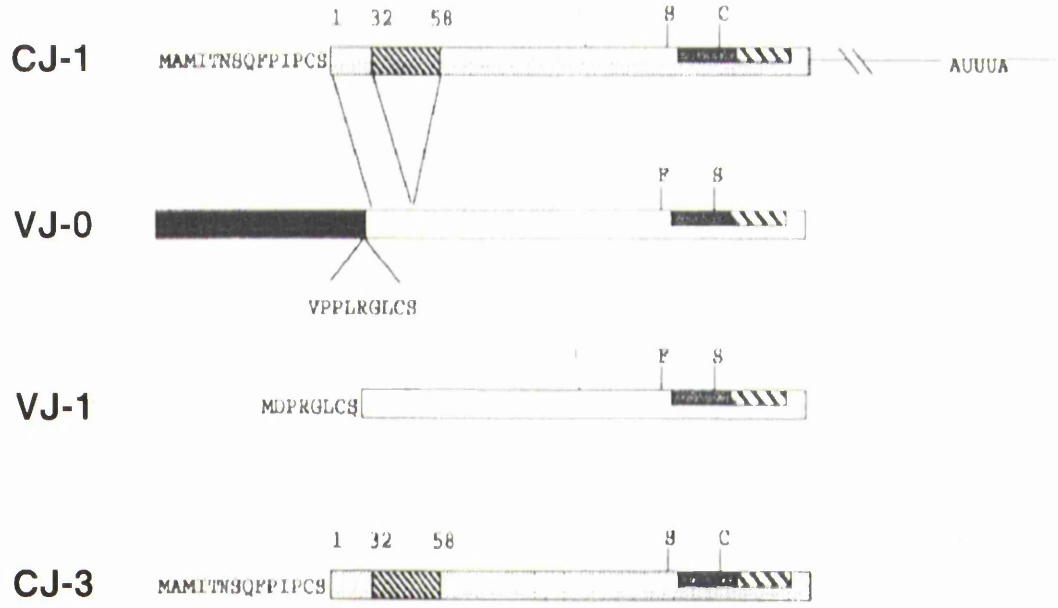


FIGURE 5.20

V.D.3. RESULTS

V.D.3a Transfections into Different Cell Backgrounds

As shown in Figure 5.19, the CAT activity of pCATb-cJ-400 was severely depressed in c-Jun- and v-Jun-overexpressing CEFs. The individual bars represent the mean value for three independent observations, corrected for plasmid DNA uptake from Hirts supernatants. The repression was specifically directed at the *c-jun* promoter sequences as transfections with the pCAT-Basic plasmid alone produced low background levels of CAT activity throughout. This contrasted with the consistently high levels of CAT activity exhibited by co-transfections of Gal4/VP16 and Gal4/CAT, and indicated that gene repression was not a general feature of c-Jun- and v-Jun-overexpressing cells.

To determine the significance of c-Jun- and v-Jun-dependent repression of the *c-jun* promoter, CAT activities were grouped according to cell type, and expressed as a proportion of the mean activity found in normal CEFs (Table 5.B). The true mean value was calculated within 95% confidence intervals for each group of nine observations using a statisitical "t-test". True means of greater than 1.0 indicate a significant elevation in CAT activity relative to normal cells, whereas true mean values of less than 1.0 indicate the reverse. As shown in Table 5.B, values of less than 1.0 were calculated for each cell type under both growth conditions analysed. From this it was concluded that the observed repression of the *c-jun* promoter, in the presence of high levels of c-Jun or v-Jun protein, was statistically significant within a 95% confidence interval.

FIGURE 5.19

Panels A, B & C:
Autoradiographs of typical TLC plates used for measuring CAT activity of pCATb - cJ400 (panel A), pCAT - Basic (negative control; panel C), and Gal4/CAT + Gal4/VP16 (positive control; panel B), in independent cell backgrounds (Figure 5.18A).

A "Hirts" analysis of genomic DNA, isolated from pCATb-cJ400 transfected cells, is illustrated directly below panel A. Each lane represents the contents of a single 60mm tissue culture dish. The DNA was extracted and digested in the presence of HindIII and XbaI to release ~ 4360bp pCAT - Basic plasmid vector sequences (denoted by a blue arrowhead). The samples were resolved on a 1% agarose gel with Bacteriophage λ (HindIII digested) markers (left hand lane). The Southern Blot was probed against a random primed oligolabelled pCAT - Basic probe linearized with XbaI. Lanes 1-12 on the Southern Blot correspond to Lanes 1-12 on the pCATb - cJ400 TLC plate.

Panel D:
Expression of pCATb-cJ400 in separate c-Jun and v-Jun expressing cell backgrounds. Each value represents a mean of three dishes within the same experiment. The experiment was performed three times with similar results. The CAT activity is expressed relative to a negative control representing parallel transient transfections in the presence of the promoterless pCAT - Basic vector sequences. The mean values were derived from the experiment described above in panels A and B.

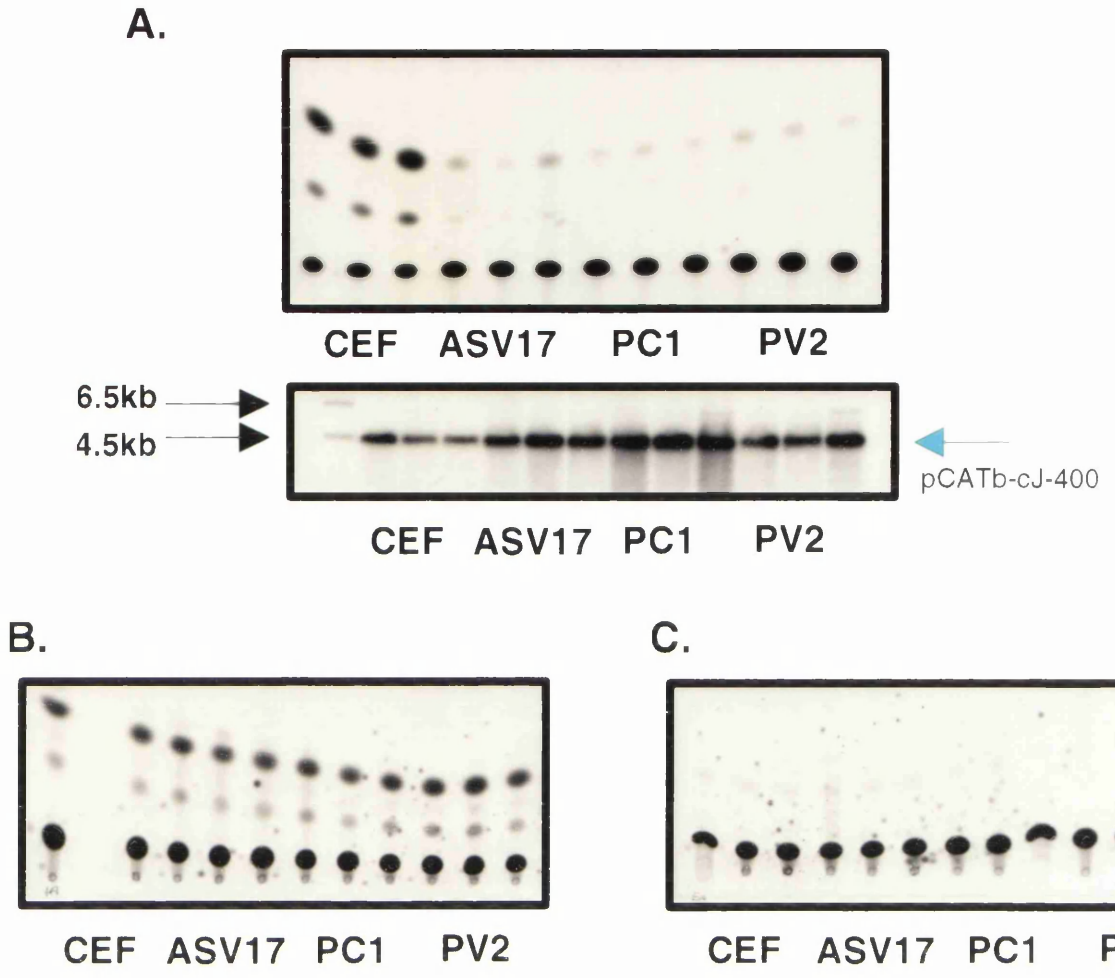

A.
CEF ASV17 PC1 PV2
6.5kb
4.5kb
pCATb-cJ-400
CEF ASV17 PC1 PV2
B.
CEF ASV17 PC1 PV2
C.
CEF ASV17 PC1 PV2
D.
% CAT Conversion
2.5
2
1.5
1
0.5
0
CEF ASV17 PC1 PV2

FIGURE 5.19

TABLE 5.B

(1)

-- Test Activity as a Proportion of the Control (CEF) Mean -

-

Single Transfections:	ASV17	PC-1	PV-2
Assay			
1	0.051	0.018	0.009
	0.018	0.056	0.04
	0.05	0.02	0.032
2	0.037	0.023	0.043
	0.036	0.047	0.029
		0.028	0.127
3	0.336	0.041	0.085
	0.058	0.052	0.108
	0.076	0.031	0.119
95% Confidence Interval	-0.004 - 0.1695	0.02413 - 0.04610	0.0318 - 0.0998

(2)

-- Test Activity as a Proportion of the Control (CEF)

Mean --

Single Transfections:	ASV17	PC-1	PV-2
Assay			
1	0.093	0.8	0.211
	0.121	0.051	0.110
	0.89	0.174	0.047
2	0.017	0.076	0.115
	0.090	0.050	0.132
	0.024	0.056	0.078
3	0.024	0.042	0.069
	0.069	0.033	0.052
	0.044		0.070
95% Confidence Interval	-0.0620 - 0.3669	-0.0591 - 0.3796	0.0495 - 0.1047

V.D.3b Co-Transfecions

Co-transfections of pCATb-cJ-400 and pCAT-Basic with c-Jun and v-Jun expression plasmids also led to a c-Jun- and v-Jun-dependent repression of CAT activity, although in this case parallel transfections using pHSV-B gal revealed a similar trend in the levels of B-galactosidase activity (Figure 5.21; Table 5.C). The expression of B-galactosidase is driven by the HSV-2 IE-5 gene promoter in the pHSV-Bgal reporter plasmid. The HSV-2 IE-5 gene promoter contains no recognizable AP-1 DNA binding sites, suggesting that c-Jun- and v-Jun-associated repression of B-galactosidase activity, was mediated through an indirect effect (Gaffney *et al*, 85). Indirect repression is dependent on high levels of protein expression (section I.B.6b). To investigate whether the Jun associated repression of the HSV-2 IE-5 gene promoter was a titratable activity, and distinct from that mediating the repression of the *c-jun* promoter, co-tranfections were repeated with 0.1x and 0.05x dilutions of the c-Jun and v-Jun expression plasmids. As shown in Figure 5.21, the titration failed to completely abolish the repression of either enzyme activity. Under these conditions, the trends in CAT and B-galactosidase activity were virtually identical, indicating that Jun-mediated repression of the *c-jun* and the HSV-2 IE-5 gene promoters was not represented by two seperable activities at these c-Jun and v-Jun concentrations.

FIGURE 5.21

Transcriptional repression of c-jun (pCATb-cJ400) and HSV-IE5 (pHSV-B gal) promoters in the presence of high levels of exogenous c-Jun and v-Jun proteins. Parallel trends in activity were observed in the presence of 2ug (panel A: lanes 3-6), 0.2ug (panel B: lanes 3-6) and 0.1ug (panel B: lanes 7-10) of pRc/RSV c-Jun and v-Jun expressing plasmids (Figure 5.20). The values represent a mean of three independent dishes within the same experiment. The experiment was performed three times using different concentrations of pRc/RSV plasmids.

Panel A:

Lane	Reporter Plasmid		c-Jun / v-Jun Expressing Plasmid
	pCATb-cJ400	pHSV-Bgal	
1	+	-	-
2	+	+	-
3	+	+	pRc/RSV
4	+	+	pRc/RSV VJ-0
5	+	+	pRc/RSV VJ-1
6	+	+	pRc/RSV CJ-3

Panel B: Transient transfections were carried out as above using 0.2ug (lanes 3-6) or 0.1ug (lanes 7-10) of pRc/RSV expression plasmids. Otherwise lanes 3-6 in panel A correspond to lanes 3-6 and lanes 7-10 of panel B.

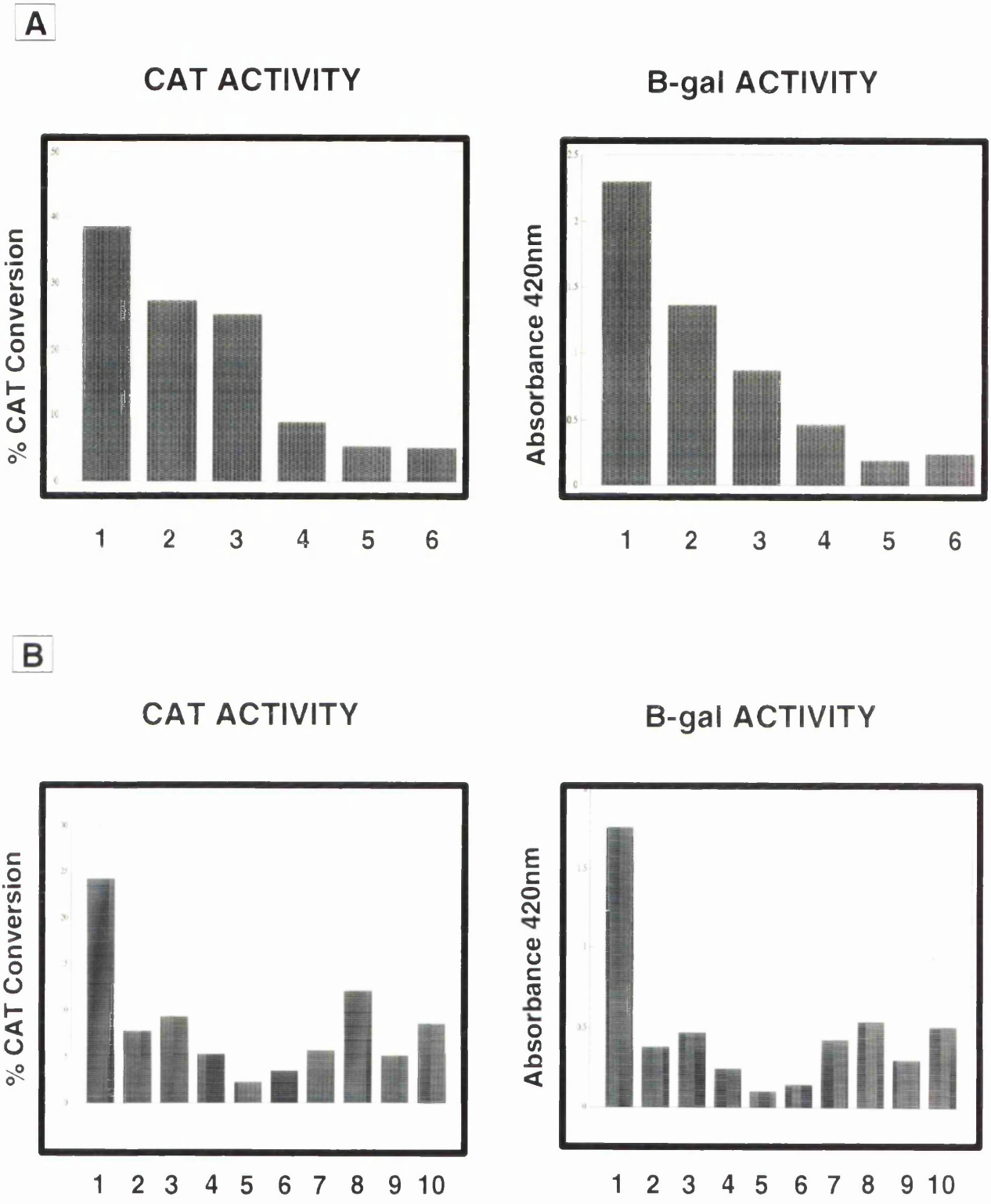

A
CAT ACTIVITY
B-gal ACTIVITY
% CAT Conversion
Absorbance 420nm
1 2 3 4 5 6
B
CAT ACTIVITY
B-gal ACTIVITY
% CAT Conversion
Absorbance 420nm
1 2 3 4 5 6 7 8 9 10

FIGURE 5.21

TABLE 5.C

(I)

DISH	REPORTER PLASMID pCATb-cJ400	REPORTER PLASMID pHSV-βgal	EXPRESSION PLASMID	μ Enzyme Activity CAT (% conv)	μ Enzyme Activity β-gal (A420nm)	Z Enzyme Activity CAT (%conv)	Z Enzyme Activity β-gal (A420nm)
1	+	-	-	**38.50**	2.30	**3.81**	0.040
2	+	+	-	**27.40**	1.36	**1.68**	0.063
3	+	+	**pRc/RSV** (x1)	**25.30**	0.87	**3.32**	0.064
4	+	+	**VJ-0** (x1)	**8.89**	0.47	**0.78**	0.019
5	+	+	**VJ-1** (x1)	**5.29**	0.19	**0.78**	0.003
6	+	+	**CJ-3** (x1)	**5.09**	0.24	**0.12**	0.032

(2)

DISH	REPORTER PLASMID pCATb-cJ400	REPORTER PLASMID pHSV-βgal	EXPRESSION PLASMID	μ Enzyme Activity CAT (% conv)	μ Enzyme Activity β-gal (A420nm)	σ Enzyme Activity CAT (%conv)	σ Enzyme Activity β-gal (A420nm)
1	+	-	-	**24.20**	1.76	**1.51**	0.136
2	+	+	-	**7.71**	0.38	**0.10**	0.048
3	+	+	**pRc/RSV** (x 0.1)	**9.29**	0.47	**0.78**	0.065
4	+	+	**VJ-0** (x 0.1)	**5.33**	0.24	**0.17**	0.009
5	+	+	**VJ-1** (x 0.1)	**2.37**	0.10	**0.32**	0.008
6	+	+	**CJ-3** (x 0.1)	**3.50**	0.14	**0.90**	0.023
7	+	+	**pRc/RSV** (x 0.05)	**5.65**	0.43	**0.14**	0.032
8	+	+	**VJ-0** (x 0.05)	**12.00**	0.54	**0.082**	0.061
9	+	+	**VJ-1** (x 0.05)	**5.09**	0.30	**1.00**	0.024
10	+	+	**CJ-3** (x 0.05)	**8.48**	0.51	**1.24**	0.041

CHAPTER VI - DISCUSSION (2).

CHAPTER VI - DISCUSSION

FUNCTIONAL ANALYSIS OF THE c-JUN PROMOTER

VI.A. c-JUN AUTO-REPRESSION IS MEDIATED PRIMARILY AT THE LEVEL OF TRANSCRIPTION

The auto-repressing properties of c-Jun and v-Jun were initially inferred from the dramatic down-regulation of endogenous c-Jun protein in c-Jun- and v-Jun-overexpressing CEFs (Figure 3.13A). RNase protection analysis demonstrated that v-Jun auto-repression was primarily directed at the level of transcription (Figure 5.1). *c-jun* mRNA levels are controlled by both transcriptional and post-transcriptional regulatory mechanisms (section IV.A.1). The precise contribution made by post-transcriptional regulation was not investigated, but run-off analysis was performed to examine the effect of v-Jun on the rate of endogenous *c-jun* expression. The sensitivity of the run-off assay was hampered by weak hybridization, due to the short length of the *c-jun*-specific probe (c-jun(1)), but clearly showed that a significant proportion of v-Jun-dependent auto-repression was mediated through a down-regulation of endogenous *c-jun* transcription (Figure 5.2).

Transcriptional auto-repression has been observed for other members of the immediate early gene family, including *c-myc* and *c-fos* (Grignani *et al*, 90; Rivera *et al*, 90). An additional effect of v-Jun was its concomitant repression of endogenous *c-fos* expression (Figure 5.2). The functional significance of this was not investigated, but it is intruiging that c-Fos is a major dimerization partner of c-Jun, and that both proteins have been

implicated in the regulation of each others expression (Herr *et al*, 94, Konig *et al*, 89; Rivera *et al*, 90; section VI.E.).

VI.B. REGULATORY ELEMENTS IN THE c-JUN PROMOTER

Sequence and restriction endonuclease mapping analysis identified the proximal promoter sequences contained within the genomic avian *c-jun* clone (Figure 5.4 & 5.5A). The region included approximately 200bp upstream of a putative transcriptional start site identified in an independently derived avian *c-jun* sequence (Nishimura *et al*, 88). A comparison of *c-jun* promoter sequences from different animal species revealed a remarkable degree of conservation over the proximal promoter region (Figure 5.8A). The homology included recognized *c-jun* regulatory elements such as the proximal junTRE and adjacent junRSRE, and extended over a number of previously uncharacterized sites such as the putative initator sequence and TATA-like box. The significance of the conserved sequences in the vicinity of the proximal promoter was supported by DNase I hypersensitive site analysis, which was performed as an initial step towards identifying potential regulatory sites in the *c-jun* promoter. Two sites of DNase I hypersensitivity were identified in chromatin prepared from *c-jun*-expressing, exponential CEFs (Figure 5.7B). HS-2 was located upstream of the 5' HindIII cloning site and may represent an unrelated regulatory element or a long range enhancer sequence. The close proximity of HS-1 to the 5' HindIII cloning site was more informative and suggested that sequences in the vicinity of the proximal promoter may be involved in regulating *c-jun* transcription in these cells.

VI.C. DIFFERENCES IN c-JUN PROMOTER SITE OCCUPANCY IN NORMAL AND ASV17-TRANSFORMED CEFs

Since the *c-jun* proximal promoter sequences exhibited a high degree of homology with other species, it was of interest to determine whether any specific changes in promoter site occupancy correlated with auto-repression. An in vitro DNA binding analysis of independent conserved sequences in the *c-jun* promoter demonstrated that the majority were occupied under *c-jun*-expressing (normal CEFs) and *c-jun*-repressing (ASV17-transformed CEFs) conditions (Figure 5.8A & 5.8B). The high level of occupancy suggested that multiple sequence specific binding sites contribute to the regulation of *c-jun* expression and that gross changes in *c-jun* promoter site occupancy are not associated with auto-repression in vivo. Two exceptions to the overall pattern of binding were provided by the previously characterized proximal junTRE and junRSRE regulatory elements, which were differentially occupied depending on the transcriptional status of the *c-jun* gene.

- junTRE Binding Activity

The junTRE binding activity in normal CEFs was represented by an apparently homogenous retarded band but contained only a proportion of c-Jun-reactive protein complexes (Figure 5.14B). This, together with the low proportion of Fos/FRA-containing complexes, suggested that distinct subsets of TRE-binding proteins recognize the junTRE and colTRE in

normal CEFs (compare Figure 5.14B & 3.16B). Differences between junTRE and colTRE binding activites in vivo have been described in several independent reports (Herr *et al*, 94; Muegge *et al*, 93; van Dam *et al*, 93). In particular, c-Jun/ATF-2 (or ATF-2-related) heterodimers have been isolated from HeLa and NIH3T3 cells by their specificity for the proximal and distal junTRE binding sites in vitro (Herr *et al*, 94; van Dam *et al*, 93). c-Jun/c-Fos heterodimers express a high affinity for the colTRE but, in contrast to ATF-containing heterodimers, have been isolated as low affinity proximal junTRE binding complexes in vitro (Herr *et al*, 94). The precise composition of junTRE binding activity in normal CEFs was not examined but the predominant or exclusive expression of c-Jun in primary CEFs (Figure 3.16A), together with the ability of c-Jun to heterodimerize with ATF-2 and recognize CRE and CRE-related elements such as the junTRE (Chatton *et al*, 94; Hai *et al*, 91), strongly supports a contribution by ATF-2 or ATF-2-related heterodimers.

The junTRE binding activity in ASV17-transformed CEF extracts was quantitatively and qualitatively distinct from that of normal cells (Figure 5.9A). Two junTRE-specific binding complexes were identified that resembled colTRE complexes II and III in terms of their electrophoretic mobility in non-denaturing acrylamide gels (compare Figure 3.15 & 5.9A). The electrophoretic resemblance was confirmed by antibody analysis which demonstrated an identical pattern of reactivity at the junTRE and colTRE using ASV17-transformed CEF extracts (compare Figure 3.16A & 5.13). This observation led to the proposal that v-Jun directs the down-regulation or inactivation of multiple junTRE-specific proteins, and thereby facilitates an exclusive occupancy of junTRE binding sites with v-Jun-containing complexes.

The junTRE-specific binding patterns of normal and ASV17-transformed CEFs were confirmed by DNase I footprinting analysis of the *c-jun*

promoter. The junTRE was protected from DNase I digestion using extracts derived from either normal or ASV17-transformed CEFs, but the protected sequence was more clearly defined when *c-jun* promoter fragments were incubated in the presence of normal CEF extracts (Figure 5.9B). A relatively weak pattern of protection was observed using an equivalent concentration of ASV17-transformed CEF extract. The pattern of binding correlated with that detected by EMSA analysis, and did not reflect the high levels of p65 gag-v-Jun expression detected in ASV17-transformed CEFs (Figure 3.5). Overexpression of the v-Jun oncoprotein, together with the apparently exclusive presence of v-Jun-containing complexes at the junTRE, supports the hypothesis that v-Jun-dependent repression of *c-jun* is mediated, at least in part, by an exclusive replacement of endogenous junTRE binding complexes with low affinity v-Jun-containing alternatives.

- junRSRE Binding Activity

A single junRSRE binding activity in normal CEFs was absent in ASV17-transformed cell extracts (Figure 5.10A). DNase I footprinting analysis with normal CEF extracts revealed an extended region of protection centred over the junRSRE and half of the adjacent junTRE binding site. A comparable pattern of protection was observed using ASV17-transformed CEF extracts (Figure 5.10B). This was attributed to an additional junRSRE binding complex detected in both cell types (Figure 5.10A).

The junRSRE binding activity in normal CEFs was completely disrupted by an antiserum raised against an RSRF family protein, RSRF-C4 (Figure 5.14A). The RSRF transcription factors were isolated by their sequence homology to the SRF over the DNA binding domain, and specifically recognize RSRE regulatory elements in vitro and in vivo (Pollock *et al*, 91).

Homology between the RSRFs and the SRF extends over a 56aa region described as the MADS BOX (Pollock *et al* 91). The MADS BOX characterizes all SRF and SRF-related proteins and is essential for high affinity DNA binding and the recruitment of accessory proteins that maximize the transcriptional response (Hill *et al*, 93; Treisman *et al*, 92; Wynne *et al*, 92). The recruitment domain is located at the carboxyl-terminus of the MADS BOX and is poorly conserved between the SRF-related proteins. This has led to the hypothesis that specific accessory factors interact with different SRF-related proteins in vivo. In support of this proposal the mammalian transcription factor SRF and the yeast regulatory protein MCM1 interact with distinct accessory factors in vivo but contain DNA binding domains that are 70% identical. The SRF- and MCM1-specific accessory factors have been proposed to influence the choice of binding site and thereby contribute to the related but distinct binding affinities of both proteins (Dalton *et al*, 93; Marais *et al*, 93; Pollock *et al*, 91; Wynne *et al*, 92).

Accessory protein interactions are less well defined for the RSRF transcription factor family. Like the SRE, the RSREs are commonly associated with non-related sequence-specific binding sites that contribute to RSRF-dependent transactivation (Flemington *et al*, 90; Zhu *et al*, 93). This, in addition to reports of cooperative interactions between the RSRFs and helix-loop-helix proteins during muscle-specific gene expression (Yu *et al*, 92; Zhu *et al*, 93), suggests that accessory protein interactions may be important regulators of RSRF function in vivo. In support of this proposal, characterization of the junRSRE binding activity in normal CEFs identified the presence of a Fos/FRA-related protein in the RSRF-containing protein complex (Figure 5.14A). A relationship between Fos/FRA and the RSRFs has not been previously reported, but interactions between monomeric Fos proteins and unrelated transcription factors are well documented in the

literature (Masuda *et al*, 93; Stein *et al*, 93(i); Stein *et al*, 93(ii)). The precise nature of the putative Fos/FRA-related component was not investigated in this analysis. FRA2 has been identified as the most abundant Fos family TRE binding protein in primary CEFs (Suzuki *et al*, 94), but transcriptional expression of *c-fos* was detected in exponential CEF cultures that were equivalent to those used to prepare non-denatured whole cell extracts for EMSA analysis (Figure 5.2). The nature of the Fos/FRA-related component may be important for the temporal regulation of jun-RSRF activity in vivo, and for the interaction between the RSRF protein complex and the junRSRE. c-Fos for example is rapidly and transiently induced in reponse to cell stimulation, whereas FRA2 exhibits slower and more prolonged kinetics of expression (Kovary *et al*, 91; Kovary *et al*, 92). Both transcription factors bind consensus TRE half sites in vitro, as heterodimers with the Jun proteins (Suzuki *et al*, 91), but their affinity for TRE-like varient sequences has not been determined.

VI.D. c-JUN AUTO-REPRESSION CAN BE REPRODUCED IN FUNCTIONAL ASSAYS

Transient CAT-expression assays confirmed the auto-repressing properties of c-Jun and v-Jun. A proximal *c-jun* promoter fragment was dramatically and significantly down-regulated in c-Jun- and v-Jun-overexpressing CEFs. The repression was specific to the proximal *c-jun* promoter sequences and a direct consequence of c-Jun and v-Jun overexpression (Figure 5.19, Table 5.B). An equivalent response was obtained using c-Jun and v-Jun expression plasmids and the *c-jun* promoter-CAT construct to co-transfect normal CEF cultures (Figure 5.20

& 5.21). However, under these conditions an unrelated promoter-CAT construct lacking detectable TRE binding sites was also down-regulated, suggesting that the repression represented a squelching artefact of non-physiological levels of c-Jun and v-Jun expression.

VI.E. POSSIBLE MECHANISMS OF JUN-MEDIATED AUTO-REPRESSION

The results presented in section V.C. describe changes in occupancy at the junTRE and adjacent junRSRE that correlate with transcriptional repression of the *c-jun* gene. The differences in promoter site occupancy were observed in extracts prepared from ASV17-transformed CEFs which express virtually undetectable levels of endogenous c-Jun protein (Figure 3.5). An apparently equivalent down-regulation of endogenous c-Jun was demonstrated in parallel cultures of high c-Jun-expressing CEFs (Figure 3.13A), suggesting that auto-repression is a consequence of high Jun expression and not dependent on additional qualitative mutations in the v-Jun oncoprotein. The levels of p39 c-Jun detected in normal CEFs are consistently lower than those of exogenous p65 gag-v-Jun in ASV17-transformed cells (Figure 3.3A). This, together with a mechanism of quantitative auto-repression, may provide an explanation for the relatively slow decline in *c-jun* mRNA following serum stimulation and the persistence of *c-jun* mRNA in serum deprived CEFs compared to their v-Jun-transformed counterparts (Figure 5.1).

Two mechanisms are described below that could account for concentration dependent auto-repression of the *c-jun* gene.

- Mutually Exclusive Binding to Adjacent Regulatory Elements

In vitro DNA binding and cross-competition analysis suggested that the junTRE and junRSRE regulatory elements in the *c-jun* promoter were not simultaneously occupied in normal CEFs (Figure 5.11 & 5.12). A pattern of mutually exclusive binding was supported by the converse effects of exogenous c-Jun and bzipFos on junTRE and junRSRE binding in vitro. The junRSRE binding activity was completely disrupted in the presence of c-Jun and bzipFos whereas the binding activity to the junTRE was markedly enhanced (Figure 5.17B). A parallel effect was not observed using a minimal junRSRE-containing oligonucleotide, suggesting that the junTRE binding site is required for the displacement but not the binding of the junRSRE-specific protein complex (Figure 5.17A).

Mutually exclusive patterns of DNA binding are commonly associated with gene regulation in vivo. They function as a concentration-dependent switch between transcriptional induction and repression and often control opposing physiological mechanisms such as differentiation and proliferation. Expression of the osteocalcin gene, for example, triggers the onset of osteoblast differentiation, and is regulated by the mutually exclusive binding of Vitamin D3 and Jun/AP-1. A hormone reponse element within the osteocalcin gene promoter presents overlapping binding sites for Vitamin D3 and Jun/AP-1, which independently activate and repress osteocalcin gene expression respectively, depending on the relative concentrations of each within differentiating and proliferating cells (Owen *et al*, 90; Schule *et al*, 90(i)).

The pattern of binding at the junTRE and junRSRE was unlike that of the osteocalcin gene promoter in that both complexes were present, and capable of binding to the *c-jun* promoter, in normal, exponentially growing CEFs (Figure 5.11A). *c-jun* mRNA was detected under these conditions of

growth (Figure 5.1), suggesting that a dual pattern occupancy, at the junTRE and junRSRE binding sites, is compatible with *c-jun* expression in vivo. A single pattern of occupancy was observed in ASV17-transformed CEFs (Figure 5.12). The absence of junRSRE binding, correlated with a dramatic down-regulation of *c-jun* mRNA (Figure 5.1). These observations have led to the proposal that *c-jun* expression is regulated by the balance of occupancy at the junTRE and junRSRE binding sites, with the absolute levels of *c-jun* expression reflecting the amount of junRSRE binding activity present within the cell. If only one site can be occupied at any one time then it must be presumed that different cells within a culture are either occupied at the junTRE or at the junRSRE. This, together with the asynchronous growth of exponential cultures suggests that there may be heterogeneity of occupancy at the junTRE and junRSRE that is possibly regulated during the cell cycle.

Exclusive occupancy of the junTRE was characteristic of ASV17-transformed CEFs. The pattern correlated with repression of endogenous *c-jun* and may represent an extreme consequence of mutually exclusive binding not observed in normal asynchronous cultures. Expression of an RSRF-related protein was unaffected in ASV17-transformed CEFs (Figure 5.15). The same antiserum recognized the junRSRE binding complex in normal CEFs (Figure 5.14A), suggesting that v-Jun down-regulates *c-jun* expression through a physical inhibition of the junRSRF DNA binding activity.

Competitive binding at the junTRE and junRSRE regulatory elements would support this hypothesis. The competitive model proposes that high levels of the v-Jun oncoprotein facilitate exclusive junTRE occupancy in ASV17-transformed CEFs, that physically inhibits binding to the adjacent junRSRE and thereby down-regulates *c-jun* expression. The model is complicated by the composition of junTRE binding complexes in normal

and ASV17-transformed cells. Only a proportion of the junTRE binding activity in normal cells was recognized by c-Jun-specific antisera (Figure 5.14B). The junTRE binding activity in ASV17-transformed CEF extracts, in contrast, was virtually entirely composed of v-Jun-containing complexes (Figure 5.13). These observations have led to the proposal that one consequence of v-Jun overexpression is the replacement of endogenous junTRE binding complexes with v-Jun-containing alternatives. Qualitative differences in junTRE binding activity may simply facilitate mutually exclusive binding in vivo. Alternatively they may express more active functions that contribute to the autoregulatory properties of the v-Jun oncoprotein.

- Sequestration

An alternative model for Jun-mediated auto-repression favours a Jun-specific disruption of junRSRE binding activity in vivo. The model proposes that high levels of v-Jun (or c-Jun) sequester a factor required for the formation of the junRSRE binding complex. Jun-specific sequestration would provide an explanation for the dramatic down-regulation of endogenous c-Jun protein in c-Jun- and v-Jun-expressing cells (Figure 3.13A) in spite of the high and equivalent levels of RSRF protein detected in both cell types (Figure 5.15), and the qualitatively distinct junTRE binding activities exhibited by normal (c-Jun-expressing) and ASV17-transformed (v-Jun-expressing) CEFs (Figure 5.9A).

Previous reports have identified the importance of a functional leucine zipper domain for c-Jun-dependent auto-repression in vivo (Castellazzi *et al*, 91). A leucine zipper-dependent interaction has not been reported between c-Jun and the RSRF proteins, but is widely recognized to mediate heterodimerization between the Jun and Fos transcription factor

families (Ransome *et al*, 89). A Fos/FRA-related component was identified within the junRSRE-specific RSRF complex in normal CEFs (Figure 5.13A). If this component was essential for optimal DNA binding activity of the RSRF proteins on the junRSRE, its sequestration through specific dimerization with excess v-Jun (or c-Jun) could effectively weaken the affinity of the junRSRE/RSRF interaction and thereby displace the RSRF-containing protein complex.

Addition of exogenous c-Jun to the in vitro DNA binding reactions effectively supported this hypothesis. The addition induced a displacement of junRSRE binding activity that was accompanied by a concomitant enhancement in junTRE binding. The elevated junTRE binding activity was attributed to the appearance of a novel Jun-Fos/FRA heterodimer due to a Jun-dependent sequestration of RSRF-specific Fos/FRA-related accessory factors. A contribution from exogenous c-Jun homodimers was excluded as a parallel effect at the junTRE was not observed using ASV17-transformed CEF extracts (Figure 5.17B). Addition of exogenous bzipFos failed to disrupt the integrity of the junRSRE binding activity in vitro. This was presumably due to the inability of Fos transcription factors to homodimerize and was used as further evidence to support the existence of a Fos/FRA-related RSRF-accessory factor. Exogenous v-Jun was not included in the analysis but was predicted to induce an equivalent disruption of junRSRE binding as v-Jun contains an identical leucine zipper domain to the c-Jun protein (Maki *et al*, 87; Nishimura *et al*, 88).

A role for Fos/FRA proteins in the formation of the junRSRE binding complex suggests a novel interactions between the Fos and RSRF protein families. The Fos proteins have been identified in multiple cases of transcriptional cross talk but are commonly associated with an additional Jun component in vivo. Jun proteins have been reported to directly displace Fos in interactions mediated through the Fos leucine zipper

domain (Stein *et al*, 93(ii)), or to physically participate in the multiprotein complex as an AP-1 heterodimer (Boise *et al*, 93; Jain *et al*, 92; Konig *et al*, 89; Masuda *et al*, 93; Wang *et al*, 94). The proposed interaction between the junRSRF and a Fos/FRA-related protein did not involve an additional Jun component (Figure 5.14A). Consequently, a Fos-specific functional domain is presumed to direct a productive interaction in vivo. The contribution made by Fos/FRA DNA binding to the overall integrity of the junRSRE binding complex was not investigated, but the highly conserved spacing of junTRE and junRSRE regulatory elements in *c-jun* promoter sequences of different species suggests that additional contacts between Fos/FRA-related proteins and the DNA may be significant (Figure 5.8A). The junRSRE binding activity was detected using a minimal junRSRE-containing oligonucleotide, spanning the entire junRSRE and half of the adjacent junTRE. TRE binding sites represent palindromic sequences of two overlapping half sites that are independently recognized by Jun and Fos proteins in the AP-1 heterodimer (Abate *et al*, 90(i); Risse *et al*, 89). Consequently, the Fos/FRA - RSRF interaction could be strengthened by additional contacts between the Fos/FRA-related proteins and the adjacent TRE half site. The displacement of junRSRE binding complexes by full length c-Jun protein together with the ability of c-Jun to contact specific bases within TRE half sites suggests that the DNA may provide a secondary anchoring function rather than the primary stimulus for the RSRF:Fos/FRA interaction. The assymetrical nature of palindromic TRE sequences presumably optimizes the affinity of Fos/FRA:DNA interactions in the presence of the complete element, and may provide an explanation for the low level of cooperativity between junTRE and junRSRE binding sites in vitro (Figure 5.11A & 5.11B).

These observations have led to the proposal that the Fos/FRA-related protein may be required for the formation of the junRSRE binding complex

and that the primary interaction is strengthened by additional contacts with adjacent DNA sequences. A titratable Fos/FRA-related accessory factor would provide a Jun-sensitive switch for the regulation of *c-jun* expression and possibly contribute additional transactivation activity to the RSRF-related proteins, analagous to the function of Elk-1 within the SRF ternary complex (Hill *et al*, 93; Marais *et al*, 93).

The models outlined above provide alternative, but not necessarily mutually exclusive, mechanisms for the correlation between *c-jun* expression and occupancy of the junRSRE binding site that is observed in the absence of detectable changes in RSRF protein expression. The Competitive Binding Model proposes that junTRE and junRSRE binding factors compete for overlapping regulatory elements within the *c-jun* promoter thereby mediating a concentration-dependent switch in gene expression. High levels of Jun protein, for example, would direct an increase in junTRE binding activity and a concomitant passive displacement of junRSRE binding factors on the *c-jun* promoter (Figure 5.22A). In the Sequestration Model for auto-repression high levels of c-Jun or v-Jun are proposed to actively displace junRSRE binding complexes through a Jun-mediated sequestration of a Fos/FRA-related junRSRF-accessory factor. A sequestration mechanism would be sensitive to the absolute levels of Jun expression and indirectly facilitate an exclusive pattern of occupancy at the junTRE (Figure 5.22B).

Observations such as the element of cooperativity between the junTRE and the junRSRE and the qualitative differences in junTRE occupancy in ASV17-transformed CEFs, support a mechanism of Jun-mediated auto-repression that includes both Jun-dependent sequestration and mutually exclusive binding. Consequently, an alternative model has been proposed to account for the cumulative results presented in this report. In the

Combined Model for auto-repression, a primary consequence of high c-Jun or v-Jun expression is the Jun-dependent sequestration of junRSRF-specific accessory factors. The concomitant displacement of junRSRE binding complexes from the *c-jun* promoter is then proposed to release the junTRE binding site and promote a corresponding increase in junTRE occupancy by cell-type-specific factors that may be significant for subsequent transrepression and the physical exclusion of further junRSRE binding activity (Figure 5.22C).

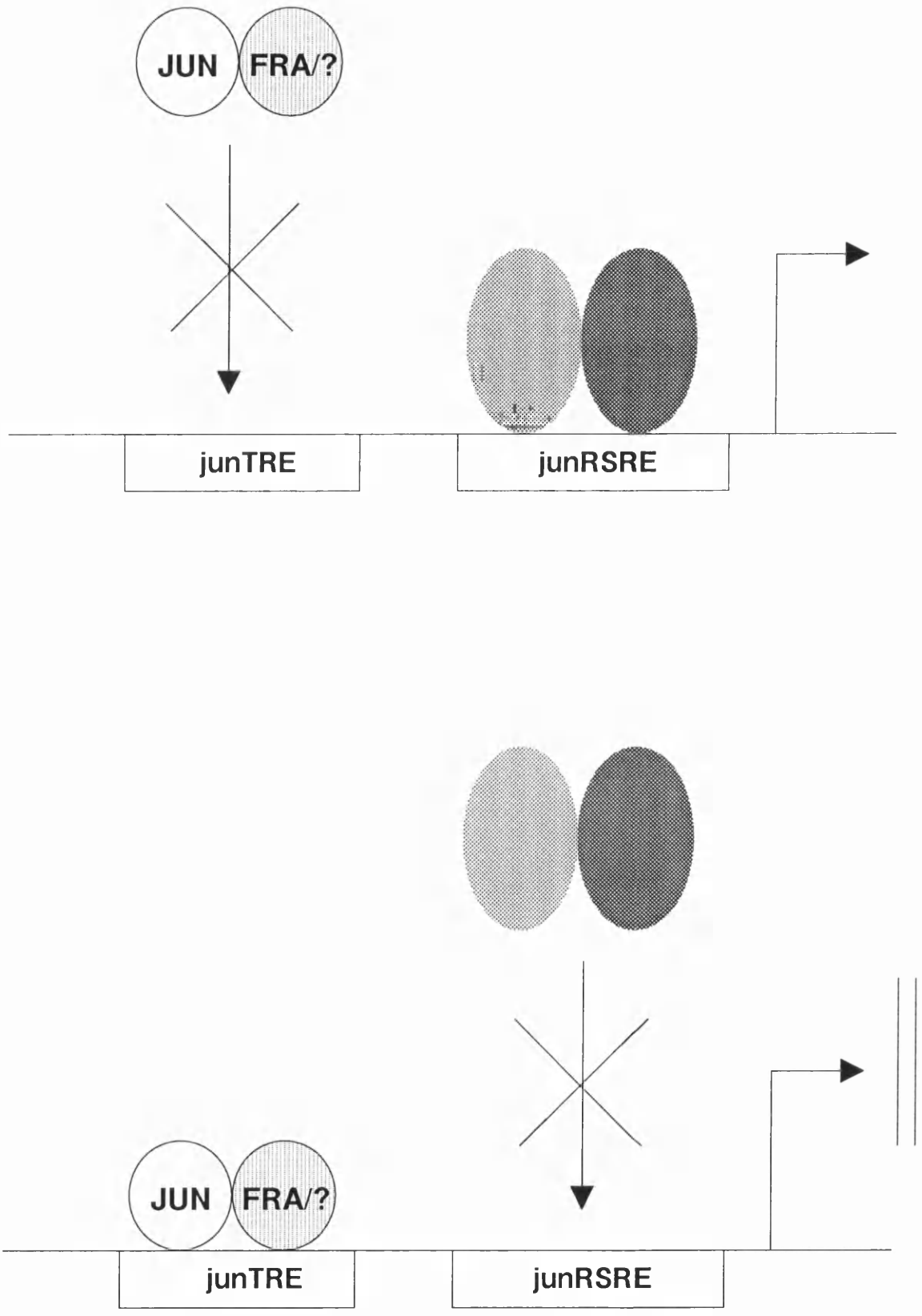
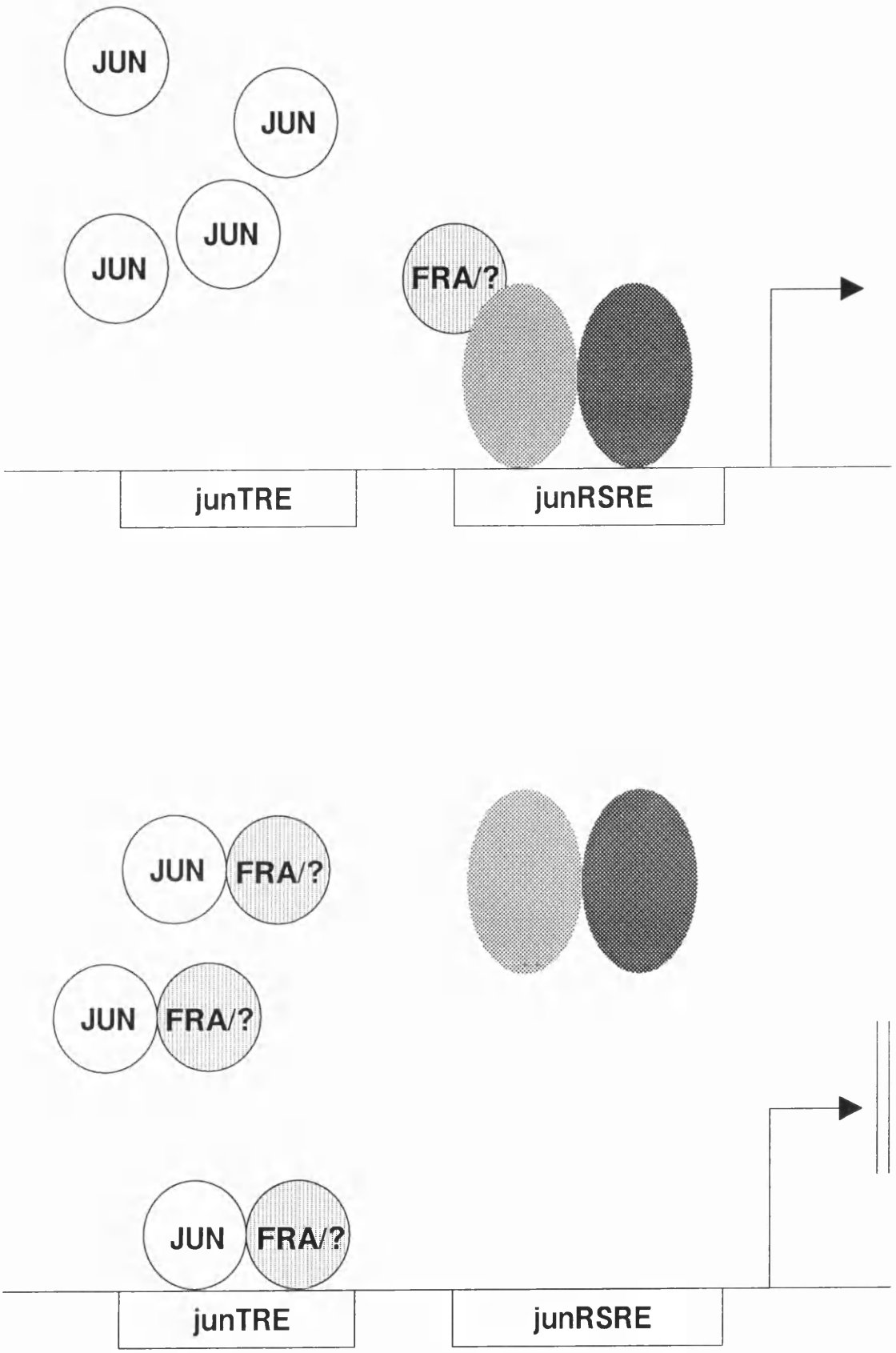


FIGURE 22.A


JUN
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FRA/?
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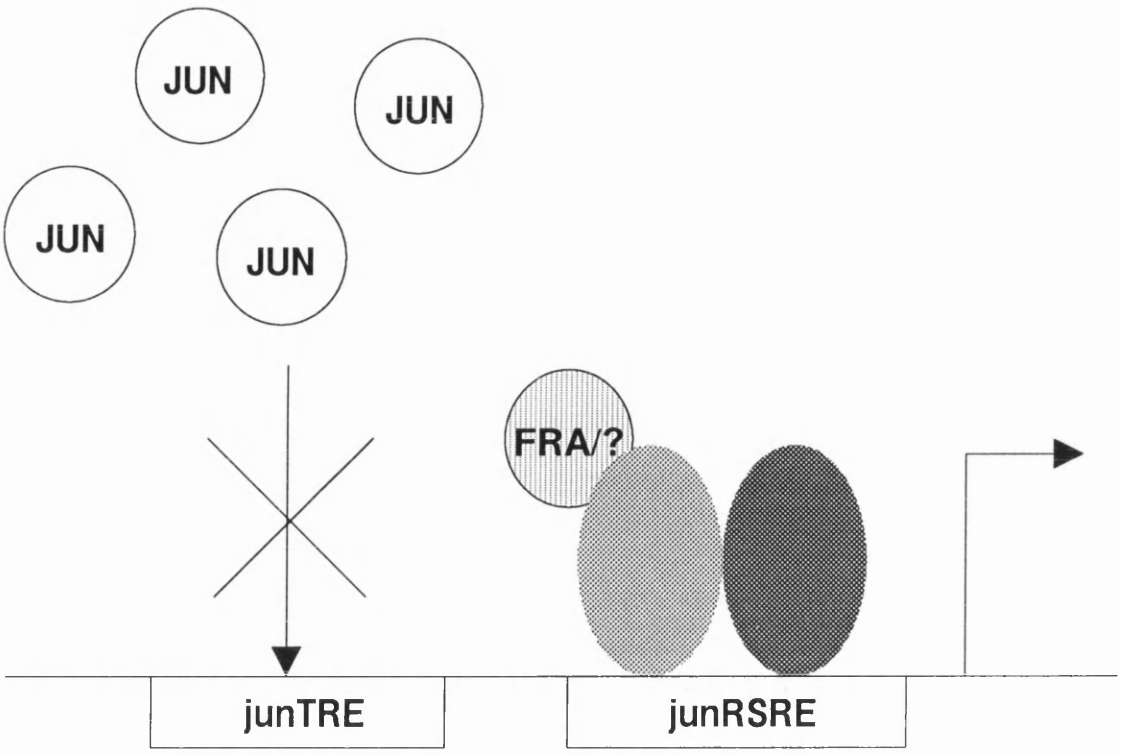
FIGURE 5.22B


JUN
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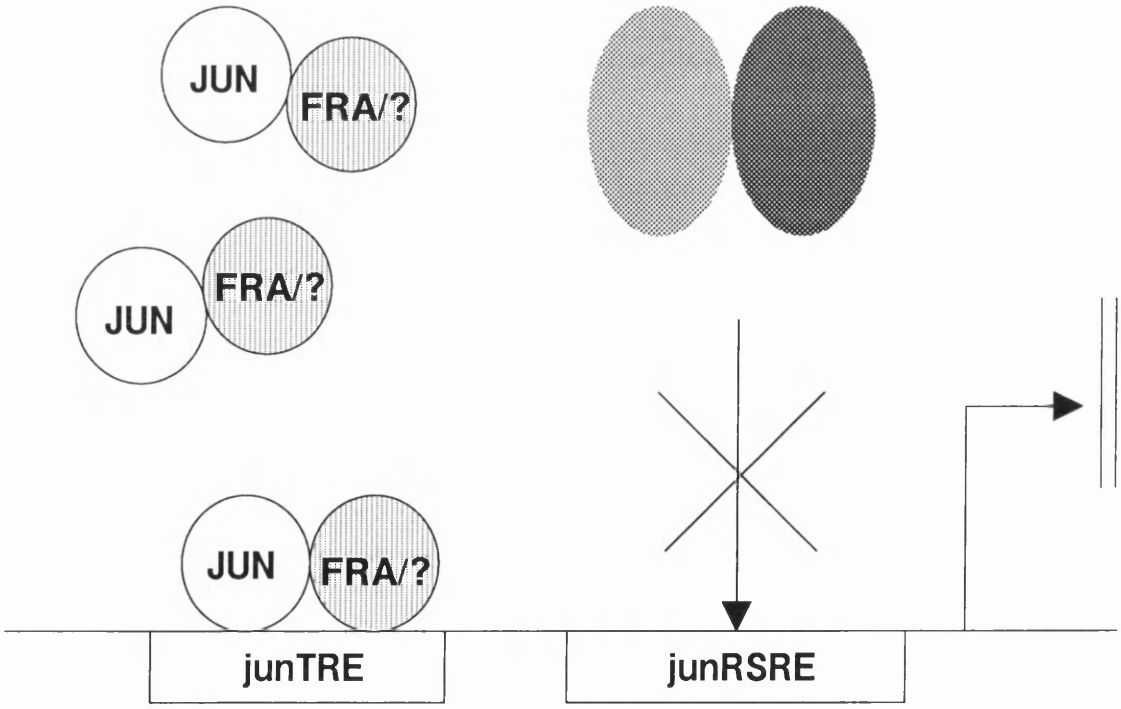
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FIGURE 5.22C

CHAPTER VII - SUMMARY.

CHAPTER VII - SUMMARY.

c-jun mRNA was induced with rapid and transient kinetics in response to serum stimulation. The pattern of expression was characteristic of other immediate early genes and dependent on transcriptional and post-transcriptional levels of regulation. Additional translational and post-translational regulatory mechanisms were proposed to contribute to the distinct kinetics of c-Jun protein expression under these conditions. Expression of endogenous p39 c-Jun was dramatically and specifically extinguished in the presence of the v-Jun oncoprotein, irrespective of the growth status of the cells. Overexpression of exogenous c-Jun induced an equivalent response indicating that auto-repression was mediated through high levels of c-Jun or v-Jun expression and not dependent on additional qualitative mutations in the v-Jun oncoprotein. The consequences of Jun-mediated auto-repression for endogenous AP-1 DNA binding activity were directly related to the apparently exclusive expression of c-Jun in normal CEF cultures. The down-regulation of endogenous c-Jun in ASV17-transformed cells was associated with a replacement of c-Jun-containing AP-1 DNA binding complexes with v-Jun-containing alternatives. The absence of auxiliary Jun family proteins may facilitate the displacement and thereby contribute to the unique transforming activity of v-Jun in avian cells.

v-Jun-mediated auto-repression was primarily directed at the level of transcription. The response was reproduced by transient CAT expression assays which confirmed the importance of high c-Jun and v-Jun expression and proximal *c-jun* promoter sequences for functional down-regulation in vivo. To identify potential regulatory elements in the proximal *c-jun* promoter, specific sequences were selected on the basis of

homology between different animal species and examined for changes in DNA binding activity that correlated with the down-regulation of endogenous c-Jun expression. A high level of promoter site occupancy was observed in normal *c-jun*-expressing CEFs. The overall pattern was unchanged in ASV17-transformed CEFs with the exception of the adjacent junTRE and junRSRE regulatory elements which were differentially occupied depending on the transcriptional status of the *c-jun* gene. The junTRE was exclusively occupied by v-Jun-containing complexes in ASV17-transformed CEFs. A junRSRE-binding complex was consistently absent in spite of high and equivalent levels of RSRF protein expression in normal and v-Jun transformed cells. The identification of an RSRF-related protein binding to the junRSRE in normal CEFs suggested that the exclusive pattern of occupancy in ASV17-transformed CEFs was mediated through a physical inhibition or disruption of junRSRE binding activity. Mutually exclusive binding or a Jun-mediated sequestration of RSRF-specific accessory factors were proposed as possible explanations for the absence of junRSRE binding activity in ASV17-transformed cells. The identification of a Fos/FRA-related protein in the RSRF-related protein complex supported the requirement for accessory factor interactions that could be disrupted by high levels of c-Jun or v-Jun. The competitive pattern of binding at the junTRE and junRSRE together with the qualitative differences in junTRE binding complexes in ASV17-transformed cells, suggested that both mechanisms may be important for the physical disruption of junRSRE binding activity in vivo and the concomitant transrepression of the *c-jun* gene.

CHAPTER VIII - REFERENCES.

CHAPTER VII - REFERENCES

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Amendment To Chapter VI

During the oral examination of this thesis some discrepancies were raised between the in vivo footprinting data and the in vitro gel shift analysis that will be addressed in more detail here.

In particular, a junRSRE-specific complex was identified by gel retard analysis that was common to both normal and ASV17-transformed CEF extracts (Figure 5.10). DNase 1 footprinting analysis over the junRSRE regulatory element corroborated the in vitro data and indicated that the junRSRE was occupied under both c-jun expressing (normal) and c-jun repressing (ASV17-transformed) conditions. In addition, it was suggested that the upper junRSRE-specific protein complex actually represented a doublet that was both Fos- (upper band) and FRA2- (lower band) reactive (Figure 5.14). Neither complex was considered completely absent from extracts prepared from ASV17-transformed CEFs (Figure 5.10).

In the light of these observations the models proposed in the final chapter of this thesis cannot be considered, and indeed were never intended, as absolute explanations of the data. They do not account for the presence of a junRSRE-specific complex under c-jun repressing conditions, or the quantitative differences in junTRE binding activity between normal and ASV17-transformed CEF extracts (Figure 5.9). In addition, the sequestration model for Jun-mediated auto-repression was primarily based on experiments using high concentrations of bacterially expressed, and therefore unmodified, c-Jun to disrupt junRSRE-specific binding complexes in vitro (Figure 5.17). Consequently, the models can only be considered as hypothetical proposals that may account for some, but not all, of the in vitro observations. Their relevance in vivo remains to be addressed through functional assays such as the CAT expression assays described in chapter V.D.