Studies of the Transcriptional Control of the Human c-fos Proto-oncogene.

Iain Matthew Morgan

Thesis submitted for the degree of Doctor of Philosophy in the University of Glasgow, being an account of research conducted at the Beatson Institute for Cancer Research, Glasgow.

September, 1989.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To Mum and Dad.

CONTENTS. PAGE

ACKNOWLEDGEMENTS.

I would like to thank my supervisor, Dr. George D. Birnie, for his advice and encouragement throughout this project and for many hours of useful discussion. In addition I am indebted to several colleagues at the Beatson Institute for their advice and technical expertise. In particular I would like to thank Drs. Mark Plumb, Robert Brown, Dave Gillespie and Margaret Frame.

I would also like to express my gratitude to the Medical Research Council and the Cancer Research Campaign for funding this project.

Thanks also to Margaret Blair who assisted in the preparation of this manuscript.

Finally, thanks to Linda for her constant patience and support throughout the years.

ABBREVIATIONS.

A, C, G, T,	Adenine, Cytosine, Guanine, Thymidine
AP-1	Activator Protein 1
ATF	Adenovirus Transcription Factor
bp	base pair(s)
β-gal.	β-galactosidase
^{o}C	degrees centigrade
CsCl	Caesium Chloride
cm, mm, μm	centimetre, millimetre, micrometre
CIP	Calf Intestinal Phosphatase
$CaCl_2$	Calcium Chloride
c-fos	Cellular fos gene
c-Fos	Cellular Fos protein
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DMS	Dimethyl sulphate
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
EtBr	Ethidium Bromide
EDTA	Ethylenediamine Tetra-Acetic Acid
EGTA	Ethyleneglycol Tetra Acetic Acid
FGF	Fibroblast Growth Factor
FCS	Foetal Calf Serum
G418	Geneticin
g, μg, ng	gram, microgram, nanogram
xg	Times gravity
kbp	Kilobase pairs

kD	kilodalton
LTR	Long Terminal Repeat
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
mRNA	messenger Ribonucleic acid
Ci	microcuries
M, mM	Molar, millimolar
MOPS	3-(N-Morpholino)propanesulphonic acid
NGF	Nerve Growth Factor
O.D.	Optical Density
PBS	Phosphate Buffered Saline
PPP	Platelet Poor Plasma
r.p.m.	Revolutions per minute
RNAse	Ribonuclease
SRE	Serum Response Element
SRF	Serum Response Factor
SV40	Simian Virus 40
NaAc	Sodium acetate
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
SDS	Sodium Dodecyl Sulphate
TPA	12-O-teetradecanoyl-13-phorbol acetate
tRNA	transfer Ribonucleic acid
TBRS	Tumour bearing rat serum
U.P.E.	Upstream Promoter Element
v-fos	viral fos gene
v-Fos	viral Fos protein
v/v	volume to volume

w/v	weight to volume
$ZnCl_2$	Zinc chloride

LIST OF FIGURES. PAGE

LIST OF TABLES. PAGE

ABSTRACT

Studies of the Transcriptional Control of the Human c-fos Proto-oncogene.

The c-fos gene is the cellular counterpart of the transforming genes of several retroviruses. Its transcription and expression is increased in many types of different cells following extra-cellular stimuli, and this increase is both rapid and transient in most cases. Many studies have focussed on the transcriptional control of the c-fos proto-oncogene. These studies have characterised an element (the serum response element - SRE) that was initially found to increase transcription from the c-fos promoter following serum stimulation of quiescent cells and subsequently has been shown to activate transcription from the c-fos promoter following a variety of extra-cellular stimuli in a variety of different cell types.

A computer-aided search of the human c-fos sequence revealed a sequence directly 3′ of the SRE that shows similarity to the DNA binding sites for the transcription factors AP-1 and ATF. These proteins bind to their sequences and confer phorbol ester and cyclic AMP responsiveness upon adjacent promoters. The purpose of this project was to re-examine the role of the SRE in stimulating transcription from the c-fos promoter, to characterise the transcriptional properties of the fosATF/AP-1 binding site that lies adjacent to the SRE, and to investigate any interaction that may take place between these two sequences in controlling transcription from the c-fos promoter.

Results obtained confirm that the SRE confers serum and phorbol ester responsiveness upon the c-fos promoter. They also show that the fosATF/AP-1 sequence forms a weaker complex with an AP-1/ATF protein than previously characterised AP-1/ATF binding sites, and that this sequence activates transcription from the c-fos promoter in growing cells. The fosATF/AP-1 binding site also confers serum and phorbol ester responsiveness upon the c-fos promoter. In quiescent cells the juxtaposed SRE and fosATF/AP-1 sequences contribute to a lower level of transcription from the c-fos promoter than does the SRE itself. This suggests an interaction between the proteins binding to these two sequences. Separating the SRE and fosATF/AP-1 sequences resulted in elevated levels of expression in quiescent cells and also resulted in higher levels of transcription from the c-fos promoter following stimulation of quiescent cells with serum or phorbol esters. It is concluded that there is a complex mechanism of interaction between the proteins that bind to the adjacent SRE and fosATF/AP-1 sequences and that there may be several proteins involved in this interaction.

CHAPTER 1 : INTRODUCTION

INTRODUCTION PAGE

1.1 Isolation and Structure of Viral and Cellular Fos Genes

1.1.1 A fos sequence is the transforming gene of FBJ-MSV

In 1966 a viral complex was isolated from a spontaneous osteosarcoma in a 260-day-old CFI mouse (1). This complex consisted of two viruses, FBJ-MLV (Finkel, Biskis, Jinkins murine leukaemia virus) which is a replication-competent helper virus and FBJ-MSV (FBJ murine osteosarcoma virus) which is replication-defective (2,3). When the FBJ viral complex was injected into neo-natal mice neoplasms developed at several sites and displayed bone-like properties (4). To identify the transforming sequence of the FBJ viral complex, presumably present in FBJ-MSV since this virus is replication-defective, the two viruses present in the complex were isolated separately in tissue culture (5,6). FBJ-MLV was isolated by diluting our the FBJ-MSV (it is present in a 10^3 to 10^4-fold excess over FBJ-MSV), and FBJ-MSV by the establishment of non-virus producing rat cells. These non-producer rat cells were injected into rats and serum was taken from those that developed tumours. This tumour-bearing-rat-serum (TBRS) identified, in immunoprecipitation reactions, two proteins present in FBJ-MSV transformed cells that were virus specific. These were a 55kD phosphoprotein (p55) which was the product of the transforming gene of FBJ-MSV, and a 39kD cellular protein (p39) which forms a complex with the viral transforming product (7).

The genome of FBJ-MSV was isolated from the non-producer rat cells used to raise the serum which identified the gene product (8). When

the plasmid containing the FBJ-MSV DNA sequence, pFBJ2, was transfected into rat 208F cells it induced morphological transformation. The transforming gene of FBJ-MSV was called v-fos (FBJ murine osteosarcoma virus) and a probe specific for this gene was taken from pFBJ-2 and used to probe Southern blots of cellular DNA from chicken, mink, mouse and human. All four species displayed a band when probed with v-fos suggesting that the transforming gene of FBJ-MSV has a cellular counterpart that is reasonably conserved, as would be expected if FBJ-MSV had transduced a cellular gene as its oncogenic sequence.

There have been two modes of viral oncogene expression discerned in cells infected with acutely oncogenic retrovisuses such as FBJ-MSV. The acquired cellular sequence, fos in this case, may be expressed as an independent viral gene product (9) or, alternatively, as a fusion protein containing peptides derived from the viral structural sequences (10). To determine which of these two possibilities was true for FBJ-MSV the DNA complimentary to the RNA of its helper virus, FBJ-MLV, was also cloned (11) as well as murine and human cellular fos genes, and comparisons were made between these sequences (and that of FBJ-MSV (12-14)). Heteroduplex analysis between FBJ-MSV and FBJ-MLV revealed that the FBJ-MSV genome had 6.8kb of the helper virus RNA deleted and replaced with 1.7kb cellular sequence thus creating v-fos. Heteroduplex analysis between FBJ-MSV and the mouse c-fos gene revealed five regions of homology separated by four regions of non-homology. The first three regions of discontinuity were due to introns in the mouse DNA while the fourth corresponds to a 104bp deletion from the v-fos gene (see Figure 1). The human and

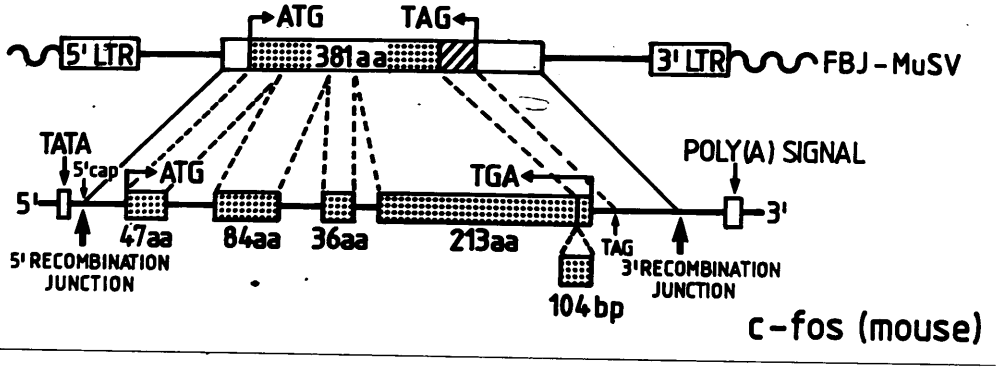


Figure 1. From c-fos to v-fos.

Molecular architecture of FBJ-MSV proviral DNA and mouse v-fos gene. FBJ-MSV is shown at the top. The large open box indicates the acquired cellular sequences; solid bars, initiation and termination coldons of v-Fos proteins; the hatched box, the C-terminal 49 amino acids of v-Fos protein encoded in a different reading frame because of deletion of 104bp of c-fos sequence; bold lines, 5′ and 3′ recombination junctions. In the c-fos gene below, the stippled boxes are the exons. The 104bp sequence that has been deleted in v-fos is indicated by a triangle. Unlike the v-Fos protein, the c-Fos protein terminates at a TGA codon. The position of the TAG codon which acts as a chain terminator for v-fos protein is shown by an arrow. This diagram is taken from I. M. Verma, Nature, 308, p317, 1984.

mouse c-fos genes have an almost identical structure with approximately 90% nucleotide sequence homology and this is why one can assume that the 104bp have been deleted from the v-fos sequence and not added to that of the c-fos. The results of the heteroduplex analysis showed that the v-fos gene present in FBJ-MSV was expressed as a single protein and not as a viral hybrid (e.g. gag-fos). This confirms earlier work which showed that the candidate product of FBJ-MSV, p55, could not be immumoprecipated with any virus-specific antibodies (6).

1.1.2. Two other retroviruses contain fos sequences responsible for transformation.

A viral complex was isolated from a ^{90}Sr-radiation-induced bone tumour in an X/Gf mouse (15) which contained two viruses, FBR-MSV (Finkil, Biskis, Reilly osteosarcoma virus) which is replication-defective and therefore presumably codes for the transforming gene, and FBR-MLV (FBR-murine leukaemia virus) which is the replication-component helper virus (16). FBR-MSV induced tumours are similar in pathology and histological appearance to those induced by FBJ-MSV and indeed FBR-MSV was shown to encode fos sequences which were presumably involved in the transformation properties of this retrovirus (17). The FBR-MSV genome was isolated from non-viral producing rat cells in a similar manner to FBJ-MSV (8) and sequenced which allowed comparison between the two fos-containing retroviruses and the mouse c-fos gene (18,19). TBRS used to identify p55, the product of FBJ-MSV, immunoprecipitated a 75kD protein that was also

immunoprecipitated using anti-gag serum. This suggested that the product of FBR-MSV was a gag-fos fusion product and this was confirmed upon the sequencing of FBR-MSV which was shown to contain an open reading frame that encoded the gag-fos fusion protein. Interestingly, the sequence also showed an additional cellular sequence, termed fox, that was present as the carboxy-terminal portion of the FBR-MSV transforming protein and thus this protein was called $p75^{gag-fos-fox}$. On comparison with the mouse c-fos sequence it was shown that FBR-MSV lacks the sequence that codes for the first 24 amino acids and the last 98 amino acids of the 380 amino acid mouse c-Fos product. In addition the coding sequence for $p75^{gag-fos-fox}$ has sustained three internal deletions, one within the gag sequence and the other two within the fos sequence.

The third, and so far the last, transforming retrovirus found to code for a Fos-related protein was isolated from a spontaneous nephroblastoma in an adult chicken (20). This retrovirus was called NK24 and, like FBJ-MSV and FBR-MSV, was replication-defective and so was isolated along with a replication-competent retrovirus. Upon re-injection of this virus into chickens tumours developed consisting of fibrobasts and osteoblasts with a degree of calcification, and in one particular chicken a nephroblastoma developed. A non-virus producing quail cell line was established and from these cells a 100kD protein was immunoprecipitated using anti-gag serum, suggesting that the transforming protein of this virus is a gag-fusion product. Upon isolation and sequencing of the NK24 genome this was confirmed as the virus contains an open reading frame that encodes a gag-Fos fusion protein of 100kD. On comparison of the fos sequence present

in NK24 with the chicken c-fos gene (21) it was observed that the oncogene product of NK24 contained a truncated version of the chicken c-fos gene as it lacks the nucleotides coding for the first 45 amino acids of the c-Fos protein.

1.2. Fos Proteins and Their Transforming Properties

1.2.1. Size and distribution of cellular and viral Fos proteins within the cell.

Immunoprecipitation and immunoblotting techniques using Fos-specific antibodies have been employed to identify the intracellular location of viral and cellular Fos proteins. These techniques detected the presence of Fos proteins (both cellular and viral) exclusively within the nucleus and immunoprecipitation of the Fos proteins co-precipitated a cellular protein, p39 (7, 18, 22, 23). p39 is complexed with the viral and cellular Fos proteins and is not antigenically related to them since denaturation of cell lysates completely abolishes the immunoprecipitation of p39 whereas the precipitation of the Fos proteins is unaffected.

The v-fos gene of FBJ-MSV has a 104bp deletion near the 3'-terminus when compared to the mouse c-fos gene and this deletion is out of frame resulting in $p55^{v\text{-}Fos}$ protein being 381 amino acid residues in length whereas c-Fos is 380 amino acids. The out-of-frame deletion means that the carboxy-terminal 48 amino acids of the c-Fos protein is completely different to the carbox-terminal 49 amino acids of the v-Fos protein (13). The preceding 332 amino acids, prior to the 104bp deletion region, show only 5 amino acid differences between the v-Fos and c-Fos proteins. To determine whether the different carboxy-terminals present in the v-Fos and c-Fos proteins leads to different protein populations, a pulse-labelling experiment was carried out with cells expressing either the c-Fos or v-Fos proteins

(22). After 15 minutes of labelling both cell lines, immunoprecipitation studies detected identically sized proteins of 54kD which were shown to smear up to around 55kD (p55). However, thereafter the pattern produced by the two cell-lines became very different. After 2 hours of labelling, in the cells expressing c-Fos protein, products of 57, 60 and 62kD could be detected by immunoprecipitation studies. The cells expressing v-Fos contained a product of 55kD that is presumably derived from the 54kD protein by some slight modification. At this point it is necessary to define a general rule for the nomenclature of viral and cellular Fos proteins. v-Fos will be used when describing $p55^{v\text{-}fos}$ (or in relation to any of the other transforming viral proteins) and c-Fos protein will be used to describe the heterogeneous populaton of modified forms of c-Fos (57, 60 and 62kD).

Because the c-Fos and v-Fos proteins have different carboxy termini it was suspected tha the differences in the protein populations were due to differential phosphorylation of the respective proteins on their carboxy-terminal regions. There have been conflicting reports regarding this possibility. The first report suggested that the differential modification of v-Fos and c-Fos was not due to their different carboxy-termini (24). This study involved the analysis of the protein products of transfected plasmids encoding the v-Fos protein, the c-Fos protein and c-Fos carboxy-terminal truncated proteins. These studies were carried out in E.coli and S.cerevisiae cells and in the prokaryotic cells the c-Fos protein was not modified suggesting that the modification process is eukaryotic-specific. In S.cerevisiae cells the modification of the c-Fos protein took place

with both the intact gene and also with the deletion mutants that had the carboxy-terminal sequences removed, suggesting that these sequences were not responsible for the modifications of the c-Fos protein in these cells. Another report provided a conflicting set of results (25). Treatment of cell extracts containing c-Fos or v-Fos proteins with alkaline phosphatase showed that most of the c-Fos protein modifications were due to phosphorylation. The c-Fos protein was 4- to 5-fold more highly phosphorylated than the v-Fos protein and comparison of tryptic peptide fragments from ^{32}P-labelled proteins indicated that, although the two proteins have several tryptic phosphopeptides in common, the c-Fos protein contains unique major phosphopeptides. These were tentatively localised to the carboxy-terminal 20 amino acids of the c-Fos protein.

1.2.2. The c-Fos protein is a transforming protein.

Not surprisingly it was originally thought that the altered carboxy terminus of the v-Fos protein was responsible for the transformation properties of FBJ-MSV. Indeed, the introduction of the c-fos gene into fibroblasts failed to induce the morphological transformation of these fibroblasts. However, when chimaeric plasmids were made containing viral (FBJ-MSV) and cellular (mouse) fos sequences, the c-Fos protein was shown to be capable of transforming fibroblasts (26). Two manipulations of the c-fos gene were required to activate its transformation properties.

i) The transcription of the gene had to be driven by a viral LTR.

ii) Sequences in the 3′ translated and 3′ untranslated regions

had to be disturbed.

These two manipultaions presumably lead to a greater level of transcription of the c-fos gene and a stabilisation of the mRNA, both of which would lead to increased levels of c-fos mRNA and therefore increased levels of c-Fos protein. A sequence in the 3′ untranslated region downstream from the translation stop codon and upstream from the polyadeylation site has been identified as being important in suppressing the oncogenic potenital of the c-fos gene (27). Removal of this sequence leads to the activation of the transforming potential of the c-fos gene (providing transcription is driven by a strong promoter such as a viral LTR). Similar alterations in the human c-fos gene are required to activate its transforming potential, and it was also shown with the human gene that the removal of the 3′ untranslated sequence from c-fos did indeed result in greater levels of c-fos RNA due to increased mRNA stability (28).

Upon analysis of the c-Fos protein present in rat 208F fibroblasts transformed with c-fos (both human and mouse) it was noted that the protein was less modified than its normal cellular counterpart and also that its half-life was reduced to 30 minutes (instead of 2 hours). The c-Fos protein that is responsible for transformation in these cells is similar in size to that found in v-fos transformed cells (22). One explanation for this could be that in the cells expressing endogenous c-Fos protein phosphorylation cascades which modified the c-Fos protein could be operating, and these cascades may not be operating in the c-fos transformed fibroblasts leading to a slowing down in the modification process.

To date, there have been no reports of c-fos sequences being the transforming gene in tumours isolated from any species. However, the human c-fos gene maps to chromosome 14, band q24.3-q31 (29) and this region has a high frequency of aberrations in connection with various types of human neoplasia (30). The activation of the c-fos transforming potential would require the deletion of the 3′ sequences responsible for mRNA destablisation and the addition of a strong transcriptional activator in the vicinity of the gene and these two criteria would most easily be met by a chromosomal translocation. Such translocations have been implicated in activating the transforming potential of the c-myc and bcr-abl genes (31, 32). However it has not yet been shown that these altered sequences can cause cellular transformation and their precise role in the transformation process is not yet clear. It will be of interest to see if any such translocations can be identified involving the c-fos gene resulting in the activation of its transforming potenetial.

To determine whether c-fos sequences can induce malignant transformation in animals, transgenic mice populations were established. These mice had the mouse c-fos sequences under the transcriptional control of the metallothionein promoter (33) or the $H2\text{-}K^{6}$ (the murine major histocompatibility complex) promoter (34). Interestingly, the c-fos 3′ untranslated sequence which destabilises the c-fos mRNA had to be removed from the c-fos sequences to obtain elevated levels of expression of the exogenous c-fos sequences in the transgenic mice. None of the c-fos transgenic mice established developed malignancies similar to those developed with FBJ-MSV although the c-Fos protein did interfere with normal bone development

when expressed in bone tissue. Visible swelling on the tibia of both hind legs could be detected in certain transgenic mice, although all of them displayed an aberrant bone formation upon histological examination and the phenotype of the bone aberrations was generally inherited. However, as mentioned above, these lesions were not malignant and failed to progress to tumours even after ten months.

The H2-k^6 promoter allowed for a wider spectrum of tissue expression for the exogenous c-fos sequences (although they did not cause expression of c-fos sequences in bone tissue), including haematopoietic cells which did not express the exogenous c-fos sequences under the transcriptional controls of the metallothionein promoter. These mice displayed aberrant thymus development due to increased levels of epithelial cells which resulted in altered T-cell development (possibly due to abnormal levels of hormones coming from the epithelial cells), resulting in immune deficiency. However, once again these mice showed no sign of malignancies due to the exogenous c-fos sequences.

Many reasons can be thought of for the failure of the exogenous c-fos sequences to cause malignancy. Conclusions can only be drawn from these transgenic experiments when the viral sequences and chimaeric v-fos/c-fos sequences are also used to establish transgenic mice and the effects of these sequences upon for example, bone structure and development, are determined.

1.2.3. FBJ-MSV and FBR-MSV have different biological properties.

The two acutely transforming murine retroviruses encoding fos sequences are FBJ-MSV and FBR-MSV which code for two different proteins : $p55^{v\text{-}fos}$ and $p75^{gag\text{-}fos\text{-}fox}$, respectively. The FBJ-MSV fos gene consists entirely of cellular fos sequences, the only difference between the viral and cellular genes being a deletion at the 3′ terminus of the c-fos gene resulting in different carboxy terminals for the v-Fos and c-Fos proteins (13) (see 1.2.1.). The FBR-MSV transforming fos sequence is very different from that of FBJ-MSV as it has truncated 5′ and 3′ ends (which have been replaced by other viral and cellular sequences) and contains several deletions and point mutations. These different protein structures lead to different biological properties for the two proteins with FBR-MSV possessing a more potent transforming activity in rat fibroblasts than FBJ-MSV (18), even although both viruses can induce tumours when injected into newborn mice. This observation was confirmed in a subsequent report (35) which also established that the gag sequences present in $p75^{gag\text{-}fos\text{-}fox}$ were not responsible for the increased transforming ability of this protein. It was also observed that FBR-MSV could establish low-passage mouse connective tissue cells as well as cause their transformation (36). This showed that the transforming protein of FBR-MSV also had immortalising properties. Although FBJ-MSV could also transform these low-passage mouse connective tissue cells the resultant transformed cells did not become established. This shows that FBJ-MSV does not possess immortalising properties and it was also observed that this virus induced smaller foci after a longer latency period than did FBR-MSV.

The different biological properties of the FBR-MSV gene product compared with that of FBJ-MSV appear to be directly related to structural alterations, since similar expression levels and turnover rates are observed with both proteins (22, 37). A comprehensive panel of FBJ/FBR/c-fos chimeric genes was used to characterise those structural alteration in Fos protein that are responsible for enhancing its transforming potential (37). This analysis yielded three major results:

1. An FBR-MSV specific amino acid exchange (glu to val at amino acid position 138) introduced into FBJ protein activates the immortalising potential of the resultant protein.
2. Replacement of glu-175 with lys in FBR-MSV (representing an FBJ-MSV specific amino acid exchange) leads to an approximately 80% decrease in the number of foci induced.
3. Replacement of the two small in-frame deletions in the C-terminal half of the FBR-MSV protein by the corresponding fos sequences reduces the transforming efficiency to approximatley 25% of that of the wild type protein.

So, it was possible to dissociate the enhanced transforming potential of FBR-MSV from its immortalising properties as any alterations which decrease its transforming efficiency do not affect its immortalising properties, and the point mutation which abolishes its immortalising properties has very little effect upon its enhanced transformation properties.

Although FBR-MSV and FBJ-MSV have different transforming properties there is evidence to suggest that both oncogenic proteins of these

retroviruses induce transformation through similar mechansism (38). This evidence has come from the study of revertants of FBJ-MSV transformed rat cells. Revertants of these cells, isolated by mutagenesis, are refractory to re-transformation with either FBJ-MSV or FBR-MSV (as well as other oncogenes). Results from this study suggest that the revertants carry mutations in cellular effector genes (those genes that are the target for oncogenic proteins) and therefore any failure to re-transform these cells suggests that the potential transforming proteins act through simialr cellular sequences.

1.3. c-fos Expression During Development and Differentiation.

1.3.1. Expression of c-fos during mouse and human development.

To determine whether c-fos is expressed in a tissue-type or stage-specific manner the level of c-fos transcripts were analysed in the embryo and extra-embryonal tissues of mouse and humans (39-42). In 10 day-old mouse foetuses there are higher levels of c-fos transcripts present in the placenta than in either the embryo proper or extra-embryonal tissues and these high levels persist until day 18, remaining both relatively high and constant throughout gestation. The level of c-fos transcripts is > 15-fold higher in the separated outer portion of the midgestation placenta (relatively undifferentiated) than in the inner moiety (predominantly differentiated). Both foetal (trophoblast) and maternal (decidua) moieties of the placenta are rapidly proliferating tissues and the mouse trophoblast has been considered a "pseudo-malignant" tissue as it grows invasively into the uterine epithelium or any other surrounding tissue. In the amniotic tissue the level of c-fos transcripts increases during gestation and in 18-day conceptuses the abundance of the c-fos transcripts in the amnion (which increases 2-fold from 12 to 18 days) approaches the levels of v-fos transcripts present in virus-transformed cells. These levels of c-fos expression were similar in human amniotic and chorionic tissues, and the rest of the human tissues also followed a similar pattern of c-fos expression to that of the mouse.

When a peptide antibody was raised against a c-fos sequence and used to determine the levels of c-Fos protein in the embryonic and extra-embryonic tissues the antibody reacted with the nuclei of cells approximately in proportion to the level of c-fos transcipts detected in the cells (43). It was also possible to detect immunoreactive material in the nuclei of most foetal and adult tissues using relatively high concentrations of antibodies, even though c-fos transcript levels were present at very low levels in most of these tissues. In all cases where the antibody reacted, representing the c-Fos protein, the reaction occurred in the nucleus and the previously identified cellular protein p39 could be co-precipitated from cells expressing the c-Fos protein which is similar to previous findings (7).

In a further study it was observed that there were high levels of c-fos transcripts and protein present in the parietal endoderm of 13.5 day-old mouse foetuses and these levels were higher than those observed in the amnion or placenta at this stage of development (44). In a bid to mimic the in vivo situation, F9 teratocarcinoma cells (a murine embryonal-carcinoma cell line) were induced to differentiate to parietal endoderm-like cells by retinoic acid and dibutyryl cAMP treatment. However, no elevated levels of c-fos transcripts could be detected in these terminally differentiated cells although a rapid and transient increase (about 3-fold) was observed within the first 2 hours after treatment. It was suggested that the failure to observe elevated levels of c-fos transcripts in the terminally differentiated F9 cells was possibly due to the cells not receiving certain growth factors or hormones that may be responsible for the elevated levels

of c-fos transcripts observed in the parietal endoderm cells. This possibility gained further credence when it was observed that in mouse primary amniotic cells grown in normal growth medium the levels of c-Fos protein and c-fos transcripts decreased to undetectable levels after a short time (45). However, in these cells c-fos expression is inducible by, and maintained at high levels in the presence of, dialysed placenta or embryo conditioned medium (dialysis removes elevated ion concentrations which may have an effect on c-fos expression). This strongly suggested a growth factor presence in the placenta or embryo-conditioned medium that was responsible for the increased levels of c-fos expression.

In post-natal mice (1 to 3 days) low levels of c-fos transcripts could be detected in all tissues (39) although RNA extracted from 'bone' tissue (sternum, ribs and vertebrae including bone marrow, muscles and part of the placenta) and 'skin' tissue (including sub-cutaneous tissue, muscles and part of the peritoneum) showed 5 to 20-fold greater levels of c-fos transcripts than other tissues. Analysis of a 17 day-old mouse embryo by <u>in situ</u> hybridisation demonstrated that c-fos expression is restricted to the perichondrial growth regions of the cartilaginous skeleton and to the web forming mesodermal cells which are characterised by a stage-specific high growth capacity (46). No c-fos transcripts could be detected in either of these tissues in day 10 or 13 embryos suggesting that c-fos transcripts are restricted to those parts of the foetal bone having the highest growth potential at about day 17 of development. This result was confirmed by a subsequent study (47) using fos-specific antibodies which also showed c-Fos protein present in the bone tissue

of day 17 embryos.

Thus, although here is a definite stage- and tissue-specific expression of both the c-fos transcript and protein the role that c-fos expression plays in the development of mouse and human embryos remains unclear. The elevated levels do appear to coincide with tissues that are rapidly proliferating (such as the amnion and placenta) but when amniotic primary cells are plated out in normal growth medium the levels of c-fos expression become undetectable but the cells retain the potential to proliferate, suggesting that elevated levels of c-fos are not crucial to the proliferation of these cells. Perhaps c-fos expression is required for an initial burst of proliferation and the degree and timing to which it is then turned off is less relevant to particular cells. However, it is very difficult on the basis of these studies to assign a definite role to the c-fos expression in the embryo and extra-embryonal tissues. Interestingly, in certain tissues (e.g. the amnion in both mouse and humans) the levels of c-Fos protein detected are similar to those found in v-fos transformed fibroblasts. Obviously the amniotic tissue is not transformed, and amniotic cells fail to induce tumours when injected sub-cutaneously into mice. This suggests that the c-Fos protein, as it can transform fibroblasts, requires to be elevated in inappropriate cell types in order to cause transformation. Other reasons for this failure to observe transformation in the amniotic cells can be suggested such as different post-translational modifications of the c-Fos protein occurring in the amniotic cells resulting in a different c-Fos protein population, perhaps preventing the accumulation of c-Fos

protein that is capable of transformation. One fact that would argue for the latter possibility is that elevated levels of c-fos expression are observed in bone tissue which is the target for the transforming FBJ-MSV and FBR-MSV retroviruses. However, it is possible that the virus infects different cells to those expressing the c-Fos protein, and it is also possible tha the v-Fos proteins transform simply because of their elevated expression levels.

1.3.2 Role of c-fos expression in haematopoietic cell differentiation.

Cellular proto-oncogenes were suspected of playing a pivotal role in crucial biological functions such as differentiation because of their ability, or that of their viral counterparts, of causing cellular transformation when expressed in an aberrant fashion. To examine the role of c-fos in haematopoietic cell differentiation the levels of c-fos expression were examined in several haematopoietic cell-lines (many of which can be induced to differentiate by chemical inducers) and in isolated populations of normal and leukaemic blood cells. These studies provided confusing and sometimes contradictory results with several reports arguing for and against a crucial role for c-fos expression in the differentiation of haematopoietic cells.

Most of the evidence indicating that c-fos expression does play a crucial role in haematopoietic cell differention has come from studies of leukaemic cell-lines and leukaemic blood cell populations. These were studied as leukaemias represent different lineages of blood cells maturation arrested at various stages prior to terminal

differentiation, and many of the leukaemic cell-lines can be induced to terminally differentiate, usually by chemical inducers. Two leukaemic cell-lines that have been employed extensively in these studies are the promonocytic cell-line U-937 (48) and the promyelocytic cell-line HL-60 (49), both of which can be induced to differentiate to macrophage-like cells after treatment with TPA (50, 51). Treatment of U-937 and HL-60 cells with TPA results in a rapid induction of c-fos expression which peaks around 30 minutes after treatment and decreases thereafter; although the levels of expression do remain elevated in comparison to basal levels for at least 90 hours (52 - 55). Treatment of HL-60 cells with DMSO or retinoic acid results in differentiation to granulocyte-like cells (56), but no induction of c-fos expression is observed following these treatments. Supporting the link of c-fos expression to macrophage differentiation is the observation that c-fos expression in HL-60 cells decreases upon "de-differentiation" of macrophage-like cells (57). This observation was made with sub-lines of HL-60 cells treated with 1,25-dihydroxy-vitamin D_3 which results in the cells sticking to the plastic culture dishes and gaining macrophage like properties which resulted in an increased expression of c-fos. However, upon removal of 1,25-dihydroxy-vitamin D_3 from the medium the cells apparently "de-differentiated" losing their macrophage-like properties and regaining those of undifferentiated HL-60 cells. This "de-differentiation" is accompanied by a decrease in c-fos expression to undetectable levels suggesting that c-fos expression is involved in the differentation of HL-60 cells to macrophages.

In leukaemic blood cell populations c-fos expression is restricted to

acute leukaemias with monocytic phenotypes in most reports, although samples representing precursors of granulocyte-like cells have also been reported as showing elevated levels of c-fos expression (59-61). Primary cells from some of the leukaemic samples could be induced to differentiate with TPA into monocytic-like cells and these inductions were accompanied by an increase in the levels of c-fos expression, once again suggesting that c-fos expression may be linked to the monocyte-macrophage differentiation pathway.

The above results indicate that c-fos expression may play a role in the monocyte-macrophage differentiation pathway. However, there have been several reports suggesting that this is not the case. The murine myeloid leukaemia cell-line WEH1-3B (D^+ subline) (62) can be induced to differentiate into macrophage-like cells after treatment with granulocyte-colony-stimulating factor plus actinomycin D (63) and c-fos expression is not elevated in these cells until 48 hours after treatment when most of the cells have already differentiated. This expression at later stages of macrophage development does not preclude a role for c-fos expression in the maturation of macrophages. More direct evidence against the idea of c-fos expression playing a crucial role in the macrophage differentiation pathway has come from studies of HL-60 and U-937 cells. It has been shown that c-fos expression is neither sufficient nor necessary for the differentiation of these two cell-lines to macrophage-like cells (64, 65). When U-937 cells were treated with a protein kinase C agonist (diacyl glycerol), or serum deprived and then serum stimulated, c-fos expression is induced in a similar fashion to that obtained following TPA treatment, but this induction does not result

in an induction of macrophage-like differentiation. A similar treatment was also given to HL-60 cells resulting in a rapid increase in c-fos expression but no differentiation to macrophage-like cells. These results showed that the induction of c-fos expression was not sufficient by itself to induce macrophage-like differentiation in either of these cell-types. HL60 sub-lines that were resistant to induction of differentiation by TPA were employed to analyse whether c-fos expression was necessary for the macrophage differentiation pathway. When these sub-lines were treated with 1,25-dihydroxy-vitamin D_3 they differentiated into macrophage like cells but did not show the rapid induction of c-fos expression. Similarly, when HL-60 cells were treated with TPA in the presence of retinal (an inhibitor of protein kinase C) the induction of c-fos expression is rapidly reduced although the cells differentiate to macrophage-like cells in a normal fashion. Both of these results suggest that c-fos expression is not necessary for the macrophage-like differentiation of HL-60 cells.

In normal blood cell populations there is confusion in the reported levels of c-fos expression in monocytes and macrophages in comparison with other cell-types. Several studies suggest that c-fos expression is indeed restricted to monocyte and macrophage cells (61, 66) whereas others have identified elevated levels of c-fos expression in granulocytes when compared to monocytes and macrophages (67, 69). The reason for these discrepancies is not clear but could involve the different methods of isolation of the blood cell populations which may result in different micro-environments containing different growth factors which may have variable effects on c-fos expression.

However, in summary it would seem that c-fos expression is not linked exclusively to marcophage-like differentiation and indeed is apparently not necessary or sufficient for such differentiation. It would seem that the induction of the c-fos expression in haematopoietic cells is more the cause of the stimulus on the cell rather than the differentiation that results from these stimulations. Several pieces of evidence support this hypothesis.c-fos expression can be induced in terminally differentiated mouse peritoneal macrophages by treatment with CSF-1 (53, 70) and by activation of protein kinase C (71). c-fos expression can also be induced in HL-60 cells induced to differentiate to granulocyte-like cells following treatment with dibutyryl cAMP (88) showing that c-fos expression is not restricted to the macrophage-like differentiation of these cells. So, it would seem that c-fos expression can be induced in haematopoietic cells by many external stimuli and that this expression is most likely due to the external-stimuli and is not linked to any particular differentiation pathway.

1.3.3 Role of c-fos expression in the differentiation of embryonal carcinoma cell lines.

The strongest evidence implying a role for c-fos expression in the differentiation process has come from studies on murine embryonal carcinoma cell lines (72). These cells have been used as a model system to study differentiation and development as they can be induced to differentiate into a variety of cell types by treatment with chemical inducers. Upon transfection of plasmid constructs which lead to constitutive expression of either the human or mouse

c-fos genes into the embryonal carcinoma cell line F9 (73), morphologically altered cells appear which express specific markers characteristic of differentiated cells (74). The expression of c-fos in these cells is usually very low (75) and it was established that the appearance of markers indicating differentiation of the cells was due to increased levels of c-fos mRNA and protein. These morphologically differentiated cells were not recognisable as any previously identified cell-type and did not express a complete set of differentiation markers characteristic of any previously identified differentiated cell. These observations suggest that the increased exogenous c-fos expression leads to the expression of certain differentiation markers but that terminal differentiation to an identifiable phenotype may require the expression of other genes as well as c-fos. Interestingly, F9 cells can be induced to differentiate with retinoic acid treatment without any elevated levels of c-fos expression at any time point and constructs efficiently expressing anti-sense c-fos RNA failed to prevent the differentiation of these F9 cells (76). However, as mentioned in 1.3.1, treatment of F9 cells with retinoic acid plus dibutyryl cAMP leads to a rapid but transient induction of c-fos expression which may be due to the added dibutyryl cAMP or a combination of effects between the two inducers (44). So, the studies on c-fos expression in differentiating F9 cells suggest that expression of this gene is neither sufficient by itself nor necessary to produce recognisable differentiated cells although high levels of expression certainly do affect the morphology of these cells.

When the plasmid constructs transfected into F9 cells have c-fos expression under the control of an inducible metallothionein promoter (77) it was observed that increasing the c-fos expression 100-fold (by treatment with cadmium) in the transfected cells did not result in any progression in the differentiated state of the F9 cells, suggesting that the induction of differentiation markers by c-fos expression is not dose dependent. These constructs were transfected into other embryonal carcinoma cell lines which yielded different results. After expression of exogenous c-fos, P19 cells (a murine embryonal carcinoma cell line (78)) showed expression of differentiation markers similar to those detected in F9 cells although the cell line PC13 (another murine embryonal carcinoma cell line (73)) failed to express any such morphological differentiation markers. An explanation for this observation could be that F9 cells have less pronounced stem cell characteristics than either P19 or PC13 cells as the F9 cells show higher levels of EGF receptor and transferrin expression (79), suggesting that these cells are more mature than P19 or PC13 cells. So, this evidence suggests that certain events have taken place in F9 cells, but not PC13 cells, that may be required for the exogenous c-fos expression to cause its observed differentiation effects. c-fos expression can be detected in P19 cells at later stages of differentiation (after 12 days of treatment) following treatment with DMSO or DMSO plus retinoic acid (80, 81), so it is rather surprising that exogenous c-fos expression should have such an effect on the immature undifferentiated P19 cells.

Although the evidence is once again confusing these studies with embryonal carcinoma cell lines provide the strongest evidence linking c-fos expression to differentiation. The apparent presence of a sub-set of differentiation markers on the altered cells would suggest that c-fos expression on its own is not capable of inducing a terminal differentiation process, but rather it requires co-operation with other genes which are as yet unidentified. This is not surprising as it is not to be expected that expression of one single gene should control a complex process such as differentiation.

1.3.4 c-fos expression in differentiating PC12 cells and in neuronal tissues in vivo.

PC12 cells are a rat pheochromocytoma cell line (82) that can be induced to differentiate into neurite-like cells by treatment with nerve growth factor (NGF) (82) or to chromaffin-like cells upon treatment with dexamethasone (83) NGF acts on a specific receptor on these cells and increases c-fos expression both rapidly and transiently with a peak in expression occuring after 30 minutes before becoming undetectable again by 120 minutes (84, 86). PC12 cells represent an asynchronously growing population of cells and the induction of differentiation by NGF leads to a cessation or reduction in the rate of cell division. Several other extra-cellular stimuli will induce rapid and transient c-fos expression in PC12 cells and some of these stimuli result in the differentiation of PC12 cells to neurites while others do not lead to any recognisable differentiation. So, although c-fos expression can be induced when PC12 cells differentiate to neurite-like cells (induction by

dexamethasone treatment to chromaffin-like cells does not induce c-fos expression) this induction is not sufficient for differentiation. In PC12 cells stably transfected with a plasmid encoding an inducbile N-ras oncogene the cells can be induced to differentiate by NGF to neurite-like cells when N-ras expression is induced, but this induction occurs without the familiar rapid and transient increase in c-fos expression (87). Together these results suggest that c-fos expression is neither sufficient nor necessary for the differentiation of PC12 cells to neurite-like cells, which is a similar conclusion to that obtained with haematopoietic cells (see sub-Section 1.3.2.). As with the haematopoietic cells it seems more likely that the induction of c-fos expression in PC12 cells is a response to the extra-cellular stimuli and does not play a crucial role in the differentiation of these cells. Several observations agree with this hypothesis such as the ability of NGF to re-induce rapid and transient c-fos expression in NGF-induced differentiated PC12 cells. NGF can also induce c-fos expression in PC12 cells induced to differentiate to chromaffin-like cells, even after 5 days of dexamethasone treatment.

Although c-fos expression is not crucial to the differentiation of PC12 cells it does seem to play a role in neuronal tissues in vivo. It was observed that a calcium ion influx into PC12 cells induces rapid and transient expression of c-fos (88, 89). Calcium influxes are a normal aspect of neuronal physiology in vivo and so studies of c-fos expression were extended to determine whether this expression could be induced in the neuronal tissues of intact animals (90 -98). Most of these studies involved the analysis of c-fos expression in

mouse or rat brain by RNA analysis or by immunohistochemical analysis. Several stimuli were shown to induce rapid and transient c-fos expression in certain areas of the brain and these included treatment with the convulsant drug Metrazole or electrical stimulations. The role of c-fos expression in response to such treatments is not clear, but it is thought that c-fos may co-operate with other gene products in the long-term adaptive response of these c-fos expressing neuronal cells. This could involve an increase in particular cell-surface receptors, an increase in certain intracellular proteins, or an increase in specific proteins excreted from the affected cells.

1.4 c-fos Expression is Induced by a Wide Variety of Extra-cellular Stimuli.

1.4.1 c-fos expression in stimulated quiescent fibroblasts.

The induction of c-fos expression has been described previously (sub-Sections 1.3.2. and 1.3.4.). These inductions involved the treatment of asynchronously growing haematopoietic cell lines or the rat pheochromocytoma cell line PC12 with growth factors, phorbol esters and other agents. These stimulations resulted in a rapid and transient induction of c-fos expression in most cases, although in HL-60 cells treated with TPA the level of c-fos expression remains above basal levels for at least 90 hours post-stimulation. No clear function can be assigned to these inductions but it seems most likely that they are related to the stimulus to the cell rather than to any biological process, such as differentiation, that occurs following stimulation. Because cellular oncogenes (or their viral counterparts) can cause cellular transformation (99) it was thought that in normal cellular functions their products may be involved in biological processes such as growth and differentiation. No conclusive link has been made between c-fos expression and differentiation, so its role in growth was examined along with that of other oncogenes.

The system chosen to examine the role of oncogene expression in growth has frequently been the stimulation of quiescent fibroblasts with growth factors. Most of these studies have employed murine 3T3 fibroblast cell lines which are immortal but not transformed, and

upon serum deprivation they can enter a state (Go) in which cell division and growth stops and many metabolic processes are reduced (100). The addition of serum to these serum-deprived (quiescent) cells initiates a complex series of events (G_1 phase) which culminates 14 to 20 hours later in DNA synthesis (101). Nuclei of Balb/c 3T3 cells were isolated at various times following stimulation of quiescent fibroblasts with serum and nuclear run-off transcription assays (102, 103) were carried out to determine whether the stimulation of these cells alters the transcription rate of any proto-oncogenes (104, 107). Seventeen proto-oncogenes were analysed but only c-fos, and to a lesser extent c-myc (which also encodes a nuclear protein (108)) were shown to increase their transcription rates in response to serum stimulation. An increase in the transcription of the c-fos gene could be detected within 5 minutes of stimulation, peaked around 10 minutes, started decreasing after 15 minutes and returned to basal levels within 30 minutes. This increase in transcription was due entirely to polymerase II transcription as α-aminitin (a polymerase II inhibitor) completely abolished any increase in c-fos transcription following serum stimulation. This increase in c-fos transcription was reflected in the levels of c-fos mRNA detected in 3T3 cells following stimulation, which peaked at around 20 minutes, decreased by 30 minutes and became undetectable after 60 minutes. The c-Fos protein showed a similar induction as it becomes detectable after 30 minutes, peaks in expression around 60 minutes before becoming undetectable after 120 minutes. As reported previously the c-Fos protein is present as a heterogenous population ranging in size from 55 to 62kD and it is also found exclusively in the nucleus. c-myc transcription increases

at a later time point than c-fos and this transcription peaks at around 30 minutes before decreasing and becoming undetectable after 60 minutes. c-myc transcription increases at a later time point than c-fos and this transcription peaks at around 30 minutes before decreasing and becoming undetectable after 60 minutes. This later switch on of c-myc transcription results in c-myc mRNA expression peaking at around 60 minutes before decreasing thereafter.

The increase in the transcription rate (and mRNA levels) of c-fos in quiescent fibroblasts can be induced by individual mitogens such as growth factors and phorbol esters as well as by whole serum. PDGF, FGF and TPA all increase c-fos transcription with similar timing although the degree of the transcriptional increase is less marked than that obtained with serum. Certain factors, such as EGF and insulin, fail to induce the familiar induction in c-fos transcription. The implications of these results will be discussed in the following sub-Section (1.4.2.). Infection of quiescent fibroblasts with SV40 stimulates the cells to enter S phase (109). This infection results in an increase in transcription of c-fos and c-myc genes, and seems to induce growth stimulating activities as medium taken from SV40 infected quiescent fibroblasts can activate other quiescent cells to re-enter the cell cycle.

Cultivation of quiescent 3T3 cells for 4 hours with the protein synthesis inhibitor cycloheximide resulted in a slightly increased level of both c-fos and c-myc mRNA. Moreover, stimulation of quiescent 3T3 cells with serum in the presence of cycloheximide led to the accumulation of both c-fos and c-myc mRNAs to concentrations

that were several-fold higher than the maximum levels observed in the absence of cycloheximide. This increase in c-fos mRNA levels is probably due to an increased rate of transcription of the gene (110) and also an increase in the stability of the mRNA suggesting that cycloheximide acts to prevent the synthesis of both a transcriptional repressor protein and a protein that acts to enhance the degradation of the c-fos mRNA. Another protein synthesis inhibitor, anisomycin, has a similar effect in these cells (110). Interestingly, the transcriptional control of c-myc seems to operate through a different mechanism in PC12 cells (110) as in these cells c-myc expression is not superinduced following NGF stimulation (c-fos expression is), and indeed its usual increase in transcription following this stimulation is abolished by cycloheximide and anisomycin. This suggests that there may be tissue-specific mechanisms involved in the transcriptional control of proto-oncogenes such as c-myc. The transcription of a gene coding for a major excreted protein (MEP) is induced with similar timing to that of c-myc in quiescent fibroblasts by treating proliferating fibroblasts with TPA (111) MEP is also elevated in transformed cells but its transcription cannot be super-induced by cycloheximide treatment of TPA-stimulated fibroblasts. Transcription of c-fos is also stimulated by TPA in proliferating fibroblasts (to a lesser extent than in quiescent fibroblasts) and can be superinduced by cycloheximide treatment. This suggests that in fibroblasts there is a set of genes, such as c-fos and c-myc, that can be transcriptionally activated by TPA without prior protein synthesis but that another set of genes, including the MEP gene, require prior protein synthesis.

Because of the large transient increase in c-fos expression in th Go to G_1 phase of the cell-cycle, the levels of c-fos expression throughout the cell cycle were determined (112). Cells grown in 10% foetal calf serum (FCS) represent an asynchronously growing population and any specific fluctuation in c-fos expression throughout the cell-cycle might be masked. To overcome this problem cells were synchronised by chemical methods and it was shown that the levels of c-fos expression throughout the normal cell-cycle remain barely detectable, with no fluctuations occuring. It was also observed that the induction of c-fos expression can occur at any time in the cell-cycle as after only 2 hours of serum deprivation (when the cells are not in a quiescent state as estimated by ^{3}H-thymidine incorporation) c-fos expression can be induced by the addition of serum. This induction becomes maximal after serum deprivation for 6 hours (when the cells are still not in a quiescent state), with longer periods of serum deprivation failing to increase the degree of c-fos induction any further (112).

Nucleosomes associated with transcriptionally active DNA are in a relatively unfolded state in comparison to those associated with transcriptionally inactive DNA (113). This observation has been exploited to isolate these unfolded nucleosomes by an affinity chromatography procedures (114). The c-fos gene is present in the unfolded nucleosome fraction isolated from the chromatin of stimulated quiescent fibroblasts (115) and this association persists when c-fos mRNA levels are superinduced with protein synthesis inhibitors. This observation supports previous studies which suggested that the superinduction of c-fos expression by these

protein sysnthesis inhibitors is at least in part due to a prolonged transcriptional activation (106, 107, 110). There are several topoisomerase II sites (116, 117) around the c-fos gene (118) which could be responsible for the "relaxing" of the DNA and thus allowing the transcriptional machinery access to the c-fos gene. So, it is possible that topoisomerase II is involved in the mediation of mitogen-induced c-fos transcription.

1.4.2 The c-fos gene may be a 'competence' gene.

Serum contains two types of growth factors that function sequentially to stimulate mitosis in density-arrested Balb/c-3T3 cells, and possibly other anchorage-dependent cell-types as well (119). Platelet-derived growth factor (PDGF) is the serum component that renders quiescent Balb/c-3T3 cells 'competent' to respond to progression factors in platelet poor plasma (PPP) (120). In the studies described in the previous sub-Section (1.4.1.) examining the change of c-fos expression in response to growth factors, PDGF and fibroblasts growth factor (FGF) could both induce c-fos expression with similar timing and to similar levels as whole serum whereas EGF and insulin could only very weakly induce c-fos expression (although it has been reported that a combination of the two can induce c-fos expression (121)) (114). Interestingly PDGF and FGF are 'competence' factors that enable the cells to become competent for DNA synthesis while EGF and insulin are 'progression' factors that require the cells to be in a competent state before the cells can progress into the S-phase of the cell-cycle. Because of these oberservations a series of studies were carried out investigating the role of c-fos

expression in the competent state of quiescent fibroblasts.

After a brief treatment of quiescent fibroblasts with PDGF, which induces c-fos expression, the cells remain competent for several hours (122). The addition of PPP, which lacks PDGF, allows these cells (but not those untreated with PDGF) to progress through G_1 and enter S-phase. A reduction in the response to PPP of PDGF treated cells occurs 8 hours after PDGF treatment and the half-life of the competent state is estimated to be 16 hours suggesting that the expression of c-fos (the c-Fos protein is undetectable 2 hours after PDGF treatment) is not responsible for the maintenance of the competent state although this expression could certainly be involved in the establishment of this state. Basic calcium phosphate crystals acts as a 'competence' factor (123) and also induce c-fos expression (124) and there are several other studies reporting the induction of c-fos expression with a host of different competence factors on different cell lines (125-127) strongly suggesting that the expression of c-fos may be related to the establishment of the competent state in these cells.

It has been shown that quiescent Balb/c-3T3 cells enter the cell-cycle and reach S-phase when a confluent monolayer is wounded in the presence of PPP, indicating that wounding is equivalent to treating these cells with a competence factor. The expression of c-fos has been analysed in such a situation (128, 129). The scratching of a monolayer of NIH 3T3 cells results in the induction of c-fos expression in an area surrounding the wound up to five cell-widths away from the scratch. This induction can be detected by

antibodies (129) and can occur in cells that were completely serum deprived for 50 hours before wounding or in cells that were grown in 10% FCS throughout the treatment, suggesting that any induction of c-fos expression is due to the wound on the monolayer rather than an increased access of growth factors from the medium to the cells. The observation that wounding resulted in the competent state in the cells surrounding the wound led to the analysis of c-fos expression in regenerating tissue in vivo and c-fos expression has indeed been detected in damaged brain tissue (94) and during liver regeneration at a period corresponding to cell proliferation (130).

All of the evidence described above strongly indicated that c-fos expression plays a role in the attainment of the competent state of certain cells, suggesting that this expression is related to the growth of the cells. To establish whether this was the case two studies were carried out using plasmid constructs coding for inducible anti-sense fos sequence (131, 132). Previous studies had shown that anti-sense RNA production in cells can prevent the translation of the targeted mRNA (133). In both of these studies the anti-sense fos sequence was under the control of the mouse mammary tumour virus (MMTV) promoter which was induced by the addition of dexamethasome to the medium. When the transfected cells were selected in the presence of dexamethasone there was a 90-96% decrease in the number of colonies obtained compared to a selection carried out in the absence of dexamethasone, this suggested that the inhibition of c-Fos protein production was lethal to the growth of the cells. The transfected cells were also unable to proliferate when they were made quiescent and stimulated with PDGF plus

progression factors in the presence of dexamethasone and no c-Fos protein was detected in the treated cells suggesting strongly that the expression of c-Fos is necessary for the proliferation of these cells.

The injection of Fos antibodies into cells can stop the synthesis of DNA and therefore prevent the cells from progressing through the S-phase of the cell-cycle (134). This evidence, when taken together with the points mentioned above, strongly suggests that the expression of c-fos plays an important, if not crucial, role in the proliferation of certain cell types and that this role could be the establishment (although not the maintenance) of the competent state in the relevant cells.

1.4.3. Intracellular pathways leading to the induction of c-fos expression.

Various reports have been described which detail the activation of c-fos transcription by a variety of extra-cellular stimuli (see sub-Section 1.3.2, 1.3.4., 1.4.1, 1.4.2). Several studies have attempted to identify the intracellular pathways that many of these extra-cellular stimuli operate through. TPA, which activates c-fos transcription in a variety of cell types, directly activates protein kinase C which can also be activated by diacyl glycerol which is produced at the cell membrane in response to growth factor-induced processes (135, 136). This activation of protein kinase C presumably triggers a phosphorylation cascade that results in the induction of c-fos transcription (52-55, 137-140). Growth factors also seem to

employ a protein kinase C independent cellular pathway to activate transcription as depletion of protein kinase C in quiescent fibroblasts does not abolish the increase in c-fos transcription following growth factor stimulation, although the induction is certainly attenuated in comparison to that obtained with normal cells (141).

An increase in intracellular levels of cAMP, which activates the cAMP-dependent protein kinase A, can also induce c-fos transcription in a variety of cell types (137, 142-144). Again, this activation presumably results in a phosphorylation cascade that activates c-fos transcription. Interestingly, in mouse bone marrow derived macrophages and in a mouse macrophage cell line P388D, an increase in intracellular levels of cAMP results in a stable induction of c-fos expression which peaks after 6 hours and remains elevated even after 12 hours of treatment. This is the only reported case of a stable induction of c-fos expression which is the result of a stable increase in transcription.

An influx of calcium ions also results in an increase in c-fos transcription. This phenomenon has been described previously for PC12 cells (88, 89) and A23187, a calcium ionophore, can also induce c-fos expression in a variety of other cell types (137, 140, 141, 145). This chemical can also induce c-fos transcription in protein kinase C depleted cells (although the induction is attenuated) and can act in an additive fashion to increase c-fos transcription. These observations suggest that the calcium ion influx caused by A23187 can activate c-fos transcription through activation of protein

kinase C-dependent and -independent pathways in a similar manner to that reported for growth factors.

So, there would appear to be three main intracellular pathways that lead to the induction of c-fos transcription in a variety of cell-types:-

1) activation of a protein kinase C-dependent pathway;
2) activation of a cAMP-dependent protein kinase A pathway;
3) activation of a protein kinase C-independent pathway that perhaps involves the mobilisation of calcium ions.

However, there are other intracellular mechanisms which can be employed to induce c-fos transcription. For example, injection or expression of a ras oncoprotein in quiescent fibroblasts results in an induction of c-fos expression (146) which seems to employ part of the growth factor induced intracellular signalling pathway (147, 148). Several hormones also activate c-fos transcription (138, 149, 150) and although some of these operate through previously mentioned intracellular pathways there have been suggestions that estrogen bound to its receptor can bind directly to DNA regions upstream from the c-fos gene to activate transcription (150).

1.5. Post-transcriptional Regulation of c-fos Expression.

The induction of c-fos expression can be superinduced in many cell-types by treatment of stimulated cells with inhibitors of protein synthesis, particularly cycloheximide, prior to or during the stimulation (105, 107, 110, 115, 127, 149). This superinduction gives elevated levels of c-fos expression for prolonged periods of time, and is due to a relatively small increase in transcription of the c-fos gene and a stabilisation of the c-fos mRNA leading to an increased half-life. Studies have been carried out to determine the sequences and mechanism(s) that lead to the post-transcriptional control of c-fos mRNA expression.

An indication of the sequences involved in the regulation of c-fos mRNA stability came from studies examining the activation of the transforming potential of the c-fos gene (26-28). These studies showed that the c-fos gene could transform fibroblasts if the 3′ untranslated region of the c-fos mRNA was replaced with an unrelated sequence, and these alterations led to a stabilisation of the c-fos mRNA and therefore to an accumulation of c-Fos protein (28). Also, in transgenic mouse studies (33) this same sequence had to be replaced with an unrelated sequence in order to obtain expression of c-fos mRNA in the mouse tissues. These studies therefore pointed to sequences in the 3′ untranslated region of the c-fos mRNA between the translation stop codon and the polyadenylation site being involved in the regulation of c-fos mRNA stability. Several studies have been carried out to examine this possibility (151-155). The half-life of c-fos mRNA in a variety of cell-types was determined by treating

cells with the transcription inhibitor actinomycin D and was found to range from 10 to 20 minutes. In transfection studies with hybrid genes containing fragments of the c-fos 3′ untranslated region attached to reporter genes it was shown that this sequence was capable of markedly reducing the half-life of previously stable mRNAs (such as globin (151)), and removal of the 3′ untranslated region from the c-fos gene led to an elevated and prolonged accumulation of c-fos transcripts following stimulation of c-fos expression. Cycloheximide treatment led to an increase in the half-life of the endogenous c-fos gene as well as activating transcription by as much as 9-fold (154) in certain cells.

The region in the 3′ untranslated region of the c-fos mRNA that is responsible for the destabilisation of the c-fos mRNA is AU-rich and similar sequences can be found in other 3′ untranslated regions of mRNAs that have short half-lives including those of c-myc and many inflammatory mediators (for a list see 156, 157). When this sequence is taken from the human lymphokine gene GM-CSF and added to the 3′ end of a construct coding for β-globin mRNA, the resultant mRNA becomes as unstable as that of GM-CSF itself suggesting that this sequence is solely responsible for determining the stability of the GM-CSF mRNA (157). Further studies using these AU-rich sequences of other inflammatory mediator genes have revealed that this sequence can destabilise other reporter mRNAs (such as CAT (158)). In vitro transcribed RNA containing these AU-sequences are preferentially degraded when they are incubated with nuclear protein extracts suggesting that there is an RNAse present in the nucleus of certain cells that selectively degrade mRNAs containing the AU-sequence

(158).

The reports described so far suggest that there is a family of short-lived mRNAs that contain an AU-rich sequence in their 3′ untranslated regions that is responsible for the selective degradation of these mRNAs by a nuclear ribonuclease. The fact that cycloheximide treatment of cells results in a stabilisation of these mRNAs suggests that this ribonuclease is extremely labile and requires protein synthesis to maintain its control over the expression of these short-lived mRNAs. However, such a hypothesis has been contradicted by several pieces of evidence. In a monocytic tumour cell-line (159) GM-CSF is over-expressed as a result of the stabilisation of its mRNA as hybrid constructs containing the GM-CSF 3′ untranslated region are stable in this cell line in comparison to others. When similar c-fos and c-myc hybrid constructs were transfected into this cell line they are degraded as expected. Therefore, GM-CSF mRNA is specifically stabilised in this cell-line, suggesting that there is no universal pathway leading to the degradation of all mRNAs that contain the AU sequence in their 3′ untranslated region. Several studies examining the induction of c-fos expression in response to a mitogenic stimulation have suggested that there is a decrease in the size of the mRNA throughout the induction period prior to its final degradation (151, 153). The role of this mRNA shortening has been examined with regard to mRNA stability (160). Cycloheximide treatment of NIH 3T3 cells 30 minutes after stimulation with serum resulted in an immediate stabilisation of c-fos mRNA and prevented the shortening of the mRNA (which is due to sequential degradation of the poly(A) tail) suggesting that c-fos

mRNA degradation required on-going translation for shortening of the mRNA and for degradation of the mRNA. This was proved by transfection studies using various plasmid constructs where it was shown that the AU-rich sequence is required for the removal of the poly (A) tail from the c-fos mRNA during translation, and any removal or mutation of this AU sequence led to a stabilsation of the c-fos mRNA and a cessation in the degradation of the poly (A) tail. This argues against the idea of a universal ribonuclease that is present in the cytoplasm and can degrade mRNAs containing the AU sequences. The most likely model for c-fos mRNA (and related mRNAs) degradation involves the degradation of the mRNA by a ribonuclease attached to the polysomes. This would recognise a sequence or configuration resulting from the AU-rich sequence and would remove the poly(A) tail from these mRNAs. The de-adenylated RNA could then be degraded by endonucleases. Cycloheximide prevents translation by freezing polysomes on mRNA and could lead to a stabilisation of c-fos mRNA not by preventing synthesis of a labile ribonuclease, but by preventing access of a polysome-associated ribonuclease. In the monocytic tumour mentioned above (159) it could be hypothesised that a further protein recognises the GM-CSF mRNA on the polysomes and prevents access of the degradative ribonuclease that removes the poly (A) tail.

1.6. Transcriptional Control of c-fos Expression.

1.6.1 Identification and definition of cis-acting transcriptional control elements.

The first DNA sequences implicated in the role of transcriptional control were identified upstream from prokaryotic genes (161), where conserved DNA sequences which bind RNA polymerase are located at about 10 and 35bp upstream of the initiation of transcription. In addition to these two sequences further upstream sequences were responsible for the negative and positive regulation of individual gene expression which bind repressor or activator proteins (162). This level of complexity in the prokaryotic transcriptional control mechanism seemed to be sufficient to control all gene expression. The situation in higher eukaryotes at first seemed very similar to that found in prokaryotes with the identification of a conserved sequence 30bp upstream from the transcriptional initiation site. This sequence, termed the TATA box, is very similar in sequence to the Pribnow box situated 10bp upstream from prokaryotic gene transcriptional initiation sites. In addition to the TATA box many eukaryotic genes contained a further conserved sequence called the CAAT box which is situated upstream from the TATA box and is typical of an upstream promoter element (UPE) (163).

Many of the earlier studies carried out to examine eukaryotic transcriptional control involved studies of DNA tumour viruses, with the major discoveries coming from studies on the genome of the simian virus, SV40 (164). This tumour virus has a complex mechanism

controlling the transcription of its early gene which encodes a tumour antigen. This gene has a TATA box followed by three 21bp repeats, each of which contain two GC-rich hexanucleotide sequences which are involved in transcriptional control. The TATA box can be deleted from this upstream sequence without any serious effect on either the rate or site of transcription initiation (which is not the case with eukaryotic cellular genes) while only two of the six GC-hexanucleotide sequences are required for the propagation of viable virus. These 21bp repeats bind the cellular protein Sp-1 (165) which specifically interacts with the GC-rich sequences. This protein has also been found to interact with similar upstream sequences present in cellular genes (for a review see 166). These GC-hexanucleotide elements can be classed as UPEs.

Further upstream from the 21bp repeats there are two copies of a 72bp element that enhances transcription from heterologous promoters (167-169). These are distinct from previously described cis-acting transcriptional control sequences as they enhance transcription when placed in either orientation upstream of a promoter, and can operate over long distances when placed either upstream or downstream from a promoter. This element was the first described and defined enhancer element. Following the discovery of this element many enhancer elements were identified in viral and cellular genes (for reviews see 170-173) and these elements fulfill the following strict criteria:

a) strong activation of transcription of linked gene from the correct initiation site;
b) activation of transcription independent of orientation;
c) ability to function over long distances (more than 1000bp),

whether from an upstream or downstream position relative to the transcription initiation site.

Since the discovery of the enhancer element present in the SV40 genome there have been precise definitions of the sequences involved in enhancer function. However, these are too many to consider here and a brief description of the characterisation of the SV40 enhancer region will serve as a paradigm for other enhancers, both viral and cellular. Characterisation of the SV40 enhancer has come about from studies of mutant viruses isolated from CV-1 cells in culture and from studies using synthetic enhancer elements with inserted mutations (174). These studies showed that a single-copy of the 72bp repeat was sufficient for propagation of the virus, and that the area incorporating enhancer activity in fact spanned 100bp (175). Revertants of mutant viruses and point mutation studies revealed that the enhancer can be separated into three separate regions (A, B and C) (175, 176), each of which fail to enhance transcription on its own but which do so when either duplicated or paired with other elements within the enhancer. The great complexity of this 100bp regions was realised with the observation that multimers of A, B and C sequences showed different cell-type specific enhancer properties (177, 178), and indeed a further element outwith the 100bp was also shown to confer cell-type specificity on transcription (179).

So, the SV40 enhancer consists of at least 3 independent enhancer elements which co-operate with duplication of their own sequence, or with copies of other enhancer elements to enhance transcription. Some of these enhancer elements display cell-type specific enhancer

properties which is the situation for many cellular enhancer elements (for a review see 173). However, within the individual enhancer elements there exists a further degree of complexity (180, 182). A, B and C are 15bp to 20bp in size and are bipartite, being composed of sub-units called enhansons which can be duplicated or interchanged to create new enhancer elements. These enhansons are obviously very short, being some 8 to 10bp in size. It is possible to differentiate between an enhanson and an enhancer element by the following criteria:

a) some enhanson elements do not enhance activity when present as multimers although they can enhance transcription when linked to a single copy of another enhanson which by itself is incapable of enhancing transcription.

b) enhanson elements are very sensitive to spacing between them. Insertions of 1 or 2bp decreases the enhancing activity of duplicated enhansons and insertions of 5bp or more completely abolishes any enhancer activity. This is not true of the enhancer elements A, B and C in SV40, which can be separated by over 100bp while retaining the capacity to co-operate and enhance transcription.

The discovery of enhansons led to the characterisation of very complex level of organisation present in the SV40 enhancer. This enhancer seems to be arranged in a hierarchical order, with the enhansons being the basic elements that lead to the formation of enhancer elements, which then co-operate with other enhancer elements to enhance transcription. To date this is the most complex organisation of any characterised enhancer, and probably reflects

the wide range of cells that can be infected by SV40. Perhaps this pattern of enhancer organisation evolved to allow the virus such flexibility in its host range.

Many cellular enhancer sequences have been characterised and several of these enhancers share sequence motifs with the SV40 enhancer (183, 184, for a review see 166). These cellular enhancers have also been shown to be short DNA sequences, although the complexity observed with the SV40 enhancer has not been found in any cellular enhancer described to date. Like the enhancer elements present in SV40 many of these cellular enhancer sequences display a restricted cell-type activity (for a review see 173).

1.6.2 The c-fos gene has an inducible enhancer.

As described in the previous sub-Section (1.6.1.) there are at least two types of transcriptional control elements: the upstream promoter elements (UPEs) and the enhancer elements. The c-fos gene has a traditional TATA box sequence 30bp upstream from the transcription initiation site although it does not contain any Sp1 binding sequences (165) or CAAT box sequences (164) which are the two most common UPEs found in other cellular genes. However, two sequences in the promoter regions of the mouse and human c-fos genes bind nuclear proteins (185, 186). One of these sequences was common to both the mouse and human c-fos genes, situated at -60bp relative to the c-fos mRNA cap site, and probably binds the cAMP response element binding protein CREB (187). The deletion of these sequences, particularly the CREB protein binding site, significantly reduces the

basal activity of the respective promoters. Figure 2 details the transcriptional control elements of the c-fos gene.

To examine the transcriptional control of the c-fos gene, promoter and upstream sequences were cloned into a plasmid vector upstream of the prokaryotic reporter gene chloramphenicol acetyl transferase (CAT) (188) and the resultant vectors transfected into murine NIH 3T3 cells (189, 190). These studies revealed the presence of an element between -400bp and -200bp relative to the c-fos mRNA CAP site that enhanced the transcription of these constructs 10-fold. DNAse 1 hypersensitivity studies revealed the presence of a DNAse 1 hypersensitivity site at -290bp $\pm$ 40bp; in this region there is an element of dyad symmetry (for a review see 191) and so it was thought that this sequence present upstream (centred around -308bp) from the c-fos gene may be involved in transcriptional control.

Many studies have revealed the transcriptional activation of the c-fos gene in quiescent fibroblasts stimulated by agents such as serum, growth factors and phorbol esters. In a bid to identify cis-acting elements that may be involved in this transcriptional activation transfection studies were carried out with the entire human c-fos gene in murine fibroblasts. The human c-fos mRNA can be distinguished from the endogenous mouse mRNA by ribonuclease protection assays (151). These studies revealed that the dyad symmetry element (DSE) was responsible for the transcriptional activation of the c-fos gene in response to serum stimulation and this element operates in either orientation and when placed 1.6kbp downstream from the c-fos gene. Thus, the element satisfies the

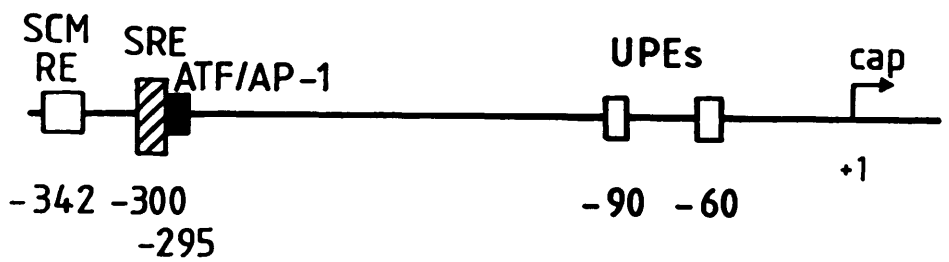


Figure 2. Transcriptional control elements of the human c-fos gene.

This figure details the previously described transcriptional control elements of the human c-fos gene. Cap : transcription initiation site (+1). UPEs: upstream promoter elements. ATF/AP-1 : adenovirus transcription factor site/ activator protein 1 site. SRE : serum response element. SCM-RE : v-sis conditioned medium response element.

criteria of an enhancer and is therefore an inducible enhancer element. It was called the serum response element (SRE). When this element was replaced by either the SV40 or Moloney murine leukaemia virus enhancers the transcription of the c-fos gene became ubiquitous; that is, it failed to stop in serum deprived cells or to increase upon serum stimulation. These results indicate that the SRE may not be classified as a classical enhancer. The SRE also stimulates transcription from a heterologous promoter (β-globin) in response to serum (151), although this stimulation is lower than that observed with the intact c-fos promoter indicating that there may be an interaction between sequences in the c-fos promoter and the SRE.

The c-fos gene is not the only gene to contain the DSE present in the SRE. Two other genes that were transcriptionally activated during the Go to G_1 transition in fibroblast cell lines were isolated and found to contain sequences very similar to the SRE (192). Actin genes are also transcriptionally activated in the transition of fibroblasts from Go to G_1 (104). Many actin genes have been found to contain a CArG box that bears a close sequence homology to the c-fos SRE and apparently binds the same cellular protein (193 - 199). The CArG box is found in actin genes present in Xenopus laevis (193) and humans (196), indicating that the transcriptional control mechanism operating through the SRE sequence is highly conserved throughout evolution. The CArG sequence present in the X. laevis cytoskeletal actin gene can act as an enhancer element when a synthetic copy is inserted upstream from a X. laevis cytoskeletal actin promoter - CAT construct, and can also respond to serum in a similar fashion to the c-fos SRE when placed upstream from the c-fos promoter. These

studies demonstrate tha the SRE (CArG) sequence can operate as a constitutive enhancer element and/or an inducible enhancer element, and is probably involved in the co-ordinated transcriptional activation of a set of genes in growth-factor stimulated fibroblasts. The expression of the actin genes which contain the CArG (SRE-like) boxes is controlled in a cell-type-specific manner. However, it seems unlikely that this sequence on its own can dictate this restricted expression as c-fos is not expressed in a similar manner therefore it is probable that the CArG boxes co-operate with other cis-acting DNA elements present in the promoter sequences of these genes to confer the cell-type-specific expression.

1.6.3. The c-fos serum response element binds a nuclear protein.

The c-fos SRE forms a complex with a nuclear protein (called the serum response factor (SRF)) present in extracts of a variety of cell-types (185,193, 200 - 204) including HeLa, mouse 3T3s, PC12, WEHI and in X. laevis oocytes. In all of these cell-types the induction of c-fos transcription makes no difference to the affinity that the SRF has for the SRE, suggesting that the activiation of c-fos transcription probably results from the modification of the SRF that is already bound to the SRE. This would require no new protein synthesis prior to c-fos transcription and therefore agrees with the observation that the stimulation of c-fos transcription occurs in the presence of inhibitors of protein synthesis. Mutations in the SRE that abolish SRE binding in vitro also prevents the SRE inducing c-fos transcription in transfection studies, confirming that the SRE-SRF complex is responsible for the transcriptional activation of

the c-fos gene.

There has been one report detailing the induction of binding of a nuclear protein to the SRE (204). Treatment of the human epidermal cell-line A431 (which overexpresses the EGF receptor) with EGF results in the induction of c-fos expression (139). Before EGF treatment these cells do not possess a protein with SRE binding activity. However, 20 minutes after treatment a nuclear protein (presumably the SRF) binds to the SRE which is responsible for the transcriptional activation of the c-fos gene. This protein remains bound to the SRE for a period extending beyond the transcriptional shut-down of the c-fos gene following EGF treatment. This suggests that there is not a simple "on-off" mechanism activating and then repressing c-fos transcription, but that other proteins are involved which may modify the already bound SRF or bind directly to other DNA-elements to repress transcription.

To characterise further the complex that forms between the SRE and SRF, the SRF protein was purified (205 - 207). It was found to be a phosphoprotein (208) of molecular weight 67kD (p67) (205, 207) or 62kD (p62) (206). The discrepancy in the measured molecular weight of the protein is probably due to the use of different techniques for determining its size rather than there being two different proteins, since the source of crude protein extract and method of purification was similar in each report. A cDNA coding for the SRF has been isolated (209). The SRF gene encodes two mRNAs that are both induced following serum stimulation of quiescent fibroblasts, irrespective of the presence of inhibitors of protein synthesis. This suggests that

the SRF gene, along with c-fos and others (210, 211), is a memeber of the cellular immediate-early genes whose expression is elevated almost immediately following the stimulation with growth factors of cells resting in Go. The SRF binds to the SRE as a homodimer which is not surprising as the SRE is a dyad symmetry element. There is a short stretch of the SRF protein which is capable of both forming homodimers and binding to the SRE. This region bares significant homology to a region present in the S.cerevisiae protein PRTF/GRM, which can also bind to the SRE, and is involved in control of cell-type-specific gene expression (212-214). No significant homology between SRF and PRTF/GRM exists outwith this region. It has also been shown that another nuclear protein of 62kD is involved in the SRF-SRE complex (this protein is distinct from the p62 that was isolated as the SRF (205, 215). This protein cannot by itself bind to the SRE but it can join a SRF-SRE complex and it also requires three bp upstream of the DSE for binding to the SRF-SRE complex. Significantly, if these three bp are removed, the 62kD protein fails to complex, and the resultant DSE is very poor at responding to serum stimulation in transfection studies.

So, the SRF is expressed in many different cell-types and appears to be highly conserved throughout evolution. It is a phosphoprotein and it seems possible that the mode of activation of this transcription factor involves phosphorylation of sequences present in the protein that show homology to previously identified phosphorylation sights. It also appears to co-operate with a further nuclear protein (of 62kD) in mediating the transcriptional activation of c-fos following serum stimu lation.

1.6.4. The c-fos SRE responds to a variety of extra-cellular stimuli.

Although the c-fos SRE was first identified as inducing c-fos transcription in response to the stimulation of quiescent fibroblasts with serum (151), there have been many studies carried out detailing this elements response to a wide vairety of extra-cellular stimuli in several cell-lines. These studies have indicated that there are several intracellular pathways that activate c-fos transcription through the element and also that there is a degree of cell-type specificity involved in the transcriptional control exerted by the SRE.

Treatment with TPA results in the induction of c-fos transcription through the SRE in 3T3 cells (216 - 218), PC12 cells (186, 220), HeLa cells (185, 216) and Chinese hamster ovary (CHO) cells (203). However, in A431 cells TPA treatment does not induce the transcription of c-fos through this element, and indeed fails to activate transfected constructs containing up to 2kbp of upstream c-fos sequence. This indicates that, in these cells, there are alternative cis-acting DNA elements responsible for the transcriptional activation of the c-fos gene that have as yet not been identified (185). In PC12 cells the calcium ionophore A23187 induces c-fos expression through the DNA element centred at -60bp relative to the mRNA CAP site (which binds the CREB protein (187)) that is conserved between the mouse and human gene (186). In A431 and HeLa cells this agent fails to induce the transcription of transfected constructs containing up to 2kbp of c-fos upstream

sequence indicating that there is a difference in the control of c-fos transcription in response to A23187 in HeLa and A431 cells compared to PC12 cells. A previous sub-Section (1.4.3.) described the activiation of c-fos transcription by several intracellular pathways. The SRE seems to respond to at least two of these pathways to activate c-fos transcription. Firstly, TPA treatment leads to the induction of c-fos transcription in many cell-types and this induction requires the presence of an intact SRE. TPA operates solely through protein kinase C and when this enzyme is depleted from the cell (by chronic exposure to TPA) serum or growth factors can stimulate c-fos transcription through the SRE. This stimulation is attenuated but shows that c-fos transcription can be induced through the SRE by protein kinase C -dependent and -independent pathways. The independent pathway is not known but is not due to the mobilisation of calcium ions as the calcium ionophore A23187 fails to activate c-fos transcription through this sequence. Similarly, activation of c-fos transcription through the cAMP-dependent protein kinase A pathway does not require an intact SRE. Mutations in this element that abolish the response of transfected constructs to serum and TPA do not affect the induction of transcription in response to elevated levels of cAMP (218). NGF (185, 219), insulin (203) and U.V. irradiation (218) induce c-fos transcription through the SRE in PC12, CHO and NIH 3T3 cells respectively, but the intracellular pathways involved in the cellular response to these factors is unknown.

There is a further DNA element in the c-fos promoter region that can activate transcription in response to serum (220). This element is

downstream from the SRE and contains several repeated sequences similar to those found in the HSP 70 promoter region which is also responsive to serum (219). This element does not seem to be additive with the SRE in its response to serum, and can function as a serum-response element in the absence of the SRE, indicating that no co-operation occurs between the two elements. This element was called SRE-2 and it also stimulates transcription in response to NGF treatment of PC12 cells (219). A sequence upstream of the SRE binds a nuclear protein following treatment of 3T3 cells with v-sis conditioned medium which contains proteins similar in activity to PDGF (222). The function of this inducible binding is not clear as c-sis or PDGF treatment of cells increases the transcription of the c-fos gene mainly through the SRE (216, 217).

So, the SRE is the target of many extra-cellular stimuli and intracellular pathways that lead to the activation of c-fos transcription. Some of these pathways clearly differ between cell-types but it is not yet known what leads to this cell-type variation in response to extra-cellular stimuli. The SRF also seems to interact with the protein that binds to the CREB binding site in the c-fos promoter as deletion of this sequence markedly reduces the transcriptional response of the c-fos promoter to serum or TPA stimulation.

1.6.5. cAMP stimulation of c-fos transcription.

There are a number of eukaryotic genes that are transcriptionally activated by cAMP stimulation and the transcriptional control of

these genes has been the focus of many studies in recent years (for a review see 223). The first study to identify an element responsible for the induction of gene expression by cAMP came from transfection analysis of the promoter region of the rat phosphoenolpyruvate carboxykinase gene which revealed a short 12bp element present in the region that was sufficient to confer cAMP inducibility that was also present in the promoter region of other cAMP regulated region (224 and references therein). This led to the study of the transcription control regions of many other cAMP inducible genes. These studies clearly revealed a region of DNA homology between all of these genes that can confer cAMP inducibilty on their respective promoters. This element was called the cAMP responsive element (CRE) (225 - 230). This CRE is a small palindromic element of 8bp with the consensus sequence TGACGTCA. It is often duplicated to form inverted repeats in several cAMP inducible promoter regions and this duplication increases the transcriptional response of these genes to cAMP stimulation (229). These repeats are often separated by 10bp, i.e. one turn of a DNA helix, and insertion of sequences disturbing this periodicity affects the response of the duplicated elements to cAMP stimulation CREs can act as enhancer elements as they increase the basal level of transcription from their respective genes in most cases, and can operate in an orientation and distance independent manner.

The CRE has also been implicated in tissue-specific expression of the human glycoprotein α-subunit gene (226). The CRE can confer cAMP inducibility on the promoter region of this gene in many cell-types, but the contribution this element makes to basal transcription is

restricted. Deletion of the CRE prevented the tissue-specific expression of this promoter but the control of the tissue-specific expression was found to depend upon a sequence upstream from the CRE which binds a protein factor in a tissue-specific manner (which the CRE does not - this element binds a protein in a ubiquitous fashion (226)). Thus, the protein binding to the upstream element must co-operate with the protein binding to the CRE to activate transcription of this gene in a tissue-specific manner as neither sequence by itself can confer tissue-specific expression. Although the CRE by itself cannot confer tissue-specific expression the cAMP inducibility via this element is cell-type-specific (230). This sequence will confer cAMP inducibility on a heterologous promoter in PC12 cells but not in HeLa cells which reflects the cAMP induction of the endogenous gene transcription in these different cell-types. All of these results indicate that the CRE will co-operate with other DNA sequences in order to confer basal and induced transcription from heterologous promoters.

The transcription of the c-fos gene is activated rapidly following treatment of certain cell-type with analogues of cAMP or adenylate cyclase agonists (for a summary see sub-Section 1.5.3.). The c-fos promoter region has an element situated at -60bp relative to the mRNA start site that bears a close homology to the previously identified CREs. When c-fos promoter sequences upstream from -60 are deleted c-fos-CAT gene hybrids retain the ability to respond to cAMP in PC12 and JEG 3 cells (231). Also, the sequence around -60 can confer cAMP inducibility upon a heterologous promoter and both of these results indicate that the sequence around -60 in the c-fos promoter region

(which is conserved between the mouse and human c-fos genes) is a CRE. A further study revealed that the c-fos promoter region has several cAMP inducible DNA elements including the SRE, the -60 element and two other sequences (232). These elements do not operate additively as deletion of one has no effect on the degree of induction obtained following cAMP stimulation. The reason for this failure to operate in an additive fashion could be that all of the CREs contribute to basal levels of expression, therefore deletion of one CRE decreases the basal level of transcription resulting in a similar induction of transcription via the remaining elements following cAMP stimulation.

The nuclear protein that binds to the CRE of the set of cAMP inducible genes was purified by DNA affinity chromatography and this protein can footprint on the c-fos -60 CRE (187). This protein is phosphorylated after cAMP stimulation of PC12 cells (233) and is phosphrylated *in vitro* when incubated with the catalytic subunit of cAMP-dependent protein kinase. Interestingly, injection of this catalytic sub-unit into PC12 cells transfect with CRE containing constructs stimulates their transcription to similar levels as those obtained following cAMP treatment (234). Similarly in *in vitro* transcription assays addition of the catalytic sub-unit increases transcription from a CRE containing construct to a greater extent than treating the nuclear extracts with cAMP (235). This *in vitro* transcription induction operates only with PC12 cell nuclear extracts and not with those from HeLa cells which reflects the *in vivo* transfection results (230).

Study of the human proenkephalin gene transcriptional control region has led to some significant discoveries with regard to the stimulation of eukaryotic transcription by cAMP. This gene is stimulated following treatment of cells with cAMP and was found to contain an element upstream from the promoter that could confer basal, cAMP-induced and TPA-induced transcription on the proenkephalin gene promoter (236). This element can operate as an enhancer as it enhances transcription (both basal and induced) in an orientation and distance-independent manner. This sequence contains binding sites for several nuclear proteins (237) one of which, ENKTF-1, binds to an element that thas not been previously implicated in the response of any other cAMP-inducible genes. This protein binds to a DNA sequence called ENKCRE-1 which is situated 10bp upstream from another transcription element termed ENKCRE-2 which binds a factor that can be competed for by sequences from previously identifed CREs (237). Mutations of either of these elements results in a drastic reduction in basal and inducible transcription indicating they act synergistically to affect transcription from the proenkephalin promoter (238). The c-fos upstream region contains a sequence that is identical to that found in ENKCRE-2 and this sequence is continuous with the previously characterised SRE. Interestingly this sequence is one of the four sequences that were identified in the c-fos upstream region that can increase transcription from the c-fos promoter in response to cAMP (232). There exists a clear difference therefore between the activity of this sequence when situated upstream from different promoters. In the proenkephalin gene it is very poor at responding to cAMP treatment when the ENKCRE-1 sequence is effectively removed, but in

the case of the c-fos promoter this sequence can respond to cAMP stimulation resulting in transcriptional activation.

In summary, the c-fos gene has four cAMP inducible DNA elements, with the sequence at -60 and the sequence that is identical to that found in the proenkephalin enhancer complexing with the nuclear protein CREB that binds to other CREs. This latter sequence, TGCGTCAG, resembles the CRE consensus sequence but it also closely resembles that for the transcription factor AP-1 (239) which induces the transcription of a set of genes in response to TPA treatment. This sequence can bind purified AP-1 protein in vitro but apparently is also capable of binding the factor that complexes with CREs (238). These two proteins are distinct from one another (see later in sub-Section 1.7.4.) and it is possible that in vivo this c-fos sequence is capable of complexing with either of the proteins to increase c-fos transcription in response to cAMP (CREB) or TPA (AP-1). It is also of interest to note that the stimulation of c-fos transcription in PC12 cells in response to the calcium ionophore A23187 operates through the -60 CRE (234) as this induction is cAMP-dependent (240).

1.6.6. TPA stimulation of c-fos gene transcription.

A cis-acting DNA element that activates transcription from adjacent promoters in response to TPA treatment of cells was identified in the upstream regions of genes that are transcriptionally activated by this agent. These genes were identified by differential screening of stimulated and unstimulated human primary fibroblasts (241). One

such was the human metallothionein IIA gene (hMTIIa) which can be transcriptionally activated by serum growth factors and activators of protein kinase C (242). It contains five distinct transcriptional control elements in the 5'-flanking region of the gene that mediate specificity and regulation of transcription (243, 244). The collagenase gene is also transcriptionally activated by TPA and transfection studies revealed a short DNA element in the 5' flanking sequence that is responsible for this activation. This element operates in either orientation and up to 2kbp 3' of the gene indicating that this sequence is an inducible enhancer element. One of the basal level enhancers present in the hMTIIa gene promoter region has a stretch of homology with a sequence within this collagenase transcriptional control region that is also present in the P-motif of the SV40 enhancer (see sub-Section 1.6.1.). The SV40 enhancer is a TPA-inducible enhancer element (245) and analysis of the constitutive parts of this complex enhancer revealed that all of the previously described enhancer elements (A, B and C, see sub-Section 1.6.1.) responded to TPA stimulation resulting in transcriptional activation (246). The P-motif which is present in the 3' region of the 72bp SV40 enhancer also acts as an enhancer element and is inducible by TPA treatment. The DNA sequence that is conserved between the SV40 P-motif, the hMTIIa transcriptional control region and the collagenase element that activates transcription in response to TPA, TGAG/CTCA, binds a HeLa cell nuclear protein in DNase I footprinting studies (247). This element was capable of activating transcription in <u>in vivo</u> transcription assays and any mutations of the consensus sequence that resulted in a failure to activate <u>in vitro</u> transcription also failed to compete for

the protein binding to this sequence in DNase I footprinting experiments. This indicated that the protein binding to this sequence was responsible for the transcription activation via this sequence and was called activator protein 1 (AP-1).

Synthetic copies of TPA-responsive sequences (called the TRE-TPA responsive elements) conferred TPA inducibility on heterologous promoters (248) and this short sequence operates as an inducible enhancer element as it operates in an orientation independent manner and when placed either upstream or downstream from a heterologous promoter. Interestingly, following TPA treatment of HeLa cells, the DNA binding activity of AP-1 is increased 3 to 4-fold but this activation does not involve fresh protein synthesis as the same phenomenon can be observed in cells treated with cycloheximide during the TPA treatment. This indicates tht TPA treatment leads to a post-translational modification of pre-existing protein leading to an increased DNA binding activity. It is possible that phosphorylation of the protein is involved in this activation as phosphatase treatment reduces the DNA-binding properties of this protein (249). AP-1 was purified using a DNA-affinity procedures and was shown to footprint the SV40, hMTIIa and collagenase TREs (239) and the polyoma α-enhancer also has an AP-1 binding site and is responsive to TPA stimulation (250). AP-1 sequences are also present in an element 700bp upstream from the vimentin gene promoter that can act as a basal level enhancer and can also activate transcription in response to both serum and TPA stimulation (251).

The sequence in the c-fos promoter region that is continuous with the SRE and activates transcription in response to cAMP (see sub-Section 1.6.4.) TGCGTCA, centred at position -296, bears a close homology to the AP-1 consensus sequence, TGAG/CTCA. It has been mentioned previously that this sequence can bind purified AP-1 (238) and it has also been reported that the polyma α-enhancer can compete for the factor binding to this sequence in a DNase 1 footprinting assay (250) and, as mentioned above, this viral enhancer contains an AP-1 binding site. Transfection analyses of c-fos promoter mutants with this sequence inserted revealed that it can activate transcription in response to both TPA and EGF stimulation almost to the same levels as those obtained with constructs containing the SRE (252). The time course for the induction by the AP-1 like sequence is similar to that observed with the SRE, being both rapid and transient, which is different from the induction observed with previously mentioned AP-1 binding sites that required overnight stimulation by TPA to increase transcription. It was also noted that this sequence is not additive with the SRE in the TPA or EGF induction of c-fos transcription perhaps because there is a mutual exclusion of the proteins binding to these sequences in vivo resulting in only one of them contributing to the transcriptional activation of the c-fos gene in response to extra-cellular stimuli.

This sequence in the c-fos promoter, TGCGTCA, is therefore responsive to TPA, EGF and cAMP stimulation and apparently binds the AP-1 protein (238) in vitro. This agrees with a report showing that AP-1 consensus sequences can activate transcription in response to both TPA and cAMP stimulation (253). In this study the collagenase AP-1

sequence was used and insertion of a G residue into this sequence coverts it into a CRE that fails to activate transcription in response to TPA although the native sequence responds to both cAMP and TPA stimulation with approximately equal effect. So, there exists a degree of "cross-talk" between the TPA and cAMP responses, and c-fos AP-1-like sequence falls into a category of transcriptional response elements that will activate transcription in response to either of these extra-cellular stimuli. Therefore the c-fos promoter region contains at least two upstream sequences that will activate transcription in response to TPA and four upstream sequences that will activate transcription in response to cAMP, with the SRE and AP-1-like element being responsive to both.

1.6.7. Viral proteins transcriptionally activate the c-fos gene.

Adenoviruses encode a protein, Ela 13s, that is responsible for the transcriptional activation of adenovirus early genes (for a review see 254). This viral trans-activating protein (for a review see 255) can also transcriptionally activate eukaryotic cellular genes such as the HSP70 and β-tubulin genes (256, 257), and its role in the transcriptional activation of several cellular oncogenes has been examined (258). This study revealed that the Ela 13s protein transcriptionally activates the c-fos promoter through a sequence that includes the previously identified SRE and AP-1-like element and that deletion of these sequences abolishes any trans-activation of the c-fos promoter by the Ela 13s protein. However, a further study contradicted this observation as it reported that the Ela 13s protein (termed Ela 13s hereafter) could trans-activate several promoters

through a specific TATA box sequence (TATAA), which included that of the c-fos gene (259). In this study a c-fos reporter gene construct containing only 53bp of upstream sequence was used in the co-transfection studes and this sequence contains none of the previously identified DNA elements involved in the transcription regulation of the human c-fos gene. The reason for the difference between these two sets of results is not clear but two different cell lines were employed in the transfection studies and it is conceivable that this difference is responsible for the conflicting results.

It is of interest to note here that Ela 13s trans-activates the adenovirus early promoters through sequences that are very similar to that of the CRE (260). Several studies have attempted to determine whether an identical cellular protein binds to the adenovirus early promoter sequences and to CREs and whether Ela 13s and cAMP can trans-activate these sequences in transfection studies (261 - 264). In vitro DNA binding studies have shown by competition analysis that the CREs of several genes can compete for the proteins that bind to the adenovirus early gene promoters, and in transfection studies, when CREs are co-transfected with constructs containing the adenovirus promoters, then both basal and Ela stimulated transcription is reduced. These results suggested that identical proteins are involved in cAMP stimulation of transcription and in Ela 13s trans-activation of adenovirus early promoters. Ela 13s and cAMP can synergistically activate transcription of the adenovirus early promoters of intact viruses that have infected tissue culture cells (265). However, in transfection studies it was shown that the CRE-like sequences present in the adenovirus early gene promoters is

not responsible for this synergistic-activation (262) suggesting that other sequences in the adenovirus early gene promoters are involved in this synergistic activation. Attempts to purify the cellular protein that binds to these adenovirus early gene promoters, called ATF (activating or adenovirus transcription factor) or CREB (cAMP responsive element binding protein) led to the purification of several proteins ranging in size from 72,000 to 40,000 daltons (187, 261, 263, 266, 267). It is certain that the CREB/ATF binding site is capable of binding more than one nuclear protein, suggesting that perhaps cAMP and Ela 13s trans-activate promoter through this sequence employing different proteins, all of which can bind to the recognition sequence.

There have been two further reports of viral trans-activators activating transcription from the c-fos promoter (268, 269). Bovine leukaemia virus (BLV) encodes a transcriptional activator called $p38^{tax}$ which activates viral transcription through three ATF sequences present in the U3 region of the viral LTR (268). This protein trans-activates several other constructs containing adenovirus early gene promoters and cAMP responsive gene promoters through regions that contain ATF-like sequences. It activates the c-fos promoter through sequences that include the SRE and AP-1-like element (-361 to -225), and removal of this sequence abolishes the trans-activation of the c-fos promoter by this protein. Neither this viral trans-activatior nor Ela 13s can trans-activate the transcription of the c-fos gene through the protein that binds to the c-fos -60 element. This element has been shown previously to stimulate transcription in response to cAMP and to bind the purified

protein CREB (187). The failure of this sequence to activate c-fos transcription in response to treatment with the viral trans-activators may be due to a lack of other proteins binding in the c-fos promoter region that are required for viral trans-activation (operating in a co-operative manner) or the fact that the c-fos -60 element binds a number of the CREB/ATF family of protiens that is unresponsive to viral trans-activators. The other viral trans-activiating protein that can transcriptionally activate the c-fos gene is the human T-cell leukaemia virus 1 (HTLV-1) encoded protein tax_1. Co-transfection experiments revealed two elements upstream from the c-fos promoter that were capable of responding to tax_1. The first element (-372 to -324) contained the DNA element that binds a protein after v-sis conditioned medium stimulation of 3T3 cells (222) and the second element contains the SRE and the AP-1-like sequence.

These studies have identified areas of the c-fos upstream region that are responsive to viral trans-activators but have not clearly delineated the precise sequences involved (e.g. is it the SRE or the AP-1-like element that activates transcription in response to these viral proteins?). In one case (259) the c-fos TATAA box has been identified as being essential in the transcriptional response of c-fos to Ela 13s, but for the remaining cases further studies must be carried out to identify the precise DNA sequences involved.

1.7. The c-Fos Protein is a Transcription Factor.

1.7.1. Evidence suggesting a role for Fos proteins in transcriptional regulation.

The fact that Fos proteins are found exclusively in the nucleus suggests that these proteins may play a role in the regulation of transcription. Further circumstantial evidence for such a role came from studies showing that v-Fos and c-Fos proteins are associated with the isolated nuclei from various cell lines (270, 271). In these studies digestion of the chromatin with DNAse released Fos proteins along with the cellular protein p39 which is associated with Fos proteins (7) from the isolated nuclei whereas treatment with ribonuclease failed to cause this release. More direct evidence suggesting a role for Fos proteins is transcriptional regulation came from studies of v-fos transformed fibroblasts where it was observed that both the transcription and the expression of the α_1 (III) collagen gene is elevated (272) and in co-transfection experiments with α_1 (III) collagen promoter-CAT and v-fos expression plasmid constructs the v-Fos protein elevated the levels of transcription from the α_1 (III) collagen gene promoter region (273). The levels of expression of the c-Fos protein seemed to reflect the levels of expression of certain major histocompatibility (MHC) genes in several haematopoietic cell lines and in metastatic lung carcinoma cell lines suggesting that the c-Fos protein controlled the expression of the MHC genes at the transcription level (274, 275).

Although the above evidence indicated that Fos proteins do regulate transcription the argument that the Fos proteins are acting through an intermediate protein to activate transcription could not be dismissed. More direct evidence implicating Fos proteins in transcriptional regulation came when it was observed that c-Fos protein is part of a nucleoprotein complex that binds to a DNA element upstream from the adipocyte lipid binding protein aP2 (276, 277). This gene is inactive in pre-adipocyte cells due to the repression of its transcription by the protein complex that binds to a sequence upstream from the aP2 transcription initiation site called the fat-specific-element 2 (FSE-2). Antibodies to the c-Fos protein disrupt the formation of the nucleoprotein complex with this sequence in gel mobility shift assays and specifically immunoprecipitate protein complexes covalently bound to FSE-2 DNA by U.V. crosslinking. Further evidence implicating c-Fos proteins in transcriptional repression has come from studies on the transcriptional control of the c-fos gene itself. The fact that c-fos transcription is not shut-down following its induction by serum stimulation of quiescent fibroblasts in the presence of cycloheximide (105, 107, 110, 115, 127, 149) suggested that a protein synthesised following stimulation is involved in shut-down of c-fos transcription. The c-Fos protein itself has been identified as a possible candidate for this repression in two independent studies (220, 278). Firstly, co-transfection of a construct coding for the c-Fos protein with c-fos promoter-CAT constructs prevented the activation of c-fos transcription in response to serum stimulation from both the region containing the SRE and the region containing the sequence homology with the HSP 70 promoter SRE (the HSP 70 transcriptional activation

by serum stimulation is also repressed by the c-Fos protein). Secondly, c-Fos protein mutants that contain foreign carboxy terminals prevent the shut-down of c-fos transcription following serum stimulation and it is thought that these aberrant proteins operate by complexing with cellular factor that interact with the native c-Fos protein to shut-down transcription. These mutant proteins are dominant in their effect and prevent transcriptional shut-down of transfected c-fos promoter-reporter gene plasmids and of the endogenous c-fos gene. It is interesting to note that the v-Fos protein, which has a different carboxy-terminus from that of the c-Fos protein (13), fails to repress the transcriptional activation of the c-fos promoter in response to serum in co-transfection experiments. This has an interesting implication for the transformation mechanism of the v-Fos protein. Perhaps it operates by preventing the transcription repression of a set of genes by the native c-Fos protein leading to cellular transformation. However, this cannot be the only mechanism by which Fos proteins trigger cellular transformation as the c-Fos protein itself, if expressed to high enough levels, can also cause transformation.

The c-Fos and v-Fos proteins can positively regulate transcription when attached to promoter regions in yeast cells (279). This study used plasmid constructs encoding hybrid proteins containing Fos (both viral and cellular) and the bacterial DNA binding protien Lex A. Lex A binds to operator sequences upstream from transcriptional initiation sites and represses transcription in prokaryotic cells. The fusions, Lex A-c-fos and Lex A-v-fos, encoded the DNA binding domain of the Lex A protein and the coding sequences for the fos

genes and these constructs activated transcription of plasmid constructs transfected into yeast cells which contained operator sequences in their promoter region. This study showed that Fos proteins could activate transcription in eukaryotic cells when bound to DNA upstream from a transcription initiation site.

1.7.2. Fos protein forms complexes with the AP-1 DNA sequence.

The previous section detailed several reports implicating the Fos protein in the regulation of transcription. A target DNA sequence for the Fos protein was discovered by analysis of the FSE-2 sequence which contains a consensus recognition sequence for the previously described transcription factor AP-1 (see sub-Section 1.6.6.). Further DNA-protein interaction analysis using the FSE-2 sequence showed that the DNA binding activity of the nucleoprotein complex that binds to this sequence could be increased by preparing protein extracts from the nuclei of serum-stimulated cells that contain elevated levels of Fos protein (280). This suggested further that the Fos protein plays a pivotal role in the complex that binds to this sequence and that perhaps the c-Fos protein interacts with the previously purified AP-1 protein to regulate transcription. Using several other AP-1 consensus containing sequences a further study employing *in vitro* DNA-protein interaction studies revealed that in all cases the c-Fos protein is involved in the nucleoprotein complex that binds to this sequence (281). Transfection studies using AP-1-binding sequence containing reporter plasmids and Fos protein expressing constructs co-transfected into tissue culture cells revealed that the c-Fos protein can stimulate transcription from the

AP-1 containing promoters (282 - 278) and that the over-expression of c-Fos protein can repress transcription from the c-fos promoter. This observation agrees with previous reports showing that the c-Fos protein can auto-regulate its own transcription by repressing transcription following stimulation (220, 278). All of the evidence described so far directly implicating c-Fos protein in transcriptional regulation has suggested that this protein interacts with the transcription factor AP-1. Several studies have been undertaken to prove that this is the case, but it is important that the discovery of the AP-1 encoding gene is described at this point.

In 1985 a virus was isolated from a spontaneous chicken tumour; it was called avian sarcoma virus (ASV) 17 (285). This virus has a genome of 3.4kb (286) and is defective in replication as it encodes a gag-fusion protein which contains cellular sequences called jun (287). The gag-jun region encodes a fusion protein of 65kD ($p65^{gag-jun}$) and this sequence can transform chick embryo fibroblasts. The amino acid sequence derived from the nucleotide sequence shows homology to the yeast transcription factor GCN4 (288) which is a component of the general control system of amino acid suynthesis in yeast (289). GCN4 has a functional domain in the carboxy-terminal region that is responsible for the specific DNA binding properties of this protein and the homology to the jun sequence is restricted to this region. GCN4 has an acidic stretch in the centre of the molecule which is responsible for its transcription activation properties and the Jun protein contains two such acidic stretches in its amino-terminal region. The carboxy-terminal region of Jun can replace that of GCN4 in a fusion protein that is capable

of complementing GCN4 minus yeast mutants. This fusion protein also requires the DNA-binding domain of the Lex A protein but in this case the Lex A sequence facilitates dimerisation of the GCN4-Jun fusion protein which then binds to the GCN4 recognition sequence. Intact GCN4 binds to its recognition sequence as a dimer (290).

The DNA element to which the GCN4 dimers bind in yeast cells is identical to that of the AP-1 binding site suggesting that Jun and AP-1 protein may be related. To test this hypothesis two antibodies were raised against peptides of Jun sequences. These two antibodies can precipitate the $p65^{gag-jun}$ protein (which is exclusively nuclear) and can specifically react with DNA-affinity-purified AP-1 p47 in immunoblots (291). Further evidence supporting the relationship between Jun and AP-1 came when it was observed that Jun protein synthesised from a human c-jun clone showed identical footprinting properties as DNA-affinity purified AP-1 (291, 292). In addition, the $p65^{gag-jun}$ fusion protein also protected the same sequences in footprinting assays as purified AP-1 (287). Cellular DNA sequences corresponding to that of the viral jun have been isolated (291, 292). The main difference between the cellular and viral protein sequences is a 27 amino acid deletion from the cellular sequence to form the viral sequences although it is not yet clear whether this deletion contributes to the transformation properties of the viral sequences. However, all of the above evidence suggests tha the cellular Jun DNA sequence encode the transcription factor AP-1. This was the first nuclear oncogene that had its cellular function identified.

Another interesting aspect of the c-jun gene is that, like c-fos, it is a number of the "immediate-early" cellular gene family that are transcriptionally activated following stimulation of cells in the Go phase of the cell-cycle (293 - 297). Like c-fos, c-jun can also be transcriptionally activated by tumour growth factor-β (TGF-β) (298 - 300), tumour necrosis factor-α (TNF-α) (301, 302) and the c-Ha-ras oncogene (146, 147, 303). However, c-fos and c-jun are not always co-induced as following membrane depolarisation of PC12 cells transcription of c-fos is induced but that of c-jun is not (304). So, although both c-fos and c-jun are members of the "immediate-early" family of cellular genes and regulate transcription through the same sequence their transcriptional control is not identical. This observation suggests that different stimuli may transcriptionally activate distinct sets of "immediate-early" genes leading to the distinctive effects of each individual stimulus.

A combination of in vivo and in vitro techniques were used to establish the relationship between the c-Fos and c-Jun proteins that presumably co-operate in binding to the AP-1 recognition sequence (305 - 309). Immunoprecipitation of Fos proteins results in the co-precipitation of a cellular protein p39 (7). Further precipitation of these Fos-immunoprecipitated proteins with Jun-specific antibodies results in the specific precipitation of p39 (305, 306). The p39 protein eluted from a gel is capable of binding to the AP-1-recognition sequence further suggesting that p39 is c-Jun protein. p39 and c-Jun (DNA-affinity purified AP-1) proteins also show identical tryptic peptide maps and all of these observations are consistent with the cellular Fos associated protein (FAP) p39 being

c-Jun. *In vitro* translated Jun protein (from a rat cDNA clone) has a low level of DNA binding activity which can be detected only at high concentrations of protein (308). *In vitro* translated Fos protein (once more from a rat cDNA clone) has no DNA binding activity but it acts co-operatively with the Jun protein to give enhanced DNA-binding activity which is due to a stabilisation of the protein-DNA complex. v-Jun protein can also complex with the c-fos protein (309). These *in vitro* DNA-protein interaction studies agreed with co-transfection experiments performed with the mouse embryonal carcinoma cell line F9 (305, 306). This cell line lacks any detectable AP-1 binding activity (310) making it an ideal model for the *in vivo* study of the Fos-Jun interactions. Transfection of a reporter construct containing an AP-1 recognition sequence with a c-Jun protein expression construct into F9 cells results in elevated levels of expression from the AP-1 containing reporter construct. However, co-transfection with a c-Fos protein expression construct along with the reporter plasmid and c-Jun protein expression construct leads to a 100-fold increase in expression from the AP-1 containing promoter. This agreed with the *in vitro* studies showing the Jun-protein can indeed interact with the AP-1 site but that Fos protein greatly increases this interaction.

So, it has been established that c-Fos and c-Jun proteins interact to activate transcription and co-operate in DNA binding. The next step was to identify the mechanism by which these two proteins interact and to try and identify DNA-binding and transcriptional activation domains.

1.7.3 The mechanism of the interaction between Fos and Jun proteins.

When considering the mechanism of interaction between Fos and Jun proteins it was of interest to note that deletion of the carboxy-terminal region from the v-Jun protein prevented any interaction with Fos protein taking place (309). In this region, which has sequence homology with the DNA-binding domain of GCN4 (288), there exists a motif that has been implicated in the DNA binding properties of certain proteins called the leucine zipper. c-Jun, c-Fos (and their viral counterparts), C/EBP (311, 312), c-myc (313) and GCN4 all contain this region. Leucine zippers are amphipathic β-helices that contain 4 or 5 leucine residues at 7-residue intervals which locates the leucines at approximately the same rotational position along the helix with leucine side chains forming a linear crest that extrudes from the helical axis. Dimerisation is thought to be mediated by hydrophobic interactions between the leucine side chains of two leucine zipper domains, hence the term "zipper". GCN4 binds to its recognition sequence as a dimer (290) and this dimerisation requires the presence of the carboxy-terminal sequences containing the leucine zipper domain. This observation suggested that perhaps Fos and Jun proteins may also interact through this domain that seems to be involved in protein-protein interactions. To determine whether this is the case a series of mutation and deletion studies were carried out with the Fos and Jun proteins in a bid to identify the specific domains involved in the interaction of these proteins.

These studies involved analysis of the interaction of *in vitro* translated Fos and Jun proteins (both mutant and wild type) and also involved the analysis of the DNA-binding potential of these proteins (314 - 320). *In vitro* translated c-Jun protein can bind very weakly to an AP-1 site in the absence of c-Fos protein (309, 316, 318, 319). However if this protein is mixed with *in vitro* translated c-Fos protein (or is co-translated) then Fos-Jun complexes form that have a much higher affinity for the AP-1 recognition site (the precise degree of this increase in affinity is variable between labs but is never less than 10-fold and is usually much greater) (308, 314, 316, 318, 319). This presented the ideal model to examine the role of the leucine zipper in both the protein-protein interactions between Fos and Jun and in DNA-binding. The c-Fos protein is the only one as yet reported that has the leucine zipper domain in the centre of the protein molecule - all other zipper-containing proteins have this domain at their carboxy termini. Deletions in the c-Fos protein from the carboxy-terminus and the amino-terminus that retain the zipper domain are still capable of complexing with c-Jun protein and binding to AP-1 recognition sequences (314 - 317, 319). Point mutations that replace one of the leucine residues in the c-Fos zipper domain generally do not greatly affect interaction with the c-Jun protein, nor does replacing the amino acids between the leucine resideues. However, mutations of two or more leucines or insertions which upset the periodicity of the leucine residues abolishes interactions with c-Jun protein (314 - 320). The region of the c-Fos protein that contains the leucine zipper is conserved in the v-Fos sequence and studies with v-Fos constructs containing similar point mutations and deletions were used in transformation assays. It is interesting to

note that the loss of interaction between v-Fos protein and c-Jun protein is mirrored by the inability of the Fos mutant constructs to transform fibroblasts. This suggests that the interaction with the Jun protein is an essential aspect of transformation by Fos protein. As mentioned previously, the Jun protein also contains a leucine zipper domain and mutation studies on this sequence revealed that the periodcity and frequency of the leucine residues are essential for the complexing of Jun protein with that of Fos (319). The Fos and Jun proteins containing double mutations in the leucine zipper domain were used to show that the proteins joined by the leucine zipper do so in a parallel configuration (319, 321), suggesting that the interaction between the zipper regions involved a "coiled-coil" structure (322).

Taking into consideration all of the above evidence it seems likely that Fos and Jun heterodimers are formed via an interaction between the respective leucine-zipper domains. However, this does not explain how the resultant heterodimers binds to its DNA recognition sequence (an AP-1 site). To the amino-terminal site of the Fos protein leucine zipper there is a stretch of amino acids that make up a basic domain that is favourable for the formation of an α-helix. This basic domain is conserved to a certain extent in the c-Jun protein and most of the other zipper domain containing proteins suggesting that this region may be involved in the interactions of these proteins with their DNA target sites. To test this hypothesis mutation studies were carried out that altered the content of the basic amino acid stretch and the resultant mutant proteins were analsysed for their DNA-binding properties (315, 318 - 320).

Mutations that appreciably alter this region of the Fos protein results in the abolition of the DNA binding properties of the Fos-Jun heterodimers complex. These mutant Fos proteins can still complex with the Jun proteins through their leucine zipper domains but fail to bind DNA suggesting that the zipper domain by itself is not sufficient to induce the DNA binding properties of the Fos-Jun heterodimer. Rather, it seems that the zipper regions interact with one another and perhaps juxtapose the basic domains of the two proteins allowing them to bind to the AP-1 consensus sequence which is a relatively small recognition sequence.

So, the c-Fos (and v-Fos) protein has two regions that are responsible for the interaction of this protein with DNA - the leucine zipper domain and the basic amino acid stretch domain. It is interesting to note here that certain c-Fos mutant proteins that retain the ability to complex with the c-Jun protein and bind to DNA fail to activate transcription in co-transfection assays with F9 cells (316). These mutant proteins have their carboxy terminal region deleted (which has been implicated in transcriptional activation) and this result suggests that the transcriptional activating properties of the c-Fos protein is not essential to its transforming properties (carboxy-terminal truncated Fos proteins can still transform fibroblasts (317)). This suggests that the Fos protein has three crucial domains that are involved in transcriptional activation: the leucine zipper, the basic domain and the carboxy-terminal region of the protein.

1.7.4. c-fos and c-jun are members of gene families.

To date, c-Fos has been implicated in transcriptional activation (272 - 275, 279, 282 - 284), transcriptional repression (276, 277, 323) and transcriptional shut-down (220, 278). The c-jun gene is positively regulated by its own product through an upstream AP-1 binding site suggesting that the Fos protein may also be involved in the transcriptional activation of this gene. The pleiotropic effects of the c-Fos protein on transcriptional regulation suggest that perhaps there may be more than one c-fos-like gene and protein. This hypothesis was given support by the observation that c-Fos antibodies can react with a whole series of Fos-related antigen (FRAs) proteins, some of which are increased in abundance following serum-stimulation (323). The first such gene to be isolated encoding one of these proteins was called fra-1 (324). The Fra-1 protein has extensive amino-acid homology with the c-Fos protein including the leucine zipper and basic domains that are essential to the DNA binding properties of the c-Fos protein (see sub-Section 1.7.3.). The transcription of this gene can also be induced by a variety of extra-cellular stimuli (although these only represent a sub-set of those that can activate c-fos transcription) and the mRNA of Fra-1 contains the motif in the 3′ untranslated sequence that has been identified as being responsible for the short half-life of many mRNAs (see sub-Section 1.5.1.). In vitro translated Fra-1 protein can form complexes with c-Jun protein and can co-operate in binding to AP-1 recognition sites due to the leucine zipper and basic domains present in this protein (325). Another FRA gene has been isolated, fos-B, which also has a conserved leucine zipper and basic domain (326) and contains the mRNA

destabilising motif in its 3′ untranslated region. As with Fra-1, in vitro translated Fos B protein can form complexes with c-Jun protein, and can co-operate in the binding to the AP-1 recognition sequence Fos B can also increase the transcriptional activation caused by constructs expressing c-Jun protein when they are co-transfected with an AP-1 sequence containing reporter plasmid into quiescent NIH 3T3 cells.

c-jun related genes have also been isolated from cDNA libraries. Jun B is transcriptioanlly activated following stimulations of quiescent cells, and contains the mRNA destablising sequence in its 3′ untranslated sequence as well on the leucine zipper and basic domain (327). Jun-B protein can complex with c-Fos protein (and Fos-B protein (326)) and bind to AP-1 recognition sites (328). Another c-jun related gene is jun D (329) whose transcription is not affected by the external stimulation of quiescent fibroblasts - it remains at a constant, relatively elevated level, throughout stimulation. This gene has also conserved the leucine zipper and basic domain. It can form complexes with c-Fos protein that bind to AP-1 sites and can co-operate with c-Fos protein in in vivo co-transfection experiments to activate the transcription of constructs containing AP-1 sites (329). c-Jun, Jun-B and Jun-D proteins can all bind to AP-1 recognition sites in the absence of c-Fos protein (328). It is also interesting to note that these proteins can also form complexes with oligonucleotides containing the CRE consensus sequence, although the affinity of the Jun proteins for this sequence is not affected by the present of c-Fos protein (328). DNA-affinity purification of the CRE binding protein (CREB/ATF) resulted in the co-purification of AP-1

proteins can bind to CRE consensus sequences (330). This showed that AP-1 and CRE binding proteins are antigenically related (which is not surprising since their DNA recognition sites are so similar), and this was confirmed upon isolation of a cDNA encoding the CREB/ATF protein (331). The protein encoded by this cDNA contains a leucine zipper and a basic domain and shows a degree of homology to Jun proteins in the region. It is not yet known whether c-Fos protein can complex with CREB/ATF protein and co-operate in the binding to the CRE sequence.

The Fos and Jun proteins seem to be highly evolutionary conserved as antigenically related proteins are present in Drosophila embryo nuclear extracts (332). These proteins were purified on an AP-1 DNA affinity column, and display the sequence specificity and transcription activation properties of the mammalian protein complexes that binds to AP-1 sequences. An Epstein-Barr Virus (EBV) encoded trans-activating protein also bears homology to the c-Fos protein in certain regions (333). However, this homology does not include the leucine zipper and, although this protein can activate EBV early gene transcription through an AP-1 sequence, it also activates through other sequecnes (334). The precise mechanism of transcriptional activation by this protein is not known but is seems unlikely that it will include interaction with Jun-like proteins as no leucine zipper is present.

So, a very complex mechanism for transcriptional regulation can be imagined when considering the Fos and Jun protein families. All of the genes encoding these proteins are not transcriptionally regulated

in an identical fashion suggesting that the sub-sets of proteins activated by specific extra-cellular stimuli may be responsible for the pleiotropic response to these stimuli. This could be due to the transcriptional regulation of different sub-sets of genes by the different combinations of Fos-Jun heterodimers that may be formed following specific stimuli. The mechanism by which Fos-Jun heterodimers regulate transcriptional activation, repression and shut-down is not clear but one could imagine general ways in which these proteins produce their pleiotropic effects. For instance, the response of a promoter to Fos-Jun heterodimers could depend upon the members of the protein families that form the compexes with the AP-1 binding sites, which may be dictated by the nucleotide sequence overlapping with the AP-1 sequence. Alternatively, the specific effect on transcriptional regulation of the Fos-Jun heterodimers may depend on the interaction these proteins have with proteins that are bound to the promoter (or further upstream) regions of the AP-1 sequence containing genes. Further studies will be required to clarify this situation, but this problem has certainly been made easier by the isolation of several members of the fos and jun gene families.

1.7.5. Hierarchical control of Fos-Jun transcriptional activation.

It is clear from the evidence described so far that the fos and jun gene families play an important role in mediating the response of cells to external stimuli. This mediation presumably involves the transcriptional activation of a number of genes that are as yet mostly unidentified. The control of Fos-Jun interactions resulting

in transcriptional activation seems to form a hierarchical structure which starts with the transcriptional stimulation of the c-fos and c-jun genes themselves. This results in protein synthesis which then form heterodimers through the leucine-zipper and these then bind to a DNA recognition sequence via the basic domains present in both proteins. These DNA-bound heterodimers then activate (or repress) gene transcription. The control of each step of this pathway that results in transcriptional activation is obviously very important, but perhaps the most crucial control is exerted at the level of gene transcription.

The purpose of this project was to analyse the transcriptional control of the human c-fos proto-oncogene. To do this regions of c-fos upstream sequence were placed upstream from the prokaryotic CAT reporter gene and the effect these sequences had upon the transcription of the CAT gene was assayed following transfection of these constructs into CT3 cells (a derivative of NIH 3T3 cells). Oligonucleotides were inserted upstream from the c-fos promoter in the c-fos-CAT constructs to determine the effect precise sequences have upon transcription from the c-fos promoter. These c-fos-CAT constructs were used not only for the analysis of c-fos transcriptional control elements in growing cells, but also to identify sequences that could confer serum or TPA responsiveness upon the c-fos promoter following stimulation of quiescent CT3 cells with the respective extra-cellular stimuli. These investigations concentrated on examining the previously characterised SRE and the sequence continuous with this element which I have called the fosATF/AP-1 sequence, which had not been previously studied at the

outset of this project. Attempts were also made to identify any synergistic control of transcription that these two sequences may exert on the c-fos promoter.

CHAPTER 2 : MATERIALS AND METHODS

MATERIALS AND METHODS PAGE

2.1. Materials

2.1.1. General Suppliers.

All chemicals used were of Analar grade and supplied by BDH Chemicals, Poole, Dorset unless otherwise stated.

All radioisotopes were obtained from Amersham International, Amersham, Buckinghamshire.

Restriction endonucleases and enzymes employed in the manipulation of DNA were purchased mainly from BRL Gibco (Europe), Paisley, and Boerhinger-Mannheim (BCL), Lewes, East Sussex.

Tissue culture medium, supplements and serum were obtained from Gibco (Europe) Paisley; or Flow Laboratories, Herts. Sterile disposable plastic flasks of growth area 25cm^2, 75cm^2 and 175cm^2 and roller bottles of 800cm^2 used in the culture of animal cells were supplied by Falcon, Oxford and Nunc, Paisley.

Ingredients of bacterial media such as Tryptone, Yeast Extract and Agar were obtained from Difco Laboratories, Surrey.

2.2. General Procedures.

2.2.1. Preparation of total cellular RNA from cultured animal cells.

2.2.1. a) Phenol method.

All RNA prepared by this method was made from monolayer cells. The culture medium was removed from the flasks and the cells washed with PBS. Following removal of the PBS 10ml of phenol saturated with lysis buffer (0.3m sodium acetate 0.5% SDS (Na_2), 5mM EDTA pH8.0) was added to the flask along with 10ml lysis buffer. The flask was then gently rotated for 5 minutes and the resultant mixture transferred to a 50ml Falcon tube and tumbled for 5 minutes. A half-volume of chloroform:isoamylalchol (in a ratio of 24:1 v/v) was then added and the mixture was tumbled for a further 10 minutes. This solution was then spun at 2,700 r.p.m. (1,220xg) for 10 minutes at room temperature in an IEC Centra-8R centrifuge. The upper phase was removed and placed in a fresh 50ml Falcon tube. Again, 10ml of chloroform:isoamylalcohol (24:1 v/v) was added to this mixture which was then tumbled and centrifuged as before. This process was repeated one more time. After this third spin the upper phase was placed into a fresh 50ml Falcon tube and mixed with two volumes of 100% ethanol. This solution was incubated at $-20^{o}C$ for 1 hour and then spun at 2700 r.p.m. (1,220xg) for 10 minutes at $4^{o}C$. The pellet was then resuspended in 1 to 2ml of sterile de-ionised H_2O and an equal volume of 4M Li Cl added to the solution. This mixture was then incubated overnight at $4^{o}C$ and then spun at 10,000 r.p.m. for 60 minutes at $4^{o}C$ in a Sorval RC-2B Superspeed centrifuge using an H8-4

rotor. The pellet was resuspended in 400µl of 3mM $MgCl_2$. This solution was then treated with 20 units of RNAse-free DNAse 1 for 30 minutes at room temperature. This was then extracted with an equal volume of a phenol-chloroform (ratio of 1:1) solution saturated with 10mM Tris HCl pH7.5 and 1mM EDTA pH8.0. The resulting mixture was then spun at 10,000 r.p.m. (8,160xg) for 5 minutes at room temperature in an eppendorf centrifuge 5415 and the aqueous phase removed and placed in a fresh test tube. This was then extracted twice with an equal volume of a chloroform:isoamylalcohol mix and the aqueous phase removed and placed in a fresh test tube. A tenth volume of 3M sodium acetate was then added followed by 2.5 volumes of 100% ethanol. The resultant mixture was then placed at $-20^{o}C$ overnight and the following day spun at 10,000 r.p.m. (8,160xg) for 15 minutes at $4^{o}C$ in an eppendorf centrifuge 5415 which pellets the precipitated RNA. The pellet was then drained and washed with 500µl of 70% ethanol and then spun at 10,000 r.p.m. (8,160xg) for 5 minutes in an eppendorf centrifuge 5415. This step was repeated once and the pellet freeze dried for 15 minutes. It was then resuspended in an appropriate volume of de-ionised H_2O and the optical density determined in order to calculate the concentration of the solution (E :1cm, 260. 1 : 00 - 40µg/ml). All aqueous solutions used in this protocol were treated with diethyl pyrocarbonate (which destroys any RNAses present) and autoclaved (15 p.s.i. for 15 minutes).

2.2.1. b) RNAzol Method.

RNAzol solution was bought from Biogenesis Limited, Bournemouth, England. RNA was prepared by this method based on that of the

manufacturer (335) with the following modifications. Medium was removed from monolayers of cells which were then washed with PBS. The cells were then removed from the plastic culture surface by treatment with a 0.25% trypsin solution and taken up in 10ml of PBS. This mixture was then spun at 1,000 r.p.m. (850xg) for 5 minutes at room temperature in an MSE Centaur 2 centrifuge. The resulting pellet was then taken up in RNAzol and the manufacturer's instructions were then followed.

2.2.2. Analytical gel electrophoresis of DNA.

Depending on the size of the expected DNA fragment restricted DNA was electrophoresed through an agarose or polyacrylamide gel. DNA samples either in restriction enzyme buffer or in distilled H_2O (following ethanol precipitation) were mixed with one-tenth volume of gel loading dye (0.1% bromophenol blue, 30% sucrose, 10mM Tris HCl pH 8.0 and 0.1mM EDTA pH 8.0). The samples were then electrophoresed either in:

a) a horizontal 1% (usually) agarose (BRL) gel in 1 x T.B.E. (0.089M Tris-borate, 0.089M Boric acid and 0.002M EDTA pH 8.0) or 1 x T.A.E. (0.04M Tris-acetate and 0.001M EDTA pH 8.0). The DNA was visualised under U.V. light following staining in a 0.1µg.ml solution of ethidium bromide (EtBr).

b) a verical neutral polyacrylamide gel (5% acrylamide, 0.18% bis-acrylamide, 0.08% ammonium persulphate and 0.05% N,N,N,N-tetramethyl-ethylene diamine (TEMED)). The DNA was visualised as in a).

Running conditions varied with convenience.

2.2.3. Isolation of DNA from agarose gels.

2.2.3. a) Phenol method.

A low melting point agarose gel was run and visualised as in sub-Section 2.2.2. A slice of gel containing the DNA fragment of interest was cut out and weighed, mixed with 2 volumes of 1 x T.B.E. and heated at 70°C for 10 minutes. After this incubation an equal volume of ice cold phenol (saturated with T.E.) was added to the tube and the two phases mixed together. The tube was then centrifuged for 5 minutes at 10,000 r.p.m. (8,160xg) in an eppendorf centrifuge 5415 and the aqueous phase placed in a fresh eppendorf tube. The phenol extraction was repeated and was followed by two chloroform:isoamylalcohol (1:24 v/v) extractions. The aqueous phase was then taken and the DNA precipitated by the addition of a tenth volume of 3M sodium acetate and 2.5 volumes of 100% ethanol. The tube was placed on dry ice/ethanol for 10 minutes, or placed at -20°C for at least 1 hour, and was then spun at 10,000 r.p.m. (8,160xg) for 15 minutes at 4°C in an eppendorf centrifuge 5415. The resultant pellet was then washed with 500µl of 70% ethanol and freeze dried for 15 minutes. The DNA pellet was then taken up in an appropriate volume of de-ionised H_2O.

2.2.3.b) Geneclean method.

An agarose gel was run in 1 x T.A.E. and visualised as in sub-Section 2.2.2.. A slice of gel containing the DNA fragment of interest was then cut out and weighed. Thereafter the Geneclean protocol (336)

was followed in order to isolate the DNA. Geneclean was obtained from BIO 101, La Jolla, California.

2.2.4. Isolation of DNA from neutral polyacrylamide gels.

Bands from 4-6% neutral polyacrylamide gel were excised and transferred to a heat sealed blue tip (1ml) plugged with siliconised glass wool. The gel slice was crushed with a hypodermic needle and incubated overnight at 37°C in 200µl of gel elution buffer (1% SDS and 0.3M NaAc). The DNA was eluted by piercing the bottom of the sealed tip and was collected by gravity. A further 200µl of gel elution buffer was added and collected. The solution was then spun at 10,000 r.p.m. (8,160xg) for 2 minutes at room temperature in an eppendorf centrifuge 5415 to pellet any polyacrylamide debris. The supernatant was placed in a fresh test tube and two volumes of ethanol were added. The sample was incubated at -80°C (dry ice and ethanol) for 15 minutes and then spun at 10,000 r.p.m. (8,160xg) for 15 minutes at 4°C. The DNA pellet was washed with 500µl of 70% ethanol and freeze dried for 15 minutes. An appropriate volume of de-ionised H_2O was used to resuspend the DNA pellet.

2.2.5. Preparation of double stranded oligonucleotides.

Single stranded DNA oligonucleotides were made on an Applied Biosystems 381A DNA synthesiser. The oligonucleotides were removed from the synthesis column by elution with 3 x 1ml of ammonia. This solution was incubated at 55°C overnight to deprotect the oligonucleotides. To further purify the oligonucleotide an Applied

Biosystems oligonucleotide purification cartridge (OPC) was used (337). To anneal complementary single stranded oligonucleotides both were incubated at a concentration of 0.05M in 1 x T.E. This solution was then heated to 90°C and allowed to cool slowly to less than 30°C. This results in the formation of double-stranded oligonucleotides at a concentration of 0.1M. To check that the annealing had worked the double-stranded oligonucleotides (along with single-stranded oligonucleotides for comparison) were run on a neutral 8% polyacrylamide gel.

2.2.6. Denaturing polyacrylamide gel electrophoresis.

Denaturing polyacrylamide gel electrophoresis was carried out using a vertical acrylamide gel (5% acrylamide, 0.2% bis-acrylamide, 1 x T.B,E., 8M urea, 0.08% ammonium persulphate and 0.05% N,N,N,N-tetramethyethylene diamine (TEMED)). The gel was set between glass plates (20cm x 45cm) separated by 0.5mm spacers and joined together using Sylglas tape. The running conditions varied between techniques.

2.2.7. Large scale preparation of plasmid DNA.

This technique was based on that described by Birnboim and Doly (338) although several modifications were adopted. A 10ml culture of L-Broth was set up overnight following inoculation from a glycerol stock. This culture was used to inoculate 500ml of L-Broth (1ml of the overnight culture was used) which was incubated overnight at 37°C in an orbital shaking incubator. The following day the bacterial

cells were pelleted by centrifuging at 7,000 r.p.m. for 5 minutes at 4°C in a Sorvall RC-5B Superspeed centrifuge using a GS-3 rotor. The pellet was well drained and then resuspended in 18ml of lysis buffer (25mM Tris HCl pH8.0, 10mM EDTA pH8.0, 50mM glucose and 5mg/ml lysozyme) and incubated on ice for 30 minutes. Following this incubation 32ml of alkali buffer (0.2M NaOH, 1% SDS) was added to the mixture which was then incubated on ice for a further 45 minutes. This solution was then mixed with 16ml of 5M potassium acetate (60ml 5M potassium acetate 11.5ml glacial acetic acid and 28.5ml H_2O per 100ml) and incubated on ice for 15 minutes. This mixture was then centrifuged at 8,000 r.p.m. for 5 minutes at 4°C in a Sorvall RC-5B Superspeed centrifuge using a GS-3 rotor. The supernatant was poured through gauze to remove any lumps and mixed with 0.6 volumes of isopropanol. The mixture was incubated at room temperature for 15 minutes and then centrifuged at 8,000 r.p.m. for 10 minutes at room temperature in a Sorvall RC-5B Superspeed centrifuge in a GSA rotor. The pellet was then drained and taken up in 5ml of 50mM Tris HCl pH8.0, 10mM EDTA pH8.0 before being mixed with 5.5g of CsCl. The CsCl was dissolved in the plasmid solution and 0.5ml of ethidium bromide (10mg/ml) was added. The refractive index of this solution was adjusted to 1.3915 $\pm$ 0.001 (H_2O = 1.3303) and the tubes containing the plasmid mix balanced using paraffin oil. The solution was then centrifuged at 40,000 r.p.m. for at least 36 hours at 15°C in a Sorvall Ultracentrifuge using a T-1270 rotor. Following this centrifugation the plasmid DNA forms a distinct band which is clearly visible in U.V. light. This band was removed from the gradient using a 50µl Supracaps disposable micro pipette attached to a 2ml disposable syringe. The plasmid solution was placed in a bijoux

bottle and extracted with CsCl-saturated isopropanol to remove the ethidium bromide. This extraction was carried out 3 to 4 times in order to remove completely the ethidium bromide. The plasmid solution was then dialysed against T.E. (10mM Tris HCl pH7.5 on 1mM EDTA pH8.0) at 4°C for at least 6 hours (using a Sartorius colloidion bag). The plasmid DNA was precipitated by mixing with 0.3M sodium acetate and 2.5 volumes of 100% ethanol, and incubating at -20°C overnight. After this incubation the plasmid DNA was pelleted by centrifuging at 8,000 r.p.m. for 15 minutes at 4°C in a Sorvall RC-2B Superspeed centrifuge using an HB-4 rotor. The pellet was drained and washed with 70% ethanol and centrifuged at 8,000 r.p.m. for 5 minutes at 4°C in a Sorvall RC-2B Superspeed centrifuge using an HB-4 rotor. The plasmid DNA pellet was drained and air dried before being resuspended in 0.5 to 1ml of de-ionised H_2O. This plasmid DNA solution was placed in a 15ml test tube and its $O.D._{260}$ value determined. It could then be diluted to an appropriate concentration with de-ionised H_2O.

2.3. Northern Blot Analysis of Total Cellular RNA.

2.3.1. Electrophoresis and transfer of RNA to nitrocellulose.

Volumes of RNA solutions containing the required quantity of RNA were freeze dried. These were then resuspended in 5µl de-ionised H_2O and 25µl of sample buffer (0.38 ml formamide, 0.08ml 10 x MOPS, 0.12ml formaldehyde, 0.05ml de-ionised H_2O, 0.05ml glycerol and 0.1% bromophenol blue) was then added. To this mixture 1µl of ethidium bromide (1.0mg/ml) was added. These samples were then

electrophoresed through a 1% agarose gel which contained 2% formaldehyde and 1 x MOPS (0.2M morpholinopropanesulfonic acid (ph7.0), 50mM sodium acetate, 5mM EDTA, pH8.0). Following electrophoresis the gel was soaked in 20 x SSC for 20 minutes. The electrophoresed RNA was transferred directly on to nitrocellulose membrane (Sartorius 0.10μm) by blotting in 20 x SSC (3M NaCl, 0.3M sodium citrate pH7.0) as described previously (339). The blotting apparatus was set up as described by Southern (340). The nitrocellulose was soaked in 10 x SSC for 30 minutes prior to blotting overnight. At the completion of transfer the nitrocellulose was air dried and then baked for 2 hours at 80°C to immobilise the RNA.

2.3.2. Synthesis of labelled DNA probe and hybridisation procedures.

To probe the nitrocellulose filters 20-50ng of DNA was labelled with ^{32}P using the random priming technique (341). The fos probe used was the 1kbp PstI fragment from pFos I (8) which contain v-fos sequences. The β_2-microglobulin probe was the 0.55kbp PstI fragment from a cDNA which contains human β_2-microglobulin sequences (342). Both DNA fragments were isolated from 1% agarose gels (1 x T.A.E.) by the Geneclean method.

Prior to hybridisation the nitrocellulose filters were placed in a polythene bag and pre-hybridised with pre-hybridisation buffer (50% formamide, 500μg/ml sheared salmon sperm DNA, 10μl/ml poly A, 10μg/ml poly C, 0.1% SDS, 5 x Denhardt's solution, 5 x SSC, 50mM sodium phosphate buffer pH7.0 and 1% glycine) at 42°C overnight in a shaking

water bath. Following pre-hybridisation the filter was then hybridised with the ^{32}P-labelled DNA probes. This involved heating appropriate amounts of the labelled DNA probe (10^6d.p.m./ml hybridisation buffer) to 100^{o}C for 10 minutes and then adding this to hybridisation buffer (50% formamaide, 10% dextran sulphate, 100µg/ml sheared salmon sperm DNA, 10µg/ml poly A, 10µg/ml poly C, 0.1% SDS, 5 x SSC, 1 x Denhardt's solution and 20mM soldium phosphate buffer pH7.0). This mixture was then incubated with the pre-hybridised nitrocellulose filter in a polythene bag overnight at 42^{o}C in a shaking water bath.

Following the hybridisation the filter underwent a series of washes at 65^{o}C in a shaking water bath: 2 x 30 minutes with 2 x SSC, 0.1% SDS; 2x30 minutes with 0.5xSSC, 0.1% SDS; 2x30 minutes with 0.1xSSC, 0.1% SDS. At the completion of washing filters were placed in polythene bags and autoradiogrphed with Kodak "XAR" of "X-S" X-ray film using Cronex "lightning plus" intensifying screens in Harmer X-ray cassettes. After exposure at -70^{o}C the films were developed in a Kodak ME-1A automatic X-ray processor.

2.4. Molecular Cloning.

2.4.1. Preparation of recombinant DNA molecules.

Vector DNA for the synthesis of recombinant DNA molecules was prepared in the following manner. The plasmid vector was linearised with the appropriate restriction endonuclease (in certain cases the vector was digested with two restriction endonucleases), run on an

agarose gel and then purified. The linearised vector DNA was then treated with Klenow enzyme to form blunt-ends if necessary. In certain cases it was also necessary to phosphatase the linearised vector DNA and this was carried out with calf intestinal phosphatase.

Insert DNA was prepared by digesting the appropriate recombinant plasmid with restriction endonuclease(s). The digestion mixture was then run on an agarose gel and the relevant DNA fragment purified from the agarose gel.

All restriction endonuclease digestions were carried out according to the manufacturer's instructions. To phosphatase DNA the DNA was treated with 0.1 units of calf intestinal phosphatase (CIP-BCL) in phosphatase buffer (0.05M Tris HCl pH9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$ and 1mM spermidine) for 30 minutes at 37°C. Following this another 0.1 units of CIP was added to the reaction mix which was incubated at 37°C for a further 30 minutes. After phosphatase treatment the reaction mix was incubated at 65°C for 15 minutes to destroy the CIP. The reaction mix was then run on a 1% agarose gel and the vector DNA isolated from the agarose gel. Klenow treatment was carried out using the random priming kit (345) that was used to ^{32}P-label probe DNA (see sub-Section 2.3.2.), with the exception that the ^{32}P-labelled nucleotide used in this reaction was replaced by the corresponding unlabelled nucleotide. Once more the reaction mix was run on a 1% agarose gel and the vector DNA isolated from the agarose gel.

Following the preparation of the vector and insert DNA recombinant

DNA molecules were made by ligation reactions. In the ligation reactions approximately 20ng of the linearised vector plamid DNA was incubated with approximately 100ng of insert DNA with 1 unit of T4 DNA ligase (BCL) in ligation reaction mix (0.07M Tris HCl pH7.6, 5mM $MgCl_2$, 5mM DTT and 1mM ATP). This provided a high ratio of insert DNA ends to linearised vector plasmid DNA ends which favours the insertion of the insert DNA fragment into the linearised vector plasmid DNA. The ligation reaction was incubated overnight at room temperature.

2.4.2. Preparation of competent JM83 bacterial cells.

A glycerol stock of JM83 E.coli cells was used to inoculate 10ml of L-Broth. This was incubated at 37°C overnight in an orbital shaking incubator. The following morning 1ml was taken from this overnight culture and added to 100ml of L-Broth which was incubated at 37°C in an orbital shaking incubator. When the $O.D._{600}$ of the culture was approximately 0.6 it was split between two 50ml Falcon tubes. The bacterial cells were pelleted by centrifuging the culture at 2,000 r.p.m. (560xg) for 5 minutes at 4°C in an IEC Centra-8R centrifuge. The cells were resuspended in 10mM $MgSO_4$ (10mls in each 50ml Falcon tube) and incubated on ice for 20 to 30 minutes. Following this incubation the bacterial cells were pelleted once more by centrifuging the suspension at 2,000 r.p.m. (560xg) for 5 minutes at 4°C in an IEC Centra-8R centrifuge. The pelleted cells were then resuspended in 50mM $CaCl_2$ (10ml total for both 50ml Falcon tubes) and incubated on ice for at least 30 minutes. Following this procedure the cells were competent; i.e. they were capable of being transformed

by circular plasmid DNA molecules.

2.4.3. Transformation of competent JM83 bacterial cells.

One-half of the ligation reaction was placed in a fresh, sterile 1.5ml test-tube and 100µl of the competent JM83 cells was added to this test-tube. This mixture was incubated on ice for 20 minutes. Following this the test-tube was incubated at 42°C for 2 to 3 minutes and then allowed to stand at room temperature for 10 minutes. The reaction mix was then mixed with 800µl of L-Broth and incubated at 37°C for 45 to 60 minutes. Following this incubation an appropriate aliquot of each culture was spread onto L-Broth agar plates containing ampicillin (L-Broth, 1.5% agar, 200µg/ml ampicillin). The culture was allowed to dry onto the agar plate which was then incubated overnight at 37°C.

2.4.4. Screening of bacterial colonies.

Any bacterial colonies that form after the overnight incubation at 37°C contain circular plasmid DNA molecules that confer the ampicillin resistance to the bacterial cells. To determine which of the colonies contained plasmids with the desired DNA fragments inserted into them, small scale plasmid preparations were carried out to isolate sufficient plasmid DNA for restriction endonuclease analysis. Bacterial colonies were picked from the agar plates and used to inoculate 2ml of L-Broth containing 200µg/ml penicillin. This was then incubated overnight at 37°C. The following morning a 1.5ml test-tube was filled with this overnight culture and spun for

30 seconds in a microcentrifuge to pellet the bacterial cells. The pellet was then resuspended in 100µl of solution I (50mM glucose; 25mM Tris HCl pH8.0, 10mM EDTA pH8.0, 4mg/ml lysozyme) and incubated for 5 minutes at room temperature. After this 200µl of solution II (0.2M NaOH and 1% SDS) was added and the mixture incubated on ice for 5 minutes after gentle mixing. 150µl of solution III (3M pottasium acetate) was then added and the mixture vortexed and placed on ice for 5 minutes. The mixture was then centrifuged for 5 minutes at room temperature in a microcentrifuge. The resultant supernatant was placed in a fresh test-tube and an equal volume of a phenol-chloroform mix (1 : 1 v/v, saturated with T.E.) was added and the mixture vortexed. This solution was then centrifuged for 5 minutes at room temperature in an eppendorf centrifuge 5415. The upper aqueous phase was then placed in a fresh 1.5ml test-tube and the plasmid DNA was precipitated with two volumes of 100% ethanol. This mixture was incubated at room temperature for 2 minutes and then centrifuged for 2 minutes at room temperature in an eppendorf centrifuge 5415. The resultant pellet was resuspended in 30µl of sterile de-ionised H_2O. For each restriction endonuclease reaction 5µl of the plasmid preparation was used and in the final 30 minutes of the digestion reaction 1µl of 10mg/ml RNAse A was added to degrade the RNA present in the plasmid preparation.

2.4.5. Sequencing of plasmid DNA.

All plasmid DNA sequenced was prepared using the CsCl method described in 2.2.7. The DNA (3 to 5 µg) was denatured by treating with 0.2M NaOH for 5 minutes (a total volume of 20µl). After this

incubation 8µl of 5M ammonium acetate was added along with 100µl of 100% ethanol. This mixture was incubated at -70°C for 10 minutes and then centrifuged for 15 minutes at 4°C in an eppendorf centrifuge 5415. The pellet was washed with 70% ethanol and freeze dried for 15 minutes. The dried pellet was resuspended in 9µl of 1 x Sequenase buffer (from the Sequenase kit (343)) and 1µl of sequencing primer was added to this solution (approximately 5 to 10ng of DNA). This mixture was heated at 65°C and then allowed to cool very slowly to below 30°C. This solution was then ready for sequencing and the protocol and reagents used for this are described in the Sequenase kit (343). The samples were electrophoresed through a 5% denaturing polyacrylamide gel which was then dried. The dried gel was autoradiographed with Kodak X-AR of X-S X-ray film using Cronex "lightning plus" intensifying screens in Harmer X-ray cassettes. After exposure at -70°C the films were developed in a Kodak ME-1A automatic X-ray processor.

2.5. Transfection of Cultured Animal Cells.

2.5.1. Cell lines and culture conditions.

All of the cell lines used throughout this project were monolayer cell lines: the mouse fibroblast cell line CT3 (a derivative of NIH 3T3 cells) (344), the human epithelial cell line HeLaS3 (345), the mouse fibroblast cell line LATK$^-$ (346) and the mouse embryonal carcinoma cell line F9 (77). These were all grown in Special Liquid Medium (SLM-Gibco), 10% foetal calf serum, 2mM glutamine and 37.5µg/ml penicillin apart from F9 cells. F9 cells were grown in

Dulbecco's Modification of Eagle's Medium (DMEM; NBL, Cramington), 10% foetal calf serum, 2mM glutamine and 37.5µg/ml penicillin. One additional step was required when culturing F9 cells and this involved gelatinising the tissue culture flasks. This was carried out by covering the plastic surface of the culture flasks with a 0.1% gelatin solution (in de-ionised H_2O) and leaving them for 5 to 10 minutes. Following this the gelatin solution was removed and the flasks allowed to air dry (under sterile conditions).

To passage each cell line the medium was removed from the monolayers which were then washed with PBS. A volume of trypsin solution (2.5% trypsin, 0.2M NaCl, 0.01M trisodium citrate, 0.01M tricine (N-tris-hydroxymethyl) methylglycine and 0.005% phenol red) was then added to the washed monolayer and "waved" over the monolayer for 30 seconds. The trypsin solution was removed and the monolayers left at room temperature for 5 to 10 minutes. The flask was tapped gently which dislodged the monolayer of cells from the plastic and these cells were taken up in 10ml of medium. An aliquot of the cells was counted in a Coulter Counter to estimate the concentration of the cells in the 10ml of medium. Cells were aliquoted into fresh tissue culture flasks along with fresh medium to the required cell density. The flask was incubated at $37^{o}C$ in 5% CO_2.

2.5.2. Calcium phosphate co-precipitation of plasmid DNA.

Plasmid DNA was introduced into cultured cells by the calcium phosphate co-precipitation technique of Graham and van der Eb (347) as modified by Wigler et al (348). The precipitates were made under

sterile conditions in Bijoux bottles. For each precipitate, two bijouxs were used. To one bijoux 2ml of 125mM $CaCl_2$ were added followed by the plasmid DNA. To the second bijoux 2ml of 2 x HBS (hepes buffered saline: 2 x HBS; 140mM NaCl, 25mM HEPES (N-2-hydroxyethylpiper-ozined-N-2-ethanesulphonic acid), 0.75mM disodium hydrogen phosphate, pH7.1) were added. The DNA-$CaCl_2$ solution was added dropwise to the 2 x HBS solution (which was agitated at the same time). Following the addition the mixture was returned to the first bijoux and allowed to stand at room temperature for 30 minutes. During this period the calcium phosphate precipitate forms which was divided between at least two flasks of cells.

2.5.3. Transient transfection procedure.

The cells to be transfected were set up at the required density the day before addition of the transfection cocktail. The following day (usually 20 to 24 hours later) the calcium phosphate precipitate was added to the cells. The following morning (12 to 16 hours later) the medium was removed from the cells which were then washed with PBS and refed with the desired medium.

The following afternoon (30 to 36 hours later) the cells were harvested. The medium was removed and the monolayer washed with PBS and then incubated at room temperature or 37°C with a covering (approximately 1ml) of TEN buffer (0.04M Tris HCl pH7.4, 1mM EDTA and 0.15M NaCl). This detaches the cells from the flask which were taken up in the TEN buffer and placed in a 1.5ml test-tube. The cells were pelleted by spinning in an eppendorf centrifuge 5415 for 2 minutes.

2.5.4. Stable transfection procedure.

The cells to be transfected were set up at the required density the day before the addition of the transfection cocktail. The following day (usually 20 to 24 hours later) the calcium phosphate precipitate was added to the cells. The calcium phosphate precipitate used in the stable transfection experiments contained high molecular weight eukaryotic cellular DNA (as a carrier) and plasmid DNA containing the neomycin resistance gene which confers G418 resistance on the transfected cells. The following morning the medium was removed and the cells washed with PBS and refed with medium containing 10% foetal calf serum. Two days later the transfected cells were removed from the plastic by treating them with trypsin (see sub-Section 2.5.1.). The cells were then counted and replated into $10cm^2$ tissue culture petri dishes and fed with medium containing 10mg/ml G418. The cells were incubated in this medium for 10 to 14 days after which time colonies of resistant cells become visible. The medium was removed from the petri dishes and individual colonies were taken up in a small quantity of medium and placed into a flask containing medium with 1mg/ml G418. The flask was incubated until the cells were confluent whereupon they were trypsinised and passaged as individual cell lines. These cell lines were grown in the presence of 1mg/ml G418.

2.6. Transcription Assays of Transfected Plasmid DNA.

2.6.1. Preparation of cell protein extracts.

Protein extracts were made from transfected cells in the following manner. The cell pellets obtained following harvest of transfected cells (see sub-Section 2.5.3.) were resuspended in 200µl of 0.2M Tris HCl pH7.8. The cells were then placed on dry ice for 5 minutes and then incubated at 37°C for 5 minutes. This cycle was repeated another two times and following this freeze thawing the ruptured cell suspension was spun for 10 minutes in a microcentrifuge. The resulting pellet was removed from the test-tube leaving behind the protein containing supernatant. When required the protein concentration of these extracts was determined using a Biorad Protein Assay kit (349) by following the manufacturer's instruction.

2.6.2. β-galactosidase assay.

This assay was based on the assay described by Miller (350). An aliquot of the protein extract was placed in a 1.5ml test-tube containing 500µl of solution I (60mM disodium hydrogen orthophosphate, 40mM sodium dihydrogen orthophosphate, 10mM KCl, 1mM $MgCl_2$ and 50mM β_2-mercaptoethanol). To this mixture 100µl of solution II (2mg/ml ONPG (o-nitrophenyl galactoside), 60mM disodium hydrogen orthophosphate, 40mM sodium dihydrogen orthophosphate,) was added and the test-tube incubated at 37°C for 15 to 60 minutes. The samples were removed from the 37°C incubation and 250µl of 1M Na_2CO_3 was added to terminate the reaction. The final solution has a yellow colour and the optical density of this solution at 595mm was

determined. Thus, β-galactosidase activity was expressed in $O.D._{595}$ units.

2.6.3. Chloramphenicol acetyl transferase assay.

This assay was based on the assay described by Gorman et al (188). An aliquot of the protein extract (2.6.1) was placed in a 1.5ml test-tube and 1µl of ^{14}C-labelled chloramphenicol was added. Acetyl coenzyme A was then added to a concentration of 5mM which starts the reaction. The reaction was incubated at 37°C for 45 to 60 minutes and then extracted with 500µl of ethyl acetate. This mixture was centrifuged for 30 seconds in an eppendorf centrifuge 5415 and the ethyl acetate (which forms the upper phase) placed in a fresh 1.5ml test-tube. The ethyl acetate was then evaporated using either a speedy-vac centrifuge or a freeze drier. The ^{14}C-chloramphenicol and its acetylated derivatives were taken up in 20µl of ethyl acetate and spotted on to a 20cm x 20cm thin layer chromatography 0.25mm silica gel plate. The sample was allowed to dry on to the plate before it was placed into a solvent tank containing 95% chloroform and 5% methanol. The plate was chromatographed for 30 to 45 minutes and then taken out of the solvent tank and air dried for 2 to 3 minutes. The plates were autoradiographed with Kodak "XAR" X-ray film using Cronex "lightning plus" intensifying screens in Harmer X-ray cassettes. After exposure at -70°C the films were developed in a Kodak ME-1A automatic X-ray processor. The radioactive spots indicating the position of the labelled and unlabelled ^{14}C-chloramphenicol were cut out from the TLC plate and placed in a

liquid scintillation vial along with 5ml of Ecoscint liquid scintillation fluid. These vials were counted in an LKB 1217 Rackbeta liquid scintillation counter and the percentage conversion of the ^{14}C-chloramphenicol to its acetylated derivatives calculated.

2.6.4. S1 analysis

A double-stranded DNA probe was used in the S1 analyses (351) carried out in this project. This probe was similar to the one used by Fisch et al (252). The plasmid containing the DNA fragment of interest was linearised by restriction endonuclease digestion and treated with calf intestinal phosphatase (50µg of plasmid DNA was used for each preparation. The linearised de-phosphorylated plasmid DNA was run on a 1% agarose gel and isolated using the geneclean protocol (sub-Section 2.2.3.b)). This plasmid was then end-labelled by treating with polynucleotide kinase. The DNA was incubated with 1 x kinase buffer (50mM Tris HCl pH7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine and 0.1mM EDTA), 50µCi of ^{32}P-labelled ATP and ten units of polynucleotide kinase (BCL) at 37^{o}C for 30 minutes. The DNA was extracted from this reaction mix using the Geneclean protocol and digested with another restriction endonuclease to generate the labelled DNA fragment. This mixture was electrophoresed through a neutral polyacrylamide gel and the gel exposed to a Kodak "X-AR" film for 1 to 3 minutes which was then developed in a Kodak ME-1A automatic X-ray processor. The labelled DNA fragment was extracted from the polyacrylamide gel and precipitated and resuspended in H_2O to the desired concentration.

RNA used in the S1 analysis experiments was prepared using the RNAzol technique. The RNA and double-stranded labelled DNA probe were mixed together and freeze dried. The freeze dried pellet was then dissolved in 10µl of formamide hybridisation buffer (80% formamide, 0.4M NaCl, 0.04M PIPES (piperazine-N_1N_1-bis [2-ethanesulfonic acid]; 1,4-piperazine-diethanesulfonic acid) pH6.4 and 1mM EDTA pH8.0) which was overlayed with 20µl of light liquid paraffin. This tube was incubated at 90 to 100°C for 10 minutes and then transferred to a water bath set at 44°C where it was incubated overnight. The following day 235µl of ice-cold S1 buffer (0.25M NaCl, 0.03M sodium acetate pH4.6, 1mM $ZnSO_4$ and 200µg/ml sheared salmon sperm DNA) was added to the tube which was then incubated on ice after mixing the solutions together. To this tube, 100 units of S1 (BCL) were added and the tube incubated at 37°C for 30 minutes. Following this incubation the reaction mix was mixed with an equal volume of phenol-chloroform (1:1 ratio, saturated with 1 x T.E.) and then spun for 5 minutes in an eppendorf 5415 centrifuge. The aqueous phase was placed in a fresh 1.5ml test-tube and the nucleotides were precipitated with 2 volumes of 100% ethanol, 0.2M sodium acetate in the presence of 10µg yeast RNA carrier. This tube was incubated at -80°C for 10 minutes and the nucleic acids pelleted by spinning for 10 minutes at 4°C in an eppendorf centrifuge. The pellet was given a wash with 70% ethanol and freeze dried for 15 minutes and redissolved in a 4µl of 1 x T.E.. This was mixed with 4µl of formamide dye mix (95% (v/v) formamide, 0.1% (w/v) Xylene cyamol FF, 0.1% (w/v) bromophenol blue) and the mixture heated at 90°C for 3 minutes. The samples were then electrophoresed through a denaturing polyacrylamide gel. Following electrophoresis the polyacrylamide gel was dried and

autoradiographed with Kodak X-AR or X-S X-ray film using Cronex "lightning plus" intensifying screens in Harmex X-ray cassettes. After exposure at -70°C the films were developed in a Kodak Me-1A automatic X-ray processor.

2.6.5. Primer extension analysis.

The oligonucleotide used in the primer extension assays (352) was end-labelled with gamma-^{32}P-ATP using polynucleotide kinase in a similar fashion to that described in sub-Section 2.6.4. The labelled oligonucleotide was ethanol precipitated in the presence of yeast carrier tRNA and electrophoresed through a denaturing polyacrylamide gel. Following electrophoresis the gel was exposed to a Kodak X-AR film for 1 to 3 minutes which was then developed in a Kodak Me-1A automatic X-ray processor. The polyacrylamide gel fragment containing the labelled oligonucleotide was incubated in 1xT.E. at 37°C overnight which elutes the labelled oligonucleotide from the gel. The oligonucleotide containing T.E. was extracted with phenol-chloroform and the oligonucleotide ethanol precipiated in the presence of yeast tRNA.

The required amount of RNA was freeze dried and the pellet was resuspended in 8µl of 10mM Tris HCl pH7.9, 1mM EDTA PH8.0 containing the end-labelled oligonucleotide. To this solution 2µl of 1M Tris HCl pH7.9, 400mM EDTA pH8.0, 1.25m KCl was added. This was incubated at 65°C for 60 minutes to bind the primer to the RNA. This was brought to room temperature and diluted with 25µl of 1 x reverse transcriptase buffer (20mM Tris HCl pH8.7, 10mM $MgCl_2$, 5mM DTT,

10µg/ml actinomycin D and 0.5mM dNTPs) and 10 units of reverse transcriptase (BCL) were added. This mxiture was incubated at 42°C for 60 minutes. Following this incubation the reaction mix was precipitated with 300µl of 100% ethanol and incubated at -20°C overnight. The following day the nucleic acids were pelleted by centrifugation for 10 minutes at 4°C in an eppendorf 5415 centrifuge and the pellet washed with 70% ethanol. The pellet was freeze dried and then dissolved in 4µl of formamide dye mix (sub-Section 2.6.4.). This mixture was electrophoresed through a denaturing polyacrylamide gel which was then dried. The dried gel was autoradiographed as previously described (sub-Section 2.6.4.).

2.7. Analysis of DNA-Protein Interactions.

2.7.1. Preparation of nuclear protein extracts from cultured animal cells.

This protocol was carried out with slight modification (353) to that described by Emerson and Felsenfeld (354). Prior to harvesting, cells for nuclear protein extract preparation were grown in 800cm^2 plastic roller bottles (4 to 8 bottles were used for each preparation). The cells were removed from the roller bottles by treatment with glass beads. The medium was poured off and several hundred glass beads (2-3mm in diameter) were added to the roller bottle which retained a small amount of medium and the bottle rotated gently. The rubbing of the glass beads detaches the cells from the plastic, and they were collected in a centrifugation bottle along with the remaining medium. The cells were then pelleted by

centrifuging at 2,000 r.p.m. (560xg) for 15 minutes at 4°C in an IEC Centra-8R centrifuge. The cells were then resuspended in 200ml of PBS + protease inhibitors (0.5mM phenyl methylsulfonyl flouride, 0.5mM benzamidine, 1µg/ml pepstatin A, 1µg/ml aprotinin and 1µg/ml leupeptin) and then pelleted as before. The cells were lysed by resuspending them in 200ml of TMS (0.13M sucrose, 2.5mM $MgCl_2$ and 5mM Tris HCl pH7.5) + 0.25% triton + protease inhibitors + 0.1mM EGTA pH7.9. The nuclei were then pelleted by centrifuging as before and then washed twice with 200ml of TMS + protease inhibitors + 0.1mM EGTA pH7.9. After pelleting the nuclei following the second wash they were resuspended in 100ml of TMS + protease inhibitors + 0.1mM EGTA pH7.9. Aliquots of the resuspended nuclei were taken and sonicated in the presence of 1.0M NaOH. The DNA concentration of this solution was estimated and following pelleting of the intact nuclei, they were resuspended in TMS + protease inhibitors + 0.1mM EGTA pH7.9 to an equivalent concentration of 5 to 10mg/ml DNA. To this suspension 4M NaCl was added dropwise to a concentration of 0.35M while the suspension was sitting on ice. This solution was then centrifuged in a Sorvall RC-5B Superspeed centrifuge using an S5-34 rotor at 17,000 r.p.m. for 20 minutes at 4°C. The supernatant was removed and centrifuged in an IEC/860 Ultracentrifuge using an SB-405 rotor at 35,000 r.p.m. for 60 minutes at 4°C. The supernatant was removed and mixed with ammonium sulphate (3.4g per 7.5ml) and incubated for 60 minutes on ice. This mixture was then centrifuged in a Sorvall RC-5B Superspeed centrifuge using an S5-34 rotor at 17,000 r.p.m. for 30 minutes at 4°C. The resulting pellet was then resuspended in E_{50} buffer (50mM ammonium sulphate, 20mM Hepes pH7.9, 5mM $MgCl_2$, 0.1mM EDTA pH8.0, 0.1% Brij 35 (Sigma), 0.1mM DTT,

protease inhibitors and 20% glycerol (v/v)) to a concentration of 4 to 5 ml to 100mg DNA (previously calculated). This solution was then dialysed overnight at 4°C against 1 litre of storage buffer (50mM NaCl 20mM Hepes pH7.9, 5mM $MgCl_2$, 0.1mM EDTA pH8.0, 1mM DTT, protease inhibitors and 20% glycerol (v/v)). The following morning the solution was centrifuged in an IEC/B60 Ultracentrifuge using an SB-405 rotor at 35,000 r.p.m. for 60 minutes at 4°C and the resulting supernatant aliquoted into 1.5ml test-tubes and stored at -70°C.

2.7.2. Gel mobility shift assay.

This assay was based on that carried out by Strauss and Varshavsky (355). Double-stranded oligonucleotides (200ng) containing putative DNA binding sequences for nuclear proteins were labelled with 20uCi of gamma-^{32}P-ATP using polynucleotide kinase (sub-Section 2.6.4.). Following incubation at 37°C for 30 minutes the labelled oligonucleotides were ethanol precipitated in the presence of yeast tRNA and then electrophoresed through an 8% neutral polyacrylamide gel. Following electrophoresis the gel was exposed to a Kodak X-AR film for 1 to 3 minutes which was then developed in a Kodak ME-1A automatic X-ray processor. The polyacrylamide gel fragment containing the labelled oligonucleotide was incubated in 1 x T.E. at 37°C overnight which elutes the labelled oligonucleotide from the gel. The following morning the acrylamide was removed from the T.E. which then contained the labelled oligonucleotide in solution.

In the gel mobility shift assay itself 1µl of the labelled oligonucleotide was incubated with 4-5µl of the nuclear protein

extract (2.7.1.) along with 3µg of poly (dI-dC) (Pharmacia) and 100ng of any competitor oligonucleotide to be used. The reaction was made up to 25µl with retard buffer (10mM Tris HCl pH7.5, 50mM NaCl, 5mM $MgCl_2$, 1mM DTT, 1mM EDTA pH8.0 and 5% glycerol (v/v)). The reaction mixture was incubated at room temperature for 15 to 20 minutes and then electrophoresed through a 4-5% neutral polyacrylamide gel. Following electrophoresis the gel was dried and autoradiographed with Kodak "X-AR" or "X-S" X-ray film using Crone "lightning plus" intensifying screens in Harmer X-ray cassettes. After exposure at -70°C the films were developed in a Kodak ME-1A automatic X-ray processor.

CHAPTER 3 : RESULTS

RESULTS PAGE

3.5. E1a trans-activation of the c-fos gene via upstream sequences.

3.5.1. Co-transfection of E1a 12s and13s plasmid constructs with c-fos-CAT constructs into CT3 cells

3.5.2. Trans-activation of control plasmids by the E1a 13s gene product.

3.5.3. Co-transfection of c-fos-CAT and E1a plasmids into CT3 cells and correction for the trans-activation of the internal control plasmid by E1a 13s.

3.1. Construction of c-fos-CAT Hybrid Plasmids.

To analyse the transcriptional activation of the human c-fos gene c-fos-CAT hybrid plasmids were made which contained the CAT gene with various c-fos upstream sequences inserted to drive the transcription of the CAT gene. These constructs were then used in transfection experiments to identify enhancer sequences and/or stimulus responsive sequences.

Figure 3 shows the CAT containing plasmid used for the construction of the c-fos-CAT hybrid plasmids, pB9, and also details sequences upstream from the c-fos promoter previously identified as having a role in the transcriptional control of this gene. pB9 contains a promoterless CAT gene which contains several unique restriction enzyme sites (shown in Figure 3) that were employed to make recombinant constructs.

3.1.1. Construction of pB9+fp1400, pB9+fp402 and pB9+fp116.

Figure 4 shows the construction of these recombinants in a flow diagram. The blunt-ended NaeI fragment from the c-fos gene (-1400 to +42 in Figure 1) was isolated following a NaeI digestion of pc-fos(human)-1 (11), electrophoresis through a 1% agarose gel and then phenol extraction of the fragment from the agarose. pB9 was digested with BamHI and the overhanging ends filled in by a Klenow enzyme reaction to give a linearised plasmid with blunt ends. This linearised pB9 DNA was then treated with calf intestinal phosphatase to prevent self-ligation in any ligation reactions. The c-fos NaeI

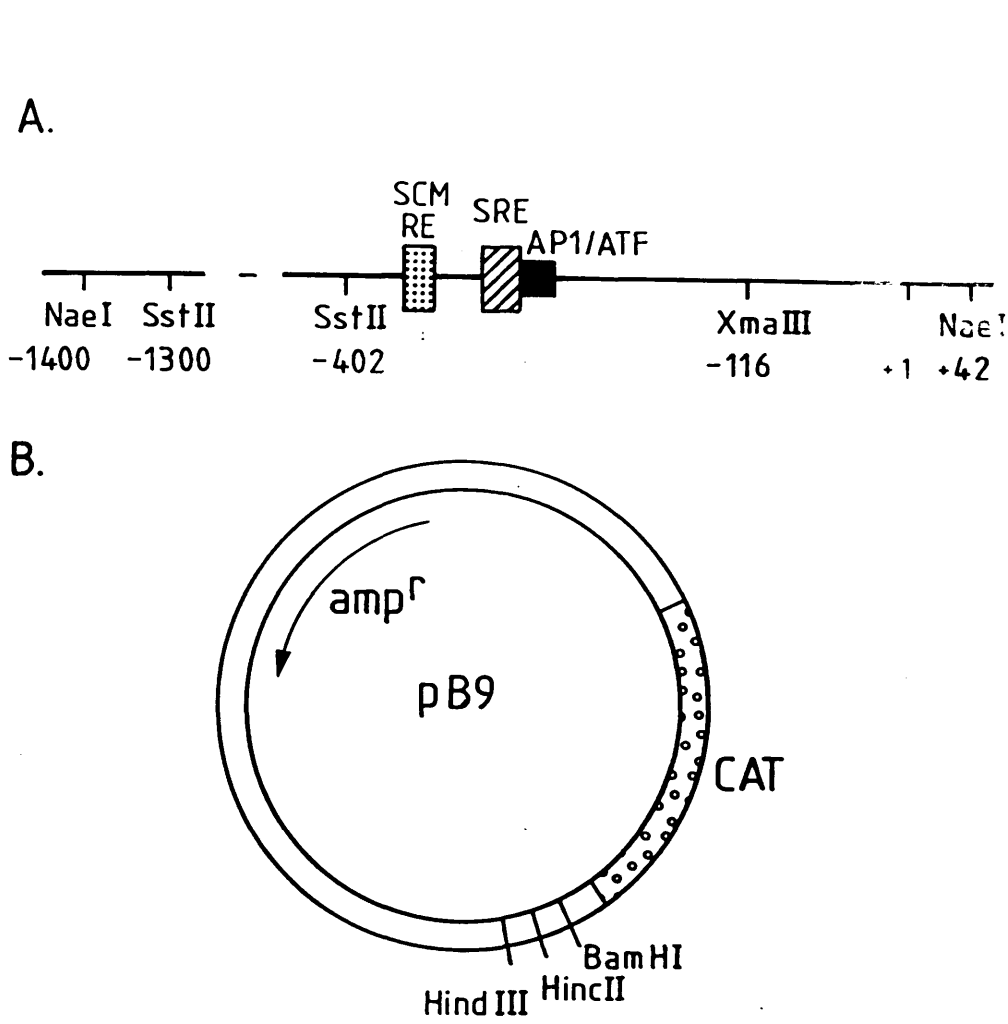


Figure 3. Sequences and recombinant used in the construction of c-fos-CAT hybrid plasmid constructs.

A. This figure details the upstream sequences of the c-fos gene which have been previously identified or postulated as having roles in the transcriptional control of this gene (1.6.). The restriction enzyme sites shown are the ones used in the cloning of these sequences upstream of the CAT gene present in pB9, details of which are given in the text. The numbers represent the position of the sequences, in bp, relative to the CAP site (+1). SCM RE : sis-conditioned medium response element. SRE : serum response element. AP-1/ATF : activator protein 1/adenovirus transcription factor binding site.

B. A representation of pB9 which contains a promoterless CAT gene. The restriction enzyme sites shown are those used in the synthesis of the various c-fos-CAT constructs described in the text. This plasmid was a gift from Dr. J. Lang and was derived by deletion of the promoter present in pLW2 (356). amp^r : ampicillin resistance gene.

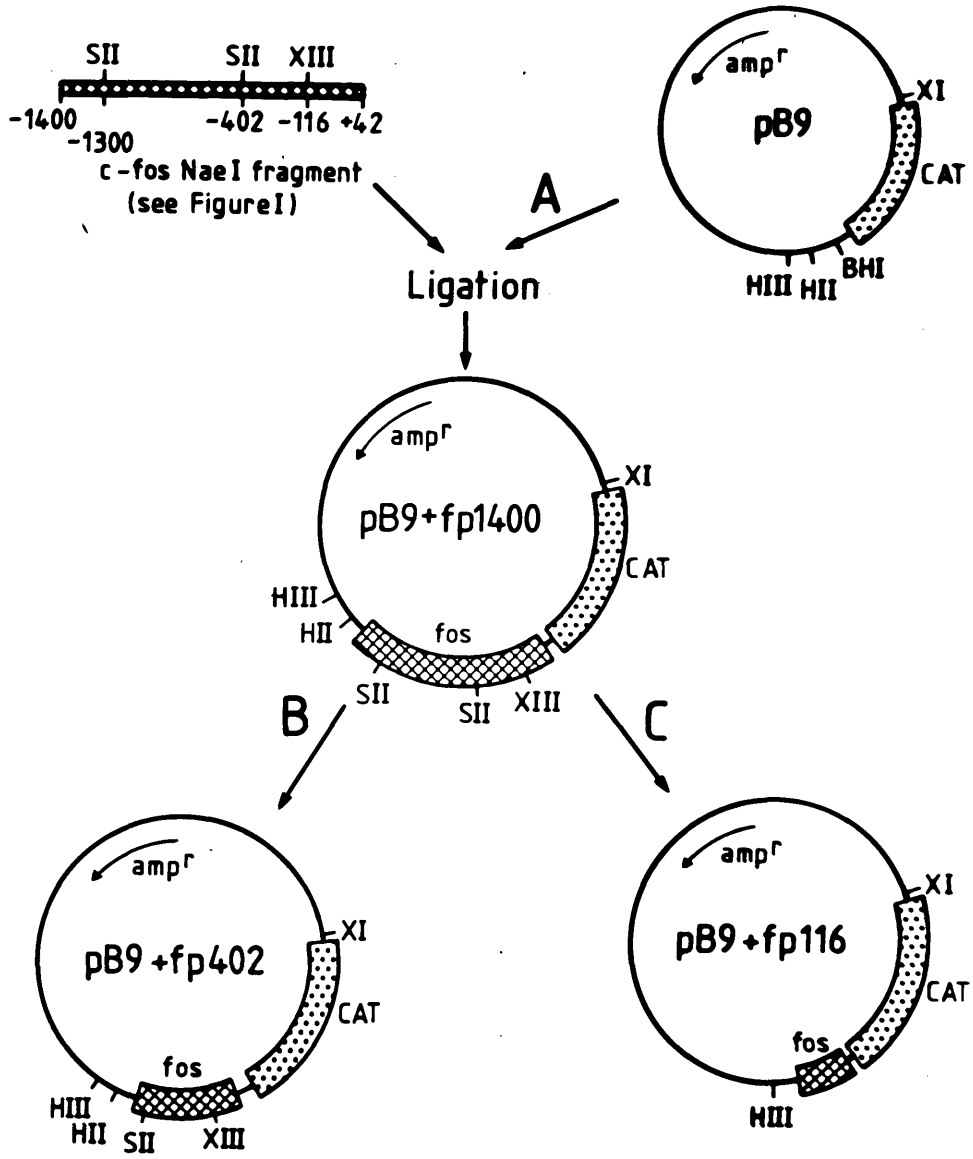
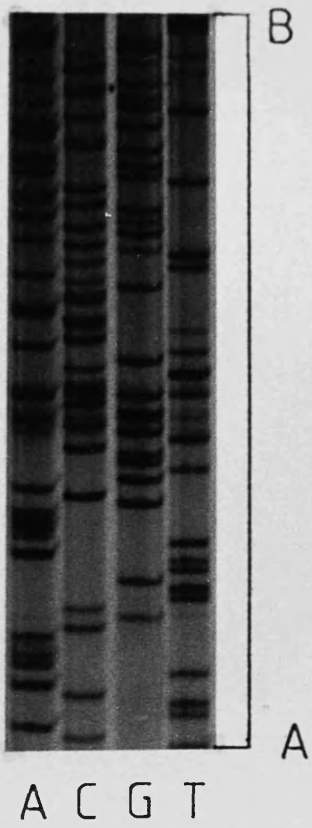


Figure 4. Construction of the c-fos-CAT recombinants pB9+fp1400, pB9+fp402 and pB9+fp116.

The construction of these recombinants is described in detail in the text. All of the reactions mentioned (restriction enzyme digestion, Klenow treatment, phosphatase treatment, ligation) are described in Section 2.4.1. amp^r : ampicillin resistance gene. A : BHI digestion, Klenow filled in, treated with CIP. B : Deletion of the SII to SII fragment. C : Digestion with XIII and HII, Klenow filled in, ligated together. SII : SstII. XIII : XmaIII. XI : XbaI. BHI : Bam HI. HII : HincII. HIII : Hind III. These constructs are not drawn to scale.

fragment was then inserted into this construct in a ligation reaction to give pB9+fp1400 (fos promoter and 1400bp of upstream sequence). The orientation of the inserted fragment was determined by restriction endonuclease analysis.

Two further c-fos-CAT recombinants were made by deleting fragments from pB9+fp1400. First, pB9+fp402, which retains all previously identified transcriptional control elements, was made by deleting the SstII fragment from pB9+fp1400 (-1300 to -402 in Figure 3). The DNA of pB9+fp1400 was digested with SstII and the fragments separated on an agarose gel. The residual CAT-containing plasmid DNA was isolated from the agarose gel by the phenol extraction method. The ends of this DNA were then self-ligated to form pB9+fp402. Second, pB9+fp116, in which all previously identified transcriptional control elements (apart from those involved in basal promoter activity) have been deleted, was made by deleting from the XmaIII site 116bp upstream from the c-fos CAP site to the HincII site 6bp 5' of the BamHI site in pB9. HincII generates blunt ends upon digestion of DNA, but XmaIII creates overhanging ends which were filled in by a Klenow enzyme reaction. This gives a blunt-ended linear recombinant and the ends were ligated together to form pB9+fp116. This construct was sequenced using Sequenase enzyme and this sequence is shown in Figure 5. The c-fos and CAT sequence shown in this Figure agrees 100% with previously published sequences (12, 361).



A TCATTCATAAAACGCTTATAAAAGCAGTGGCTGCGGCGCCTCGTACTCCAACCGCATCT

FOS TCATTCATAAAACGCTTATAAAAGCAGTGGCTGCGGCGCCTCGTACTCCAACCGCATCT

GCAGCGAGCAACTGAGAAGCCAAGACTGAGCC GATCCG TCGAGAATTTT B

FOS GCAGCGAGCAACTGAGAAGCCAAGACTGAGCC

CAT TCGAGAATTTT

Figure 5. Sequence of pB9+fp116.

4µg plasmid DNA was denatured as described in Section 2.4.5. The denatured plasmid was then annealed with the reverse Universal primer and sequenced as instructed in the Sequenase kit (343). Following the termination of the sequencing reactions the reactions were then electrophoresed through a 5% denaturing polyacrylamide gel which was then dried and autoradiographed. The sequence from A to B is shown and compared with previously published c-fos and CAT sequences.

3.1.2. Construction of c-fos-CAT recombinants with inserted oligonucleotides.

A common method used to analyse the role of short DNA elements thought, or known to be involved in transcriptional control is to insert oligonucleotides containing the sequence of interest upstream from a promoter and a reporter gene and then analyse in transfection studies the influence the inserted oligonucleotide has upon the transcription of the reporter gene from the promoter. This method was used to study the effects of sequences upstream from the c-fos promoter. A schematic diagram of how these oligonucleotides were inserted upstream from the c-fos promoter is given in Figure 6. pB9+fp402 was digested with XmaIII and then HindIII and the digestion mixture ran on an agarose gel. The linear recombinant with the HindIII to XmaIII fragment deleted was isolated by the Geneclean method (2.2.3.b)). Pairs of complementary oligonucleotides representing the sequences to be investigated were synthesised with HindIII and XmaIII compatible ends. These were then ligated into the pB9+fp402 HindIII to XmaIII deleted linear recombinant to give a construct containing the c-fos promoter upstream of the CAT gene with the oligonucleotide inserted 116bp upstream from the c-fos CAP site. This mehtod of construction had two advantages over previously published experiments where oligonucleotides inserted had similar restriction enzyme compatible ends.

1) Only a single copy of the oligonucleotides will be inserted upstream from the c-fos promoter.

2) The oligonucleotide inserted will be in a known orientation.

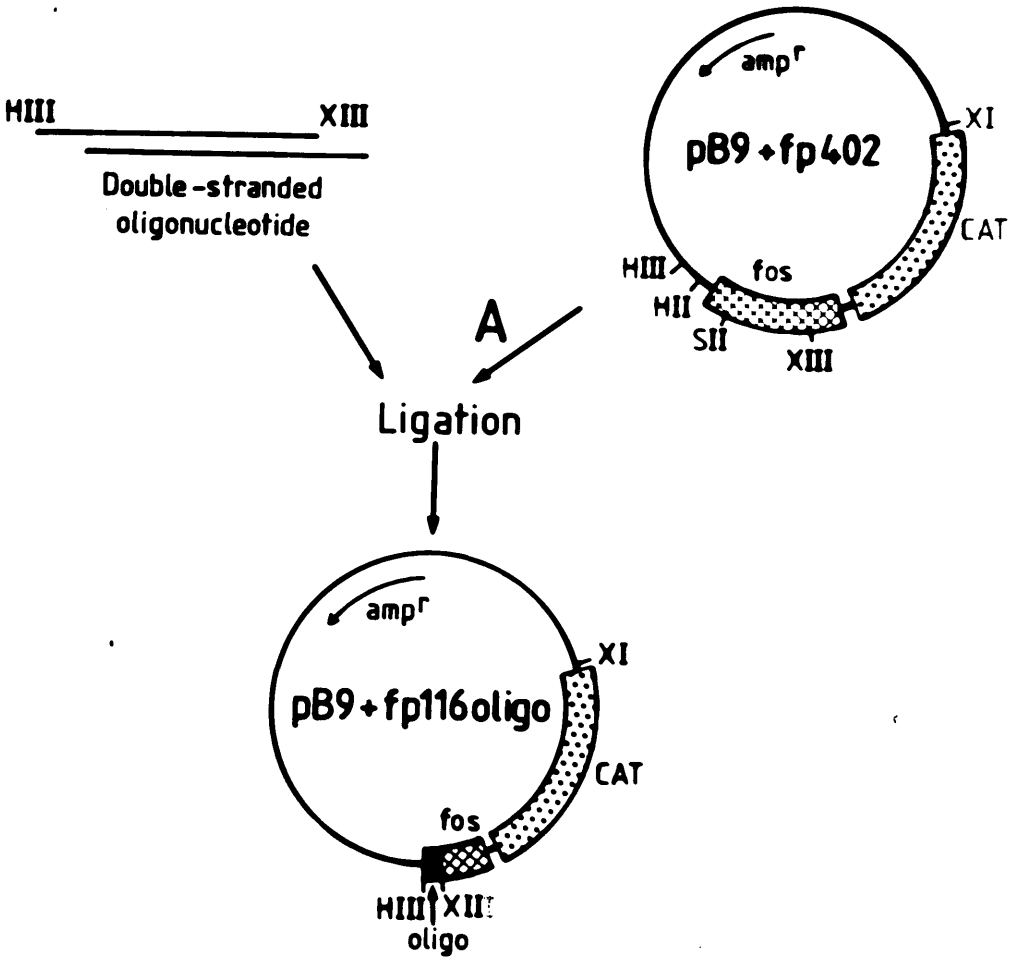


Figure 6. A schematic diagram representing the insertion of oligonucleotides upstream from the c-fos promoter in c-fos-CAT hybrid recombinants.

This diagram represents the insertion of oligonucleotides containing transcriptional control sequences upstream of the c-fos promoter in c-fos-CAT recombinants. The sequences of the oligonucleotides and names of individual constructs are given in Figure 7. All of the reactions mentioned (restriction enzyme digestion, ligation) are described in sub-Section 2.4.1. amp^r : ampicillin resistance gene. A : HIII to XIII deletion. HIII : HindIII. XIII : XmaIII. XI : XbaI. SII : SstII. HII : HincII. These constructs are not drawn to scale.

Several constructs were synthesised using this protocol with the oligonucleotides inserted containing different sequences. These sequences, representation of sequencing gels of the constructs, and names of the constructs are all given in Figure 7. Sequencing was carried out using the Sequenase enzyme.

3.1.3. Analysis of the transcriptional start site of the parental c-fos-CAT construct.

On synthesis of recombinant constructs for transcriptional analysis it is important to determine that the construct is initiating transcription from the correct position. For this purpose S1 and primer extension analyses were carried out with RNA prepared from $LATK^-$ cells stably transfected with pCN+fp1400. This construct, pCN+fp1400, is based on pB9+fp1400 except it also contains the neomycin resistance gene.

The synthesis of pCN+fp1400 is described diagrammatically in Figure 8 pB9+fp1400 was digested with HindIII and XbaI and the resulting fragment containing the c-fos and CAT sequences was isolated from an agarose gel using the phenol method. This sequence was then ligated into HindIII and XbaI double digested pUN-1 which contains the neo-resistance gene inserted into a BamHI site, and the resultant recombinant was named pCN+fp1400. pUN-1 was synthesised by inserting the BamHI fragment containing the neo-resistance gene from p61-C-24 (a kind gift from Dr. J. Lang) into the BamHI site of pUC18. The neo-resistance gene present in p61-C-24 is under the control of a thymidine kinase (TK) promoter, and has a TK polyadenylation site

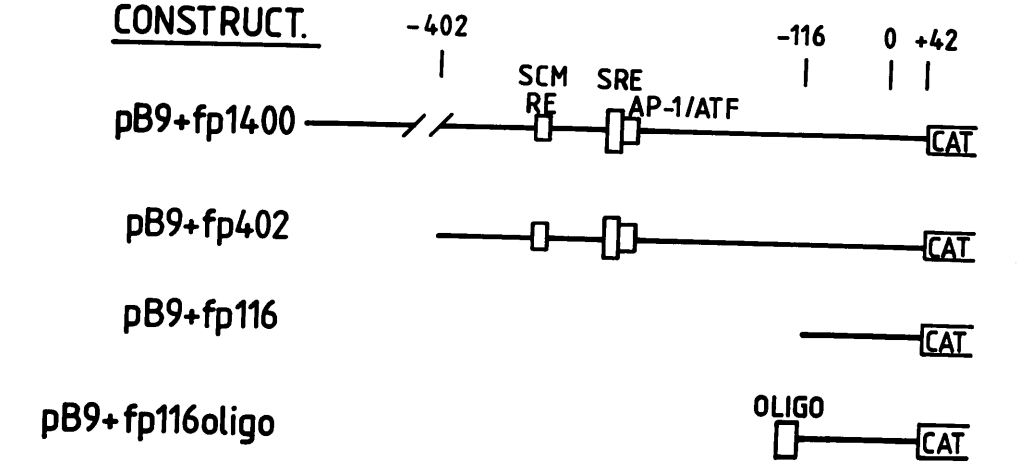
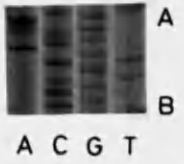
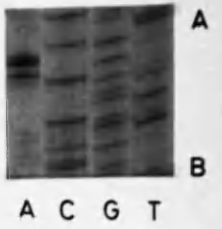
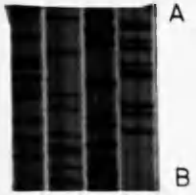
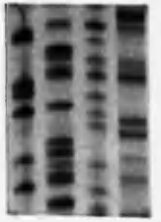
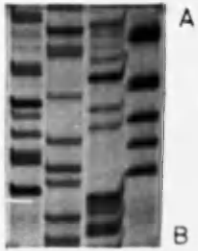
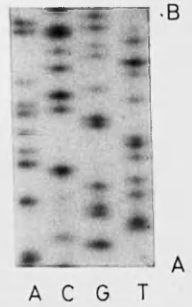
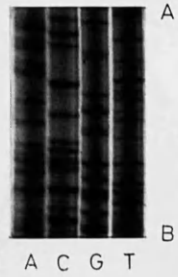


Figure 7. The sequence of oligonucleotide containing c-fos-CAT constructs.

A:A schematic diagram indicating where the oliginucleotides have been inserted with respect to the c-fos promoter.
B:4µg of the respective c-fos-CAT construct was denatured as described in sub-Section 2.4.5. The denatured plasmid was then annealed with a 35bp CAT oligonucleotide which anneals 24bp 3′ to the start of the CAT gene sequence and sequenced as instructed in the Sequenase kit (343). Following the termination of the sequencing reactions the reactions were heated at 65°C for 2 minutes in the presence of formamide dye mix. The reactions were then electrophoresed through a 5% denaturing polyacrylamide gel which was then dried and autoradiographed. The sequence of the inserted oligonucleotides are shown along with representative sequencing gels. A to B denotes the sequence from the HindIII to the XmaIII site resepctively.

NAME OF CONSTRUCT	pB9+fp116ATF	pB9+fp116SRE-ATF	pB9+fp116MUT-ATF
OLIGONUCLEOTIDE SEQUENCE	AGCTTCTGCGTCAGCCAGC AGACGCAGTCGGTCGCCGG	AGCTTGGATGTCCATATTAGGACATCTGCGTCAGCCAGC ACCTACAGGTATAATCCTGTAGACGCAGTCGGTCGCCGG	AGCTTCTGCACCAGCCAGC AGACGTGGTCGGTCGCCGG
REPRESENTATIVE SEQUENCING GEL			

NAME OF CONSTRUCT	pB9+fp116SRE-AP-1	pB9+fp116E3-ATF	pB9+fp116E3AP-1
OLIGONUCLEOTIDE SEQUENCE	AGCTTGGATGTCCATATTAGGACATCTGAGTCAGCCAGC ACCTACAGGTATAATCCTGTAGACTCAGTCGGTCGCCGG	AGCTTCGGGCGGCTTTCGTCACAGGGTGCGGTC AGCCCGCCGAAAGCAGTGTCCCACGCCAGCCGG	AGCTTCCGAAGTTCAGATGACTAACTCAGGGC AGGCTTCAAGTCTACTGATTGTGTCCCGCC
REPRESENTATIVE SEQUENCING GEL			

NAME OF CONSTRUCT	pB9+fp116SRE-5bp-ATF	pB9+fp116SRE-10bp-ATF
OLIGONUCLEOTIDE SEQUENCE	ACGTTGGATGTCCATATTAGGACATCCGATCTGCGTCAGCCAGC ACCTACAGGTATAATCCTGTAGGCTAGACGCAGTCGGTCGCCGG	AGCTTGGATGTCCATATTAGGACATCCGATCCAGTGTGCGTCAGCCAGC ACCTACAGGTATAATCCTGTAGGCTAGGTCACACGCAGTCGGTCGCCGG
REPRESENTATIVE SEQUENCING GEL		

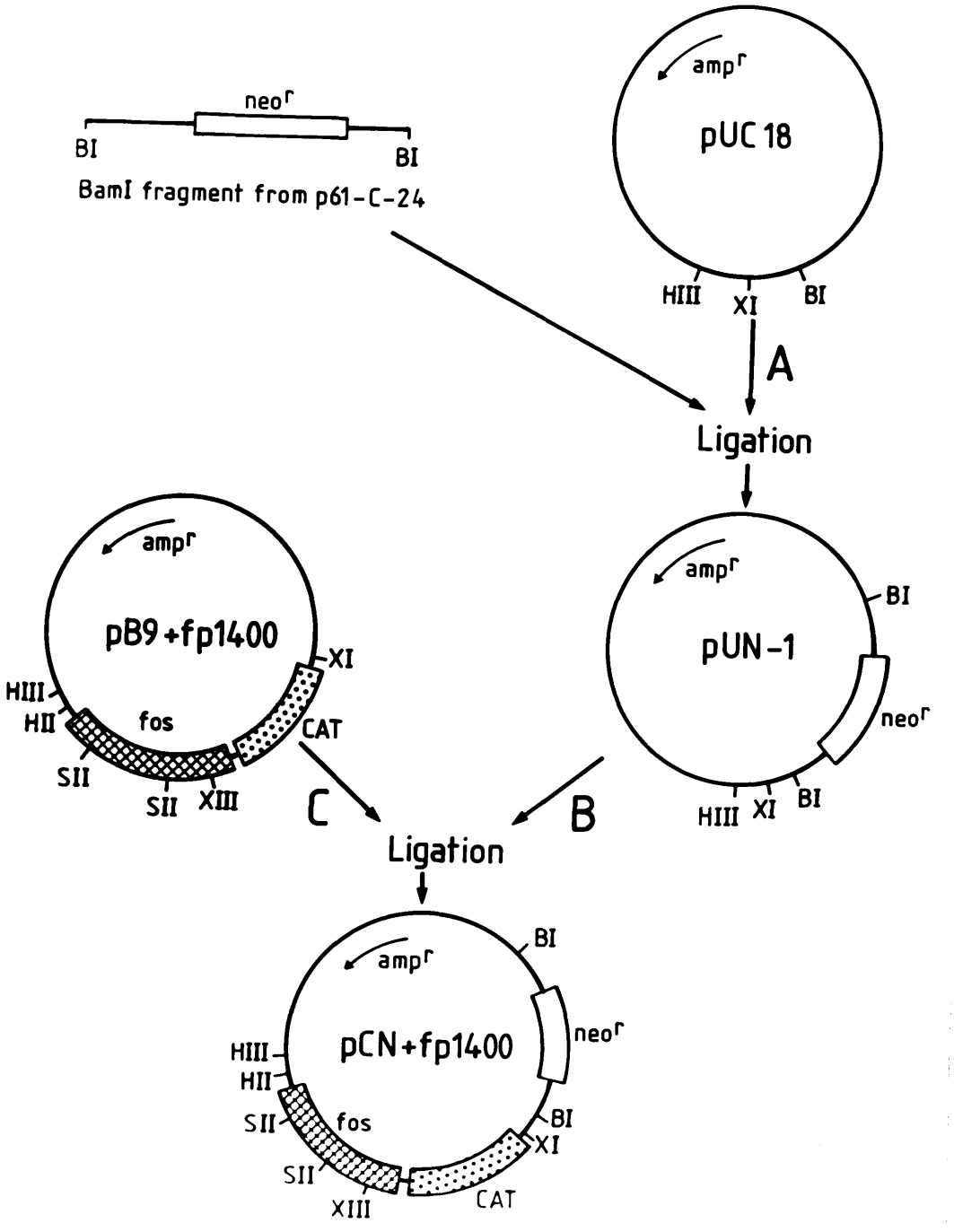


Figure 8. Construction of pUN-1 and pCN+fp1400.

The construction of these recombinants is described in detail in the text. All of the reactions mentioned (restriction enzyme digestion, phosphatase treatment, ligation) are described in sub-Section 2.4.1. amp^r : ampicillin resistance gene neo^r : neomycin resistance gene. A : BHI digestion, CIP treatment. B. HIII, XI double digest. C: Digestion with HIII, XI followed by isolation of the fragment containing the c-fos-CAT sequences. BI : BamHI. HIII : HindIII. XI : XbaI. HII : HincII. SII : SstII. XIII : XmaIII. These constructs are not drawn to scale.

inserted 3′ of the gene, all of which is present in the BamHI fragment cloned into pUC18.

This construct, pCN+fp1400, was transfected into LATK$^-$ mouse fibroblasts (2.5.2.) as follows. The cells were plated out at $5x10^5$ cells 16 to 24 hours prior to transfections. 16 hours after the addition of the Ca PO_4 precipitate which contained 1µg of pCN+fp1400 and 20µg of genomic carrier DNA per flask the cells were washed with PBS and refed with medium containing 10% FCS. 36 hours later these cells were trypsinised from the flask, counted, and replated into $10cm^2$ petri dishes with medium containing 800µg/ml G418 and 10% FCS. 10 days later G418 resistant colonies were clearly visible and 6 were picked, grown up, and assayed for CAT activity (2.6.3.). There was a great deal of variation in CAT activity obtained with the clones and RNA was prepared from that which gave the highest CAT activity so that analysis of the transcriptional start site of pCN+fp1400 could be carried out. This stable clone was called L-fp1400-4. Figure 9 shows the results of a primer extension analysis experiment with RNA prepared from L-fp1400-4. The largest primer extension product obtained in this experiment was 110bp (estimated by analysis of the sequencing reaction run on the same gel). The size of this product is as expected if pCN+fp1400 is initiating transcription at the correct position. A number of other products can also be observed in this primer extension analysis experiment which are probably due to premature termination of the reverse transcriptase reaction.

To confirm that pCN+fp1400 can initiate transcription at the correct

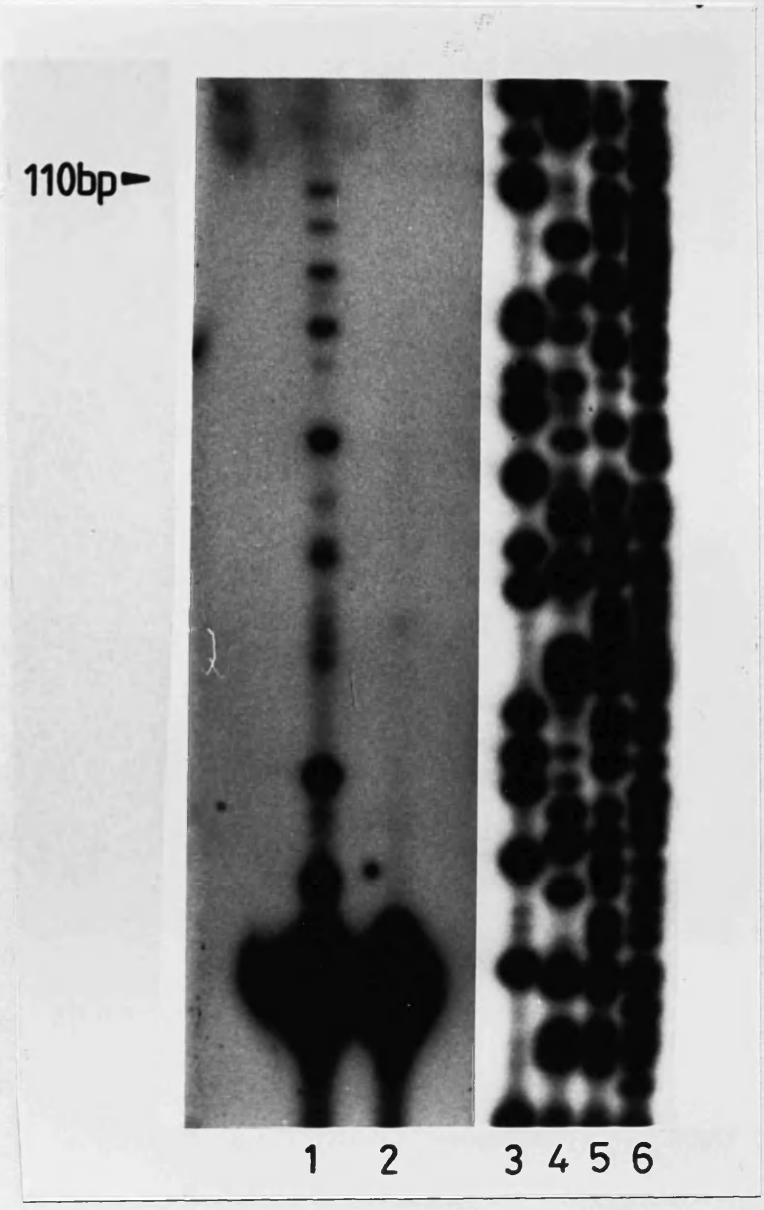


Figure 9. Primer extension analysis of RNA extracted from L-fp1400-4.

The CAT sequence oligonucleotide used in the sequencing of oligonucleotide containing c-fos-CAT constructs was used in this assay (see Figure 7). This oligonucleotide was end-labelled with ^{32}P-ATP using polynucleotide kinase and annealed to 30µg of RNA prepared from L-fp1400-4. This mixture was then treated with reverse transcriptase as described in sub-Section 2.6.5. The resulting nucleic acids were electrophoresed through a 5% denaturing polyacrylamide gel which was dried and autoradiographed. Lanes 1 and 2 represent reverse transcriptase reactions carried out with 30µg of L-fp1400-4 RNA and 30µg of yeast tRNA respectively. Lanes 3 to 6 represent a sequencing reaction (A, C, G, T respectively) carried out with Universal primer and control M13 DNA which was used as a size marker for the primer extension products.

position S1 analysis was carried out with RNA prepared from L-fp1400-4 (Figure 10). The protected fragment obtained with the L-fp1400-4 RNA is 302bp in size and this is what is expected if pCN+fp1400 is initiating transcription at the correct position.

In an attempt to characterise the transcriptional start site of other c-fos-CAT constructs RNA was prepared from transiently transfected CT3 cells. These transiently transfected cells were stimulated with serum or TPA after serum deprivation in an identical fashion to the experiments described in sub-Section 3.4.2. The cells were transfected with 20µg of the c-fos-CAT constructs and 10µg of pLW2 which was designed to act as a transfection efficiency control. Following preparation of the RNA (total cellular) 30µg was taken and used in S1 analysis experiments identical to those described in Figure 10. The same probe was used in the transient analysis as was used for analysis of the L-fp1400-4 RNA. Unfortunately these experiments failed to detect CAT transcripts presumably due to the instability of this mRNA.

So, the primer extension and S1 analyses both show that the parental c-fos-CAT construct, pCN+fp1400, is capable of initiating transcription from the correct position. In all of the manipulations carried out with pB9+fp1400 (from which pCN+fp1400 was derived) to generate further c-fos-CAT constructs, the c-fos-CAT junction is not affected, so these other constructs should also initiate transcription from the correct position.

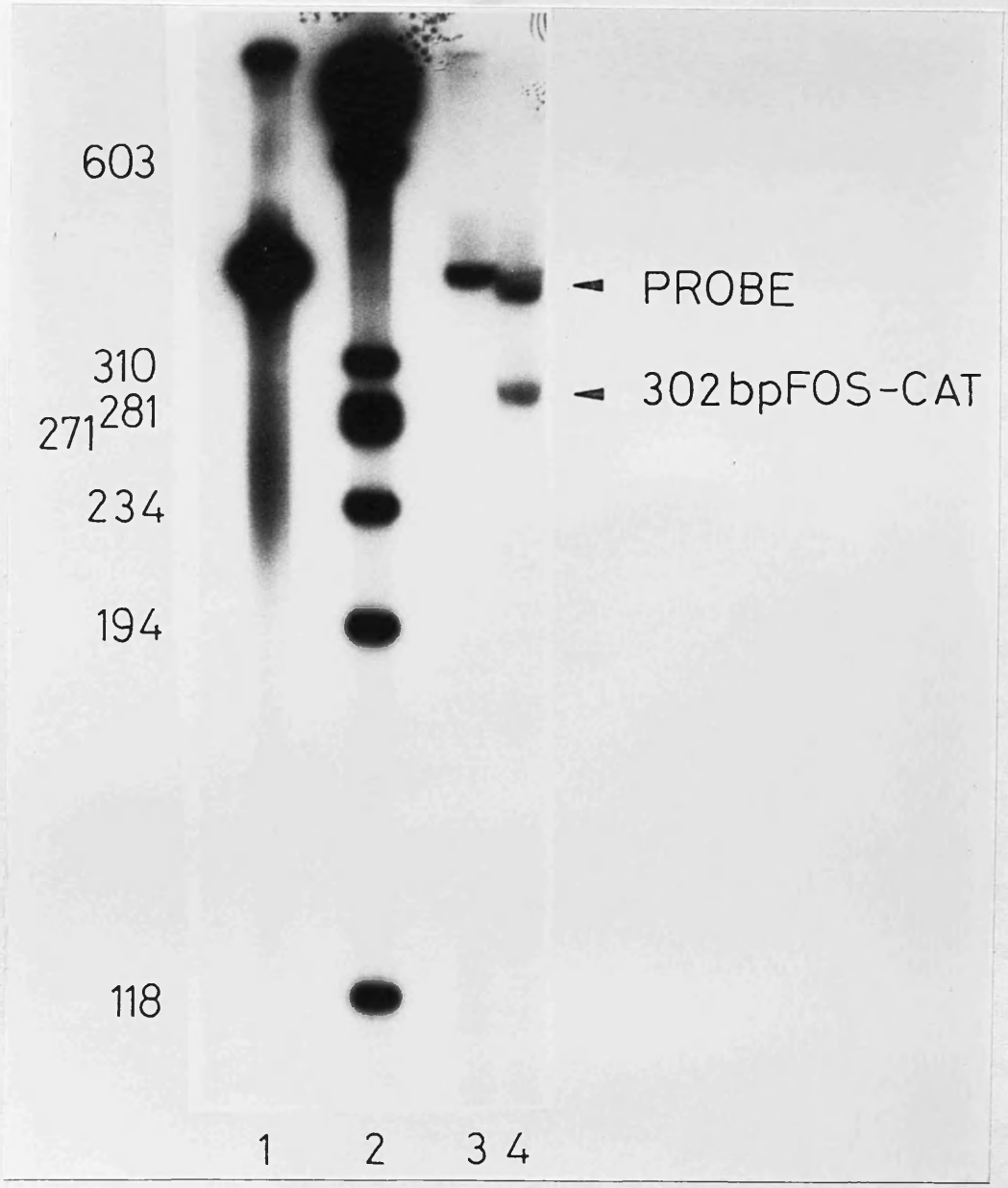


Figure 10. S1 analysis of L-fp1400-4 RNA.

The probe used in this assay was an end-labelled double-stranded DNA probe. This probe was generated by digestion of pB9+fp402 with EcoRI. This linearised plasmid was then phosphatased and labelled with gamma-^{32}P-ATP by treatment with polynucleotide kinase. The labelled, linearised plasmid was then digested with BssHII and the labelled fragment isolated from a neutral polyacrylamide gel. This double-stranded DNA probe was then used in S1 reactions which are described in sub-Section 2.6.4. Following the S1 reaction the nucleic acids were electrophoresed through a 5% denaturing polyacrylamide gel which was then dried and autoradiographed. Lanes 3 and 4 represent S1 reactions carried out with 30µg of yeast tRNA and 30µg of L-fp1400-4 RNA respectively. Lane 2 represents PhiX HaeIII markers end-labelled with gamma-^{32}P-ATP by treatment with polynucleotide kinase. Lane 1 shows the undigested probe. Marker sizes in bp are given down the right hand side of the figure.

3.2.1. Transcriptional activity of fos-CAT plasmid constructs in CT3 cells.

The purpose of the experiments shown, described and quantified in this section was to identify sequences upstream from the c-fos promoter that contribute to the transcriptional activity of the c-fos promoter. In all of the experiments described in this section proliferating CT3 cells in medium containing 10% FCS were transfected as described in sub-Section 2.5.2., washed with PBS the following day and fed with fresh medium containing 10% FCS. These cells were harvested 30-36 hours later and protein extracts prepared (2.6.1.) for β-gal assays (2.6.2.) and CAT assays (2.6.3.).

The first experiment compared the degree of transcription obtained with several c-fos-CAT hybrid plasmid constructs and this experiment is shown in Figure 11 and quantified in Table 1. Comparing the results obtained with pB9+fp116 and pB9+fp402 it can be seen that deleting the sequences containing the previously reported enhancer elements (190) of the c-fos gene between positions -116bp and -402bp reduced transcription between 13- and 24- fold. pB9+fp116SRE, the construct containing the oligonucleotide insertion representing the SRE, give 7- to 16- fold greater transcription than pB9+fp116 and this agrees with published results (190) suggesting that the SRE can behave as an enhancer of transcription when inserted upstream from the c-fos promoter. Interestingly, the construct that contains the previously unstudied ATF/AP-1-like sequence, pB9+fp116ATF, gives a 4- to 5- fold greater transcription than pB9+fp116. pB9+fp116SRE-ATF increases transcription to levels similar to those obtained with

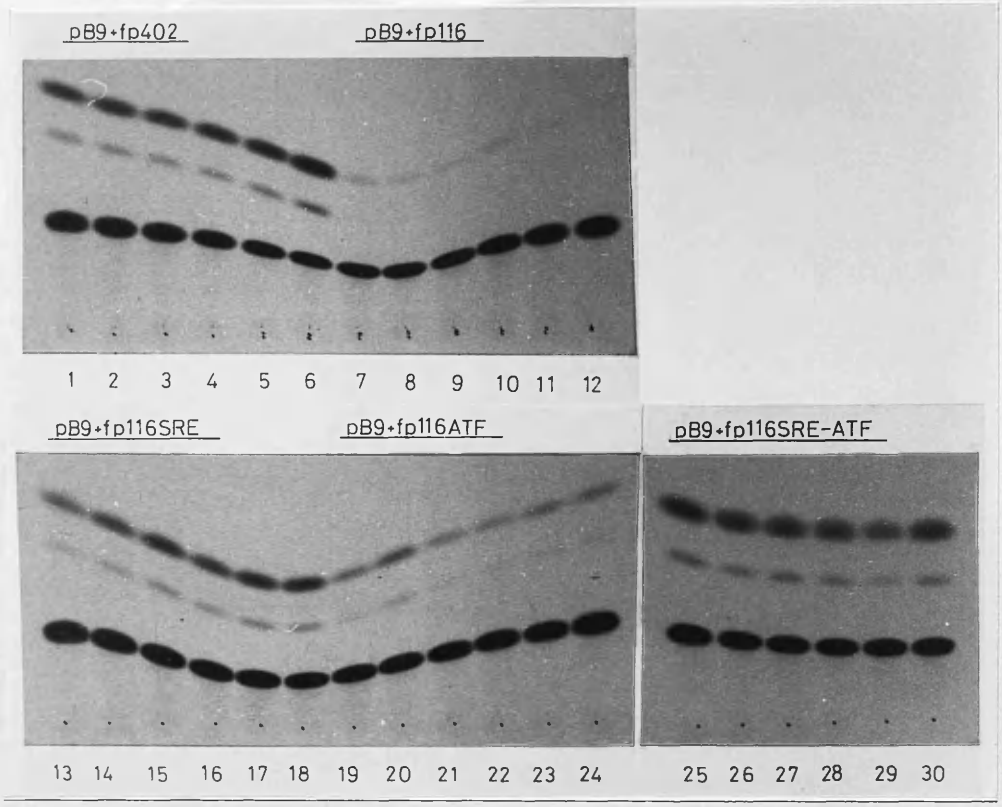


Figure 11. Transcriptional activity of c-fos-CAT plasmid constructs in CT3 cells.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp402 (lanes 1 to 6), pB9+fp116 (lanes 7 to 12), pB9+fp116SRE (lanes 13 to 18), pB9+fp116ATF (lanes 19 to 24) and pB9+fp116SRE-ATF (lanes 25 to 30). The experiment shown above is quantified in Table 1 and is Experiment B. Experiments A, B and C all gave similar results and therefore only one of them is shown in this figure. In Experiment B the cells were plated out at 3 different densities prior to transfection. The first two lanes with each construct represent cells plated out at $5x10^5$ cells per F75 flask, the second two 10^6 cells per F75 and the third part $1.5x10^6$ per F75. In Experiment A the CT3 cells were plated out at $5x10^5$ cells per F75 and in C at $1.5x10^6$ per F75. 16 to 24 hours later the transfection cocktail was added to each flask (2.5.2.) and 16 hours after the addition of the cocktail cells were washed with PBS and then refed with medium containing 10% FCS. 36 hours later the cells were harvested. In Experiments A and B transfection cocktails were divided into 2 flasks to form duplicates, and each duplicate sample is beside the other in this figure (and also in Table 1) i.e. 1 and 2 are duplicates, 3 and 4 are duplicates etc. In Experiment C all of the samples come from different cocktails. In Experiment A and B 7.5µg of c-fos-CAT plasmid was added to each flask in the transfection cockctail along with 10µg of HSV-β-gal plasmid while in Experiment C 7.5µg of each plasmid was added per flask. β-gal assays were performed on aliquots from each extract and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

	PLASMID CONSTRUCT				
	pB9+fp116	pB9+fp402	pB9+fp116SRE	pB9+fp116ATF	pB9+fp116SRE-ATF
EXPERIMENT A	2.0 3.0 1.7 1.2	36.1 49.8 36.1 44.4	48.7 31.5 38.3 25.9	7.1 6.3 13.4 9.4	75.7 32.8 61.7 41.6
AVERAGE CONVERSION	2.2	41.9	36.1	9.1	53.0
STNDARD DEVIATION	0.7	3.4	9.8	3.0	19.2
FOLD INCREASE	1.0+\-0.3	19.1+\-1.5	16.4+\-4.5	4.1+\-1.4	24.1+\-8.7
EXPERIMENT B	7 1.2 8 0.8 9 0.8 10 0.7 11 0.8 12 0.6	1 17.0 2 16.9 3 14.7 4 17.7 5 20.6 6 26.0	13 8.9 14 9.6 15 10.8 16 10.2 17 11.2 18 11.3	19 3.1 20 5.7 21 3.0 22 2.3 23 4.9 24 3.3	25 16.3 26 15.0 27 14.1 28 15.7 29 9.6 30 18.1
AVERAGE CONVERSION	0.8	18.8	10.3	3.7	14.8
STANDARD DEVIATION	0.3	4.1	1.3	1.4	2.9
FOLD INCREASE	1.0+\-0.4	23.5+\-5.1	12.9+\-1.6	4.6+\-1.8	18.5+\-3.6
EXPERIMENT C	0.6 1.1	9.2 9.6 17.0	4.4 2.2 11.2	2.3 2.3 9.8	8.5 6.5 3.1
AVERAGE CONVERSION	0.9	11.9	5.9	4.8	6.0
STANDARD DEVIATION	0.3	4.5	4.8	4.3	2.8
FOLD INCREASE	1.0+\-0.3	13.2+\-5.0	6.6+\-5.3	5.3+\-4.8	6.7+\-3.1

Table 1. Transcriptional activity of c-fos-CAT plasmid constructs in CT3 cells.

This table quantifies the CAT activity obtained in the experiments shown and described in Figure 11. Numbers represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated forms in each extract. The fold increase represents the ratio of the average percentage conversion (and standard deviation) obtained with the c-fos-CAT constructs to that obtained with pB9+fp116. In Experiment B the numbers 1 to 36 correspond to lanes 1 to 36 of Figure 11.

pB9+fp116SRE. This is not surprising since the fosATF/AP-1 sequence increases transcription only 4 to 5 fold and therefore any contribution this sequence may make to the transcriptional activity of pB9+fp116SRE-ATF may be masked by the much stronger transcriptional activitor, SRE.

So, the previously unstudied fosATF/AP-1 sequence gives a weak (4- to 5- fold) increase in transcription in comparision to the promoter-only construct, pB9+fp116. To confirm that the transcriptional activation is due to the inserted fosATF/AP-1 sequence and not to the creation of a transcriptional activator due to oligonucleotide insertion an identical construct to pB9+fp116ATF was made except for two mutations in the AP-1/ATF like sequence of the inserted oligonucleotide (see sub-Section 3.1.2.). This construct, called pB9+fp116MUT-ATF, was assayed along with pB9+fp116 and pB9+fp116ATF in transfection experiments similar to those described previously. These experiments are shown in Figure 12 and quantified in Table 2. As predicted, pB9+fp116MUT-ATF shows no increase in transcription when compared to pB9+fp116 showing that it is the fosAP-1/ATF sequence that is responsible for the transcriptional increase obtained with pB9+fp116ATF.

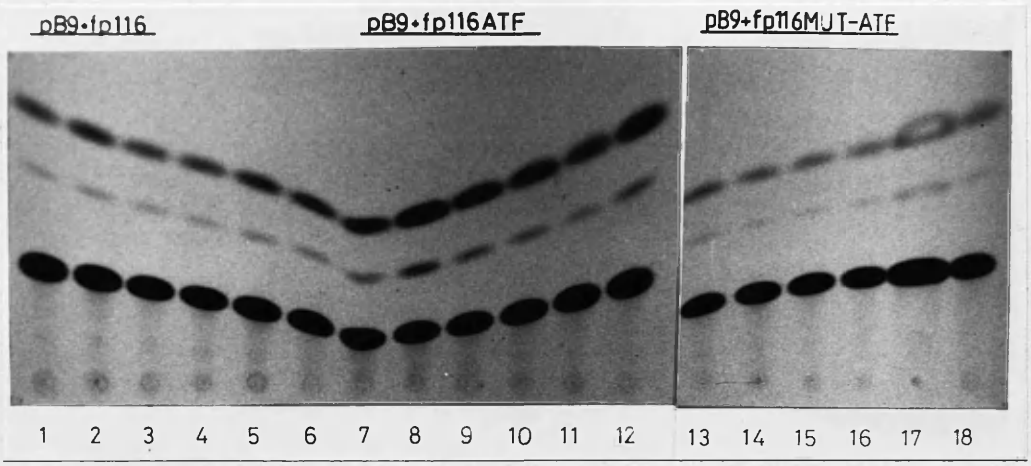


Figure 12. Comparison of transcriptional activity obtained with pB9+fp116, pB9+fp116ATF and pB9+fp116MUT-ATF.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp116 (lanes 1 to 6), pB9+fp116ATF (lanes 7 to 12) and pB9+fp116MUT-ATF (lanes 13 to 18). The experiment shown above is quantified in Table 2 and is Experiment A. Experiments A and B gave similar results and therefore only one of them is shown in this figure. In Experiments A and B the CT3 cells were plated out at 10^6 cells per F75 flask; 16 to 24 hours later the transfection cocktail was added to each flask (2.5.2.), and 16 hours after the addition of the cocktail cells were washed with PBS and then refed with medium containing 10% FCS. 36 hours later the cells were harvested. In both of these experiments transfection cocktails were split into 2 flasks to form duplicates, and each duplicate sample is beside the other in this figure (and also in Table 2) i.e. 1 and 2 are duplicates, 3 and 4 are duplicates etc. In Experiment A 8µg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 4µg of HSV-β-gal plasmid while in Experiment B 10µg and 5µg respectively were used. β-gal assays were performed on aliquots from each extract and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

	PLASMID CONSTRUCT		
	pB9+fp116	pB9+fp116ATF	pB9+fp116MUT-ATF
EXPERIMENT A	1 4.4 2 5.0 3 3.9 4 4.2 5 4.7 6 7.1	7 9.7 8 19.0 9 12.6 10 11.3 11 8.8 12 16.0	13 4.0 14 2.2 15 3.0 16 3.6 17 4.6 18 4.3
AVERAGE CONVERSION	4.9	12.9	3.6
STANDARD DEVIATION	1.1	3.9	1.0
FOLD INCREASE	1.0+\-0.2	2.6+\-0.8	0.7+\-0.2
EXPERIMENT B	- 4.2 6.4 4.9 2.6 1.8	17.9 8.2 15.6 8.8 6.7 8.6	- - 4.5 1.8 2.4 3.5
AVERAGE CONVERSION	4.0	11.0	3.1
STANDARD DEVIATION	1.8	4.5	1.0
FOLD INCREASE	1.0+\-0.4	2.8+\-1.1	0.8+\-0.3

Table 2. Comparison of transcriptional activity obtained with pB9+fp116, pB9+fp116ATF and pB9+fp116MUT-ATF.

This table quantifies the CAT activity obtained in the experiments shown and described in Figure 12. Figures represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated forms in each extract. The fold-induction represents the ratio of the average percentage conversion (and standard deviation) obtained with the c-fos-CAT constructs to that obtained with pB9+fp116. In experiment A the numbers 1 to 24 correspond to lanes 1 to24 of Figure 12.

In an attempt to determine whether the fosATF/AP-1 sequence complexes with AP-1 or ATF protein(s) a series of experiments was carried out comparing the fosATF/AP-1 sequence with previously characterised AP-1 and ATF binding sites .

3.3. Comparison of the fosATF/AP-1 sequence with previously characterised AP-1 and ATF sequences.

Two approaches were adopted in an effort to identify the protein factor(s) responsible for stimulating transcription from the c-fos promoter through interaction with the fos ATF/AP-1-like sequence. The first of these approaches was the gel mobility shift (or gel retardation) assay which was used to identify protein(s) present in HeLaS3 nuclear protein extract able to bind to the fosATF/AP-1 sequence. An alternative approach was to compare the expression of c-fos-CAT constructs containing previously characterised AP-1 and ATF sequences in cell lines which have widely different levels of AP-1 or ATF activity.

3.3.1. Protein binding studies on the fosATF/AP-1 sequence.

Oligonucleotides representing the fosATF/AP-1 sequence, an AP-1 sequence and an ATF sequence (both taken from the adenovirus type 5 E3 promoter which had been characterised previously (267) were synthesised for use in gel mobility shift assays. The AP-1 and ATF sequences, termed E3-AP-1 and E3-ATF, can be seen in 3.1.2. These sequences were end-labelled with polynucleotide kinase (2.6.4.) and incubated with HeLaS3 nuclear protein extracts, before resolving the

resultant mixture by polyacrylamide gel electrophoresis. Cross-competition between the sequences was carried out by including 1000-molar excess of cold competitor oligonucleotide in the incubation reaction. Figure 13 represents a gel mobility shift assay using the three mentioned oligonuceotides and the HeLaS3 nuclear protein extract. As can be seen from this figure the fosATF/AP-1 sequence binds a protein (lane 1) that is specifically competed out by its own cold oligonucleotide (lane 2). The mobility of this retarded complex is more similar to that seen with the E3-AP-1 sequence (lane 5) than that seen with the E3-ATF sequence. However, when the E3-AP-1 sequence is used as a competitor for the protein binding to the fosATF/AP-1 sequence (lane 3) it seems to shift the complex from the mobility of an AP-1 complex to that seen with E3-ATF. The fos ATF/AP-1 sequence is a poor competitor for the proteins that bind to the E3-AP-1 sequence (lane 6) and the E3-ATF sequence (lane 10) suggesting that it is a weak binding site for one of the two proteins, or that it binds a relative of these proteins. The pattern of proteins binding to the E3-AP-1 and E3-ATF sequences are similar to those previously published (267) as are the competition results obtained in this figure. In summary, the fosATF/AP-1 sequence seems to preferentially bind an AP-1 like protein but also seems to be able to bind an ATF like protein when the AP-1 proteins are "removed" from the nuclear protein extract by competitor AP-1 sequences.

To confirm that the protein forming the complex with the oligonucleotide containing the fosATF/AP-1 sequence is actually binding to the sequence that is similar to AP-1 and ATF a gel

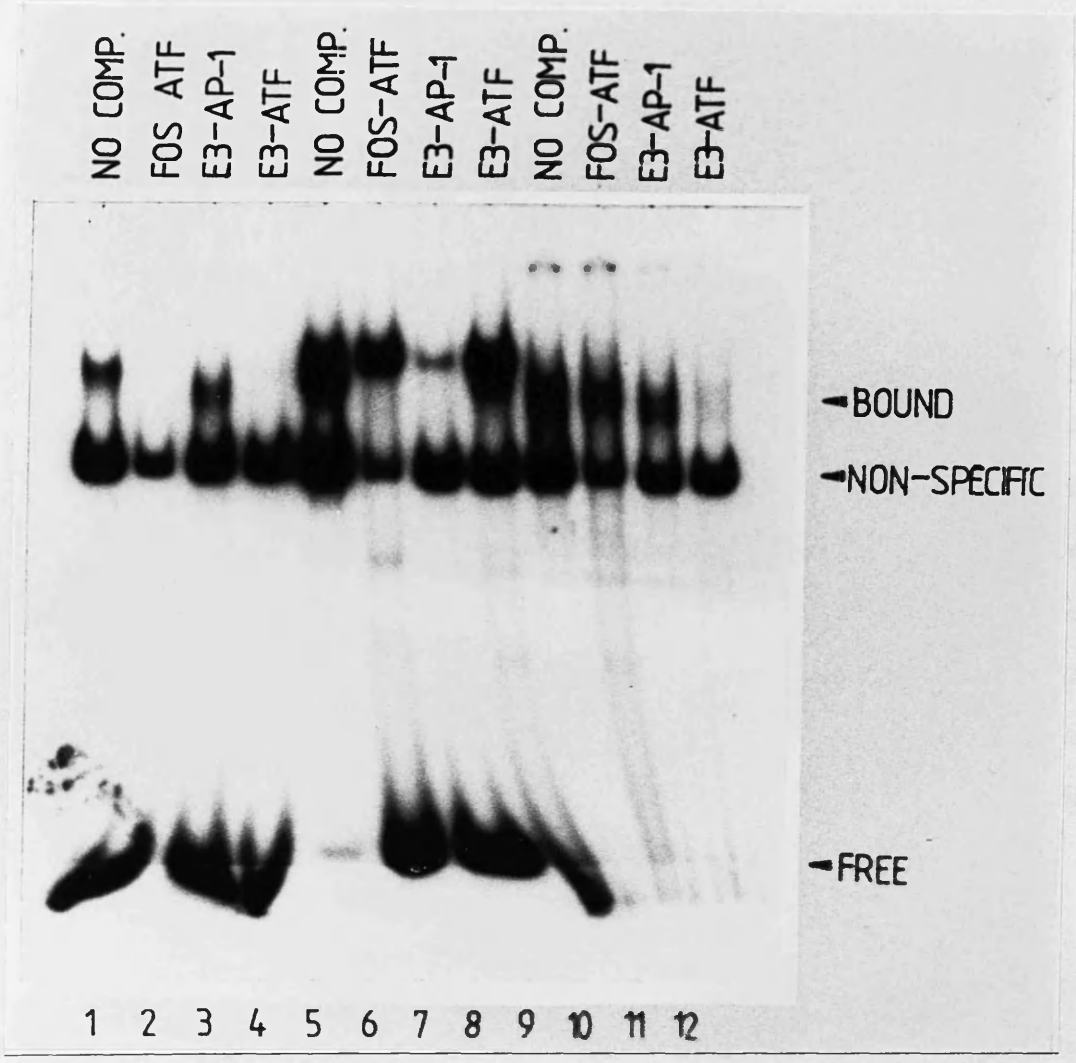


Figure 13. A gel mobility shift assay involving the incubation of fosATF/AP-1, E3-AP-1 and E3-ATF sequences with HeLaS3 nuclear protein extract.

Gel mobility shift assay using end-labelled oligonucleotides representing fosATF/AP-1 (lanes 1 to 4), E3-AP-1 (lanes 5 to 8) and E3-ATF (lanes 9 to 12) sequences. The oligonucleotides were incubated with equivalent amounts of HeLaS3 nuclear protein extract for 20 minutes at room temperature before the reaction mixture was electrophoresed through a 5%, 0.5xTBE, polyacrylamide gel (2.7.2.). Competitor oligonucleotides used are shown above each lane and are co-incubated with the labelled oligonucleotide-HeLaS3 nuclear protein extract mix. 0.1ng of probe used per reaction. 100ng competitor DNA used in each lane where indicated. No comp : no competitor.

mobility shift assay was carried out using labelled fosATF/AP-1 oligonucleotide. This oligonucleotide and one containing the fosATF/AP-1 sequence with two mutations in it were used as competitor oligonucleotides in increasing concentrations. The sequence of this mutated fosATF/AP-1 oligonucleotide is given in sub-Section 3.1.2. and is the same sequence that was inserted upstream from the c-fos promoter to form the construct pB9+fp116 MUT-ATF. This experiment is shown in Figure 14. As can be seen, when fosATF/AP-1 sequences are used as a competitor for binding to the fosATF/AP-1 sequence reduction in binding occurs with a 50-fold molar excess and elimination of any band representing specific protein binding occurs when a 1000 molar excess of this competitor is used. However, the mutant fosATF/AP-1 sequence fails to compete even partially for the proteins that bind to the fosATF/AP-1 sequence and the complex persists even at 2000 molar excess of competitor. This demonstrates that the fosATF/AP-1-like motif contributes directly to complex formation.

Because the fosATF/AP-1 sequence is adjacent to and continuous with the previously described SRE in the endogenous c-fos upstream sequence it was important to determine whether complexes can form on both elements simultaneously or whether they are mutually exclusive. A gel mobility shift assay with labelled oligonucleotides containing sequences representing the SRE, fosATF/AP-1 and SRE-ATF (fosATF/AP-1) was carried out to answer this question. This experiment is shown in Figure 15. Unfortunately the quality of the SRE and SRE-ATF containing oligonucleotides was poor resulting in competition of the non-specific band (compare the uniformity of the non-specific band in

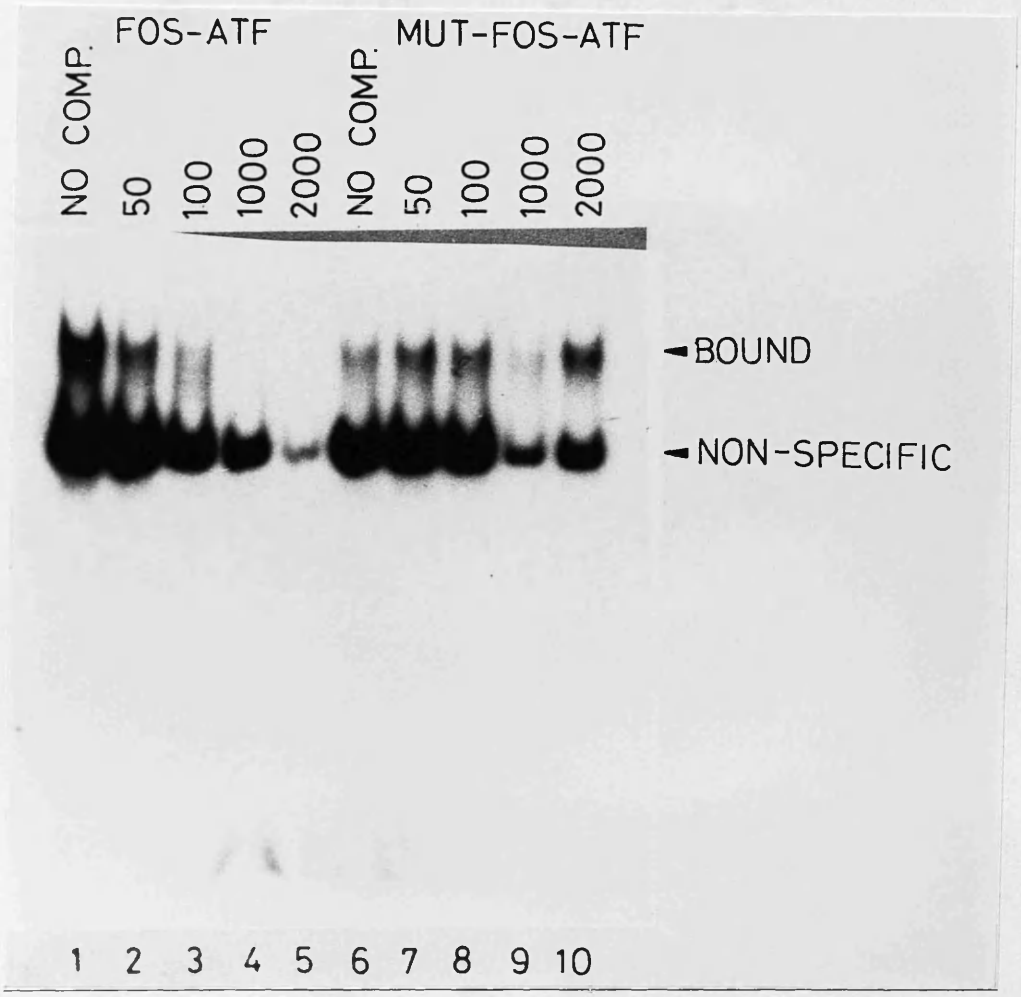


Figure 14. Competition for the HeLaS3 nuclear protein that binds to the fosATF/AP-1 sequence with fosATF/AP-1 and mutated fosATF/AP-1 sequences.

Gel mobility shift assay using an end-labelled oligonucleotide containing the fosATF/AP-1 sequence (lanes 1 to 10). The oligonucleotide was incubated with equivalent amounts of HeLaS3 nuclear protein extract for 20 minutes at room temperature before the mixture was electrophoresed through a 5%, 0.5xTBE, polyacrylamide gel (2.7.2.). Competitor oligonucleotides are indicated at the top of the figure and the numbers above each lane represent the excess molar concentration of competitor used in each reaction. 0.1ng of probe used per reaction. No comp : no competitor.

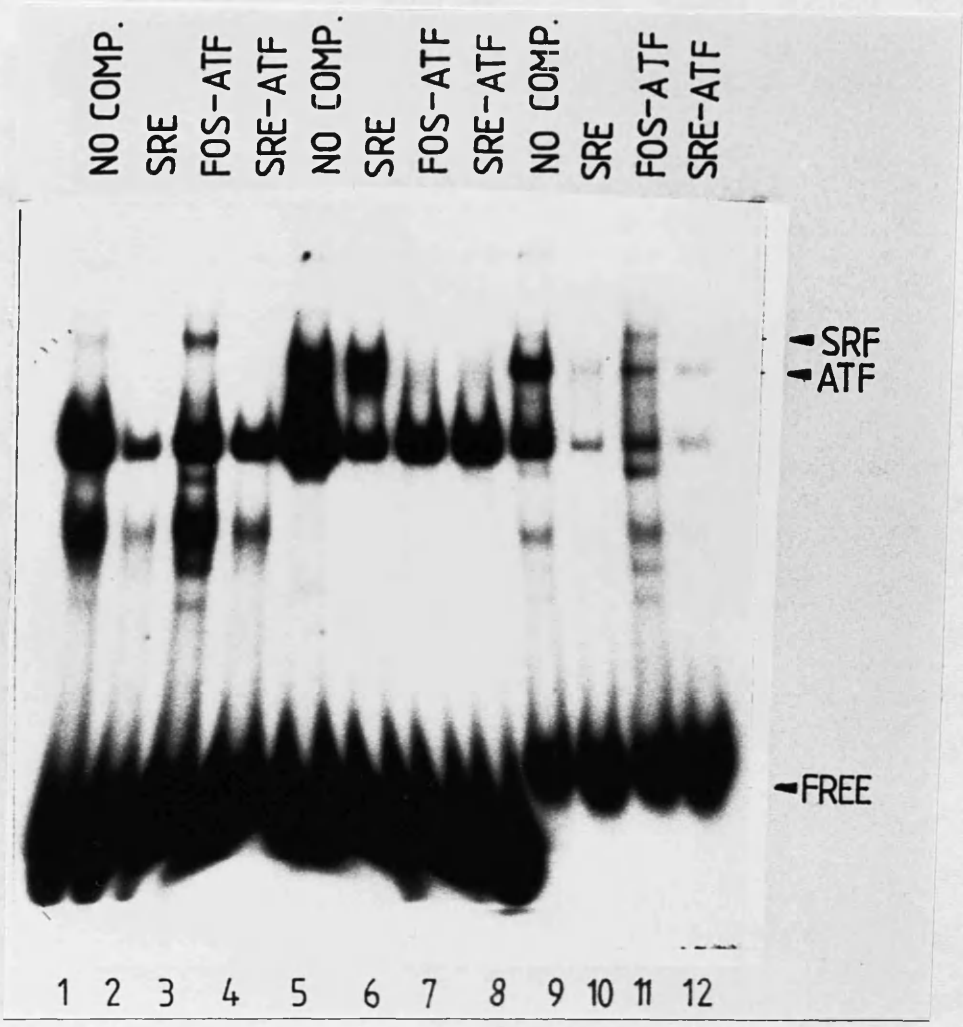


Figure 15. A gel mobility shift assay involving the incubation of fosATF/AP-1, SRE and SRE-ATF sequences with HeLaS3 nuclear protein extract.

Gel mobility shift assay using end-labelled oligonucleotides representing the SRE (lanes 1 to 4), fosATF/AP-1 (lanes 5 to 8) and SRE-ATF (lanes 9 to 12) sequences. The oligonucleotides were incubated with equivalent amounts of HeLaS3 nuclear protein extract for 20 minutes at room temperature before the reaction mixture was electrophoresed through a 5%, 0.5xTBE, polyacrylamide gel (2.7.2.). Competitor oligonucleotides used are shown above each lane and are co-incubated with the labelled oligonucleotide, HeLaS3 nuclear protein extract mix. 0.1ng of probe used per reaction. 100ng of competitor DNA used in each lane where indicated. No comp : no competitor.

Figure 13 and that seen in Figure 15). However, several points may still be made from this experiment. The oligonucleotide containing the SRE sequence binds a protein in the HeLaS3 nuclear protein extract to form a complex with a greater mobility shift than that obtained with the fosATF/AP-1 containing oligonucleotide (compare lanes 1 and 5). This is expected since the protein that binds the SRE sequence, the serum response factor (SRF) is 67kD (206, 207, 208) in size whereas the AP-1/ATF family of proteins range in size from 39 to 47 kD (330). The oligonucleotide containing the SRE-ATF sequence forms two protein complexes (lane 9). The upper one involves the binding of the SRF since it is competed out by the oligonucleotide containing the SRE sequence. The lower complex would appear to bind a portion of the complex that forms with the fosATF/AP-1 containing oligonucleotide (compare lanes 5 and 9) suggesting that the presence of the SRF has an influcence over the protein(s) that can bind to the fos-ATF/AP-1 sequence in this oligonucleotide. It might be expected that a complex representing both the SRF and the AP-1/ATF like protein binding together onto the oligonucleotide containing the SRE-ATF sequence would be observed. The failure to observe such a complex in Figure 15 may be due to the insensitivity of the gel mobility shift technique.

In all three of the gel mobility shift assays described so far (Figures 13, 14 and 15) there have been suggestions that the fosATF/AP-1 sequence is capable of binding more than one protein. In Figure 13 it forms two complexes (lanes 1 and 3) similar in mobility to those observed with AP-1 and ATF containing oligonucleotides. In Figure 14, increasing concentrations of fosATF/AP-1 competitor DNA

seems to enable the fosATF/AP-1 sequence to bind a number of proteins (lane 3) while in Figure 15 the presence of the SRE sequence in the SRE-ATF containing oligonucleotide seems to limit the width of the complex forming with the fosATF/AP-1 sequence (compare lanes 5 and 9). To determine whether, as is suggested by Figure 13, the fosATF/AP-1 sequence can form complexes with both AP-1 and ATF-like proteins a gel mobility shift assay was carried out in which the fosATF/AP-1 containing oligonucleotide was incubated with HeLaS3 nuclear protein extract and a 1000 molar excess of E3-AP-1 containing oligonucleotide was co-incubated. Increasing amounts of either fosATF/AP-1 or E3-ATF containing oligonucleotides were also co-incubated in this experiment which is shown in Figure 16. As in Figure 13 the E3-AP-1 containing competitor oligonucleotide leads to the formation of a new complex (compare lanes 1 and 2). When E3-ATF containing oligonucleotide is co-incubated in increasing concentrations with the E3-AP-1 containing oligonucleotide competitor it competes for the protein shifted down the gel by the E3-AP-1 competitor sequences more efficiently then the fosATF/AP-1 sequence containing oligonucleotide. Both competitor DNAs (fosATF/AP-1 and E3-ATF) do evenutally compete for this complex although at these concentrations (500 molar excess) it would be expected that these competitors would abolish formation of the retarded complex with or without the presence of a 1000 molar excess of E3-AP-1 oligonucleotide. However, this experiment does suggest that the E3-AP-1 competitor DNA allows the fosATF/AP-1 sequence to bind a protein that is similar to that which forms a complex with the E3-ATF oligonucleotide.

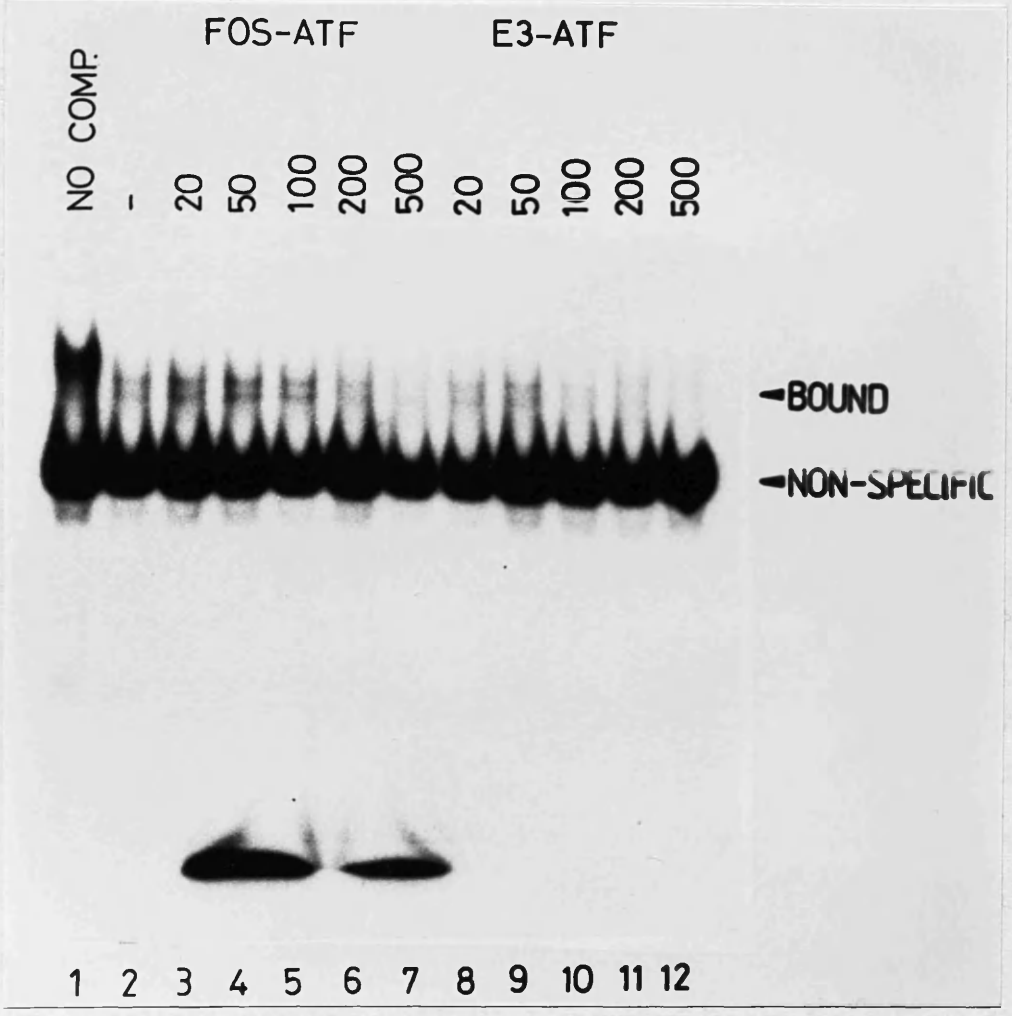


Figure 16. Competition for the HeLaS3 nuclear protein that binds to the fosATF/AP-1 sequence by co-incubation with E3-AP-1 and either fosATF/AP-1 or E3-ATF containing oligonucleotides .

Gel mobility shift assay using an end-labelled oligonucleotide containing the fosATF/AP-1 sequence (lanes 1 to 12). The oligonucleotide was incubated with equivalent amount of HeLaS3 nuclear protein extract for 20 minutes at room temperature before the mixture was electrophoresed through a 5%, 0.5xTBE polyacrylamide gel (2.7.2.). In lanes 2 to 12 1000 molar excess of E3-AP-1 oligonucleotide was co-incubated with the reaction mix. Other competitor oligonucleotides co-incubated are indicated at the top of the figure and the numbers above each lane represent the excess molar concentration of the competitor oligonucleotides used in each reaction. 0.1ng probe used per reaction. No comp : no competitor.

In Figure 13 it can also be seen that the fosATF/AP-1 oligonucleotide competes very poorly for the protein that binds to and forms a complex with the E3-ATF oligonucleotide even at a 1000 molar excess, although the E3-ATF oligonucleotide can completely abolish any formation of a complex with the fosATF/AP-1 oligonucleotide. Because the competitor concentrations in this experiment were in 1000 molar excess it is impossible to determine whether the E3-ATF oligonucleotide can compete as efficiently (or identically) as the fosATF/AP-1 oligonucleotide for the protein-DNA complex formed with the fosATF/AP-1 oligonucleotide. To determine whether this is the case a gel mobility shift assay was carried out which used increasing concentration of fosATF/AP-1 and E3-ATF oligonucleotides as competitor DNAs with labelled fosATF/AP-1 oligonucleotide. This experiment is shown in Figure 17. The oligonucleotide containing the fosATF/AP-1 sequence competes slightly more efficienctly for the protein that forms the complex with the labelled fosATF/AP-1 oligonucleotide than the E3-ATF oligonucleotide (compare lanes 6 and 8). It is also interesting to note that at certain concentrations of fosATF/AP-1 competitor oligonucleotide several proteins appear to form complexes with the fosATF/AP-1 sequence (lanes 4 and 5). This is similar to the pattern observed in Figure 14, lane 3.

In summary, Figures 13 to 17 indicate that the fosATF/AP-1 sequence is capable of binding a protein from a HeLaS3 nuclear protein extract that is related to the AP-1/ATF family. Because it forms a complex similar in mobility to that formed with the E3-AP-1 oligonucleotide it would suggest that the fosATF/AP-1 sequence prefentially binds an AP-1-like protein (Figure 13), but can bind a protein and form a

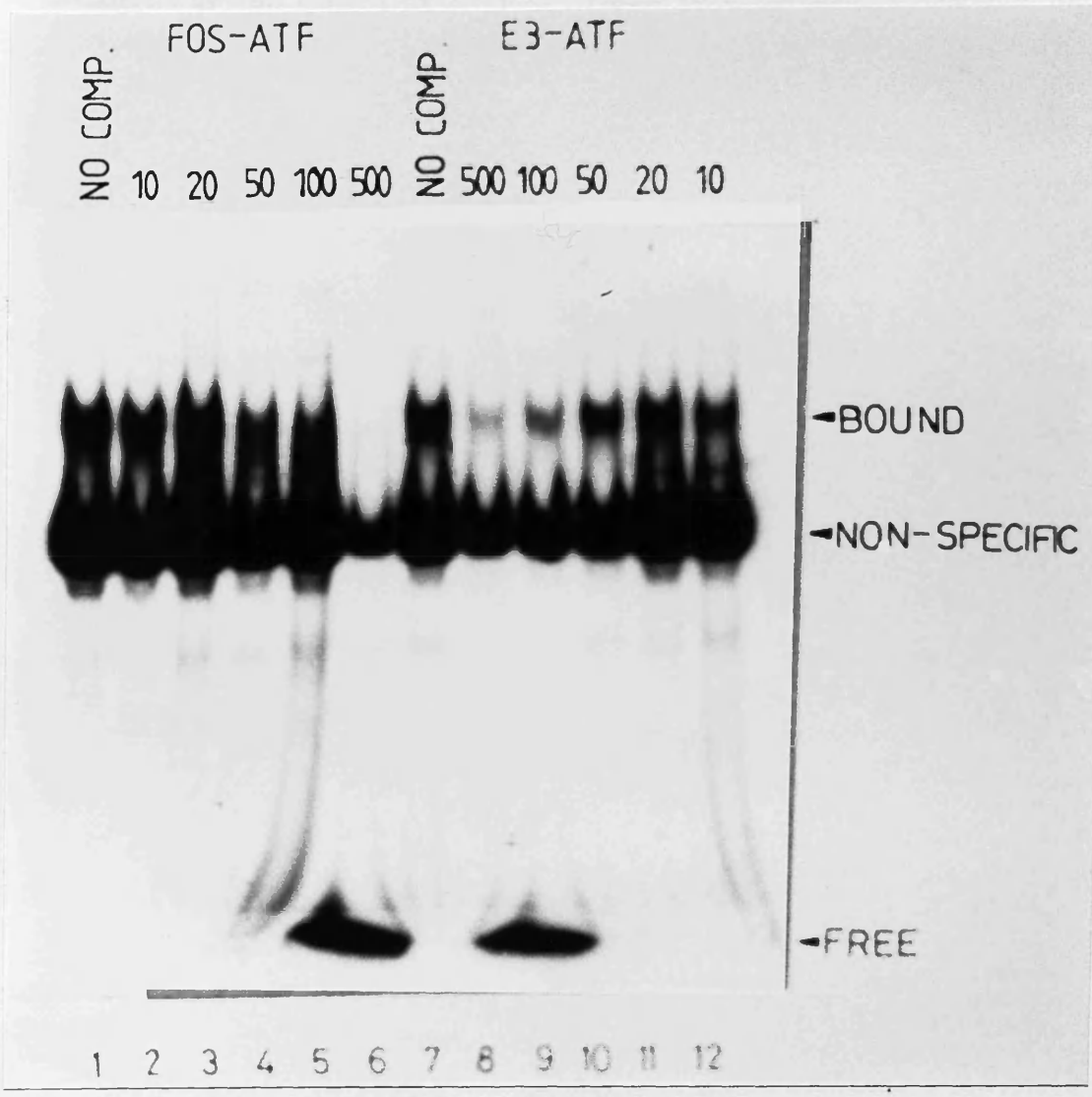


Figure 17. Competition for the HeLaS3 nuclear protein that binds to the fosATF/AP-1 sequence with fosATF/AP-1 and E3-ATF sequences.

Gel mobility shift assay using an end-labelled oligonucleotide containing the fosATF/AP-1 sequence (lanes 1 to 12). The oligonucleotide was incubated with equivalent amounts of HeLaS3 nuclear protein extract for 20 minutes at room temperature before the mixture was electrophoresed through a 5%, 0.5xTBE, polyacrylamide gel (2.7.2). Competitor oligonucleotides are indicated at the top of the figure and the numbers above each lane represent the excess molar concentration of competitor used in each reaction 0.1ng of probe was used in each reaction. No comp : no competitor.

complex similar to that formed with the E3-ATF oligonucleotide when AP-1 protein(s) are effectively removed from the nuclear protein extract by an excess of E3-AP-1 competitor oligonucleotide. The fosATF/AP-1 sequence is a poor competitor for the proteins that form complexes with the E3-AP-1 and E3-ATF oligonucleotides, suggesting that it is perhaps a weak binding site for which ever protein binds to this sequence, or alternatively that it binds a particular member of the AP-1/ATF family. To compare the transcriptional activity of these sequences with the fosATF/AP-1 sequence the E3-AP1 and E3-ATF oligonucleotides were inserted upstream from the c-fos promoter (3.1.2.) and transfection experiments carried out to compare the contribution that these sequences make to transcription from the c-fos promoter.

3.3.2. Comparison of the transcriptional activation obtained with c-fos-CAT constructs containing fosATF/AP-1 E3-AP-1 and E3-ATF sequences inserted upstream from the c-fos promoter.

Transfection experiments similar to those carried out in Section 3.2. were carried out to compare the degree of transcriptional activation obtained with pB9+fp116ATF, pB9+fp116E3-AP-1 and pB9+fp116E3-ATF (3.1.2.). This experiment involved the transfection of these constructs into proliferating CT3 cells, washing the cells the following morning and growing them for a further 36 hours in medium containing 10% FCS before cell harvest. This experiment is shown in Figure 18. Once again, pB9+fp116ATF give 3 to 4 fold greater CAT activity than that obtained with pB9+fp116 which agrees with previous results (see Tables 1 and 2). pB9+fp116E3-ATF and pB9+fp116E3-AP-1

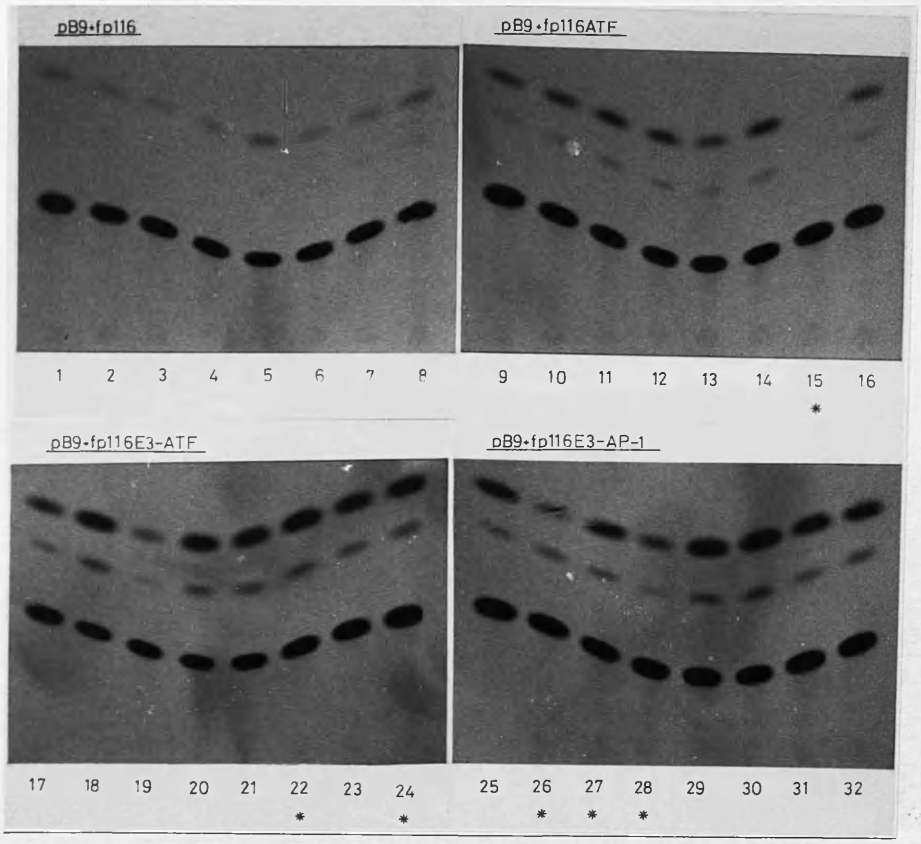


Figure 18. Comparison of the transcriptonal activation obtained with c-fos-CAT constructs containing fosATF/AP-1, E3-AP-1 and E3-ATFsequences inserted upstream from the c-fos promoter.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp116 (lanes 1 to 8), pB9+fp116ATF (lanes 9 to 16), pB9+fp116E3-ATF (lanes 17 to 24) and pB9+fp116E3-AP-1 (lanes 25 to 32). The experiment shown here is quantified in Table 3. The cells were plated out at 10^6 per F75 flask. 16 to 24 hours later the transfection cocktail was added to each flask (2.5.2.) and 16 hours after the addition of the cocktail cells were washed with PBS and then refed with medium containing 10% FCS. 36 hours later the cells were harvested. In this experiment transfection cocktails were split into 2 flasks to form duplicates, and each duplicate sample is beside the other in this figure i.e. 1 and 2 are duplicates, 3 and 4 are duplicates etc. 10µg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 10µg of HSV-β-gal plasmid. β-gal assays were performed on aliquots from each extract and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

* These CAT assays had incorrect amounts of extract used and are therefore not included in Table 3.

	PLASMID CONSTRUCT			
	pB9+fp116	pB9+fp116ATF	pB9+fp116E3-ATF	pB9+fp116E3-AP-1
	1 2.0 2 1.5 3 1.1 4 1.4 5 2.9 6 1.3 7 1.4 8 2.6	9 3.9 10 5.9 11 8.5 12 8.0 13 5.3 14 7.3 15 - 16 5.2	17 19.6 18 17.6 19 18.2 20 15.3 21 25.1 22 - 23 25.1 24 -	25 24.6 26 - 27 - 28 - 29 21.7 30 27.3 31 12.4 32 18.2
AVERAGE CONVERSION	1.8	6.3	20.2	20.8
STANDARD DEVIATION	0.6	1.7	3.8	6.0
FOLD INDUCTION	1.0+\-0.3	3.5+\-0.9	11.2+\-2.1	11.6+\-3.3

Table 3. Comparison of the transcriptional activation obtained with c-fos CAT constructs containing fosATF/AP-1, E3-AP-1 and E3-ATF sequences inserted upstream from the c-fos promoter.

This table quantifies the CAT activity obtained in the experiment shown and described in Figure 18. Numbers represent the percentage ^{14}C-chloramphenicol converted to mono-acetylated forms in each extract. The fold increase represents the ratio of the average percentage conversion (and standard deviation) obtained with the c-fos-CAT constructs to that obtained with pB9+fp116. The numbers 1 to 32 correspond to lanes 1 to 32 of Figure 18.

both give 11-fold greater activity than that obtained with pB9+fp116 suggesting that the E3-ATF and E3-AP-1 sequences are stonger transcriptional activators than the fosATF/AP-1 sequence. This correlates with the gel mobility shift assay shown in Figure 3 where it can be seen that the fosATF/AP-1 oligonucleotide probably binds a member of the AP-1/ATF family of proteins weakly in comparison to the complexes formed with the E3-AP-1 and E3-ATF sequences.

So, all of the evidence obtained so far suggests that the fosATF/AP-1 sequence binds a member of the AP-1/ATF family of proteins and that perhaps this protein is distinct from those which form complexes with the E3-AP-1 and E3-ATF sequences. In a further effort to indicate that the protein binding to the fosATF/AP-1 sequence in gel mobility shift assays and activating transcription via this sequence in transfection studies is a member of the ATF/AP-1 family of proteins transfection experiments were carried out with the murine teratocarcinoma cell line, F9. This cell line has previously been shown to contain no PEA-1 (the muringe homologue of AP-1) (310) activity in footprinting studies. Upon differentiation of this cell line PEA-1 activity becomes detectable by footprinting experiments using the polyoma α-enhancer.

3.3.3. Transcriptional properties of fos-CAT constructs transfected into the murine embryonal carcinoma cell line, F9.

The transfection experiments described in this section were carried out with F9 cells that were grown in medium containing 10% FCS throughout the experiment, and were then harvested and protein

extracts prepared for β-gal (2.6.2.) and CAT (2.6.3.) assays. Three to four times more plasmid than in the previous transfection studies was used per flask in these transfection studies because the transfection efficiency with F9 cells is lower than that obtained with CT3 cells. Figure 19 shows the results obtained with transfection experiments carried out with F9 cells, and Table 4 quantifies these experiments. The striking feature of these experiments when compared to those carried out with CT3 cells is the absence of differential expression of c-fos-CAT constructs containing sequences that enhance transcription in CT3 cells when compared to the promoter-only c-fos-CAT construct, pB9+fp116. The possible exception to this is pB9+fp402 which gives at best a 2-fold increase in CAT activity over that obtained with pB9+fp116. This is compared to a 20-fold increase in CAT activity obtained with transfection experiments in CT3 cells. pB9+fp116E3-AP-1 gives no increase in transcription over pB9+fp116 and this was expected since F9 cells had previously been shown to lack any AP-1 activity and it is not surprising that pB9+fp116E3-ATF and pB9+fp116ATF (the construct containing the fosATF/AP-1 sequence) did not give any increase in transcription since the proteins that bind to these sequences are probably members of the same family as the AP-1 proteins. However, it had not been previously established that the SRE fails to increase transcription in these cells, presumably due to a lack of the SRF. The increase in transcription obtained with pB9+fp402 (only 2-fold) could be due to another protein(s) contributing towards transcription from a previously unidentified cis-acting transcriptional control element(s), or it could be that there are low levels of SRF and the protein that binds to the fosATF/AP-1 sequence and that these operate

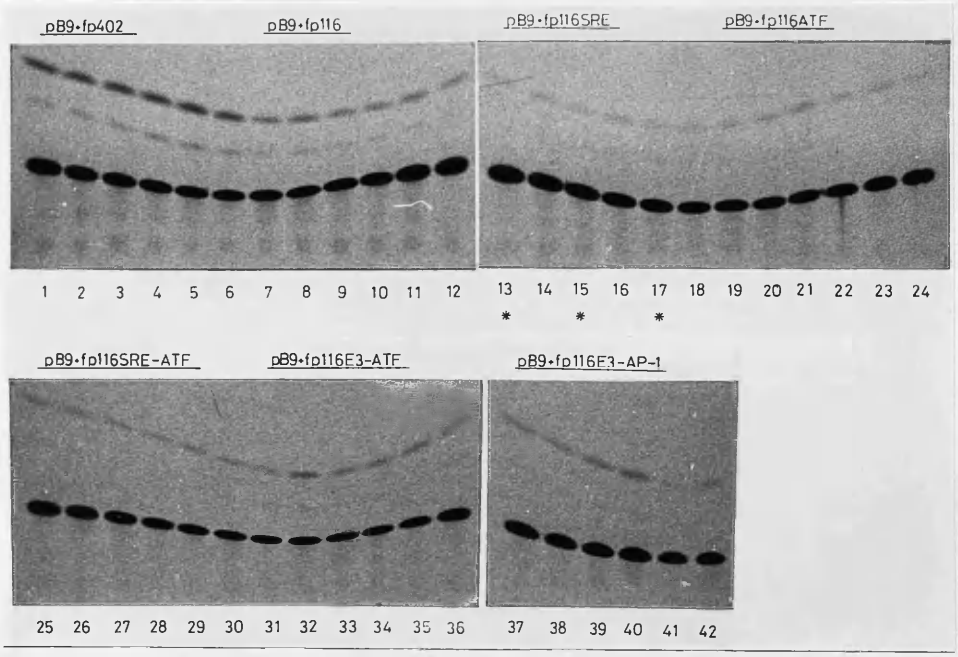


Figure 19. Transcriptional properties of c-fos-CAT constructs transfected into the murine teratocarcinoma cell line, F9.

CAT assays carried out with cell extracts prepared from F9 cells transfected with pB9+fp402 (lanes 1 to 6), pB9+fp116 (lanes 7 to 12), pB9+fp116SRE (lanes 13 to 18), pB9+fp116ATF (19 to 24), pB9+fp116SRE-ATF (lanes 25 to 30), pB9+fp116E3-ATF (lanes 31 to 36) and pB9+fp116E3-AP-1 (lanes 37 to 42). The experiment shown above is quantified in Table 1 and represents Experiment B. Experiments A and B gave similar results and therefore only one of them is represented in this figure. In both experiments 10^6 cells were plated out the day before transfection. 3 hours before transfection the cells were refed with medium containing 10% FCS. The cocktail was then added and 8 hours later removed and the cells were then given a 2 minute shock with 15% glycerol and then washed and refed with medium containing 10% FCS. The cells were harvested 24 hours later. In Experiment B the transfection cocktails were split into 2 flasks to form duplicates, and each duplicate sample is beside the other in this figure i.e. 1 and 2 are duplicates, 3 and 4 are duplicates, etc. In Experiment A one transfection cocktail was split into 3 flasks to form triplicates and another was split into 2 flasks to form duplicates. In Experiments A and B 30μg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 20μg of HSV-β-gal plasmid. β-gal assays were performed on aliquots from each extract and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

* These CAT assays had incorrect amounts of extract used and are therefore not included in Table 3.

	PLASMID CONSTRUCT						
	pB9+fp116	pB9+fp402	pB9+fp116SRE	pB9+fp116ATF	pB9+fp116SRE-ATF	pB9+fp116E3-ATF	pB9+fp116E3-AP-1
EXPERIMENT A	17.6 11.8 15.4 15.6 12.0	29.6 25.7 17.4 24.5 21.3	17.7 11.1 10.5 8.9 6.8	11.7 11.2 16.3 10.4 11.2	- - - - -	12.5 11.3 18.1 8.6 9.1	12.4 21.6 11.6 6.3 6.1
AVERAGE CONVERSION	14.5	23.4	11.0	12.2	-	11.9	11.6
STANDARD DEVIATION	2.4	6.2	4.1	2.1	-	3.9	6.3
FOLD INCREASE	1.0+\-0.2	1.6+\-0.4	0.8+\-0.3	0.8+\-0.1	-	0.8+\-0.3	0.8+\-0.4
EXPERIMENT B	7 2.6 8 2.3 9 1.7 10 2.2 11 1.7 12 2.2	1 4.4 2 5.3 3 4.3 4 4.8 5 5.7 6 4.7	13 - 14 0.8 15 - 16 1.2 17 - 18 1.2	19 1.7 20 1.5 21 1.3 22 0.8 23 1.1 24 1.5	25 1.2 26 1.6 27 1.0 28 1.8 29 1.7 30 1.2	31 2.2 32 2.3B 33 2.2 34 1.8 35 2.0 36 1.4	37 1.8 38 3.5 39 2.1 40 2.1 41 1.6 42 2.1
AVERAGE CONVERSION	2.1	4.9	1.1	1.3	1.4	2.0	2.2
STANDARD DEVIATION	0.2	0.6	0.2	0.2	0.4	0.2	0.7
FOLD INDUCTION	1.0+\-0.1	2.3+\-0.3	0.5+\-0.1	0.6+\-0.1	0.7+\-0.2	1.0+\-0.1	1.0+\-0.3

Table 4. Transcriptional properties of c-fos-CAT constructs transfected into the murine embryonalcarcinoma cell line F9.

This table represents the CAT activity obtained in the experiment shown and described in Figure 19. Figures represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated forms in each extract. The fold-increase represents the ratio of the average percentage conversion (and standard deviation) obtained with the c-fos-CAT constructs to that obtained with pB9+fp116. The numbers 1 to 42 correspond to lanes 1 to 42 of Figure 19.

more efficiently when their cis-acting transcriptional control elements are the correct distance from the c-fos promoter.

It was of interest to determine whether there were any proteins present in the F9 cells that would bind to the AP-1/ATF sequences that were transcriptionally inactive in the F9 cells as it could be argued that their transcriptional inactivity is not due to the absence of an activator protein but due to the presence of a protein that binds to these sequences and prevents transcriptional activation. For this purpose F9 nuclear protein extracts were made and gel mobility shift assays carried out using end-labelled oligonucleotides containing the AP-1/ATF sequences. This experiment is shown in Figure 20. Surprisingly the fosATF/AP-1, E3-ATF and E3-AP-1 sequences all form complexes which can be competed out by the corresponding cold oligonucleotide. Unlike the gel mobility shift assay with the HeLaS3 nuclear protein extract shown in Figure 13 the fosATF/AP-1 sequence seems to bind a protein and form a complex that is more like that formed with the E3-ATF sequence than the E3-AP-1 sequence. This suggests that the proteins in the F9 nuclear protein extracts that bind to these sequences may be different from those in the HeLaS3 nuclear protein extract. It is surprising that the AP-1/ATF sequences form complexes with proteins present in the F9 nuclear protein sequences since these sequences do not increase transcription from the c-fos promoter in transfection experiments with c-fos-CAT constructs in these cells (Figure 19 and Table 4). There could be two explanations for this observation.

1) The proteins from the F9 nuclear protein extracts that form

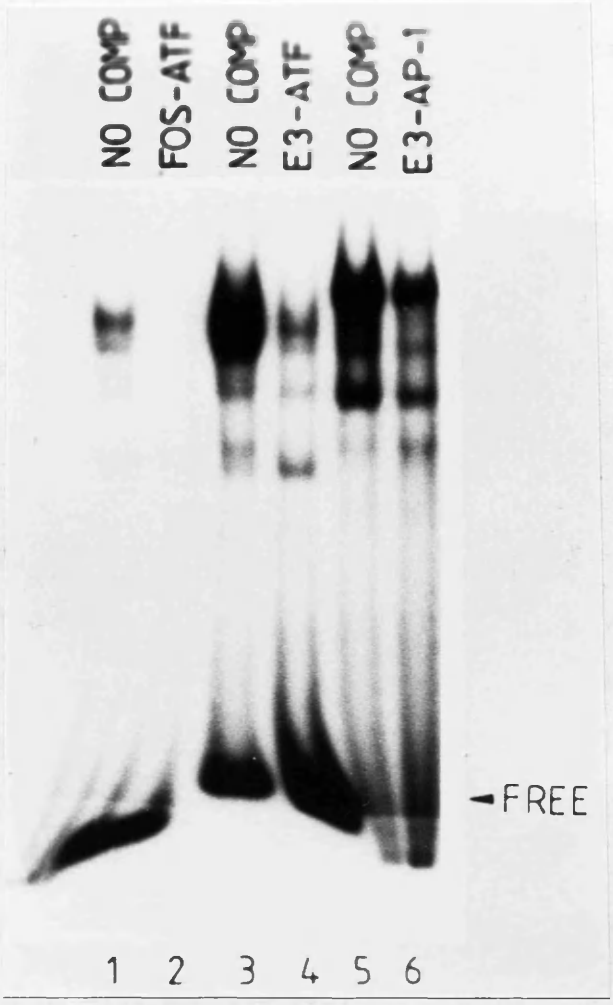


Figure 20. A gel mobility shift assay involving the incubation of fosATF/AP-1, E3-AP-1 and E3-ATF sequences with F9 nuclear protein extract.

Gel mobility shift assay using end-labelled oligonucleotides representing fosATF/AP-1 (lanes 1 and 2), E3-ATF (lanes 3 and 4) and E3-AP-1 (lanes 5 and 6) sequences. The oligonucleotides were incubated with equivalent amounts of F9 nuclear protein extract for 20 minutes at room temperature before the reaction mixture was electrophoresed through a 5%, 0.5xTBE, polyacrylamide gel (2.7.2.). Competitor oligonucleotides used are shown above each lane and were co-incubated with the labelled oligonucleotide, F9 nuclear protein extract mix. 0.1ng of probe used per reaction. 100ng of competitor DNA used in each lane where indicated. No comp : no competitor.

complexes with the AP-1/ATF sequences are different from those responsible for the transcriptional activation observed in CT3 cells transfected with c-fos-CAT constructs containing these sequences. These proteins could either prevent binding of transcriptional activators, or are themselves transcriptional repressors.

2) The F9 cells had undergone some form of differentiation prior to preparation of the nuclear protein extract, thus inducing the production of AP-1 like proteins.

The second explanation seems unlikely as the cells were cultured for only four days without passaging prior to harvesting and preparation of the nuclear protein extract, and the morphology of the cells did not change prior to harvesting as would be expected if the cells had "spontaneously" differentiated. This period of culture was similar to that used in the transfection experiments. To try and establish whether the proteins from the F9 and HeLaS3 nuclear protein extracts that form complexes with the AP-1/ATF sequences are different gel mobility shift assays involving pre-inclubation of the nuclear protein extracts with Fos antibodies prior to the gel mobility shift reactions were carried out.

3.3.4. Gel mobility shift assays with nuclear proteins extracts pre-incubated with Fos antibody.

The proteins that form complexes with AP-1 sequences include Fos protein(s) (280, 281, 307 - 309) and it is possible to disrupt the formation of the complex by pre-incubating the nuclear protein extract with a Fos antibody prior to carrying out the gel mobility

shift reactions. Such an experiment with HeLaS3 nuclear protein extract and AP-1/ATF sequences is shown in Figure 21 where the protein extracts were pre-incubated with Fos antibody overnight prior to carrying out the gel mobility shift assay reactions. As can be seen the Fos antibody pre-incubation disrupts the formation of the complex with the E3-AP-1 oligonucleotide (lane 2) and this agrees with previously published results. However, the inclusion of the Fos peptide to which the Fos antibody was raised in the pre-incubation with the nuclear protein extract does not seem to reverse the disruption of this complex as it has done in previously published results (lane 3). The reason for this is not clear but perhaps the Fos peptide has been "inactivated" in some way, perhaps due to degradation. The pre-incubation of the extract with the Fos antibody does not disrupt the formation of complexes with the E3-ATF or fosATF/AP-1 sequences. The lanes in this gel mobility shift assay show that the proteins are slightly more degraded than in the previous assays, and this is not surprising since the protein extracts were incubated overnight at $4^{o}C$.

Unfortunately, this experiment proved to be inconsistent when it was repeated, and when repeated with F9 and HeLaS3 nuclear protein extracts failed to produce a similar result, even with the HeLaS3 extracts. So, it was impossible to differentiate between the proteins from the F9 and HeLaS3 nuclear protein extracts that form complexes with the AP-1/ATF sequences.

One further attempt was made to try and establish whether an AP-1 or

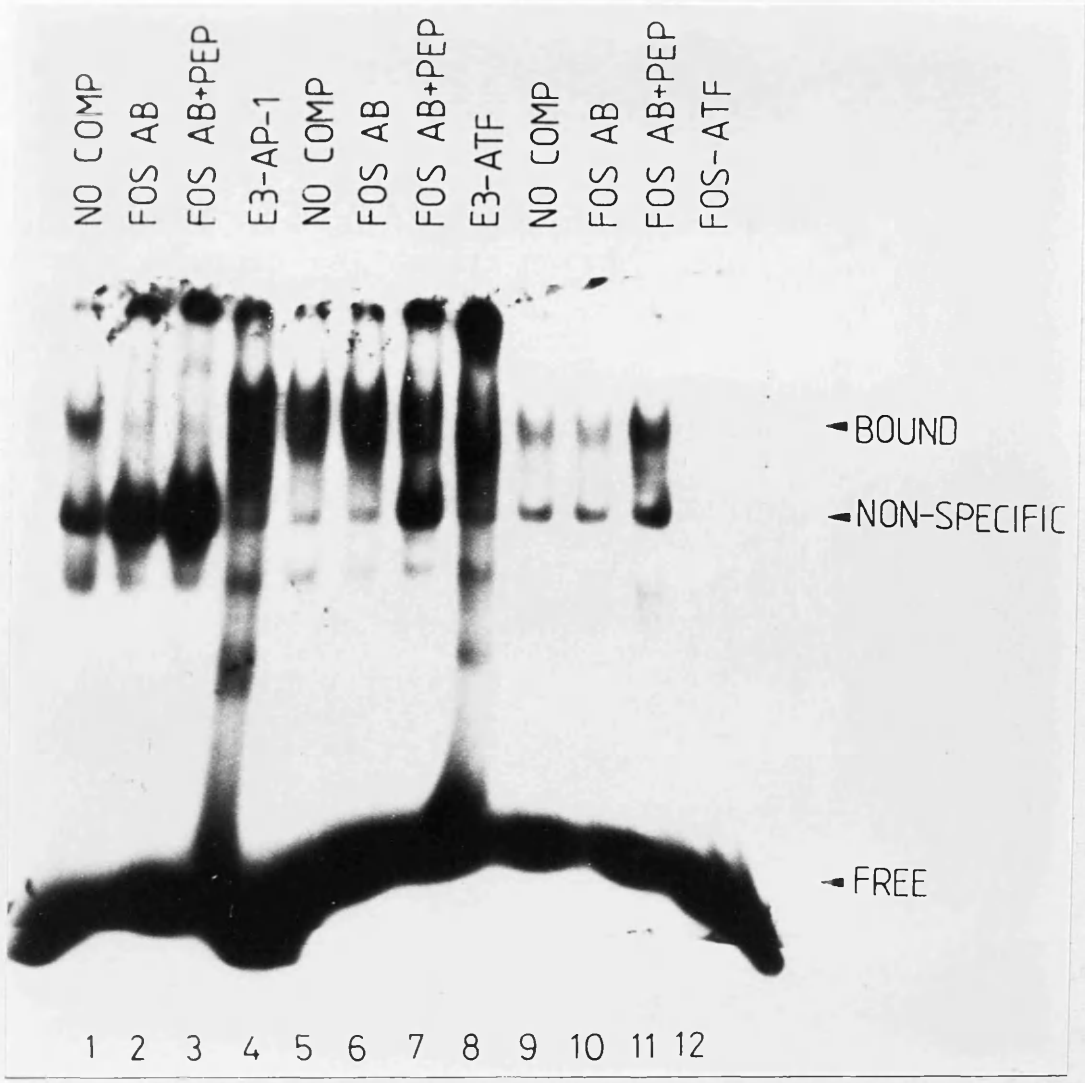


Figure 21. Gel mobility shift assay with HeLaS3 nuclear protein extracts pre-incubated with Fos antibody.

Gel mobility shift assay using end-labelled oligonucleotides representing E3-AP-1 (lanes 1 to 4), E3-ATF (lanes 5 to 8) and fosATF/AP-1 (lanes 9 to 12) sequences. Equivalent amounts of HeLaS3 nuclear protein extract were incubated overnight with or without Fos antibody or Fos antibody plus Fos peptide at 4°C. Fos antibody and Fos peptide treatment is indicated above the relevant lanes (FOSAB: Fos antibody; FOSAB+PEP: Fos antibody plus peptide). The oligonucleotides were then incubated with these mixtures at room temperature for 20 minutes before the reaction mixture was electrophoresed through a 4%, 0.5xTBE, polyacrylamide gel. Competitor oligonucleotides used are indicated above the relevant lanes and are co-incubated with the labelled oligonucleotide-protein mix. 0.1ng of probe used per reaction. 100ng of competitor DNA used in each lane where indicated. No comp : no competitor.

an ATF like protein binds to the fosATF/AP-1 sequence. So far the evidence has been conflicting with this sequence appearing to form a complex with a protein that shows a similar mobility to the complex formed with the E3-AP-1 oligonucleotide, but the Fos antibody experiment failed to disrupt the complex with the fos-ATF/AP-1 sequence while disrupting that which formed with the E3-AP-1 sequence. In order to try and establish whether this sequence binds an AP-1 or ATF like protein a gel mobility shift assay was carried out with *in vitro*-translated Fos and Jun proteins.

3.3.5. Gel mobility shift assay with in vitro translated Fos and Jun proteins.

The *in vitro* translated Fos and Jun proteins (a kind gift from Dr. M. Frame) used in this experiment were co-translated in a rabbit reticulocyte lysate using RNA transcribed from human cDNAs of fos and jun (314). A gel mobility shift assay using this *in vitro* translated protein is shown in Figure 22. As can be seen the two oligonucleotides which contain AP-1 sequences, E3-AP-1 and SRE-AP-1 (3.1.2.) form complexes with the *in vitro* translated proteins that are stronger than those formed with the other oligonucleotides. The SRE-AP-1 sequence seems to form an additional complex to that formed with the E3-AP-1 sequence. Because the AP-1 sequence in the SRE-AP-1 oligonucleotide is different from that in the E3-AP-1 oligonucleotide it is difficult to determine whether this additional complex is due to the presence of the SRE or the fact that the sequences are different. It should also be noted that the mobility of the complex formed between the *in vitro* translated proteins and the AP-1

Figure 22. Gel mobility shift assay using in vitro translated Fos and Jun proteins.

Gel mobility shift assay using end-labelled oligonucleotides representing fosATF/AP-1 (lanes 1 and 2), E3-AP-1 (lanes 3 and 4, lanes 11 and 12), E3-ATF (lanes 5 and 6), SRE-AP-1 (lanes 7 and 8) and SRE-ATF (lanes 9 and 10) sequences. In lanes 1 to 10 equivalent amount of in vitro translated Fos and Jun proteins were incubated with the oligonucleotides before the mixture was electrophoresed through a 5%, 0.5xTBE, polyacrylamide gel (2.7.2.). In lanes 11 and 12 equivalent amounts of HeLaS3 nuclear protein extract were used in the reactions. Competitor oligonucleotides used are shown above each lane and are co-incubated with the labelled oligonucleotide-protein mix. 0.1ng of probe used per reaction 100ng of competitor DNA used in each lane where indicated. No comp : no competitor.

sequences is different from that formed between the HeLaS3 nuclear protein extract and these sequences (compare lanes 3 and 7 with lane 11). The reason for this is not clear. The fosATF/AP-1 sequence forms a complex with the in vitro translated proteins although the mobility of this complex is much greater than that formed with HeLaS3 nuclear protein extract. The E3-ATF sequence also forms a complex with the in vitro translated proteins that shows a mobility similar to that formed with the AP-1 sequences, although the binding is much weaker. Interestingly, the SRE-ATF sequence forms a complex with the in vitro translated protein that is similar in mobility to the complex formed with the AP-1 sequences suggesting that the SRE sequence has some influence upon the formation of this complex since the fosATF/AP-1 sequence alone forms a complex that migrates much faster.

The experiments described so far have identified transcriptional control elements upstream from the human c-fos promoter and have attempted to characterise the proteins that bind to these sequences in gel mobility shift assays. It was decided to determine whether these sequences can activate transcription in response to extra-cellular stimuli such as serum or TPA, as has already been established for the SRE (185, 200, 201, 203, 204 216 - 218).

3.4. Identification of transcriptional control elements upstream from the c-fos promoter that are responsive to serum and TPA stimulation.

As described in Section 1.6., treatment of serum-deprived fibroblasts with mitogens results in transcriptional activation of the c-fos gene (104 - 107). Transfection studies using plamid constructs containing upstream c-fos sequences linked to a reporter gene revealed the presence of the SRE 300bp upstream from the c-fos CAP site which can respond to various mitogens such as serum, growth factors and phorbol esters to activate transcription from the c-fos promoter (185, 200, 203, 216 - 218). It was decided to further examine the role of the SRE in the transcriptional activation of the c-fos gene in response to serum and TPA. Previous studies had characterised AP-1 sequences as being both TPA and serum response elements (see sub-Section 1.6.6.). For this reason the role of the fosATF/AP-1 sequence in the activation of transcription from the c-fos promoter in response to serum and TPA stimulation was also examined. The possibility of an interaction between the proteins binding to the continuous SRE and fosATF/AP-1 sequences was also examined.

Transfection studies with the c-fos-CAT constructs described previously (see sub-Section 3.1.) were carried out to examine the role of various *cis*-acting DNA elements in the transcriptional activation of the c-fos promoter in response to stimulation of serum-deprived fibroblasts with mitogens. Once again the CT3 cell line was used in these transfection experiments as it is a derivative of NIH 3T3 cells which were used (along with Balb/c-3T3 cells) in

previous transfection experiments which identified DNA elements upstream from the c-fos promoter responsive to serum and TPA stimulation. In all of the transfection experiments carried out in this section the CT3 cells were plated out 16 to 24 hours prior to transfection and grown in medium containing 10% FCS. Sixteen hours after transfection the cells were washed with PBS and refed with medium containing 0.5% FCS. The cells were then maintained in 0.5% FCS for between 36 and 48 hours before stimulation (or not) with medium containing 15% FCS or medium containing 100ng/ml TPA with 0.5% serum. The cells were then harvested and protein extracts prepared for β-gal (2.6.2.) and CAT (2.6.3.) assays.

Before proceeding with transfection experiments Northern blot analyses were carried out with RNA prepared from serum deprived CT3 and HeLaS3 cells stimulated with either serum or TPA to confirm that the transcription of the endogenous c-fos gene is increased in response to these treatment.

3.4.1. Northern blot analyses of RNA prepared from serum and TPA stimulated CT3 and HeLaS3 cells.

The serum stimulated RNA samples used in this section were extracted from cells that were serum-deprived by being grown in medium containing 0.5% serum for 30 hours (by which time they were considered quiescent) prior to stimulation with medium containing 15% FCS. The cells were then harvested for RNA preparation at the indicated time intervals after addition of this medium. TPA stimulation was carried out on both proliferating cells (ie those

grown in medium containing 10% FCS prior to the TPA stimulation) and serum-deprived cells. The method of RNA extraction is detailed in each figure legend and described in sub-Section 2.2.1.

Figure 23 shows Northern blots carried out with the serum stimulated samples from the CT3 and HeLaS3 cells and probed with a v-fos fragment from pFos1 (2.3.2.) (A). The induction of the c-fos mRNA in the two cell lines displays similar kinetics to previously published results with the induction being both rapid and transient, peaking between 30 and 45 minutes and decreasing by 60 minutes and becoming undetectable after 120 minutes. The sizes of the bands detected (2.5 and 2.6kb in CT3 and HeLaS3 samples, respectively) are larger than previously reported c-fos mRNA (2.2kb and 2.3kb). The reason for this could be that the gels used in the electrophoresis of the RNA were small and could have led to inaccuracies in the measurement of the size of the bands. Figure 23B shows the same filters used in 23A re-probed with β_2-microglobulin to check that the distribution of the RNA between the lanes was equal.

Figure 24A shows Northern blots of RNA samples prepared from cells treated with 100ng/ml TPA. These cells were proliferating (ie were growing in medium containing 10% FCS) at the time of stimulation and no c-fos induction can be observed in these RNA samples. This was not entirely unexpected as previously reported TPA stimulations of c-fos expression had on some occasions been carried out on proliferating cells (as in Figure 24) and on other occasions on serum deprived cells. Presumably this variability is due to the different types of NIH 3T3 (or other 3T3 cell types) that were used in the labs

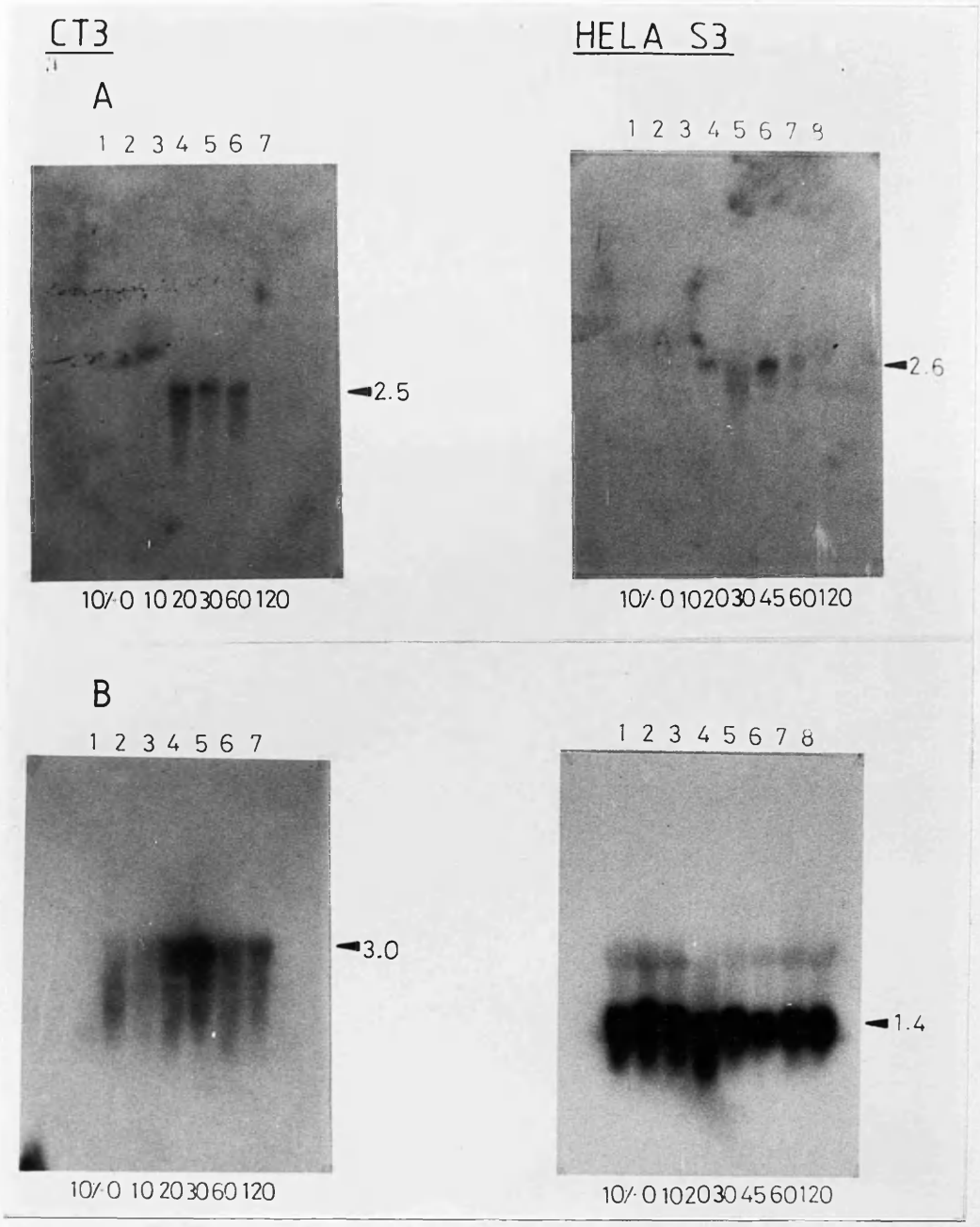


Figure 23. Induction of c-fos expression by serum stimulation of quiescent CT3 and HeLaS3 cells.

Northern blot analyses of RNA extracted from serum stimulated CT3 and HeLaS3 cells. In A the filters were probed with a 1kbp PstI fragment from pFos1 (v-fos). The size of the bands are indicated to the right of each blot. Figures at the top of each blot represent the lane numbers and the figures at the bottom the time in minutes between serum stimulation and cell harvest. 10% : RNA extracted from proliferating cells. In B the filters were re-probed with β_2-microglobulin to check the distribution of the RNA between the lanes. The RNA used in these blots was prepared using the phenol extraction method (2.2.1 a)). 15µg of RNA used per lane.

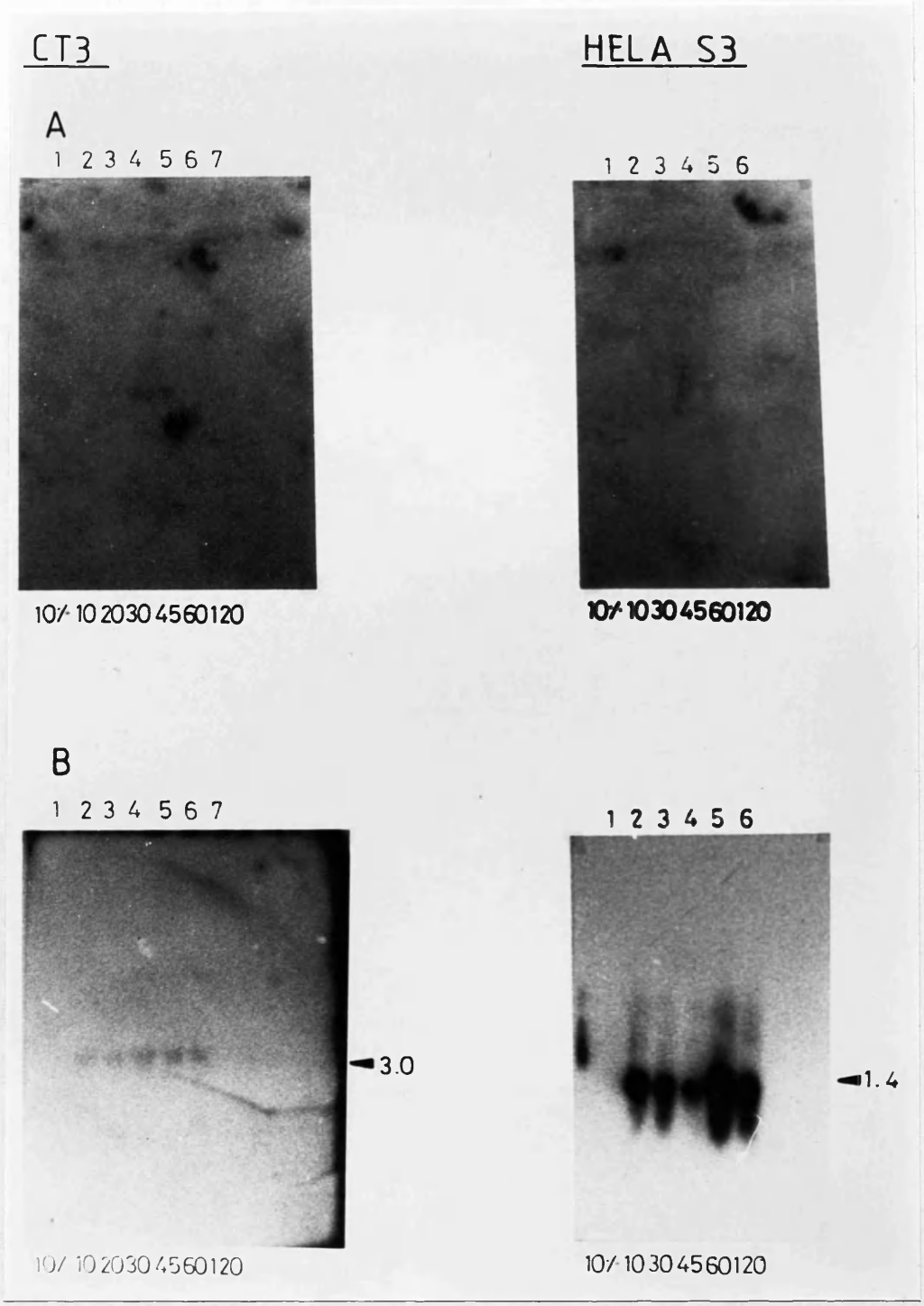


Figure 24. Failure of TPA to stimulate c-fos expression upon stimulation of proliferating CT3 and HeLaS3 cells.

Northern blot analyses of RNA extracted from proliferating CT3 and HeLaS3 cells stimulated with 100ng/ml TPA. In A the filters were probed with a 1kbp Pst1 fragment from pFos1 (v-fos). The figures at the top of each blot represent the lane number and the figures at the bottom the time in minutes between TPA stimulation and cell harvest. In B the filters were re-probed with β_2-microglobulin to check the distribution of the RNA between the lanes. The RNA used in these blots was prepared using the pheonol extraction method (2.2.1.a)). 15µg of RNA used per lane.

reporting these conflicting results. Figure 24B shows the same filters used in 24A re-probed with β_2-microglobulin to check that the distribution of the RNA between the lanes was consistent. The next step taken was to stimulate serum-derprived cells with medium containing 100ng/ml TPA and 0.5% FCS. The cells were made quiescent in the same manner as that described for the serum-stimulation experiment (Figure 23). The cells were then treated with the medium containing 100ng/ml of TPA (and 0.5% FCS) and RNA prepared from the cells at several intervals after the stimulation. The result of this experiment, shown in Figure 25A, shows that c-fos expression is induced in quiescent fibroblasts after stimulation with 100ng/ml TPA. This induction is very similar to previous studies as it peaks between 30 and 45 minutes and returns to basal levels after 60 minutes. The size of the c-fos transcript detected in this experiment, 2.3kb, is also similar to previously published results. Figure 25B shows the same filter used in 25A re-probed with β_2-microglobulin to check that the distribution of the RNA between the lanes was consistent.

From these Northern blot results it was established that in the transfection studies examining the induction of c-fos expression by TPA, the stimulations should be carried out with serum deprived fibroblasts.

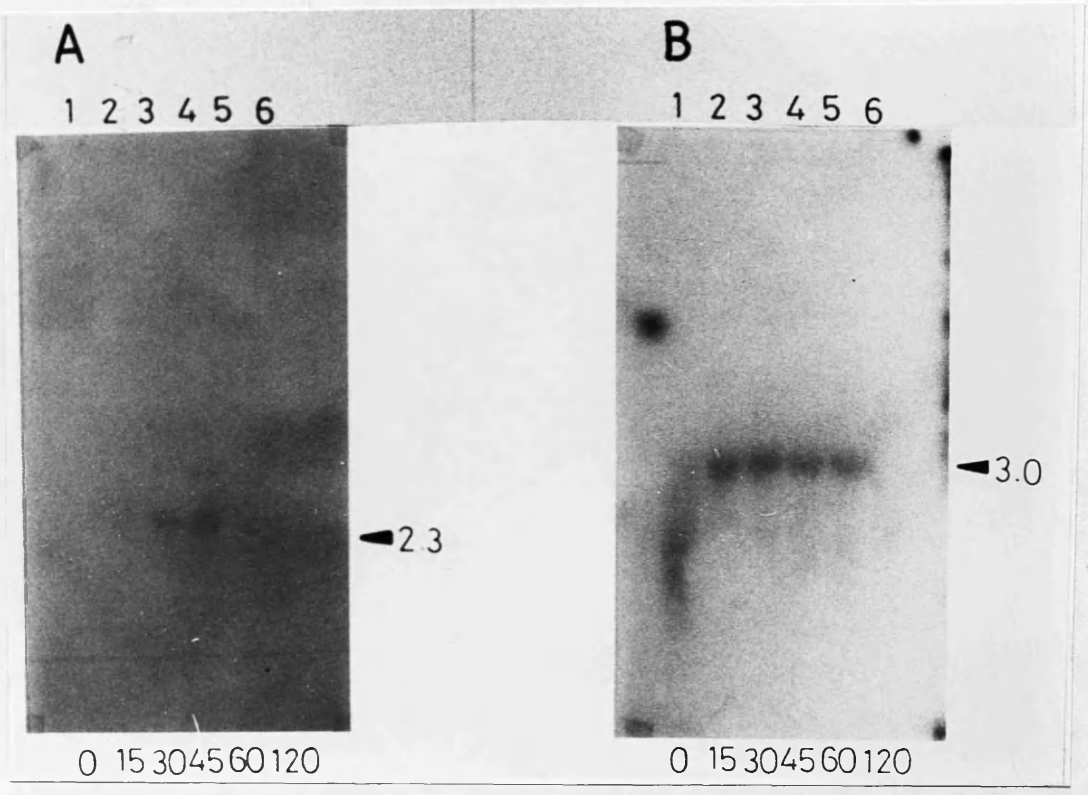


Figure 25. Induction of c-fos expression by TPA stimulation of quiescent CT3 cells.

Northern blot analysis of RNA extracted from serum deprived CT3 cells stimulated with 100ng/ml TPA. In A the filter was probed with a 1kbp Pst1 fragment from pFos1 (v-fos). The size of the bands are indicated to the right of each blot. Figures at the top of the blot represent the lane numbers and the figures at the bottom the time in minutes between TPA stimulation and cell harvest. In B the filter was re-probed with β_2-microglobulin to check the distribution of the RNA between the lanes. The RNA used in this blot was prepared using the RNAzol method (2.2.1.b)). 15µg of RNA used per lane.

3.4.2. Short-term serum or TPA stimulation of serum-deprived CT3 cells transfected with c-fos-CAT constructs.

In all of the experiments detailed in this section proliferating CT3 cells were transfected as described in 2.5.2., washed the following day and fed with medium containing 0.5% FCS. These cells were left for 36 hours and then stimulated with 15% FCS or 100ng/ml TPA as described, and harvested at various times thereafter.

A preliminary experiment with CT3 cells transfected with pB9+fp402, the c-fos-CAT construct that contains 400bp of intact upstream sequence which includes the SRE, is shown in Figure 26. Unfortunately this experiment (and others) was not quantified although it can clearly be seen that there is no increase in CAT activity with serum stimulated samples when compared to serum deprived samples. Indeed, the levels of CAT activities are about the same in serum-deprived and serum-stimulated cells and in those grown in 10% FCS up until cell harvest (+). This suggests that the serum deprivation of the CT3 cells has failed to shut down transcription from the c-fos promoter. Three reasons were thought of for this failure to observe the expected serum response:-

1) The c-fos-CAT construct is defective i.e. it does not initiate transcription from the correct position.

2) The cells were not quiescent prior to stimulation.

3) The density of the cells prior to stimulation plays a role

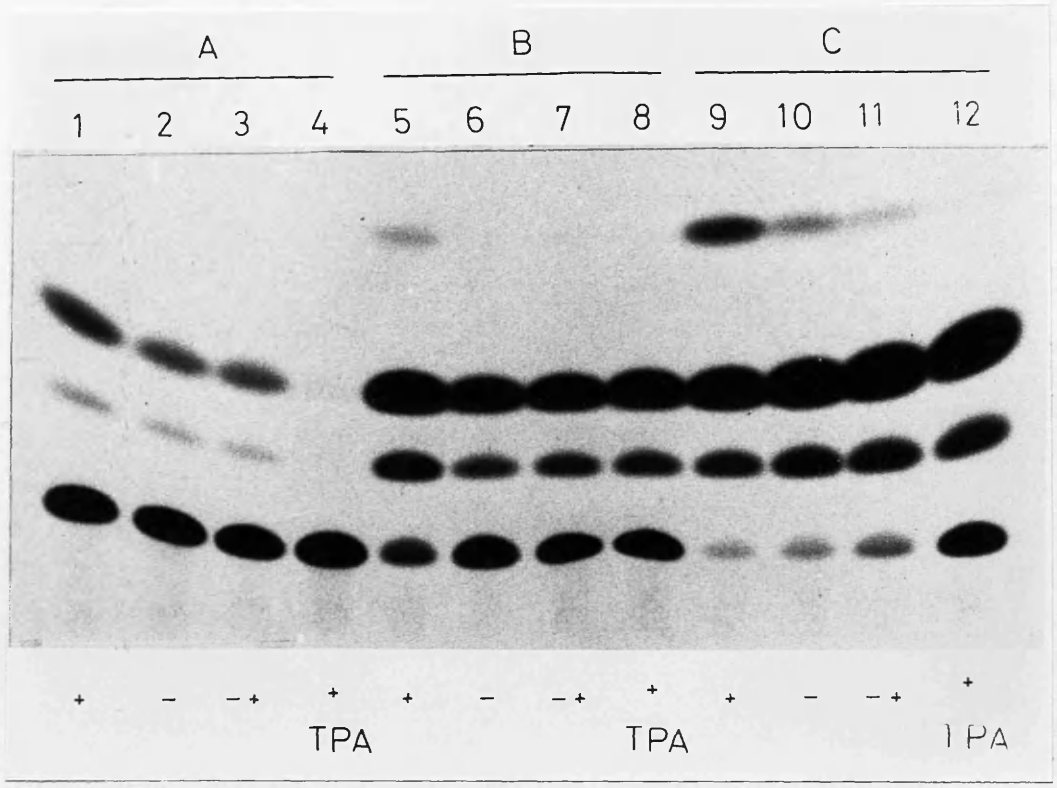


Figure 26. Serum and TPA stimulation of CT3 cells transfected with increasing amounts of pB9+fp402.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp402. 16 to 24 hours after plating the cells out at 10^6 per F75 flask the transfection cocktail was added to the flasks (2.5.2.). 16 hours after the addition of the cocktail cells were washed with PBS and then refed with medium containing either 10% FCS or 0.5% FCS. All cells were harvested 48 hours later. A represents a set of 4 flasks transfected with aliquots from the same transfection cocktail, each flask being transfected with 6µg of pB9+fp402. Similarly, the flasks in B and C were transfected with 15µg and 30µg of pB9+fp402 respectively. Lanes 1,5 and 9 represent extracts from cells grown in 10% FCS (+) whiles lanes 2, 6 and 10 represent extracts from cells grown in 0.5% serum (-). Lanes 3, 7 and 11 represent cells grown in 0.5% serum and treated with medium containing 15% FCS for one hour prior to harvest (- +). Lanes 4, 8 and 12 represent cells grown in 10% serum and treated with 100ng/ml of TPA one hour prior to cell harvest (+ TPA). The protein concentration of each cell extract was estimated using the Biorad Assay (2.6.1.) and equivalent amounts of protein were used in each CAT assay.

in the response of the c-fos-CAT constructs to serum.

Point 1 seems unlikely for two reasons. Firstly, primer extension and S1 analyses of the parent c-fos-CAT construct, pCN+fp1400 (Figure 7 and 8, 3.1.3.), demonstrated that the transcription initiation from this construct is occuring at the correct position. Secondly, transfection with constructs containing the SRE results in a clear increase in transcription as compared to constructs lacking this sequence (Figure 1, Table 1, 3.2.1.) which is in agreement with previously published results. Point 2 also seems unlikely since serum deprivation for 36 hours has previously been described as sufficient to induce quiescence in these cells (104). Also, microscropic examination of the cells used in these experiments (an example of which is shown in Figure 26) showed the serum-deprived cells to be less dense than those grown in serum throughout the experiment, suggesting that they had stopped growing.

To test the third possibility, that cell density is important in the response of the c-fos-CAT constructs to serum stimulation, CT3 cells were plated out at three different densities, transfected with pB9+fp402 or pB9fp116SRE, and then either serum-starved or serum-starved and then stimulated with medium containing 15% FCS for 1 hour prior to being harvested. The result of this experiment is shown in Figure 27 and quantified in Table 5. In the CAT assays carried out with extracts prepared from CT3 cells transfected with pB9+fp402 it can be seen that there is no increase in CAT activity in serum-stimulated samples as compared to serum-deprived samples at the two lower cell densities. At the higher cell density a 2-fold

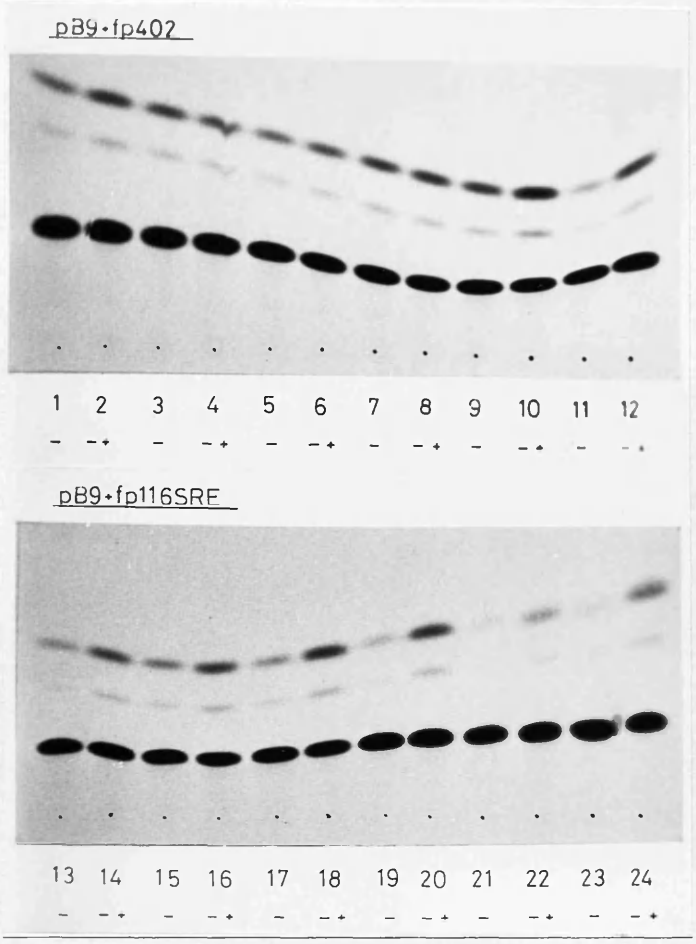


Figure 27. The effect of cell density upon the serum stimulation of c-fos-CAT constructs in serum-deprived CT3 cells.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp402 (1 to 12) or pB9+fp116SRE (13 to 24). Cells were plated out at three different densities : A; $5x10^5$ cells per F75 flask : B; 10^6 cells per F75: C; $1.5x10^6$ cells per F75. 16 to 24 hours after plating the cells out the transfection cocktail was added to each flask (2.5.2.). 16 hours after the addition of the cocktail cells were washed with PBS and then refed with medium containing 0.5% serum. All cells were harvested 36 hours later. Transfection cocktails were made and divided between two flasks to form duplicates and one flask of cells from each pair remained serum deprived until cell harvest (-) while the other was stimulated with medium containing 15% FCS for 1 hour prior to cell harvest (-+). 7.5µg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 7.5µg of HSV-β-gal plasmid. β-gal assays were carried out on aliquots of the cell extract and equivalent amount of β-gal activity were used in each CAT assay.

	PLASMID CONSTRUCT			
CELL DENSITY	pB9+fp402		pB9+fp116SRE	
	-	-+	-	-+
$5x10^5$	1 8.3 3 10.2	2 11.8 4 8.9	13 3.5 15 4.1	14 7.2 16 11.0
AVERAGE CONVERSION	9.3	10.4	3.8	9.1
STANDARD DEVIATION	1.3	1.5	0.4	2.7
FOLD INDUCTION	1.0+\-0.1	1.1+\-0.2	1.0+\-0.1	2.4+\-0.7
$1x10^6$	5 6.1 7 9.6	6 8.8 8 12.8	17 3.1 19 2.2	18 12.3 20 9.8
AVERAGE CONVERSION	7.9	10.8	2.7	11.1
STANDARD DEVIATION	2.1	2.8	0.6	1.8
FOLD INDUCTION	1.0+\-0.3	1.4+\-0.4	1.0+\-0.2	4.1+\-0.7
$1.5x10^6$	9 11.0 10 3.7	10 17.9 12 11.8	21 1.7 23 2.0	22 4.7 24 13.6
AVERAGE CONVERSION	7.4	14.9	1.9	9.2
STANDARD DEVIATION	5.0	4.0	0.2	6.1
FOLD INDUCTION	1.0+\-0.7	2.0+\-0.5	1.0+\-0.1	5.0+\-0.3

Table 5. The effect of cell density upon the serum stimulation of c-fos-CAT constructs in serum starved CT3 cells.

This table quantifies the CAT activity obtained in the experiment shown and described in Figure 21. Figures represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated forms in each extract. The fold-induction represents the ratio of the average percentage conversion (and standard deviation) obtained with the serum stimulated samples (-+) to the average percentage conversion obtained with the corresponding serum deprived samples (-). The numbers 1 to 36 correspond to lanes 1 to 36 of Figure 27.

induction of CAT activity is observed with the serum stimulated samples, although the standard deviations of the results obtained with both the serum-starved and serum-stimulated samples are rather large making it difficult to interpret this result and to decide if it is significant. However, with pB9+fp116SRE an increase in CAT activity can be observed in serum stimulated samples as compared to serum-deprived samples. The fold-increase in CAT activity is significant at all cell densities, although the fold-induction of CAT activity in the serum stimulated samples increases with increasing cell density (2.4 fold-induction at the lowest cell density compared to a 5-fold induction at the highest cell density). So, both constructs give results which suggest that the density of the cells in the culture into which the c-fos-CAT constructs are transfected plays an important role in the detection of a serum response in CAT assays carried out with extracts prepared from serum-deprived and serum-stimulated cells.

To determine whether other c-fos-CAT constructs were serum responsive, a transfection experiment similar to the one described in Figure 27 was carried out, the cells being plated out at the highest density used in the previous experiment, i.e. $1.5x10^6$ per F75 flask, the day before transfection. The result of this experiment is shown in Figure 28 and quantified in Table 6. An experiment where the cells were plated out at $7x10^5$ cells per F25 is also quantified in Table 6 and represents Experiment B. In Experiment A the results obtained with pB9+fp402 confirms that at a high cell density the transcription of this construct is induced following serum stimulation. However in Experiment B pB9+fp402 failed to show a

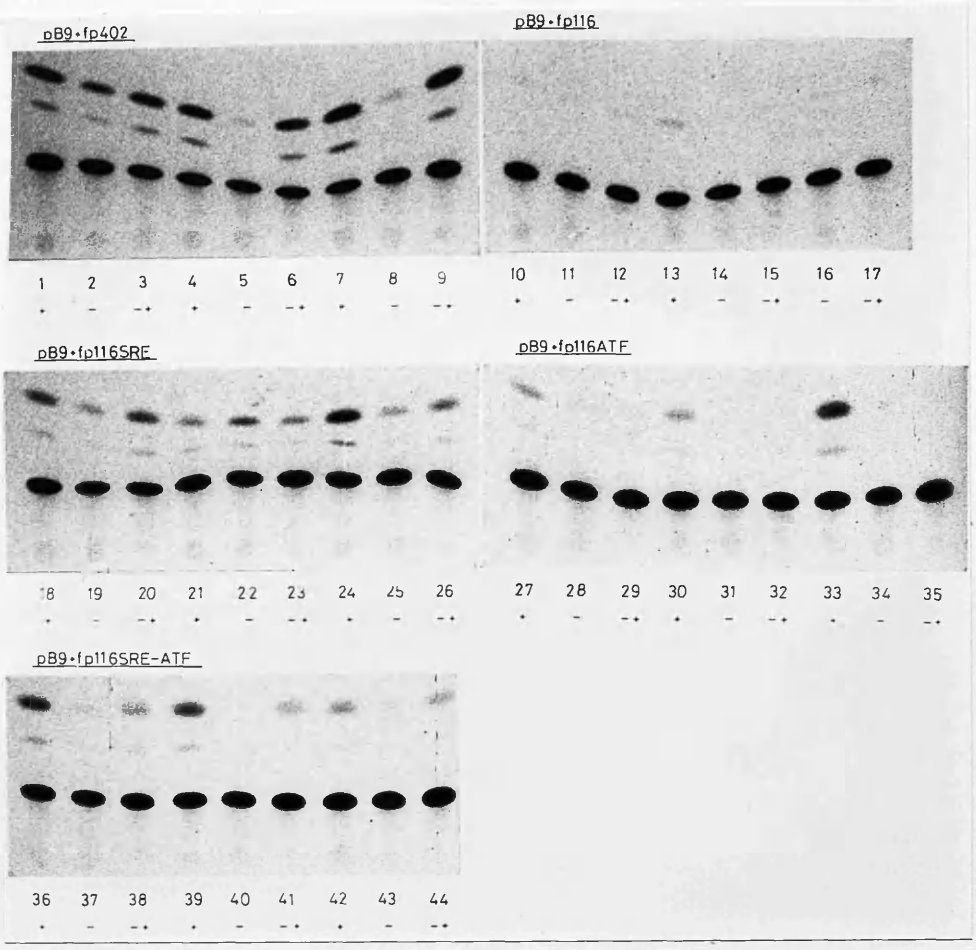


Figure 28. Serum stimulation of c-fos-CAT constructs in high density serum deprived CT3 cells.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp402 (1 to 9), pB9fp+116 (10 to 17), pB9+fp116SRE (18 to 26), pB9+fp116ATF (27 to 35) or pB9+fp116SRE-ATF (36 to 44). The experiment shown above is quantified in Table 6 and represents Experiment A. Experiments A and B gave similar results and therefore only one of them is represented in this figure. In Experiment A the cells were plated out at 1.5×10^6 cells per F75 tissue culture flask while in Experiment B they were plated out at 7 10 cells per F25. 16 to 24 hours later the transfection cocktail was added to each flask (2.5.2.). 16 hours after the addition of the cocktail cells were washed with PBS and then refed with medium containing either 0.5% FCS or 10% FCS. All cells were harvested 36 hours later. Transfection cocktails were made and aliquoted into 3 flasks to form triplicates. One flask from each triplicate was grown in serum (10%) throughout the experiment (+), one was serum deprived for 36 hours before harvest (-) and the other was serum starved but stimulated with medium containing 15% FCS for 1 hour prior to cell harvest (-+). 7.5µg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 7.5µg of HSV-β-gal plasmid. β-gal assays were performed on aliquots from each extract, and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

	PLASMID CONSTRUCT								
	pB9+fp402			pB9+fp116			pB9+fp116SRE		
CONDITION OF CELLS	+	-	-+	+	-	-+	+	-	-+
EXPERIMENT A	1 9.2 4 9.6 7 17.0	2 5.5 5 0.6 8 1.3	3 10.9 6 8.7 9 13.1	10 0.6 13 1.1	11 0.3 14 0.4 16 0.3	12 0.3 15 0.3 17 0.5	18 4.2 21 2.2 24 11.2	19 1.7 22 3.8 25 2.2	20 4.1 23 2.3 26 3.2
AVERAGE CONVERSION	11.9	2.5	10.9	0.9	0.3	0.4	5.9	2.6	3.2
STANDARD DEVIATION	6.4	2.6	2.2	0.3	0.1	0.1	4.8	1.0	0.9
FOLD INCREASE		1.0+\-1.0	4.4+\-0.8		1.0+\-0.3	1.3+\-0.3		1.0+\-0.4	1.2+\-0.3
EXPERIMENT B	5.8 4.9 7.5	4.0 4.2 4.4	4.4 4.5 6.1	0.6 1.1 0.7	0.4 0.6 0.7	0.5 0.8 0.6	1.9 6.5 3.4	3.2 1.3 1.3	3.7 3.5 2.0
AVERAGE CONVERSION	6.1	4.2	5.0	0.8	0.6	0.6	3.9	1.9	3.1
STANDARD DEVIATION	1.1	0.2	1.0	0.3	0.2	0.2	2.4	1.2	0.7
FOLD INDUCTION		1.0+\-0.0	1.2+\-0.2		1.0+\-0.3	1.0+\-0.3		1.0+\-0.6	1.6+\-0.4

PLASMID CONSTRUCT									
	pB9+fp116ATF				pB9+fp116SRE-ATF				
CONDITION OF CELLS	+		-	-+	+		-		-+
EXPERIMENT A	27 2.3 30 2.3 33 9.8		28 0.5 31 0.3 34 0.8	29 0.5 32 0.4 35 0.5	36 8.5 39 6.5 42 3.1		37 0.9 40 0.3 43 0.8		38 3.4 41 2.6 44 2.3
AVERAGE CONVERSION	4.8		0.5	0.5	6.0		0.7		2.8
STANDARD DEVIATION	4.3		0.3	0.1	3.0		0.3		0.2
FOLD INDUCTION			1.0+\-0.6	1.0+\-0.2			1.0+\-0.4		4.0+\-0.3
EXPERIMENT B	0.8 0.8 0.9		0.2 0.4 0.4	0.4 0.2 0.4	2.6 2.3 4.1		0.9 0.6 1.0		1.7 2.5 1.7
AVERAGE CONVERSION	0.8		0.3	0.3	3.0		0.8		2.0
STANDARD DEVIATION	0.3		0.1	0.1	1.0		0.4		0.1
FOLD INDUCTION			1.0+\-0.3	1.0+\-0.3			1.0+\-0.5		2.5+\-0.2

Table 6. Serum stimulation of c-fos-CAT constructs in high density serum deprived CT3 cells.

This table quantifies the CAT activity obtained in the experiment shown and described in Figure 28. Figures represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated forms in each extract. The fold-induction represents the ratio of the average percentage conversion (and standard deviation) obtained with the serum stimulated samples(-+) to the average percentage conversion obtained with the corresponding serum deprived samples (-). The numbers 1 to44 correspond to lanes 1 to 44 of Figure 28. In experiment B greater amounts of extract were used with the pB9+fp116 samples leading to the comparatively elevated levels of activity.

response following serum stimulation which contradicts the results obtained in Experiment A. The reason for this is not clear but perhaps the density of the cells used in Experiment B was too low preventing pB9+fp402 from showing a serum response. This would agree with the results obtained with the experiment shown in Figure 27 which suggested that only at a high density of cells would pB9+fp402 show a serum response. In Experiments A and B pB9+fp116SRE fails to show a serum response and this is surprising as in the previous experiment (Figure 27) a serum response could be observed with this construct, even at a low cell density. The reason for this failure is unclear but could be related to the relatively high levels of activity in the serum deprived samples. This contrasts with pB9+fp116SRE-ATF which gives a consistent serum response in both experiments. It should be noted that pB9+fp116SRE-ATF gives a level of basal expression in the serum deprived samples that is 2- to 3-fold lower than that obtained with pB9+fp116SRE. pB9+fp116 shows no increase in CAT activity in response to serum and this is as expected since no serum-responsive cis-acting DNA elements are present in this construct. Interestingly, pB9+fp116ATF is sensitive to serum deprivation as in the serum starved samples the level of CAT activity is much reduced when compared to that obtained with samples taken from cells grown in 10% FCS throughout the experiment. The fosATF/AP-1 sequence is not responsive to serum as serum stimulation of cells transfected with pB9+fp116ATF gives no increase in CAT activity.

A general feature observed with all the constructs was a reduction in transcriptional activity when the cells were serum deprived compared

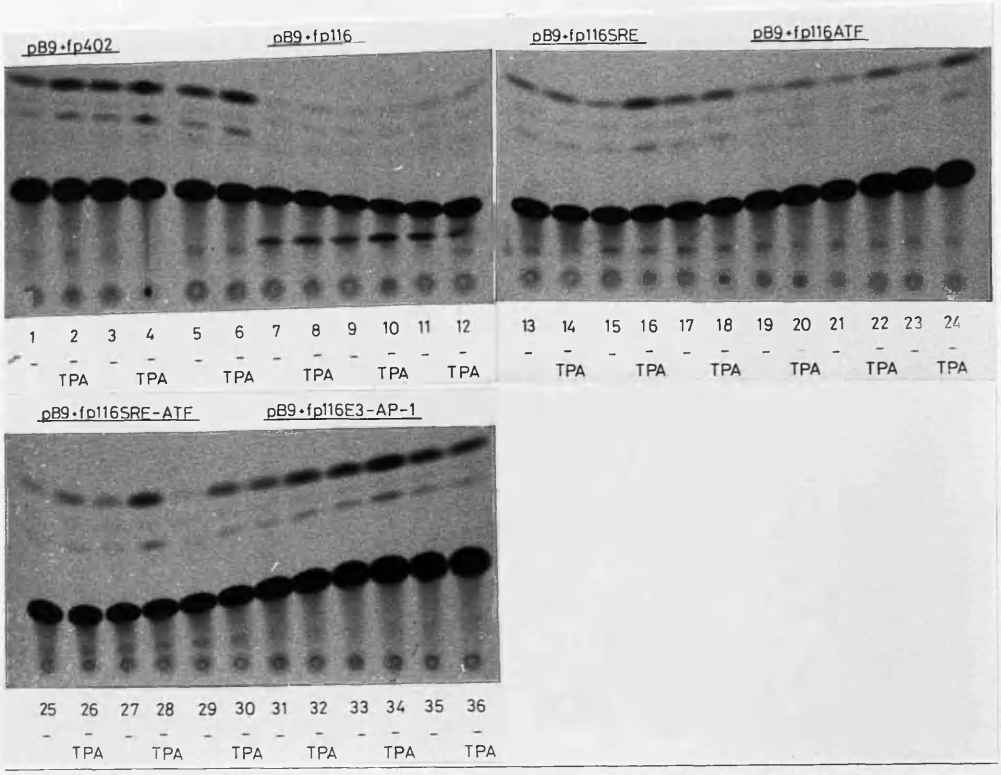


Figure 29. TPA stimulation of c-fos-CAT constructs in high density serum deprived CT3 cells.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp402 (1 to 6), pB9+fp116 (7 to 12), pB9+fp116SRE (13 to 18), pB9+fp116ATF (19 to 24), pB9+fp116SRE-ATF (25 to 30) and pB9+fp116E3-AP-1 (31 to 36). The cells were plated out at 1.5×10^6 cells per F75 tissue culture flask and 16 to 24 hours later the transfection cocktail was added to each flask (2.5.2.). 16 hours after the addition of the cocktail cells were washed with PBS and then refed with medium containing 0.5% FCS. All cells were harvested 36 hours later. Transfection cocktails were made and divided into 2 flasks to form duplicates and one flask of cells from each pair remained serum deprived until cell harvest (-) while the other was stimulated wiht medium containing 100ng/ml TPA, 0.5% FCS for 1 hour prior to cell harvest (- TPA). 7.5µg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 7.5µg of HSV-β-gal plasmid. β-gal assays were carried out on aliquots from each cell extract and in this experiment differing amounts of β-gal activity were taken for CAT assays. Aliquots representing 0.11 $O.D._{420}$ β-gal units were taken from extracts representing cells transfected with pB9+fp402, pB9+fp116SRE, pB9+fp116SRE-ATF and pB9+fp116E3-AP-1, while 0.17 $O.D._{420}$ β-gal units were taken from extracts representing cells transfected with pB9+fp116ATF and 0.25 $O.D._{420}$ β-gal units were used from extracts representing cells transfected with pB9+fp116.

	PLASMID CONSTRUCT											
TREATMENT OF CELLS	pB9+fp402				pB9+fp116				pB9+fp116SRE			
	-		-TPA		-		-TPA		-		-TPA	
	1	1.2	2	2.8	7	0.3	8	0.3	13	0.6	14	0.8
	3	1.9	4	4.2	9	0.3	10	0.4	15	0.4	16	2.2
	5	1.6	6	2.5	11	0.3	12	0.3	17	0.8	18	1.1
AVERAGE CONVERSION	1.6		3.2		0.3		0.3		0.6		1.4	
STANDARD DEVIATION	0.4		0.7		0.0		0.1		0.2		0.6	
FOLD INDUCTION	1.0+\-0.3		2.0+\-0.4		1.0+\-0.0		1.0+\-0.3		1.0+\-0.3		2.3+\-1.0	
	PLASMID CONSTRUCT											
TREATMENT OF CELL	pB9+fp116ATF				pB9+fp116SRE-ATF				pB9+fp116E3-AP-1			
	-		-TPA		-		-TPA		-		-TPA	
	19	0.4	20	0.7	25	0.5	26	0.9	31	1.0	32	2.1
	21	0.5	22	0.9	27	0.8	28	2.3	33	2.1	34	3.5
	23	0.4	24	1.5	29	0.3	30	1.3	35	2.2	36	1.7
AVERAGE CONVERSION	0.4		1.0		0.5		1.5		1.8		2.4	
STANDARD DEVIATION	0.1		0.4		0.3		0.7		0.5		1.1	
FOLD INDUCTION	1.0+\-0.3		2.5+\-1.0		1.0+\-0.6		3.0+\-1.4		1.0+\-0.3		1.3+\-0.6	

Table 7. TPA stimulation of c-fos-CAT constructs in high density serum deprived CT3 cells.

This table quantifies the CAT activity obtained in the experiment shown and described in Figure 29. Figures represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated forms in each extract. The fold induction represents the ratio of the average percentage conversion (and standard deviation) obtained with the TPA stimulated samples (-TPA) to the average percentage conversion obtained with the corresponding serum deprived samples (-). The numbers 1 to 36 correspond to lanes 1 to 36 of Figure 29.

to the activity obtained with cells grown in 10% FCS throughout the experiment. This is not a general feature of all enhancer/upstream promoter elements as the HSV-β-gal construct (used an an internal control) gives equivalent levels of β-gal activity in serum-deprived and serum-stimulated cells and cells grown in 10% FCS throughout the experiment. To determine whether any of the c-fos sequences increase transcription from the c-fos promoter in response to TPA stimulation of cells an experiment similar to that detailed in Figure 28 was carried out. It had been shown previously that with CT3 cells the cells must be serum deprived and then TPA stimulated (Figure 24 and 25) to induce the expression of the endogenous c-fos gene and therefore in the transfection experiment the cells were treated as described for Figure 28 only they were stimulated with medium containing 100ng/ml TPA (and 0.5% FCS) for one hour before cell harvest. This experiment is shown and described in Figure 29 and quantified in Table 7. The transfection efficiency in this experiment was poor leading to low levels of β-gal activity obtained in β-gal assays carried out with aliquots from the transfected cell extracts. This resulted in a variation between the c-fos-CAT constructs of the amount of β-gal activity used in the CAT assasy, the details of which are given in Figure 29.

The SRE has been resported by other investigators to activate transcription when cells are stimulated with TPA (185) and so it was expected that constructs containing this element would give an increase in CAT activity in response to TPA stimulation. As expected pB9+fp116 showed no increase in transcription in response to TPA stimulation as it contains no putative TPA responsive elements; also

as expected pB9+fp402, pB9+fp116SRE and pB9+fp116SRE-ATF gave a 2 to 3-fold increase in CAT activity in response to TPA stimulation as these constructs all contain the SRE. The fosATF/AP-1 site had not been previously reported as being TPA responsive at the time of this experiment and here it can be seen that TPA stimulation leads to a 2.5-fold increase in CAT activity with pB9+fp116ATF. In this experiment (Figure 29 and Table 7) the E3-AP-1 sequence, present in pB9+fp116E3-AP-1, does not increase CAT activity upon TPA stimulation. Previous experiments identfying AP-1 as being a putative TPA responsive element involved the overnight stimulation of serum deprived fibroblast cells with TPA (248) and so perhaps it is not suprising that this sequence does not respond to TPA after stimulation of quiescent fibroblasts for 1 hour.

Several major points can be made from the short-term (1 hour) serum and TPA stimulation experiments described so far:

i) Cells must be plated out at a high density prior to transfection in order to observe a serum response, at least with the construct containing the 400bps intact c-fos upstream sequence, pB9+fp402 (Figure 17 and Table 5).

ii) Constructs containing the SRE are both serum and TPA responsive (Figure 28 and 29).

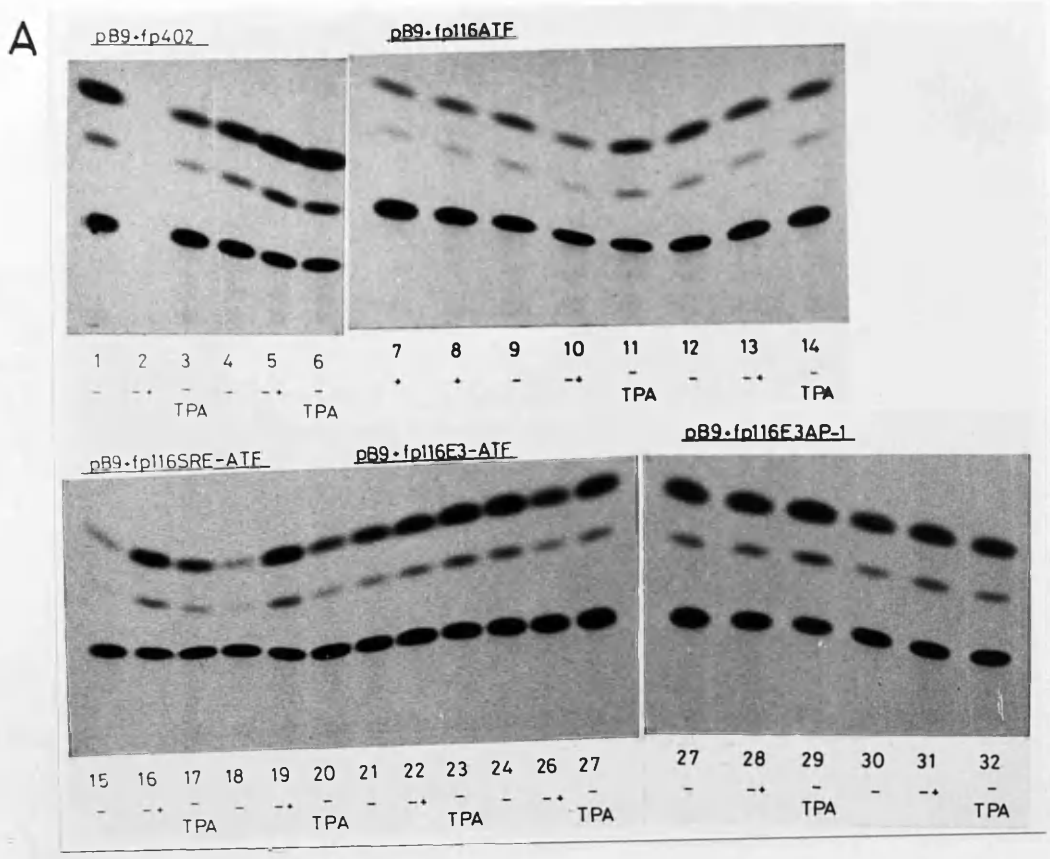
iii) Transcriptional activation of the c-fos promoter by the fosATF/AP-1 sequence (present in pB9+fp116ATF) is shut down in serum deprived cells (Figure 28).

iv) In these short-term stimulation experiments the fosATF/AP-1 sequence does not increase transcription in response to serum stimulation, but does in response to TPA stimulation (Figure 28 and

29).

v) An AP-1 site (present in pB9+fp116E3-AP-1) does not increase transcription from the c-fos promoter in response to a short-term stimulation with TPA (Figure 29).

In Figure 29 the transfection efficiency in the experiment shown was very low in comparison to other experiments. It was therefore decided that this experiment should be repeated in order to better characterise the fosATF/AP-1 sequence as being TPA responsive folllowing a 1 hour TPA stimulation. For this purpose further transfection experiments were carried out that were similar to those carried out in Figures 27 to 29. Transfection cocktails were divided into three flasks that allowed for a serum deprived sample, a serum deprived and serum stimulated sample and a serum deprived and TPA stimulated sample. This allows for a direct comparison between the serum and TPA stimulated samples. In all of these experiments pB9+fp116SRE-ATF was included which acts as a positive control as in the previous serum and TPA stimulation experiments this construct gave the highest and most consistent inductions following serum or TPA stimulation. Two such experiments are shown in Figure 30 in which the cells were plated out at 2 different densities to determine whether cell density influences the transcriptional properties of other c-fos-CAT constructs. Unfortunately an error was made in cutting up the plates for counting the amount of ^{14}C-chloramphenicol converted to mono-acetylated forms and so this experiment was not quantified. However by examining the CAT assay plates by eye several points may be made. The results clearly do not agree with the


A
pB9·fp402
pB9·fp116ATF
1 2 3 4 5 6
7 8 9 10 11 12 13 14
TPA
pB9·fp116SRE-ATF
pB9·fp116E3-ATF
pB9·fp116E3AP-1
15 16 17 18 19 20 21 22 23 24 26 27
27 28 29 30 31 32

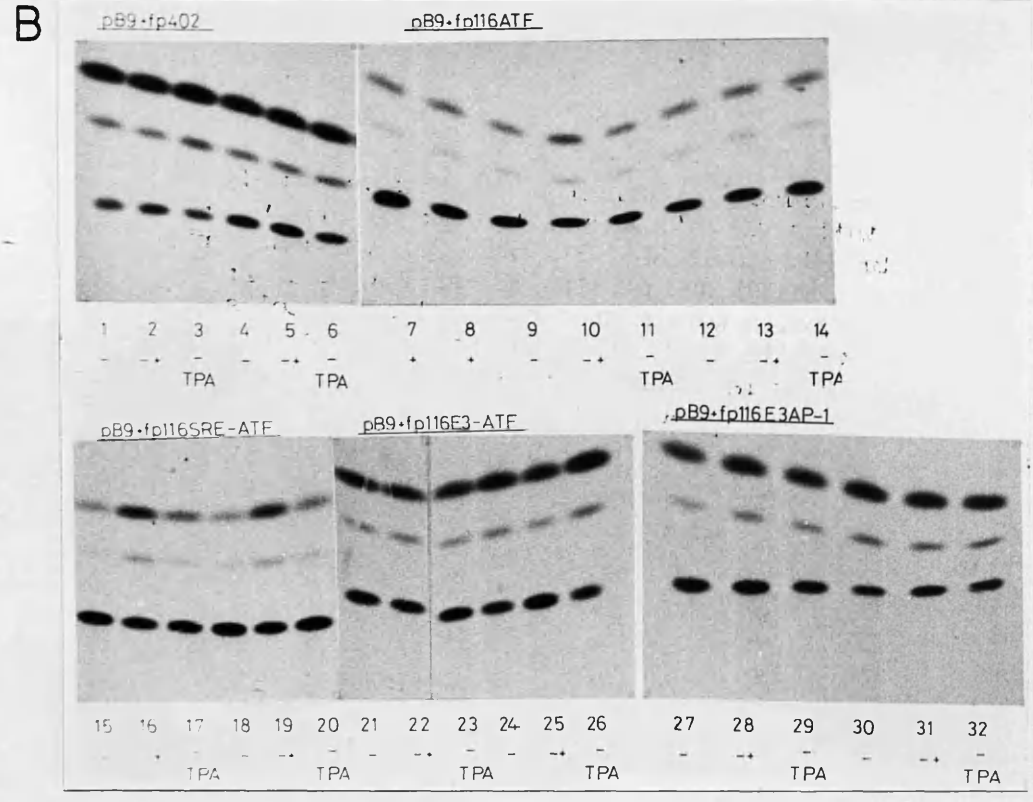

B
pB9·fp402
pB9·fp116ATF
1 2 3 4 5 6
7 8 9 10 11 12 13 14
TPA
pB9·fp116SRE-ATF
pB9·fp116E3-ATF
pB9·fp116E3AP-1
15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32

Figure 30. Serum and TPA stimulation of c-fos-CAT constructs in two densities of serum deprived CT3 cells.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp402 (lanes 1 to 6; A and B), pB9+fpATF (7 to 14; A and B), pB9+fp116SRE-ATF (15 to 20; A and B), pB9+fp116E3-ATF (21 to 26; A and B) and pB9+fp116E3-AP-1 (27 to 32; A and B). In Figure 30A the cells were plated out at 1.5×10^6 cells per F75 tissue culture flask while in 30B the cells were plated out at 10^6 per F75 and 16 to 24 hours later the transfection cocktail was added to each flask (2.5.2.) 16 hours after the addition of the cocktail cells were washed with PBS and then refed with medium containing 0.5% FCS (or 10% FCS in lanes 7 and 8; A and B). All cells were harvested 36 hours later. Transfection cocktails were made and divided into 3 flasks to form triplicates. One flask from each triplicate was grown in 0.5% FCS up until cell harvest (-), one was serum stimulated with medium containing 15% FCS for 1 hour prior to cell harvest (- +), and the other stimulated with medium containing 100ng/ml TPA; 0.5% FCS for an identical period prior to cell harvest (- TPA). In lanes 7 and 8 (A and B) the cells were transfected with aliquots from the same cocktail and grown in medium containing 10% FCS until cell harvest. 10µg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 10µg of HSV-β-gal plasmid. β-gal assays were performed on aliquots from each extract, and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

previous observation that at a high cell density (i.e. when cells are plated out at $1.5x10^6$ cell per F75 flask prior to transfection) a serum and/or TPA response can be observed with pB9+fp402 (Figures 27, 28 and 29). In this experiment clearly no such responses occur: the CAT activity is high even in the serum-deprived samples resulting in a failure to observe any increase in CAT activity in response to the stimulations. This is similar to the results obtained with the experiment described in Figure 26. With pB9+fp116ATF there is also an increase in the levels of CAT activity obtained in the serum deprived cells in comparison to previous experiments. This increase has resulted in a failure to observe any increase in CAT activity by this construct following TPA stimulation. The construct which contains the SRE and fosATF/AP-1 sequences inserted on an oligonucleotide upstream from the c-fos promoter, pB9+fp116SRE-ATF, is the only construct to give an increase in CAT activity in reponse to serum or TPA stimulation. The serum response obtained with this construct was greater than that obtained with TPA (compare lanes 22 and 25 with lanes 23 and 26) and this agrees with studies of the transcriptional stimulation of the endogenous c-fos gene where the increase in transcription obtained with growth factor or serum stimulation is greater than that obtained with TPA stimulation (106, 218). The results obtained when the cells were plated out at a lower cell density prior to transfection were identical to those obtained with the higher density (compare Figure 30A with Figure 30B) with pB9+fp116SRE-ATF again being the only construct to give an increase in CAT activity upon serum or TPA stimulation. The results obtained from the experiment described in Figure 30 are puzzling when Figures 27, 28 and 29 are considered in which pB9+fp402 gave an increase in

transcription in response to serum or TPA stimulation, whereas in Figure 30 no such response is observed. Also, the shut-down in transcription from pB9+fp116ATF was not as marked as in previous experiments, again suggesting that there was a difference between the two sets of experiments. Several possibilities were thought of to explain these conflicting results.

i) The actual density of the cells upon stimulation with serum or TPA may have been markedly different in this experiment in comparison to the other. In the experiment shown in Figure 30 the cells plated out at $1x10^6$ per F75 prior to transfection were 30% confluent prior to serum or TPA stimulation while the cells plated out at 1.5×10^6 per F75 were about 40% confluent. These estimations were made by eye and are therefore not particularly accurate although they may be used as a rough guideline. In the previous experiments (shown in figures 27, 28 and 29) no estimation of cell denisty was made prior to stimulation and cell harvest. It is possible that the cells were at different densities prior to stimulation as CT3 cells demonstrated variable growth rates throughout this project, possibly due to different batches of serum. It is not known whether different batches of serum were used in the experiments described in Figures 27 to 30. The toxicity of the transfection cocktails added to the cells may also have varied between experiments leading to varying degrees of cell death and hence varying densities of cells prior to stimulation.

ii) Perhaps the CT3 cells used in the transfection experiment described in Figure 30 were transformed leading to them becoming

insensitive to serum deprivation. However this seems unlikely as the cells used failed to form foci, even when allowed to grow to confluence, and they also showed varying growth rates in 10% FCS and 0.5% FCS with those growing in 10% FCS being more dense than those grown in 0.5% FCS suggesting that the cells were sensitive to serum deprivation.

iii) Perhaps the density of the cells prior to plating out for transfection is important, possibly because it dictates the growth of the cells after replating. However, this would seem unlikely as no particular attention was given to the density of the cells prior to setting up any of the transfection experiments described in Figures 26 to 30. Basically, the cells were grown until they were sub-confluent and taken for re-plating when it was thought they would become confluent by the following day if they were not passaged. So, the density of the cells prior to plating out for transfection should not have been markedly different between experiments.

To determine whether the density of the cells prior to stimulation played a crucial role in obtaining an increase in transcription in response to serum or TPA with the pB9+fp402 and pB9+fp116ATF constructs several transfection experiments were carried out in which the cells were plated out at different densities prior to transfection. By this time the CT3 cells were being grown in a different batch of serum from that used in any of the previous transfection experiments. In this serum the doubling time of the CT3 cells was greatly reduced in comparison to those obtained with other batches of serum. Figure 31 shows an example of one of these

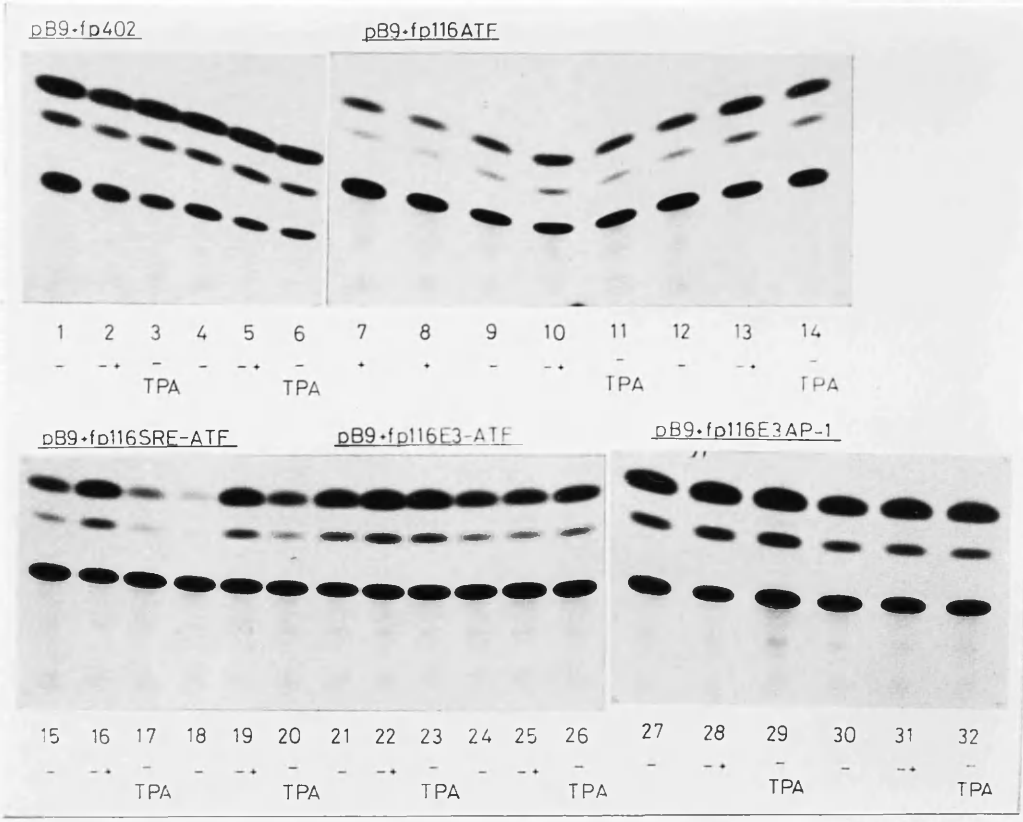


Figure 31. Serum and TPA stimulation of c-fos-CAT constructs in high density serum deprived CT3 cells.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp402 (lanes 1 to 6), pB9+fp116ATF (lanes 7 to 14), pB9+fp116SRE-ATF (lanes 15 to 20), pB9+fp116E3-ATF (lanes 21 to 26) and pB9+fp116E3-AP-1 (lanes 27 to 32). The experiment shown above is quantified in Table 8 and respresents Experiment B. Experiments A, B and C all gave similar results and therefore only one of them is represented in this figure. In Experiment A the CT3 cells were plated out at 10^6 per F75 while in B they were plated out at $5x10^5$ cells per F75 and in C at $1.2x10^6$ per F75. 16 to 24 hours later the transfection cocktail was added to each flask (2.5.2.) and 16 hours after the addition of the cocktail cells were washed with PBS and then refed with medium containing 0.5% FCS (or 10% FCS in lanes 7 and 8). In Experiments A and B the cells were harvested 36 hours later while in C the cells were harvested 48 hours later. Transfection cocktails were made and divided into 3 flasks to form triplicates. One flask from each triplicate was grown in 0.5% FCS up until cell harvest (-), one was serum stimulated with medium containing 15% FCS for 1 hour prior to cell harvest (- +), and the other stimulated with medium containing 100ng/ml TPA; 0.5% serum for an identical period prior to cell harvest (- TPA). In lanes 7 and 8 the cells were transfected with aliquots from the same cocktail and grown in medium containing 10% FCS until cell harvest. In Experiment A 5µg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 5µg of HSV-β-gal plasmid while in Experiments B and C 10µg of each plasmid was added per flask. β-gal assays were performed on aliquots from each extract, and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

	PLASMID CONSTRUCT								
	pB9+fp116SRE-ATF			pB9+fp116E3-ATF			pB9+fp116E3AP-1		
TREATMENT OF CELLS	-	-+	-TPA	-	-+	-TPA	-	-+	-TPA
EXPERIMENT A	0.7 0.8	4.2 3.0	4.0 2.3	2.4 0.7	3.5 0.2	3.1 1.1	7.1 -	4.9 -	6.7 -
AVERAGE CONVERSION	0.8	3.6	3.2	1.6	1.9	2.1	-	-	-
STANDARD DEVIATION	0.0	1.2	1.2	1.1	2.3	1.4	-	-	-
FOLD INDUCTION	1.0+\-0.0	5.1+\-1.6	4.6+\-1.6	1.0+\-0.4	1.2+\-1.4	1.3+\-0.9	1.0	0.7	0.9
EXPERIMENT B	15 11.0 18 1.6	16 35.2 19 25.0	17 4.7 20 10.4	21 33.0 24 17.3	22 34.7 25 12.9	23 34.1 26 16.4	27 47.4 30 30.7	28 64.4 31 34.2	29 37.5 32 27.3
AVERAGE CONVERSION	6.3	30.1	7.6	25.2	23.8	25.3	39.1	49.3	32.4
STANDARD DEVIATION	6.6	7.2	3.8	10.9	19.2	12.3	11.5	21.4	7.2
FOLD INCREASE	1.0+\-1.0	4.8+\-1.1	1.2+\-1.1	1.0+\-0.4	0.9+\-0.8	1.0+\-0.5	1.0+\-0.3	1.3+\-0.5	0.8+\-0.2
EXPERIMENT C	5.8	55.7	14.1	60.8 9.2	59.8 18.7	81.8 19.0	41.5 21.4	58.0 39.3	65.3 39.3
AVERAGE CONVERSION	-	-	-	35.0	39.3	50.4	31.5	48.7	52.3
STANDARD DEVIATION	-	-	-	36.4	28.9	44.4	14.0	12.8	18.4
FOLD INCREASE	1.0	9.6	2.4	1.0+\-1.0	1.1+\-0.8	1.4+\-1.3	1.0+\-0.4	1.5+\-0.4	1.7+\-0.6

	PLASMID CONSTRUCT							
	pB9+fp402			pB9+fp116ATF				
TREATMENT OF CELLS	-	-+	-TPA	-	-+	-TPA	+
EXPERIMENT A	3.4 1.9	5.8 1.4	11.7 2.5	0.8 0.6	1.2 0.6	1.2 0.7	2.5 2.0
AVERAGE CONVERSION	2.7	3.6	7.1	0.7	0.9	1.0	2.3
STANDARD DEVIATION	0.8	2.5	6.5	0.1	0.4	0.3	0.4
FOLD INDUCTION	1.0+\-0.1	1.3+\-0.9	2.6+\-2.4	1.0+\-0.1	1.3+\-0.6	1.4+\-0.4	-
EXPERIMENT B	1 56.5 4 64.3	2 53.1 5 82.3	3 76.8 6 75.6	9 8.4 12 8.2	10 7.9 13 5.0	11 17.0 14 5.6	7 36.3 8 39.0
AVERAGE CONVERSION	60.4	67.7	76.2	8.3	6.5	11.3	37.7
STANDARD DEVIATION	5.5	20.6	0.8	0.1	1.7	8.1	1.9
FOLD INDUCTION	1.0+\-0.1	1.1+\-0.3	1.3+\-0.0	1.0+\-0.0	0.8+\-0.2	1.4+\-1.0	-
EXPERIMENT C	26.2 37.0	45.3 57.2	47.6 55.4	20.5 8.0	18.1 4.4	33.3 6.5	43.8 18.0
AVERAGE CONVERSION	31.6	51.3	51.5	14.3	11.3	19.9	30.9
STANDARD DEVIATION	7.6	7.8	5.5	8.7	9.6	19.0	18.2
FOLD INDUCTION	1.0+\-0.2	1.6+\-0.2	1.6+\-0.2	1.0+\-0.6	0.8+\-0.7	1.4+\-1.3	-

Table 8. Serum and TPA stimulation of c-fos-CAT constructs in high density serum-deprived CT3 cells.

This table quantifies the CAT activity obtained in the experiments shown and described in Figure 21. Figures represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated forms with each extract. The fold induction represents the division of the average percentage conversion (and standard deviation) obtained with the serum stimulated (-+) and the TPA stimulated (-TPA) samples by the average percentage conversion obtained with the corresponding serum deprived samples (-). The numbers 1 to 38 correspond to the lane numbers in Figure 30.

transfection experiments while three independent experiments are quantified in Table 8. It is clear that the results obtained with the experiment shown in Figure 31 and quantified in Table 8 are similar to those shown in Figure 30 i.e. the previously observed increase in CAT activity in response to serum or TPA stimulation of transfected cells (Figure 27 to 29) was not seen with pB9+fp402,nor was the transcriptional shut-down or TPA stimulation of pB9+fp116ATF observed. Once more pB9+fp116SRE-ATF gave an increase in CAT activity in response to both serum or TPA stimulation with serum providing the greater stimulation as predicted. In Experiment A and C described in Figure 31 the cells were confluent prior to stimulation and harvest while in Experiment B the cells were sub-confluent. This would suggest that the density of the transfected cells prior to stimulation was not a crucial factor in obtaining an increase in CAT activity with the c-fos-CAT constructs in response to serum or TPA stimulation. The failure to repeat the serum or TPA stimulation of pB9+fp402 or to observe the complete transcriptional shut-down with pB9+fp116ATF contradicts previous experiments. The major difference between the earlier and later experiments was that a different batch of serum was used in both sets of experiments which may have resulted in the anomalous results. These results therefore failed to confirm the fos ATF&AP-1 sequence can activate transcription from the c-fos promoter following a 1 hour TPA stimulation. The failure to observe such an induction in the latter experiments could be explained by the comparitively high levels of CAT activity in the serum deprived cells masking any increase in CAT activity following a 1 hour stimulation with TPA.

3.4.3. Overnight serum or TPA stimulation of CT3 cells transfected with fos-CAT constructs.

As described in 3.4.2. it had become impossible to obtain an increase in CAT activity with the c-fos-CAT constructs (apart from pB9+fp116SRE-ATF) in response to serum or TPA stimulation of transfected, serum deprived CT3 cells. The reason for this failure is unclear but stems from the base level CAT activity prior to stimulation being too great, thus apparently masking any increase in transcription that may be occuring due to serum or TPA stimulation. To get round this problem it was decided that the transfected, serum-deprived CT3 cells should be stimulated with serum or TPA for 12 hours prior to cell harvest. This length of stimulation has been used previously to identify AP-1 sequences as being responsive to TPA stimulation (251) and these experiments also involved the transfection of CAT constructs into fibroblasts, and serum deprivation prior to stimulation.

The results of transfection experiments in which the length of stimulation of serum deprived, transfected CT3 cells was increased to 12 hours are quantified in Table 9 and shown and described in Figure 32. Essentially these experiments were identical to the previous short-term stimulation experiments, apart from the 12 hour stimulations prior to cell harvest. pB9+fp402, the construct with 402bp of intact c-fos upstream sequence 5′ of the CAT gene, consistently responded to TPA stimulation over this time period giving a 3- to 4-fold increase in CAT activity. However, the serum stimulation experiments carried out with cells transfected with this

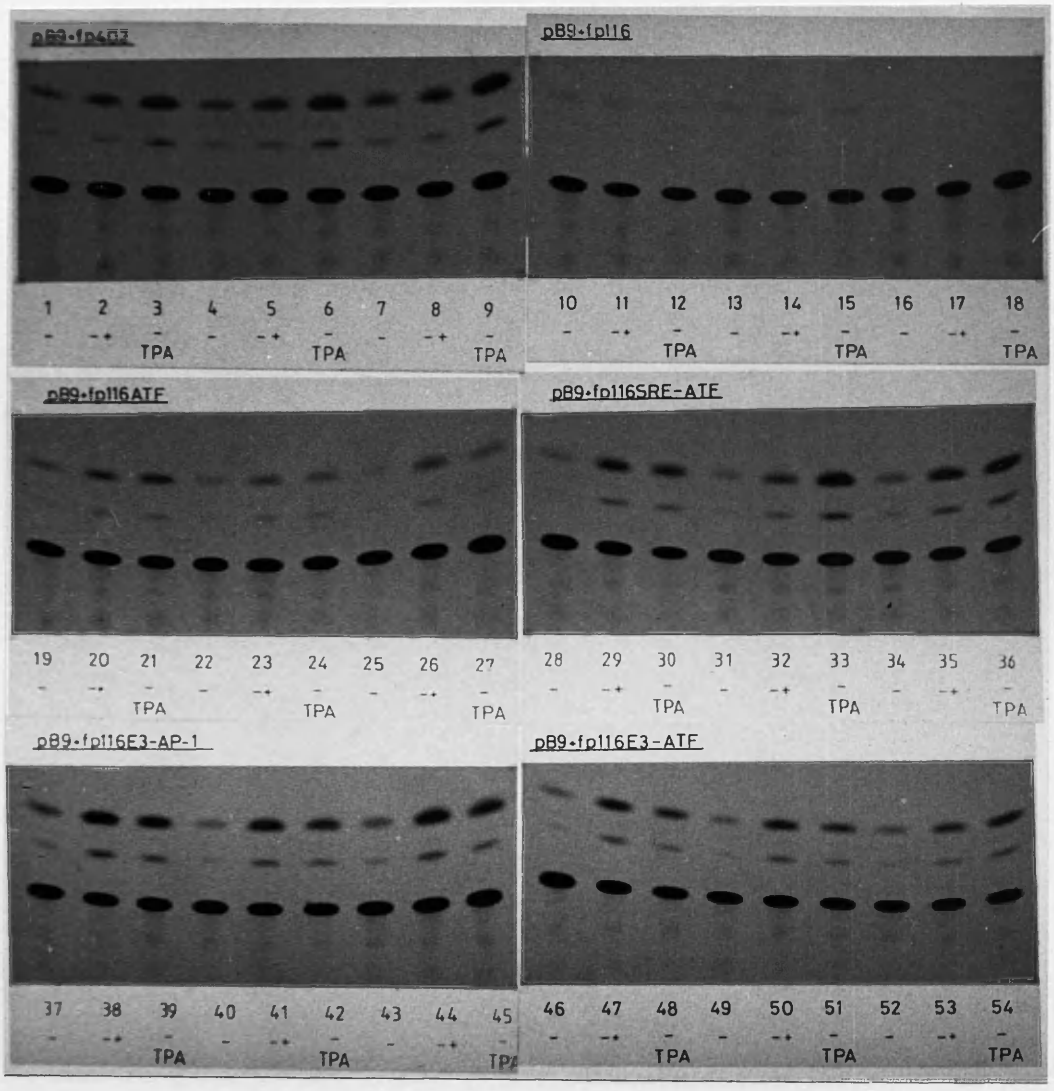

pB9-fp402
pB9-fp116
pB9-fp116ATF
pB9-fp116SRE-ATF
pB9-fp116E3-AP-1
pB9-fp116E3-ATF
TPA

Figure 32. Overnight serum or TPA stimulation of serum deprived CT3 cells transfected with c-fos-CAT constructs.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp402 (lanes 1 to 9), pB9+fp116 (lanes 10 to 18), pB9+fp116ATF (lanes 19 to 27), pB9+fp116SRE-ATF (lanes 28 to 36), pB9+fp116E3-AP-1 (lanes 37 to 45) and pB9+fp116E3-ATF (lanes 46 to 54). The experiment shown in this figure is quantified in Table 9 and represents Experiment B. Experiment A, B and C all gave similar results and therefore only one of them is represented in this figure. In all three experiments the CT3 cells were plated out at 10^6 per F75 flask and 16 to 24 hours later the transfection cocktail was added to each flask (2.5.2.) and 16 hours after the addition of the cocktail the cells were washed with PBS and then refed with medium containing 0.5% FCS. The cells remained in 0.5% FCS for 36 hours and any stimulations were then given and the cells harvested 12 hours later. Transfection cocktails were made and divided into 3 flasks to form triplicates. One flask from each triplicate was grown in 0.5% FCS up until cell harvest (-), one was serum stimulated with medium containing 15% FCS for 12 hours prior to cell harvest (- +), and the other stimulated with medium containing 100ng/ml TPA; 0.5% FCS for an identical time period prior to cell harvest (- TPA). In Experiment A 10μg of c-fos-CAT plasmid was added to eqch flask in the transfection cocktail along with 10μg of HSV-β-gal plasmid while in Experiments B and C 5μg of each plasmid was added per flask. β-gal assays were performed with aliquots from each extract, and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

* These CAT assays had incorrect amounts of extract used and are therefore not represented in Table 9.

Table 9. Overnight serum or TPA stimulation of serum deprived CT3 cells transfected with c-fos-CAT constructs.

This table quantifies the CAT activity obtained in the experiments shown and described in Figure 32. Figures represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated forms with each extract. The fold-induction represents the division of the average percentage conversion (and standard deviation) obtained with the serum stimulated (-+) and TPA stimulated (-TPA) samples by the average percentage conversion obtained with the corresponding serum deprived samples (-). The numbers 1 to 54 correspond to the lane numbers in Figure 32.

	PLASMID CONSTRUCT								
	pB9+fp402			pB9+fp116			pB9+fp116ATF		
TREATMENT OF CELLS	-	-+	-TPA	-	-+	-TPA	-	-+	-TPA
EXPERIMENT A	15.3 14.9	83.3 60.5	83.7 27.5	- -	- -	- -	4.5 2.2	10.9 4.2	18.0 7.2
AVERAGE CONVERSION	15.1	71.9	60.6	-	-	-	3.4	7.5	12.6
STANDARD DEVIATION	0.3	16.1	20.4	-	-	-	1.4	4.9	7.6
FOLD INDUCTION	1.0+\-0.0	4.8+\-1.1	4.0+\-1.4	-	-	-	1.0+\-0.4	2.2+\-1.4	3.7+\-2.2
EXPERIMENT B	1 2.0 4 2.9 7 3.9	2 3.3 5 4.4 8 4.9	3 7.8 6 7.2 9 13.2	10 0.6 13 0.7 16 0.4	11 0.7 14 1.3 17 0.3	12 0.9 15 0.5 18 -	19 1.2 22 1.0 25 1.0	20 1.9 23 2.6 26 3.3	21 3.4 24 2.0 27 2.2
AVERAGE CONVERSION	2.9	4.2	9.6	0.5	0.8	0.7	1.1	2.6	2.5
STANDARD DEVIATION	0.8	0.8	3.0	0.1	0.4	0.3	0.1	0.7	0.9
FOLD INDUCTION	1.0+\-0.3	1.4+\-0.3	3.3+\-1.0	1.0+\-0.2	1.6+\-0.8	1.4+\-0.6	1.0+\-0.1	2.4+\-0.6	2.3+\-0.8
EXPERIMENT C	4.7 11.6 5.7	12.1 9.7 5.1	29.2 26.3 29.3	- - -	- - -	- - -	2.6 3.3 2.8	9.3 11.8 5.6	8.1 5.5 7.5
AVERAGE CONVERSION	7.3	9.2	28.4	-	-	-	2.9	8.9	7.0
STANDARD DEVIATION	3.8	3.3	2.5	-	-	-	0.4	3.1	1.6
FOLD INDUCTION	1.0-\+0.5	1.3+\-0.5	3.9+\-0.3	-	-	-	1.0+\-0.1	3.9+\-1.1	2.4+\-0.6

	PLASMID CONSTRUCT								
		pB9+fp116SRE-ATF			pB9+fp116E3AP-1			pB9+fp116E3-ATF	
TREATMENT OF CELLS	-	-+	-TPA	-	-+	-TPA	-	-+	-TPA
EXPERIMENT A	3.6 3.0	22.2 23.1	19.2 12.9	5.3 4.5	16.4 5.9	25.2 13.4	5.1 4.6	11.8 6.1	26.5 17.9
AVERAGE CONVERSION	3.3	22.7	16.1	4.9	11.2	19.4	4.9	9.0	22.2
STANDARD DEVIATION	0.4	0.6	4.1	0.6	7.3	8.3	0.3	3.8	6.1
FOLD INDUCTION	1.0+\-0.1	6.9+\-0.2	4.9+\-1.2	1.0+\-0.1	2.3+\-1.5	3.9+\-1.7	1.0+\-0.1	1.8+\-0.8	4.5+\-1.2
EXPERIMENT B	28 1.5 31 1.2 34 1.7	29 8.5 32 11.7 35 9.3	30 5.4 33 4.8 36 7.2	37 3.2 40 1.8 43 2.9	38 7.2 41 7.8 44 5.3	39 16.3 42 9.5 45 7.4	46 1.6 49 1.2 52 1.6	47 4.7 50 4.2 53 9.0	48 6.3 51 4.8 54 7.0
AVERAGE CONVERSION	1.5	9.8	5.8	2.6	6.8	11.1	1.5	6.0	5.7
STANDARD DEVIATION	0.3	1.9	1.2	0.9	1.0	4.5	0.2	2.5	2.7
FOLD INDUCTION	1.0+\-0.2	6.7+\-1.3	3.9+\-0.8	1.0+\0.3	2.6+\-0.4	4.3+\-1.7	1.0+\-0.1	4.0+\-1.7	3.8+\-1.8
EXPERIMENT C	1.5 2.1 2.6	11.9 24.2 11.2	7.1 6.5 6.3	4.6 8.3 5.5	12.3 25.2 14.0	13.8 15.3 11.4	1.3 2.2 -	18.1 7.3 -	14.3 4.2 -
AVERAGE CONVERSION	2.1	15.3	6.6	6.1	17.2	13.5	1.8	12.7	9.3
STANDARD DEVIATION	0.3	7.3	0.9	2.0	6.4	2.0	0.2	7.6	7.0
FOLD INDUCTION	1.0+\-0.1	7.5+\-3.5	3.1+\-0.4	1.0+\-0.3	2.8+\-1.0	2.2+\-0.3	1.0+\-0.1	7.0+\-4.2	5.2+\-3.9

construct provided inconsistent results. In Experiment A (Table 9) a 5-fold induction is observed but in the proceeding two experiments no such induction was detectable. An increase in CAT activity is also obtained in response to serum or TPA stimultion with pB9+fp116ATF, with serum giving a 2- to 3-fold induction and TPA a 2- to 4-fold induction. It was expected that TPA stimulation might give an increase in CAT activity since short-term stimulation with TPA indicated an increase in CAT activity with this construct (Figure 29 and Table 7). However, it was not expected that serum would induce this construct since in a short-term stimulation experiment no increase in CAT activity is observed with pB9+fp116ATF (Figure 28 and Table 6). pB9+fp116SRE-ATF once more, as with the short-term stimulations, consistently gives the greatest fold-inductions with a 7-fold increase in CAT activity with serum and a 3- to 4-fold increase with TPA. The two "foreign" sequences from the adenovirus E3 promoter, E3-ATF and E3-AP-1, both give an increase in CAT activity in response to both serum and TPA stimulation when placed upstream from the c-fos promoter. These sequences are present in pB9+fp116E3-ATF and pB9+fp116E3AP-1, giving a 2- to 7- and 2- to 3-fold increase respectively in CAT activity in response to TPA. These results are as expected for pB9+fp116E3AP-1 as the AP-1 sequence has been previously shown to be responsive to both TPA and serum (251, 254) stimulation. However, the results obtained with pB9+fp116E3-ATF were rather surprising since it has been shown that ATF sequences (although not the one used here) do not respond to TPA stimulation, but to cAMP stimulation (256). Indeed the protein that binds to the ATF sequence is very similar or identical to the cAMP response element binding protein, CREB (264).

In Experiment B cells were transfected with pB9+fp116, and stimulated with either serum or TPA (after serum deprivation). As can be seen from Figure 32 and Table 9, no increase in CAT activity is obtained with this construct in response to serum or TPA stimulation. This shows that any of the effects observed in these experiments in response to serum or TPA stimulation are due to the sequences inserted upstream from the c-fos promoter.

The overnight stimulation experiments identified several sequences that were responsive to both serum and TPA stimulation. Interestingly, pB9+fp116SRE-ATF gave the greatest and most consistent fold-inductions in both short-term and long-term stimulation experiments. Also in the short term stimulation assays described in Figure 28 pB9+fp116SRE-ATF gave lower levels of CAT activity than pB9+fp116SRE in the serum deprived cells. These results suggested that that there may be an interaction occuring between the proteins complexing with the fosATF&AP-1 sequences to influence transcription from the c-fos promoter.

3.4.4. Short-term serum or TPA stimulations investigating a possible interaction between the proteins complexing with the c-fos SRE and ATF/AP-1 sequences.

The results obtained with the short-term and long-term stimulation assays suggested that there may be an interaction between the proteins complexing with the SRE and fosATF/AP-1 sequences. Two main pieces of evidence suggested that this may be the case:-

1) As mentioned above, pB9+fp116SRE-ATF gave lower levels of CAT activity in serum deprived cells than pB9+fp116SRE. This led to a more consistent serum and TPA response

2) The two sequences are continuous with one another in the endogenous c-fos gene sequences with not a bp separating them. This is also the case with two other immediate-early genes (192) suggesting a function for this juxtaposition of transcriptional control elements.

Because of these observations transfection experiments were carried out to investigate the possibility on an interaction between the two (or more) proteins complexing with these sequences.

In order to determine whether the SRE and fosATF/AP-1 sequences require to be continuous to exert their transcriptional control, two further c-fos-CAT constructs were synthesised: pB9+fp116SRE-5bp-ATF and pB9+fp116SRE-10bp-ATF. These constructs contain the SRE and fosATF/AP-1 sequences separated by the insertion of 5bp and 10bp of random sequence. Another c-fos-CAT construct was made, pB9+fp116SRE-AP-1, to determine whether the fos-ATF/AP-1 sequence is crucial for the transcriptional control exerted by the SRE and fosATF/AP-1 sequences. In pB9+fp116SRE-AP-1 the fosATF/AP-1 is replaced by a consensus AP-1 binding site. These three constructs, along with pB9+fp116SRE-ATF, were used in transfection experiments where the cells were serum deprived as previously described and serum

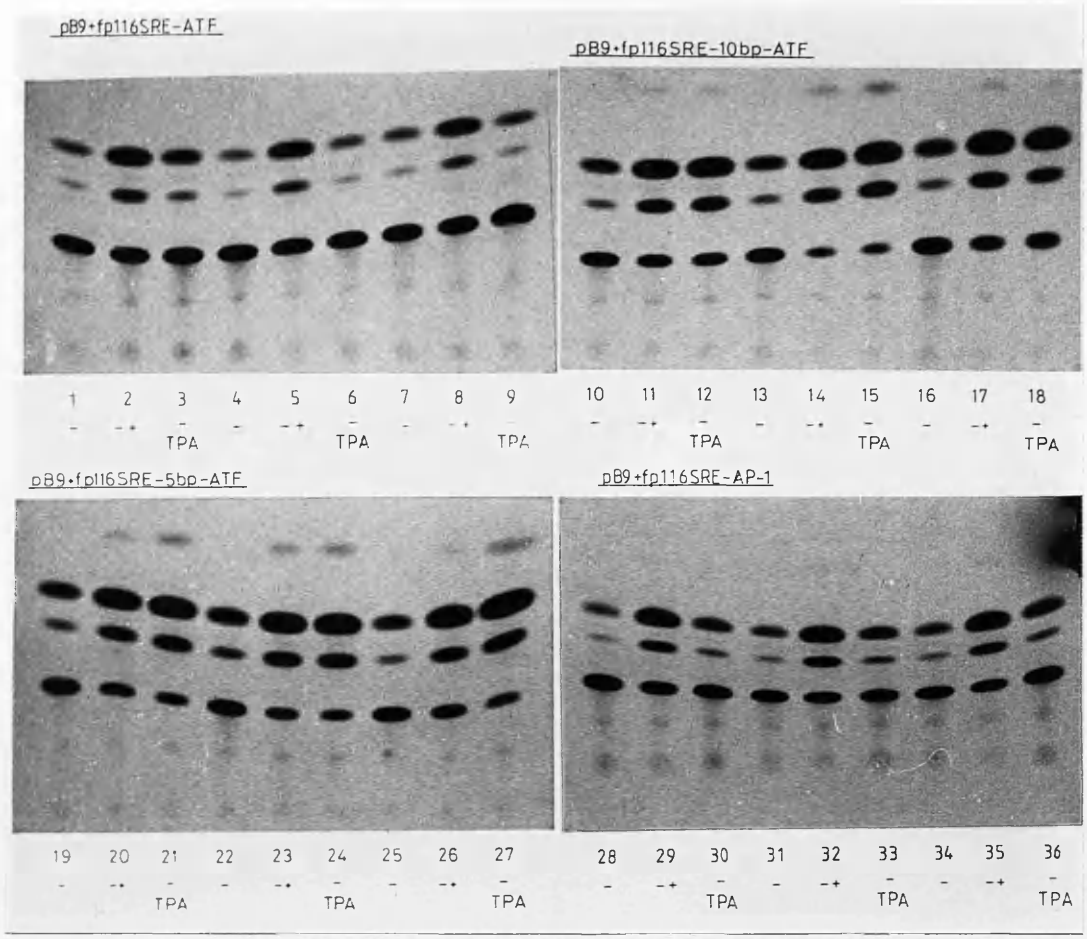


Figure 33. Short-term serum or TPA stimulations of cells transfected with c-fos-CAT constructs designed to investigate interactions between SRF and fosATF/AP-1.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp116SRE-ATF (lanes 1 to 9), pB9+fp116SRE-10bp-ATF (lanes 10 to 18), pB9+fp116SRE-5bp-ATF (lanes 19 to 27) and pB9+fp116SRE-AP-1 (lanes 28 to 36). The experiment shown in this Figure is quantified in Table 10 and represents EXperiment B. Experiments A and B gave similar results and therefore only one of them is represented in this Figure. In both of these experiments the CT3 cells were split 1 in 5 16 to 24 hours prior to transfection. 16 hours after the addtion of the transfection cocktail cells were washed with PBS and then refed with medium containing 0.5% FCS. The cells were serum starved for 36 hours before cell harvest. Transfection cocktails were made and divided into 3 flasks to form triplicates. One flask from each triplicate was grown in 0.5% FCS up until cell harvest (-), one was serum stimulated with medium containing 15% FCS for 12 hours prior to cell harvest (- +), and the other stimulated medium containing 100ng/ml TPA; 0.5% FCS for an identical time period prior to cell harvest (-TPA). In Experiments A and B 5μg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 5μg of HSV-β-gal plasmid. β-gal assays were performed with aliquots from each extract, and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

	PLASMID CONSTRUCT								
	pB9+fp116SRE-ATF			pB9+fp116SRE-10bp-ATF			pB9+fp116SRE-5bp-ATF		
TREATMENT OF CELLS	-	-+	-TPA	-	-+	-TPA	-	-+	-TPA
EXPERIMENT A	12.5 7.3 9.0	65.8 59.4 53.5	21.3 34.5 19.1	14.3 10.2 10.3	50.0 50.6 49.5	51.9 58.5 45.3	42.5 55.7 21.7	92.5 79.4 58.2	96.3 77.0 78.3
AVERAGE CONVERSION	9.6	59.6	25.1	11.6	50.0	51.9	40.0	76.7	83.9
STANDARD DEVIATION	2.7	5.9	7.7	2.6	3.3	6.6	17.0	17.3	10.4
FOLD INDUCTION	1.0+\-0.3	6.2+\-0.6	2.6+\-0.8	1.0+\-0.2	4.3+\-0.3	4.5+\-0.6	1.0+\-0.4	1.9+\-0.4	2.1+\-0.3
EXPERIMENT B	1 5.0 4 2.4 7 7.7	2 35.1 5 28.8 8 24.6	3 7.4 6 3.8 9 4.1	10 12.9 13 12.3 16 13.2	11 76.9 14 95.1 17 86.4	12 79.5 15 93.4 18 76.2	19 20.4 22 18.4 25 14.7	20 86.9 23 90.8 26 79.5	21 93.3 24 94.8 27 96.6
AVERAGE CONVERSION	5.0	29.5	5.1	12.8	86.1	83.0	17.8	85.7	94.9
STANDARD DEVIATION	2.7	5.3	2.0	0.5	9.6	9.6	3.2	6.4	1.7
FOLD INDUCTION	1.0+\-0.5	5.9+\-1.1	1.0+\-0.4	1.0+\-0.0	6.7+\-0.8	6.5+\-0.8	1.0+\-0.2	4.8+\-0.4	5.3+\-0.1

PLASMID CONSTRUCT			
	pB9+fp116SRE-AP-1		
TREATMENT OF CELLS	-	-+	-TPA
EXPERIMENT A	5.0 4.7 5.7	46.8 33.3 38.2	10.1 10.9 12.3
AVERAGE CONVERSION	5.1	39.4	11.1
STANDARD DEVIATION	0.9	7.1	1.1
FOLD INDUCTION	1.0+\-0.2	7.7+\-1.4	2.2+\-0.2
EXPERIMENT B	28 2.5 31 4.5 34 4.7	29 44.1 32 53.6 35 66.9	30 6.1 33 8.3 36 6.7
AVERAGE CONVERSION	3.9	53.2	7.0
STANDARD DEVIATION	1.2	20.0	1.4
FOLD INDUCTION	1.0+\-0.3	13.6+\-5.1	1.8+\-0.4

Table 10. Short-term serum or TPA stimulation of cells transfected with c-fos-CAT constructs designed to investigate interactions between SRE and fosATF/AP-1.

This table quantifies the CAT activity obtained in the experiments shown and described in Figure 33. Figures represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated and di-acetylated forms with each extract. The fold-induction represents the ratio of the average percentage conversion (and standard deviation) obtained with the serum stimulated (-+) and the TPA stimulated (-TPA) samples to the average percentage conversions obtained with the corresponding serum deprived samples(-). In experiment B the numbers (1 to 36) correspond to the lane numbers in Figure 33.

or TPA stimulated for 1 hour prior to cell harvest. The results of such experiments are shown and described in Figure 33 and quantified in Table 10. Unfortunately, in Experiment A all of the pB9+fp116SRE-ATF samples and the first 2 sets of 3 from the pB9+fp116SRE-5bp-ATF samples had a different batch of ^{14}C-chloramphenicol used in the CAT assays. This batch was less active than the batch used for the rest of the experiment and led to higher percentage conversions than would have been obtained had the second batch been used throughout (for example, compare the results obtained with the last set of 3 with the first 2 with pB9+fp116SRE-5bp-ATF in Table 10). However, many points can still be made from these experiments, particularly since the standard deviations were relatively small throughout Experiment B. In the cells that remained serum deprived throughout these experiments it can be seen that the constructs which have the insertions between the SRE and fosATF/AP-1 sequences show an increased level of CAT activity when compared to pB9+fpSRE-ATF. The 10bp insert increases levels approximately 2-fold while the 5bp insert increases it around 3-fold. The CAT activity obtained with serum stimulated samples representing pB9+fp116SRE-10bp-ATF and pB9+fp116SRE-5bp-ATF is greater than that obtained with pB9+fp116SRE-ATF but the inductions are similar because the basal levels obtained with pB9+fp116SRE-10bp-ATF and pB9+fp116SRE-5bp-ATF in the serum deprived samples are greater than those obtained with pB9+fp116SRE-ATF. In response to TPA stimulation, pB9+fp116SRE-10bp-ATF and pB9+fp116SRE-5bp-ATF give fold-inductions of CAT activity that are similar to those obtained with serum stimulation. This is different from the results obtained with pB9+fp116SRE-ATF where once again the response to TPA

stimulation is significantly less than the response to serum stimulation. Interestingly pB9+fp116SRE-AP-1, the c-fos-CAT construct which contains the SRE sequence directly beside an AP-1 sequence, behaves in an almost identical fashion to pB9+fp116SRE-ATF. The levels of CAT activity obtained with the serum deprived samples are equivalent and stimulations with serum or TPA gave very similar inductions. The results obtained with pB9+fp116SRE-AP-1 suggest that the SRF, the protein that binds to the SRE, influences or dictates the protein that binds to the AP-1/ATF site beside it.

To determine whether pB9+fp116SRE-10bp-ATF and pB9+fp116SRE-5bp-ATF give a greater level of CAT activity in proliferating cells in comparison to pB9+fpSRE-ATF a transfection experiment was carried out in which the cells were grown in medium containing 10% FCS up until cell harvest.

3.4.5. Comparison of the transcriptional activity obtained with pB9+fpSRE-10bp-ATF,pB9+fp116SRE-5bp-ATF and other c-fos-CAT constructs in proliferating CT3 cells.

A transfection experiment similar to those carried out in Section 3.2. was carried out to compare the levels of CAT activity obtained with various c-fos-CAT constructs. This experiment involved the transfections of the constructs into proliferating CT3 cells and maintaining these cells in medium containing 10%FCS up until cell harvest. This experiment is shown and described in Figure 34 and quantified in Table 11. Unfortunately in this experiment the samples representing pB9+fp116 and pB9+fp116SRE were lost and so the

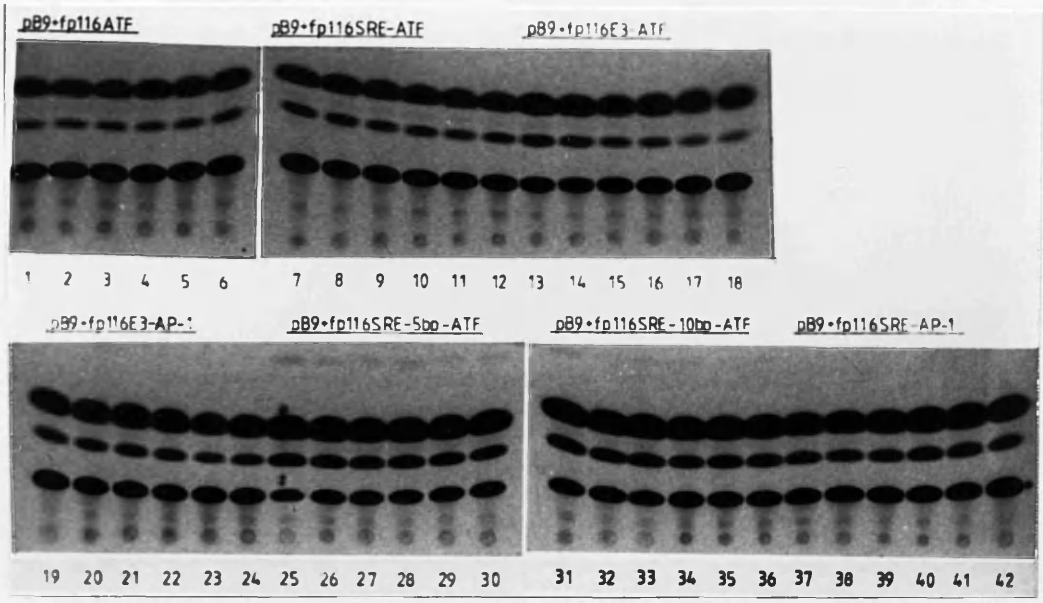


Figure 34. Comparison of transcriptional activity obtained with pB9+fp116 SRE-5bpATF and pB9+fp116SRE-10bp-ATF and other c-fos-CAT constructs in proliferating CT3 cells.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp116ATF (lanes 1 to 6), pB9+fp116SRE-ATF (lanes 7 to 12), pB9+fp116E3-ATF (lanes 13 to 18), pB9+fp116E3-AP-1 (lanes 19 to 24), pB9+fp116SRE-5bp-ATF (lanes 25 to 30), lanes pB9+fp116SRE-10bp-ATF (lanes 31 to 36) and pB9+fp116SRE-AP-1 (lanes 37 to 42). The experiment shown in this figure is quantified in Table 11. 16 to 24 hours prior to transfection the CT3 cells were plated out at $7x10^5$ per F75 tissue culture flask. 16 hours after the addition of the transfection cocktail the cells were washed with PBS and refed with medium containing 10% FCS. 36 hours later the cells were harvested. Transfection cocktails were split between two flasks to form duplicates and each duplicate sample is beside the other in this figure i.e. 1 and 2 are duplicates, 3 and 4 are duplicates etc. 10μg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 10μg of HSV--β-gal plasmid. β-gal assays were performed with aliquots from each extract, and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

	PLASMID CONSTRUCT					
	pB9+fp116ATF	pB9+fp116SRE-ATF	pB9+fp116E3-ATF	pB9+fp116E3AP-1		
	1 7.9 2 11.7 3 8.0 4 10.7 5 9.8 6 11.2	7 18.1 8 20.9 9 16.3 10 16.5 11 14.3 12 16.9	13 40.4 14 34.7 15 19.0 16 13.7 17 8.4 18 7.7	19 21.4 20 23.7 21 32.4 22 24.6 23 15.0 24 21.1		
AVERAGE CONVERSION	9.9	17.2	20.7	23.0		
STANDARD DEVIATION	1.5	1.9	13.7	5.8		
FOLD INDUCTION	1.0+\-0.2	1.7+\-0.2	2.1+\-1.4	2.3+\-0.6		
	PLASMID CONSTRUCT					
	pB9+fp116SRE-5bpATF	pB9+fp116SRE-10bp-ATF	pB9+fp116SRE-AP-1			
	25 87.5 26 66.4 27 63.5 28 75.5 29 59.4 30 61.9	31 67.6 32 51.9 33 68.8 34 42.2 35 50.1 36 47.8	37 36.9 38 29.5 39 28.7 40 44.3 41 38.1 42 22.3			
AVERAGE CONVERSION	69.0	54.7	33.3			
STANDARD DEVIATION	10.9	11.1	7.9			
FOLD INCREASE	7.0+\-1.1	5.5+\-1.1	3.4+\-0.8			

Table 11. Comparison of transcriptional activity obtained with pB9+fp116SRE-5bp-ATF and pB9+fp116SRE-10bp-ATF and other c-fos-CAT constructs in proliferating CT3 cells.

This table quantifies the CAT activity obtained in the experiment shown and described in Figure 34. The figures represent the percentage of ^{14}C-chloramphenicol converted to mono- and di-acetylated forms with each extract. The fold-increase represents the ratio of the average conversion obtained with pB9+fp116ATF to all the average conversions and standard deviations obtained with the other c-fos-CAT constructs. The numbers (1 to 42) correspond to the lane numbers in Figure 34.

experimental results had to be standardised using pB9+fp116ATF as the construct that is least active. This does not enable comparison with previous experiments carried out to examine promoter and/or enhancer elements upstream from the c-fos promoter (Table 1 and 2), but probably more importantly does allow for comparison within this experiment. The most striking feature of this experiment is the greatly elevated levels of CAT activity obtained with pB9+fp116SRE-10bp-ATF and pB9+fp116SRE-5bp-ATF compared to that obtained with pB9+fp116SRE-ATF. These two constructs give approximately 3-fold greater CAT activity with the 5bp construct giving the slightly higher levels. Indeed, these differences are very similiar to those observed when these constructs are assayed for CAT activity in cells that have been serum deprived prior to cell harvest (see Table 10, particularly Experiment B). From Table 11 it can also be seen that pB9+fp116SRE-AP-1 gives a 2-fold greater level of CAT activity when compared to the level obtained with pB9+fp116SRE-ATF. This increase is probably due to the AP-1 sequence present in the former which is a much stronger transcriptional activator than the fosATF/AP-1 sequence (see Table 3).

3.5. Ela trans-activation of the c-fos gene via upstream sequences.

The Ela gene of adenoviruses codes for one RNA transcript which is then processed to form two, therefore producing two proteins called 12s and 13s (for a review see 257). The 13s protein contains a domain that is responsible for the trans-activation of viral and cellular genes, and this domain is deleted from the 12s protein (for a review see 258). Previous reports of co-transfection experiments with Ela encoding constructs and c-fos-CAT constructs provided conflicting results with one report suggesting that sequences around the SRE were responsible for the trans-activation of the c-fos gene by the Ela 13s protein, while another identified sequences in the promoter region as being responsible for the trans-activation (261, 262). In an effort to determine which of these reports were correct transfection experiments were carried out in which c-fos-CAT constructs were co-transfected (or not) with plasmid constructs coding for the 12s and 13s Ela gene products (kind gifts from Dr. N.C. Jones, ICRF, London) (358). It was also hoped that it would be possible to identify exact sequences which were responsive to Ela 13s trans-activation.

3.5.1. Co-transfection of Ela 12s and 13s plasmid constructs with c-fos-CAT constructs into CT3 cells.

To examine trans-activation by Ela 13s, a transfection experiment similar to those described in Section 3.2. was carried out, apart from the Ela 12s and 13s plasmid constructs being co-transfected with the c-fos-CAT constructs. This experiment is shown and described in

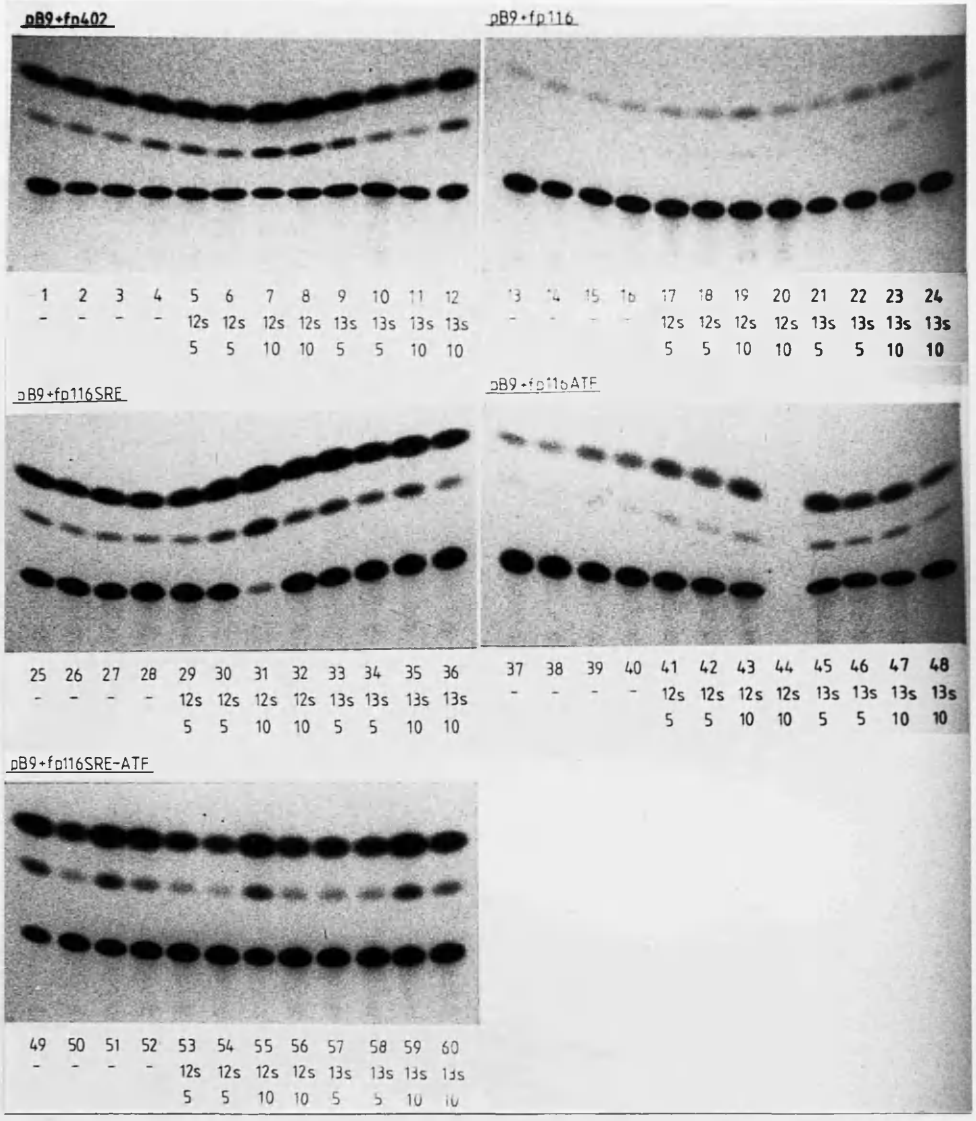

pB9+fp402
1 2 3 4 5 6 7 8 9 10 11 12
- - - - 12s 12s 12s 12s 13s 13s 13s 13s
5 5 10 10 5 5 10 10
pB9+fp116
13 14 15 16 17 18 19 20 21 22 23 24
- - - - 12s 12s 12s 12s 13s 13s 13s 13s
5 5 10 10 5 5 10 10
pB9+fp116SRE
25 26 27 28 29 30 31 32 33 34 35 36
- - - - 12s 12s 12s 12s 13s 13s 13s 13s
5 5 10 10 5 5 10 10
pB9+fp116ATF
37 38 39 40 41 42 43 44 45 46 47 48
- - - - 12s 12s 12s 12s 13s 13s 13s 13s
5 5 10 10 5 5 10 10
pB9+fp116SRE-ATF
49 50 51 52 53 54 55 56 57 58 59 60
- - - - 12s 12s 12s 12s 13s 13s 13s 13s
5 5 10 10 5 5 10 10

Figure 35. Co-transfection of Ela 12s and 13s plasmid constructs with c-fos-CAT constructs into CT3 cells.

CAT assays carried out with cell extracts prepared from CT3 cells transfected with pB9+fp402 (lanes 1 to 12), pB9+fp116 (lanes 13 to 24), pB9+fp116 SRE (lanes 25 to 36), pB9+fp116ATF (lanes 37 to 49) and pB9+fp116SRE-ATF (lanes 59 to 60). Ela plasmids were co-transfected with the c-fos-CAT constructs in some of the lanes and these are indicated by 12s and 13s written under the relevant lanes. The numbers under the 12s and 13s notations indicate the amounts (in µgs) of the Ela plasmid co-transfected. This experiment is quantified in Table 12. The CT3 cells were plated out at $5x10^5$ per F75 flask and 16 to 24 hours later the transfection cocktail was added to each flask (2.5.2.) and 16 hours after the addition of the cocktail the cells were washed with PBS and then refed with medium containing 10% FCS. 36 hours later the cells were harvested. Transfections cocktails were split between two flasks to form duplicates and each duplicates sample is beside the other in this figure i.e. 1 and 2 are duplicates, 3 and 4 are duplicates, etc. 7.5µg of c-fos-CAT plasmid was added to each flask in the transfection cocktail along with 10µg of 116V-β-gal plasmid (and the Ela plasmid where indicated). β-gal assays were performed with aliquots from each extract, and a volume from each extract representing equivalent amounts of β-gal activity was taken and used in each CAT assay.

	PLASMID CONSTRUCT								
	pB9+fp402			pB9+fp116			pB9+fp116SRE		
CO-TRANSFECTED PLASMID	-	12s	13s	-	12s	13s	-	12s	13s
	1 36.1 2 49.8 3 36.1 4 44.4	5 41.9 6 37.7 7 73.4 8 64.0	9 40.0 10 17.1 11 37.1 12 63.1	13 2.0 14 3.0 15 1.7 16 2.2	17 2.3 18 3.1 19 4.0 20 2.1	21 5.8 22 6.3 23 8.5 24 6.4	25 48.7 26 31.5 27 38.3 28 25.9	29 29.1 30 54.7 31 95.5 32 42.2	33 66.3 34 41.6 35 51.2 36 31.6
AVERAGE CONVERSION	41.6	54.2	39.4	2.2	2.9	6.8	36.0	55.4	47.7
STANDARD DEVIATION	6.7	17.4	18.7	0.7	0.7	0.7	10.3	28.6	14.7
FOLD INDUCTION	1.0+\-0.2	1.3+\-0.4	0.9+\-0.4	1.0+\-0.4	1.3+\-0.3	3.1+\-0.3	1.0+\-0.3	1.5+\-0.8	1.3+\-0.4

	PLASMID CONSTRUCT					
	pB9+fp116ATF			pB9+fp116SRE-ATF		
CO-TRANSFECTED PLASMID	-	12s	13s	-	12s	13s
	37 7.1 38 6.3 39 13.4 40 9.4	41 12.2 42 11.1 43 13.6 44 -	45 23.1 46 17.1 47 19.2 48 13.2	49 75.7 50 32.8 51 61.7 52 41.6	53 24.1 54 14.8 55 57.3 56 24.5	57 27.3 58 19.7 59 40.1 60 26.0
AVERAGE CONVERSION	9.1	12.3	18.2	52.3	30.2	28.3
STANDARD DEVIATION	3.0	1.3	3.8	21.6	18.6	8.4
FOLD INDUCTION	1.0+\-0.3	1.4+\-0.1	2.0+\-0.4	1.0+\-0.4	0.6+\-0.4	0.5+\-0.2

Table 12. Co-transfection of E1a 12s and 13s plasmid constructs with c-fos-CAT constructs into CT3 cells.

This table quantifies the CAT activity obtained in the experiment shown and described in Figure 35. The figures represent the percentage of ^{14}C-chloramphenicol converted to mono- and di-acetylated forms with each extract. In this table the results obtained with the different amounts of co-transfected E1a plasmid constructs have been taken to represent equivalent samples as no appreciable difference can be detected between the two sets of results. The fold-increase represents the ratio of the average conversion obtained with the E1a minus samples (-) to the average conversions and standard deviations obtained with the samples that had E1a plasmid constructs co-transfected. The numbers 1 to 60 correspond to lanes 1 to 60 of Figure 35.

Figure 35 and quantified in Table 12. In this experiment no increase in CAT activity is obtained when the Ela 13s construct is co-transfected with any of the c-fos-CAT constructs containing the SRE (pB9+fp402, pB9+fp116SRE and pB9+fp116SRE-ATF). With the construct that has had the SRE and fosATF/AP-1 site removed, pB9+fp116, a 3-fold increase in CAT activity is obtained with the samples that were co-transfected with the plasmid coding for the Ela 13s product. It can be seen that when pB9+fp116ATF is co-transfected with the Ela 13s construct that there is a 2-fold increase in CAT activity. It is perhaps not surprising that the Ela 13s protein trans-activates the c-fos promoter through the fosATF/AP-1 sequence since it can trans-activate through AP-1 and ATF sequences (270), which this fos sequence is certainly related to.

The inductions obtained in the experiment shown in Figure 35 are rather low in comparison to these previously reported (262) using similar c-fos-CAT constructs where pB9+fp116 would have been expected to give a 12-fold increase in CAT activity when co-transfected with the Ela 13s plasmid. The reason for this discrepancy soon became clear. In a further transfection experiment repeating the co-transfection of pB9+fp116 and pB9+116ATF with Ela plasmid constructs it was observed that the HSV-β-gal plasmid was being trans-activated by the Ela 13s gene product. On examination of the results obtained the β-gal assays carried out with aliquots from the extracts used in Figure 35 it became clear that the HSV-β-gal plasmid was also trans-activated in this experiment.

3.5.2. Trans-activation of control plasmids by the Ela 13s gene product.

Any trans-activation of the transfection efficiency control plasmid (such as HSV-β-gal) is obviously going to lead to inappropriate aliquots being taken from the cell extract for use in the CAT assays. By the time the trans-activation of the HSV-β-gal plasmid construct was detected several transfection experiments had been carried out and the results of the β-gal assays performed with aliquots of the cell extracts from these transfection experiments are shown in Table 13. After this phenomenon had been identified an identical experiment to that described in Figure 35 was carried out using a β-gal construct with an SV40 promoter as an internal control (359) (HSV-β-gal was derived from this construct by the insertion of the HSV promoter present in pLW2 (356)). The β-gal assay results obtained with this construct are also given in Table 13 and it can be seen that this construct is also trans-activated by the Ela 13s gene product. It is difficult to determine whether the trans-activation of this construct is less than that observed with HSV-β-gal since only one experiment was carried out with the SV40-β-gal construct, and as can be seen with the results obtained with HSV-β-gal the levels of induction are variable.

In an attempt to obtain a suitable internal control plasmid co-transfection experiments were carried out with a further three plasmid constructs. These three constructs all contained the human growth hormone (HGH) gene with its transcription driven by three different promoter. These were the TK (364) α-globin and PBG

PLASMID CONSTRUCT	EXPERIMENT	n	NO E1a	n	12s	n	13s
HSV-ß-GAL	A	20	0.418	20	0.505	20	1.165
	FOLD INDUCTION		1.00		1.24		2.86
HSV-ß-GAL	B	8	0.137	16	0.078	14	0.211
	FOLD INDUCTION		1.00		0.57		1.50
SV40-ß-GAL	C	20	0.082	19	0.081	20	0.153
	FOLD INDUCTION		1.00		0.99		1.87
HSV-ß-GAL	D	18	1.037	20	0.730	20	1.713
	FOLD INDUCTION		1.00		0.70		1.65
HSV-ß-GAL	E	6	0.076	12	0.070	12	0.187
	FOLD INDUCTION		1.00		0.92		2.46
HSV-ß-GAL	F	19	0.097	20	0.117	20	0.539
	FOLD INDUCTION		1.00		1.21		5.56

Table 13. Trans-activation of β-gal plasmid constructs by the E1a 13s gene product

The results shown in this table all involve co-transfection experiments in which the β-gal constructs and the E1a constructs were added to the cells in the same transfection cocktails. One such typical experiment is described in Figure 35. The figures represent the average $O.D._{420}$ obtained with β-gal assays (2.6.2.) carried out with similar cell extracts. n is the number of similar cell extracts used in each experiment. No E1a; β-gal constructs added to the cells in the absence of E1a constructs: 12s and 13s; β-gal constructs added to the cells in the presence of constructs coding for the E1a products 12s and 13s respectively. The fold-increase represents the ratio of the average $O.D._{420}$ obtained with the no E1a samples to the average $O.D._{420}$ obtained with the samples that had E1a plasmid constructs co-transfected i.e. 12s and 13s.

deaminase gene promoters (both kind gifts from Dr. J. Frampton). The constructs containing the first two promoters gave results in preliminary co-transfection experiments which suggested that both promoters were trans-activated by the Ela 13s gene product (data not shown) while the third construct, which contained the PBG-deaminase promoter, did not show any expression. This is not surprising since PBG-deaminase is a red blood cell specific gene. This inability to obtain a suitable internal control plasmid for Ela co-transfection experiments led to the adoption of a strategy in which the results obtained with the internal control constructs were adjusted to correct for the trans-activation by Ela 13s.

3.5.3. Co-transfection of c-fos-CAT and Ela plasmids into CT3 cells and correction for the trans-activation of the internal control plasmid by Ela 13s.

In Table 13 Experiment A represent β-gal assays carried out with aliquots from the cell extracts used for the CAT assays carried out in the experiment shown in Figure 35. It can be seen that the Ela 13s product gives a 3-fold increase in β-gal activity when compared to assays carried out with extracts that had no Ela plasmid co-transfected. However, the average $O.D._{420}$ in these β-gal assays is rather high and so they were repeated with less extract to ensure that there was no possibility of the β-gal assays going off the linear scale. The results of these repeat β-gal assays are given in Table 13 Experiment F where it can be seen that the Ela 13s gene product gives a 6-fold increase in β-gal activity. The CAT assays carried out in the experiment shown in Figure 35 were repeated, only the amount of extract used in each assay was adjusted to compensate for the 6-fold trans-activation of the HSV-β-gal control plasmid. This experiment is shown and described in Figure 36 and quantified in Table 14. As can be seen from Table 14 the correction for the trans-activation of the control plasmid increases the induction with pB9+fp116 and pB9+fp116ATF to around 7-fold. This induction is more like that observed in previous publications (262). However, pB9+fp402 also gives around a 7-fold induction in this experiment. This is probably due to the rather low levels of CAT activity obtained with the samples that had no Ela plasmid contructs co-transfected as with 12s co-transfected (which is not trans-activating protein) a 3 to 4-fold induction is observed. So, the trans-activation of

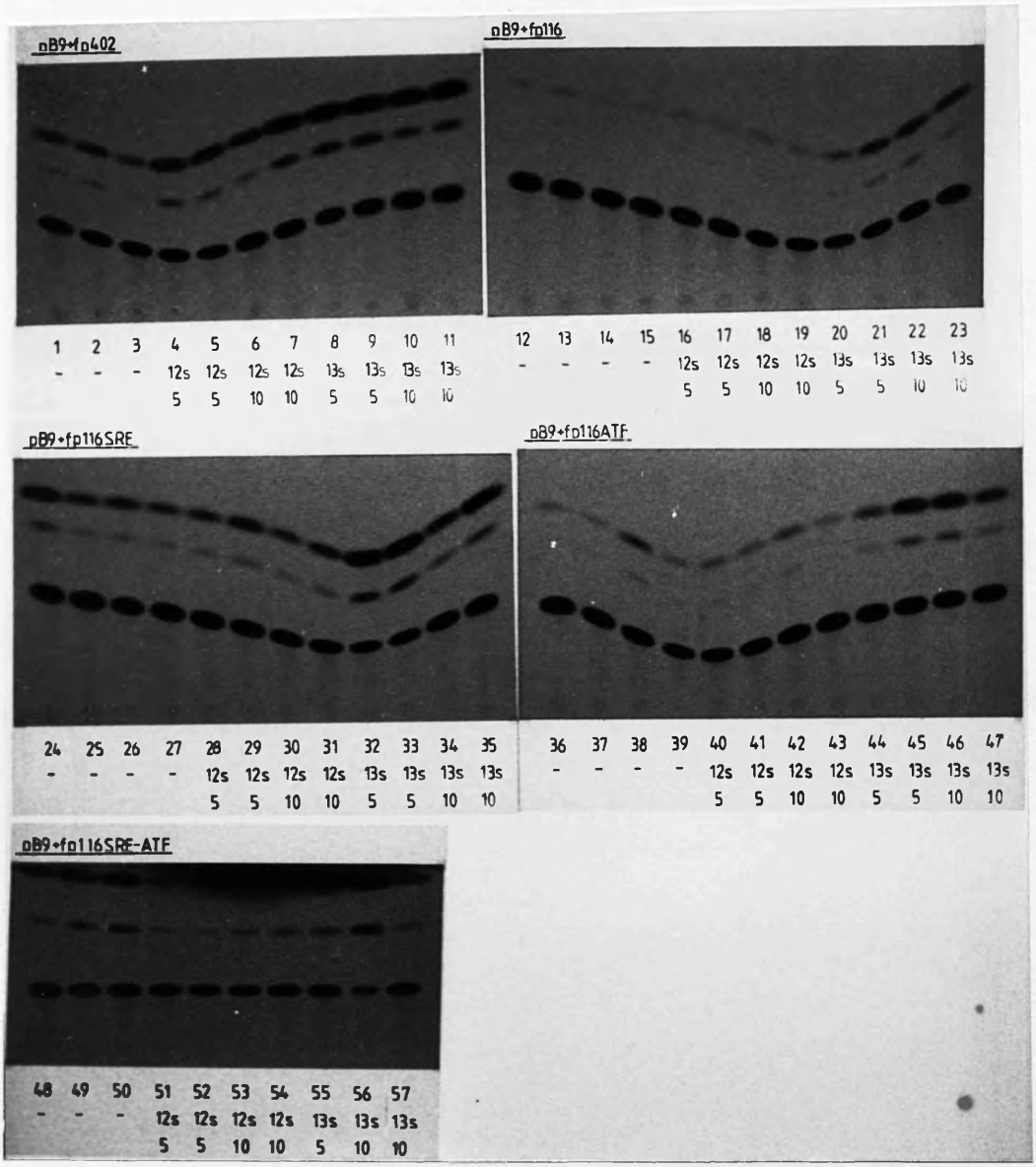


Figure 36. Co-transfection of c-fos-CAT and Ela plasmids into CT3 cells and correction for the trans-activation of the internal control plasmid by Ela 13s.

This figure is essentially identical to Figure 25 apart from one significant difference i.e. the amount of extract used in each CAT assay is corrected to compensate for the trans-activation of the HSV-β-gal internal control plasmid by the Ela 13s gene product (see test in details). It should also be noted that lanes 1, 50 and 58 from Figure 35 are missing in this figure because of a lack of cell extract. This experiment is quantified in Table 14.

	PLASMID CONSTRUCT								
	pB9+fp402			pB9+fp116			pB9+fp116SRE		
CO-TRANSFECTED PLASMID	-	12s	13s	-	12s	13s	-	12s	13s
	1 6.0 2 9.5 3 5.1	4 21.1 5 23.6 6 12.2 7 41.4	8 66.0 9 58.2 10 31.2 11 42.0	12 1.1 13 1.6 14 0.9 15 1.1	16 1.5 17 1.5 18 1.6 19 1.2	20 5.3 21 10.3 22 7.8 23 11.7	24 28.2 25 8.2 26 14.9 27 7.4	28 8.2 29 15.3 30 6.9 31 8.6	32 44.1 33 44.5 34 14.8 35 29.7
AVERAGE CONVERSION	6.9	24.6	49.4	1.2	1.5	8.8	14.7	9.8	33.3
STANDARD DEVIATION	2.2	12.2	15.5	0.1	0.2	2.7	9.6	3.6	14.0
FOLD INCREASE	1.0+\-0.3	3.6+\-1.8	7.2+\-2.2	1.0+\-0.1	1.3+\-0.2	7.3+\-2.3	1.0+\-0.7	0.7+\-0.2	2.3+\-1.0

	PLASMID CONSTRUCT					
	pB9+fp116ATF			pB9+fp116SRE-ATF		
CO-TRANSFECTED PLASMID	-	12s	13s	-	12s	13s
	36 1.6 37 1.5 38 5.7 39 1.9	40 1.9 41 2.1 42 3.4 43 1.6	44 5.8 45 23.5 46 28.0 47 13.3	48 14.1 49 29.2 50 25.6	51 8.2 52 8.7 53 19.8 54 16.0	55 22.5 56 90.0 57 9.3
AVERAGE CONVERSION	2.7	2.3	17.7	23.0	13.2	40.6
STANDARD DEVIATION	2.0	0.6	9.9	7.7	5.6	43.3
FOLD INCREASE	1.0+\-0.7	0.9+\-0.2	6.6+\-3.7	1.0+\-0.3	0.6+\-0.2	1.8+\-1.9

Table 14. Co-transfection of c-fos-CAT and E1a plasmids into CT3 cells and correction for the trans-activation of the internal control plasmid by E1a.

This table quantifies the CAT activity obtained in the experiment shown and described in Figure 36. The figures represent the percentage of ^{14}C-chloramphenicol converted to mono-acetylated forms with each extract. In this table the results obtained with the different amounts of co-transfected E1a plasmid constructs have been taken to represent equivalent samples as no appreciable difference can be detected between the two sets of results. The fold-increase represents the ratio of the average conversion obtained with the E1a minus samples (-) to the average conversions and standard deviations obtained with the samples that had E1a plasmid constructs co-transfected. The numbers 1 to 57 correspond to lanes 1 to 57 of Figure 36.

pB9+fp402 by 13s is probably only around 2-fold. the same levels of trans-activation of pB9+fp116SRE and pB9+fp116SRE-ATF by Ela 13s co-transfection were also observed.

In a subsequent experiment involving the co-transfection of HSV-β-gal plasmid with Ela plasmids, the 13s producing construct trans-activated HSV-β-gal 35-fold (data not shown). Because of this wide variability in the degree of trans-activation by Ela 13s of the HSV-β-gal construct it was decided that the adoption of the correction strategy should be stopped. Bio-rad protein estimations (2.6.1) were carried out on some extracts and equivalent amounts of protein used in each CAT assay. However, the variability in CAT activity between equivalent samples was large using this technique, presumably due to the variation in transfection efficiencies (data not shown). It was therefore decided that this technique was also of no use. So, because of the difficulties of obtaining an internal transfection efficiency control and because no other methods yielded satisfactory results it was decided to stop further experimental work involving the trans-activation of the c-fos-CAT constructs by Ela gene containing plasmids. However, preliminary results did indicate that the Ela 13s protein trans-activates c-fos expression through sequences within 116bp upstream of the mRNA CAP site (which contains the basal promoter elements) and not through the SRE or fosATF/AP-1 sequences (which are 300bp upstream from the mRNA CAP site).

CHAPTER 4 : DISCUSSION

DISCUSSION PAGE

4.1. Identification of cis-acting Transcriptional Control Elements.

It is possible that gene expression may be regulated at a variety of steps that lead to the production of the protein encoded by a particular gene. The control may come at the transcriptional, post-transcriptional, translational or post-translational levels, or it may involve a combination of these steps. In recent years great efforts have been made to characterise the transcriptional control of eukaryotic genes as the initiation of transcription is probably the most important step in the regulation of gene expression.

A combination of *in vivo* and *in vitro* techniques have been employed to identify *cis*-acting DNA sequences involved in the transcriptional control of eukaryotic gene expression. DNA sequences to which nuclear proteins bind have been identified by *in vivo* and *in vitro* methods (sub-Section 4.1.1.), whereas *in vivo* studies employing transfection techniques (sub-Section 4.1.2.) have been used as functional tests of the interaction of specific DNA sequences with putative transcription regulatory factors.

4.1.1. Identification of cis-acting transcriptional control elements by protein binding studies.

It was originally thought that eukaryotic gene transcription was controlled by the interaction of protein factors with DNA sequences in or around the genes. This idea was supported by the observation that DNAse 1 hypersensitivity in chromatin is most frequently confined to discrete domains in the 3'- or 5'-flanking regions of

genes (361), and is usually correlated with the expression of those genes (362, 363). These DNAse1 hypersensitive sites are formed due to the presence of protein factors that bind to the relevant DNA sequences resulting in an increased accessibility for the nuclease. This observation was exploited to characterise further the precise DNA sequences involved in DNA-protein interactions. The DNase1 footprinting assay was developed which involved the digestion of a labelled-DNA fragment that had been incubated in vitro with cellular protein extracts (364). This digestion results in a "ladder" when the recovered DNA is fractionated by electrophoresis through a denaturing gel, but any region protected from DNase1 digestion (by protein binding) results in gaps appearing in the "ladder". These gaps were called footprints and represent DNA sequences involved in DNA-protein interactions. This technique has been used extensively to analyse many transcriptional control regions (for example see 365) and has proved an extremely useful tool in the identification of precise DNA sequences that are involved in the control of transcription. A modification of this technique employs the enzyme exonuclease III (366). This enzyme, a 3′ to 5′ exonuclease, degrades a double-stranded DNA fragment progressively from both 3′ termini until the substrate becomes single-stranded and resistant to further digestion. Specific protein binding blocks the passage of exoIII and generates fragments that remain resistant under limiting digestion conditions. This technique is more sensitive than DNase1 footprinting as exonuclease resistant fragments can be positively identified even if they occur as a small fraction of the total DNA population. In summary both of these footprinting techniques are useful in delineating regions of DNA that bind protein factors and

are therefore putatively involved in transcriptional control.

The identification of specific, relatively short DNA sequences putatively involved in transcriptional control led to a more extensive use of the gel mobility shift (or gel retardation) assay (355). Gel mobility shift assays involve the incubation of labelled DNA fragments or oligonucleotides with protein extracts followed by electrophoresis through a neutral gel. If a protein-DNA complex is formed its mobility will be slower than that of the labelled DNA which allows for detection of the complex. This assay is quicker and easier to perform than either of the two previously mentioned assays and is useful in determining the affinity a particular protein has for its DNA recognition sequence and also for cross-competition experiments with other DNA sequences. As with the footprinting assays, this technique identifies DNA sequences that bind protein factors and are therefore putatively involved in transcriptional control.

The protein-DNA interaction assays described so far have involved *in vitro* techniques which involve the incubation of specific DNA elements/fragments with prepared protein extracts. The *in vivo* genomic sequencing technique (367) can be modified for *in vivo* footprinting. This technique involves the addition of dimethylsulphate (DMS) to the cells of interest before harvest, isolating the DNA, digesting it with a restriction enzyme and cleaving it with piperidine at the methylated residues. Electrophoresis of this DNA through a denaturing gel and hybridisation with a specific probe identifies sequences that were

protected from methylyation and therefore presumably had bound nuclear protein at the time of the addition of DMS to the cells. This technique has been used in the analysis of protein-DNA interactions occurring in the region of several genes (368, 369). DMS methylates guanine residues and this chemical also has been employed in in vitro DNA-protein interaction studies to characterise the points of contact a particular protein has with its recognition sequence (for example see 370). Recently, this technique was used to analyse the c-fos SRE and showed that two cellular proteins can recognise this sequence and that each protein has different points of contact with the SRE (371).

All of the techniques detailed so far have led to the identification of short DNA elements that interact with putative transcriptional control protein factors. This observation resulted in the purification of many of these transcription factors by DNA affinity chromatography (372). A technique that was helpful in characterising these proteins was the in vitro transcription assay (373), although this technique has also been used to characterise particular protein extracts. This assay analyses the effect of a purified protein (or protein extract) on the transcription in vitro of a DNA template and can therefore be used to characterise the specific effects of purified protein (or protein extracts) on transcription.

Following characterisation of DNA sequences that complex with protein factors it is necessary to determine the effects these sequences have upon transcription in vivo, and for this purpose transfection experiments have proved informative.

4.1.2. Functional analysis of transcriptional control elements.

To define transcriptional control elements by functional tests, plasmid constructs can be transfected into eukaryotic cells. These plasmids contain the promoter and upstream sequences of interest (sequences 3' of transcription initiation can also be assayed using this technique) linked to a reporter gene whose product can be assayed following the harvesting of the transfected cells. The transcription assays determine the influence that the promoter and upstream sequences exert over the transcription of the reporter gene.

An alternative method for inserting exogenous DNA into eukaryotic cells is to infect the cells with retroviruses (374) containing the exogenous DNA sequences of interest. However, this technique has limited uses for the analysis of transcriptional control for several reasons. Firstly, when a cell is infected by a retrovirus low copy numbers of the foreign DNA sequences are incorporated into the cell which makes it difficult to assay for any reporter protein (or RNA). Secondly, many retroviruses have a limited cellular host range which often makes it impossible to compare results between species. Thirdly, the retroviral genome is larger than that of a plasmid making cloning into, and manipulation of, the retroviral DNA extremely difficult. Fourthly, upon infection by a retrovirus the cells containing the retrovirus must be selected for making it difficult to carry out transient transfection studies to analyse transcriptional control. When all of these facts are considered it is clear that the transfection technique has many advantages over retroviral infection as a method for analysing the control of

eukaryotic gene transcription.

There are two types of transfection studies that can be employed in the analysis of transcriptional control : stable and transient. Stable transfections involve the transfection of the transcription assay plasmid along with a plasmid containing a drug resistance gene, the most commonly used of which is the neomycin resistance gene (which confers resistance to the drug G418). Following transfection, cells that have taken up the DNA can be selected for by incubating the cells in medium containing the drug to which the co-transfected gene confers resistance. The cells are then incubated for 10 to 14 days whereupon colonies are formed representing individual cells that have been transfected with the plasmid DNA. These colonies can then be grown up and the cloned cells analysed or, more frequently, the colonies are pooled and then grown up. In this way variations due to differences in the sites at which the exogenous DNA has been incorporated are averaged. In transient transfection studies a population of cells is transfected with the transcription assay plasmid and harvested 48 hours later and then prepared for transcription assays.

The transient and stable transfection techniques have certain advantages and disadvantages which must be considered before adopting a particular strategy. The major disadvantage of the transient transfection technique is the requirement for an internal control of transfection efficiency. Each population of cells transfected with an identical amount of plasmid shows a different degree of DNA uptake. To correct for this variation in transfection efficiency a

plasmid must be co-transfected with the transcription assay plasmid, this co-transfected plasmid also encoding a gene whose product can be assayed. It is also important that the transcription of the internal control gene is not sensitive to the experimental conditions e.g. if the cells are stimulated in some manner it is important that the transcription of the internal control gene is not affected by such stimulations. Following the internal control assay which corrects the variation in transfection efficiency, equivalent amounts of cell extract are used in the transcription assay. This technique operates well in checking for variations in transfection efficiency but there is still a degree of variation in the data produced. Thus it is still necessary to have a number of "identical" independent samples within each transfection experiment and to repeat these experiments at least once to confirm any results obtained. Another drawback of the transient technique is that only a sub-population of the cells to which the transfection cocktail is added will take up DNA and it is possible that this sub-population represents a sub-set of cells, e.g. at a certain point in their growth cycle. It is also possible that each of these cells will take up several hundred copies of the transcription assay plasmid (not all of which will be nuclear) which could titrate out particular nuclear protein factors that may act on the transfected plasmid DNA. However, the transient transfection technique does enable a large variety of plasmid constructs to be studied in a reasonably short time, and is thus amendable to detailed studies of the effects of differences in transcriptional control elements.

Stable transfections obviate the requriement for an internal control plasmid used for correcting variations in transfection efficiency as once the population of stably transfected cells is established this really represents a "new" cell line. Populations of the stably transfected cells can therefore be plated out and harvested for transcription assays without further transfection which saves time if these cells are going to be used in many different experiments. If a pool of transfected cells are taken together to form the stable cell line this should average out the variation in copy number and/or insertion sites that would be present in each individual clone and would lend to a continuity between experiments carried out with the stable cell line. However, these stable transfection experiments are time consuming (transfected cells must be selected for between 10 and 14 days) particularly when many different plasmid constructs are to be analysed.

So, when selecting a model for in vivo analysis of transcriptional control it is necessary to balance the advantages of the stable and transient transfection techniques. Most investigations employ the transient transfection technique simply because it quickly produces results that are rapidly repeatable. Both of these techniques have been used in recent years to analyse the control of transcription and they have identified many cis-acting DNA transcriptional control elements (for reviews see 170 - 173).

Upon harvesting of the transfected cells there are many transcription assays that can be employed to determine the transcriptional control exerted by the sequences under study upon the reporter gene. These

assays can be divided into two different categories : the protein assays and the RNA assays. Belonging to the former group is the CAT assay (188), the β-galatosidase assay (350) and the human growth hormone assay (360); and to the latter group S1 analysis (351), RNAase protection analysis (375) and primer extension analysis (352). Although the advantages and disadvantages of each independent technique will not be discussed here, a comparison between protein and RNA assays will be made.

Protein assays are generally rapid and simple but do contain certain features that are open to criticism:

1) Protein assays cannot determine whether the construct under examination initiates transcription from the correct position.

2) If the protein being assayed is particularly stable in comparison to its mRNA, small amounts of mRNA may lead to the accummulation of an unrepresentative quantity of the protein. Although this would not significantly alter gross differences between constructs, it may mask more subtle effects upon transcription.

3) Enhancer elements have been discovered previously in intron sequences (376) but it is impossible to assay the effect upon transcription of sequences 3′ of the transcription initiation site of a gene in their endogenous positions using protein assays.

The best way to circumvent these problems is to employ RNA assays. These lead to the determination of the site of transcription initiation, obviate the problem relating to protein-mRNA stability and can allow for the assay of the effects of sequences 3′ of the

site of transcription initiation. This last point can be overcome using intact genes in the transfection assays (using a different species of eukaryotic cells from that of the gene under analysis to permit the endogenous and exogenous sequences to be differentiated), or by using deletions of the gene under examination. These assays also have the advantage that they are one step closer to the level of transcription and circumvent any possibility of post-transcriptional or translational control leading to artefactual results. RNA analysis can employ, as well as intact and deleted genes as mentioned, fusion plasmids similar to those used in the protein assays. This has the advantage of comparing the results obtained with protein assays with those obtained with RNA analysis assays, but has the disadvantage of not permitting the assay of sequences 3′ to the site of transcription initiation in their endogenous positions. The major disadvantage of the RNA assays is that they are more time consuming and technically more difficult than their protein counterparts. Also, the RNA under analysis may be relatively unstable making it difficult to detect in the RNA assays.

As with the transient and stable transfection techniques described earlier it is a matter of choosing the correct transcription assay for the particular study being undertaken. It is often beneficial to employ both a protein assay and an RNA analysis assay when assaying for transcriptional control elements in vivo. Protein assays can be employed for obtaining preliminary results which can then be confirmed with an RNA assay.

4.1.3. Transfection technique and transcription assays adopted in this project.

The previous two sub-Sections (4.1.1. and 4.1.2.) have detailed a variety of *in vivo* and *in vitro* techniques that can be used for the analysis of the control of eukaryotic gene transcription. This sub-Section will describe a selection of these techniques that were employed in this project for the analysis of c-fos transcriptional control.

Previous studies on the control of the c-fos gene transcription have employed a combination of *in vivo* and *in vitro* techniques and have identified several DNA elements involved in the transcriptional control of this gene (see Section 1.6.). All of these elements are present in the promoter and upstream region of the c-fos gene. These investigations used footprinting studies and gel mobility shift assays to identify DNA elements that bind nuclear proteins, and transfection studies using various transcription assays were employed to analyse the influence these sequences exert upon c-fos transcription *in vivo*. RNA analysis assays have been carried out using RNA prepared from mouse cells transfected with the entire human c-fos gene (including upstream sequences) (151) or with c-fos upstream sequences fused to the CAT gene (232). CAT assays were also used in these studies (190) and in certain cases the CAT assays were complemented with RNA analysis assays (220). These studies identified a serum inducible protein binding enhancer element centered 308bp upstream from the transcription initiation site of c-fos and the results obtained with the CAT assays reflected those

obtained with the RNA analysis assays.

The majority of these studies employed the transient transfection technique using calcium phosphate precipitates added to mouse fibroblast cells. In this project a similar system was adopted and the cell line used, CT3, was a derivative of NIH 3T3 cells which were used extensively in the previous analyses. The advantageous aspects of the transient transfection technique, compared to the stable transfection technique, also influenced the choice. This was important in this project as several of the c-fos-CAT constructs were made only after obtaining results in preliminary experiments that suggested the need for the production and assay of such constructs.

As mentioned earlier, previous reports with c-fos-CAT constructs showed that results obtained with CAT assays mirrored those obtained with RNA analysis of the same construct (220). Since the CAT assay is quicker and easier to do than any of the RNA analysis assays it was decided that this would be the major transcription assay employed throughout this project. For this purpose, many c-fos-CAT hybrid plasmid constructs were made (see Section 3.1.). To check for the variation in transfection efficiencies in these transient transfection assays a plasmid encoding the β-galactosidase gene was co-transfected with the c-fos-CAT constructs. The protein produced by this plasmid can be assayed for using the β-galactosidase assay and equivalent amounts of β-galactosidase activity were used in the CAT assays. Because CAT assays cannot determine whether the c-fos-CAT constructs initiate transcription from the correct position attempts were made to carry out S1 analysis on RNA prepared from

cells transfected with these constructs. Although such analysis failed on RNA prepared from cells transiently transfected with the c-fos-CAT constructs, such analysis using RNA prepared from on LATK$^-$ cell line stably transfected with pCN+fp1400 was successful. The S1 analysis carried out with the RNA prepared from the stably transfected LATK$^-$ cells showed that pCN+fp1400 initiates transcription from the correct position. Since all of the other c-fos-CAT constructs were directly related to this construct it seems reasonable to assume that all of the c-fos-CAT constructs initiate transcription from the correct position.

Upon identification of a region in DNA that is involved in the control of the transcription of a gene it is usual to attempt to characterise the precise DNA sequence that is responsible for this control. In vivo protein binding studies on the DNA region identify sequences involved in DNA-protein interaction and are therefore putatively responsible for the control of transcription. To analyse these sequences in vivo an oligonucleotide containing the sequence of interest is synthesised and inserted upstream from the relevant promoter that has had its corresponding endogenous sequence deleted. These constructs are then analysed in transfection and transcription assay experiments to determine the effect the inserted oligonucleotide has upon transcription from the relevant promoter. This technique has been used extensively in characterising relatively short DNA sequences (10 to 20bp) that are involved in basal and inducible transcriptional activation. The data obtained in such experiments must be interpreted with caution particularly if the DNA sequence under analysis has been moved from its endogenous position

(usually it is moved closer to its promoter). This may result in a failure of the protein binding to the sequence to interact with other DNA-binding proteins that have had their recognition sequences deleted in the synthesis of the oligonucleotide containing construct, or in a failure to interact with DNA binding proteins present in the promoter region of the gene under analysis due to an inability of the truncated DNA to form the correct secondary structure. It is also important to check that the insertion of the oligonucleotide does not create a new transcriptional control element in the oligonucleotide plasmid overlap region. The best way of checking that this is not the case is to carry out mutation analysis to determine that it is the protein binding site present in the oligonucleotide that is responsible for exerting the transcriptional control. Such studies have been used in characterising DNA sequences involved in the control of c-fos transcription. The major element characterised by these studies is the SRE (see sub-Section 1.6.2.) which was shown to be a serum inducible enhancer element capable of conferring serum inducibility upon the c-fos promoter when a single copy was inserted upstream from the promoter. In a study of the element downstream from the SRE that bears close homology to the DNA sequeces which bind the transcription factors AP-1 and ATF (which I have termed fosATF/AP-1), a single copy of this element was capable of conferring TPA and EGF responsiveness on the c-fos promoter (252). Previous studies of other AP-1 and ATF sequences (see sub-Sections 1.6.5. and 1.6.6.) also showed that a single copy of such sequences is capable of exerting transcriptional control. Because of these observations it was decided that in this project single copies of oligonucleotides containing putative transcriptional control elements would be

inserted upstream from the c-fos promoter in c-fos-CAT constructs. The effects of these sequences upon transcription from the c-fos promoter were assayed for in transient transfections followed by CAT assays. As a single copy of the relevant sequences were considered to be sufficient to control transcription from the c-fos promoter, the oligonucleotides containing these sequences were inserted upstream from the c-fos promoter in an orientation dependent manner. This was done by giving the oligonucleotides different restriction enzyme sites at their 5' and 3' termini and inserting them into a c-fos-CAT construct that had been digested with the relevant restriction enzymes.

So, the transfection/transcription assay techniques employed throughout this project seemed reasonable when compared to those used in previous studies that have investigated the control of c-fos transcription. However, all data obtained using these techniques must be interpreted with caution as they occasionally produce results that are difficult to explain. For example, in a previous study on the fosATF/AP-1 sequence (252) this sequence was shown to confer TPA and EGF responsiveness on the c-fos promoter only in stably transfected cells while transient transfection studies failed to identify this element as a transcriptional control element. This observation suggested caution in interpretation of transfection/transcription assay data. Two independent reports produced conflicting reports on the serum inducibility of c-fos-CAT constructs transfected into mouse fibroblast cells (189,220). In both cases the CAT assay was used as the transcription assay and the c-fos-CAT constructs were similar in structure. In stably

transfected NIH 3T3 cells these constructs failed to show a significant increase in CAT activity following serum stimulation of serum deprived transfected cells (189), while a similar treatment of transiently transfected NIH 3T3 cells resulted in a significant increase in CAT activity following serum stimulation (220). The only major difference between these studies was the transfection technique and it is not known why this should contribute towards the anomalous results obtained. Some conflicting results were also obtained in this project using the transfection/transcription assay technique (see sub-Section 3.4.2.). Initial transfection experiments in CT3 cells failed to identify the SRE containing c-fos-CAT construct pB9+fp402 as being serum responsive. The reason for this failure was not clear as Northern blot analysis showed that the endogenous gene is induced following serum stimulation of these cells. Three possible explanations were thought of for the failure to observe a serum response with the c-fos-CAT construct:-

1) The c-fos-CAT construct was defective.
2) The CT3 cells were not quiescent prior to stimulation.
3) The density of the cells prior to stimulation is crucial.

It seems unlikely that the c-fos-CAT construct are defective as they do show variations in transcriptional activity similar to those reported previously, and the parent c-fos-CAT construct does initiate transcription from the correct position in stably transfected cells. It also seems unlikely that the cells were not quiescent prior to serum stimulation as they were serum deprived for at least 30 hours prior to stimulation and harvest. This length of time has been used

previously in rendering mouse fibroblasts into a quiescent state. The density of the cells prior to stimulation and harvesting for Northern blot analysis, and prior to stimulation and harvesting for transcription assays, was not noted. It was therefore possible that cell density played a crucial role in the serum stimulation of c-fos expression. Results obtained with transfection experiments with the cells at different densities prior to stimulation and harvest showed that the cell density did play a crucial role in obtaining a serum response with the c-fos-CAT constructs. The higher the density of the cells the greater the response of the c-fos-CAT constructs to serum stimulation. This observation provided an explanation for the failure to observe a serum response with the pB9+fp402 in the preliminary transfection experiments, i.e. the cells may not have been at a high enough density prior to serum stimulation. It could also explain the anomaly mentioned earlier in the previously published reports of serum stimulation of NIH 3T3 cells transfected with c-fos-CAT constructs i.e. the cells may have been at different densities in the two sets of experiments prior to serum stimulation. The results obtained with the high density of cells was repeated another twice. However, following these two subsequent experiments it became impossible to obtain a serum stimulation of pB9+fp402 even though the transfected cells were at a high density prior to serum stimulation. The results were similar to those obtained in the earliest experiments : there was a failure to shut down transcription upon serum deprivation of the cells. Several batches of frozen cells were grown up and used in subsequent experiments, but still no serum response could be detected with the transfected c-fos-CAT constructs. The major difference between the previous experiments that gave a

serum response with pB9+fp402 and the subsequent ones that failed to do so was that a different batch of serum was used in the growth of the cells used in the different sets of transfection experiments. The precise effect these different batches of serum have upon the growth of the cells cannot be determined but it is possible that this difference may be responsible for the anomalous results obtained in the transfection experiments.

So, no matter the care taken in the choice of transfection technique and transcription assay, it is important to remember that data obtained in these studies must be interpreted with caution. However, these techniques have been used extensively in the characterisation of cis-acting transcriptional control elements and may be considered crucial in the analysis of the control of eukaryotic gene transcription.

4.2. The c-fos Gene has Several cis-acting Transcriptional Control Elements.

The techniques discussed in Section 4.1. have resulted in the identification of many transcriptional control elements (for reviews see 170 - 173). These elements can be separted into two categories: the upstream promoter elements (UPEs) and the enhancer elements. The UPEs contribute towards the basal levels of transcription from promoters and are located close to the transcription initiation site (usually within 100bp upstream). The enhancer elements have different properties which include the ability to activate transcription in an orientation and distance independent manner and to exert transcriptional control over heterologous promoters (sub-Section 1.6.1.).

Previous studies on the control of c-fos transcription identified an element 300bp upstream from the transcription initiation site that can behave as an enhancer element and also activate transcription in response to serum stimulation of quiescent cells (see sub-Section 1.6.2.). Two UPEs have also been characterised in the human c-fos promoter region which contribute to basal levels of transcription and form complexes with nuclear proteins. The experiments carried out in this project further investigated the role of the SRE in c-fos transcription, and also characterised a sequence that is continuous with the SRE that bears homology to previously identified transcription factor binding sites. This element was identified by a computer-aided search of the human c-fos sequence. It bears close homology to the previously characterised AP-1 and ATF proteins

binding sites (see sub-Section 1.6.5. and 1.6.6.). These two binding sites differ by only 1bp in their consensus sequences and it is therefore difficult to determine whether a similar sequence binds AP-1 or ATF simply by analysing its base composition. AP-1 binding sites are capable of conferring TPA or serum responsiveness upon heterologous promoters (see sub-Section 1.6.6.). As the c-fos gene is transcriptionally activated following treament of cells with serum or TPA, it was of interest to try and identify the protein that complexes with the fosATF/AP-1 sequence, and to establish whether this element plays a role in activating transcription from the c-fos promoter following serum or TPA stimulation.

4.2.1. Identification of cis-acting elements regulating c-fos transcription.

Transient transfection experiments carried out in this project with c-fos-CAT constructs detected the presence of an enhancer of transcription upstream from position -116bp (relative to the c-fos transcription initiation site). Downstream from position -116bp retains the previously characterised UPEs (185) which contribute to a basal level of c-fos transcription. Both of these observations, and the degree of enhancement (approximately 20-fold), agree with a previously published report using similar c-fos-CAT constructs (190). This report identified an enhancer element present between 200bp and 400bp upstream from the c-fos transcription initiation site. The SRE and fosATF/AP-1 sequences are both within this region and are 100% conserved between the human, mouse and chicken c-fos genes. Previous reports have characterised the SRE as an enhancer element (see

sub-Section 1.6.2.). When the c-fos SRE is placed upstream from the X.laevis promoter that has had its SRE deleted, it enhances transcription when placed in either orientation. This element also activates transcription from the c-fos promoter upon serum stimulation of quiescent cells when placed in either orientation upstream or downstream from the c-fos promoter (151). At the beginning of this project the fosATF/AP-1 element had not been previously studied, but has subsequently been shown to induce transcription from the c-fos promoter in response to stimulation with EGF or TPA (252).

In this project the effect of the SRE and fosATF/AP-1 sequences on transcription from the c-fos promoter were assayed in growing cells. When the SRE was placed 116bp upstream from the c-fos promoter it increased transcription approximately 10-fold in growing cells. This is in agreement with the previous report identifying the SRE as an enhancer element in growing cells (190). When the fosATF/AP-1 sequence was inserted in an identical position to the SRE upstream from the c-fos promoter it increases transcription approximately 4-fold, which is a relatively low increase in comparison to other AP-1/ATF binding sites (see sub-Section 1.6.5. and 1.6.6.). Mutation of the fosATF/AP-1 element abolished the observed increase in transcription showing that this increase was specific for the fosATF/AP-1 sequence and not because of the synthesis of a transcriptional control element due to the insertion of the oligonucleotide containing the fosATF/AP-1 sequence. When the SRE and fosATF/AP-1 sequences are inserted together upsteam of the c-fos promoter it is difficult to determine whether these sequences act in

an additive fashion to activate transcription as the fosATF/AP-1 sequence contributes to the activation of transcription at a relatively low level. One fact that is clear from the transfection studies using the c-fos-CAT constructs with the inserted oligonucleotides is that they fail to activate transcription to the same degree as the full 400bp c-fos upstream region. This observation suggests one of two things :

1) The 400bp of c-fos upstream sequence contains further cis-acting DNA elements that contribute towards the transcriptional activation from the c-fos promoter.
2) The SRE and fosATF/AP-1 sequences activate transcription better when situated in their endogenous positions.

It is not clear which of these possibilities applies to this discrepancy. However, footprinting analyses of the c-fos promoter region have not revealed any protein binding sites in this region that are not present in the construct containing the oligonucleotide with the SRE and fosATF/AP-1 sequences. This suggests that the latter possibility may be the one that applies in this situation. A similar result was obtained when the c-fos SRE was placed upstream from the X.laevis actin gene promoter (193) - this construct does not restore endogenous levels of transcription when compared to the activity obtained with the intact promoter region containing the SRE in its correct position. This again suggests that the SRE and fosATF/AP-1 sequences may activate transcription better when situated in their endogenous positions. The reason for this reduction in transcriptional activating properties of cis-acting DNA elements when

they are moved from their endogenous position may be due to a failure of the proteins binding to these sequences to co-operate fully with other proteins bound in the promoter region. Such interactions have previously been proposed for the SRE (185). When one of the UPEs present in the promoter region of the c-fos gene is deleted, the induction of c-fos transcription by the SRE in response to external stimuli is reduced in comparison to that obtained with an intact c-fos promoter. In vitro binding studies have also shown that the transcription factor ATF co-operates in the formation of the proteins binding to the TATA box of an adenovirus gene promoter (377). This co-operation leads to quicker formation of such complexes which allows access for polymerase II. Obviously, if such binding sequences are moved from their endogenous positions this may affect the rate of formation of the complex at the TATA box and thus affect the rate of binding of polymerase II and therefore affect the rate of transcription from the relevant promoter.

4.2.2. The fosATF/AP-1 element binds a member of the AP-1/ATF protein family.

Although the fosATF/AP-1 element activated transcription when placed upstream of the c-fos promoter, this did not prove that a member of the AP-1/ATF protein family was responsible for this activation. To try and determine whether a member of this family was responsible for the transcriptional activation due to the fosATF/AP-1 element a combination of in vitro and in vivo studies were carried out.

Two previous reports had suggested that the fosATF/AP-1 sequence

could bind an AP-1-like protein. Footprinting studies of the c-fos upstream region showed that the protein binding to the fosATF/AP-1 sequence could be competed out by an α-enhancer sequence from the polyoma virus which contains an AP-1 binding site (250). In vitro translated AP-1 protein (from a c-jun clone) was also shown to be capable of complexing with the fosATF/AP-1 sequence (238). However, neither of these studies determined the precise nature of the protein that would bind to this sequence if it were incubated with a nuclear protein preparation, nor did they determine the strength of the protein binding in comparison to other AP-1/ATF elements. Gel mobility shift assays were employed in this project to investigate the protein(s) binding to the fosATF/AP-1 sequence.

An initial mobility shift assay using HeLaS3 nuclear protein extract demonstrated that the fosATF/AP-1 sequence formed a specific complex with a nuclear protein. The mutant fosATF/AP-1 sequence that was used in the transfection studies failed to compete for this protein forming the complex, agreeing with the observation that the fosATF/AP-1 sequence is responsible for the transcriptional activation observed in the in vivo studies. To determine whether this protein was a member of the AP-1/ATF protein family a gel mobility shift assay was carried out using previously characterised AP-1 and ATF sequences as competitors. These sequences were taken from the adenovirus 5 E3 gene promoter region and were characterised as AP-1 and ATF sites using gel mobility shift assays with other AP-1 and ATF sequeces as competitors (267). In a gel mobility shift assay using these sequences and the fosATF/AP-1 sequence, the complex formed with the fosATF/AP-1 sequence shows a similar mobility to that

formed with the E3-AP-1 sequence (the E3-ATF complex has a slightly quicker mobility). However, both the E3-AP-1 and E3-ATF sequences can compete for the protein that forms the complex with the fosATF/AP-1 sequence which in turn fails to compete for the proteins that form the complexes with the E3-AP-1 and E3-ATF sequences. Since the E3-AP-1 and E3-ATF sequences can partially compete for each others' binding protein (267), these results suggest that the fosATF/AP-1 sequence forms a weak complex with an AP-1 like protein. One interesting aspect of this particular study was the observation that, in the presence of excess E3-AP-1 competitor, the fosATF/AP-1 sequence forms a new complex that has a mobility shift similar to that observed with the E3-ATF sequence. This observation suggests that the fosATF/AP-1 sequence may be capable of forming a complex with more than one particular nuclear protein. This phenomenon is not unique as it has been shown previously that the octamer sequence is capable of binding at least two different nuclear proteins : oct1 (378) and oct2 (379). Like the ATF and AP-1 protein these two proteins have shared motifs that are involved in DNA binding and transcriptional activation (380). More recently it has also been shown that the c-fos SRE can form complexes with at least two nuclear proteins (371). Only one of the genes encoding these proteins has as yet been isolated, so it is not possible to determine whether they also represent a family of DNA-binding proteins which have shared motifs. So, these mobility shift assays showed that the fosATF/AP-1 sequence can bind a member of the AP-1/ATF protein family. They did not make clear which of these two proteins complex with the sequence, and indeed it is possible that the fosATF/AP-1 sequence may complex with a previously unidentified member of this family.

In a bid to characterise further the fosATF/AP-1 element the transcriptional activation properties of this sequence was compared with those of the E3-AP-1 and E3-ATF sequences. This study involved the transient transfection study of c-fos-CAT constructs with oligonucleotides containing the AP-1/ATF sequences inserted in an identical position upstream from the c-fos promoter. The results of this experiment showed that the E3-AP-1 and E3-ATF sequences are more potent transcriptonal activators than the fosATF/AP-1 sequence when all three are placed individually upstream from the c-fos promoter. This agreed with the gel mobility shift assays which showed that the fosATF/AP-1 sequence weakly binds a member of the AP-1/ATF protein family.

So, these in vivo and in vitro studies together suggested that the fosATF/AP-1 sequence forms a weak complex with a nuclear protein that is a member of the AP-1/ATF protein family. Because the gel mobility shift assays showed that the complex formed with the fosATF/AP-1 sequence was similar in mobility to that formed with the E3-AP-1 sequence, a gel mobility shift assay was carried out with nuclear protein extract pre-incubated with Fos antibody. Previous studies have shown that such a pre-incubation interferes with, or abolishes, the AP-1 complex formation (276, 277) which contains Fos protein. Such an assay carried out in this project showed that the pre-incubation with the Fos antibody did indeed interfere with the formation of the E3-AP-1-protein complex, but failed to do so with the fosATF/AP-1 complex or the E3-ATF-protein complex. This again suggests that the fosATF/AP-1 does not bind previously characterised AP-1 protein, and the pattern of the mobility shift had already

suggested that it does not bind a previously characterised ATF protein. It is possible that the fosATF/AP-1 sequence complexes with c-Jun protein homodimers rather than with c-Jun-c-Fos heterodimers (see Section 1.7.). If this were the case the Fos antibody pre-incubation would fail to interfere with such binding. c-Jun homodimers complex weakly with AP-1 sites (in comparison to c-Fos-c-Jun heterodimers), which also fits in with the observations made with the fosATF/AP-1 sequence. However, it is impossible to determine whether this is the case without similar pre-incubation experiments using a c-Jun antibody which was not available in this project. A gel mobility shift assay with in vitro co-translated Fos and Jun proteins also showed that the fosATF/AP-1 sequence formed a complex that was different in mobility to those formed with either the E3-AP-1 or E3-ATF sequences.

In a bid to characterise further the fosATF/AP-1 element as complexing with a member of the AP-1/ATF family of proteins transfection experiments were carried out with F9 embryonal carcinoma cells. Previous studies examined the role that PEA-1 (the murine homologue of AP-1) plays in the transcriptional control of the polyoma α-enhancer in this cell line (250, 310, 381). This enhancer element contains a PEA-1 (AP-1) protein binding sequence and is transcriptionally inactive in F9 cells.

Protein binding studes on the polyoma α-enhancer revealed that there were very low levels of PEA-1/AP-1 present in undifferentiated F9 cells, but following differentiation (when the α-enhancer becomes active) protein binding to this sequence can be easily detected. The

polyoma α-enhancer also contains a further protein binding site for a factor called PEA-2. This site overlaps with that of the PEA-1/AP-1 site and functions as a transcriptional (378) repressor. Following differentiation of F9 cells this factor becomes undetectable. This observation suggested that the polyoma α-enhancer may be inactive in F9 cells because of the repressor activity of PEA-2 rather than any lack of PEA-1 factor. It seems likely that there is a balance between the expression of these two factors that controls the transcriptional activity of the polyoma α-enhancer on the differentiated and undifferentiated F9 cells.

Studies of other AP-1 binding sites (such as that of the collagenase gene) have also shown that AP-1 elements are transcriptionally inactive in F9 cells. These sites do not contain overlapping PEA-2 binding sites. In the studies carried out in this project the E3-AP-1, E3-ATF, fosATF/AP-1 and SRE elements were all transcriptionally inactive in F9 cells. Not all transcriptional control elements tested were inactive in these cells as the HSV-β-gal plasmid gives a relatively high level of activity. The results obtained with the AP-1/ATF elements were not surprising as previous reports showed these to be inactive in F9 cells, but it has not been previously reported that the SRE is transcriptionally inactive in these cells. It is perhaps not surprising that this is the case as experiments examining the interaction between Fos and Jun proteins showed that co-transfection of Fos expressing constructs greatly enhanced the trans-activation properties of the Jun protein on constructs containing the AP-1 recognition sequence (305, 306). This suggested that in the F9 cells there was little or no Fos protein

present prior to these co-transfection experiments. Although the enhancer elements were transcriptionally inactive in the F9 cells there is a basal level of expression from the c-fos promoter. Only the c-fos-CAT construct containing the intact 400bp of c-fos upstream sequence showed an elevated level of expression in comparison to the c-fos promoter only construct. This increase was much smaller than that observed in CT3 cells (2-fold in comparison to 20-fold) which is not surprising considering the SRE and fosATF/AP-1 sequences are transcriptionally inactive in the F9 cells. The small increase in the level expression could be due to further transcriptional control elements present in the intact c-fos upstream sequence that has been deleted from the oligonucleotide containing constructs. Alternatively it could be because any residual transcription factors present in the F9 cells (such as the SRF) operate better when their target sequences are situated in the correct position with respect to the c-fos promoter. This observation is similar to the results obtained with transfected CT3 cells. In these cells the intact 400bp of upstream activates transcription to a greater degree than any of the oliognucleotide containing c-fos-CAT constructs and a discussion of the possible explanations for this phenomenon is given in sub-Section 4.2.1.

To show that the transcriptional inactivity of the AP-1/ATF elements in the F9 cells was due to a lack of trans-activating proteins a gel mobility shift assay was carried out. Surprisingly, this assay revealed that in F9 nuclear protein extracts there were proteins present that were capable of forming complexes with the E3-AP-1, E3-ATF and fosATF/AP-1 sequences. This observation contrasted

sharply with the transcription analysis and contradicted previously published work which showed that in a gel mobility shift assay no AP-1 binding activity could be detected (310). Several reasons can be thought of to explain this anomaly. It is possible that in preparation of the nuclear protein extracts from the F9 cells a certain step caused pre-existing inactive AP-1 proteins to be modified resulting in an activation of their binding properties. It should also be noted that the oligonucleotide sequences used in the mobility shift assay shown here were different from those used in previously published studies. It is therefore possible that the proteins forming complexes with the AP-1/ATF elements may not necessarily be classical AP-1/ATF-like proteins , but could be proteins that are either incapable of activating transcription or perhaps act as transcriptional repressors. Evidence to support the idea that these proteins are not classical AP-1/ATF proteins comes from comparing the pattern of mobility shifts obtained between F9 nuclear protein extracts and HeLaS3 nuclear protein extracts. The patterns are slightly different suggesting that it is different proteins forming complexes with the AP-1/ATF elements in the F9 and HeLaS3 nuclear protein extracts. However, it must be remembered that F9 and HeLa cells were established from different species which may also contribute to any differences observed in the proteins complexing with the AP-1/ATF sequences.

The original idea of studying the transcriptional control elements in F9 cells was to further characterise the fosATF/AP-1 sequences as a member of the AP-1/ATF family. Although this element does indeed behave in a similar fashion to the E3-AP-1 and E3-ATF sequences, the

SRE is also transcriptionally inactive in these cells. This suggest that there are many transcriptional control elements that are inactive in F9 cells and that this property is not exclusive to AP-1/ATF like elements. So, although this series of experiments failed to add substantial weight to the idea that the fosATF/AP-1 sequences complexes with a member of the AP-1/ATF family of proteins, they did not argue against this idea.

In a further bid to show that the fosATF/AP-1 sequence binds a member of the AP-1/ATF protein family in vivo, co-transfection experiments were carried out with a plasmid construct encoding the adenovirus 5 Ela 13s trans-activating protein (358). This protein can transcriptionally activate adenovirus early genes through AP-1 and ATF binding sequences (267). Unfortunately, in this project the Ela 13s protein trans-activated several transfection efficiency control plasmids and the inability to obtain a suitable internal control plasmid made it impossible to obtain conclusive results from these studies. However, the preliminary results that were obtained suggested that the Ela 13s protein activates c-fos transcription through sequences in the c-fos gene promoter region which agrees with a previous publication which suggested that the c-fos TATA box sequence is Ela 13s responsive (259). The results obtained here also contradict a previous publication (258) which suggested that the region including the SRE was Ela 13s responsive. The study identifying the c-fos TATA box as an Ela 13s responsive element also showed that the sequence including the SRE was not Ela 13s responsive. These variations may be due to the different cells and plasmid contructs used in the different labs. The limited results

obtained in this project also suggested that the fosATF/AP-1 sequence was Ela 13s responsive. However, this sequence was not compared with the E3-AP-1 or E3-ATF sequences and therefore no conclusions concerning the nature of the protein binding to the fosATF/AP-1 sequence in vivo can be made from these experiments.

In conclusion, the experiments carried out in this project confirmed that the SRE acts as an enhancer of c-fos transcription in growing cells. They also identified a further cis-acting transcriptional control element upstream from the c-fos promoter, the fosATF/AP-1 sequence. This sequence binds a member of the AP-1/ATF protein family and behaves as an AP-1/ATF sequence in the embryonal carcinoma cell line F9. However, it is a relatively weak transcriptional activator when compared with previously identifed AP-1 and ATF sequences.

4.3. Serum and TPA Response Elements Upstream from the Human c-fos Promoter.

The pleiotropic effects of different extra-cellular stimuli upon cells comes about through the expression of unique sets of genes. In recent years great efforts have been made to isolate these genes and to then analyse their transcriptional control. It was of great interest to determine whether genes activated in a co-ordinated fashion following a specific extra-cellular stimuli were transcriptionally activated by a similar mechanism. Such studies have resulted in the characterisation of the transcriptional control mechansisms of many genes in response to steroid hormones (for a review see 382), phorbol esters (see sub-Section 1.6.6.), cAMP (see sub-Section 1.6.5.), certain viral proteins (see sub-Section 1.6.7.), interferon (383, 384) and serum or serum growth factors (see sub-Section 1.6.2.). In all of these cases sets of genes that are transcriptionally activated in a co-ordinated fashion share a common mechanism of transcriptional activation. This involves cis-acting DNA elements upstream from the gene promoters that confer responsiveness and are common to all of the co-ordinately regulated genes. These elements bind proteins (trans-acting factors) that are responsible for the transcriptional activation. There seems to be three basic mechanisms for protein-DNA interaction that leads to the co-ordinated transcriptional activation of unique sets of genes:

1) In the case of steroid hormones (and retinoic acid (385)) the hormone binds to its receptor which then moves into the nucleus to complex with the relevant DNA response element (382).

2) Viral proteins activate transcription through pre-existing cellular factors. The mechanism involves the modification of cellular transcription factors, but the exact mechanism has not yet been characterised for any viral trans-activating protein.

3) Activation of intra-cellular pathways leading to the transcriptional activation of sets of genes. This mechanism applies to TPA, cAMP and serum or serum growth factors. A description of these pathways is given in sub-Section 1.4.3.

Studies of the transcriptional control of the c-fos gene have shown that all of the extra-cellular stimuli mentioned so far are capable of, or have been implicated in, activation of c-fos transcription (see Section 1.6.). The cis-acting DNA elements and nuclear proteins involved in these transcriptional activations have been characterised in many cases. The majority of the analyses investigating the control of c-fos transcription have been carried out with transfected murine fibroblast cells that were made quiescent and then stimulated with the relevant extra-cellular stimuli which results in the activation of endogenous c-fos transcription. The major cis-acting DNA transcriptional control elements identifed by such studies is the SRE positioned 300bp upstream from the c-fos transcriptional initiation site, which can activate transcription in response to a variety of extra-cellular stimuli including serum or serum growth factors, TPA and cAMP (see Section 1.6.). The fosATF/AP-1 element can confer EGF and TPA responsiveness upon the c-fos promoter and can also function as a cAMP response element (232). In this project the role of the SRE in the activation of c-fos transcription was investigated further, as was the fosATF/AP-1 element which was also

compared with the E3-AP-1 and E3-ATF sequences.

4.3.1. The fosATF/AP-1 sequence acts as a serum and TPA response element.

The previous study on the fosATF/AP-1 sequence characterising this element as being EGF and TPA responsive involved 1 hour stimulations of quiescent cells transfected with a c-fos CAT construct containing this element (252). As described in sub-Section 4.1.2. such short-term stimulations proved variable in this project. To overcome the inability to observe consistent serum or TPA responses in such stimulations, overnight stimulations were carried out on quiescent cells transfected with c-fos-CAT constructs containing the fosATF/AP-1, E3-AP-1 and E3-ATF elements. Overnight stimulations had been used previously in characterising AP-1 sequences as being TPA response elements (248). The implications of the limited results obtained with the 1 hour stimulations are discussed later.

The data discussed in the previous Section (4.2.) characterised the fosATF/AP-1 element as an activator of c-fos transcription which could complex with a member of the AP-1/ATF family of proteins in vitro. However, these studies failed to determine whether an AP-1 or ATF protein complexed with this element, or whether a previously uncharacterised member of the ATF/AP-1 protein family was involved in this complex formation. Previous reports showed that AP-1 sites could confer both serum or TPA responsiveness upon heterologous promoters (251), while ATF sites failed to do so (253). It was reasoned that the characterisation of the fosATF/AP-1 element as

being a serum or TPA response element would therefore help in determining which protein (either AP-1 or ATF) could complex with this sequence in vivo. The overnight stimulation assays carried out in this project characterised the fosATF/AP-1 element as being both serum and TPA responsive; both inductions activated c-fos transcription through this element to a similar degree. The E3-AP-1 element also acted as a serum or TPA response element when placed upstream from the c-fos promoter. Again, the degree of induction of c-fos transcription was similar for each treatment and these results agreed with previously published results showing AP-1 sites as being able to confer both serum and TPA responsiveness upon heterologous promoters. Surprisingly, the E3-ATF sequence could also confer serum or TPA responsiveness upon the c-fos promoter. The protein that complexes with this sequence, ATF, is indistinguishable from the cAMP response element binding protein (187) which binds to CREs and activates transcription in response to cAMP. It has been previously reported that the CREs were incapable of conferring TPA responsiveness upon promoters (253). Two reasons may be thought of to explain this discrepancy:

1) The E3-ATF sequence has not been characterised in vivo as being a CRE. So, although in vitro competition experiments with previously characterised CREs suggested that both elements complex with an identical protein (267), this may not be the case. They both may complex with different proteins that have equal affinities for both sites.

2) The sequences in the c-fos promoter may influence the response of the E3-ATF sequence to the serum and TPA stimulations. A previous

study showed that sequences flanking AP-1 or CRE sites influence the degree of transcriptional activation these elements confer in response to extra-cellular stimuli. In this case these sequences may also contribute to the specificity of the response. It is also possible that proteins that are bound to the c-fos promoter region also contribute towards the specificity of the response of the inserted oligonucleotide.

These results once again suggest that the fosATF/AP-1 sequence complexes with a member of the AP-1/ATF family of proteins in vivo, but also once again fail to differentiate between which protein is involved in this complex. In the previous Section (4.2.) it was suggested that the fosATF/AP-1 element may complex with a previously uncharacterised member of the AP-1/ATF protein family as it complexes with a nuclear protein in vitro and does not behave as either a classical AP-1 or ATF element. Also the fosATF/AP-1 sequence is a poorer transcriptional activator than either the E3-AP-1 or E3-ATF sequences. The limited results obtained with the short-term (1 hour) stimulations in this project also indicate that the fosATF/AP-1 element behaves in a different fashion from the E3-AP-1 element. The previously reported study on the fosATF/AP-1 element (252) showed that it could confer TPA responsiveness upon the c-fos promoter after a 1 hour stimulation. Classical AP-1 sites were characterised as being TPA response elements following 12 hours stimulations (248). In this project it was shown that the fosATF/AP-1 element could confer TPA responsiveness on the c-fos promoter following a 1 hour stimulation, but that the E3-AP-1 sequences failed to do so. This result once again suggests that the fosATF/AP-1 does not behave as a

classical AP-1 element, although it must be interpreted with some caution as subsequent experiments failed to repeat this observation. This was possibly because the basal level of transcription due to the fosATF/AP-1 sequence became relatively high in the subsequent experiments and thus possibly masked any increase in activity following the 1 hour TPA stimulation. It would be of obvious interest to determine whether the fosATF/AP-1 sequence could confer TPA responsiveness upon a heterologus promoter as other AP-1 elements are capable of doing so (see sub-Section 1.6.6.).

One other interesting feature obtained from the limited number of short-term stimulation experiments was the inability of the fosATF/AP-1 sequence to confer serum responsiveness upon the c-fos promoter following a 1 hour stimulation. The enhanced transcription from the c-fos promoter due to the fosATF/AP-1 element was completely abolished in quiescent cells, but transcription failed to increase following the 1 hour stimulation. So, the protein complexing with the fosATF/AP-1 sequence in vivo can only activate transcription in short-term stimulation assays following TPA treatment. The significance of this result is unclear.

Whatever the complexity of the situation, it is clear that the fosATF/AP-1 sequence can confer serum and TPA responsiveness upon the c-fos promoter, and that this responsiveness does not follow an identical pattern to that obtained with the E3-AP-1 sequence. These observations, taken together with those made in the previous Section (4.2.), suggest that it is possible that the fosATF/AP-1 sequence does not behave as a classical AP-1 element.

4.3.2. A possible interaction between the proteins binding to the SRE and fosATF/AP-1 sequences.

Previous reports have characterised the SRE as being able to confer both serum and TPA responsiveness upon the c-fos promoter following short-term stimulations (see sub-Section 1.6.2.). A preliminary experiment carried out in this project confirmed that the SRE could confer serum responsiveness upon the c-fos promoter, and this reponse increased with increasing cell density. In subsequent experiments where the SRE and SRE, fosATF/AP-1 sequences were assayed in the same experiments it was observed that the SRE, fosATF/AP-1 sequence showed a more consistent serum response. The reason for this seemed to be the low levels of CAT activity obtained with the SRE, fosATF/AP-1 construct in the quiescent cells in comparison to those obtained with the construct containing the SRE alone. This observation suggested a possible interaction between the proteins complexing with the SRE and fosATF/AP-1 sequences. This was perhaps not surprising as two other immediate early genes also contain the juxtaposed SRE and fosATF/AP-1 sequences in their putative transcriptional control regions (192). So in this project the possibility of such an interaction between the SRE and fosATF/AP-1 elements was analysed.

Before moving on to discuss the co-operation between the two elements in controlling transcription from the c-fos promoter it is worthwhile mentioning the results obtained with the c-fos-CAT construct containing an intact 402bp of c-fos upstream sequence. In overnight or short-term stimulations of cells transfected with this construct there was a failure to obtain a consistently reproducible serum or

TPA induction of CAT activity. This seemed anomalous as this construct contains the SRE and fosATF/AP-1 sequences which together in oligonucleotide-containing constructs gave consistent and reproducible inductions of CAT activity following serum or TPA stimulation. Several explanations were thought of for this failure:

1) The intact 402bp contains a transcriptional activation sequence that has been deleted from the oligonucleotide containing c-fos-CAT constructs.
2) Sequence overlapping the SRE-fosATF/Ap-1 element infl u ences the way these elements behave in their endogenous positions.
3) The SRE-fosATF/AP-1 sequences behave in a different fashion in the oligonucleotide containing construct, pehaps due to different interactions with proteins bound to the c-fos promoter region due to different spacing.

A detailed discussion of these possibilities is given in sub-Section 4.2.1. with relation to the results obtained in growing cells. This discussion is also applicable to the results obtained in the serum and TPA stimulation assays and it is impossible to determine which of these possibilities may be responsible for the anomalous results obtained with the intact c-fos upstream region and the oligonucleotide containing c-fos-CAT construct.

In vitro protein binding studies carried out in this project and in other work (215) suggest that the SRE and fosATF/AP-1 elements bind their respective proteins in a mutually exclusive manner. A recent publication (386) has also detailed in vitro footprinting studies on

these two sequences which also suggested a mutually exclusive pattern of protein binding to these sequences. However the same authors have also recently published a report of an in vivo genomic footprinting study showing that proteins bind to these sequences simultaneously (387). Although the mutually exclusive binding would suggest that the proteins complexing with the SRE and fosATF/AP-1 elements do not co-operate in controlling c-fos transcription it is perhaps misleading to extrapolate results with on vitro protein binding studies to the in vivo situation. Indeed, the results obtained with the transfection experiments in this project do suggest an interaction between the proteins complexing with these two sequences, and the in vivo genomic footprinting result supports this hypothesis.

To investigate the possible interaction between the proteins complexing with the SRE and fosATF/AP-1 sequences oligonucleotides that contained the two sequences separated by 5bp and 10bp inserts were inserted into c-fos-CAT constructs. These two inserts represent a half-helical and a full-helical turn of DNA respectively. The idea behind these insertions was to attempt to disrupt any interactions occurring between the proteins that complex with the SRE and fosATF&AP-1 sequences. Obviously, the insertion of a half-helical turn of DNA between the sequences will place the protein recognition sites on opposite sides of the DNA helix in comparison to the endogenous sequence, while the insertion of a full-helical turn should restore both sites to their endogenous sides. It was anticipated that the 5bp insertion would severely disrupt any interactions taking place between the proteins complexing with the SRE and fosATF/AP-1 sequences, while the 10bp sequence may restore

the interaction phenomenon. A similar study had been carried out previously with two elements involved in the synergistic transcriptional activation of the preproenkephalin gene promoter in response to cAMP stimulation (238). One of these elements contained a sequence identical to the fosATF/AP-1 sequence. In their endogenous positions these two sequences were separated by 11bp, representing a full-helical turn. Increasing this spacing to 21bp decreased the synergistic response to cAMP stimulation 2-fold, while reduction of the spacing to 6bp reduced the response a further 2-fold. The most dramatic decrease was obtained with insertion of 14bp between the two elements which severely reduces both basal and cAMP regulated expression by these two elements. This showed that increasing the spacing by a half-helical turn severely interfered with the transcriptional control properties exerted by these two elements.

Separating the SRE and fosATF/AP-1 elements had three major effects that were observed in transfection studies. Firstly, the basal levels of transcription from the c-fos promoter in the serum deprived cells increased approximately 3-fold with the 5bp insertion construct and 2-fold with the 10bp insertion construct in comparison to the levels obtained with the contiguous sequences. This level of increase is similar to that observed between the constructs containing the SRE, fosATF/AP-1 and the SRE alone. This suggested that the fosATF/AP-1 sequence must lie directly beside the SRE in order that the two sequences exert their control over transcription that leads to low levels of basal activity. Secondly, upon serum stimulation the level of transcription with the insert elements is approximately 3-fold

greater than that observed with the two contiguous sequences. Thirdly, the level of transcription obtained with the separated elements following TPA stimulation is similar to the levels obtained following serum stimulation. This is clearly different from the results obtained with the contiguous sequences where the serum response was always significantly greater than the TPA response. Two recent reports, published after the completion of these experiments, corroborate the results obtained (386,388). In one of the publications (388) it was observed that removal of the fosATF/AP-1 sequence from the SRE resulted in a 3-fold increase in basal levels of transcription. This report suggested that Fos-Jun heterodimers were responsible for this transcriptional shut-down by the fosATF/AP-1 sequence as anti-sense fos sequences increased the levels of transcription from a construct containing the SRE-fosATF/AP-1 sequences to levels similar to those obtained with the SRE alone. In the other publication (386) it was reported that separating the SRE and fosATF/AP-1 sequences resulted in an increased basal level of transcription in quiescent cells and an increase in transcription following serum stimulation when compared to the continuous sequences. Neither of these reports examined the transcriptional control exerted by the separated sequences following TPA stimulation. Another interesting result was obtained in this project when a consensus AP-1 binding site was juxtaposed with the SRE replacing the fosATF/AP-1 sequence. This construct behaved in an identical fashion to that containing the contiguous SRE and fosATF/AP-1 sequences. This again strongly suggests an interaction between the proteins complexing with these sequences as an AP-1 element on its own shows a relatively elevated level of transcription in quiescent cells in

comparison to the SRE-fosATF/AP-1 containing c-fos-CAT construct.

I would like to propose a hypothesis to explain the complex phenomenon observed in this project and by others (386,388). In the quiescent cells basal levels of transcription from the c-fos promoter are increased upon removal of the fosATF/AP-1 sequence from beside the SRE or by increasing the spacing between these two sequences by 5bp or 10bp. Also, mutations of the SRE which prevent proteins binding to this sequence lead to an increase in transcription from the c-fos promoter, probably due to the fosATF/AP-1 sequence (386). All of these results suggest an interaction between the two proteins binding to these sequences, although it could be argued that a further protein binds to these adjacent sequences in quiescent cells and blocks binding of the SRF and/or AP-1/ATF elements. There are two possible ways in which these proteins may interact to repress c-fos transcription : either by a direct protein-protein interaction or by the involvement of a third protein that recognises the proteins bound to these sequences and interacts with them to repress transcription. It is proposed here that a combination of a third protein and the influence of the protein(s) complexing with the SRE are responsible for the low basal levels of transcription observed with the continuous SRE and fosATF/AP-1 sequences. The construct containing the SRE juxtaposed with the consensus AP-1 site behaves in a similar fashion to the construct containing the continuous SRE and fosATF/AP-1 sequences. This suggests that the protein(s) complexed with the SRE must influence the protein(s) complexing with the consensus AP-1 site as an AP-1 site on its own leads to comparatively elevated levels of transcription from the c-fos promoter in quiescent

cells. It is possible that bound SRF prevents complexing of Fos-Jun heterodimers with the AP-1 sequence. It is also possible that the same proteins complex with the AP-1 site as with the fosATF/AP-1 site due to the influence of the bound SRF. Although it is not known what these proteins may be it seems reasonable to suppose that they are members of the AP-1/ATF family of proteins. The third protein postulated to be involved in the SRF-AP-1/ATF complex, and therefore regulation of transcription from the c-fos promoter, may be the Fos protein itself. Two pieces of evidence support this hypothesis. Firstly it has been reported that anti-sense fos sequences have a similar effect upon transcription from the c-fos promoter as removal of the fosATF/AP-1 sequence (388). Secondly, in this project Fos antibody did not disrupt the protein complex with the fosATF/AP-1 sequence (while it did with an AP-1 sequence) which contradicted a previous publication (220). The gel mobility shift experiments detailed in this paper used a DNA fragment that contained the SRE and fosATF/AP-1 sequences. So, if the Fos protein does not make a direct contact with the DNA of the fosATF/AP-1 sequence but depends upon recognition of the proteins binding to the SRE and fosATF/AP-1 sequence this would explain the discrepancy between these two results. Obviously, separation of the two sequences, which results in an increase in basal levels of transcription, may be due to the failure of the third protein, putatively Fos, to recognise the separated proteins.

When the SRE and fosATF/AP-1 sequences are separated not only is there an increase in the basal levels of transcription but there is also an increase in the levels of transcription following serum

stimulation. The reason for this phenomenon is not clear but could again be due to the presence of a third protein that can interact with the proteins complexing with the SRE and fosATF/AP-1 sequences, or to a modified interaction between the bound proteins. In this project the separation of the SRE and fosATF/AP-1 sequences also resulted in an increase in transcription following TPA stimulation to levels similar to those obtained following serum stimulation. This is different to the results obtained with the contiguous sequences where the serum stimulation was always greater than the TPA stimulation. As well as postulating a role for a third protein in this stimulation and&or a modification of the interaction between the proteins complexing with the SRE and fosATF/AP-1 sequences it is also possible that the protein bound to the fosATF/AP-1 sequence contributes towards the TPA stimulation. This would agree with results obtained in this project which indicated that the fosATF/AP-1 sequence on its own can activate transcription from the c-fos promoter following TPA stimulation.

Interesting results were also obtained when the SRE, consensus AP-1 and SRE-insert-fosATF/AP-1 containing constructs were assayed for their transcriptional properties in growing cells. In these cells the SRE and consensus AP-1 elements seem to act in an additive fashion to activate c-fos transcription. This suggests that the proteins complexing with these two sequences in growing cells are different (or modified) when compared to the proteins complexing with these sequences in quiescent cells where it was postulated that the proteins bound to the SRE influenced the protein(s) complexing with the AP-1 site. It was also of interest to note that the constructs

containing the separated SRE and fosATF/AP-1 sequences gave a 3-fold higher transcriptional activity than did the construct containing the two contiguous sequences. Again it may be postulated that this is due to the involvement of a third protein or because of a modification in the interaction between the proteins recognising these sequences.

4.3.3. The c-fos gene has a complex mechanism of transcriptional control.

The results obtained in this project and published by others demonstrate that the c-fos gene has a complex mechanism of transcriptional control. This project has detailed two *cis*-acting transcriptional control elements that activate transcription from the c-fos promoter in growing cells and following serum or TPA stimulation of serum-deprived cells. Other studies have also identified further transcriptional control sequences which activate c-fos transcription in response to a host of extra-cellular stimuli. Results obtained in this project also suggest a novel form of interaction between two *cis*-acting transcriptional control elements (the SRE and fosATF/AP-1 sequences). When these two sequences are placed together they maintain a low level of transcription from the c-fos promoter in quiescent cells. However, separation by as little as 5bp, or removal of the fosATF/AP-1 sequence leads to an increase in transcription from the c-fos promoter in quiescent cells. This suggests that these two sequences may be regarded as enhansons which co-operate to repress transcription from the c-fos promoter in quiescent cells. Enhansons were first identified in the SV40 enhancer where they were shown to co-operate with other enhansons to activate

transcription from an adjacent promoter. As with the SRE and fosATF/AP-1 sequences the SV40 enhansons lose their co-operative control over transcription when they are separated by as little as 5bp.

Further studies would be required to establish the precise mechanism of transcriptional control exerted by the contiguous SRE and fosATF/AP-1 sequences and to determine whether there is any interaction between the proteins that recognise these sequences. These would involve the delineation of the precise sequences required for the transcriptional control exerted by these two elements i.e. are the entire SRE and fosATF/AP-1 sequences required? Can the fosATF/AP-1 sequence be replaced by sequences other than the consensus AP-1 sequence analysed in this project? The spacing between the two elements may also be crucial : in this project insertion of 5bp and 10bp between the elements interfered with their transcriptional control but the effects of smaller or larger insertions were not examined.

The results presented here also suggest that the protein(s) bound to the SRE influence the protein(s) binding to the contiguous AP-1/ATF element in quiescent cells. For example, an AP-1 binding site when placed beside the SRE behaves in a different fashion to an independent AP-1 binding site. To establish whether this is the case it is possible that the use of antibodies in in vitro DNA-protein interaction studies would be capable of determining whether different proteins recognise the AP-1 site when it is with or without the SRE. A long-term goal following the studies on the proteins recognising

these sequences would be to determine whether there is any physical interaction between the proteins. Previous studies have shown that the Fos and Jun proteins interact via a leucine zipper domain and it would be of interest to identify similar interactions occurring between the proteins complexing with the SRE and fosATF/AP-1 sequences, and if possible to identify the domains involved in such interactions.

CHAPTER 5 : REFERENCES

1. Finkel, M.P., Biskis, B.O., and Jinkins, P.B., (1966), Science, 151, pp698-701.
2. Levy, J.A., Hartley, J.W., Rowe, W.P., and Huebner, R.J., (1973), J. Natl. Can. Inst., 51, pp525-539.
3. Levy, J.A., Kazan, P.L., Reilly Jr., C.A., and Finkel, M.P., (1973), J. Virol. 26, pp11-15.
4. Wood, H.M., and Young, D.M., (1976), Can. Res., 36, pp3985-3992.
5. Curran, T., and Teich, N., (1982), Virology, 116, pp221-235.
6. Curran, T., and Teich, N., (1982), J. Virol., 42, pp114-122.
7. Curran, T., van Beveren, C., Ling, N., and Verma, I.M., (1985), Mol. Cell. Biol., 9, pp167-172.
8. Curran, T., Peters, G., van Beveren, C., Teich, N.M., and Verma, I.M., (1982), J. Virol., 44, pp674-682.
9. Brugge, J.S., and Erikson, R.L., (1977), Nature 269, pp346-348.
10. Witte, O.N., Rosenberg, N., Paskind, M., Shields, A., and Baltimore, D., (1978), Proc. Natl. Acad. Sci., 75, pp2488-2492.
11. Curran, T., MacConnell, W.P., van Straaten, F., and Verma, I.M., (1983), Mol. Cell. Biol., 3, pp914-921.
12. van Straaten, F., Muller, R., Curran, T., van Beveren, C., and Verma, I.M., (1983), Proc. Natl. Acad. Sci., 80, pp3183-3187.
13. van Beveren, C., van Straaten, F., Curran, T., Muller, R., and Verma, I.M., (1983), Cell, 32, pp1241-1255.
14. Verma, I.M., (1984), Nature 308, p317.
15. Finkel, M.P., Reilly, C.A., Biskis, B.O., and Greco, I.L., (1973), In "Colston papers, Proceedings 24th Symposium of the Colston Research Society", Vol.24, pp353-366.
16. Lee, C.K., Chan, E.W., Reilly, C.A., Pahake, V.A., Rockus, G., and Finkel, M.P., (1979), Proc. Soc. Exp. Biol., 162, pp214-220.
17. Michiels, L., Maisin, J.R., Pedersen, F.S., and Merregairt, J.,

(1984), Int. J. Cancer 33, pp511-517.

18. Curran, T., and Verma, I.M., (1984), Virology, 135, pp218-228.

19. van Beveren, C., Erani, S., Curran, T., and Verma, I.M., (1984), Virology, 135, pp229-243.

20. Nishizawa, M., Goto, N., and Kawai, S., (1987), J. Virol. 61, pp3733-3740.

21. Fujiwara, K.T., Ashida, K., Nishina, H., Iba, H., Miyajima, N., Nishizawa, M., and Kawai, S., (1987), J. Virol., 61, pp4012-4018.

22. Curran, T., Miller, A.D., Zokas, L., and Verma, I.M., (1984), Cell 36, pp259-268.

23. Franza, B.R., Sambucetti, L.C., Cohen, D.R., and Curran, T., (1987), Oncogene, 1, pp213-221.

24. Sambucetti, L.C., Schuber, M., Kramer, R., Crawl, R., and Curran, T., (1986), Gene 43, pp69-77.

25. Barber, J.R., and Verma, I.M., (1987), Mol. Cell. Biol. 7, pp2201-2211.

26. Miller, A.D., Curran, T., and Verma, I.M., (1984), Cell, 36, pp51-60.

27. Meijlink, F., Curran, T., Miller, A.D., and Verma, I.M., (1985), Proc. Natl. Acad. Sci., 82, pp4987-4991.

28. Lee, W.M.F., Lin, C., and Curran, T., (1988), Mol. Cell. Biol., 8, pp5521-5527.

29. Eckstrand, A.J., and Zech, L., (1987), Exp. Cell Res., 1690, pp262-266.

30. Mitelman, F., (1983), Cytogenet. Cell. genet., 36, pp1-2.

31. Battey, J., Moulding, C., Taub, R., Murphy, W., Stewart, T., Potter, H., Lenoir, G., and Leder, P., Cell, 34, pp779-787.

32. Shtivelman, E., Lifshitz, B., Gale, R.P., and Canaani, E.,

(1985), Nature, 315, pp550-554.

33. Ruther, U., Garber, C., Komirowski, D., Muller, R., and Wagner, E.F., (1987), Nature, 325, pp412-416.

34. Ruther, U., Muller, W., Suanita, T., Tokuhisa, T., Rajewsky, K., and Wagner, E.F., (1988), Cell, 53, pp847-856.

35. Miller, A.D., Verma, I.M., and Curran, T., (1985), J. Virol., 55, pp521-526.

36. Jenuwein, T., Muller, D., Curran, T., and Muller, R., (1985), Cell, 41, pp629-637.

37. Jenuwein, T., and Muller, R., (1987), Cell, 48, pp647-657.

38. Zarbl, H., Latreille, and Jolicoeur, P., (1987), Cell, 51, pp357-369.

39. Muller, R., Slamon, D.J., Tremblay, J.M., Cline, M.J., and Verma, I.M., (1982), Nature, 299, pp640-644.

40. Muller, R., Verma, I.M., and Adamson, E.D., (1983), EMBO J., 2, pp679-684.

41. Muller, R., Tremblay, J.M., Adamson, E.D., and Verma, I.M., (1983), Nature, 304, pp454-456.

42. Adamson, E.D., Muller, R., and Verma, I.M., (1983), Cell. Biol. Int. Rep., 7, pp557-558.

43. Adamson, E.D., Meek, J., and Edwards, S.A., (1985), EMBO J., 4, pp941-947.

44. Mason, I., Murphy, D., and Hogan, B.L.M., (1985), Differentiation, 30, pp76-81.

45. Muller, R., Muller, D., Vertier, B., Bravo, R., and Herbst, H., (1986), EMBO J., 5, pp311-316.

46. Dony, C., and Gruss, P., (1987), Nature, 328, pp711-714.

47. de Togni, P., Niman, H., Raymond, V., Sawchenko, P., and Verma, I.M., (1988), Mol. Cell. Biol., 8, pp2251-2256.

48. Sundstrom, C., and Nilsson, K., (1976), Int. J. Can., 17, pp565-577.

49. Collins, S.J., Gallo, R.C., and Gallagher, R.E., (1977), Nature, 270, pp347-349.

50. Nilsson, K., Forsbeck, K., Gidlund, M., Sundstrom, G., Toterman, T., Sullstrom, J., and Vinge, P., (1981), Hematol. Blood Transfus., 26, pp215-221.

51. Rovera, G., Santoli, D., and Dansky, C., (1979), Proc. Natl. Acad. Sci., 76, pp2779-2783.

52. Mitchell, R.L., Zokas, L., Schreiber, R.D., and Verma, I.M., (1985), Cell, 40, pp209-217.

53. Muller, R., Curran, T., Muller, D., and Guilbert, L., (1985), Nature, 314, pp546-548.

54. Calabretta, B., (1987), Mol. Cell. Biol., 1, pp769-774.

55. Barzilay, J., Kushtai, G., Plaksin, D., Feldman, M., and Eisenbach, L., (1987), Leukaemia, 1, pp198-204.

56. Collins, S.J., Ruscetti, F.W., Gallagher, R.E., and Gallo, R.C., (1978), Proc. Natl. Acad. Sci., 75, pp2458-2462.

57. Studzinski, G.P., and Brelin, Z.S., (1987), J. Natl. Can. Inst., 79, pp67-76.

58. Brelin, Z.S., and Studzinski, G.P., (1986), J. Cell. Biol., 102, pp2234-2243.

59. Mairlio, F., Testa, U., Sposi, N.M., Perrini, M., Pelosi, E., Bordignon, C., Amadori, S., Mandell, F., and Peschle, C., (1987), Blood, 69, pp160-164.

60. Pinto, A., Coileta, G., Vecchio, L.D., Rosati, R., Attadia, V., Cimino, R., and Colombatti, A, (1987), Blood, 70, pp1450-1457.

61. Lee, J., Mehta, K., Blick, M.B., Gutterman, J.U., and Lopez-Berestein, G., (1987), Blood, 69, pp1542-1545.

62. Metcalf, D., and Nicola, N.A., (1982), Int. J. Cancer, 30, pp773-780.

63. Gonda, T.G., and Metcalf, D., (1984), Nature, 310, pp249-251.

64. Mitchell, R.L., Henning-Chubb, C., Huberman, E., and Verma, I.M., (1986), Cell, 45, pp497-504.

65. Calabretta, B., (1987), Mol. Cell. Biol. 7, pp769-774.

66. Larsson, L.G., Gray, H.E., Totterman, T., Pettersson, U., and Nilsson, K., (1987), Proc. Natl. Acad. Sci., 84, pp223-227.

67. Heidorn, K., Kriepe, H., Radzun, H.J., Muller, R., and Parwaresch, M.R., (1987), Blood, 70, pp456-459.

68. Sariban, E., Mitchell, T., Ranbaldi, A., and Kufe, D.W., (1988), Blood, 71, pp488-493.

69. Kriepe, H., Radzun, H.J., Heidorn, K., Parwaresh, M.R., Berrier, B., and Muller, R., (1986), Differentiation, 33, pp56-60.

70. Muller, R., Muller, D., and Guilbert, L., (1984), EMBO J., 3, pp1887-1890.

71. Radzioch, D., Boltazzi, B., and Varesio, L., (1987), Mol. Cell. Biol. 7, pp595-599.

72. Mintz, B., and Fleischman, R.A., (1981), Adv. Can. Res., 34, pp221-278.

73. Bernstine, E.G., Hooper, M.L., Grandchamp, S., and Ephrussi, B., (1973), Proc. Natl. Acad. Sci., 70, pp3899-3903.]

74. Muller, R., and Wagner, E.F., (1984), Nature, 311, pp438-442.

75. Muller, R., (1983), Cold Spring Harbor Conf. Cell Prolif. 10, pp451-468.

76. Levi, B-Z, and Ozato, K., (1988), Genes Dev. 2, pp554-566.

77. Ruther, U., Wagner, E.F., and Muller, R., (1985), EMBO J., 4, pp1775-1781.

78. McBurney, M.W., and Rogens, B.J., (1981), Dev. Biol., 89,

pp503-508.

79. Adamson, E.D. and Hogan, B., (1984), Differentiation, 27, pp152-157.

80. Edwards, S.A., and Adamson, E.D., (1986), Exp. Cell Res. 165, pp473-480.

81. Pony, C., and Gruss, P., (1988), Differentiation, 37, pp115-122.

82. Greene, L.A., and Tischler, A.S., (1976), Proc. Natl. Acad. Sci., 73, pp2424-2428.

83. Schubert, D., LaCorbiere, M., Klier, F.G., and Steinbach, J.H., (1980), Brain Res. 190, pp67-69.

84. Curran, T., and Morgan, J.I., (1985), Science, 229, pp1265-1268.

85. Kruijer, W., Schubert, D., and Verma, I.M., (1985), Proc. Natl. Acad. Sci., 82, pp7330-7334.

86. Milbrandt, J., (1986), Proc. Natl. Acad. Sci., 83, pp4789-4793.

87. Guerrero, I., Pellicer, A., and Burnstein, D.E., (1988), Bioch. Biophys. Res. Comm., 150, pp1185-1192.

88. Morgan, J.I., and Curran, T., (1986), Nature 332, pp552-555.

89. Curran, T., and Morgan, J.I., (1986), Proc. Natl. Acad. Sci., 83, pp8521-8524.

90. Morgan, J.I., Cohen, D.R., Hempstead, J.L., and Curran, T., (1987), Science, 237, pp192-197.

91. Hunt, S.P., Pini, A., and Evan, G., (1987), Nature, 328 pp632-634.

92. Sagar, S.M., Sharp, F.R. and Curran, T., (1988), Science, 240, pp1328-1331.

93. Robertson, R.M., Dragunow, M., and Robertson, H.A., (1988), Mol. Brain Res., 4, pp259-262.

94. Dragunow, M., and Robertson, H.A., (1988), Brain Res., 455, pp295-299.

95. Dragunow, M., and Robertson, H.A., (1988), Brain Res., 440, pp252-260.

96. White, J.D., and Gall, C.M., (1987), Mol. Brain Res., 3, pp21-29.

97. Kacznarck, L., Siedlecki, J.A., and Danysz, W., (1988), Mol. Brain Res., 3, pp183-186.

98. Rupert, C. and Willie, W., (1987), MOl. Brain Res., 2, pp51-56.

99. Bishop, J.M., (1985), Cell, 42, pp23-38.

100. Pardee, A.B., Dubrow, R., Humlin, J.L., and Kletzein, R.F., (1978), Ann. Rev. Biochem., 47, pp715-750.

101. Todaro, G.J., Lazar, G.K., and Green, H., (1965), J. Cell. Comp. Phys., 66, pp325-324.

102. McKnight, G.S., and Palmiter, R., (1979), J. Biol. Chem., 254, 9050-9058.

103. Groudine, M., Peretz, M., and Weintraub, H., (1981), Mol. Cell. Biol. 1, pp281-288.

104. Greenberg, M.E., and Ziff, E.B., (1984), Nature, 311, pp433-438.

105. Muller, R., Bravo, R., Bruckhardt, J., and Curran, T., (1984), Nature, 312, pp716-720.

106. Kruijer, W., Cooper, J.A., Hunter, T., and Verma, I.M., (1984), Nature, 312, pp711-716.

107. Cochran, B.H., Zullo, J., Verma, I.M., and Stiles, C.D., (1984), Science, 226, pp1080-1082.

108. Hann, S.R., Abrams, H.D., Rohrschneider, L.R., and Eisenman, R.N., (1983), Cell, 34, pp789-798.

109. Momke, M., Quaiser, A., Muller, D., and Montenarh, M., (1988), Oncogene, 3, pp151-158.

110. Greenberg, M.E., Hermanowski, A.L., and Ziff, E.B., (1986), Mol. Cell. Biol., 6, pp1050-1057.

111. Rubin, M.S., Doherty, P.J., and Gottesman, M.M., (1986), Proc. Natl. Acad. Sci., 83, pp357-360.

112. Bravo, R., Burckhardt, J., Curran, T., and Muller, R., (1986), EMBO J., 5, pp695-700.

113. Prior, C.P., Cantor, C.R., Johnson, E.M., Littau, V.C., and Allfrey, V.G., (1983), Cell, 34, pp1033-1042.

114. Allegra, P., Sterner, R., Clayton, D.F., and Allfrey, V.G., (1987), J. Mol. Biol., 196, pp379-388.

115. Chen, T.A., and Allfrey, V.G., (1987), Proc. Natl. Acad. Sci., 84, pp5252-5256.

116. Wang, J.C., (1985), Ann. Rev. Biochem., 54, pp665-697.

117. Vosberg, H.P., (1985), Curr. Top. Microb. Immunol., 114, pp 19-102.

118. Darby, M.K., Herrera, R.E., Vosberg, H-P, and Nordheim, A., (1986), EMBO J., 5, pp2257-2265.

119. Scher, C.D., Shepard, R.C., Antoniades, H.N., and Stiles, C.D., (1979), Biochim. Biophys. Acta, 560, pp217-241.

120. Stiles, C.D., Capone, G.T., Scher, C.D., Antoniades, H.N., Van Wyke, J.J., and Pledger, W.J., (1979), Proc. Natl. Acad. Sci. 76, pp1279-1283.

121. Reb Vard, M., Leibovitch, S., Jullien, M., Talha, S., and Harel, L., (1987), Exp. Cell Res., 172, pp432-438.

122. Breavo, R., Burckhardt, J., and Muller, R., (1985), Exp. Cell Res., 160, pp540-543.

123. Cheung, H.S., Van Wyk, J.J., Russell, W.E., and McCarty, D.J., (1986), J. Cell Physiol., 128, pp143-148.

124 Cheung, H.S., Mitchell, P.G., and Pledger, W.J., (1989), Can. Res., 49, pp134-138.

125. Andrews, C.K., Varma, S., and Ebner, K.E., (1987), Biochim.

Biophys. Acta, 909, pp231-236.

126. Takosuta, T., Ishibashi, S., and Ide, T., (1987), Biochim. Biophys. Acta, 909, pp161-164.

127. Colletta, G., Cirafici, A.M., and Vecchio, G., (1986), Science, 233, pp458-460.

128. Wichelhaus, O., Olek, K., Wappenschmidt, C., and Wagener, C., (1987), J. Clin. Chem. Clin. Biochem., 25, pp419-421.

129. Verrier, B., Muller, D., Bravo, R., and Muller, R., (1986), EMBO J, 5, pp913-917.

130. Connal, M., Tichonicky, L., Guguen-Guillouzo, C., Corcas, D., Raymondjean, M., Paris, B., Kruh, J., and Defor, N., (1985), Exp. Cell Res., 160, pp427-434.

131. Nishikura, K., and Murray, J.M., (1987), Mol. Cell. Biol., 7, pp639-644.

132. Holt, J.T., Gopal, T.V., Moulton, A.D., and Nienhaus, A.W., (1986), Proc. Natl. Acad. Sci., 83, pp4794-4798.

133. Melton, D.A., (1985), Proc. Natl. Acad. Sci., 82, pp144-148.

134. Riabowol, K.T., Vosatka, R.J., Ziff, E.B., Lamb, N.J., and Feramisco, J.R., (1988), Mol. Cell. Biol., 8, pp1670-1676.

135. Nishizuka, Y., (1984), Nature, 308, pp693-698.

136. Nishizuka, Y., (1986), Science, 233, pp305-312.

137. Tsuda, T., Hamamon, Y., Yamashita, T., Fukumoro, Y., and Takai, Y., (1986), FEBS, 208, pp39-42.

138. Doglio, A., Dani, C., Grimaldi, P., and Ailhand, G., (1989), Proc. Natl. Acad. Sci., 8, pp1148-1152.

139. Bravo, R., Burckhardt, J., Curran, T., and Muller, R. (1985), EMBO J., 4, pp1192-1197.

140. Kawahara, Y., Sunako, M., Tsuda, T., Fukuzaki, H., Fukumoto, Y., and Takai, Y., (1988), Biochem. Biophys. Res. Comm., 150,

pp52-59.

141. Bravo, R., Neuberg, M., Burckhardt, J., Almendral, J., Wallich, R., and Muller, R., (1987), Cell, 48, pp251-260.

142. Tsuda, T., Fukumoto, Y., Hamamoni, Y., Yamashita, T., and Takai, Y., (1987), J. Biochem., 102, pp1579-1583.

143. Chaplinski, T.J., and Niedel, J.E., (1982), J. Clin. Invest., 70, pp953-964.

144. Mehmet, H., Sinnet-Smith, J., Moore, J.P., Evan, G.I., and Rozengust, E., (1988), Oncogene Res., 3, pp281-286.

145. Shikanuma, M., Kuraki, T., and Nose, K., (1987), Eur. J. Biochem., 164, pp15-19.

146. Stacey, D.W., Watson, T., Kung, H-F, and Curran, T., (1987), Mol. Cell. Biol., 7, pp523-527.]

147. Lin, A.H., Grappi, V.E., and Gorman, R.R., (1988), Mol. Cell. Biol. 8, pp5052-5055.

148. Zullo, J., and Faller, D.V., (1988), Mol. Cell. Biol., 8, pp5080-5085.

149. Stumpo, D.J., and Blackshear, P.J., (1986), Proc. Natl. Acad. Sci. 83, pp9453-9457.

150. Weisz, A., and Bresciani, F., (1988), Mol. Endo., 2, pp816-824.

151. Treisman, R., (1985), Cell, 42, pp889-902.

152. Rahmsdorf, H.J., Schontal, A., Angel, P., Litfin, M., Ruther, U., and Herrlich, P., (1987), Nucl. Ac. Res., 15, 1643-1659.

153. Fort, P., Rech, J., Vie, A., Piechaczyk, M., Bonnieu, A., Jeanteur, P., and Blanchard, J-M., (1987), Nucl. Ac. Res. 15, pp5657-5667.

154. Sariban, E., Luebbers, R., and Kufe, D., (1988), Mol. Cell. Biol., 8, pp340-346.

155. Kubnick, K.S., and Howman, D.E., (1988), Mol. Cell. Biol., 8,

pp3244-3250.

156. Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shiner, S., and Cerami, A., (1986), Proc. Natl. Acad. Sci., 83, pp1670-1674.

157. Shano, G., and Kamen, R., (1986), Cell, 46, pp659-667.

158. Reeves, R., Elton, T.S., Nissen, M.S., Lehn, D., and Johnson, K.R., (1987), Proc. Natl. Acad. Sci., 84, pp6531-6535.

159. Schuler, G.D., and Cole, M.D., (1988), Cell, 55, pp1115-1122.

160. Wilson, T., and Treisman, R., (1988), Nature, 336, pp396-399.

161. Siebenlist, U., Simpson, R.B., and Gilbert, W., (1980), Cell, 20, pp269-281.

162. Hochschild, A., Irwin, N., and Ptashne, M., (1983), Cell, 32, pp319-325.

163. Dynan, W.S., and Tjian, R., (1985), Nature, 316, pp774-778.

164. McKnight, S., and Tjian, R., (1985), Cell, 46, pp795-805.

165. Dynan, W.S., and Tjian, R., (1983), Cell, 35, pp79-87.

166. Jones, N.C., Rigby, P.W., and Ziff, E.S., (1988), Genes Dev., 2, pp267-281.

167. Banerji, J., Ruscori, S., and Schaffner, W., (1981), Cell, 27, pp299-308.

168. Moreau, P., (1981), Nucl. Acids Res., 9, pp6047-6068.

169. Fromm, M., and Berg, M., (1983), Mol. Cell. Biol., 3, pp991-999.

170. Serfling, E., Jasin, M., and Schaffner, W., (1985), TIG, pp224-230.

171. Rogers, B.L., and Saunders, G.F., (1985), Bioessays, 4, pp62-65.

172. Voss, S.D., Schlokat, U., and Gruss, P., (1986), TIBS, 11, pp287-289.

173. Maniatis, T., Goodbourn, S., and Fischer, J.A., (1987), Science, 236, pp1237-1244.

174. Wasylyk, B., (1988), CRC Crit. Rev. Biochem., 23, pp77-120.

175. Zenke, M., Grundstrom, T., Matthes, H., Winteerith, M., Schatz, C., Wildeman, A., and Chambon, P., (1986), EMBO J., 5, pp387-397.

176. Herr, W., and Clarke, J., (1986), Cell, 45, pp461-470.

177. Ondek, B., Shepard, A., and Herr, W., (1987), EMBO J., 6, pp1017-1025.

178. Schirm, S., Jimcay, J., and Schaffner, W., (1987), Genes Dev., 1, pp65-74.

179. Petterson, M., and Schaffner, W., (1987). Genes Dev. 1, pp962-972.

180. Ondek, B., Gloss, L., and Herr, W., (1988), Nature, 335, pp40-45.

181. Fromental, C., Kanno, M., Nomiyama, H., and Chambon, P., (1988), 54, pp943-953.

182. Davidson, I., Xiao, J-H., Rosales, R., Straub, A., and Chambon, P., (1988), Cell, 54, pp931-942.

183. Bohmann, D., Keller, W., Dale, T., Scholar, H.R., Tebb, G., and Mattaj, I.W., (1987), Nature, 325, pp268-272.

184. Sturn, R., Baumrucker, T., Franza Jr., B.R., and Herr, W., (1987), Genes Dev., 1, pp1147-1160.

185. Fisch, T.M., Prywes, R., and Roeder, R., (1987), Mol. Cell. Biol. 7, pp3490-3502.

186. Sheng, M., Dougan, S.T., McFadden, G., and Greenberg, M.E., (1988), Mol. Cell. Biol., 8, pp2787-2795.

187. Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H., and Montminy, M.R., (1988), Nature, 334, pp494-498.

188. Gorman, C., Moffat, L.F., and Howard, B.H., (1982), Mol. Cell. Biol., 2, pp1044-1051.

189. Renz, M., Neuberg, M., Kurz, C., Bravo, R., and Muller, R. (1985), EMBO J., 4, pp3711-3716.

190. Deschamps, J., Meijlink, F., and Verma, I.M., (1985), Science, 230, pp1174-1177.

191. Pabo, C., and Sauer, R., (1984), Ann. Rev. Biochem., 63, pp293-321.

192. Chavrier, P., Zerial, M., Lemaire, P., Almendral, J., Bravo, R., and Charnay, P., (1988), EMBO J., 7, pp29-35.

193. Mohun, T., Garret, N., and Treisman, R., (1987), EMBO J., 6, pp667-673.

194. Gustafson, T.A., Miwa, T., Boxer, L.M., and Kedes, L., (1988), Mol. Cell. Biol., 8, pp4110-4119.

195. Frederickson, R.M., Michian, M.R., Iwamoro, A., and Miyamoto, N.G., (1989), Nucl. Acids Res. 17, pp253-270.

196. Kakunaga, T., Makino, K., Kawamoto, T., Sugihama, H., Omita, S., and Niwa, H., (1988), In Cell Differentiation, Genes and Cancer, 92, pp90-101. Eds T. Kakunaga et al.

197. Minty, A., and Kedes, L., (1986), Mol. Cell. Biol., 6, pp2125-2136.

198. Miwa, T., Boxer, L.M., and Kedes, L., (1987), Proc. Natl. Acad. Sci., 84, pp6702-6706.

199. Miwa, T., and Kese, L., (1987), Mol. Cell. Biol. 7, pp2803-2813.

200. Treisman, R., (1986), Cell 46, pp567-574.

201. Greenberg, M.E., Siegfried, Z., and Ziff, E.B., (1987), Mol. Cell. Biol., 7, pp1217-1225.

202. Gilman, M., Wilson, R.N., and Weinberg, R.A., (1986), Mol. Cell. Biol., 6, pp4305-4316.

203. Stumpo, D.J., Stewart, T.N., Gilman, M.Z., and Blackshear, P.J., (1988), J. Biol. Chem., 263, pp1611-1614.

204. Prywes, R., and Roeder, R.G., (1986), Cell, 47, pp777-784.

205. Treisman, R., (1987), EMBO J., 6, pp2711-2717.

206. Prywes, R., and Roeder, R.G., (1987), Mol. Cell. Biol. 7, pp3482-3489.

207. Schroter, H., Shaw, P.E., and Nordheim, A., (1987), Nucl. Acids Res. 15, pp10145-10158.

208. Prywes, R., Dutta, A., Cromlish, J.A., and Roeder, R.G., (1988), Proc. Natl. Acad. Sci., 8, pp7206-7210.

209. Norman, C., Runswick, M., Pollock, R., and Treisman, R., (1988), Cell, 55, pp989-1003.

210. Lau, L.F., and Nathans, D., (1985), EMBO J., 4, pp3145-3151.

211. Almendral, J.M., Sommer, D., MacDonald-Bravo, H., Burckhardt, J., Perera, J., and Bravo, R., (1988), Mol. Cell. Biol. 8, pp2140-2148.

212. Bender, A., and Sprague Jr., G.F., (1987), Cell 50, pp681-691.

213. Keleher, C.A., Gouetta, C., and Johnson, A.D., (1988), Cell, 53, pp972-936.

214. Passmore, S., Maine, G.T., Elble, R., Christ, C., and Tye, B.K., (1988), J.Mol. Biol., 204, pp593-606.

215. Shaw, P.E., Schroler, H., and Nordheim, A., (1989), Cell, 56, pp563-572.

216. Siegfried, Z., and Ziff, E.B., (1989), Oncogene, 4, pp3-11.

217. Gilman, M.Z., (1988), Genes Dev., 2, pp394-402.

218. Buscher, M., Rahnsdorf, H.J., Litfia, M., Karin, M., and Herrlich, P., (1988), Oncogene, 3, pp301-311.

219. Visvader, J., Sassone-Comsi, P., and Verma, I.M., (1988), Proc. Natl. Acad. Sci., 85, pp9474-9478.

220. Sassone-Corsi, P., and Verma, I.M., (1988), Nature, 334, pp314-319.

221. Wu, B.J., Kingston, R.E., and Morimoto, R.I., (1986), Proc. Natl. Acad. Sci., 83, pp629-633.

222. Hayes, T.E., Kirchen, A.M., and Cochran, B.H., (1987), Proc. Natl. Acad. Sci., 84, pp1271-1276.

223. Roesler, W.J., Vandenbork, G.R., and Hanson, R.W., (1988), J. Biol. Chem., 263, pp9063-9066.

224. Short, J.M., Wynshaw-Bons, A., Short, H.P., and Hanson, R.W., (1986), J. Biol. Chem., 261, pp9721-9726.

225. Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G., and Goodman, R.H., (1986), Proc. Natl. Acad. Sci., 83, pp6682-6686.

226. Delegeane, A.M., Ferland, L.H., and Mellon, P.L., (1987), Mol. Cell. Biol., 7, 3994-4002.

227. Quinn, P.G., Wong, T.W., Magnuson, M.A., Shabb, J.B., and Granner, D.K., (1988), Mol. Cell. Biol., 8, pp3467-3475.

228. Jameson, J.L., Jaffe, R.C., Deutsch, P.J., Albanese, C., and Habener, J.F., (1988), J. Biol. Chem., 263, pp9879-9886.

229. Fink, J.S., Verhuve, M., Kasper, S., Tsukada, T., Mander, G., and Goodman, R.H., (1988), Proc. Natl. Acad. Sci. 85, pp6662-6666.

230. Kanei-Ishii, C., and Ishii, S., (1989), Nucl. Acids Res., 17 pp1521-1536.

231. Sassone-Corsi, P., Viswater, J., Ferland, L., Mellon, P.L., and Verma, I.M., (1988), Genes Dev. 2, pp1529-1538.

232. Fisch, T.M., Prywes, R., Celeste Simon, M., and Roeder, R.G., (1989), Genes Dev., 3, pp198-211.

233. Montminy, M.R., and Bilezikjian, L.M., (1987), Nature, 328, pp175-178.

234. Riabowol, K.T., Fink, J.S., Gilman, M.Z., Walsh, D.A., Goodman, R.H., and Feramisco, J.R., (1988), Nature, 336, pp83-86.

235. Nakagawa, J., van der Ahe, D., Pearson, D., Hemmings, B.A., Shibahora, S., and Nagamine, Y., (1988), J. Biol. Chem., 263, pp2460-2468.

236. Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E., and Goodman, H.M., (1986), Nature 323, pp353-356.

237. Hyman, S.E., Comb, M., Lin, Y-S, Pearlberg, J., Green, M.R., and Goodman, H.M., (1988), Mol. Cell. Biol., 8, pp4225-4233.

238. Comb, M., Mermod, N., Hynan, S.E., Pearlberg, J., Ross, M.E., and Goodman, H.M., (1988), EMBO J., 7, pp3793-3805.

239. Lee, W., Mitchell, P., Tjian, R., (1987), Cell, 49, pp741-752.

240. Ran, W., Dean, M., Levine, R.A., Henhle, C., and Campisi, J., (1986), Proc. Natl. Acad. Sci., 83, p8216-8220.

241. Angel, P., Poting, A., Mallick, U., Rahmsdorf, H.J., Scharpp, M., and Herrlich, P., (1986), Mol. Cell. Biol., 6, pp1760-1766.

242. Imbra, R.J., and Karin, M., (1987), Mol. Cell. Biol., 7, pp1358-1363.

243. Haslinger, A., and Karin, M., (1985), Proc. Natl. Acad. Sci., 82, pp8572-8576.

244. Karin, M., Haslinger, A., Heguy, A., Deitlin, T., and Cooke, T., (1987), Mol. Cell. Biol., 7, pp606-613.

245. Imbra, R.J., and Karin, M., (1986), Nature, 323, pp555-558.

246. Chiu, R., Imagawa, M., Imbra, R.J., Bockoven, J.R., and Karin, M., (1987), Nature, 329, pp648-651.

247. Lee, W., Haslinger, A., Karin, M., and Tjian, R., (1987), Nature, 325, pp368-372.

248. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P., and Karin, M., (1987), Cell, 49, pp729-739.

249. Jones, R.H., Moreno, S., Nurse, P., and Jones, N.C., (1988),

Cell, 53, pp659-667.

250. Piette, J., and Yaniv, M., (1987), EMBO J., 6, pp1331-1337.

251. Rittling, S.R., Coutinho, L., Amram, T., and Kolbe, M., (1989), Nucl. Acids Res., 17, pp1619-1633.

252. Fisch, T.M., Prywes, R., and Roeder, R.G., (1989), Mol. Cell. Biol. 9, pp1327-1331.

253. Deutsch, P.J., Hoeffler, J.P., Jameson, J.L., and Habener, J.F., (1988), Proc. Natl. Acad. Sci., 85, pp7922-7926.

254. Berk, A.J., (1986), Ann. Rev. Genet., 20, pp45-79.

255. Moran, E., and Matthews, M.B., (1987), Cell, 48, pp177-178.

256. Stein, R., and Ziff, E., (1984), Mol. Cell. Biol., 4, pp2797-2801.

257. Kao, H-T., and Nevins, J.R., (1983), Mol. Cell. Biol. 3, pp2058-2065.

258. Sassone-Corsi, P., and Borrelli, E., (1987), Proc. Natl. Acad. Sci., 84, pp6430-6433.

259. Simon, M.C., Fisch, T.M., Benecke, B.J., Nevins, J.R., and Heintz, N., (1988), Cell, 52, pp723-729.

260. Lee, K.A., and Green, M.R., (1987), EMBO J., 6, pp1345-1353.

261. Lin, Y-S., and Green, M.R., (1988), Proc. Natl. Acad. Sci., 85, pp3396-3400.

262. Sassone-Corsi, P., (1988), Proc. Natl. Acad. Sci., 85, pp7192-7196.

263. Cortes, P., Buckbinder, L., Aleida Leza, M., Rak, N., Hearing, P., Merino, A., and Reinberg, D., (1988), Genes Dev., 2, pp975-990.

264. Hardy, S., and Shenk, T., (1988), Proc. Natl. Acad. Sci., 85, pp4171-4175.

265. Engel, D.A., Hardy, S., and Shenk, T., (1988), Genes Dev., 2,

pp1517-1628.

266. Raydhaudhuri, P., Bagchi, S., and Nevins, J.R., (1989), Genes Dev., 3, pp620-627.

267. Hurst, H.C., and Jones, N.C., (1987), Genes Dev. 1, pp1132-1146.

268. Katoh, I., Yoshinaka, Y., and Ikawa, Y., (1989), EMBO J., 8, pp497-503.

269. Fujii, M., Sassone-Corsi, P., and Verma, I.M., (1988), Proc. Natl. Acad. Sci., 85, pp5526-8530.

270. Sambucetti, L.C., and Curran, T., (1986), Science, 234, pp1417-1419.

271. Renz, M., Verrier, B., Kurz, C., and Muller, R., (1987), Nucl. Acids Res., 15, pp277-292.

272. Setoyama, C., Hatamochi, A., Peterkofsky, B., Prather, W., and de Crombrugghe, B., (1986), Biochem. Biophys. Res. Comm., 136, pp1042-1048.

273. Setoyama, C., Frunzio, R., Liau, G., Mudryj, M., and de Crombrugghe, (1988), Proc. Natl. Acad. Sci., 83, pp3213-3217.

274. Barzilay, J., Kushtai, G., Plaskin, D., Feldman, M., and Eisenbach, L., (1987), Leukemia, 1, pp198-204.

275. Kushtai, G., Barzilay, J., Feldman, M., and Eisenbach, L., (1988), Oncogene, 2, pp119-127.

276. Distel, R.J., Ro, H-S, Rosen, B.S., Groves, D.L., and Spiegelman, B.M., (1987), Cell, 49, pp835-844.

277. Distel, R.J., and Spiegelman, B.M., (1988), Prog. Clin. Biol. Res., 284, pp187-209.

278. Wilson, T., and Treisman, (1988), EMBO J., 7, pp4193-4202.

279. Lech, K., Anderson, K., and Brent, R., (1988), Cell, 52, pp179-184.

280. Rauscher III, F.J., Sambucetti, L.C., Curran, T., Distel, R.J.,

and Spiegelman, B.M., (1988), Cell, 52, pp471-480.

281. Franza Jr., B.R., Rauscher III, F.J., Josephs, S.F., and Curran, T., (1988), Science, 239, pp1150-1153.

282. Schontal, A., Herrlich, P., Rahmsdorf, H.J., and Penta, H., (1988), Cell, 54, pp325-334.

283. Lucibello, F.C., Neuberg, M., Hunter, J.B., Jenuwein, T., Schurmann, M., Wallich, R., Stein, B., Schontal, A., Herrlich, P., and Muller, R., (1988), Oncogene, 3, pp43-51.

284. Kerr, L.D., Holt, J.T., and Marrision, L.M., (1988), Science, 242, pp1424-1427.

285. Cavalieri, F., Ruslio, T., Tinoco, R., Benedict, S., Davis, C., and Vogt, P.K., (1985), Virology, 143, pp680-683.

286. Maki, Y., Bos, T.J., Davis, C., Starbuck, M., and Vogt, P.K., (1987), Proc. Natl. Acad. Sci., 84, pp2848-2852.

287. Bos, T.J., Bohman, D., Tsuchie, H., Tjian, R., and Vogt, P.K., (1988), Cell, 52, pp705-712.

288. Vogt, P.K., Bos, T.J., and Doolittle, R.F., (1987), Proc. Natl. Acad. Sci., 84, pp3316-3319.

289. Struhl, K., (1987), Cell, 48, pp295-297.

290. Hope, I.A., and Struhl, K., (1987), E,MBO J., 6, pp2781-2784.

291. Bohmann, D., Bos, T.J., Adman, A., Nishimura, T., Vogt, P.K., and Tjian, R., (1987), Science, 238, pp1386-1392.

292. Angel, P., Allegretto, E.A., Okino, S.T., Hattori, K., Boyle, W.J., Hunter, T., and Karin, M., (1988), Nature, 332, pp166-171.

293. Angel, P., Hattori, K., Smeal, T., and Karin, M., (1988), Cell 55, pp875-885.

294. Lamph, W.W., Wamsley, P., Sassone-Corsi, P., and Verma, I.M., (1988), Nature, 334, pp629-631.

295. Ryseck, R.-P., Hirai, S.I., Yaniv, M., and Bravo, R., (1988),

Nature, 334, pp535-538.

296. Quantin, B., and Breathnach, R., (1988), Nature, 334, pp538-539.

297. Ryder, K., and Nathans, D., (1988), Proc. Natl. Acad. Sci., 85, pp8464-8467.

298. Libio, E., Franceso, P.D., Gallinari, P., Testa, U., Ross, G.B., and Peschle, C., (1988), Biochem. Biophys. Res. Comm., 151, pp298-305.

299. Chambard, J-C., and Poyssegur, J., (1988), J. Cell. Phys., 135, pp101-107.

300. Pertovaara, L., Sistonen, L., Bos, T.J., Vogt, P.K., Keski-oja, J., and Alitalo, K., (1989), Mol. Cell. Biol., 9, pp1255-1262.

301 Lin, X. J., and Vilcek, J, (1987), J. Biol. Chem., 262, pp11908-11911.

302. Brenner, D.A., O'Hara, M., Angel, P., Chojkiev, M., and Karin, M., (1989), Nature, 337, pp661-663.

303. Sistonen, L., Holhaa, E., Makela, T.P., Keski-Oja, J., and Alitalo, K., (1989), EMBO J., 8, pp815-822.

304. Bastel, D.P., Sheng, M., Lau, L.F., and Greenberg, M.E., (1989), Genes Dev., 3, pp304-313.

305. Sassone-Corsi, P., Lamph, W.W., Kamps, M., and Verma, I.M., (1988), Cell 54, pp553-560.

306. Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T., and Karin, M., (1988), Cell, 54, pp541-552.

307. Rauscher III, F.J., Cohen, D.R., Curran, T., Bos, T.J., Vogt, P.K., Bohman, D., Tjian, R., and Franza Jr., B.R., (1988), Science, 240, pp1010-1016.

308. Rauscher III, R.J., Voulalas, P.J., Franza Jr., B.R., and Curran, T., (1988), Genes Dev., 2, pp1637-1699.

309. Bos, T.J., Rauscher III, F.J., Curran, T., and Vogt, P.K.,

(1989), Oncogene, 4, pp123-126.

310. Kryszke, M.-H., Piette, J., and Yaniv, M., (1987), Nature, 328, pp254-256.

311. Landschulz, W.H., Johnson, P.F., and McKnight, S.L., (1988), Science, 240, pp1759-1764.

312. Landschulz, W.H., Johnson, P.F., and McKnight, S.L., (1989), Science, 243, pp1681-1688.

313. Dang, C.V., McGuire, M., Buckmire, M., and Lee, W.M., (1989), Nature, 337, pp664-666.

314. Halazonetis, T.D., Georgopoules, K., Greenberg, M.E., and Leder, P., (1988), Cell, 55, pp917-924.

315. Kouzarides, T., and Ziff, E., (1988), Nature, 336, pp646-651.

316. Sassone-Corsi, P., Ransone, L.J., Lamph, W.W, and Verma, I.M., (1988), Nature, 336, pp692-695.

317. Schussmann, M., Neuberg, M., Hunter, J.B., Jenuwein, T., Ryseck, R.P., Bravo, R., and Miller, R., (1989), 56, pp507-516.

318. Turner, R. and Tjian, R., (1989), Science, 243, pp1689-1694.

319. Gentz, R., Rauscher III, F.J., Abate, C., and Curran, T., (1989), Science, 243, pp1695-1699.

320. Neuberg, M., Schuermann, M., Hunter, J.B., and Muller, R., (1989), Nature, 338, pp589-590.

321. O'Shea, E.K., Rutkoski, R., and Kim, P.S., (1989), Science, 243, pp538-542.

322. Crick, F.H., (1952), Nature, 170, pp882-883.

323. Franza, B.R., Sambucetti, L.C., Cohen, D.R., and Curran, T., (1987), Oncogene, 1, pp213-221.

324. Cohen, D.R., and Curran, T., (1988), Mol. Cell. Biol., 8, pp2063-2064.

325. Cohen, D.R., Ferreira, P.C.P., Gentz, R., Franza Jr., B.R., and

Curran, T., (1989), Genes Dev. 3, pp173-184.

326. Zerial, M., Toschi, L., Ryseck, R.P., Schuermann, M., Muller, R., and Bravo, R., (1989), EMBO J., 8, pp805-813.

327. Ryder, K., Lau, L.F., Nathans, D., (1988), Proc. Natl. Acad. Sci., 85, pp1487-1491.

328. Nakabeppa, Y., Ryder, K., and Nathans, D., (1988), Cell, 55, pp907-915.

329. Hirai, S.-I., Ryseck, R.-P., Mechta, F., Bravo, R., and Yaniv, M., (1989) EMBO J., 8, pp1433-1439.

330. Hai, T., Liu, F., Allegretto, E.A., Karin, M., and Green, M.R., (1988), Genes Dev., 2, pp1216-1226.

331. Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L., and Habener, J.F., (1988), Science, 242, pp1430-1433.

332. Perkins, K.K., Dailey, G.M., and Tjian, R., (1988), EMBO J., 7, pp4265-4273.

333. Umer, G., Buisson, M., Chambard, P., and Sergeant, A., (1989), EMBO J., 8, pp1447-1453.

334. Farrell, P.J., Rowe, D.T., Rooney, C.M., and Kouzandis, T., (1989), EMBO J., 8, pp127-132.

335. Biogenesis Ltd. Isolation of RNA by the RNAzol method.

336. BIO 101, Inc., Geneclean.

337. McBride, L.J., McCollum, C., Davidson, S., Efcavitch, J.W., Andrus, A., and Lombardi, S.J., (1988), Biotechniques, 6, pp362-367.

338. Birnboim, H.C., and Doly, J., (1979), Nucl. Acids Res., 7, p1513.

339. Thomas, P., (1980), Proc. Natl. Acad. Sci., 77, pp5201-5205.

340. Southern, E.M., (1975), J. Mol. Biol., 98, pp503-517.

341. Boehringer Mannheim. Random primed DNA labeling kit.

342. Suggs, S.V., Wallace, R. Bruce, Hirose, T., Kawashima, E.H., and Itakura, K., (1981), Proc. Natl. Acad. Sci., 78, pp6613-6617.

343. United States Biochemical Corporation, Cleveland, Ohio. The sequenase kit.

344. Jainchill, J.L., Aaronson, S.A. and Todaro, G.J., (1969), J.Virol., 4, pp549-553.

345. Puck, T.T., Marcus, P.I., and Cieciura, S.J., (1956), J. Exp. Med., 103, pp273-284.

346. Kit, S., Dubbs, D.R., Pierkarski, L.J., and Hsu, T.C., (1963), Expt. Cell Res., 31, pp297-312.

347. Graham, F.L., and Van der Eb, A.J., (1977), Virology, 52, pp456-467.

348. Wigler, M., Silverstein, S., Lee, L-S, Pellicer, A., Cheng, Y-C., and Azel, R., (1977) Cell, 11, p223-232.

349. Bio-Rad, Munchen, Germany. Bio-Rad Protein Assay Kit.

350. Miller, J.H., (1972), Cold Spring Harbor: Cold Spring Harbor Laboratory.

351. Favaloro, J., Treisman, R., and Kamen, R., (1980), Methods, Enzymol., 65, p718.

352. Zinn, K., DiMiao, D., and Maniatis, T., (1983), Cell, 34, pp865-879.

353. Plumb, M., Frampton, J., Wainwright, H., Walker, M., MacLeod, K., Goodwin, G., and Harrison, P., (1989). Nucl. Acids Res., 17, pp73-92.

354. Emerson, B.M., and Felsenfeld, G., (1984), Proc. Natl. Acad. Sci., 81, pp95-99.

355. Strauss, F., and Varshavsky, A., (1984), Cell, 37, pp889-901.

356. Gaffney, D.F., McLauchlan, J., Whitton, J.L., and Clements, J.B., (1985), Nucl. Acids Res., 13, pp7847-7863.

357. Alton, N.K., and Vapnek, D., (1979), Nature, 282, pp864-869.

358. Jones, N.C., Richter, J.D., Weeks, D.L., and Smith, L.D., (1983), Mol. Cell. Biol., 3, pp2131-2142.

359. Hall, C.V., Jacob, P.E., Ringold, G.M., and Lee, F., (1983), J. Mol. Appl. Gen., 2, pp101-109.

360. Selden, R.F., Howie, K.B., Rowe, M.E., Goodman, H.M., and Moore, D.D., (1986), Mol. Cell. Biol., 6, pp3173-3179.

361. Elgin, S.C.R., (1981), Cell, 27, pp413-415.

362. Wu, C., (1980), Nature, 286, pp854-860.

363. Stalder, J., Larsen, A., Engel, J.D., Dolan, M., Groudine, M., and Weintraub, H., (1980), Cell, 20, pp451-460.

364. Galas, D., Schmitz, A., (1978), Nucl. Acids Res., 5, pp3157-3170.

365. Emerson, B.M., Lewis, C.D., and Felsenfeld, G., (1985), Cell, 41, pp21-30.

367. Church, G.M., and Gilbert, W., (1984), Proc. Natl. Acad Sci., 81, pp1991-1995.

368. Kovesdi, I., Reichel, R., Nevins, J.R., (1986), Cell, 45, pp219-228.

369. Schuster, W.A., Treacy, M.N., and Martin, F., (1988), EMBO J., 7, pp1721-1723.

370. Carthew, R.W., Chocosh, L.A., and Sharp, P.A., (1987), Genes Dev., 1, pp973-980.

371. Ryan, W.A., Franza,Jr., B.R., and Gilman, M.Z., (1989), EMBO J., 8, pp1785-1792.

372. Jones, K.A., Kadonaga, J.T., Rosenfeld, P.J., Kelly, T.J., and Tjian, R., (1987), Cell, 48, pp79-89.

373. Jones, K.A., Yamamoto, K.R., and Tjian, R., (1985), Cell, 42, pp559-572.

374. Varmus, H., (1988), Science 240, pp1427-1435.

375. Zinn, K., DiMaio, D., and Maniatis, T., (1983), Cell, 34, pp865-879.

376. Gillies, S.D., Morrison, S.L., Oi, V.T., and Tonegawa, S. (1983), 33, pp717-728.

377. Morikashi, M., Hai, T., Lin, Y-S, Green, M.R., and Roeder, R.G., (1988), Cell 54, pp1033-1042.

378. Fletcher, C., Heintz, N., and Roeder, R.G., (1987), Cell, 51, pp773-781.

379. Scheidereit, C., Heguy, A., and Roeder, R.G., (1987), Cell, 51, pp783-793.

380. Sturm, R.A., and Herr, W., (1988), Nature, 336, pp601-604.

381. Wasylyk, B., Imter, J.L., Chatton, B., Schatz, C., and Wasylyk, C., (1988), Proc. Natl. Acad. Sci., 85, pp7952-7956.

382. Beato, M., (1989), Cell, 56, pp335-344.

383. Shirayoshi, Y., Burke, P.A., Appella, E., and Ozato, K., (1988), Proc. Natl. Acad. Sci., 85, pp5884-5888.

384. Tiwari, R.K., Kusari, Y., Kumar, R., and San, G.C., (1988), Mol. Cell. Biol., 8, pp4289-4294.

385. Umesono, K., Giguere, V., Glass, C.K., Rosenfeld, M.G., and Evans, R.M., (1988), Nature, 336, pp262-265.

386. Shaw, P.E., Frasch, S., and Nordheim, A., (1989), EMBO J., 8, pp2567-2574.

387. Herrera, R.E., Shaw, P.E., and Nordheim, A., (1989), Nature, 340, pp68-70.

388. Konig, H., Ponta, H., Rahmsdorf, U., Buscher, M., Schontal, A., Rhamsdorf, H.J., and Herrlich, P., (1989), EMBO J., 8, pp2559-2566.