Characterization of c-*fos* promoter binding proteins in normal and neoplastic liver cells

Ian D. de Belle

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfillment for the
Ph.D. degree in the
Ottawa-Carleton Institute of Biology

Ottawa, Ontario. Canada
1995

ISBN 0-612-19945-2

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

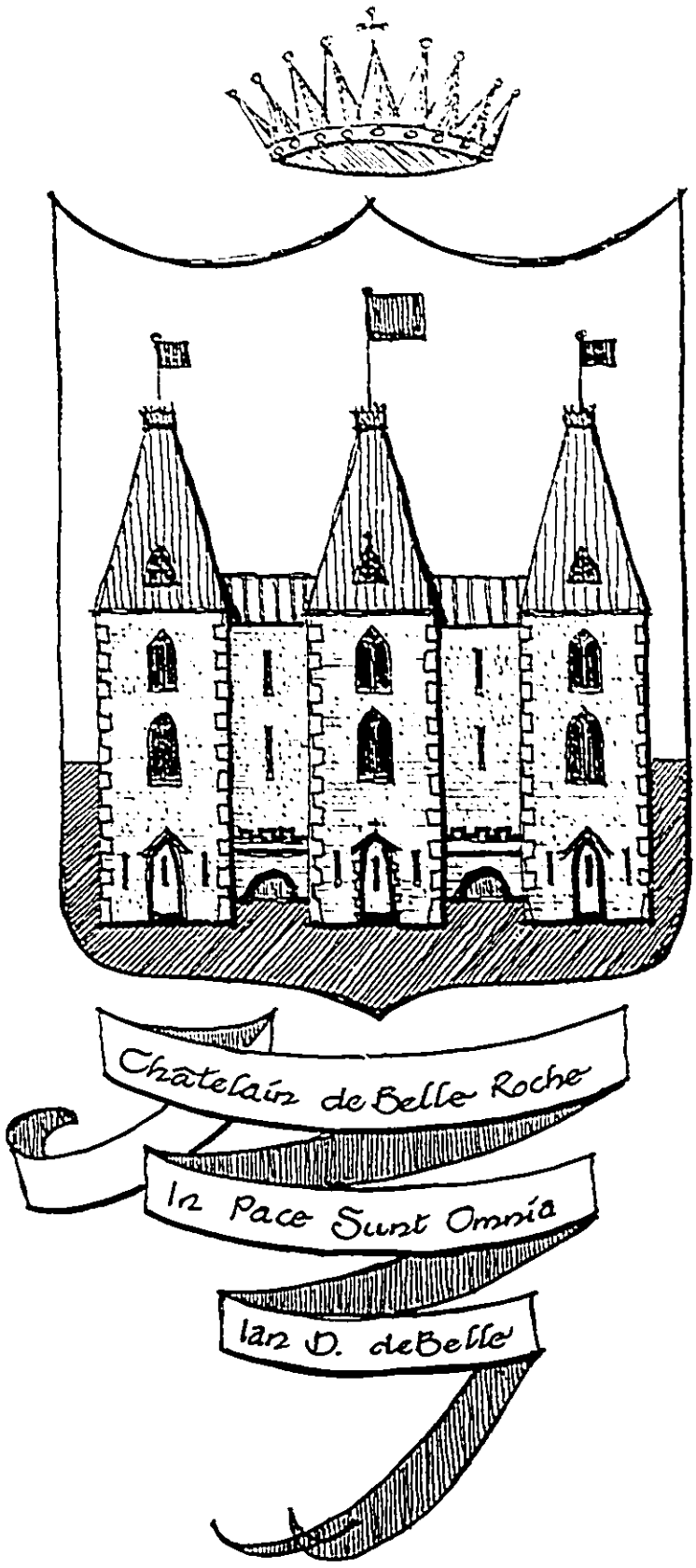

Chatelain de Belle Roche
In Pace Sunt Omnia
Ian D. deBelle

TABLE OF CONTENTS

ABSTRACT

The ability of the c-*fos* gene to respond to a variety of stimuli reflects the complexity of its regulation. Indeed, aberrant regulation can lead to cellular transformation. The fine regulation of the gene relies on the interaction of protein factors interacting with a set of regulatory elements present within the promoter of the gene in a cooperative fashion. In these studies, multiprotein complexes capable of binding to the serum response element, the cAMP response element, and *sis*-conditioned medium response element, were identified in liver cell nuclei. A subset of these proteins was able to interact with multiple regulatory elements and might be able to direct functional cooperation between them. These multiple factors could be targeted by different signalling pathways.

The behavior of these multiprotein complexes was analyzed under physiological conditions of transient c-*fos* expression in regenerating rat liver and under pathological conditions of chemically induced hepatocarcinogenesis and in solid 5123tc Morris hepatomas whereby constitutive expression of the gene occurs. Elevated expression of the c-*fos* gene in both normal and in transformed cells correlated with the loss and/or complete absence of the 47 kDa CRE-binding activity. The 47 kDa CRE-binding protein was characterized as highly CRE-specific and distinct from the other members of the CREB/CREM/ATF family of proteins. The 47 kDa CREB factor displayed DNA-binding activity in the dephosphorylated form. The functional importance of the c-*fos* promoter-binding proteins was assessed by *in vitro* transcription which suggested the presence of a transcriptional block in quiescent liver cells which was relieved in proliferating hepatocytes. The results are consistent with the identification of the 47 kDa CRE-specific DNA-binding protein as a transcriptional repressor in quiescent liver cells.

RESUME

La regulation du gene codant pour c-*fos* est complexe, et le fait que ce gene puisse repondre a divers stimuli illustrre bien cette complexite. La regulation precise de ce gene depend de l'interaction cooperative de plusiers facteurs proteiques avec une panoplie d'elements regulateurs qui sont confines au niveau du promoteur. Les etudes effectuees sur des noyeaux de cellules hepatiques demontrent l'existence de complexes multiproteiques capables de se lier aux elements de reponse au serum, a l'AMPc de meme qu'au milieu conditionne par *sis*. Certaines de ces proteines peuvent interagir avec de multiples elements de reponse et pourraient etre capables de diriger une cooperation fonctionnelle enter eux. L'existence de ces multiples facteurs a pu etre mis a jour par l'etude de diverses voies de transduction. Le comportement de ces complexes multiproteique a pu etre etudie en conditions physio;ogique lors de la regenerescence du foie de rat, alors que l'expression de c-*fos* est transitoire. Parallelement, ces etudes se sont entendues a des conditions pathologiques alors que c-*fos* est exprime constitutivement lors de l' hepatocarcinogenese induite chimiquement, de meme que chez les hepatomes solides 5123tc Morris. Une expression elevee du gene c-*fos*, tant chez les cellules normales que transformees, a pu etre associee avec la perte ou l'absence complete d'un facteur de 47 kDa capable de lier CRE. Cette proteine de 47 kDa s'est areree etre hautement specifique pour CRE et distincte des autres membres proteiques de la famille CREB/CREM/ATF. La facteur CREB de 47 kDa demontre une activite de liason a l'ADN sous la forme deposphorylee. L'importance fonctionelle des proteines capables de se lier au promoteur de c-*fos* a etre determinee par des etudes de transcription *in vitro* qui suggerent la presence d'un bloquage transcriptionel chez les cellules hepatiques quiescentes. A l'inverse, ce bloquage est leve chez les cellules en phase de proliferation. L'identification d'une represseur transcriptionnel de 47 kDa, specifique a CRE, et capable de lier l'ADN est en accord avec les resultats cites plus haut.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Marianna Sikorska, for her support and guidance throughout the course of this work. Her enthusiasm and dedication to scientific excellence will always be admired, and I am grateful to have had the opportunity to learn in the most stimulating of atmospheres. A teacher and a friend, to whom I express my deepest thanks. A person of greater integrity can not be found. I would also like to thank Dr. P. Roy Walker for his assistance and advise, which was always appreciated and will hopefully continue.

The kindness and friendship of the members of both Sikorska and Walker labs, past and present, has turned the work into fun; it made the time go by so quickly. Particular thanks go to Dr. Joanna Kwast-Welfeld for guiding me through the techniques of DNA/protein interactions; her help was invaluable and always given freely. Also, the advise of Dr. Henriette Gourdeau in the preparation and analysis of RNA is gratefully acknowledged. The special kindness and technical talents of Lucia Testolin, Julie LeBlanc, Yoga Adcharamoorthy and Agnieszka Zoledziowska must also be acknowledged.

The financial support of Dr. I.C.P. Smith is gratefully acknowledged and the help of my co-supervisor, Dr. D. Hickey, has not gone unnoticed and is appreciated. The assistance of Dr. R.H. Rixon and Rick Isaacs in setting up the resistant hepatocyte model and in handling of the animals is gratefully acknowledged. The assistance of Dr. R.H. Rixon, and Dr. S. Commons, with histological techniques and pathological assessment of hepatic foci and lesions, and Drs. P. Kozlowski and K. Butler for MRI analyses, is gratefully acknowledged. Also, the help of Mr. J. Daoust, with rabbit injections and harvesting the sera is gratefully acknowledged. Thanks also go to Dr. C. Mueller for help in setting up the *in vitro* transcription assay. The efforts of Dr. J.F. Whitfield on my behalf are also acknowledged. Special thanks to Tom Devecseri for excellent photographic work.

Finally, I thank my family for their support and encouragement; this thesis is dedicated to them.

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
AP-1	Activator protein 1
ATF	Activating transcription factor
BSA	Bovine serum albumin
C/EBP	CAAT/enhancer binding protein
cAMP	3',5'-cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CRE	3',5'-cyclic adenosine monophosphate response element
CREB	3',5'-cyclic adenosine monophosphate response element binding protein
CRE-BP1	3',5'-cyclic adenosine response element binding protein 1
CREM	3',5'-cyclic adenosine monophosphate response element modulator protein
CSF-1	Colony stimulating factor 1
C-terminal	Carboxyl terminal
DNA	Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle medium
Dex	Dexamethasone
dATP	Deoxyadenosine triphosphate
dGTP	Deoxyguanosine triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethyleneglycol-bis-(ß-amino-ethylether)N,N'-tetraacetic acid
ERK-1	Extracellular signal regulated kinase 1
FPLC	Fast protein liquid chromatography
Fra	Fos related antigen
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
Hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPX	Partial hepatectomy
IP-1	Inhibitory protein 1
LB	Luria broth
LTR	Long terminal repeat
MAP kinase	Mitogen activated kinase
MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid
O-Me-GTP	3'-O-methylguanosine5'-triphosphate
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PEPCK	Phosphoenol pyruvate carboxy kinase
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PMSF	Phenyl methyl sulfonyl fluoride
Rb	Retinoblastoma protein

RCE	Retinoblastoma control element
Ref-1	Redox factor 1
RNA	Ribonucleic acid
SCMRE	*sis*-conditioned medium response element
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SH2	Src homology region 2
SRE	Serum response element
SRF	Serum response factor
TFIID	Transcription factor IID
TRE	TPA response element
TPA	12-O-tetradecanoylphorbol 13-acetate
Tris	Tris[hydroxymethyl]aminomethane
UTP	Uridine triphosphate
VIP	Vasoactive intestinal peptide

INTRODUCTION

Historical Perspective

The search for causative agents responsible for both the generation and propagation of human tumours had its beginnings with the investigations of Sir Percival Potts in the latter half of the 16th century who noticed a correlation between the deaths of young men as a result of scrotal cancer and their occupation as chimney sweeps. Sir Percival concluded that chimney soot was the likely basis of the disease. Since that first description of an environmental carcinogen, vast amounts of scientific effort have been devoted to the elucidation of the initiating agents of various forms of cancer with the view towards disease treatment and/or prevention. Presently, it is clear that there is no single cause of the disease, but rather that cancer may arise by one or more distinct causative agents, each resulting in the conversion of the target cell to the neoplastic phenotype. Agents which have been found to produce neoplasia include environmental pollutants, radiation-induced genetic damage, DNA and retroviruses, and familial predisposition all of which may culminate in genome alterations characteristic of transformed cells. Clearly, all of these etiologic agents function through genome perturbations although the mechanisms by which their effects are achieved is far from being understood.

In 1911, Peyton Rous found that filtrates from a chicken sarcoma were capable of producing sarcomas when reintroduced into chicken hosts, providing the first evidence that a sub-cellular agent (now known as the Rous sarcoma virus), could produce a tumour in an appropriate host (1). This was the initial demonstration that a subcellu-

lar agent could direct tumour formation, and since that time the identification and characterization of a large number of tumour viruses has proven to be fertile ground for the study of cancer biology.

To a large degree the current understanding of the viral etiology of cancer has been provided by studies on the transforming properties of a large number of retroviruses (2). Genetic analyses proved that the transforming potential of RNA viruses is due to their genetic complement and these genes, responsible for the transformed phenotype, are designated oncogenes (3-5). With the discovery that viral oncogenes have sequence similarities within the normal host genome came the realization that viral oncogenes consist of transduced cellular sequences and, therefore, each cell contains within its own genome cellular versions of viral transforming oncogenes (6-8). This seminal work has been the catalyst for the identification of those genes within the genome which have the potential to be involved in the transformation process. Furthermore, the study of retrovirus biology has provided a system whereby questions concerning the function of oncogenes in the carcinogenic process could be addressed. Although each cell has oncogenic potential, specific mutations or alterations must occur in the cellular forms of the genes in order to transform a cell. It is for this reason that the cellular form of oncogenes have been called proto-oncogenes (9).

Of the imbalances produced in neoplastic cells, regardless of the causative agent(s), perhaps the most fundamental one which constitutes a defining feature of all neoplasias is the loss of control of normal cellular proliferation. This phenotype is likely to be the net result of changes in patterns of the expression of genes normally under the control of rigorous cell cycle checkpoints (10). It is not surprising, therefore,

that all of the oncogenes described so far have their effects by disrupting strategically located key control points in the transduction of extracellular signals (8).

Proto-oncogene families

Proliferative signals must culminate in changes in gene expression to generate the necessary protein requirements for cell cycle progression. Proliferation of both normal and transformed cells begins with signals emanating from the plasma membrane through receptor ligand interactions. These signals are then transduced by protein kinases which, in turn, must relay the message through to the nucleus to effect changes in gene expression. Oncogenes and their protein products occupy key points in this cascade of events. Based upon their intracellular localization and function they can be broadly categorized into three different groups.

The first of these classes are those oncogenes which have homology to cellular growth factors and their cognate surface receptors. The second group consists of oncogenes which have the ability to generate intracellular signals and/or possess protein kinase activity. The third class of oncogenes consists of nuclear transcription factors which are able to transduce cellular signals to effect changes in gene expression.

Cellular proliferation is under the influence of specific growth factors which initiate a cascade of cellular events culminating in the expression of genes required for cell cycle progression (11). In the absence of the appropriate stimulus normal cells do not proliferate, but remain in a quiescent state in the G0 phase of the cell cycle, but may re-enter the cell cycle upon growth factor stimulation (12). Transformed cells, on the other hand, have relaxed requirements for exogenous growth factors and are able to

proliferate under conditions of growth factor deprivation (13).

The first clue that viral oncogenes are related to growth factors came with the discovery that the transforming gene from the simian sarcoma virus, v-*sis*, contains a region of 109 amino acids which is highly homologous to the ß chain of platelet derived growth factor (PDGF, 14-16). It was known that PDGF is involved in the mitogenic response through the induction of gene expression (17). This led to the suggestion that aberrant gene expression seen in transformed cells might result from altered growth factor signalling (11, 18).

The discovery of partial homology between certain oncogenes and growth factor receptors has strengthened the idea that signal transmission from the extracellular environment is altered in cells transformed by these oncogenes. Accordingly, the transforming gene of the avian erythroblastosis virus, v-*erb*-B, is homologous, in part, to the epidermal growth factor receptor (EGFR, 20). It is known that EGFR contains an endogenous tyrosine kinase activity which becomes activated upon ligand binding and that the downstream phosphorylation events are associated with the transduction of mitogenic signals to the cell nucleus (21,22). Interestingly, the v-*erb*-B oncogene does not contain the extracellular ligand binding domain, but does have tyrosine kinase activity which may provide clues to the mechanism by which this oncogene stimulates continuous growth and transformation (20,23,24). Another truncated growth factor receptor is illustrated by the oncogene v-*fms* of the feline sarcoma virus, which turns out to be a shortened version of the receptor for the hemopoetic growth factor colony stimulating factor-1 (CSF-1R, 25). Some evidence suggests that v-*fms* may also function by constitutive stimulation in the absence of growth factor binding (26). Additional

homologies demonstrated between growth factors and oncogene products include partial amino acid homology between transforming growth factor ß (TGF-ß), a growth factor secreted by some transformed cells and epidermal growth factor (19).

The second grouping of oncogenes consists of those intracellular signalling proteins with protein kinase activity (27). The first oncogene shown to have tyrosine kinase activity attributed to it was the transforming protein of the v-*src* oncogene from the Rous sarcoma virus (28). Since then, several oncoproteins were shown to have intrinsic kinase activities. Several of these were shown to phosphorylate tyrosine residues reminiscent of the growth factor receptor tyrosine kinases. Examples of these are provided by transforming genes encoded by v-*abl* and v-*yes* (29,30). Interestingly, these proteins are associated with membranes and although they do not appear to be transmembrane growth factor receptors they have related tyrosine kinase domains (27). The oncogenic forms of these proteins are associated with increased or altered tyrosine kinase activity resulting from genetic mutations or truncations which occurred during retroviral transduction (29,30).

The oncogene products which possess serine/threonine protein kinase activity also belong to this group. Members of this group of kinases include v-*mos* and v-*raf* which have acquired their oncogenic potential by deletion of the regulatory region of the kinase (31-34). An association between stimulation of membrane-bound receptors and increased activity of the Raf-1 kinase suggests that the signal relay from the extracellular environment to the cytoplasm is mediated by the proto-oncogene products (35).

Other intracellular signalling proteins which are associated with retroviruses are membrane associated guanosine nucleotide-binding proteins which convert proliferative

signals through the hydrolysis of GTP (36,37). These oncoproteins are illustrated by the *ras* family of oncogenes which function in signal transduction through the cycling of their GTP and GDP bound forms of endogenous GTPase activity. Ras, when in the GTP bound form, activates adenylate cyclase leading to increased protein phosphorylation by cAMP-dependent protein kinase (PKA, 38-40). The transforming potential of v-*ras* has been shown to accompany mutations in the gene which impair the GTPase activity and thereby maintain its GTP-bound state and provides, therefore, continuous stimulation of adenylate cyclase (39,40). Recent data have demonstrated a functional and direct association between Ras and Raf proteins (41,42). The downstream effect of this association is the activation, by phosphorylation, of the mitogen activated protein (MAP) kinase signal transduction pathway providing a partial view of the hierarchy of signal transduction by these proto-oncogenes (43,44).

The third category of oncogenes encodes proteins localized to the cell nucleus which are the ultimate target for the extracellular stimuli (45). Over the last 6-8 years, a considerable effort has been made to identify nuclear oncogenes and to determine their functions. This category of oncogenes is exemplified by the *fos*, *jun*, and *myc* oncogenes (46-48). It is now generally accepted that probably all nuclear oncoproteins function as sequence specific DNA binding proteins. They interact with defined sequences present in the regulatory regions of target gene(s) and change the transcriptional activities of those genes (49-51). The full complement of genes controlled by the nuclear oncogene products is not known, however a role in the generation of cell cycle competence has been suggested by the rapid induction of nuclear proto-oncogenes in response to growth factor stimulation of quiescent cells (52-54). In fact, the induction

of several nuclear proto-oncogenes are some of the earliest detectable events following growth factor stimulation of quiescent cells re-entering the cell cycle (55).

Perhaps the key feature that connects nuclear proto-oncogenes with the signal transduction machinery is that the transcription of several nuclear proto-oncogenes proceeds in the absence of protein synthesis (56,57). Furthermore, their transcription can be stimulated by activation of protein kinase C and/or PKA pathways.
Together these observations suggest that these genes can respond to changes in the extracellular environment detected by the cytoplasmic signal transduction proteins and resulting in post-translational modification of pre-existing nuclear proteins (58-61). It is known that the oncogenic forms of the nuclear oncoproteins are able to transform cells by virtue of their overexpression as well as through increases in the half lives of their messenger RNAs (62,63). Of particular significance, enhanced expression of nuclear oncogenes is able to decrease the growth factor requirement for proliferation suggesting that cells transformed by nuclear oncogenes are no longer responsive to the extracellular environment due to an uncoupling of the normal flow of information at the point of the nuclear transducers (52-54). These oncogenic proteins, then, are forcing the proliferative response in the absence of an exogenous growth stimulus.

While the above is by no means an exhaustive documentation of all oncogenes, it suffices to illustrate the main properties of oncogene families. Clearly, with all of these factors located in their strategic places and functioning properly, an intact communication and response system is available to act upon extracellular signals through interactions from the plasma membrane, through the cytoplasm, to the nucleus leading to the appropriate changes in gene expression. Their strategic locations at the

various key points along the signal transduction pathways implicate the proto-oncogenes and their products as focal points in normal cellular growth and their oncogenic counterparts in the initiation and progression of carcinogenesis.

The *fos* Oncogene

The v-*fos* gene was first identified as the transforming gene of a murine osteosarcoma in the laboratory of Dr. Miriam Finkel who, at the time, was studying the risks of radiation induced neoplasia to workers handling materials used in the development of the hydrogen bomb and in other nuclear industries. Previous studies had linked an increase in the incidence of osteosarcoma with radium clock dial painters, and Dr. Finkel's lab was investigating the possible involvement of a viral agent responsible for radiation induced and spontaneous osteosarcomas in mice (64, reviewed in 65). Success was first achieved with the isolation of the Finkel-Biskis-Jinkins (FBJ-MSV) murine osteosarcoma retrovirus from a spontaneously occurring tumour (66). The Finkel-Biskis-Reilly (FBR-MSV) sarcoma virus was later identified in a tumour arising from an irradiated mouse and was found to be related to the FBJ-MSV, harbouring a similar transforming oncogene (67). The transforming protein from these viruses has been identified by immunological detection using antisera from FBJ-MSV infected rats and named Fos after the parent virus (*F*BJ-MSV *O*steogenic *S*arcoma virus, 68)

The v-*fos* gene encoded by the FBJ-MSV was the first *fos* gene to be cloned and was subsequently used as a probe to identify homologous sequences in the normal mouse and human genomes (67,69). Following sequencing of the genes from viral and cellular origins (70-72), a comparison between the normal and oncogenic versions of

the protein was made possible and proved to be invaluable in determining the mechanism of oncogenic activation of the v-*fos* gene. The human c-*fos* gene is a single copy gene and has been mapped to the q arm of chromosome 14 (67,73). Sequence comparison with the murine c-*fos* gene of 3.4 kb encoding a protein of 380 amino acids on chromosome 12, revealed virtual structural identity between the two genes with approximately 90% homology within the coding region. Both human and mouse genes consist of four exons coding for 47, 84, 36 and 213 amino acids respectively and both genes are transcribed, producing a mature mRNA species of 2.2 kb (65). Of the 4026 nucleotides that define the FBJ-MSV provirus, approximately 41% have been transduced from the normal c-*fos* gene. However, sequence comparisons indicate that the v-*fos* gene has undergone a deletion of 104 base pairs within a region of discontinuity between v-*fos* and c-*fos*. This deletion has resulted in a frame shift mutation such that the viral gene codes for one additional amino acid, and the C-terminal 49 amino acids are different from the C-terminal 48 residues of c-*fos*. The other transforming *fos* provirus, the FBR-MSV, encodes a 3791 nucleotide v-*fos* gene with about 19% of the sequence derived from c-*fos* sequences. There are more substantial differences between the FBR v-*fos* and c-*fos* genes, such as deletions of 24 amino acids at the N terminal, 98 amino acids at the C terminal of the protein, as well as two internal deletions of 39 and 27 base pairs. Furthermore, the 5' portion of the FBR v-*fos* gene has been fused to viral *gag* sequences and the 3' end to sequences called *fox*. Thus, the FBR v-*fos* gene encodes a 75 kDa gag-fos-fox fusion protein (71).

On its own, the c-*fos* gene is not oncogenic, but genetic manipulations enable the gene to transform cells in culture (74). The first of these requirements for transforming

potential is the addition of a constitutive enhancer such as the FBJ long terminal repeat (LTR). In addition to the enhancer element, untranslated sequences in the 3' portion of the gene must be disrupted. This 3' part of the gene contains a region rich in AU sequences which are implicated in message stability and, interestingly, these sequences are deleted in the viral *fos* genes (62,74,75). The fact that these disruptions activate the oncogenic potential of the c-*fos* gene suggests that *fos* induced transformation requires elevated expression of the gene coupled with a decrease in the turnover rate of the message. This view is strengthened by the fact that most normal tissues express very low levels of the c-*fos* gene.

Cellular Expression of the c-*fos* proto-oncogene

The description of *fos* as an oncogene prompted the examination of its expression during cell growth. Growth arrested cells normally reside in the quiescent G_0 state and can be stimulated to progress through the cell cycle by growth factors (76). Much of the proliferative control is exerted at the G_0/G_1 boundary and it has been shown that the G_0/G_1 transition requires gene expression (77). This prompted the search for the regulatory genes involved in proliferation. Cell growth-specific genes were first isolated from a fibroblast cDNA library screened by differential hybridization following PDGF stimulation (17). Since then many laboratories have employed a similar strategy to look for mitogen and tumour promoter inducible genes in their studies on growth regulation (78-80). From these studies a group of genes has emerged which share the characteristic features of being rapidly, and transiently, induced at the point of G_0/G_1 transition in the absence of *de novo* protein synthesis. Further, their messages are typically superinduced in the presence of an inhibitor of protein synthesis suggesting

that the down regulation and/or turnover of their message is protein synthesis-dependent (81). This group of genes has been called immediate early, growth response or primary response genes and has amongst its members gene products identified as extracellular matrix, cytoskeletal and nuclear proteins (reviewed in 81,82).

Most resting cells have low levels of c-*fos* expression with a few exceptions including macrophages, fetal liver cells, and placental cells (83,102). These cell types express high constitutive levels of c-*fos* , but this expression is dependent upon the continual provision of growth factor. For example, macrophages require CSF-1 and placental cells in culture need placental conditioned medium present to express c-*fos* constitutively (84). Atypical of the growth response genes, c-*fos* expression can occur in response to a variety of stimuli including serum, growth factors, cAMP, the tumour promoting phorbol ester TPA and a multitude of others which provoke, depending on the cell type and stimulus, both cellular proliferation or differentiation (65). Rapid and transient c-*fos* expression is seen upon differentiation of macrophages and monocytes by the tumour promoter TPA as well as during NGF-induced differentiation of rat PC12 pheochromocytoma cells into a neuronal morphology (84-86). Also, down regulation of c-*fos* expression is associated with the inhibition of TPA induced differentiation of monocytic cells by the synthetic glucocorticoid dexamethasone (87). c-*fos* inducibility has been extensively studied in quiescent fibroblasts stimulated to re-enter the cell cycle by growth factors (55, 65, 88, 89). The data clearly suggests a role for the c-*fos* gene in growth regulation and defines it as an immediate early gene.

Combined results from a number of studies have shown that one of the most striking and intriguing features of c-*fos* is that, typical of the early response genes, its

transcription can be induced in a very rapid and transient manner in those cell types which normally have low basal levels of expression. In fact, this inducible c-*fos* transcription is the earliest known nuclear event following growth factor stimulation with transcriptional activation of c-*fos* occurring within 5 minutes post stimulation and mRNA levels peaking at about 30 minutes and returning to basal levels by 2 hours (55,88,89). A role for Fos in the cell cycle was suggested from experiments demonstrating that antibodies to the *fos* protein or antisense c-*fos* oligonucleotides can block mitogen induced cell proliferation and DNA synthesis (90-92). However, further studies demonstrated that c-*fos* expression in fibroblasts could be induced by TPA at any point in the cell cycle and that c-*fos* expression is necessary, but not sufficient for cell cycle progression (93). The conclusions from these studies were that Fos is required for preparing or priming the cell for cell cycle re-entry and, as such, constitutes a competence factor. The requirement for other factor(s) suggests that progression factor(s) must act downstream of Fos in promoting proliferation. Transgenic mice containing the c-*fos* gene under the control of the metallothionein promoter display high levels of c-*fos* expression which could be further augmented in the presence of heavy metals. These transgenic mice have abnormal bone development with deregulated bone remodeling especially on the long bones of the leg (94,95). Thus, c-*fos* expression is associated with both G_0/G_1 transit and proliferation, and with cellular differentiation. While it was possible to induce c-*fos* expression at any point during the cell cycle in fibroblasts (93), suggesting that Fos is not specific for initiation of the proliferative response, there is no evidence that cell cycle independent expression takes place *in vivo* and this result may simply reflect the responsive nature of the gene. This possibility is

strengthened by the fact the expression of the c-*fos* gene proceeds in the absence of protein synthesis and is reliant upon trans-acting factor modification for inducibility (56).

The multiplicity of factors capable of inducing the c-*fos* gene in a rapid and transient manner coupled with its diverse effects in the cell have frustrated the search for a specific role for Fos. It seems that rather than being responsible for a single, specific event Fos is likely to have a more general role. One proposal is that Fos may act as a master switch capable of responding to a number of transient stimuli, from more than one signal transduction pathway, by changing the rate of transcription of other genes (19). In this way, extracellular signals can be transmitted to the nucleus and result in the appropriate alterations in gene expression. Thus, while the c-*fos* gene is an integral component of normal cell functioning, the v-*fos* oncogenic counterpart is capable of cellular transformation highlighting its important position in the translation of an extracellular signal.

The c-Fos Protein

The Fos protein is composed of 380 amino acids with a predicted molecular weight of about 42 kDa and has been subcellularly localized to the nucleus (70,96). The Fos protein has a half life of approximately 2 hours (88,97). The protein contains an unusually high number of proline residues (9%), and carries a net negative charge. Fos is extensively post-translationally modified by phosphorylation on serine and threonine residues which accounts for its apparent molecular weight on SDS-PAGE of 55-62 kDa (98). Fos is phosphorylated in response to TPA or serum treatment of cells, suggesting the involvement of protein kinase C in Fos phosphorylation (98), but Fos can also be

phosphorylated by cAMP dependent protein kinase as well, at least *in vitro* (99). It is likely that the phosphorylation of Fos is involved in modifying the properties of the protein and it has been demonstrated that phosphorylation may be involved in protein turnover (88), as well as in mediating transcriptional autorepression through its own promoter (100).

The nuclear localization of Fos coupled with its modification in response to signal transduction, led to the speculation that Fos is involved in genetic regulation (101). It was later shown that Fos complexes in the nucleus with a 39 kDa protein (102,103), which together are able to interact with DNA (104). The ability of a v-*fos* expression vector to stimulate transcription from a cotransfected plasmid containing the collagenase promoter was the first demonstration that Fos could stimulate transcription (105). Subsequently it was shown that a specific DNA/protein complex formed at the FSE2 region of the aP2 gene promoter coding for a lipid binding protein induced during adipocyte differentiation could be disrupted by antibodies against Fos. This was the first evidence for the participation of Fos in a transcriptional regulatory complex (106,107).

At around the same time, another nuclear oncogene was discovered from the avian sarcoma virus 17 and was called v-*jun* (108). The cellular homolog of the viral protein, c-*jun*, was recognized to contain a region of homology in its C terminus with the known yeast transactivator GCN4 (109). GCN4 was known to be a DNA binding pro tein which is involved in regulating amino acid homeostasis (110). It was found that the DNA binding site for GCN4 is strikingly similar to the binding site for the mammalian transcriptional activator AP-1 (111). This led to the idea of the possible relation-

ship between AP-1 and c-*jun* which was later confirmed both immunologically and functionally (112-114). Also, the Fos binding site within the aP2 gene promoter was identified as a sequence specific AP-1 binding site (106,107,115), prompting studies on the association between the two nuclear proto-oncogene proteins, Fos and Jun. To this end, it was found that the Fos associated protein in the ap2 promoter complex, p39, is immunologically related to Jun (116-118), demonstrating that both Fos and Jun are components of the AP-1 complex.

The AP-1 Transcriptional Activator

The eukaryotic transcription factor AP-1 (activator protein 1), was first identified as a factor that binds to and can stimulate transcriptional activity from a specific sequence of the human metallothionein IIa and SV40 early promoters (111, reviewed in 119). A great deal of interest was generated when the binding site for AP-1 was identified as the same DNA sequence that conferred phorbol ester responsiveness, implicating the involvement of AP-1 in a cellular pathway responding to tumour promoters and protein kinase C (120,121). In fact, an intact AP-1 binding site also known as the TRE (TPA response element), is necessary and sufficient for TPA responsiveness of AP-1 regulated genes (120). With the identification of the AP-1 complex as a composite dimer of proto-oncogene products, a clearer picture of the series of events associated with AP-1 activity were emerging.

The purification of AP-1 by site-specific affinity chromatography consistently resulted in a mixture of polypeptides which could not be accounted for by the presence of Fos and Jun alone, and it is now clear that both Fos and Jun are constituents of larger families of nuclear proteins with similar, but not identical properties (119,122).

Thus, the AP-1 complex can be composed of combinations of the Fos and Jun family of proteins. Recent work has shown that there are functional differences between AP-1 molecules depending upon their composition which are manifested by different DNA binding affinities and trans-acting properties (123).

Other members of the Fos family

Polyclonal antibodies to the Fos protein used in Western blotting were shown to cross-react with other polypeptides which are called Fos related antigens or Fras (124). Fra1 and Fra2 have now been cloned from cDNA libraries and both demonstrate partial sequence homology to Fos and share certain functional properties (124-126). Both Fra1 and Fra2 contain a conserved leucine zipper and basic domains, (known as bZip). They also share a high degree of homology to the c-*fos* gene in other regions known to contain conserved tertiary structure (124). In addition to their structural similarities, Fra1 has been shown to participate in complex formation with Jun family members and bind to the TRE sequence (126,128). Moreover, it has been found that the mRNA for Fra1 is serum inducible in quiescent fibroblasts albeit with slightly different kinetics from c-*fos* induction (124). Following serum stimulation of fibroblasts, Fra1 mRNA appears after the c-*fos* message and is detectable for a longer period of time. Similar induction of the Fra2 gene by TPA was demonstrated in monocytes (125). Interestingly, a sequential pattern of expression of the Fos gene family was observed after TPA addition with c-*fos* expressed first followed by *fra1* and then *fra2* suggesting that the composition of AP-1 is likely to change in an orderly fashion during the cellular response to a particular stimulus.

FosB is another member of the Fos family cloned from a cDNA library of

serum stimulated NIH-3T3 cells (127). Like the Fras, FosB shares the conserved bZip domain and an overall 70% homology with c-Fos. It also interacts with Jun and binds to the AP-1 site (128).

In addition to being a trans-activator FosB is capable of effectively transforming fibroblasts transfected with a FosB expression vector driven by either the v-*fos* LTR or the SV40 early promoter (129). In fact, under conditions of constitutive expression, FosB is a more effective transforming protein than c-Fos. Chimeric constructs between c-Fos and FosB have mapped the increased transforming potential of FosB to the 3' coding region. In c-Fos this region is thought to be responsible for the rapid turnover of the protein and this destabilizing region is absent in FosB (129).

The genomic structure of the *fos*B gene has been determined from a clone isolated from a mouse genomic library (130), and sequence analysis of this clone shows a similar promoter structure to the c-*fos* promoter suggesting the possibility that similar mechanisms are regulating these two genes. An interesting feature of *fosB* is that it was found to express two mRNA species as a result of differential splicing yielding FosB and a truncated FosB/SF (also called delta FosB). FosB/SF can also interact with Jun, but while FosB is a potent trans-activator (129), conflicting data have ascribed both activating and repressing activities to FosB/SF (131-133).

While the precise functions of the Fos family of proteins is not entirely clear, they do represent a group of evolutionarily related proteins which may have evolved to elicit different responses to stimuli.

Other members of the Jun family

As with the Fos family of proteins, there also exists a family of Jun-related

proteins all of which can participate in complex formation and specific DNA binding (119,134). Presently the Jun family consists of c-*jun*, *jun*-B, and *jun*-D all of which display several regions of sequence homology, but also display some intriguing functional differences.

The gene for c-*jun* was cloned from cDNA libraries from human fibroblasts and a human cervical carcinoma cell line (112,113), as well as from mouse cDNA libraries of serum stimulated fibroblasts (135,136).

The c-*jun* gene is both serum and TPA inducible and can activate a TRE-containing promoter which is the reflection of an increase in AP-1 activity following these stimuli (137). Transcription of the gene occurs rapidly and in the presence of protein synthesis inhibitors which categorizes c-*jun* in the group of immediate early genes. Analysis of c-*jun* genomic sequences revealed a number of interesting features including the absence of intronic sequences and the presence of a single TRE within the promoter which can bind Jun/AP-1 and stimulate its own transcription (138,139).

The *jun*-B gene was isolated from a cDNA library from serum stimulated fibroblasts screened for sequences homologous to the DNA binding domain of v-*jun* (140). JunB has an overall homology of about 95% to c-Jun which spans over at least five separate domains including the highly conserved basic leucine zipper region as well as a stretch of 65 amino acids at the N terminus of which 45 are either identical or conservative replacements (137). Like c-*jun*, *jun*-B is a member of the immediate early family of rapidly responding genes and induction of the gene does not require new protein synthesis (139). Both JunB and c-Jun can dimerize and bind to the AP-1 site with binding

activity of both proteins being dramatically increased in the presence of c-Fos or Fras (123,134).

While the regulation of the c-*jun* and *junB* genes share remarkable similarity and although the protein products of these genes bind to DNA in an identical fashion, their trans-activating properties are notably different.

Transfection of an expression vector for c-Jun is able to activate a cotransfected promoter construct containing a single TRE, but a JunB expression vector does not trans-activate this construct (141). These studies further showed that in the presence of JunB the activation of a TRE construct is nullified suggesting the JunB is a negative regulator of c-Jun function. Interestingly, JunB is able to activate a promoter construct containing multiple copies of the TRE (141). The interpretation of the variable activities of JunB is based on the fact that the activation domains of c-Jun and JunB are different and that trans-activation by JunB requires interactions between adjacently bound complexes while c-Jun does not. It is possible that JunB is able to modulate the expression of genes induced by c-Jun and/or that the two different proteins have evolved to differentially regulate genes containing TRE sequences. In addition, a clear cell type-specific pattern of expression of the *jun* family of genes exists with c-*jun* expression being limited to the lung and heart tissues of the adult mouse while *jun*-B is expressed in a wider range of tissues (142,143).

The third member of the *jun* family to be characterized is the *jun*-D gene (142,143,144). The *jun*-D gene was also cloned from a cDNA library of serum stimulated fibroblasts probed for homology to the C-terminal DNA binding domain of c-Jun.

JunD displays about 70% homology to c-Jun with the most highly conserved region encompassing the bZip domain enabling JunD to interact with other leucine zipper proteins and bind to the TRE site and activate transcription (142,143,145). In contrast to the other members of the *jun* family, however, *jun*-D is insensitive to serum or TPA stimulation of quiescent cells and is not an immediate early gene. *jun*-D is expressed at high levels in a number of adult mouse tissues and may play a distinct role in transcriptional regulation of cell type specificity (142,143).

Analysis of a chicken genomic clone of *jun*-D reveals that the gene is intronless as is c-*jun*, but that, unlike c-*jun*, within the sequenced portion of the 5' non-coding region there is no AP-1 or TRE site (144). Instead, there are two regulatory elements called CREs (cAMP response element), which are transcriptionally activated in response to elevated cAMP via PKA and CREB proteins (cAMP response element binding proteins, 146). Interestingly, JunD contains an additional serine residue within a consensus site for PKA phosphorylation which is absent from c-Jun and JunB. The lack of a destabilizing sequence in the 3' untranslated region of the message for JunD may account for the higher steady state levels of *jun*-D mRNA compared to those for the other family members.

Clearly with the combination of Jun and Fos proteins, AP-1 represents a transcriptional regulatory complex with the capability for a diversity of functions by responding to growth stimuli in different ways depending on the composition of the prevailing AP-1 molecule, perhaps in a cell type-specific manner. Furthermore, AP-1 composition changes during cell cycle progression (147,148). It was found that immediately after serum stimulation c-Fos predominates in complexes with the Jun proteins,

but at later times c-Fos is replaced with the Fra proteins. Whereas c-Fos and FosB are required for G_0/G_1 transit, the Fra proteins are required for exponential growth (149). The results from retroviral overexpression of c-*jun*, *jun*-B and *jun*-D also suggest that the Jun family members may have different roles in the regulation of cell growth (150). For example c-*jun*, and to a lesser extent *jun*-B, generated colonies in agar, but only c-*jun* supported growth in low serum. *jun*-D on the other hand is unable to support growth in low serum or develop colonies in agar and by these criteria is not a proto-oncogene.

The leucine zipper dimerization motif

Protein sequence analysis revealed that the region of homology between Jun and GCN4 consists of a domain of basic residues followed by a leucine rich domain (151). Earlier studies on the CAAT binding protein, C/EBP, showed the same motifs to be functionally significant (152). It was demonstrated that within this domain 4 leucine residues were spaced with a regular heptad repeat. Within the motif, a leucine residue was placed at position "d" of the heptad and a hydrophobic residue at position "a" giving rise to a helical structure containing hydrophobic residues aligned along one face of the helix. This motif can dimerize to a coiled coil structure reminiscent of that found in tropomyosin and the other members of the k/e/m/f (keratin/elastin/myosin/fibrin) family of fibrous proteins (153). A 33 amino acid peptide representing the dimerization domain of GCN4 has been crystallized and the structure determined to a resolution of 1.8 angstroms (154). This study showed that hydrophobic interactions and ion pairing within that region both contribute to protein dimerization. This dimerization motif, dubbed the leucine zipper, has since been shown to be a common structure present in

many proteins associating with DNA. Both Fos and Jun proteins contain the leucine repeat region and their direct association has been shown to be mediated by this motif.

The leucine zipper is indispensable for DNA binding, but it is the adjacent basic region that makes contact with the DNA in the major groove and determines the specificity of binding (155). Mutational analyses have shown that the leucine zipper and DNA binding domains are prerequisites for trans-activation since they bring the basic domains into a conformation suitable for DNA binding (156,157). Structurally, the leucine zipper forms a two stranded coiled coil which places the basic regions in apposition forming a structure able to interact with the DNA molecule in what has been described as a "scissors grip" fashion (158).

Fos and Jun leucine zipper domains are necessary for both dimerization and, therefore, DNA binding proving that this region is functionally important for both proteins (159,160). While Jun can homodimerize and bind tc the AP-1 site, Fos does not homodimerize and consequently does not bind to DNA on its own (119). Instead, Fos and Jun cooperate in DNA binding with both proteins contributing to the DNA binding potential of the complex. Interestingly, in solution the Fos/Jun heterodimers are preferentially formed and have a 10-25 fold greater affinity for the AP-1 site than does the Jun homodimers (134,161,162). In addition to having a greater affinity for DNA, the Fos/Jun heterodimers have a greater trans-activating potential (119).

Dimerization specificity has been further mapped to a region within the leucine zipper consisting of only 8 amino acid residues at positions e and g in the heptad repeat which direct the preferential association of Fos and Jun (162). Within the Fos protein, acidic amino acids at these positions provide the thermodynamic force for preferential

heterodimerization and probably prevent Fos homodimerization by destabilization of the coiled coil structure. DNA binding specificity is dictated by the basic region adjacent to the leucine zipper as determined using domain swapping experiments between Fos, Jun and CREB (cAMP response element binding protein, 163).

Regulation of AP-1 Activity

Recent work has challenged the idea that AP-1 responds solely to tumour promoters or proliferative stimuli transduced through the PKC pathway. The other major signal transduction pathway in the cell is mediated by cAMP and PKA which effect a transcriptional response via modification of CREB and CREB-related proteins (146,164,165). The CREB and related ATF (activating transcription factor), also require dimerization for DNA binding and contain the conserved bZip domains (166-168). The interaction of these two major signal transduction pathways has been demonstrated at the transcriptional level suggesting "cross talk" within signal transduction pathways. First, it has been shown that Fos and Jun proteins are able to bind to the CRE sequence and trans-activate a promoter construct containing this regulatory element (169,170). Secondly, it was shown that Jun can dimerize with the CREB related protein CRE-BP1, and the resultant heterodimers retain site specific and cooperative DNA binding to the CRE sequence (171-173). Curiously, while Jun can dimerize with CRE-BP1, Fos proteins can not (172,173), and neither Fos nor Jun can interact with CREB which is highly related to CRE-BP1 (173,174). Thirdly, increased cellular levels of cAMP are able to enhance the effects of TPA on the induction of c-*fos* expression through the TRE (175-177). Lastly, cross talk between pathways at the transcriptional level is suggested by experiments where the catalytic subunit of PKA transfected

into JEG-3, NIH 3T3 or HepG2 cells synergizes with co-transfected c-Jun in activating a TRE reporter construct by increasing TRE binding activity (178). The effect of PKA is unclear since it does not directly phosphorylate c-Jun, nor does the effect appear to be mediated through the CREB protein which is known to be activated by this kinase (165,178).

However, the effects of PKA are not consistent amongst all cell types. HeLa and COS cells can strongly activate a TRE containing promoter in the presence of PKA alone and do not show any synergism with cotransfected c-*jun* while P19EC cells do not activate a TRE containing promoter in the presence of either PKA or c-Jun. Possibly these different cell types have evolved to respond to different signal transduction pathways permitting signal differentiation amongst different cell types within an organism. One further piece of evidence that signal transduction pathways are not likely to function independently is supplied by the finding that TPA can induce the phosphorylation of adenylate cyclase (179). Further work will probably uncover additional intersection points between these pathways and it seems likely that cell signalling will soon be viewed as a series of integrated circuits (see also 180-185).

c-*jun* expression is inducible by TPA through the PKC pathway and in the absence of protein synthesis implicating protein phosphorylation in c-*jun* regulation (120). The role of protein phosphorylation in the regulation of transcription factor activity is well documented and has been shown to affect a large number of transcription factors. The phosphorylation, catalyzed by a variety of protein kinases, was shown to regulate the different properties of transcription factors (reviewed in 186). For example, transcription factor phosphorylation can affect DNA binding properties by

either increasing or decreasing the affinity for the regulatory sequence. The *trans*-activating properties can also be dramatically influenced by the status of transcription factor phosphorylation. Indeed, even the nuclear translocation of certain transcription factors is regulated by phosphorylation and in some cases more than one of these regulatory mechanisms functions within the same transcription factor.

Phosphorylation of c-Jun occurs on at least 5 separate residues of Ser or Thr, but interestingly none of these sites are phosphorylated by PKA *in vitro*. Controversy exists regarding whether or not c-Jun is a substrate for PKC with one study demonstrating that Jun can be phosphorylated by PKC *in vitro*, while the other suggests that although c-*jun* is responsive to TPA it is not a substrate for the kinase (187,188). Despite this controversy, phosphorylation of c-Jun appears to be functionally important since it has been shown that cellular transformation by c-Ha-Ras as well as other transforming oncogenes result in hyperphosphorylation of Jun and is required for oncogenic transformation (189). Specifically, phosphorylation at two serine residues located in the N-terminal activation domain of the molecule (Ser 63 and 73), is stimulated in response to oncogene transfection and increases the trans-acting activity of AP-1 (189,190,191). The Ras-activated phosphorylation of these residues is coupled with the dephosphorylation of other serine residues which affect the DNA-binding activity of c-Jun (189). Interestingly, *in vitro*, c-Jun is phosphorylated by the growth factor-stimulated MAP2 kinase (also called ERK 1), however the site(s) of phosphorylation and their effects remain to be confirmed since there are contradictory reports claiming phosphorylation at different sites in c-Jun (192-194). The additional serine, and one threonine residue mentioned above are present adjacent to the DNA binding domain of

c-Jun and influence DNA binding (195). These residues are in the phosphorylated state in resting cells and become dephosphorylated upon serum or TPA stimulation (195). The dephosphorylation of these residues results in an increase in the DNA binding activity of Jun/AP-1 and suggest that the increase in DNA binding activity of AP-1 in response to mitogens and PKC stimulation is the result of site-specific dephosphorylation.

Clearly, phosphorylation of c-Jun plays more than one key role and must involve the integration of kinase and possibly phosphatase activities. Thus, the TPA-induced dephosphorylation must be coupled with the N-terminal phosphorylation of c-Jun to achieve both DNA binding and trans-activation (196). The convergence of signal transduction pathways at the level of transcriptional activators and specifically involving AP-1 has been described recently with the identification of a negative regulator of AP-1 activity called inhibitory protein 1 (IP-1, 197). IP-1 regulates the ability of AP-1 to interact with the TRE in a phosphorylation-dependent manner. IP-1 associates with AP-1 to inhibit DNA binding activity when in its dephosphorylated form. TPA induces PKA-dependent phosphorylation of IP-1 by an uncharacterized mechanism of signal transduction cross talk. PKA phosphorylation of IP-1 relieves its inhibitory effect on AP-1 restoring its DNA binding ability. Considering these results, IP-1 is a candidate for a third messenger and convergence point for two major signalling pathways (198).

Modification of AP-1 activity can also occur in the absence of covalent modification. It is becoming clear that AP-1 may not necessarily function alone, with increasing evidence for protein/protein interactions existing at composite regulatory elements

(199). A relationship suggesting the interaction between AP-1 and the glucocorticoid receptor (GR), was initially suggested when it was found in cotransfection studies that AP-1 induced collagenase expression is downregulated in the presence of cotransfected GR (200-202). In the presence of ligand, the GR binds to a specific sequence present in target genes called the glucocorticoid response element (GRE, 203,204). The GR binds to target DNA sequences as a dimer interacting through a conserved motif called the zinc finger. DNA binding proteins interacting through zinc finger structures generally contain conserved cysteine and histidine residues which coordinate an atom of zinc juxtaposing a ß sheet and an α helix forming a "finger-like" structure (205). In the collagenase promoter, it has been shown that AP-1 induction of the gene can be down regulated by the synthetic glucocorticoid dexamethasone. Furthermore, this down regulation by dexamethasone functions through an AP-1 binding site indicating a functional interaction between AP-1 and GR (201). Subsequent experiments demonstrated that a similar situation might occur in the promoters of other genes. For example, the proliferin gene is differentially regulated by both TPA and glucocorticoids through overlapping regulatory sequences which define a composite regulatory element (206,207).

The exact nature of transcriptional regulation through this composite regulatory element is not clear since contradictory results showing induction of the proliferin gene by TPA and its repression by dexamethasone as well as cooperative activation of the gene by these agents has been reported (205, 207). The glutamine synthetase gene is also induced by glucocorticoids and the regulatory sequences responsive to glucocorticoids have been mapped to a consensus GRE (208). Interestingly for maximal gluco-

corticoid responsiveness in addition to the GRE the adjacent AP-1-like sequence must also be present providing further evidence for AP-1 and GR interactions.

A direct interaction between AP-1 and GR has been demonstrated by *in vitro* crosslinking experiments and coprecipitation using either GR or Fos/Jun antibodies (200,201,207), however others have not been able to detect these interactions (202,209). However, in one study coexpression of c-Jun and GR repressing the trans-activation at a GRE-containing promoter was reported (201). It was initially thought that the downregulation of AP-1 activity in the presence of GR might be a result of competition for DNA binding since downregulation depends upon an intact bZip domain in c-Jun (202). However, it was shown that the GR does not bind to the TRE nor does AP-1 bind to the GRE ruling out direct competition for DNA binding sites (200,209). Since the bZip domain is required for c-Jun/GR interaction, but cross binding does not occur, it is proposed that c-Jun interaction with GR probably requires an AP-1 homo or heterodimer.

In a somewhat contradictory scenario, c-Fos and GR have also been shown to interact by crosslinking experiments and immunopreciptation experiments, but these interactions do not depend upon the bZip domain of c-Fos, but rather on a region spanning residues 40-111 to which no previous function has been attributed (209). Residues 40-111 in c-Fos are poorly conserved amongst Fos family members, and are not present in FosB, which is unable to form a complex with GR (209).

Functional interaction between AP-1 and GR is also furnished by demonstrating that overexpression of c-*jun* is able to overcome the inhibition by GR (200). While there appears to be no cross competition for DNA-binding sites, it has been suggested that

AP-1 and GR mutually repress their DNA-binding activities since it has been shown that coexpressed c-Jun and GR do not bind the TRE or GRE (200), and bacterially produced c-Jun is able to dramatically reduce complex formation between a GRE binding site in COS cell extracts overexpressing the GR (202).

Again, contrasting results were seen in a separate study which showed that Dex has no effect on AP-1 binding activity (201). In an effort to clarify these apparently contradictory results a carefully controlled study was undertaken to examine the interactions of GR with AP-1 (210). These results indicate that the DNA-binding domain of GR can differentially inhibit AP-1 binding and activation of a collagenase promoter construct depending upon the AP-1 composition. A 10 fold higher concentration of GR is required to inhibit the DNA-binding activity of Jun homodimers than to inactivate Jun/Fos heterodimers. This study concluded that the Fos/Jun form of AP-1 is the preferential target for inhibition by the GR (210).

From these combined results it is apparent that regulation of AP-1 can involve multiple protein/protein interactions and that this may indeed be a major regulatory mechanism *in vivo* controlling the balance between the mutually exclusive pathways of proliferation and differentiation.

Recently a novel mechanism for the regulation of AP-1 activity has emerged and involves regulation of Fos/Jun dimerization and DNA binding activity by a redox (reduction/oxidation) mechanism (211,212). This form of regulation involves cysteine residues in Fos and Jun which are inactive when oxidized. The oxidized proteins can be activated to promote their association and DNA-binding with either high concentrations of reducing agents or with a nuclear protein called Ref-1 which increases the

DNA binding activity in the absence of reducing agents (212,213). This novel form of transcription factor regulation may be significant with respect to the transformation properties of c-Fos since v-Fos contains a serine residue in place of cysteine-154 in c-Fos (108). Expression vectors containing wild type Fos or Fos with cysteine-154 mutated to serine indicate that this mutation results in an increased transforming potential (214).

Transcriptional Regulation of the c-*fos* gene

The c-*fos* proto-oncogene has become a paradigm for the study of the regulation of immediate early genes owing to its remarkably rapid and transient response to a myriad of stimuli associated with cell growth, differentiation and other cellular responses. The ability of the c-*fos* gene to respond to these stimuli has led to the suggestion that the gene may act as a "master switch" constituting the first transcriptional signal translator and being able to direct downstream events in a coordinated fashion. As discussed earlier, this remarkably sensitive immediate early gene can be induced in the presence of protein synthesis inhibitors and is, in fact, superinduced in their presence leading to the suggestion of the involvement of a labile repressor in c-*fos* regulation (11,56).

As a potential master switch gene a great deal of effort has been undertaken to understand the mechanisms by which the gene promoter can integrate a response from divergent pathways. The first step in identifying regulatory regions within the c-*fos* promoter which might be responsible for transcriptional regulation was the identification of DNase I hypersensitive sites within the 5' region of the gene in NIH-3T3 cells (215).

DNase I hypersensitivity is thought to reflect interactions of regulatory proteins with DNA sites usually within the 5' portion of the gene (216). Upon serum stimulation of 3T3 cells the major hypersensitive region in the c-*fos* promoter did not change, but one site located around nucleotide +10 lost hypersensitivity. This was coincident with elevated expression of the gene. Similar rapid and reversible changes in DNase I hypersensitivity coincident with elevated c-*fos* expression (217) were observed in HeLa cells following serum stimulation (217). The role of chromatin structure on c-*fos* expression is further highlighted by studies which showed that the rapid induction of the gene is tightly coupled to topoisomerase I activity which controls the rate of chain elongation (218).

Initial examination of the c-*fos* gene revealed the presence of a TATA box element required for correct initiation of transcription and a polyadenylation signal in the 3' non-coding portion of the gene (72).

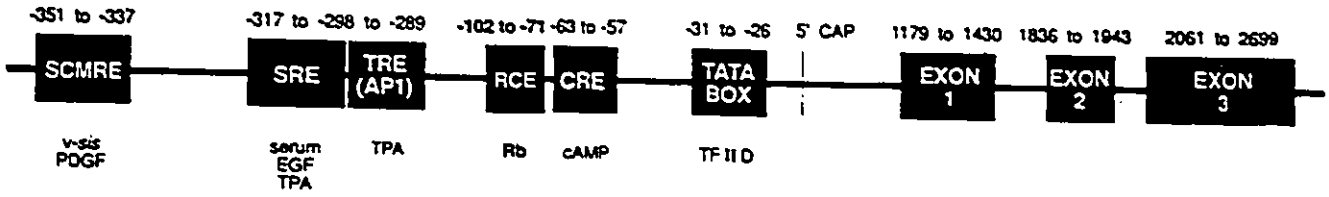
The identification of *cis*-acting sequences and their *trans*-acting factors has been addressed by a number of investigators in the past 7 years. Figure 1 presents a diagram of some of the *cis*-acting sequences identified in the c-*fos* promoter which will be discussed further, but it does not attempt to reflect the full complexity of the c-*fos* gene. Through transient transfections of c-*fos* promoter deletion mutants, the *cis*-acting regulatory element responsible for the serum inducibility of the gene was identified and characterized as having properties of an enhancer (62). This same study also determined that sequences in the 3' end of the gene encompassing both coding and flanking regions are associated with regulating the transient accumulation of the message, and thus playing an important role in regulating the half life of transcribable message.

Recent data suggests that the 3' part of the transcript functions as an RNA-mediated mRNA destabilizer which does not involve the nascent polypeptide and is presumed to function through an unidentified structural motif capable of interacting with the degradation machinery (219). This property is important in preventing constitutive availability of Fos protein and it is noteworthy that the oncogenic v-*fos* contains a frame shift mutation within this region (72). Also as pointed out earlier, oncogenic conversion of c-*fos* can be achieved, in part, by removal of these 3' sequences (74). The deletion of this part of the protein is not necessary for this effect as it has been shown that mutations in this C-terminal region are sufficient to deregulate c-*fos* expression (220).

Figure 1. Diagram of the c-*fos* promoter regulatory sequences

Several of the well characterized c-*fos* regulatory element sequences are shown as black rectangles. The position of the regulatory elements are given relative to the 5' cap site. Below each regulatory element is a partial list of stimuli or proteins which have their effects through that sequence.

Diagram of Promoter and Partial Exon Structure of the c-fos gene



Using a combination of sensitive techniques including gel mobility shift, methylation interference and S1 nuclease protection assays, several sites within the promoter have been identified as protein binding sites and have been shown to regulate c-*fos* expression (61,221,222-231). While many of these regulatory sites have not been characterized in detail, studies of the c-*fos* promoter have identified those regulatory elements which are central to the accurate regulation of the gene and will be described below. These regulatory elements include: the serum response element (SRE), mapped to position -322 to -299 relative to the transcriptional initiation site, the *sis* conditioned medium response element (SCMRE), mapped to position -351 to -336, the TPA response element (TRE), mapped to position -293 to -287, the cAMP response element (CRE), mapped to position -66 to -59 in the mouse gene.

The c-*fos* Serum Response Element: GGATGTCCATATTAGGACATCT (222)

The most extensively studied of the c-*fos* regulatory elements is a conserved sequence centered approximately 300 bp upstream of the mRNA cap site and is termed the serum response element (SRE, 62 and reviewed in 232). While it is clear that in many different cell types the SRE responds to various growth factors as well as to activators of protein kinase C (223-225), it is not certain whether the SRE is also required for c-*fos* induction in response to increases in intracellular cAMP (225). Using transient transfection of various regions of the c-*fos* promoter it was found that while the SRE was not necessary for, it could respond to cAMP suggesting that the SRE may be involved in the cAMP response (227). Others however, have found that while multiple elements in the c-*fos* promoter can respond to cAMP they all have homology

to a consensus CRE, but these do not include the SRE (226).

The SRE sequence is highly conserved, with human and mouse sequences being identical, and contains a region of hyphenated dyad symmetry and some properties associated with transcriptional enhancers such as the ability to stimulate transcription from a heterologous promoter as well as the ability to stimulate transcription independent of orientation in relation to the gene. However, unlike other enhancers the effectiveness of the SRE is influenced by distance from its normal position (62). Interestingly, SRE-like sequences called CArG boxes (derived from CC(A/T rich)GG), have also been identified in the promoters of other serum inducible genes such as the skeletal and cardiac actin genes (233,234), but no SRE sequence has been reported in the serum inducible c-*jun* gene. Thus, the SRE may represent a conserved, but not the sole mechanism for a rapid response to serum growth factors.

The identification of a protein capable of specific binding to the c-*fos* SRE has been reported (235-237). This protein has an estimated molecular weight of 67 kDa and has been called the serum response factor (SRF). The gene encoding SRF has now been cloned from a human placental cDNA library (238). The SRF cDNA clone has an open reading frame encoding a protein with a predicted molecular weight of 51,593 which suggests the possibility of post-translational modification resulting in its higher than estimated molecular weight. Indeed, SRF has been shown to be phosphorylated on serine residues *in vivo* and is a substrate for casein kinase II *in vitro* (239-243). Interestingly, the SRF gene is itself serum inducible (238), and it will be interesting to see if SRF plays a transcriptional autoregulatory role.

There is also evidence that a protein similar to SRF binds to and activates the

SRE sequence in the skeletal actin gene suggesting conservation of transcriptional regulatory mechanisms (233).

Similarities between the CArG binding factor and SRF have been suggested by their identical DNA recognition sequences, cross-binding of these factors to the CArG box and SRE, and the recognition of CArG box binding protein by an anti-SRF antibody (233,244,245). Differences in the regulation of c-*fos* and the actin genes is believed to be directed by interactions between CArG binding factor and other muscle-specific proteins (245-247).

The c-*fos* SRE also is the site for multiple protein interactions contributing to the regulatory properties of the element (248-257). It was found that SRF is related to the *Saccharomyces cerevisiae* DNA-binding protein MCM1 which is involved in directing mating type-specific transcription by interacting with a CArG box and recruiting different accessory proteins (238, 257-260). The similarity between SRF and MCM1 was exploited to develop a yeast screening procedure for the identification of proteins able to interact with SRF. This study identified a human homeobox protein called Phox1 capable of enhancing the DNA-binding properties of SRF (261). Since homeobox proteins are known to be involved in establishing cell type during development (262), the interaction of SRF with an homeobox protein may permit different interpretations to the same signal dependent upon cell type. Most, but not all of the evidence to date indicates that SRF is a positively acting transcription factor (263). Mutations in the SRE which prevent the binding of SRF also block serum inducibility of the element (188,231,263).

It has been shown in HeLa cells that serum inducibility of the c-*fos* SRE is dependent upon the ability of SRF to interact with an accessory protein called $p62^{TCF}$ (for Ternary Complex Factor, discussed below), which does not bind directly to the SRE, but rather binds to SRF (250). Interestingly, SRF is associated with the SRE both in the presence and absence of growth factors (221,222,231,249). The sole exception described to date is EGF-induced SRE binding in A431 epidermal carcinoma cells (262). This suggests the possibility that permanent occupation of the SRE by SRF and transcriptional activation may be modulated by the modification of SRF and/or its interaction with other proteins (222,249,250). Post-translational modification and/or protein/protein interactions are also indicated by the aforementioned ability of c-*fos* transcription to be stimulated in the presence of protein synthesis inhibitors (56).

A role for the SRE-bound protein complex in growth regulation has been suggested by studies which have demonstrated that $p62^{TCF}$ is a substrate for the growth factor activated MAP kinase resulting in increased ternary complex formation (264). Moreover, phosphorylation of $p62^{TCF}$ correlates with increased transcriptional activity of the c-*fos* gene (265). *In vivo* footprinting studies have shown that protein interactions at the SRE do not change with increased transcriptional activity of the gene (249), and changes in complex formation upon $p62^{TCF}$ phosphorylation as determined by gel shift analyses have been interpreted as a reversible modification of pre-existing complexes (265). Furthermore, $p62^{TCF}$ appears to be related to Elk-1 which is a member of the Ets family of proto-oncogene transcription factors which are known to interact with c-Fos to stimulate transcription from the polyoma enhancer (266,267, reviewed in 268). Binding studies have shown that Elk-1 can bind to SRF preventing complex

formation with $p62^{TCF}$ and that antibodies against Elk-1 prevent complex formation between SRF and both Elk-1 and $p62^{TCF}$ (266). These data suggest the possibility for cooperation of the *ets* and *fos* proto-oncogenes in the regulation of downstream genetic events.

Another protein containing regions of homology to Elk-1 has been identified using a yeast screening method designed to identify proteins recruited by SRF to bind to the SRE. This protein called SAP-1, for SRF accessory protein-1, was found to have DNA-binding properties similar to $p62^{TCF}$ (257).

A distinct 62 kDa protein has also been identified which, in contrast to $p62^{TCF}$, interacts directly with the SRE independently of bound SRF and has been called $p62^{DBF}$ (for Direct Binding Factor, 248). Another study reported that the multifunctional transcription factor YY1 competes with SRF for SRE binding and overexpression of YY1 represses serum inducible transcription of the c-*fos* gene (269). Recent studies have now proven that $p62^{DBF}$ is homologous to YY1 (S. Natesan and M.Z. Gilman, personal communication).

The complexity of SRE function in c-*fos* transcriptional regulation is further highlighted by the finding that it is also the sequence involved in negative regulation of the gene. It has been shown, for example, that superinduction of c-*fos* expression by cycloheximide can be mimicked by a synthetic promoter carrying the SRE sequence suggesting that labile protein(s) are repressing transcription either through interaction with the SRE and/or the protein/SRE complex (56, 270-274). Evidence in favour of the latter is suggested by the observations that mutating the SRE such that SRF binding is impaired results in the loss of cycloheximide superinduction (271,272).

The c-*fos* *sis*-Conditioned Medium Response Element: CAGTTCCCGTCAATC (230)

Slightly further upstream from the SRE and centered at position -345 another *cis*-acting element shown to be required for the responsiveness of the c-*fos* promoter to v-*sis* conditioned medium (SCMRE), and PDGF is located (230). The properties of this element have not been fully characterized to date, but it is known that the c-*fos* gene responds in a rapid and transient manner to PDGF exposure and that the SCMRE is the binding site for a nuclear protein factor(s) following induction through this element (55,88,89,230,275). Unlike the SRE, however, treatment of BALB/c 3T3 fibroblasts with either PDGF or v-*sis*-conditioned medium results in inducible binding to the SCMRE (230,275).

Inducible binding of a factor to this site has also been reported in A431 cells in response to EGF treatment suggesting that the SCMRE may respond to different growth factor stimuli (249). Furthermore, the kinetics of inducible binding correlate with transcriptional activity of the gene and transfection of SCMRE promoter mutants unable to bind to the inducible factor are also incapable of responding to purified *sis* protein (275). A recent report has demonstrated that inducible binding of a protein to the SCMRE in response to EGF can be reconstituted in a cell-free environment and that binding occurs within 20 seconds of EGF addition suggesting the possibility that the inducible factor may be activated via tyrosine phosphorylation by the EGF receptor (276). Furthermore a protein of molecular weight 91 kDa (p91), has been shown to interact directly with the EGF receptor through a SH2 domain and is phosphorylated on tyrosine upon EGF - receptor binding (277). Activated p91 is then able to stimulate

transcription from an SCMRE-containing reporter plasmid *in vitro*.

The c-*fos* TPA Response Element: ACATCTGCGTCAGCAG

Immediately downstream from the SRE, located at position -293, is another regulatory element related to the TRE sequence present in genes responsive to the phorbol ester TPA (120). The consensus TRE is an octameric sequence (TGAGTCAG), and the c-*fos* TRE contains an C/G base pair instead of the consensus A/T pair at position 3 in the octamer. As described above, the TRE is the recognition sequence for the binding of the AP-1 transcriptional regulatory complex of Fos and Jun proteins and has also been shown to be required for the full transcriptional response of the c-*fos* promoter (278).

The c-*fos* cAMP Response Element: CCCAGTGACGTAGGA

It is well established that several eukaryotic genes are induced by agents that elevate the intracellular level of cAMP (for review, see 164,278,279), and that the molecular mechanism of this induction is based on protein factors interacting with a cis-acting DNA regulatory sequence element termed the cAMP-responsive element (CRE). The presence of an 8 base pair palindromic CRE consensus sequence (TGACGTCA) is necessary but not always sufficient for cAMP induction (164).

Some cAMP-inducible genes, such as somatostatin and chorionic gonadotropin, (CGα), contain the consensus CRE sequence, whereas others have differences within the element. For example, PEPCK and proenkephalin CREs have different bases in positions 1 and 2 in the octamer, while c-*fos*, VIP and E2A are different in positions 7 and 8. The effect of these changes in the CRE on transcription has been analyzed by Deutsch *et al.*(281,282) who showed that point mutations in the first (T to A), fourth (C

to G), or eighth (A to T) positions of the CGα CRE sequence almost completely inhibited transcription. However, since the cAMP-responsive VIP gene contains thymidine in position eight of its CRE but is still active, sequences next to the CRE must also influence the transcriptional activity of these genes. Indeed, DNase protection assays with CRE promoters indicate that more than the 8 bp palindrome is engaged in protein binding (164,167,283,284).

As previously noted, the c-*fos* gene is responsive to cAMP as well as to adenylate cyclase agonists such as cholera toxin and forskolin and contains a CRE at position -60 relative to the mRNA cap site. While other sequences within the c-*fos* promoter have been identified as cAMP responsive, the element centered at position -63 is the major cAMP responsive sequence (226,227). In addition to mediating responsiveness to cAMP, the c-*fos* CRE is also critical for basal level expression of the gene (229). The c-*fos* CRE does not contain the perfect CRE consensus, but does have homology in 7 of the 8 nucleotides within the consensus octamer, with a cytosine nucleotide at position 7 being deleted in the c-*fos* CRE (72). While the CRE is known to be a factor binding site, the identification of the protein or proteins binding to the c-*fos* CRE has not been described (221). It is known, however, that the c-*fos* CRE does bind protein(s) constitutively (229).

Over the last few years a number of different protein factors, including CREB, have been isolated and shown to interact with the CRE. In fact, several distinct cDNA clones encoding CRE-binding proteins have been isolated so far, such as CREB-327 (delta) and CREB-341 (alfa) (167,168,226,285), CRE-BP1 (171,286), CRE-BP2 (287), CREM (288), CREB-2 (289) and a family of activating transcription factors (ATF)

(166,187,290). Some of these proteins, such as the CREB or CREM isoforms are derived from alternative splicing of the transcript from the same gene, whereas others are different gene products. In fact, subsequent detailed analyses of cDNA and genomic clones proved that the CREB gene alone encodes multiple mRNAs coding for proteins still uncharacterized (291, 292).

The complexity of transcriptional regulation involving the CRE is further increased by the extent of phosphorylation of individual CRE-binding proteins (165,168,291-293), as well as their ability to form homo and heterodimers with the structurally related members of the Fos/Jun family (123,166,286,296).

Other Regulatory Sequences

Recent studies have identified an additional regulatory element in the c-*fos* promoter which binds and responds to the retinoblastoma protein, Rb. The retinoblastoma control element (RCE) is located immediately 5' to the CRE in c-*fos* gene. It has been reported that the binding of Rb to the RCE represses transcription of the c-*fos* gene in mouse fibroblasts (297). However, other studies have demonstrated that Rb/RCE interaction can function either as a transcriptional repressor or as an activator depending upon cellular context suggesting the possibility of additional cell type-specific interactions at this element (298).

OBJECTIVES

The presence of multiple regulatory elements together with the potential for their cooperative interaction reflects the complexity of c-*fos* regulation. The convergence of signals from different signal transduction pathways leading to the activation of the c-*fos* gene suggests that a number of proteins capable of responding to the different stimuli must preexist in cells. Clearly, regulation of the c-*fos* gene is governed by a number of *cis*-acting sequences in its promoter, however the *trans*-acting factors directing the transcriptional activity from their cognate binding sites have not been fully characterized. In order to coordinate the accurate regulation of the gene responding to multiple signal transduction pathways, it is likely that a set of functional interactions mediated by multiple protein complexes exists at the regulatory *cis*-acting elements.

Therefore, the objective of this study was to identify and characterize proteins and protein complexes interacting with this gene promoter. Characterization of the interactions of transcription factors with each other as well as with different regulatory elements may provide some clue to the mechanism of cooperation between the elements in the regulation of c-*fos* transcription.

While the complete set of downstream events arising from c-*fos* induction is not yet clear, abundant evidence exists documenting its growth regulatory role. Elucidating the mechanisms by which the c-*fos* gene is regulated would assist in the understanding of both normal and neoplastic growth and may, in fact, provide further insight to the fundamental question of how a tumour cell escapes normal growth control.

Thus, the specific objectives of the study were the following:

- characterization of interactions of nuclear proteins from liver cells with the c-*fos* promoter
- characterization of functional properties of DNA/protein complexes and analysis of their behavior during elevated expression of the c-*fos* gene
- demonstration of the functional significance of individual proteins within these complexes and correlation of their properties with the transcription of the gene.

To address these objectives rat liver cells were chosen as the primary experimental model. The rat liver is an essentially quiescent organ, but has maintained its proliferative capacity. Thus, following 67% hepatectomy the remaining hepatocytes will proliferate synchronously in a growth compensatory fashion. This provides an excellent *in vivo* system to study the expression of growth regulatory genes such as c-*fos* in normal proliferating cells. In addition, the rat liver was used for a model of chemically induced carcinogenesis which follows a set of well defined morphological and biochemical stages of neoplasia. This model along with the fully transformed Morris hepatoma 5123tc represented the early and later stages of pathological state allowing examination of c-*fos* promoter binding proteins in preneoplastic and neoplastic liver cells. The following cell systems were employed in this study:

- quiescent rat liver cells
- proliferating cells from regenerating liver following partial hepatectomy
- preneoplastic cells from chemically altered livers in the resistant hepatocyte model
- neoplastic cells from 5123tc transplantable Morris hepatoma.

MATERIALS AND METHODS

Tissue and Cell Sources

Normal liver tissue was obtained from 200-250g male, specific pathogen-free Sprague-Dawley rats bred at the National Research Council of Canada, Ottawa, Canada. Rats were maintained on an *ad libitum* diet of Purina Rat Chow and water and a 12 h light and dark schedule. Partial hepatectomy which involved removal of 68% of the liver mass, was carried out by ligating and excising the median and left lateral lobes (299). Laparotomy was carried out in the same manner except for ligation and excision of the hepatic lobes. Liver tissue was harvested at the appropriate times following euthanasia by cervical dislocation.

The resistant hepatocyte model of Solt and Farber (300), was used to generate hyperplastic hepatic foci and nodules in male Fischer F-344 rats (Charles River Laboratories, Montreal, P.Q.). The rats were maintained on Purina rat chow and water *ad libitum* and used at a body weight of 170-200g. Hyperplastic foci and nodules were initiated by a single i.p. injection of diethylnitrosamine (DENA, Sigma Chemical Co., St. Louis, Mo.), at a dose of 20mg/100g body weight in 0.9% sterile saline. Fourteen days after DENA injection a four day regimen of gavage with 20mg/kg body weight of 2-acetoaminofluorine (2-AAF, Sigma Chemical Co., St. Louis, Mo.) dissolved in DMSO and diluted with propylene glycol to a concentration of 10 mg/mL was administered. Following the last day of 2-AAF administration hepatocyte proliferation was induced by performing 2/3 hepatectomy under halothane anesthesia as described above. At specified intervals, animals were sacrificed by cervical dislocation and livers re-

moved for histological and biochemical analyses.

Hepatomas 5123tc and 5123D were propagated in 300g male Buffalo rats (Harlem Sprague Dawley Co., Indianapolis, IN), by subcutaneous injection into the inguinal region as described (301). The original malignant (metastasized to the lung) 5123 hepatoma was produced in a female Buffalo rat that had been fed the slow acting carcinogen *N*-2-fluorenylphthalamic acid (302). The 5123tc hepatoma is derived from cells that had been passed in tissue culture and the 5123D hepatoma is a slow growing subline of the 5123 hepatoma after the 16th serial transplant generation.

Cells from the Morris Hepatoma 5123tc were placed in cell culture by plating a suspension of cells from the tumour in RPMI 1640 medium + 4 mM Hepes, pH 7.2 (Gibco BRL, Bethesda, MD.), supplemented with 10% (v/v), fetal bovine serum and 20 μg/ml gentamicin. The cells were grown at 37°C in a controlled atmosphere of 7% CO_2. The cells were split 1:18 on a weekly basis and for all experiments the cells were used at 50-70% confluence.

BALB/c 3T3 fibroblasts were obtained from Dr. J. Durkin (National Research Council, Ottawa, Canada) and were maintained in DMEM supplemented with 10% fetal bovine serum.

Synchronization of cultured cells was achieved by serum deprivation for a period of either 48 or 72 h. Following serum deprivation cells were stimulated to re-enter the cell cycle by supplying the cultures with fetal bovine serum to a final concentration of 10% (v/v).

Metabolic Labeling of Cells with ^{35}S-Methionine

Cells were grown in medium containing 10% fetal bovine serum to approximately 70-80% confluence and were then placed in complete medium in the absence of serum for the indicated period of time. 90 min before cells were to be harvested the cells were washed in medium lacking methionine and cysteine (Gibco BRL, Bethesda, MD), and then incubated at 37°C in the same Met/Cys-free medium containing 100 μCi/ml of ^{35}S-Met (Tran^{35}Slabel, 1185 Ci/mmol, ICN, Irvine, CA). After labeling, the cells were washed with PBS and then total cell lysates were prepared by incubation of the cells with 100 μl of RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) on ice for 10 min. After incubation, the lysates were centrifuged at 12,000 rpm in a microfuge and supernatants analyzed by SDS-PAGE followed by autoradiography.

Magnetic Resonance Imaging of Liver Tissue

Images were acquired on a Bruker Biospec 4.7T/30 spectrometer equipped with custom built shielded gradient coils. Rats were placed in an animal holder and kept at 37.5°C. The animals were intubated and anesthetized using 1.25% halothane. The data acquisition was gated to the ventilator which essentially eliminated all motion artifacts. Images were recorded using a standard multi-slice multi-echo sequence with an echo time TE=30 milliseconds and a repetition time T_r=2.0 seconds. The whole liver was imaged by using eight contiguous coronal slices of 2mm thickness. The effect of saturation was insignificant since the sequence was modified to - slice 1,3,5,7-T_r -slice 2,4,6,8-T_r. Total imaging time was approximately 30 min.

Tissue Histology

Slices of the remaining right lateral lobe were placed in Carnoys fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 4-6 hours, paraffin embedded and sectioned. For staining with neutral red, sections were dewaxed, rehydrated in distilled water and stained with a 0.2% solution of neutral red (Aldrich Chemical Co., Milwaukee, Wis.) at pH 5.5 for 10-15 min. For HPS staining, sections were transferred from the Carnoy's fixative to 95% ethanol and further dehydrated by transferring to increasing concentrations of ethanol and toluene. The sections were then paraffin embedded and sectioned to 4 μm thicknesses. Sections were then stained in HPS (hematoxylin dunkel, Chroma, Germany; phloxine B, Fischer Scientific, Canada; alcohol saffron de Gatenais, Chroma, Germany) following standard protocol (303). Sections were then examined by microscopy and photographed.

Nuclei Isolation Procedures

Nuclei from liver tissue were prepared by the low speed centrifugation and Triton X-100 washing method as described by Sikorska et al. (304). Briefly, after removing the liver the tissue was immediately homogenized in ice cold buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 0.25 M sucrose, 0.2 mM PMSF (Sigma Chemical Co., St Louis, MO), 0.2 mM benzamidine (Sigma Chemical Co.. St Louis,MO), in a glass homogenizer fitted with a teflon pestle. The homogenate was strained through sterile gauze and centrifuged at approximately 1,000 x g for 10 min to pellet nuclei. The pellet was gently resuspended in the same buffer and recentrifuged at the same speed. The pellet was then resuspended in the same buffer containing 1% (w/v) Triton X-100 and centrifuged at the same speed. At this point the pellet

appeared white in colour and was washed in the same buffer without Triton X-100 twice. After the final centrifugation, the nuclei appeared intact and free of contaminating cytoplasmic debris by microscopic examination.

Nuclei from cultured cells were prepared following the method of Filipski et al. (305). Cells were washed in ice cold phosphate buffered saline and harvested by gentle scraping with a rubber policeman. The cells were then centrifuged at 1,000 x g for 2 min and the cell pellet was resuspended at 10^6 cells/ 10 ml in a buffer consisting of 2 mM KH_2PO_4,/ KOH, pH 6.55, 1 mM EGTA, 0.1 mM DTT, 5 mM $MgCl_2$, 0.2 mM PMSF, 0.2 mM benzamidine, 0.15 M NaCl. The cells were then centrifuged at the same speed described above and the pellet resuspended in the same buffer containing 0.3% (w/v) Triton X-100 and left on ice for 10 min. The cells were centrifuged at the same speed and then the nuclear pellet washed twice with the same buffer without Triton X-100. After isolation the nuclei were further processed immediately and were never stored for future use.

Preparation of Nuclear Proteins for *in vitro* Transcription

Livers from control rats or from rats subjected to partial hepatectomy were used to prepare nuclear proteins for analysis by *in vitro* transcription as previously described (306). Briefly, livers were homogenized in a buffer consisting of 10 mM HEPES, pH 7.6, 12 mM KCl, 0.15 mM spermidine(Sigma Chemical Co., St Louis, MO), 0.5 mM spermine (Sigma Chemical Co., St Louis, MO), 1 mM EDTA, 2.0 M sucrose, 5% glycerol, 1% non-fat milk, 0.5 mM DTT, 0.1 mM PMSF, 1% aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzamidine (all protease inhibitors were from Sigma Chemical Co., St Louis, MO). Nuclei were then purified by centrifugation on a

sucrose cushion consisting of homogenization buffer without milk. Purified nuclei were then lysed in a buffer consisting of 10 mM HEPES, pH 7.6, 0.55 M KCl, 0.1 mM EDTA, 10% glycerol, 3 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 1% aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A. Nuclear proteins were further purified by precipitation with 0.3 g/ml of solid $(NH)_2SO_4$ and then the precipitated proteins were dialyzed against 25 mM HEPES, pH 7.6, 0.1 mM EDTA, 40 mM KCl, 1 mM DTT. After dialysis, the extract was centrifuged for 10 min at 12,000 X g
in a microfuge to remove any precipitate, an the extract was then aliquoted and stored at -80°C.

Extraction of Nuclear Proteins

Isolated nuclei were extracted with 3 volumes of ice cold 25 mM HEPES, pH 7.9 containing 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.2 mM PMSF, 0.2 mM benzamidine, 0.5 mM DTT, 0.42 M NaCl as described by Dignam et al. (307). The nuclei were suspended in the extraction buffer by pipetting up and down several times using a manual pipette and then incubated in ice for 20-30 min with occasional mixing. Extracts were centrifuged at 100,000 x g for 15 min. and the supernatants were stored in aliquots at -80°C.

Phosphorylation and Dephosphorylation of Nuclear Proteins

The phosphorylation of nuclear proteins by endogenous kinases present in the extracts by incubating nuclear proteins at 30°C for 30 min in the presence of 100 μM ATP and 5 mM $MgCl_2$ in a buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.2 mM PMSF, 0.2 mM benzamidine. In certain experiments nuclear proteins were phosphorylated by incubation in the same buffer conditions at 30°C for 15 min includ-

ing the addition of 1 U/ μg protein of catalytic subunits of cAMP-dependent protein kinase (Sigma Chemical Co., St. Louis, MO). Some experiments included the addition of 1 μCi/ 10 μl reaction volume of gamma ^{32}P-labeled ATP (3000 Ci/ mmol, NEN Dupont , Boston MA). Nuclear proteins were dephosphorylated using calf intestinal alkaline phosphatase immobilized on agarose beads (Sigma Chemical CO., St. Louis, MO). 100 μg of nuclear proteins was incubated at 37°C for 30 min in the presence of 10 mM Tris-HCl, pH 8.0, 1.0 mM $MgCl_2$, 1.0 mM $ZnCl_2$ and 2 U/ 10 μg protein of alkaline phosphatase. After incubation was complete, the enzyme was removed from the reaction mixture by centrifugation at 12,000 x g for 2 min in a micocentrifuge.

Sequence-Specific DNA Affinity Chromatography

The c-*fos* SRE and CRE affinity columns were prepared essentially as described by Kadonaga and Tjian (308). Briefly, 500 μg of each oligonucleotide was annealed and 5' phosphorylated using gamma labeled ATP (3000 Ci/mmol, NEN, Boston MA) and 10 U T4 kinase (Pharmacia, Uppsala, Sweden) in buffer supplied by the manufacturer. The labeled oligonucleotides were then ligated using T4 ligase (Gibco BRL, Bethesda, MD), and coupled to Sepharose 6MB resin (Pharmacia Biotech, 6 ml of settled volume), with a coupling efficiency of 60-75% as determined by subtracting the scintillation counts in the supernatant following coupling from the total counts added to the resin. Nuclear protein extracts (prepared as described above), were diluted with buffer A (20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 0.1% (v/v) Nonidet P-40) to a final salt concentration of 100 mM NaCl and applied directly to the column under gravity flow. Approximately 20 mg of protein was applied to the column each time. The column was

washed with buffer A containing 100 to 250 mM KCl and the bound proteins were eluted with the same buffer containing 2 M KCl. The eluates were then diluted with buffer A to a final concentration of 100 mM KCl and re-applied to the affinity column eluting the bound proteins as before to achieve two passages through the column. The final eluates were extensively dialyzed against distilled water at 4°C, freeze dried and analyzed on 10% SDS-polyacrylamide gel according to Laemmli (309).

Southwestern Blotting

Southwestern blots were performed as described by Silva et al. (310). Briefly, 100-150 μg of nuclear proteins was separated by either 8.5% or 10% SDS-PAGE and then incubated for 2.5 h at room temperature with gentle agitation in renaturing buffer consisting of 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.1 mM DTT, 4 M urea. Following renaturation, the proteins were electrotransferred at 100 mA/ 16 h onto nitrocellulose membranes (Hybond C, Amersham, Oakville, Ont.). Filters were blocked for 1 h at room temperature with 5% (w/v), non-fat milk powder in 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT and incubated for 3 h at room temperature with 0.2-0.5 μg of 3' end-labeled oligonucleotide probe (10^5 cpm/ng), and 200 μg of poly(dI-dC)-poly(dI-dC),(Pharmacia), in 20 ml of blocking buffer. After incubation, the filters were washed twice for 10 min in blocking buffer without NaCl followed by a 1 min rinse in blocking buffer without milk powder and NaCl. The filters were then air dried and autoradiographed on Kodak X-OMAT AR X-ray film. Where noted, the Southwestern blotting procedure was performed exactly as described above with the exception of the omission of the renaturation step and was directly electrotransferred after SDS-PAGE.

Gel Shift Assay

The gel shift assay was performed as described by Garner and Revzin (311) and Fried and Crothers (312). Briefly, 5 to 10 μg of nuclear proteins was pre-incubated for 10 min at room temperature in the presence of 2 μg of poly(dI-dC)-poly(dI-dC) in a buffer composed of 10 mM Tris-HCl, pH 7.6, 0.2 mM EDTA, 5% (v/v) glycerol, 2 mM $MgCl_2$, 1 mM DTT, 60 mM NaCl. Then 0.1 to 0.5 ng of ^{32}P-labeled probe was added (10-15 x 10^5 cpm), and incubation continued for a further 20 min at room temperature. DNA-protein complexes were separated by electrophoresis on a 5% polyacrylamide gel in either 6.7 mM Tris-HCl, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA or 10 mM Tris-HCl, pH 8.5, 60 mM glycine, 0.2 mM EDTA. The gels were dried on a slab gel drier and the locations of the complexes were visualized by autoradiography on Kodak XOMAT AR X-ray film. For competition using the gel shift assay, complexes were allowed to form for the first 10 min of the binding reaction and then the appropriate molar quantity of competitor was added and incubation continued for an additional 10 min.

Protein Extraction from Shifted Complexes

After the gel shift assay the wet gels were wrapped in plastic wrap and autoradiographed at 4°C overnight to locate the DNA-protein complexes. The shifted bands were excised from the gel with a razor blade and extracted overnight at 37°C in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% SDS, 0.2 mM PMSF, 0.2 mM benzamidine. Control gels were run in the absence of radiolabeled probe and the corresponding region of the gel was excised and extracted. Extracted proteins were precipitated from the supernatant with 4 volumes of ice cold acetone and collected by centrifugation at

3,000 x g for 10 min. The proteins extracted from 20 individual shifted complexes were combined, resuspended in SDS-PAGE loading buffer, re-run on a 10% SDS polyacrylamide gel and silver stained according to the manufacturers procedure (Bio-Rad Laboratories, Richmond CA.).

Western Blotting

Western blotting was performed as described (313). Antisera used to probe the Western blots consisted of polyclonal sera raised against synthetic peptide antigens described above. Antibodies against the CREB protein were a generous gift from Dr. Marc Montminy (The Salk Institute, La Jolla, CA). Briefly, 100-200 μg of protein was resolved by either 8.5% or 10% SDS-PAGE and then electrotransferred at 100 mA for 16 h in transfer buffer consisting of 88 mM Tris-HCl, pH 8.3, 192 mM glycine onto a nitrocellulose membrane (Hybond C, Amersham, Oakville, Ont.). After transfer, the membrane was blocked in a buffer containing 5 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5% (w/v) BSA (Fraction V, Sigma Chemical Co., St. Louis, MO) at room temperature for 30 min. After blocking the antiserum was added to the same buffer at the appropriate dilution and incubation continued for a further 90 min followed by a series of wash steps. Blots were first washed in the same buffer without BSA at room temperature for 10 min and then washed twice for 20 min each time in the same buffer without BSA and containing 0.05% (w/v) NP40 and 0.1% (w/v) Tween 20. The blots were then washed for 10 min in the same buffer without BSA or detergents. Immune complexes were detected by incubating the blots in the same buffer, without BSA or detergents, and 1 μCi/ml of ^{125}I-labeled Protein A (NEN Dupont, Boston, MA) with incubation at room temperature for 30 min. The blots were then

washed as above and complexes detected by autoradiography as described.

Immunoprecipitation

100-200 μg of nuclear proteins, prepared as described above, diluted in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) to a final volume of 1 ml. 100 μl of a 50% suspension of protein A sepharose beads (Sigma Chemical Co., St Louis, MO) washed in RIPA buffer were added to the protein extract and incubated at 4°C for 1 h with gentle rocking. Protein A sepharose beads were then removed by centrifugation at 12,000 rpm for 30 seconds. The supernatant was collected and then incubated at 4 °C overnight with the appropriate antibody at a concentration of 10 μl of antiserum diluted in a total volume of 1 ml. After incubation with antibodies, 100 μl of a 50 % suspension of protein A sepharose beads were added to the mixture and incubation was continued at the same temperature for a further 1 h. The beads were collected by centrifugation and then washed 4 times with RIPA buffer with centrifugation of the beads and aspiration of the supernatant between each wash. After the final wash, the beads were boiled for 5 min in SDS-PAGE sample buffer and the eluted proteins collected by centrifugation for 5 min at 12,000 rpm. Supernatants were collected and then analyzed by electrophoreseis on SDS-PAGE.

Preparation of Cellular RNA

All solutions used in the preparation of RNA were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC), overnight and then autoclaved. Buffers containing Tris-HCl, however, were prepared in DEPC-treated and autoclaved distilled water and then reautoclaved. All glassware and plastic pipette tips were autoclaved before use and

gloves were worn at all times when handling RNA. Liver sections frozen in liquid nitrogen at the time of sacrifice were used to prepare total cellular RNA by the method of Chirgwin et al. and included purification through a CsCl gradient (314). Poly(A)+ RNA was prepared using a PolyATract mRNA system IV (Promega Corp., Madison, WI). RNA was quantitated by absorbance at 260 nm based on a 40 μg/ml solution of RNA giving an absorbance at 260 nm of 1.0. Preparation of cellular RNA from cultured cells was performed as described (315). Briefly, cells were washed with ice cold phosphate buffered saline followed by gentle scraping of the cells off the flask in the same buffer. The cells were collected by centrifugation at 12,000 x g for 10 seconds 4°C and the plasma membrane was lysed by the addition of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5% (v/v) NP40 on ice for 10 min. The RNA-containing supernatant was then mixed with an equal volume of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5.0 mM EDTA, 0.2% (w/v) SDS and extracted twice with phenol:chloroform:isoamyl alcohol (50:48:2). After extraction, the RNA was precipitated by the addition of 1/20 volume of 3 M NaCl and 2 volumes of 95% ethanol overnight at -20°C. Polyadenylated RNA was selected for exactly as described above.

Northern Blotting

The RNA was then separated on a 1.2% agarose/1.0 M formaldehyde gel and transferred onto a Nytran nylon membrane (Schleicher and Schuell, Keene, NH.) by capillary action overnight in 10 X SSC (1 X SSC; 150 mM NaCl, 15 mM Na_3citrate.$2H_2O$, pH 7.0 with 1 M HCl). After transfer was complete the RNA was fixed onto the membrane by uv crosslinking with a Stratalinker 2400 (Stratagene, La Jolla, CA.). Hybridization to radiolabeled probes was done in 0.5 M $NaHPO_4$ / 1 mM

EDTA / 1% BSA / 5% SDS / 50% formamide at 42°C for 18-24 h. After hybridization blots were washed sequentially in 2X SSC / 0.1% SDS / r.t.; 0.2X SSC / 0.1% SDS / r.t.; 0.2X SSC / 0.1% SDS / 65°C. Blots were then wrapped in plastic wrap and autoradiographed at -80°C for the indicated length of time using Kodak XOMAT AR X-ray film.

Preparation of cDNA probes

The appropriate restriction fragment derived from cDNA clones harboring the gene of interest was isolated and quantitated by fluorometry (Hoefer Scientific Instruments, San Francisco, CA.) using the DNA-specific dye Hoechst 33258 (Sigma Chemical Co., St. Louis, MO.). Approximately 50-100 ng of the appropriate restriction fragment was radiolabeled using [α-^{32}P]dATP and [α-^{32}P]dGTP (800 Ci/mmol, Dupont NEN, Boston, MA) and the Multiprime DNA labeling system (Amersham Canada, Oakville, Ont.). DNA probes were routinely labeled to a specific activity of 10^9 cpm/ μg DNA. The mouse cDNA clone for c-*fos* was a generous gift from Dr. Andrew Lassar (Harvard University, Cambridge, MA). The cDNA clone for rat GST-P was a generous gift from Dr. Brian Knoll (VA Medical Center, Houston, TX). The rat cDNA clone for CREB was a generous gift from Dr. Marc Montminy (The Salk Institute, La Jolla, CA), and the human cDNA clone for α-tubulin was a generous gift from Dr. N. Cowan (New York University, N.Y., NY).

Preparation of Oligonucleotide Probes

Complementary oligonucleotides containing *Bam*H1 linkers were synthesized by the phosphorimidate method on an Applied Biosystems 380 synthesizer at the Biotechnology Research Institute (BRI, Montreal, Canada). The synthetic oligonucleotides

used in these studies are listed in Table 1 as well as their positions relative to the cap site. The oligonucleotides were used as probes or competitors in the gel shift assay and in Southwestern blots, or for binding sites in DNA sequence-specific affinity chromatography. For probes, oligonucleotides were 3'-labeled using [α-^{32}P]dATP and [α-^{32}P]dGTP (800 Ci/mmol, Dupont NEN, Boston, MA), and the Klenow fragment of DNA polymerase I (Pharmacia Biotech, Montreal, P.Q.). The labeled probes were then purified by gel filtration chromatography on NICK columns (Pharmacia Biotech), equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. The labeled probe was then precipitated in 95% ethanol at -20°C. The specific activity of the probes was approximately 10^5 cpm/ng DNA.

Preparation of Plasmid Templates for *in vitro* transcription

Templates for *in vitro* transcription were constructed by ligating oligonucleotides representing c-*fos* regulatory elements into the pBSTATA-G-free vector consisting of the albumin TATA sequences upstream of a 390 nucleotide cassette devoid of guanine nucleotides (a generous gift from Dr. C. Mueller, Queen's University, Kingston, Ont.). The vector was linearized by digestion with Bam H1 and then incubated with 5 units of calf intestinal alkaline phosphatase at 37°C for 30 min in a buffer consisting of 10 mM Tris, pH 8.3, 1 mM $MgCl_2$, 1 mM $ZnCl_2$. Following dephosphorylation the vector was incubated with 100 μg/ml of proteinase K in 5 mM EDTA and 0.5% SDS at 56°C for 30 min and then purified by extraction with phenol/chloroform and then ethanol precipitated. 1 μg of oligonucleotides consisting of the c-*fos* SRE, CRE and SCMRE (see Table 1), were prepared for ligation into the vector by 5' phosphorylation using 10U of T4 DNA kinase in the presence of 10 mM Tris, pH 7.5, 1 mM $MgCl_2$,

0.1 mM ATP at 37°C for 30 min. Phosphorylated oligonucleotides were purified by extraction with phenol/chloroform and then ethanol precipitation. Ligation reactions were performed using 0.1 μg of dephosphorylated vector and 1 μg of phosphorylated oligonucleotides in 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 0.5 mM ATP, 50 μg/ml BSA and 1U T4 ligase (Gibco, BPL, Bethesda, MD.).

Ligations were carried out at 16°C for 16 h and plasmid constructs were then used to transform competent HB101 E. coli cells (Gibco BRL, Bethesda, MD), by the $CaCl_2$-mediated transformation (315). Transformed cells were selected for on LB/agar plates containing 50 μg/ml ampicillin and single colonies were picked and grown in LB medium containing ampicillin. Mini-preparations of plasmids were purified by cell lysis in 10 mM Tris, pH 8.0, 1 mM EDTA, 0.1 N NaOH, 0.5% SDS followed by plasmid solubilization in 3 M sodium acetate, pH 5.2. Plasmid preparations were then precipitated with ethanol and, following collection by centrifugation, resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA.

Southern Blot Analysis

Plasmid constructs were confirmed by Southern blotting and hybridization with radiolabeled oligonucleotide probes representing the inserted sequences. Approximately 1 μg of plasmid purified from an isolated clone was separated on a 1% agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA). The DNA was then transferred onto a nylon membrane (Hybond N, Amersham, Oakville, Ont.) in 0.5 M NaOH, 0.4 M NaCl. After transfer was complete, the DNA was fixed to the membrane by uv crosslinking with a Stratalinker 2400 (Stratagene, La Jolla, CA.). The membrane was then hybridized to a radiolabelled oligonucleotide probe (see above for

labeling protocol), in 50% formamide, 0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 1.0% SDS, 10% dextran sulphate, 200 μg/ml denatured salmon sperm DNA at 42°C overnight. Following hybridization the blot was washed in series with 5 X SSC, 0.5% SDS, 65°C, 5 min; 0.1 X SSC, 1.0% SDS, 50°C, 20 min; 2 X SSC, room temperature, 5 min. The hybridized probe was detected by autoradiography. For re-probing, the blot was boiled for 2-3 min in distilled water containing 0.05% SDS. The blot was then exposed to X-ray film overnight to ensure complete removal of hybridized probe before hybridizing with a different probe.

***In vitro* Transcription**

Transcription reactions were performed as previously described (306). Briefly, nuclear proteins were incubated with 800 ng of template DNA and 400 ng of reference plasmid DNA in a buffer containing 10 mM HEPES, pH 7.6, 3% glycerol, 25 mM KCl, 6 mM $MgCl_2$, 0.6 mM CTP, 0.6 mM ATP, 0.04 mM UTP, 0.2 mM O-methyl GTP (Pharmacia, Uppsala, Sweden), 10 μCi α-^{32}P-UTP (800 Ci/mmol), 7U RNasin (Promega, Madison, WI) at 37°C for 30 min. Transcripts were purified by phenol/choloroform extraction and ethanol precipitation. Transcripts were analyzed by separation on a 4% acrylamide gel containing 6 M urea followed by autoradiography.

Preparation of Synthetic Peptides

The amino acid sequences of CREB and SRF, were scanned for likely antigenic sites using the University of Wisconsin Genetic Computer Group software package and the algorithm of Jameson and Wolf (316). Computer searches and antigenic determinations were performed by Dr. P.R. Walker (National Research Council, Ottawa, Cana-

da). The peptides representing amino acids 134-150 of CREB and 141-154 of SRF were synthesized by the simultaneous multiple peptide synthesis method (317). Briefly, the protected peptide resins were synthesized using a p-methyl-benzhydrylamine resin (100-200 mesh, 0.4 - 0.8 meq/g) and N-α-tertiarybutoxycarbonyl (t-boc) amino acids (Bachem Inc., Torrance, CA) by the method of simultaneous multiple peptide synthesis developed by Houghten (317). The peptides were cleaved off the resin by the conventional hydrogen fluoride/anisole procedure (318). The purity of the crude peptides were assessed by chromatography on FPLC reversed phase PepRPC HR5 column (Pharmacia, Uppsala, Sweden). The chromatograms were developed with a gradient of 0.1% CF_3COOH/H_20 and 0.1% CF_3COOH/CH_3CN. The peptides represented approximately 85% of OD_{214} absorbing material. After concentrating the peptides by freeze drying they were used directly as immunogens.

Production of Antibodies

New Zealand white rabbits were immunized with unconjugated synthetic peptides according to the following schedule. The first intramuscular injection of 0.5 mg peptide emulsified with complete Freund's adjuvant (1:1 v/v), was followed by two consecutive subcutaneous injections of peptides emulsified with incomplete adjuvant at one week intervals. The rabbits were test bled at 7 and 14 days after the last vaccination and the titer of their sera were determined by enzyme-linked immunosorbent assay as described (319).

Other Methods

Cell cycle analyses were performed on an Ortho Diagnostic Systems 2150 cytofluorograph equipped with a helium/neon laser and using propidium iodide as the fluorescent DNA probe.

SDS-PAGE was performed according to the method of Laemmli (309), and two-dimensional gel electrophoresis was performed according to the method of O'Farrell (320), using ampholines with a pH range of 4-8 (Pharmacia, Uppsala, Sweden), for isoelectric focussing.

RESULTS

Part 1: Identification and characterization of c-*fos* promoter-binding proteins from normal rat liver

Identification of Protein Complexes Interacting with c-fos Promoter Sequences

The ability of nuclear transcription factors to interact with DNA in a site-specific manner defines the mechanisms by which genetic regulation functions. Therefore, these studies were designed to identify and characterize nuclear proteins interacting with regulatory sequences within the c-*fos* promoter. Table 1 lists all of the oligonucleotides used for these studies. The oligonucleotides were synthesized with *Bam* HI linkers to facilitate their polymerization for use in the generation of sequence-specific DNA affinity columns. The linkers also provided a convenient site for the generation of the TATA G-free cassettes used for *in vitro* transcription and for the radiolabelling of the oligonucleotides using DNA polymerase. The gel shift assay has been developed to demonstrate DNA-binding activity present in nuclear protein extracts (311, 312). Nuclear proteins were prepared from normal rat liver tissue and used in the gel shift assay to detect proteins capable of interacting with c-*fos* regulatory sequences. Figure 2B showed that these extracts contained proteins that were able to interact with radiolabeled oligonucleotide sequences representing the c-*fos* *sis*-conditioned medium response (SCMRE), serum response (SRE), and cAMP response (CRE) elements. The electrophoretic mobility of each of these radiolabeled promoter elements in the non-denaturing gel was altered indicating protein/DNA interactions. Each of these elements generated multiple bands suggesting the existence of different

Table 1. List of Synthetic Oligonucleotides*

1. 5' gatcCCGCCCAGTGACGTAGGA 3'	c-*fos* CRE (-74 to -57, 226)
2. 5' gatcCAGTTCCCGTCAATCC 3'	c-*fos* SCMRE (-351 to -336, 228)
3. 5' gatcCAGGATATCCATATTAGGACATCT 3'	c-*fos* SRE (-322 to -299, 232)
4. 5' gatcCAGGATGTCCAT*CGGCT*GACATCT 3'	c-*fos* dSRE1 (253)
5. 5' gatcCAGGATGT*GGAT**CGGCTC*ACATCT 3'	c-*fos* dSRE2 (253)
6. 5' gatcTTTTGGTTACAAACTGTTCTTAAAA 3'	MMTV GRE (280)
7. 5' gatcGCGCCTCCTTGGCTGACGTCAGAG 3'	24 bp somatostatin CRE (-60 to -37, 146)
8. 5' gatcCTCCTTGGCTGACGTCAGAG 3'	20bp somatostatin CRE (-56 to -37)
9. 5' gatcTTGGCTGACGTCAGAG 3'	16bp somatostatin CRE (-52 to -37)
10. 5' gatcCATGACTCAGAGGAAAACATACG 3'	aP2 TRE (-121 to -99, 106)

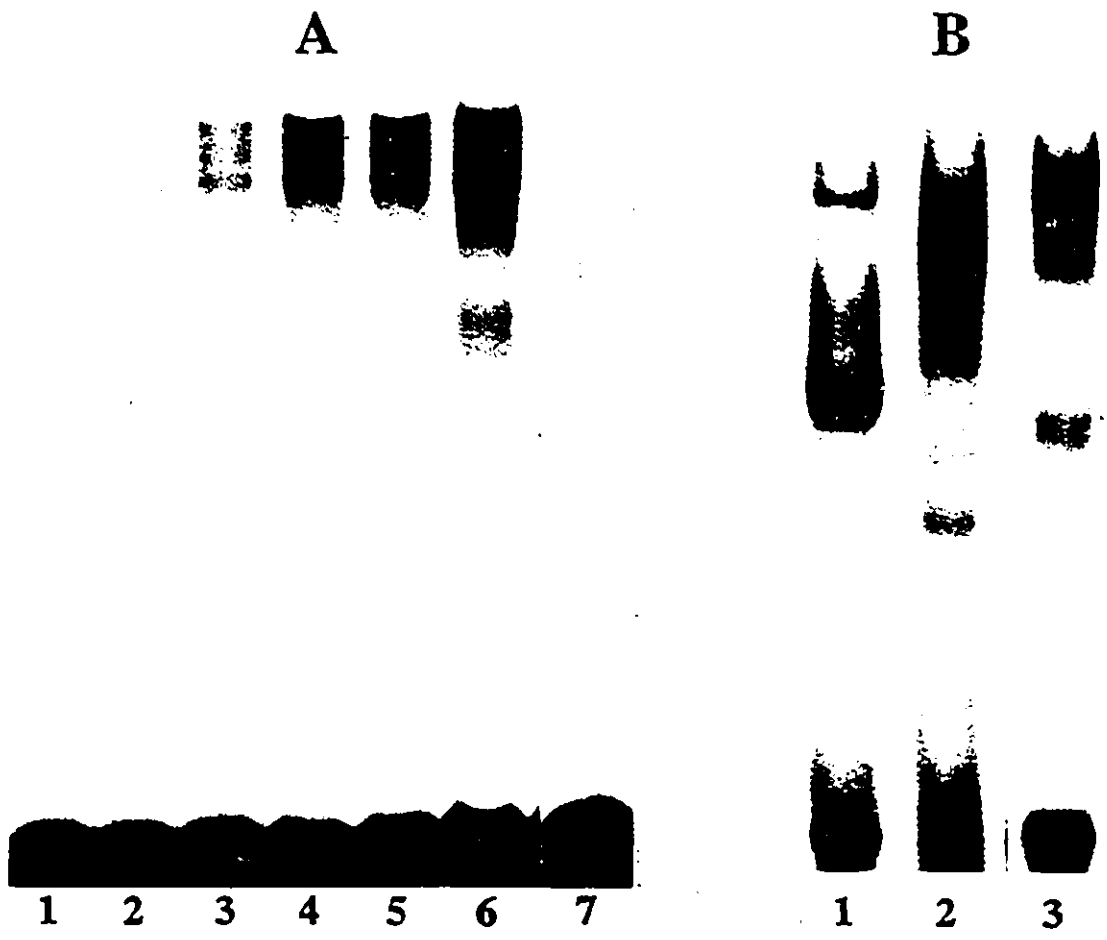
* The table shows one of the complimentary strands

Figure 2. Interaction of nuclear proteins from normal rat liver with c-*fos* promoter regulatory sequences.

Panel A: Gel shift assay with the c-*fos* cAMP response element (CRE, Table 1, sequence 1). Nuclear protein extracts were prepared from normal rat liver and incubated with 0.5 ng of ^{32}P-labeled c-*fos* CRE probe in the presence of 2 μg of poly(dI-dC) under conditions described in the Methods section.
Lane 1 - free CRE probe, lanes 2 to 6 - CRE-binding proteins from 2, 4, 6, 8, and 10 μg of nuclear proteins from normal rat liver, lane 7 - 10 μg of nuclear proteins from normal rat liver were preincubated for 10 min at 25° C with 10 μg of proteinase K before addition of the CRE probe.

Panel B: Gel shift assay with individual c-*fos* promoter regulatory elements. 15 μg of nuclear proteins from normal rat liver and 0.5 ng of appropriate ^{32}P-labeled oligonucleode probes in the presence of 2 μg poly(dI-dC) were used in these assays.
Lane 1 - *sis*-conditioned medium response element-binding proteins from normal rat liver (SCMRE, Table 1 sequence 2), lane 2 - serum response element-binding proteins from normal rat liver (SRE, Table 1 sequence 3), lane 3 - cAMP response element-binding proteins from normal rat liver (CRE, Table 1 sequence 1).

DNA-protein complexes were resolved on 5% non-denaturing polyacrylamide gels (acrylamide:bis, 29:1). Gels were dried and complexes visualized by autoradiography on Kodak XAR-5 X-ray film. The autoradiogram was exposed overnight.


A
B
1 2 3 4 5 6 7
1 2 3

protein/DNA and possibly protein/protein interactions. It was also evident that the individual regulatory elements were capable of interacting with distinct, but not necessarily unrelated, protein factors since the patterns of shifted complexes were different for individual oligonucleotide probes (Fig. 2B). The intensity of shifted bands increased with increasing amounts of nuclear proteins added (Fig. 2A, lanes 2-6). It was established that the optimal protein concentration to be used in these assays should be in range of 10-15 μg/assay. In order to prove that these complexes were indeed the result of protein/DNA interactions and not due to other components of the extract the samples were preincubated with proteinase K (Fig. 2A, lane 7). This treatment completely destroyed DNA-binding activity indicating that the shifted complexes were the result of DNA/protein interactions. For each assay the final concentration of NaCl was adjusted to 60 mM by dilution of the nuclear protein extract in the extraction buffer such that an equivalent volume of extract was added. Therefore, the results obtained with nuclear proteins from normal rat liver tissue confirmed the observations of Gilman et al. seen in extracts from cultured cell lines (221). Since the DNA sequences used in the gel shift assay represented c-*fos* regulatory elements involved in the induction of the gene (62, 221), these results also indicated that the factors responsible for c-*fos* induction preexist in quiescent, non-proliferating rat liver tissue accounting for, at least in part, the ability of the gene to respond within minutes of stimulation even in the presence of inhibitors of protein synthesis (55).

Specificity of Protein Complexes

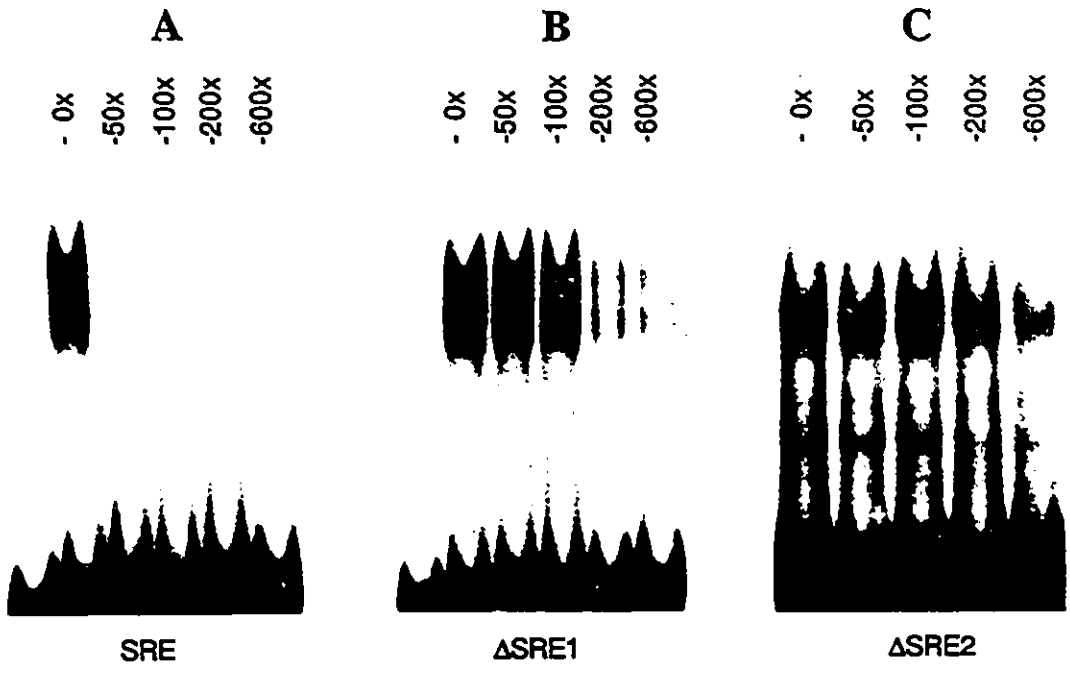
Despite the presence of the non-specific competitor poly(dI-dC)-poly(dI-dC) in the gel shift assay, it was necessary to confirm that protein complexes binding to

Figure 3. Specificity of SRE-protein interactions

Panel A: Gel shift assay with the c-*fos* SRE sequence. 10 μg of nuclear proteins from normal rat liver were incubated with 0.5 ng of ^{32}P-labeled SRE probe in the presence of 2 μg of poly(dI-dC) and the SRE-protein complexes were competed with 0-600 fold molar excess of unlabeled homologous SRE oligonucleotides.

Panel B: Gel shift assay with the c-*fos* SRE sequence. 10 μg of nuclear pro teins from normal rat liver were incubated with 0.5 ng of ^{32}P-labeled SRE probe and a 0-600 fold molar excess of the mutated unlabeled mutant (SRE1, Table 1, sequence 4).

Panel C: Gel shift assay with the c-*fos* SRE sequence. Competitions were performed as described in panels A and B, but with a 0-600 fold molar excess of the unlabeled second SRE mutant (SRE2, Table 1, sequence 5).


A
B
C
- 0x
-50x
-100x
-200x
-600x
SRE
ΔSRE1
ΔSRE2

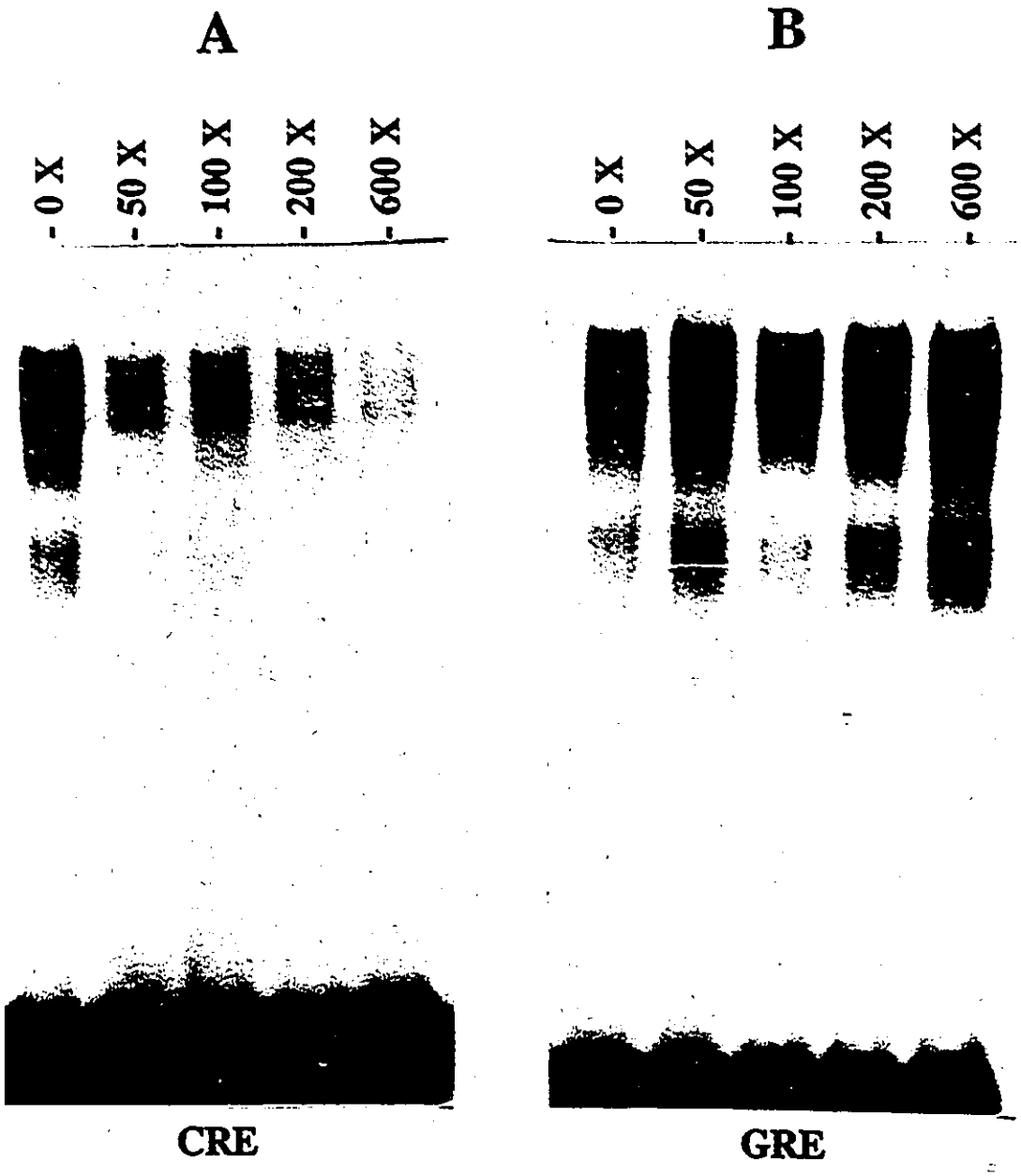
the regulatory elements were specific for that sequence. To demonstrate such a specificity, a gel shift competition analysis was performed. Complexes were first allowed to form in solution and then an increasing molar excess of non-radiolabeled competitor DNA was added. Figure 3A showed the results from the competition analysis for complex formation at the c-*fos* SRE. The addition of the unlabeled homologous sequences was able to completely abolish complex formation at a 50 fold molar excess indicating that the SRE/protein complexes were specific. It is important to note that complexes were allowed to preform and then competitor was added since addition of the probe together with unlabeled homologous competitor results in a reduction of its specific activity. Further analysis of complex specificity was undertaken by introducing two different mutations in the SRE sequence. The altered SRE sequences are shown in Table 1 and represent mutations known to interfere with protein/SRE interactions (250,254) and, therefore they should not disrupt specific complex formation. A mutation encompassing the 5' portion of the core SRE sequence was ineffective until approximately 200 fold molar excess of dSRE1 sequence was added (Fig. 3B). Further alterations in the SRE consisting of mutations at both ends of the core SRE (dSRE2), were completely ineffective even at a 600 fold molar excess (Fig. 3C). Significantly, the mutations which define the dSRE2 consist of the bases known to be critical for the binding of protein (250,254). These results demonstrated that proteins present in extracts from normal rat liver were capable of specific interactions with the c-*fos* SRE.

Similar experiments were done to assess the specificity of complexes at the other regulatory elements. Figure 4A showed that complex formation at the c-*fos* CRE with nuclear proteins from normal rat liver was also specific since an increasing molar

Figure 4. Specificity of CRE-protein interaction

Panel A: Gel shift assay with the c-*fos* CRE sequence. 10 μg of nuclear proteins from normal rat liver were incubated with 0.5 ng of ^{32}P-labeled c-*fos* CRE probe in the presence of 2 μg of poly(dI-dC) and 0 - 600 fold molar excess of unlabeled homologous CRE oligonucleotides.

Panel B: Gel shift assay with the c-*fos* CRE sequence. CRE complexes were competed as described in panel A, but with 0 - 600 fold molar excess of unlabeled non-homologous oligonucleotides representing the MMTV-GRE (Table 1, sequence 6).


A
B
- 0 X
- 50 X
- 100 X
- 200 X
- 600 X
- 0 X
- 50 X
- 100 X
- 200 X
- 600 X
CRE
GRE

quantity, (10-600 fold molar excess), of unlabeled c-*fos* CRE was able to effectively compete out the complex formation. Fig. 4B showed that competition for protein/CRE complexes was ineffective when the nonhomologous oligonucleotides representing the glucocorticoid response element (GRE, Table 1) were used as competitor. The results presented in Figures 3 and 4 validate the use of the gel shift assay to study specific interactions of nuclear proteins with DNA regulatory sequences.

Purification of Proteins Interacting with the c-*fos* SRE by Oligonucleotide Affinity Chromatography

While the gel shift assay is a useful and sensitive technique to study DNA/protein interaction, it does not provide information about the identity of the polypeptide(s) taking part in these interactions. To identify the protein(s) responsible for the SRE gel shift, sequence-specific SRE affinity chromatography was used. Affinity columns were prepared and nuclear proteins extracts were passed twice through the affinity column to purify proteins binding to the sequence. The procedure was first described by Kadonaga and Tjian (308) and is detailed in the Methods section. Figure 5 shows the purification of SRE-binding proteins from normal rat liver nuclear extract. When the protein extract was loaded onto the column in a high ionic strength buffer (250 mM KCl), only a 67 kDa protein was retained on the column and was eluted with 2.0 M KCl as shown by SDS-PAGE and Coomassie blue staining (lane 3). The effectiveness of this method of purification can be seen by comparing the total nuclear protein extract before chromatography (lane 2), with the column purified material in lane 3. The purification of a 67 kDa protein was consistent with the high affinity DNA-binding properties of SRF which was purified from HeLa cells by a similar technique and which has a

similar molecular weight by SDS-PAGE (235,236).

Although changes in c-*fos* gene expression have been observed *in vivo* under a variety of circumstances, the exact mechanism of the induction is not clear. For example, it has been shown that the transcription of c-*fos* is rapidly and transiently induced in regenerating liver 10-120 minutes after partial hepatectomy (321-323). A several fold increase in the level of c-*fos* mRNA was also observed during the development of chemically induced rat Hepatomas (324). To determine whether the changes in c-*fos* gene expression in these cellular systems result from changes in SRE-binding proteins nuclear protein extracts were obtained from both intact and proliferating rat liver tissue as well as from tumour tissue of Morris Hepatomas and analyzed for SRE binding factors using SRE affinity chromatography. When these nuclear extracts were loaded onto the column in a lower ionic strength buffer (100 mM KCl), the eluted material consisted of three proteins with molecular weights of 67, 62 and 45 kDa (Fig. 5B). The same proteins were retained by the column from nuclear extracts of quiescent and proliferating rat liver (lanes 1 and 2), as well as from Morris Hepatoma 5123tc (lane 3). A trace amount of a 72 kDa protein copurified from tumour extracts (lane 3). These results demonstrated that while the 67 kDa protein had the nighest apparent affinity for the SRE sequence, other proteins present in the extract were also able to interact with the SRE either through direct binding or by protein/protein interactions and could be identified by affinity chromatography.

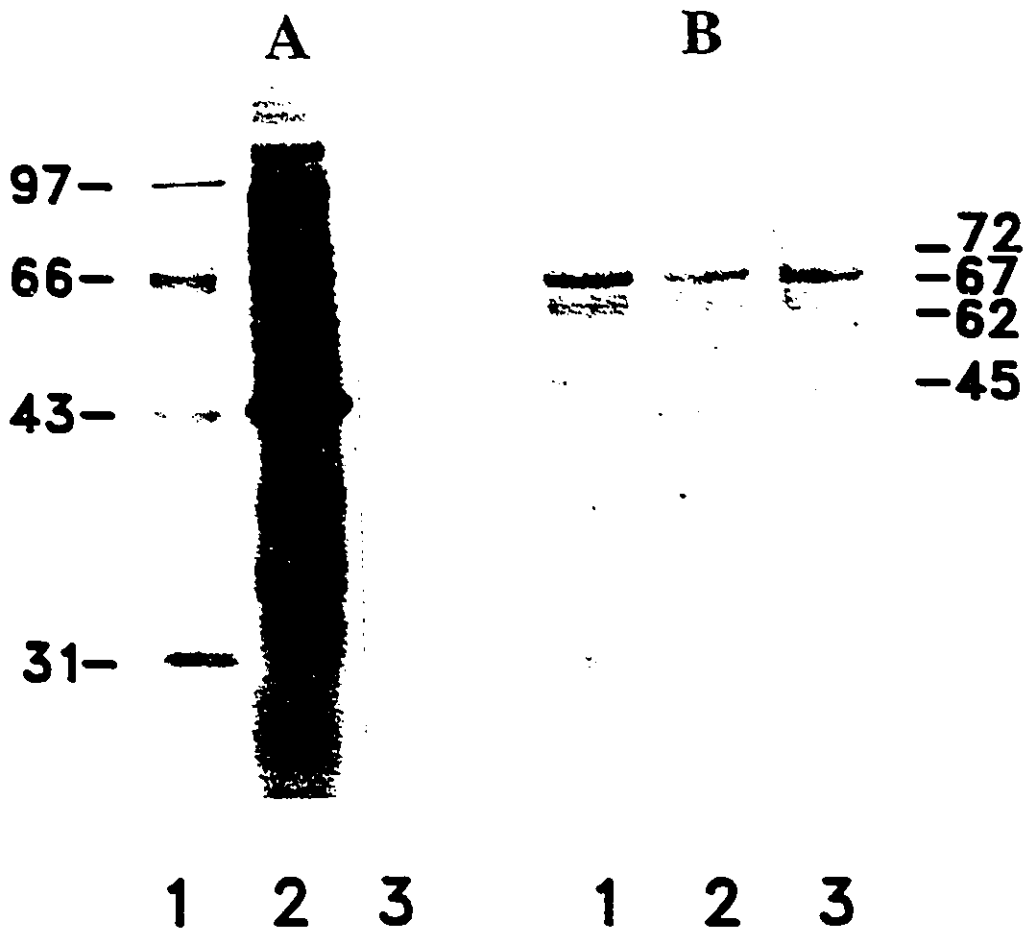
Identification of Components in the SRE/protein Complexes

Sequence-specific affinity chromatography revealed that at least four distinct polypeptides were capable of binding to the c-*fos* SRE. While this result may explain the

Figure 5. Purification of proteins interacting with the c-*fos* SRE by DNA sequence specific affinity chromatography.

Panel A: SRE sequence specific affinity chromatography of nuclear proteins from normal rat liver.
Approximately 20 mg of nuclear proteins from normal rat liver prepared in 25 mM HEPES buffer, pH 7.9 containing 1 mM EDTA, 10% glycerol, 0.1% NP40, 0.2 mM PMSF and 250 mM KCl (buffer A). Nuclear proteins were incubated with 5 μg of poly(dI-dC) and 5 μg of calf thymus DNA per mg of proteins at 4°C for 10 min. The proteins were then applied to the SRE affinity column (Table 1, sequence 3) by gravity flow. The preparation of the affinity columns is described in the Methods section. The column was washed with 3 column volumes of loading buffer and bound proteins were eluted with the same buffer containing 2.0 M KCl. The 2.0 M KCl eluate was then dialyzed against buffer A and re-applied to the affinity column. After the second pass the 2.0 M KCl eluate was dialyzed extensively against distilled water and then freeze dried. The protein profile was examined by 10% SDS-PAGE and Coomassie blue staining. Lane 1 - Bio-Rad low range molecular mass protein standards, lane 2 - total nuclear extract from normal rat liver before chromatography, lane 3 - 2.0 M KCl eluate from column.

Panel B: SRE sequence specific affinity chromatography of nuclear proteins from normal rat liver.
Affinity chromatography was performed as described for panel A except that buffer A contained 100 mM KCl. Lane 1 - 2.0 M KCl eluate from normal rat liver, lane 2 - 2.0 M KCl eluate from rat liver 30 minutes after partial hepatectomy, lane 3 - 2.0 M KCl eluate from 5123tc Morris hepatoma. Molecular masses of the proteins were calculated from a polynomial standard curve drawn through the positions of the markers and are indicated in kilodaltons.

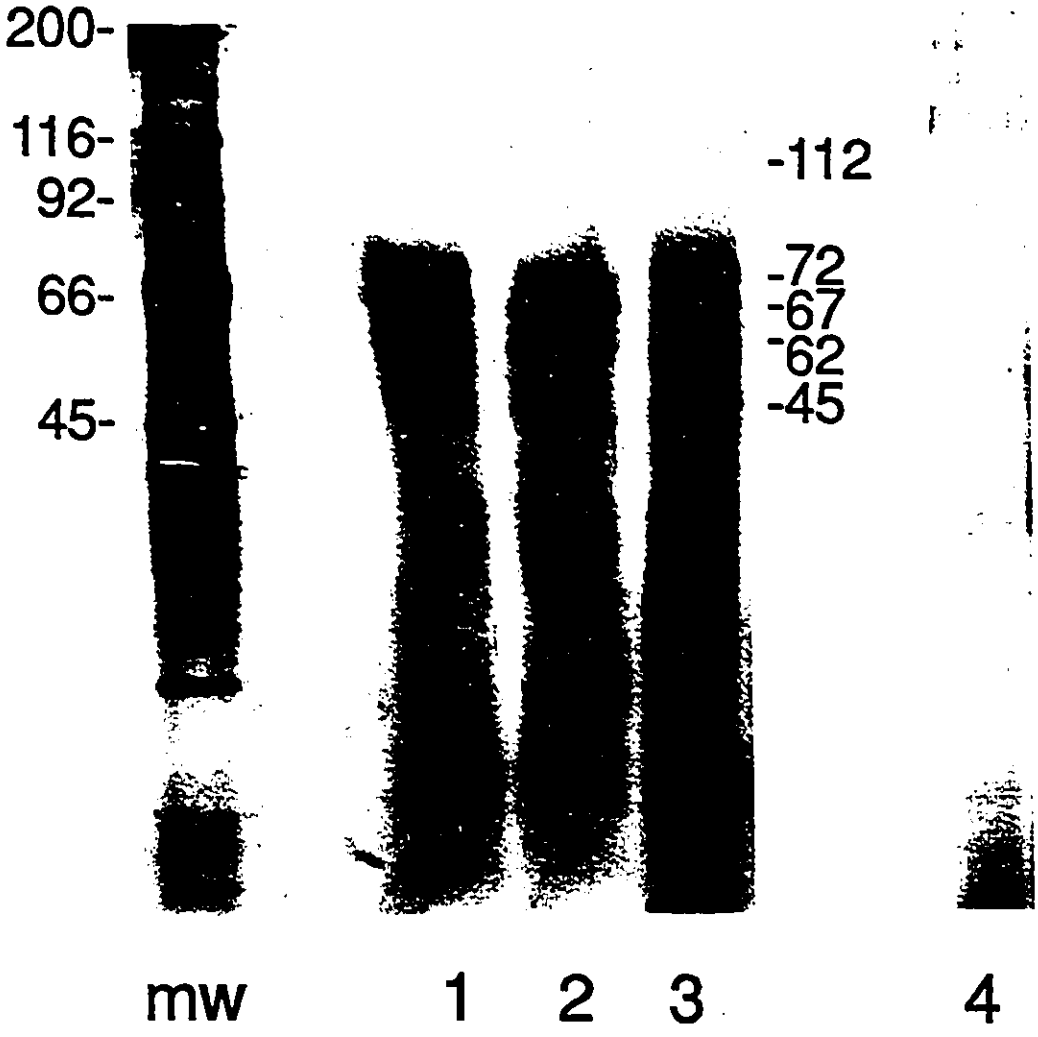

A
B
97–
66–
43–
31–
72
67
62
45
1 2 3
1 2 3

presence of multiple bands in the gel shift assay, another technique was applied to demonstrate that the proteins purified by affinity chromatography were also those responsible for the gel shift. To directly identify the protein components of the shifted SRE-protein complexes, several gel shift assays were run in parallel, the complexes from twenty lanes were excised and extracted with a buffer described in the Methods section. Individual protein components were identified by SDS gel electrophoresis followed by silver staining. Silver staining of proteins extracted from 20 separate bands was necessary since the yield of protein extracted from each gel shifted band was estimated to be approximately 1 ng. The shifted bands obtained from both normal liver and Morris hepatoma 5123tc were extracted and compared with the profile of proteins purified by affinity chromatography. In all SRE/protein complexes four major polypeptides could be identified as shown in Figure 6. Their molecular weights were 72, 67, 62 and 45 kDa which closely corresponded to the protein profiles retained by affinity chromatography. The composition of the equivalent complex from Morris Hepatoma (lane 3), was similar to those from normal (lane 1), or proliferating liver (lane 2), also consistent with the results seen by affinity chromatography. However, there was an additional polypeptide of molecular weight 112 kDa present in the SRE/protein complex from Morris hepatoma 5123tc (lane 3). While the same four polypeptides were present in the analyzed complexes, the relative amounts of the individual proteins were different in each tissue. The 67 kDa protein was the major component in rat liver complexes (lanes 1,2), whereas in complexes from tumour tissue (lane 3), the 72kDa protein was predominant. To eliminate the possibility of non-specific protein migration into the gel in the absence of SRE probe, a control experiment was performed where

Figure 6. Identification of proteins present in SRE-protein complexes

Elution of proteins from the gel shift assay with nuclear proteins from normal rat liver.
SRE-protein complexes were cut out and eluted from gel slices as described in the Methods section. The proteins were precipitated with 4 volumes of cold acetone and run on 10 % SDS-PAGE. Shown is a silver-stained gel of proteins extracted from 20 combined gel shift assays.

Lanes: mw - high range Bio-Rad size standards, lane 1 - protein eluate from normal rat liver, lane 2 - protein eluate from rat liver at 30 min after HPX, lane 3 - protein eluate from 5123tc Morris hepatoma, lane 4 - protein eluate from normal rat liver run on the gel without a DNA probe and extracted from the excised gel at the same position as the SRE-protein complex shown in lane 1 (the material from 20 lanes was combined). Molecular masses of the proteins were calculated from a polynomial standard curve drawn through the positions of the markers and are indicated in kilodaltons.


200-
116-
92-
66-
45-
-112
-72
-67
-62
-45
mw
1
2
3
4

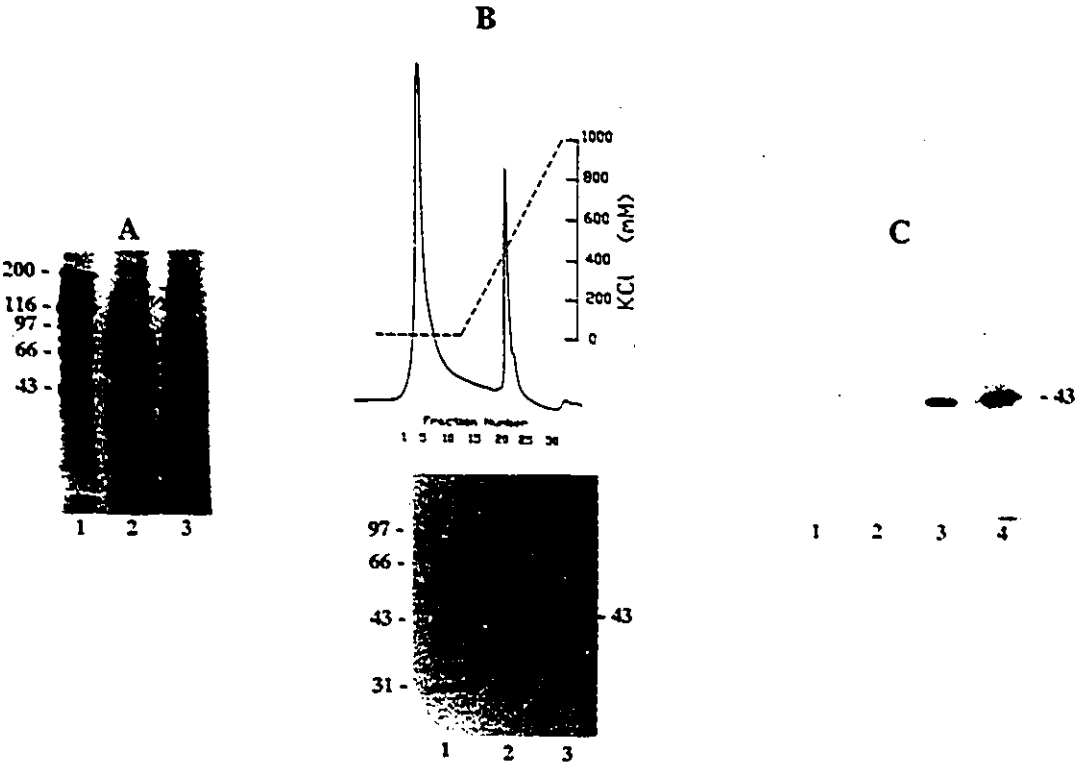
nuclear proteins from normal rat liver were electrophoresed under gel shift assay conditions, but in the absence of SRE probe. The area of the gel at the shifted complex position was excised, extracted and rerun on SDS-PAGE. Lane 4 of Figure 6 showed that in the absence of probe only a minor amount of protein entered the gel thus demonstrating that the proteins extracted from the shifted bands were bound to the SRE probe.

Purification and *in vitro* phosphorylation of proteins interacting with the c-*fos* CRE

Sequence specific affinity chromatography with the c-*fos* CRE sequence was also carried out to characterize the proteins interacting with this element and to determine whether the previously characterized CREB protein could be shown to interact with this element. The CREB protein was previously purified from rat pheochromocytoma cells using the same sequence specific affinity chromatography procedure with the binding site consisting of the CRE from the somatostatin gene (281). Figure 7A showed that after two passes of a nuclear extract prepared from normal rat liver through the affinity column, several polypeptides ranging in molecular weight from approximately 95-30 kDa bound to the c-*fos* CRE. The predominant protein eluting from the column was one with a molecular weight of 43 kDa. This is consistent with the molecular weight of the CREB protein purified previously. Furthermore, the protein profiles observed from purifications using either a c-*fos* CRE or a somatostatin CRE affinity column were similar (compare lanes 2 and 3). Since both affinity columns retained a protein of 43 kDa, further purification of this protein was undertaken. CRE affinity purified proteins were re-chromatographed on a Mono Q ion exchange column using FPLC. Two major peaks of absorbance were observed at 280 nm (Fig. 7B,top).

Figure 7. Purification and *in vitro* phosphorylation of proteins interacting with the c-*fos* CRE

Panel A: CRE sequence specific affinity chromatography of nuclear proteins from normal rat liver. 20 mg of nuclear proteins from normal rat liver prepared as described in the legend to Fig. 4 were passed through a c-*fos* CRE affinity column (Table 1, sequence 1). The proteins were prepared and were loaded onto the column in 25 mM HEPES buffer, pH 7.9 containing 1 mM EDTA, 10% glycerol, 0.1% NP40, 0.2 mM PMSF, 100 mM KCl. Bound proteins were eluted with the same buffer containing 2.0 M KCl. The eluted proteins were reapplied onto the affinity column and eluted proteins were dialyzed against distilled water, freeze dried and analyzed by 10% SDS-PAGE and stained with Coomassie blue. Lane 1 - Bio-Rad high range molecular size standards indicated in kilodaltons, lane 2 - proteins retained by the c-*fos* CRE affinity column, lane 3 - proteins retained by an affinity column prepared with the somatostatin CRE sequence (Table 1, sequence 7). **Panel B:** Further purification of CRE sequence specific affinity chromatography of nuclear proteins from normal rat liver by FPLC. Proteins binding to the DNA-affinity columns, prepared by ligating polymerized c-*fos* and somatostatin CRE sequences (Table 1, sequences 1 and 7 respectively), were further purified by FPLC ion-exchange chromatography on a Pharmacia Mono Q column (V_T=1ml) equilibrated in 20 mM Tris-HCl, pH 7.5. Proteins eluted from the CRE affinity column were dialyzed against water, freeze dried, resuspended in 20 mM Tris-HCl, pH 7.5 and applied to the Mono Q column at a flow rate of 1.0 ml/min. The column was washed with the same buffer and then eluted with a 20 ml linear gradient of 0-1.0 M KCl in 20 mM Tris-HCl, pH 7.5 (0.5 ml fractions). The fractionation was monitored by absorbance at 280 nm at 0.2 aufs. The peak eluting at about 420 mM KCl was collected, dialyzed against distilled water, freeze dried, analyzed by 10% SDS-PAGE and stained with Coomassie blue. Lane 1 - Bio-Rad low range molecular mass standards, lane 2 - Mono Q purified protein from c-*fos* CRE affinity column, lane 3 - Mono Q purified protein from somatostatin CRE affinity column. **Panel C:** *In vitro* phosphorylation of CRE-binding proteins. Approximately 200 ng of proteins purified by c-*fos* and somatostatin CRE affinity and Mono Q ion exchange chromatographies were phosphorylated *in vitro* by 10 U of the catalytic subunit of cAMP-dependent protein kinase in 50 μl of reaction mixture consisting of 20 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 0.10 mM ATP, 1 μCi gamma ^{32}P-labeled ATP (specific activity 3000 Ci/mmol). The reaction was carried out at 30°C for 15 min and it was stopped by the addition of 10 μl of SDS-stop buffer (5 % w/v SDS, 0.25 M Tris-HCl, pH 6.75, 50 % w/v glycerol, 5 mM ß-mercaptoethanol, 0.5 % w/v bromophenol blue). The protein phosphorylation was assessed by autoradiography after being resolved on 10 % SDS-PAGE. The autoradiogram was exposed overnight. Lane 1 - phosphorylation reaction in the absence of affinity purified protein, lane 2 - phosphorylation reaction in the absence of protein kinase, lane 3 - phosphorylation of c-*fos* CRE-purified protein, lane 4 - phosphorylation of somatostatin CRE-purified protein. Molecular masses were calculated from a polynomial standard curve drawn through the positions of the markers and are indicated in kilodaltons.


A
200 -
116 -
97 -
66 -
43 -
1 2 3
B
1000
800
600
400
200
0
KCl (mM)
97 -
66 -
43 -
31 -
43
1 2 3
C
43
1 2 3 4

The first peak eluted with the void volume of the column and the second peak eluted from the column at an ionic strength of 400-500 mM KCl. An identical profile was observed for the somatostatin CRE affinity purified proteins (data not shown). The fractions representing the peak eluting at 400-500 mM KCl were pooled, dialyzed against distilled water, freeze dried, and the protein patterns were examined by SDS-PAGE and Coomassie blue staining. Figure 7B (bottom), showed that the material from both c-*fos* CRE, (lane 2), and somatostatin CRE, (lane 3), columns when repurified on a Mono Q column consisted of a single polypeptide of 43 kDa. In order to characterize further this 43 kDa CRE-binding protein and compare it to the CREB factor, which has been previously shown to be phosphorylated by cAMP-dependent protein kinase, *in vitro* phosphorylation of the purified 43 kDa protein was performed. Figure 7C showed that the 43 kDa protein which interacted with both the c-*fos* and somatostatin CRE was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (lanes 3 and 4). These results indicated that the 43 kDa protein had properties similar to the CREB protein.

Further Characterization of Nuclear Proteins from Rat Liver Interacting with c-*fos* Regulatory Sequences

Comparison of the pattern of affinity purified proteins from both CRE and SRE affinity columns is shown in Figure 8A (lanes 2 and 3 respectively). There were certain similarities in the components of these profiles. For example both columns retained polypeptides of molecular weight 67 and 43 kDa, but the relative quantities of these proteins was different. The 67 kDa protein was the major protein purified by the SRE affinity column while the 43 kDa was the major component of the CRE purified

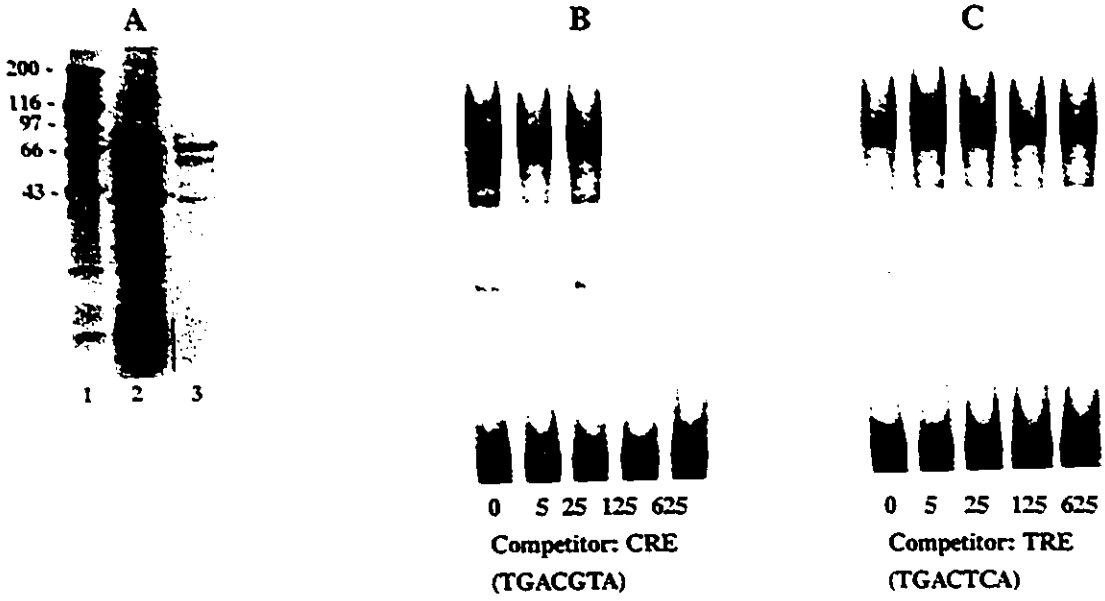
Figure 8. Non-homologous competition for c-*fos* SRE-protein complexes

Panel A: SDS-PAGE gel of purified DNA-binding proteins.
The CRE-binding and SRE-binding proteins purified by DNA sequence-specific affinity chromatography and analyzed by SDS-PAGE and Coomassie blue staining.
Lane 1 - high range Bio-Rad molecular size markers, lane 2 - CRE-binding proteins from normal rat liver, lane 3 - SRE-binding proteins from normal rat liver. Molecular masses were calculated from a polynomial standard curve drawn through the positions of the markers and are indicated in kilodaltons.

Panel B: Gel shift assay with the c-*fos* SRE sequence.
10 μg of nuclear proteins from normal rat liver were incubated at room temperature with approximately 0.5 ng (10,000-15,000 cpm), of ^{32}P-labeled c-*fos* SRE oligonucleotides in the presence of 2 μg of poly(dI-dC) and with a 0 - 625 fold molar excess of unlabeled c-*fos* CRE oligonucleotides for 30 min. Protein-DNA complexes were visualized by autoradiography with an overnight exposure time following resolution on a 5% non-denaturing polyacrylamide gel (acrylamide:bis, 29:1).

Panel C: Gel shift assay with the c-*fos* SRE sequence.
Competition for the SRE-protein complexes by the unlabeled oligonucleotide representing the TPA response element (TRE) from the ap2 gene (Table 1, sequence 8). The assay was performed exactly as described in panel B.

The amount of competitor added is indicated below each lane as a fold molar excess.


A
200 -
116 -
97 -
66 -
43 -
1 2 3
B
0 5 25 125 625
Competitor: CRE
(TGACGTA)
C
0 5 25 125 625
Competitor: TRE
(TGACTCA)

material. To test the possibility that the same proteins were capable of interacting with different regulatory elements, albeit with different relative affinities, a cross competition analysis was performed using the gel shift assay. Figure 8B showed that complexes formed at the c-*fos* SRE could, in fact, be competed with an unlabeled c-*fos* CRE at a molar excess of greater than 125 fold. This result indicated that the proteins which interacted with the SRE also had some affinity for the CRE sequence and may have indicated cross-binding of factors between these two elements. However, the complexes could not be disrupted by the TRE sequence which is highly homologous to the CRE (Fig. 8C) demonstrating the specificity of protein cross-binding between the CRE and SRE sequences.

Similar cross competition experiments were performed with complexes formed at the c-*fos* CRE as shown in Figure 9. Figure 9A is identical to Figure 8A and showed the pattern of CRE and SRE purified proteins. Whereas the CRE oligonucleotides were able to compete out the proteins capable of interacting with the SRE (Fig. 8B), the reciprocal competition was ineffective (Fig. 9C). Only a minor reduction in CRE complex formation was observed when a concentration equal to a 625 fold molar excess of unlabeled SRE was used. The complexes formed with the CRE sequence were indeed very stable and also withstood the competition from the highly homologous TRE sequence (Fig 9B). These data suggested that similar nuclear protein factors were capable of interacting with both regulatory elements consistent with the similarities between CRE and SRE affinity purified proteins.

Analysis of c-*fos* Promoter Binding Proteins by Southwestern Blotting

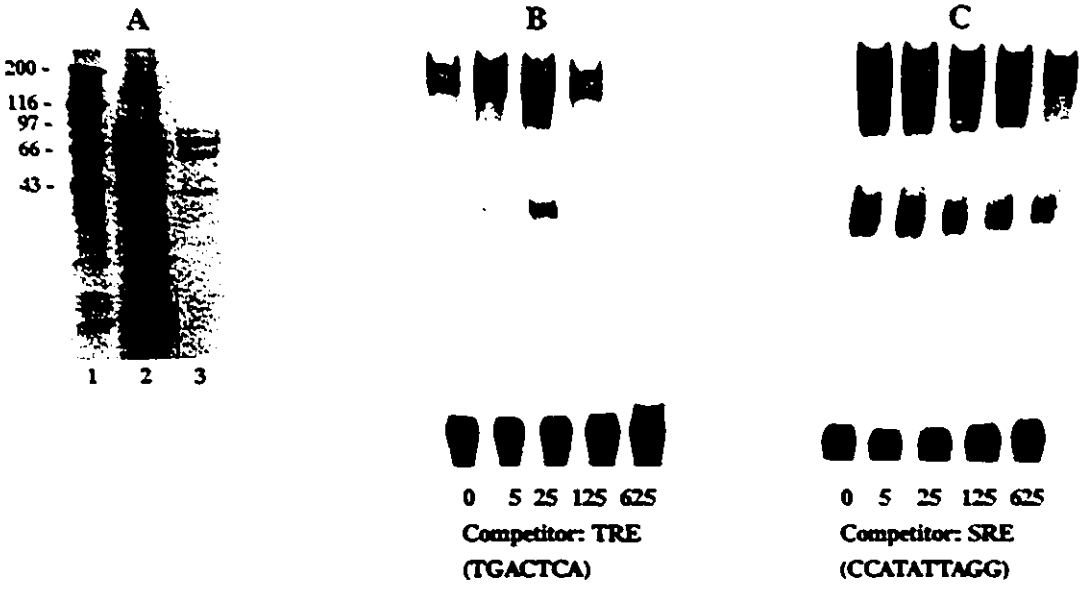
The data presented so far revealed that multi-protein complexes could be formed

Figure 9. Non-homologous competition for the c-*fos* CRE-protein complexes

Panel A: SDS-PAGE gel of purified DNA-binding proteins. The CRE-binding and SRE-binding proteins purified by DNA sequence-specific affinity chromatography and analyzed by SDS-PAGE and Coomassie blue staining. Lane 1 - high range Bio-Rad molecular size markers, lane 2 - CRE-binding proteins from normal rat liver, lane 3 - SRE-binding proteins from normal rat liver. Molecular masses were calculated from a polynomial standard curve drawn through the positions of the markers and are indicated in kilodaltons.

Panel B: Gel shift assay with the c-*fos* CRE sequence. 10 μg of nuclear proteins from normal rat liver were incubated at room temperature with approximately 0.5 ng (10,000-15,000 cpm), of ^{32}P-labeled c-*fos* CRE oligonucleotides in the presence of 2 μg of poly(dI-dC) and with a 0 - 625 fold molar excess of unlabeled TPA response element (TRE), oligonucleotides for 30 min (Table 1, sequence 8). Protein-DNA complexes were visualized by autoradiography with an overnight exposure time following resolution on a 5% non-denaturing polyacrylamide gel (acrylamide:bis, 29:1).

Panel C: Gel shift assay with the c-*fos* CRE sequence. Competition for the c-*fos* CRE-protein complexes by an unlabeled oligonucleotide representing the c-*fos* SRE. The assay was performed exactly as described in panel B.
The amount of competitor added is indicated below each lane as a fold molar excess.


A
200 -
116 -
97 -
66 -
43 -
1 2 3
B
0 5 25 125 625
Competitor: TRE
(TGACTCA)
C
0 5 25 125 625
Competitor: SRE
(CCATATTAGG)

at both the c-*fos* CRE and SRE. Furthermore, cross competition experiments suggested the possibility of a common factor(s) capable of interacting with these elements. To better understand the mechanism by which such a cross binding might occur, it was important to determine which proteins present in these complexes were direct DNA-binding factors and which were present in the complexes due to protein/protein interactions. In the case of the SRE the probability of protein/protein interactions seemed high since the DNA footprint at this element is relatively small and accounts for only two to three proteins (249). To identify DNA-binding factors the Southwestern blotting procedure was applied which has been shown to be a convenient method for this purpose. The assay involved the resolution of nuclear proteins by SDS-PAGE, their transfer to a membrane support and hybridization with a radioactive DNA probe. This technique is, therefore, useful for the detection of those DNA-binding proteins which bind to their recognition sequence as monomers or homodimers. Figure 10 shows the results of Southwestern blots performed with nuclear proteins from normal rat liver and probed with c-*fos* promoter regulatory sequences. Figure 10A showed that three major proteins of 112, 67 and 36 kDa were recognized and labeled by the SRE probe. The same extract contained five proteins of 112, 72, 56, 40 and 36 to 34 kDa which were recognized by the c-*fos* CRE probe (Fig. 10B), and two proteins of 112 and 36 kDa which were labeled by the SCMRE probe (Fig. 10C). The results revealed that the 67 kDa protein was SRE- specific, the 72, 56 and 40 were CRE-specific and the 112 and 36 kDa proteins were recognized by all three elements. While the function(s) of the 112 and 36 kDa proteins has not yet been determined, they represented candidate proteins for mediating interactions between multiple regulatory elements through their

Figure 10. Southwestern blot analysis rat liver nuclear proteins binding to c-*fos* promoter regulatory elements

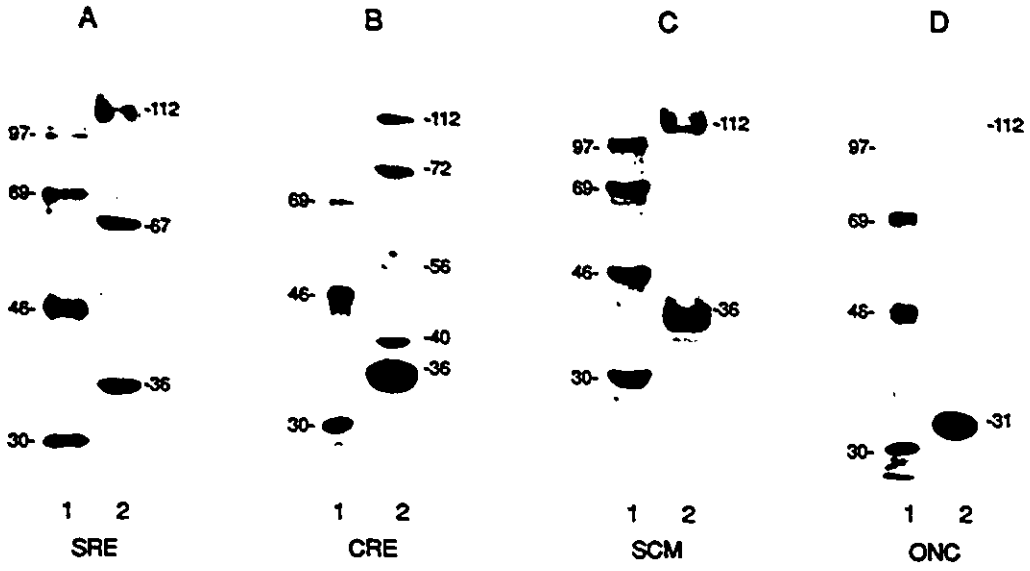
Panel A: Southwestern blot of nuclear proteins from normal rat liver binding to the c-*fos* SRE sequence.
100 μg of nuclear proteins from normal rat liver were resolved by 8.5% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (100 mA, 16 h). After transfer the filter was blocked in a buffer consisting of 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl and 5% (w/v) nonfat milk powder, treated with 25 μg/ml poly(dI-dC) and hybridized with 0.05 μg/ml of ^{32}P-labeled c-*fos* SRE oligonucleotides (2-5 x 10^6 cpm/ml) according to the procedure described in the Methods section (no urea renaturation method). Proteins labeled by the radioactive DNA probe were visualized by autoradiography.
Lane 1 - Amersham ^{14}C-labeled molecular size markers, lane 2 - c-*fos* SRE-binding proteins.

Panel B: Southwestern blot of nuclear proteins from normal rat liver binding to the c-*fos* CRE sequence.
Southwestern blot analysis was performed exactly as described in panel A except the oligonucleotide probe represented the c-*fos* CRE.
Lane 1 - Amersham ^{14}C-labeled molecular size markers, lane 2 - c-*fos* CRE-binding proteins.

Panel C: Southwestern blot of nuclear proteins from normal rat liver binding to the c-*fos* SCMRE sequence.
Southwestern blot analysis was performed exactly as described in panel A except that the oligonucleotide probe represented the c-*fos* SCMRE.
Lane 1 - Amersham ^{14}C-labeled molecular size markers, lane 2 - c-*fos* SCMRE-binding proteins.

Panel D: Southwestern blot of nuclear proteins from normal rat liver binding to a sequence derived from the oncomodulin gene.
Southwestern blot analysis was performed exactly as described in panel A except that the probe represented sequence derived from the coding region of the oncomodulin gene and consisting of a 125 bp *Eco*RI restriction fragment (Gillen et al. 1987).
Lane 1 - Amersham ^{14} C-labeled molecular size markers, lane 2 - proteins binding to the oncomodulin restriction fragment.

Autoradiograms were exposed for two days and molecular masses of DNA-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated in kilodaltons.


A
B
C
D
97-
69-
46-
30-
-112
-67
-36
-72
-56
-40
1 2
SRE
CRE
SCM
ONC
-31

relaxed DNA binding specificities. Interestingly, none of these proteins could bind a 125 bp DNA fragment representing a portion of the coding sequence from the oncomodulin gene (325), which was used as a control for nonspecific DNA binding (Fig. 10D). The oncomodulin DNA did, however, bind to a protein of molecular weight 31 kDa which most likely represented histone H1. The observation that the 112 and 36 kDa proteins were recognized by more than one c-*fos* regulatory element raised the question of their specificity and affinity for an individual element. This question has been addressed by performing the Southwestern blot using mutated SRE probes as binding sites (253). Those results have been published elsewhere (253), and indicate that, insofar as SRE binding is concerned, the 112 kDa protein is specific for the SRE since the binding to a mutated SRE (dSRE2, Table 1) is less than 20% that of the native SRE sequence (253).

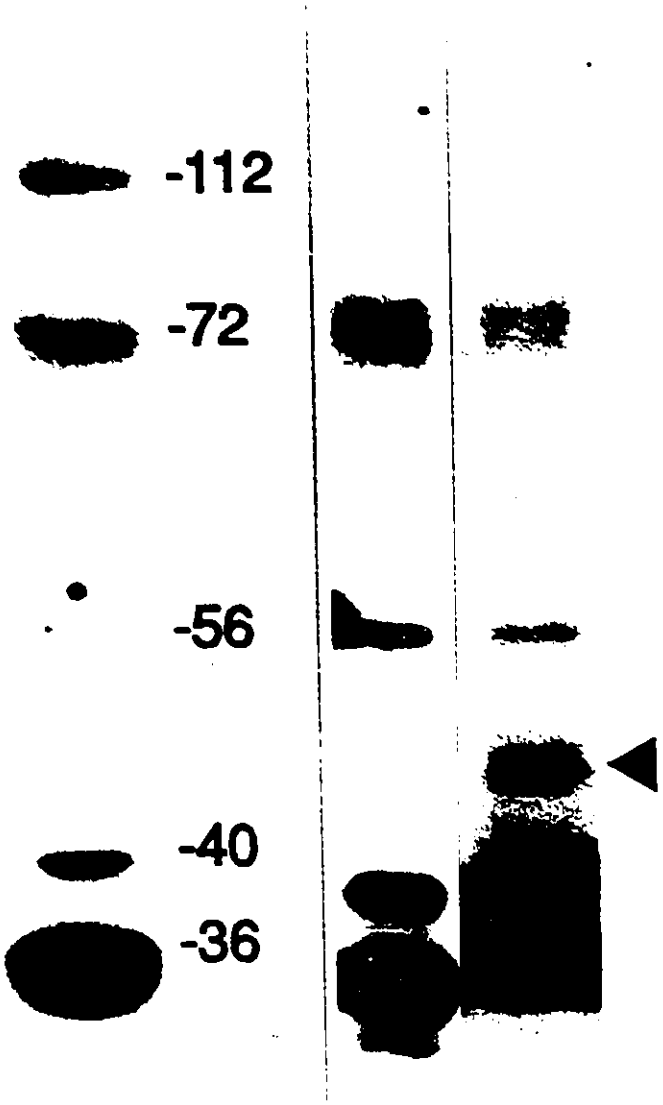
The question was also addressed whether the presence of SDS interfered with the DNA-binding activities of nuclear factors. Therefore the Southwestern blotting procedure incorporated a renaturation step following SDS-PAGE. This step involved incubation of the gel in a buffer containing 4 M urea to remove protein-bound SDS to allow partial refolding of the proteins. Figure 11 shows the results of an experiment done with nuclear extracts from normal rat liver analyzed by Southwestern blotting with and without protein renaturation for CRE-binding proteins. The renaturation step significantly reduced the affinity of the 112 kDa protein for the CRE, but also revealed two additional DNA-binding proteins of molecular weight 56 and 47 kDa which were not observed in the absence of the renaturation step (compare lanes 1 and 2).

The same nuclear protein extract was used to analyze the nuclear proteins interacting

Figure 11. Detection of CRE-binding proteins by Southwestern blotting

100 μg of nuclear proteins from normal rat liver were resolved by 8.5% SDS-PAGE and the gels were either electrotransferred directly onto a nitrocellulose membrane (lane 1), or they were incubated for 3 h in a renaturation buffer containing 4 M urea, as described in the Methods section, before being electrotransferred onto nitrocellulose filters (lanes 2 and 3). After transfer the filters were treated exactly as described in the Methods section and in Fig. 9. The hybridizations were done with either 0.05 μg/ml of ^{32}P-labeled c-*fos* CRE oligonucleotides (2-5 x 10^6 cpm/ml) (lanes 1 and 2) or with 0.05 μg/ml of ^{32}P-labeled somatostatin CRE (lane3).

Autoradiograms were exposed for two days and molecular masses of DNA-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated in kilodaltons. The arrowhead indicates the position of the 47 kDa CRE-binding protein.


-112
-72
-56
-40
-36
1
2
3

with the somatostatin CRE (lane 3). The pattern of labeled proteins was the same as with the c-*fos* CRE, but the 47 kDa protein (indicated with an arrow head), demonstrated higher affinity for the somatostatin CRE. This CRE-binding protein is highlighted and will be described further below. Since the sensitivity of CRE-binding was increased as a result of protein renaturation all subsequent Southwestern blots probed with the CRE incorporated this step unless otherwise indicated.

A similar analysis was performed for SRE binding proteins and it was found that the 112 kDa and 67 kDa SRE-binding proteins lost their DNA binding activities when the renaturation wash was included. Therefore, all subsequent Southwestern blots analyzed for SRE-binding did not include this step.

The Effect of Dephosphorylation on SRE and CRE Binding Activities

Stimulation of the c-*fos* gene occurs very rapidly and in the absence of protein synthesis, suggesting the involvement of protein modification in the mechanism of transcriptional regulation. In fact, SRF has been shown to be a phosphoprotein and SRF phosphorylation has been reported to be required for SRE binding (239,240). CREB is also a phosphoprotein whose DNA binding activity was shown to increase upon cAMP-dependent protein kinase phosphorylation (165). Therefore, the requirement for phosphorylation of the SRE and CRE-binding proteins in order to interact with the regulatory sequences was assessed. Figure 12A showed that dephosphorylated nuclear proteins did not form complexes with the SRE in the gel shift assay confirming the results of Prywes et al., (239). However, Southwestern blotting with the same SRE probe (Fig. 12B), showed that there was no difference in the DNA-binding properties of either of the two, the 67 and 112 kDa, SRE-specific factors as a result of their

Figure 12. Effect of dephosphorylation on the DNA-binding properties of the SRE- and CRE-specific proteins

Panel A: Gel shift assay with the c-*fos* SRE sequence. 10 μg of nuclear proteins from normal rat liver incubated directly with approximately 0.5 ng of ^{32}P-labeled c-*fos* SRE oligonucleotides and 2 μg of non-specific competitor poly(dI-dC) as described in the Methods section (lane 1). In parallel experiments, the proteins were first dephosphorylated by treatment with 2 units/10 μg protein of alkaline phosphatase immobilized on agarose beads at 37°C for 30 min. The beads were removed by centrifugation and the gel shift assay was performed as above (lane 2). Protein-DNA complexes were visualized by autoradiography with an overnight exposure time following resolution on a 5% non-denaturing polyacrylamide gel (acrylamide:bis, 29:1).

Panel B: Southwestern blot with the c-*fos* SRE sequence. 100 μg of nuclear proteins from normal rat liver untreated (lane 2) or dephosphorylated by alkaline phosphatase as described in Panel A (lane 3) were resolved by 8.5% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (100 mA, 16 h), treated with 25 μg/ml poly(dI-dC) and hybridized with 0.05 μg/ml of ^{32}P-labeled c-*fos* SRE oligonucleotides (2-5 x 10^6 cpm/ml) according to the procedure described in the Methods section (no urea renaturation). Proteins labeled by the radioactive DNA probe were visualized by autoradiography. Amersham ^{14}C-labeled molecular size markers are shown in lane 1.

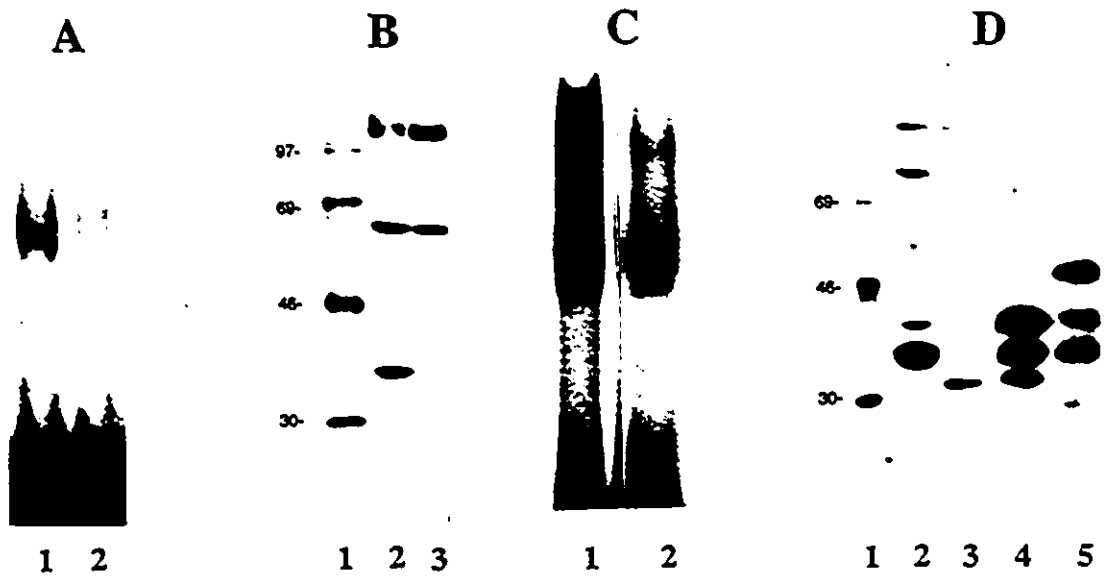
Panel C: Gel shift assay with the c-*fos* CRE sequence.

25 μg of nuclear proteins from normal rat liver incubated directly with approximately 0.5 ng of ^{32}P-labeled c-*fos* CRE oligonucleotides and 2 μg of non-specific competitor poly(dI-dC) (lane 1). The proteins were also dephosphorylated by treatment with 2 units/10 μg protein of alkaline phosphatase immobilized on agarose beads and the gel shift assay was performed as above (lane 2).

Panel D: Southwestern blot with the c-*fos* CRE sequence.

100 μg of nuclear proteins from normal rat liver untreated (lanes 2 and 4) or dephosphorylated by alkaline phosphatase as described in panel A (lanes 3 and 5) were resolved by 8.5% SDS-PAGE and the gels were either electrotransferred directly onto a nitrocellulose membrane (lanes 2 and 3) or they were incubated for up to 3 h in a renaturation buffer containing 4 M urea before being electrotransferred onto nitrocellulose filters (lanes 4 and 5). After transfer the filters were treated exactly as described in the Methods section and in the legend to Figure 10. The hybridizations were done with 0.05 μg/ml of either ^{32}P-labeled c-*fos* CRE (lanes 2 and 3), or somatostatin CRE (lanes 4 and 5) oligonucleotides. Amersham ^{14}C-labeled molecular size markers are shown in lane 1.

Autoradiograms were exposed for two days and molecular masses of DNA-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers.


A
B
C
D
97-
69-
46-
30-
1 2
1 2 3
1 2
1 2 3 4 5

dephosphorylation. The only protein that lost its SRE binding property was the 36 kDa one.

The effect of dephosphorylation on CRE-binding activity was also examined by the same methods. Figure 12C showed a dramatic decrease in CRE-binding of dephosphorylated nuclear proteins by the gel shift assay. However, in contrast to the SRE-binding factors, dephosphorylation dramatically modified the DNA-binding properties of CRE-specific factors as shown by Southwestern blotting (Fig. 12D). This study was performed using both protein non-renaturing (lanes 2 and 3), and renaturing (lanes 4 and 5) conditions. Figure 12D, lane 3 showed that all of the CRE-binding proteins except the 112 and 34 kDa proteins lost their ability to interact with the element after dephosphorylation. Similarly, examination of the CRE-binding proteins following the renaturation step revealed a significant decrease in DNA-binding of the 40, 36 and 34 kDa proteins upon dephosphorylation (Fig. 12D, lane 5). However, surprisingly the dephosphorylation resulted in the dramatic increase in CRE-binding activity of the 47 kDa protein which was barely detectable in untreated nuclear extracts.

Such a dramatic change in the DNA-binding properties of the dephosphorylated CRE-binding proteins contrasted to the properties of the 67 and 112 kDa SRE-binding proteins which did not require phosphorylation for their binding to DNA. Phosphorylation must therefore have altered their interaction with other protein factors required for complex formation and full transcriptional activation.

Identification of the 67 kDa SRE-Binding Factor

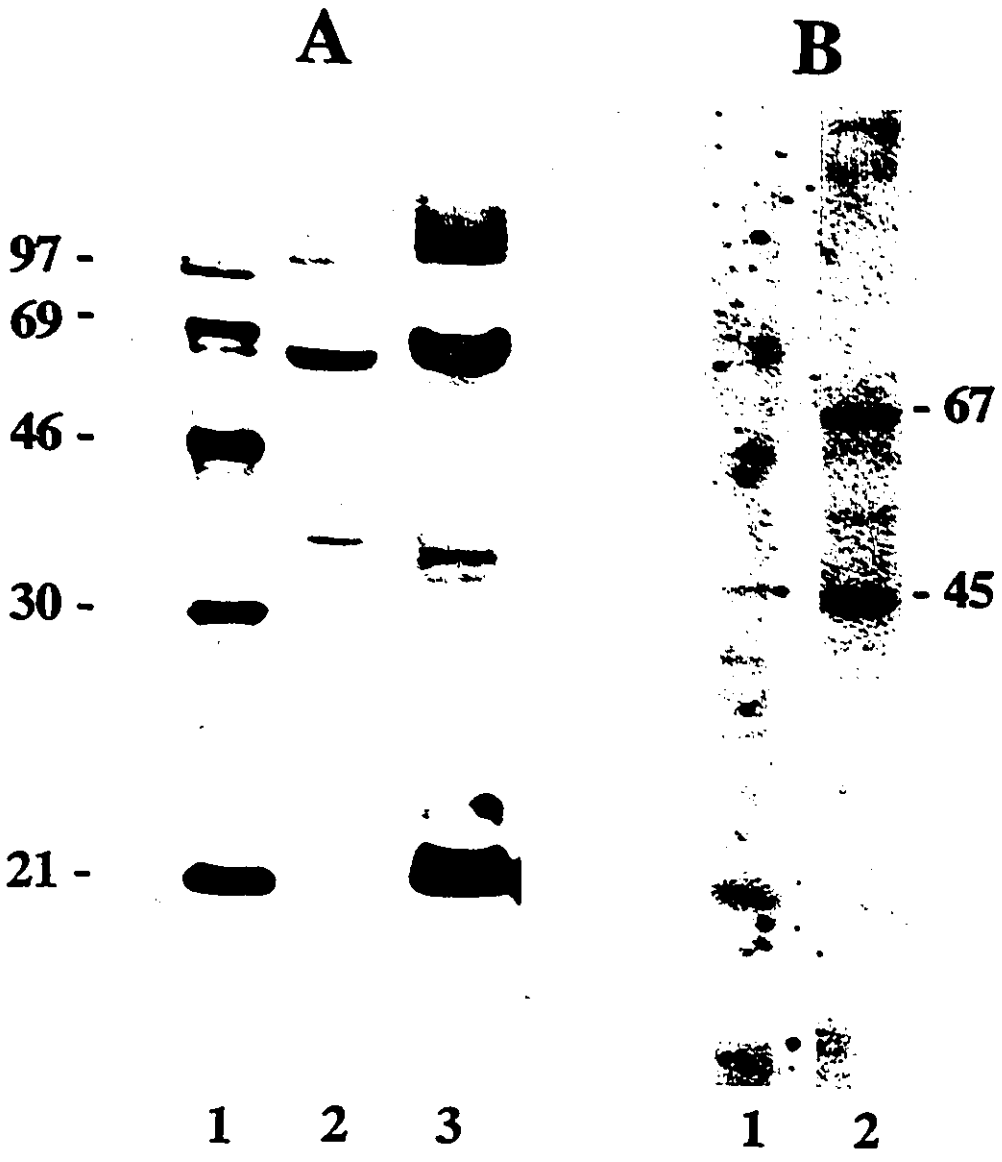
Since the 67 kDa SRE-binding protein identified here was similar in molecular weight to SRF, experiments were performed to compare the properties of these two

Figure 13. Identification of the 67 kDa SRE-binding factor

Panel A: Southwestern blot with the c-*fos* SRE sequence.
100 μg of nuclear proteins from normal rat liver (lane 2) and 200 μg of nuclear proteins from 5123tc hepatoma tumour (lane 3) were resolved by 10% SDS-PAGE and analyzed by Southwestern blotting for c-*fos* SRE-binding proteins as described in the Methods section. Amersham ^{14}C-labeled molecular size markers are shown in lane 1.
The autoradiogram was exposed for two days.

Panel B: Western blot with rabbit anti-SRF polyclonal serum raised against a synthetic peptide representing amino acid residues 141 - 154 of cloned human placental SRF (234).
100 μg of nuclear proteins from normal rat liver were resolved on 10 % SDS-PAGE followed by electrotransfer onto a nitrocellulose membrane (100mA, 16 h). The filter was blocked in 5 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5% (w/v) BSA fraction V, at room temperature for 30 min followed by an addition of 10 μl/ 10 ml of preimmune (lane 1) or anti-SRF (lane 2) serum and incubation was continued for an additional 1.5 h. Polypeptides recognized by the antiserum were detected with ^{125}I-labeled Protein A and autoradiography as described in the Methods section with a 5 day autoradiogram exposure.

Molecular sizes of proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated in kilodaltons.


A
B
97 -
69 -
46 -
30 -
21 -
- 67
- 45
1
2
3
1
2

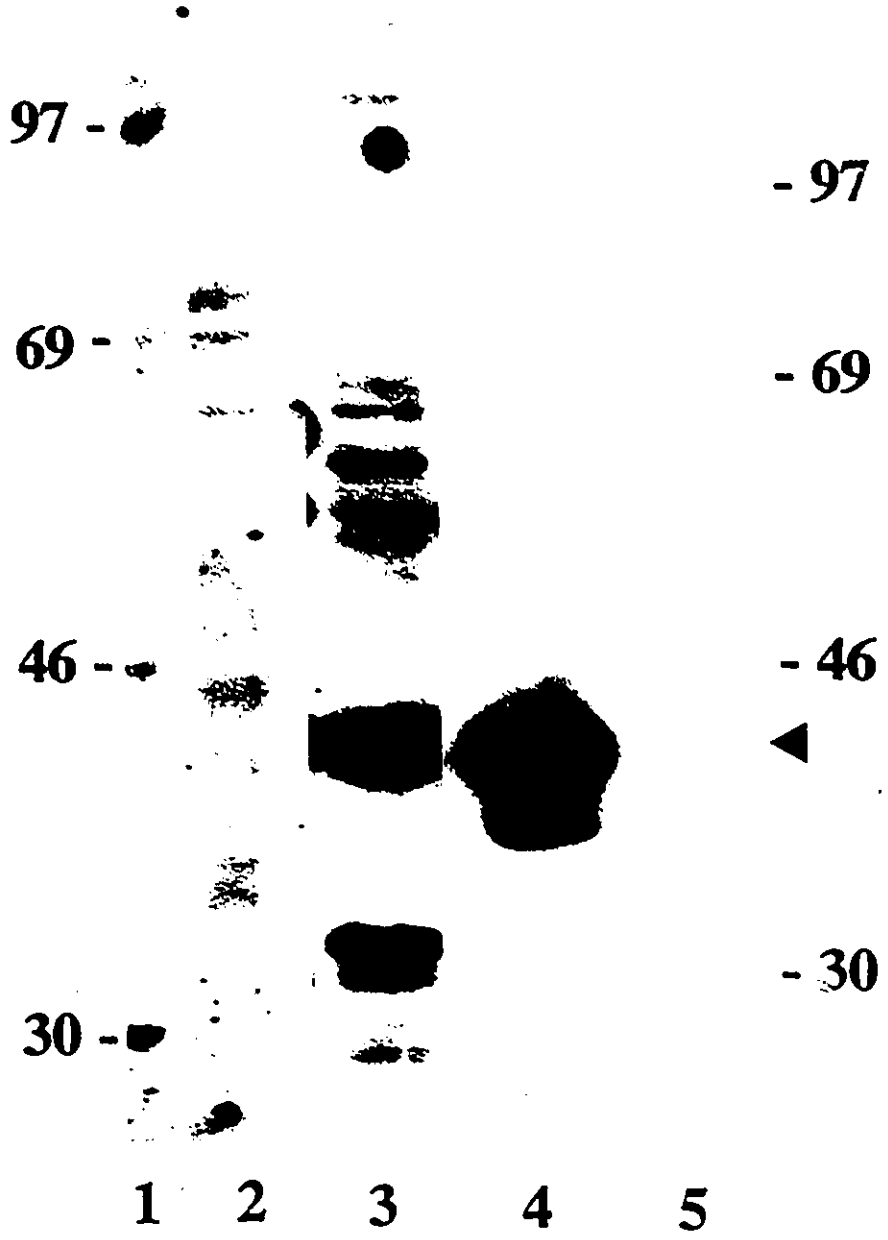
proteins. As shown by Southwestern blotting (Fig. 13A), the 112, 67 and 36 kDa SRE-binding proteins were present in nuclear extracts from normal rat liver (lane 2), as well as in the extracts from Morris hepatoma 5123tc (lane 3). In the tumour tissue, however, there was an additional SRE-binding protein of approximately 20 kDa present. A similar SRE-binding polypeptide was identified in HeLa cells and was shown to be a proteolytic fragment of the SRF protein (259). Therefore, it is possible that the 20 kDa protein seen in tumour tissue was a proteolytic fragment of the 67 kDa protein generated *in vivo*, since protease inhibitors were present at all times during the isolation and extraction of nuclei. To further probe the identity of the 67 kDa SRE-binding protein, a polyclonal antibody raised against a synthetic peptide corresponding to the DNA-binding domain of SRF was used in Western blotting. Figure 13B, shows that the antiserum recognized two proteins of molecular weights 67 and 45 kDa in nuclear extracts from normal rat liver. The molecular weight of the larger polypeptide recognized by the serum was the same as that recognized by the SRE probe on Southwestern blots strongly suggesting that this was the 67 kDa SRF factor. The 45 kDa protein, on the other hand, might represent a protein related to SRF or a related protein which had some homology with SRF within their DNA-binding domains.

Characterization of Peptide-derived anti-CREB Antiserum

To further characterize the CRE-binding proteins identified by Southwestern blotting an antiserum against residues 134-150 of CREB-327 was prepared. Results shown in Figure 14 demonstrated that the antiserum recognized a band of molecular weight 43 kDa in normal rat liver (lane 3), consistent with the molecular weight of CREB (283).

Figure 14. Characterization of peptide-derived anti-CREB serum

Western blot of nuclear proteins from normal rat liver with rabbit polyclonal anti-CREB-327/341 serum raised against a synthetic peptide representing amino acid residues 134 - 150 of cloned human placental CREB-327 (167). 200 μg of rat liver nuclear proteins (lanes 2 and 3) and 100 μl of a total bacterial lysate containing expressed CREB-341 protein (lane 4, a generous gift from M. Montminy), were resolved on 10 % SDS-PAGE followed by electrotransfer onto a nitrocellulose membrane (100mA, 16 h). After transfer the filter was blocked in 5 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5% (w/v) BSA fraction V, at room temperature for 30 min followed by an addition of 5 μl/ 10 ml of anti-CREB (lanes 3 and 4) or pre-immune serum (lane 2), and incubation was continued for an additional 1.5 h. Polypeptides recognized by the antiserum were detected with ^{125}I-labeled Protein A as described in the Methods section with a 5 day autoradiogram exposure. Amersham ^{14}C-labeled molecular size markers are shown in lanes 1 and 5.


97 -
69 -
46 -
30 -
- 97
- 69
- 46
- 30
1
2
3
4
5

This antibody also recognized bacterially expressed CREB protein (lane 4), which was obtained from an expression vector containing the full length CREB (a gift from M. Montminy). This band was not recognized by a preimmune serum (lane 2), providing proof that the peptide-derived antibody was CREB specific.

Interestingly, normal liver contained additional proteins recognized by the CREB antibody which may represent other isoforms of the CREB protein or related proteins. Considering that CREB-341 and CREB-327 are members of a large family of related transcription factors including the ATF proteins, and since the CREB-341 gene is itself alternately spliced giving rise to at least 5 distinct mRNA species (292), it seems reasonable that some of these isoforms and/or related proteins would be recognized by the polyclonal anti-CREB antiserum.

Further Characterization of CRE-Binding Proteins

To confirm that the 34-40 kDa CRE-binding proteins detectable on Southwestern blots corresponded to different isoforms of the CREB protein the pattern of liver nuclear proteins recognized by the CREB antibody and by the somatostatin CRE were compared after the proteins were resolved by two-dimensional gel electrophoresis. Figure 15A showed that the 40 and 34 kDa bands present in the one-dimensional SDS-PAGE separation (Fig. 15A, left lane) remained as single spots (indicated by the arrow heads) after two-dimensional separation. The 36 kDa band was resolved into three spots labeled I, II, and III with slightly different pI values. Interestingly, following two-dimensional protein separation in this pH range (4.0 - 8.0) the DNA-binding activity of the 47 kDa CRE-binding protein could not be detected. This suggested that either the pI of this protein is outside the range of pH separation used here or that its DNA-

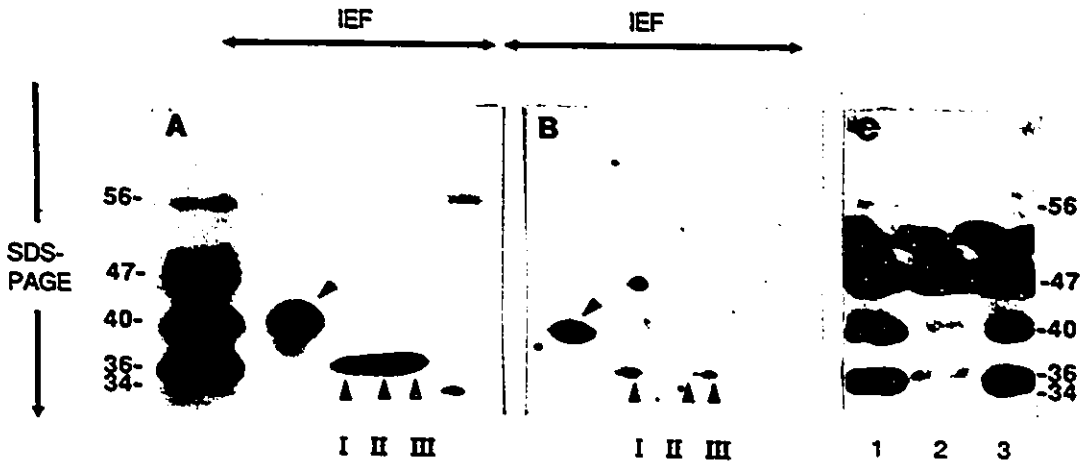
Figure 15. Identification of CREB-341/327 isoforms on Southwestern and Western blots

Panel A: Southwestern blot with the somatostatin CRE.150 μg of nuclear proteins from normal rat liver were first separated by isoelectric focusing on 4% polyacrylamide tube gels prepared with pH 3.0 - 10.0 ampholines according to O'Farrell (320), and then resolved by 8.5% SDS-PAGE. The separated proteins were electrotransferred onto nitrocellulose filters (100 mA, 16 h), and hybridized with a radiolabeled oligonucleotide probe consisting of the somatostatin CRE as described in the Methods section including urea renaturation of the proteins. Included is a one dimensional separation of the same amount of nuclear proteins by SDS-PAGE. The blot was exposed for seven days.

Panel B: Western blot with peptide-derived anti-CREB serum.150 μg of nuclear proteins were resolved by two dimensional electrophoresis as described in Panel A and blotted directly without renaturation of proteins. Western blot analysis was performed as described in the legend to Figure 14 using the same polyclonal antibody raised against residues 134 - 150 of CREB-327. The blot was exposed for nine days.

Panel C: Southwestern blot with the somatostatin CRE.250 μg of nuclear proteins were incubated in a buffer consisting of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 1% deoxycholate, 0.1% SDS, 0.25 mM PMSF at room temperature for 30 min alone (lane 1), in the presence of a 1:100 dilution of anti-CREB-327 antibody (lane 2), or the same dilution of pre-immune serum (lane 3). After incubation, Protein A-coated agarose beads were added to each mixture and antigen - antibody complexes were removed from the mixture as described in the Methods section. Proteins remaining in the mixture after antigen - antibody complex removal were resolved by 8.5% SDS-PAGE and analyzed by Southwestern blotting using a radiolabeled somatostatin CRE oligonucleotide probe as described in Panel A. The blot was exposed for seven days.

Molecular sizes of proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated in kilodaltons.


IEF
IEF
SDS-
PAGE
A
B
C
56-
47-
40-
36-
34-
I II III
I II III
1 2 3
-56
-47
-40
-36
-34

binding activity is lost as a result of the separation of components contributing to this binding. Western blotting of the proteins separated in the same manner (Fig. 15B), showed that the CRE oligonucleotide probe and the CREB antibody both recognized the same 36-40 kDa proteins (indicated by the arrow heads). The antibody also recognized proteins of molecular weight 34 and 56 kDa seen after one-dimensional separation (Fig. 15C), but the spots were difficult to reproduce from the autoradiograms since the signal weakened as a result of the two-dimensional separation. It should be noted, however, that under the experimental conditions used for Southwestern blotting (8.5% SDS-PAGE, urea renaturation) the major CREB protein which ran on 10% SDS-PAGE gels as a 43 kDa band ran with an apparently lower molecular weight of 40 kDa (Fig. 15B). The 36 kDa band could be resolved into three separate spots on both blots, indicating that they were different modifications of the same CREB isoform.

To further distinguish between the 47 kDa protein and other CREB factors, a liver nuclear extract was treated with the CREB antibody and the immunoprecipitated proteins removed by sedimentation with protein A-coated agarose beads. The remaining proteins were then resolved on SDS-PAGE and probed for CRE-binding with the somatostatin CRE. Figure 15C showed that the antibody was able to remove all but the 47 kDa CRE-binding activities (lane 2). Control lanes with proteins treated under immunoprecipitation conditions, but without the antibody or with a preimmune serum showed no changes in CRE-binding activities (lanes 1 and 3 respectively).

The experiments described here and elsewhere, indicated that several distinct proteins can bind to the CRE sequence. However, it is likely that the affinity of these proteins for the CRE are influenced by the sequences flanking the regulatory element.

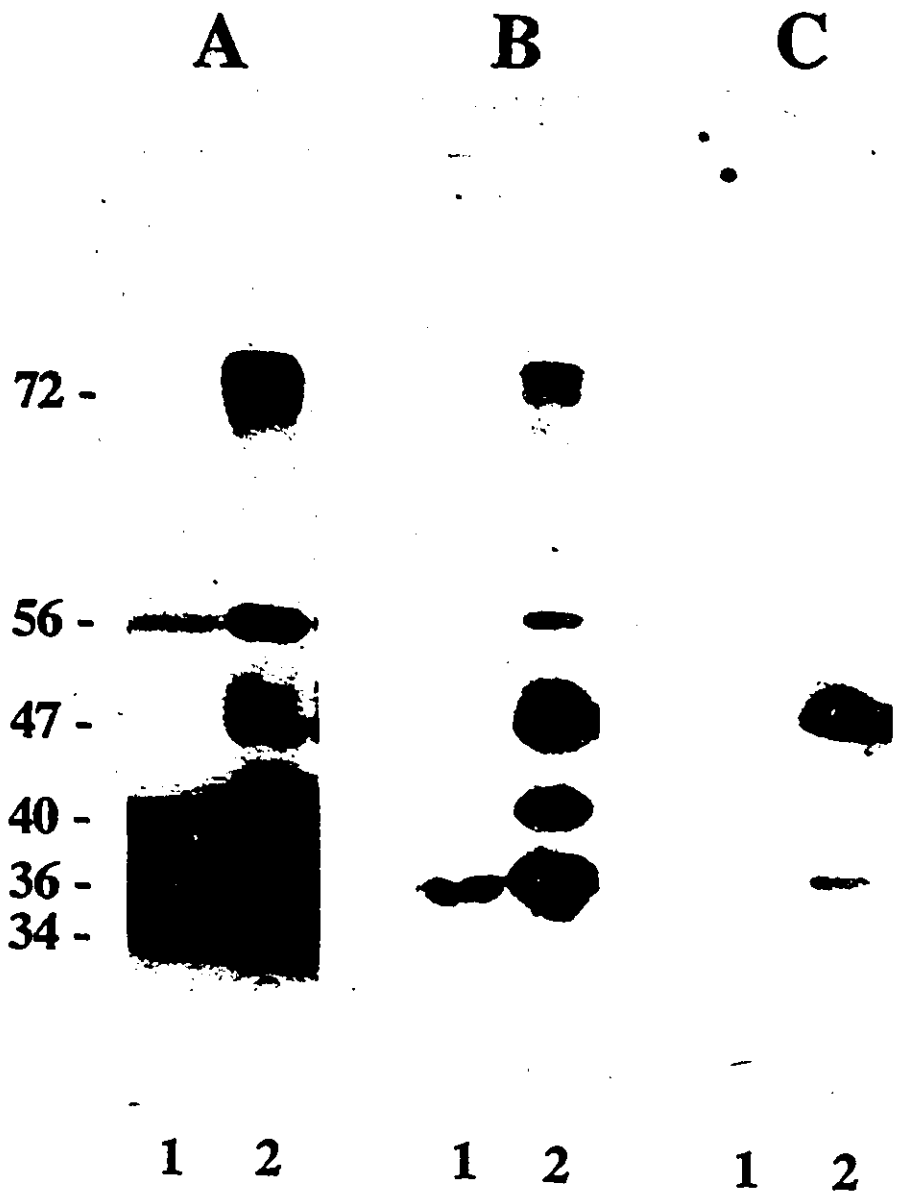
Figure 16. Effect of sequences flanking the CRE element on protein binding

Panel A: Southwestern blot with 24 bp somatostatin CRE probe (Table 1, sequence 7).
200 μg of nuclear proteins from Morris hepatoma 5123tc (lane 1), or from rat liver (lane 2) were resolved by 8.5% SDS-PAGE, renatured in a buffer containing 4 M urea and electrotransferred onto nitrocellulose. The blot was then hybridized with a radiolabeled probe consisting of the somatostatin CRE as described in the Methods section.

Panel B: Southwestern blot with 20 bp somatostatin CRE probe (Table 1, sequence 8).
Hybridization was performed exactly as described in Panel A except that the oligonucleotide probe consisted of the somatostatin CRE shortened at the 5' end by 4 base pairs.

Panel C: Southwestern blot with 16 bp somatostatin CRE probe (Table 1, sequence 9).
Hybridization was performed exactly as described in Panel A except that the oligonucleotide probe consisted of the somatostatin CRE shortened at the 5' end by 8 base pairs.

Autoradiograms were exposed for two days and molecular sizes of DNA-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated in kilodaltons.

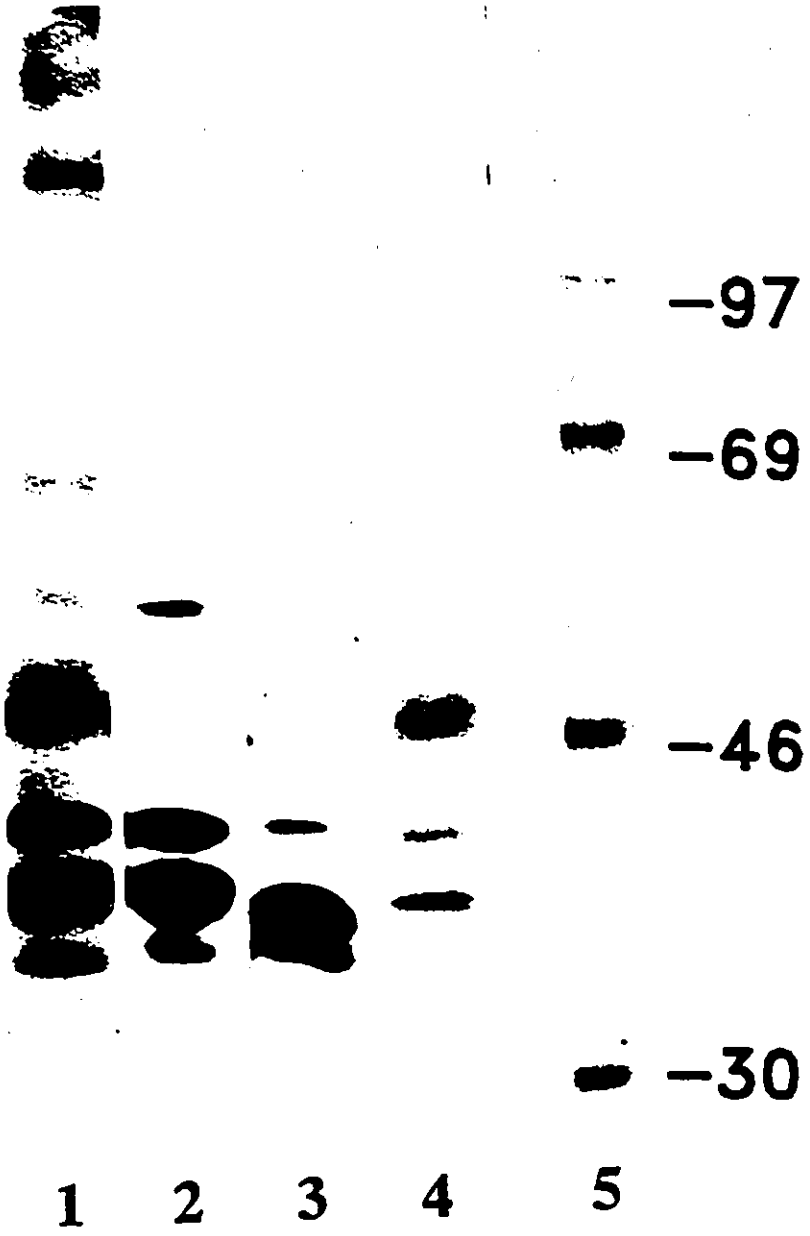

A
B
C
72 -
56 -
47 -
40 -
36 -
34 -
1 2
1 2
1 2

To find out which one of the CRE-binding proteins described here had the highest specificity for the regulatory element, nuclear extracts were probed using the somatostatin CRE containing 5' flanking sequences of various lengths (see Table 1, sequences 7-9). The longest probe was the 24 bp and the other probes were shortened from the 5' end to a 20-mer and to a 16-mer. The probes were incubated with nuclear proteins from Morris hepatoma 5123tc (Fig. 16, lane 1), and with proteins from normal rat liver (Fig. 16, lane 2). Very clearly, the labeling of all of the CRE-binding nuclear proteins, except for the 47 kDa protein depended upon the length of the CRE probe. Figure 16 A showed the complement of CRE-binding proteins recognized by the 24 bp CRE probe. Interestingly, the hepatoma tissue (lane 1), did not contain the 47 or 72 kDa CRE-binding factors. This result has been previously reported (321). Shortening of the CRE probe to 20 (Fig. 16B), or 16 (Fig. 16C), bp resulted in a decrease in the binding of all of the CRE-binding proteins with the exception of the 47 kDa CRE-binding factor from rat liver (lane 2). The data suggested that this protein had the highest specificity for the core regulatory element. Again, none of the shortened probes hybridized to a 47 kDa protein in rat hepatoma tissue.

The Southwestern blotting method was also used to compare the pattern of CRE-binding proteins present in different rat tissues. The results are summarized in Figure 17 which showed the CRE-binding proteins from normal liver (lane 1), Morris hepatoma 5123tc (lane 2), rat placenta (lane 3), and rat brain (lane 4). The liver tissue contained the highest number of CRE-binding proteins which ranged in molecular weight from 112 to 34 kDa as described earlier. The unique 47 kDa CREB protein was

Figure 17. Identification of CRE-binding proteins in different rat tissues by Southwestern blotting

150 μg of nuclear proteins from normal rat liver (lane 1), Morris hepatoma 5123tc (lane 2), placenta (lane 3) and brain (lane 4) were resolved by 8.5% SDS-PAGE and electrotransferred onto nitrocellulose for Southwestern blotting with a radiolabeled somatostatin CRE oligonucleotide probe as described in the Methods section including the protein renaturation step in buffer containing 4 M urea. The Southwestern blot was exposed for two days and molecular masses of DNA-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers (lane 5).


-97
-69
-46
-30
1
2
3
4
5

also present in rat brain (lane 4), but not in placenta (lane 3), or hepatoma tissue (lane 2). The other CRE-binding factors, 40, 36, and 34 kDa, were present in the tested tissues.

Part 2: Analysis of c-*fos* Promoter-Binding Proteins in Proliferating Cells *in vitro* and *in vivo*

Expression of c-*fos* after Serum Readdition to Morris Hepatoma 5123tc Cells in Culture

The identification of the 47 kDa CREB factor as distinct from CREB-341 or CREB-327 as well as its absence in hepatoma tissue prompted the further characterization of this protein. Since the protein was absent from tissues expressing high levels of the c-*fos* gene and which are highly proliferative, experiments were performed to determine the relationship between c-*fos* expression during the cell cycle and the DNA-binding activity of the 47 kDa protein.

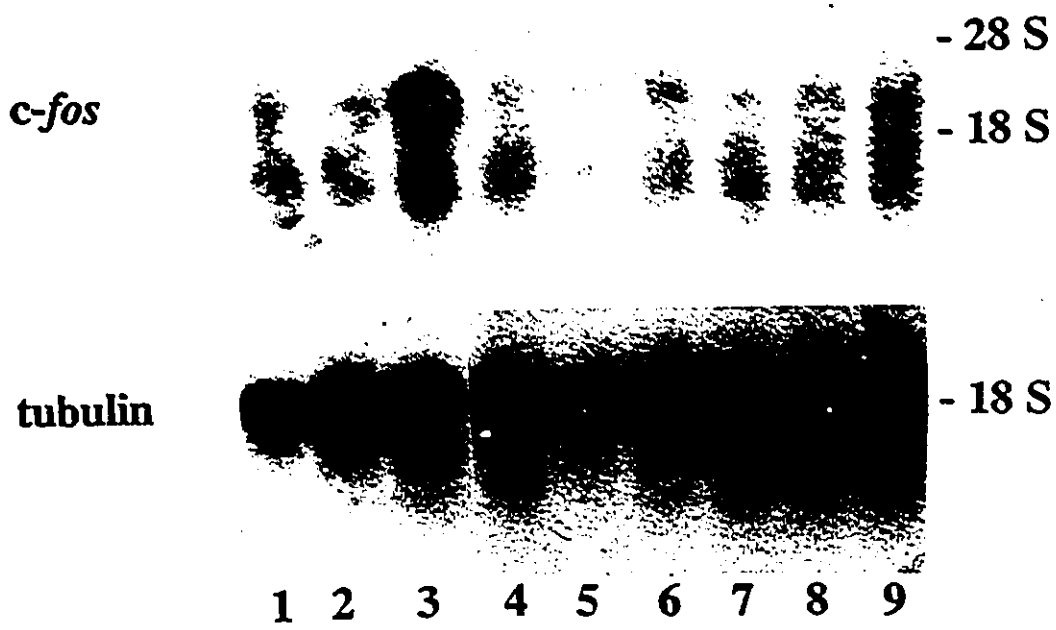
It is known that serum deprived fibroblasts become quiescent and arrested in the G_0 stage of the cell cycle (55). When this synchronized population of cells is then stimulated to re-enter the cell cycle by the readdition of serum, the expression of c-*fos* is rapidly and transiently induced. In the experiments described here, Morris hepatoma 5123tc cells were also synchronized by serum starvation for 48 h and released from the cell cycle block by serum re-addition. The cells were harvested at different times to assess the expression of c-*fos* as they progressed through the cell cycle. Figure 18 showed the Northern blot of mRNA prepared from Morris hepatoma 5123tc cells under these conditions. There was a dramatic increase in the expression of the gene 30 min

Figure 18. Expression of c-*fos* following serum stimulation of Morris hepatoma 5123tc cells

Northern blot of total cytoplasmic poly(A^+)RNA prepared from 5123tc Morris hepatoma cells and hybridized with a 2.2 kbp Hind III/ Bam HI restriction fragment of the CMV-fos cDNA.

5123tc cells were grown in RPMI medium supplemented with 10% fetal bovine serum to approximately 70% confluence and then placed in medium without a serum supplement for 48 h. After starvation, 10 % serum was readded and cells harvested at the appropriate time point according to the procedure detailed in the Methods section. Total cytoplasmic RNA was obtained and the polyadenylated RNA was selected as described in the Methods section. Approximately 1 μg of poly(A+) RNA was resolved on a 1.2% agarose gel containing 1.0 M formaldehyde and then transferred onto a nylon membrane as described in the Methods section. The membrane was hybridized with a radiolabeled c-*fos* cDNA probe with a specific activity of approximately 1-3 x 10^9 cpm/μg DNA. c-*fos* expression was visualized by autoradiography with an exposure time of two days (top panel). The same blot was then stripped by immersion in boiling distilled water for two minutes to remove the hybridized c-*fos* probed and the reprobed with a radiolabeled cDNA consisting of a 1.6 kbp Pst I restriction fragment of the kα1 clone of the human α-tubulin gene with the same specific activity. The blot was autoradiographed overnight to visualize tubulin expression (bottom panel).
Lane 1 - control asynchronously growing cells, lanes 2 to 9 - cells starved for 48 h and harvested at 15, 30, 60, 90, 120, 150, 180, 240 min after serum readdition respectively.

The positions of the 28 S and 18 S ribosomal RNA species are indicated.


- 28 S
c-fos
- 18 S
tubulin
- 18 S
1 2 3 4 5 6 7 8 9

after serum readdition (lane 3) as compared to its level in non-cycling cells (lane 1). This induction was transient, returning to basal levels by 60 min. The same Northern blot was stripped and then reprobed with a tubulin cDNA to assess RNA loading. This rapid and transient induction of c-*fos* expression is consistent with the results described for serum stimulated fibroblasts (55). Thus, the Morris hepatoma 5123tc could be placed in culture conditions and manipulated to reproducibly express the c-*fos* gene at high levels.

Analysis of CRE-binding Proteins in Morris Hepatoma 5123tc Cells Following Serum Stimulation

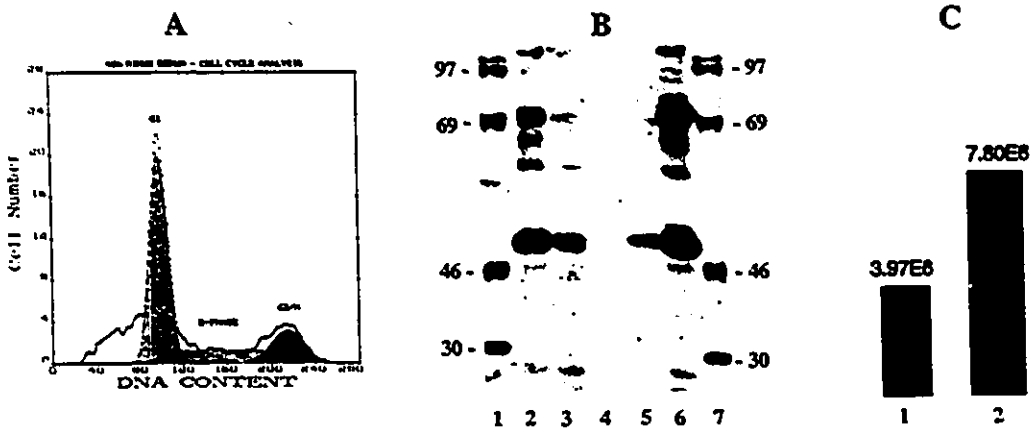
In order to determine whether serum starvation of Morris hepatoma 5123tc cells was sufficient to synchronize the cells a cell cycle analysis was performed using flow cytometry. Figure 19A showed that after 48 h of serum deprivation approximately 70-75% of the cells were arrested in the G_o/G_1. Therefore, the induction of c-*fos* expression described above (Fig. 18), reflected an event occurring in a synchronous population of cells at a specific point in their cell cycle. To determine whether the changes in c-*fos* expression in these cells could be the result of changes in the properties of DNA-binding proteins, Southwestern blotting was performed (Fig. 19B). After serum readdition the cells were harvested at different times, nuclear proteins were extracted and analyzed using the c-*fos* CRE as a probe. In control, unsynchronized cells (lane 2), and in cells synchronized by serum starvation (lane 3), two predominant CRE-binding protein species of 47 and 72 kDa were present. Following serum readdition there was a dramatic loss of CRE-binding activity such that almost no CREB factors were detectable at 1.5 h after the stimulation (lane 4). This loss, however, was transient and

Figure 19. Cell cycle progression of 5123tc Morris hepatoma cells following serum stimulation.

Panel A: Cell cycle analysis by flow cytometry.Morris hepatoma 5123tc cells were grown in culture and serum starved for 48 h as described in the legend to Fig. 18. Cells were analyzed according to their total DNA content by Flow cytometry. The abscissa shows the DNA content of the cells in arbitrary fluorescence units, and the ordinate shows the numbers of cells in each channel x 10^{-1}.

Panel B: Southwestern blot with the somatostatin CRE probe. 100 μg of nuclear proteins from 5123tc cells were resolved by 8.5% SDS-PAGE and analyzed for somatostatin CRE-binding proteins by Southwestern blotting as described in the legend to Fig. 10 and in the Methods section.
Lanes 1 and 7 - Amersham ^{14}C-labeled markers, lane 2 - control asynchronously growing cells, lane 3 - cells serum starved for 48 h, lanes 4 to 6 - cells starved for 48 h and analyzed 1.5, 3, and 6 h after serum readdition, respectively.
The autoradiogram was exposed for two days and the molecular sizes of the markers are indicated in kilodaltons.

Panel C: Total cell number determined in a Coulter counter after 48 h of serum starvation (lane 1) and 24 h after serum readdition (lane 2). The num bers above the histogram bars indicate the cell counts in one 75 cm^2 tissue culture flask.


A
B
C
Cell Number
DNA CONTENT
97 -
69 -
46 -
30 -
- 97
- 69
- 46
- 30
1 2 3 4 5 6 7
7.80E6
3.97E6
1
2

the CRE-binding activities were restored to control levels by 6 h (lane 6). Interestingly, the 47 kDa CRE-binding protein was the first activity to be restored beginning at 3 h after serum readdition while the other CRE-binding activities were not detected until later times.

Total cell numbers were counted before and 24 h after serum stimulation to assess whether serum stimulation was indeed able to drive the cells through the cell cycle. Figure 19C indicated that under these conditions, the doubling time for these cells was approximately 24 h. These results showed that stimulation of quiescent hepatoma cells to progress through the cell cycle is accompanied by both a rapid and transient induction of c-*fos* expression and by a transient loss in CRE-binding activity. The observation that the CRE-binding protein profiles differ between the solid Morris hepatoma and the same Morris hepatoma cells in culture will be addressed in a later section.

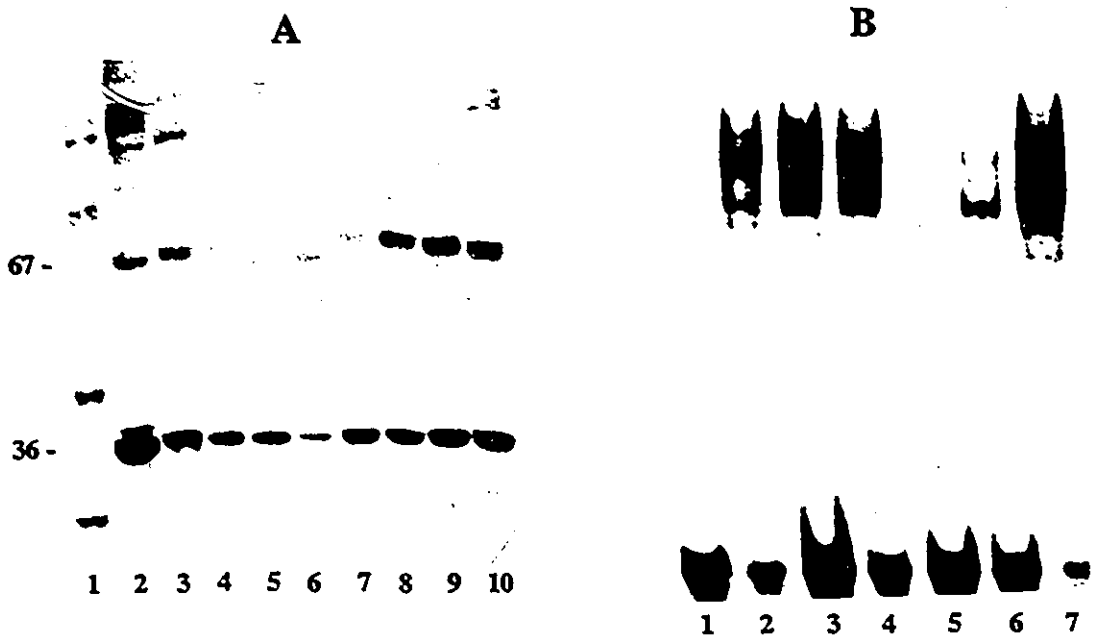
Analysis of c-*fos* SRE-Binding Proteins in Morris Hepatoma 5123tc Cells Following Serum Stimulation

The same nuclear proteins from serum stimulated Morris hepatoma cells were probed for changes in SRE-binding by Southwestern blotting and the gel shift assay. Figure 20A showed the presence of 67 kDa and 36 kDa proteins in unsynchronized 5123tc cells (lane 2), similar to those detected in normal rat liver (Fig. 13). There was little change in the SRE-binding activities at 48 h after serum starvation (lane 3), however, a transient reduction in SRE-binding activity of the 67 kDa protein was seen at 0.5 to 3 h after serum re-addition (lanes 4-7). That transient decrease in the DNA-binding activity seen by Southwestern blotting correlated with a decrease in SRE gel

Figure 20. Analysis of c-*fos* SRE-binding proteins in Morris hepatoma 5123tc cells following serum stimulation

Panel A: Southwestern blot with the c-*fos* SRE sequence.Morris hepatoma 5123tc cells were maintained without serum for 48 h and then serum was added to a final concentration of 10%. Cells were harvested at the appropriate times, nuclei isolated and nuclear proteins extracted as outlined in the Methods section. Nuclear proteins were resolved by 8.5% SDS-PAGE and electrotransferred onto a nitrocellulose filter and hybridized with a ^{32}P-labeled oligonucleotide probe representing the c-*fos* SRE as detailed in the Methods section (no urea wash method). SRE-binding proteins were visualized by autoradiography with an overnight exposure time.
Lane 1 - Amersham ^{14}C-labeled molecular size markers, lane 2 - asynchronously growing cells, lane 3 - cells starved for 48 h, lanes 4 to 10 - cells starved for 48 h and analyzed 0.5, 1, 1.5, 3, 6, 9, 24 h after serum readdition, respectively.
The molecular sizes of the major SRE-binding proteins are indicated in kilodaltons.

Panel B: Gel shift analysis with the c-*fos* SRE sequence.10 μg of nuclear proteins from Morris hepatoma cells synchronized by serum deprivation and stimulated by serum readdition (as described in panel A) were incubated at room temperature with 0.5 ng of a ^{32}P-labeled oligonucleotide probe representing the c-*fos* SRE in the presence of 2 μg of poly(dI-dC) competitor DNA for 30 min. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel as described in the Methods section and were visualized by autoradiography with an overnight exposure time.
Lane 1 - free oligonucleotide probe , lane 2 - asynchronously growing cells, lane 3 - cells starved for 48 h, lanes 4 to 7 - cells starved for 48 h and analyzed 0.5, 1.5, 3, 6 h after serum readdition, respectively.


A
B
67 -
36 -
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7

shift which also recovered to control levels by 6 h after serum stimulation (Fig. 20B). It was consistently observed that the decrease in SRE-binding activity was less pronounced under the same experimental conditions. These results combined with those shown in Figure 19 indicated that serum stimulation of Morris hepatoma 5123tc cells resulted in a transient decrease in activities of DNA-binding proteins interacting with c-*fos* regulatory sequences during the period of elevated gene expression.

In vivo Labeling of Cellular Proteins Following Serum Stimulation of Morris Hepatoma 5123tc Cells

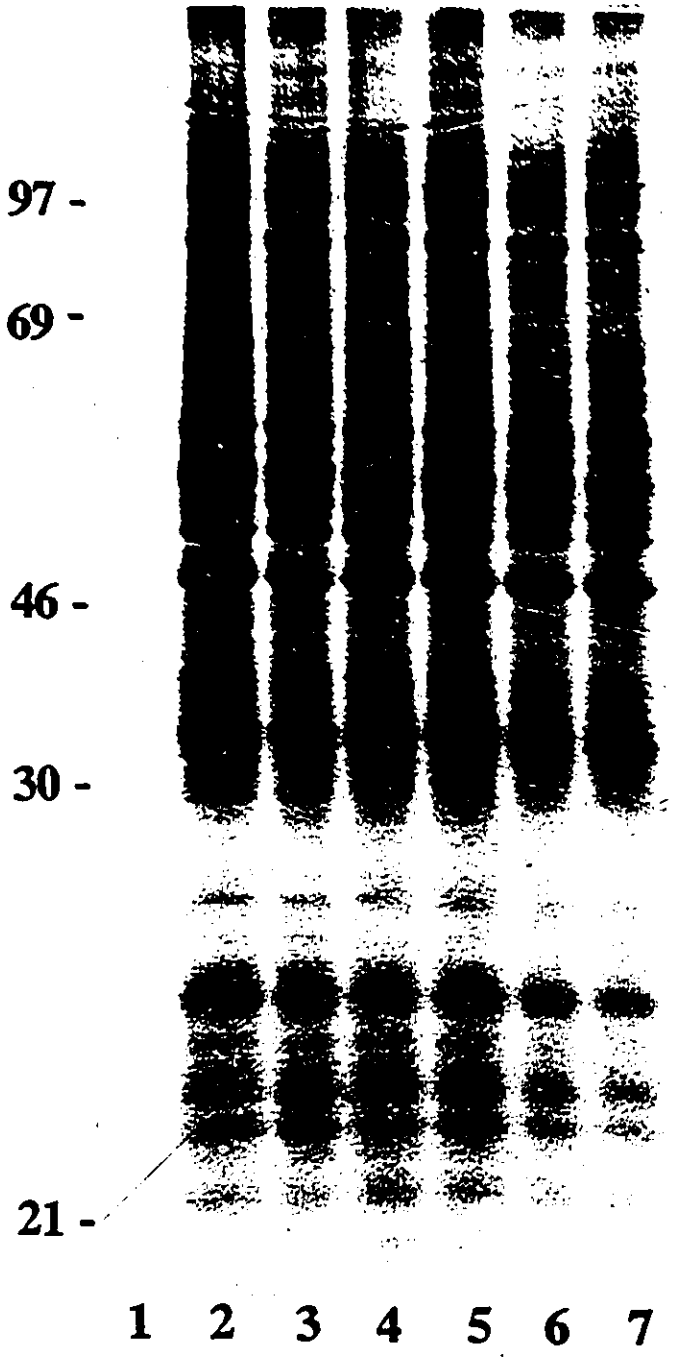
Since the loss of DNA-binding properties was observed for both the CRE and SRE sequences the possibility existed that this was a non-specific event reflecting a general loss or increased turnover of total cellular proteins. To test this possibility, serum synchronized cells were metabolically labeled with ^{35}S-methionine for 90 min The cells were lysed, total cellular proteins were resolved on SDS-PAGE, and autoradiographed. Figure 21 showed that there was no significant difference in the pattern of labeled proteins throughout the experimental period (0-9 h after serum stimulation). This result, therefore, indicated that the transient loss in DNA-binding activity was a specific event occurring as a result of serum stimulation of quiescent hepatoma cells.

The Effects of Phosphorylation and Dephosphorylation on Protein Complex Formation at the c-*fos* CRE

In vivo metabolic labeling of cellular protein revealed that there was no gross change in the level of protein synthesis in serum stimulated cells. Therefore, the changes in DNA-binding activities of nuclear proteins during cell cycle progression could have resulted from changes in their post-translational modification. To address

Figure 21. ***In vivo*** **^{35}S protein labeling of Morris hepatoma 5123tc cells stimulated by serum**

Morris hepatoma 5123tc cells were starved for 48 h and stimulated by serum readdition as described in Fig. 17. Two hours before each time point, the cells were transferred for 30 min to methionine and cysteine depleted RPMI medium, followed by the addition for a further 90 min of 200 μCi/ 75 cm^2 flask of ^{35}S-Met. After incubation with the radiolabeled methionine, cells were washed twice with ice cold PBS and then total cellular protein was harvested by lysing the plasma and nuclear membranes in 1.5 ml/flask of a buffer consisting of 50 mM Tris-HCl, pH 7.5, 1% (v/v) NP40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl for 30 min on ice. Cell lysates were collected by centrifugation at 15,000 x g for 15 min at 4°C and stored at -80°C. Approximately 100 μg of proteins were resolved by 8.5% SDS-PAGE (1 x 10^6 cpm/lane), and the gel was dried on a slab gel drier and labeled proteins were visualized by autoradiography with an exposure time of 24 h.
Lane 1 - Amersham ^{14}C-labeled molecular size markers indicated in kilodaltons, lane 2 - asynchronously growing cells, lane 3 - cells starved for 48 h, lanes 4 to 7 - cells starved for 48 h and analyzed 2, 3, 6, and 9 h after serum stimulation, respectively.


97 -
69 -
46 -
30 -
21 -
1 2 3 4 5 6 7

this question nuclear proteins from serum stimulated Morris hepatoma 5123tc cells were analyzed following incubation at 37°C with and without ATP and $MgCl_2$ to activate endogenous protein kinases. The extracts were then assessed for their ability to form DNA/protein complexes by the gel shift assay. Figure 22A showed a transient decrease in the DNA-binding properties of nuclear proteins from serum stimulated cells to form a complex with the c-*fos* CRE sequence. The results shown in Figure 22B were obtained after the samples were incubated with ATP and $MgCl_2$. Phosphorylation of nuclear proteins in these samples partially restored their ability to form complexes at the CRE. However, the composition of the restored complex must have been different from that seen in control, unstimulated cells since the pattern of shifted complexes observed up to 6 h after serum stimulation was different from that seen at 0 and 9 h. The same experiment was performed with *in vitro* dephosphorylated nuclear proteins and the results are presented in Figure 23. Dephosphorylation of nuclear proteins with alkaline phosphatase was not able to restore CRE-binding activities (Fig. 23B). In fact, this treatment significantly decreased the interaction of proteins with the CRE sequence seen in a control sample (lane 2). The result shown in lane 8 was an artifact of an inadequate amount of the CRE probe added to the sample. Thus, the transient loss in DNA-binding proteins observed in 5123tc cells could not be completely explained by changes in their status of phosphorylation/dephosphorylation.

Analysis of CREB Gene Expression in Morris Hepatoma 5123tc Cells Following Serum Stimulation

To determine whether the loss of CRE-binding activities resulted from a decrease in the transcription of genes encoding CREB proteins, RNA was prepared from

Figure 22. Effect of phosphorylation on the formation of CRE-protein complexes

Panel A: Gel shift analysis with the c-*fos* CRE sequence.Morris hepatoma 5123tc cells were synchronized by serum starvation for 48 h and stimulated by serum readdition as described in Fig. 17.
10 μg of nuclear proteins from Morris hepatoma 5123tc cells were incubated at 37°C for 10 min and then at room temperature with a ^{32}P-labeled oligonucleotide probe representing the c-*fos* CRE in the presence of 2 μg of poly(dI-dC) as described in Fig. 2. DNA-protein complexes were resolved by 5% nondenaturing polyacrylamide gel electrophoresis and visualized by autoradiography with an overnight exposure time.
Lane 1 - free oligonucleotide probe , lane 2 - asynchronously growing cells, lane 3 - cells starved for 48 h, lanes 4 to 10 - cells starved and analyzed 0.5, 1, 1.5, 2, 4, 6, or 9 h after serum stimulation, respectively.

Panel B: Gel shift analysis of *in vitro* phosphorylated nuclear proteins from Morris hepatoma cells with the c-*fos* CRE sequence.
An experiment was performed in parallel to that described in panel A, except that the nuclear proteins were incubated at 37°C for 15 min in the presence of 100 μM ATP and 5 mM $MgCl_2$ before addition of the radiolabeled c-*fos* CRE probe. Lane descriptions are identical to those given in panel A.

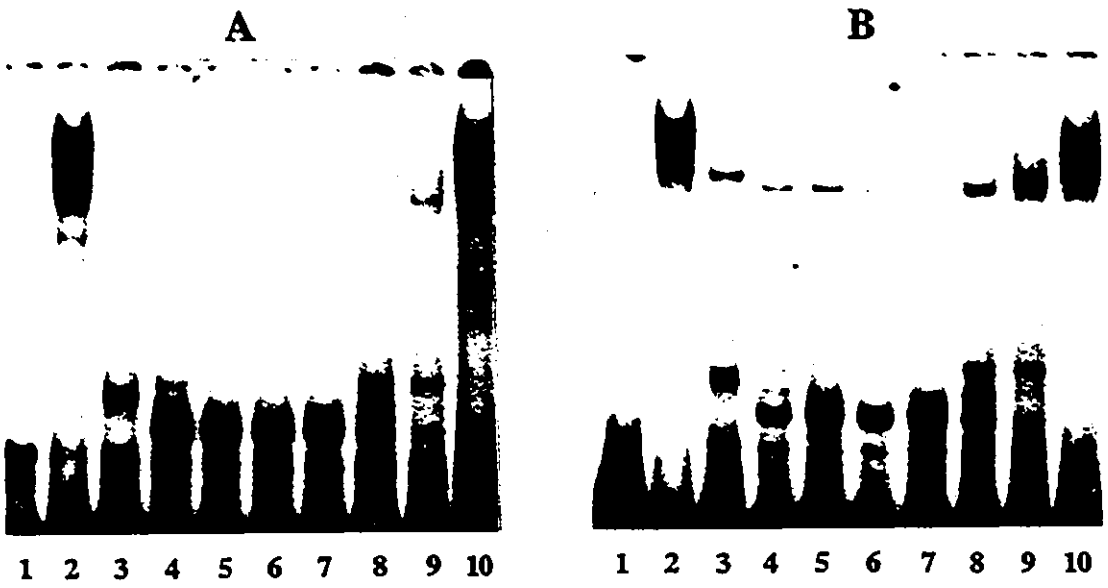
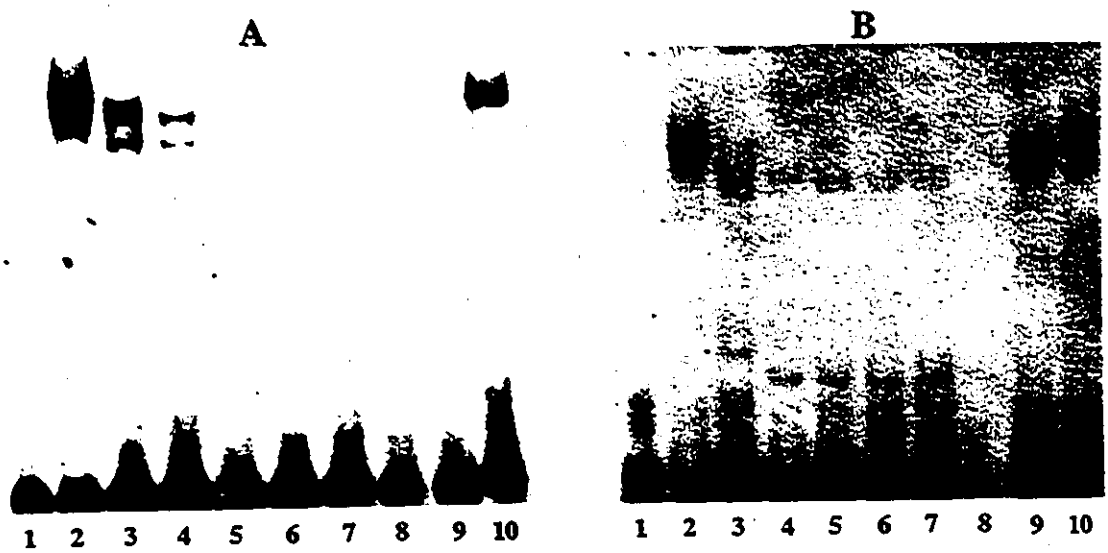

A
B
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

Figure 23. Effect of dephosphorylation on CRE-protein complexes

Panel A: Gel shift analysis with the c-*fos* CRE sequence.
An experiment was performed exactly as described in panel A of Fig. 21.
Lane 1 - free oligonucleotide probe , lane 2 - asynchronously growing cells, lane 3 - cells starved for 48 h, lanes 4 to 10 - cells starved and analyzed 0.5, 1, 1.5, 2, 4, 6, or 9 h after serum stimulation, respectively.

Panel B: Gel shift analysis of *in vitro* dephosphorylated nuclear proteins from Morris hepatoma cells with the c-*fos* CRE sequence.
An experiment was performed in parallel to that described in panel A, except that the nuclear proteins were incubated at 37°C for 10 min in the presence of 1 mM $MgCl_2$ and 5U of calf intestinal alkaline phosphatase before the addition of the radiolabeled c-*fos* CRE probe. Lane descriptions are identical to those given in panel A.


A
B
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

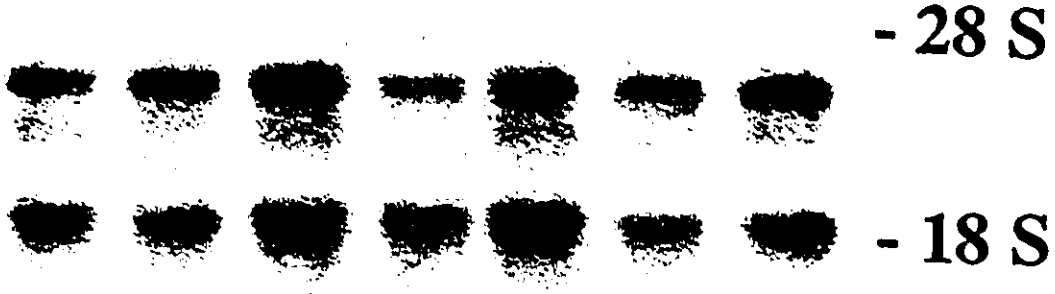
serum stimulated Morris hepatoma 5123tc cells and probed with a cDNA of CREB-341. The results are summarized in Figure 24. In these cells the CREB-341 probe hybridized with two mRNA species. However, their level did not change during cell cycle progression. The same blot was probed with a tubulin cDNA and confirmed that equal amounts of RNA were loaded in each lane (data not shown). Therefore, the transient loss in c-*fos* CRE-binding activities after serum stimulation could not simply be explained by a decrease in the transcription of the CREB genes as judged by Northern blotting.

Inducibility of the c-*fos* Gene by TPA and Serum in Hepatoma 5123tc and Fibroblasts

The changes in the c-*fos* CRE and SRE-binding proteins observed in serum stimulated 5123tc cells followed the transient induction of the gene and extended past the period of its elevated expression. To assess the functional significance of these changes, another agent, TPA, known to elevate c-*fos* expression was used to stimulate the gene in addition to serum. The experiments were designed to determine whether the gene could be induced to the level observed immediately after serum re-addition, during the period of reduced CRE and SRE-binding activities. The results are summarized in Figure 25. The characteristic pattern of transient expression of the c-*fos* gene was observed following serum stimulation of synchronized Morris hepatoma 5123tc cells (lanes 2-6). The highest expression was observed at 0.5 h after serum stimulation (lane 2). If, however, an attempt was made to further induce expression of c-*fos* by TPA during periods of transient loss of DNA-binding activities, there was essentially no response to TPA above that seen with serum alone (compare lanes 3 and 7, 4 and 8,

Figure 24. Analysis of CREB expression following serum stimulation of Morris hepatoma 5123tc cells

Northern blot analysis of total cytoplasmic poly(A)+ RNA probed with a 0.6 kbp AvaII/StuI restriction fragment of the CREB-341 gene.
Morris hepatoma 5123tc cells were starved for 48 h and stimulated by serum readdition as described in Fig. 18. Total cellular RNA was isolated, poly(A)+ RNA selected and resolved on an agarose/formaldehyde gel and transferred to a nylon membrane as described in Fig. 18. The blot was then hybridized with a ^{32}P-labeled cDNA probe of the CREB-341 gene with a specific activity of 1-3 x 10^9 cpm/μg DNA. CREB expression was visualized by autoradiography with an overnight exposure time.
Lane 1 - RNA from asynchronously growing cells, lane 2 - RNA from cells starved for 48 h, lanes 3 to 7 - RNA from serum starved cells harvested 0.5, 1, 2, 4, 8 h after serum readdition. The positions of the 28 S and 18 S ribosomal RNA species are indicated.



1 2 3 4 5 6 7

Figure 25. Expression of the c-*fos* gene in fibroblasts and hepatoma cells following serum and TPA stimulation

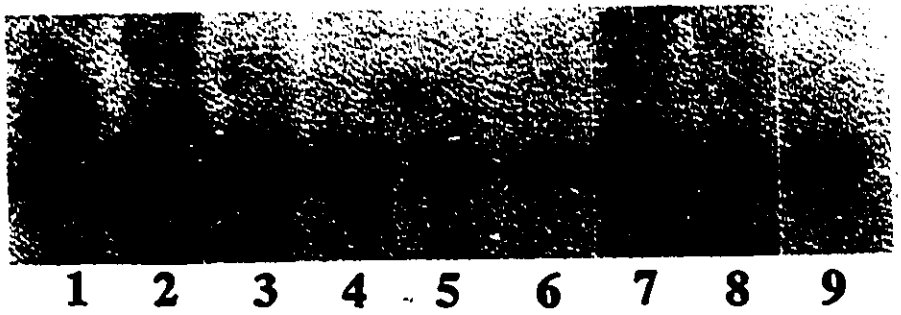
Panel A: Northern blot of cytoplasmic poly(A)+ RNA prepared from Morris hepatoma 5123tc cells in culture and hybridized with a 2.2 kbp restriction fragment of a c-*fos* cDNA.
Cells were serum starved for 48 h and then were placed in medium containing 10% (v/v) fetal bovine serum. In a parallel experiment 400 nM TPA was added to serum stimulated cells for 30 min before the cells were harvested. RNA was isolated and poly(A)+ RNA was purified as described in the Methods section. 1 μg of mRNA was then resolved on an agarose/formaldehyde gel and then transferred onto a nylon membrane for Northern blotting as described in the Methods section.

Lane 1 - mRNA from asynchronously growing 5123tc cells, lanes 2 to 6 - mRNA from serum stimulated 5123tc cells at 0.5, 1, 2, 4, and 6 h after serum readdition, lanes 7 to 9 - mRNA from serum and TPA stimulated 5123tc cells at 1, 2, and 4 h after stimulation.

Panel B: Northern blot of cytoplasmic poly(A)+ RNA prepared from BALB/c 3T3 cells hybridized with a 2.2 kbp restriction fragment of a c-*fos* cDNA.
Cells serum starved for 24 h and then were placed in fresh medium containing 10% (v/v) fetal bovine serum. In a parallel experiment 400 nM TPA was added to the medium 30 min before the cells were harvested. RNA was isolated and poly(A)+ purified according to the procedure detailed in the Methods section. 1 μg of mRNA was then resolved on an agarose/formaldehyde gel and then transferred onto a nylon membrane for Northern blotting as described in the Methods section.

Lane 1 - mRNA from cells 30 min after serum readdition, lane 2 - mRNA from cells 1 h after serum stimulation, lane 3 - mRNA from cells 1 h after serum and TPA addition, lane 4 - mRNA from cells 3 h after serum stimulation, lane 5 - mRNA from cells 3 h after serum and TPA addition, lane 6 - mRNA from cells 6 h after serum stimulation, lane 7 - mRNA from cells 6 h after serum and TPA stimulation.

A



B

5 and 9). While there may have been some induction above control levels, it is possible that this is due to incomplete synchronization of the hepatoma cells. Clearly, the attempt to stimulate expression of the gene by TPA during the transient reduction in DNA-binding activity, to the level seen at 0.5 h after serum stimulation, was unsuccessful.

To demonstrate that this effect was not confined to hepatoma cells only , the same experiments were repeated with BALB/c 3T3 cells synchronized and then stimulated by serum. As shown in Fig. 25B, lanes 1, 2, 4, and 6, c-*fos* expression displayed the same pattern of transient induction. Again the highest level of expression was seen at 0.5 h after stimulation. The addition of TPA at 1h (lane 3), 3h (lane 5), and 6h (lane 7), increased the level of c-*fos* expression above that seen in cells stimulated by serum alone (lanes 2, 4, and 6 respectively). However, in none of the TPA treated cells was the level of c-*fos* expression as high as that seen at 0.5 h after serum stimulation.

Modification of the DNA-binding Activity of the 47 kDa CREB Factor During Cell Cycle Progression

To specifically assess the role of the 47 kDa CREB factor during cell cycle progression, its DNA-binding activity was examined in Morris hepatoma 5123tc cells in response to inhibitors of different signal transduction pathways. The 5123tc Morris hepatoma cells were synchronized by serum deprivation followed by serum re-addition in the presence of the tyrosine kinase inhibitor tyrphostin or the inhibitor of cAMP-dependent protein kinase, the Walsh inhibitor. The results are summarized in Figure 26. In serum stimulated cells the DNA-binding activity of the 47 kDa CREB was downregulated at 3 h and was restored by 6 h (Fig. 26A). These cells doubled within

Figure 26. Modification of the DNA-binding activity of the 47 kDa CREB factor during cell cycle progression

Morris hepatoma 5123tc cells in culture were synchronized by serum starvation for a period of 48 h and then stimulated to reenter the cell cycle by serum readdition.

Panel A: Southwestern blot of c-*fos* CRE-binding proteins.
Cells were stimulated by the addition of 10% fetal calf serum.
Lane 1 - CRE-binding proteins from 5123tc cells at 3 h after serum stimulation, lane 2 - CRE-binding proteins from 5123tc cells at 6 h after serum stimulation.

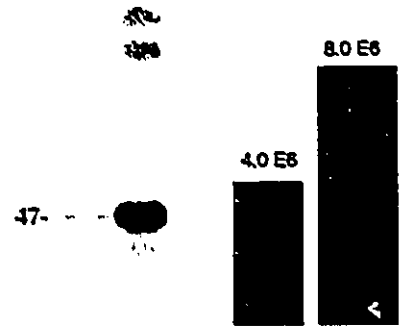
Panel B: Southwestern blot of c-*fos* CRE-binding proteins.
After 48 h of starvation 50 μM tyrphostin tyrosine kinase inhibitor together with 10 % fetal calf serum was added to the medium.
Lane 1 - CRE-binding proteins from 5123tc cells at 3 h after tyrphostin and serum readdition, lane 2 - CRE-binding proteins from 5123tc cells at 6 h after tyrphostin and serum readdition.

Panel C: Southwestern blot of c-*fos* CRE-binding proteins.
After 48 h of starvation 50 μg/ml of the Walsh inhibitor of cAMP-dependent protein kinase together with 10 % fetal calf serum was added to the medium.
Lane 1 - CRE-binding proteins from 5123tc cells at 3 h after Walsh inhibitor and serum readdition, lane 2 - CRE-binding proteins from 5123tc cells at 6 h after Walsh inhibitor and serum readdition.

In each panel 100 μg of nuclear proteins/lane were examined for CRE-binding proteins and the position of the 47 kDa CRE-binding protein is indicated. Autoradiograms were exposed overnight.

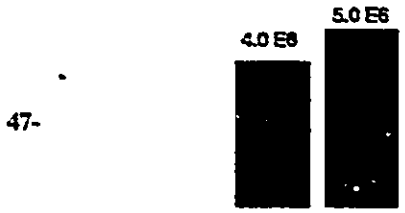
The histograms to the right of each panel represent the total cell number at time 0 and after 24 h of treatments with cell numbers given at the top of each bar.

A



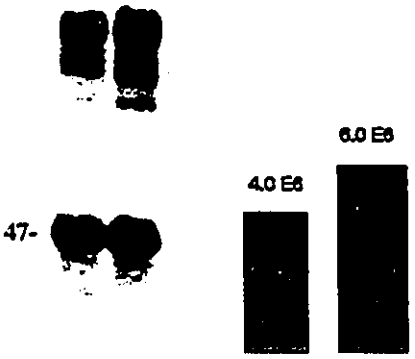
1 2

B



1 2

C



1 2

24 h as determined by cell counts. When tyrphostin was added to these cells together with serum, the 47 kDa CREB lost its CRE-binding activity. In the presence of this inhibitor, the cells did not divide (Fig. 26B). Similarly, the Walsh inhibitor of cAMP-dependent protein kinase, when added to the cells together with serum, dramatically altered the DNA-binding activity of the 47 kDa CREB. However, in the presence of this inhibitor the CRE-binding activity of this protein (as well as the 72 kDa protein), was greatly enhanced. There was no cell cycle-dependent reduction in the CRE-binding activity under these conditions. The cell cycle progression under these conditions was also retarded (Fig. 26C). These results suggested that multiple signal transduction pathways were involved in the modification of the CRE-binding properties of the 47 kDa CREB. The cAMP second messenger pathway seemed to be involved in the down regulation of its CRE-binding properties whereas a tyrosine kinase signalling pathway was required for its restoration.

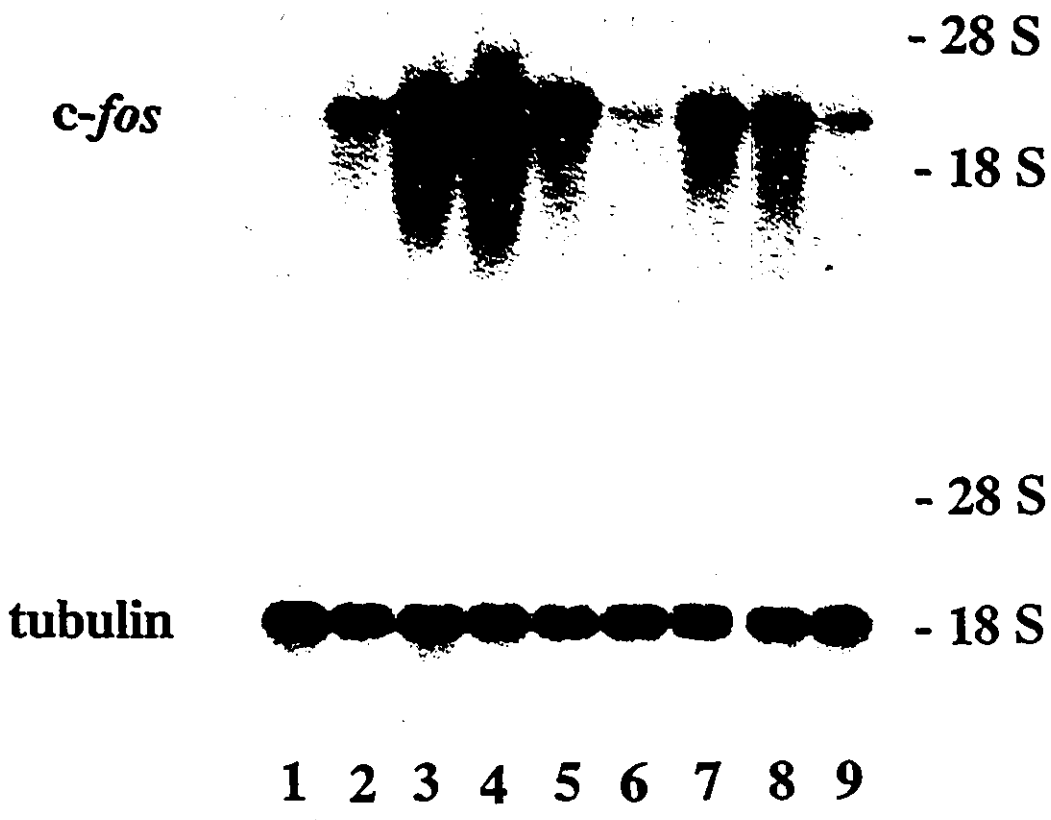
Analysis of c-*fos* Expression Following Partial Hepatectomy

To determine whether the transient cell cycle-related decrease in c-*fos* promoter-binding was only a property of cultured cells or a more generalized phenomenon, further experiments were performed using the *in vivo* experimental model of liver cell proliferation induced by partial hepatectomy. Previous studies have shown that upon partial hepatectomy (HPX), the remaining hepatocytes will proliferate in a coordinated manner with DNA synthesis occurring approximately 12-14 h after HPX. The remaining hepatocytes will continue to proliferate, and the liver will regenerate to the starting mass after about two weeks post-surgery (76). Regenerating liver has, therefore, been used as a model to dissect the regulatory events which occur in normal cell proliferation

Figure 27. Expression of c-*fos* in rat liver following partial hepatectomy

Northern blot analysis of total cellular poly(A)+ RNA probed with a 2.2 kbp Hind III/ Bam HI restriction fragment of the CMV-fos cDNA.
Rats were sacrificed at different times after HPX, their livers removed and placed immediately in liquid nitrogen until all samples were collected. RNA was prepared from the liver samples by homogenization in a buffer of 50 mM Tris-HCl, pH 7.5, containing 4 M guanidine isothiocyanate, 0.5% sarkosyl, 20 mM EDTA, 0.2 M ß-mercaptoethanol as detailed in the Methods section. RNA was purified by centrifugation through a cesium chloride gradient and then poly(A)+ RNA was selected as described in the Methods section. The RNA was then resolved on an agarose/formaldehyde gel and transferred onto a nylon membrane and hybridized with a ^{32}P-labeled c-*fos* cDNA probe. The expression of c-*fos* was visualized by autoradiography with an overnight exposure time.
Following autoradiography the hybridized probe was removed by boiling the blot in distilled water twice for two min each time. The blot was then hybridized with a cDNA probe derived from the α-tubulin gene as described in Fig. 18. After hybridization, tubulin expression was visualized by autoradiography with an exposure time of 4 h.
Lane 1 - RNA from a control rat liver, lane 2 - RNA from liver of rat at 30 min after a sham operation, lanes 3 to 5 - RNA 0.5, 1, 2 h after HPX, lane 6 - RNA from rat liver 3 h after the sham operation, lanes 7 to 9 - RNA from rat liver 3, 4 and 6 h after HPX.

The positions of the 28 S and 18 S ribosomal RNA species are indicated.


- 28 S
c-*fos*
- 18 S
- 28 S
tubulin
- 18 S
1 2 3 4 5 6 7 8 9

in vivo (76). Earlier studies have shown that c-*fos* expression is increased as early as 15 min after surgery and is maintained for 2-3 h before returning to basal expression levels. While the exact mechanism by which the induction occurs is not fully understood, it is thought to be mediated by multiple circulatory factors (76,321,322,323). Thus, as with serum stimulated fibroblasts and Morris hepatoma 5123tc cells, hepatocytes induced to progress through the cell cycle by HPX induce rapidly the expression of the c-*fos* gene (Fig. 27). Expression of the gene peaked at 60 min after HPX (lane 4), and returned to control levels by about 6 h after HPX (lane 9). While there was also some c-*fos* expression in the control laparotomized livers at 0.5 h after surgery (lane 2), this probably reflected an effect of surgical stress (326), and this expression was clearly lower than that seen in the time matched HPX liver. The Northern blot was also probed with the tubulin cDNA to demonstrate the RNA loading. The results were consistent with those of others and indicated that following HPX the expression of c-*fos* occurs rapidly and transiently returning to baseline before DNA synthesis has occurred. Furthermore, the results were similar to those seen with Morris hepatoma 5123tc cells stimulated to progress through the cell cycle by serum stimulation.

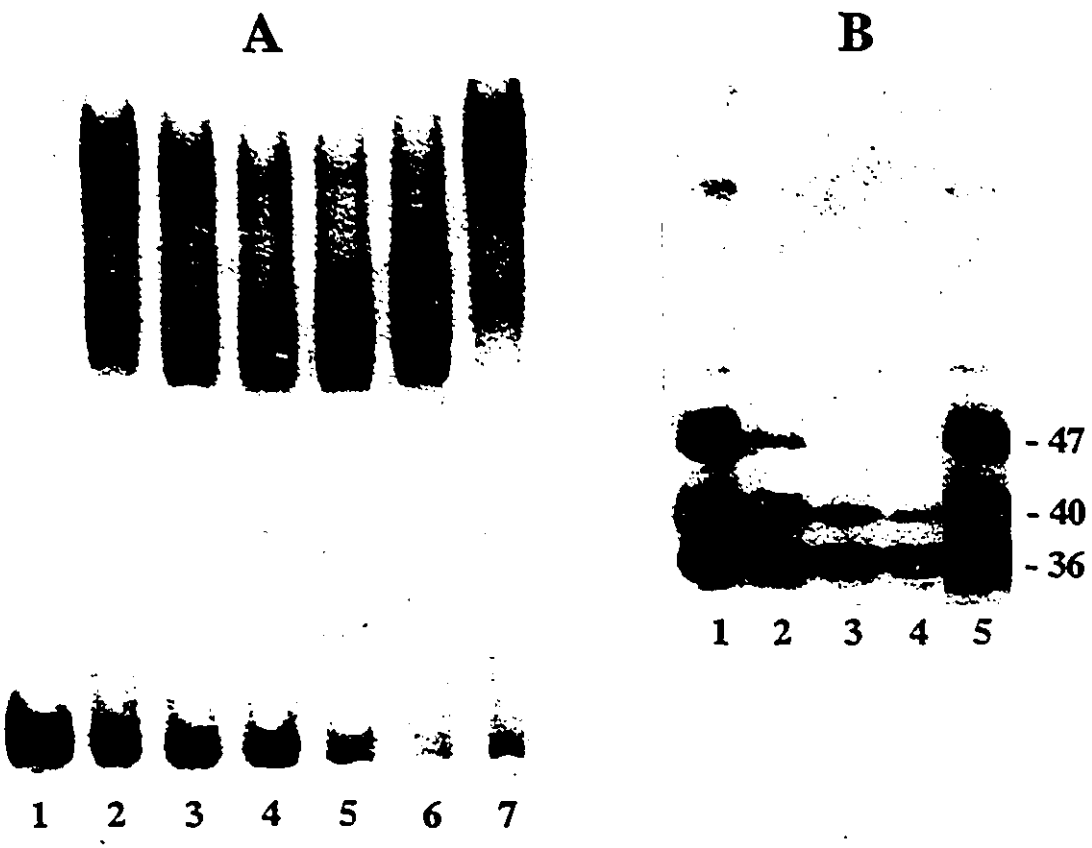
Analysis of CRE-binding Proteins Following Partial Hepatectomy

To determine whether the expression of the c-*fos* gene correlated with changes in c-*fos* CRE-binding proteins, the gel shift and Southwestern blotting procedures were used. The gel shift assay (Fig. 28A), revealed a transient change in the composition of the CRE/protein complexes observed from approximately 1.5 to 6 h after HPX (lanes 3-6). 12 h after HPX (lane 7), the CRE/protein complex was essentially the same as that seen in quiescent liver (lane 2). Changes which occurred in liver cells between

Figure 28. Analysis of CRE-binding proteins in rat liver following partial hepatectomy

Panel A: Gel shift analysis with the somatostatin CRE sequence. Rat livers were removed at the appropriate times after HPX, immediately homogenized in a 50 mM Tris-HCl, pH 7.5 buffer containing 5 mM $MgCl_2$, 25 mM KCl, 0.25 M sucrose, 0.2 mM PMSF, 0.2 mM benzamidine and liver nuclei were isolated as described in the Methods section using the Triton X-100 washing procedure. Nuclear proteins were extracted in a 20 mM Hepes, pH 7.9 buffer containing 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.42 M NaCl, 0.2 mM PMSF, 0.2 mM benzamidine.
10 μg of nuclear proteins were incubated at room temperature with a ^{32}P-labeled oligonucleotide probe representing the somatostatin CRE for 30 min. Following incubation DNA-protein complexes were resolved on a 5% nondenaturing gel and then complexes were visualized by autoradiography with an overnight exposure time.
Lane 1 - free DNA probe, lane 2 - nuclear proteins from control intact rat liver, lanes 3 to 7 - nuclear proteins from livers 1, 1.5, 3, 6, 12 h after HPX, respectively.
Panel B: Southwestern blot with the somatostatin CRE sequence.
100 μg of nuclear proteins were resolved by 8.5% SDS-PAGE and then the gel was washed with a renaturation buffer containing 4 M urea as described in Fig. 11. The proteins were electrotransferred to a nitrocellulose filter and then hybridized with a ^{32}P-labeled oligonucleotide probe representing the somatostatin CRE. Proteins binding to the probe were visualized by autoradiography after an overnight exposure.
Lane 1 - nuclear proteins from control intact rat liver, lanes 2 to 5 - nuclear proteins from livers 1.5, 3, 6, 12 h after HPX, respectively.

The molecular masses of CRE-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated in kilodaltons.


A
B
- 47
- 40
- 36
1 2 3 4 5
1 2 3 4 5 6 7

1.5 and 6 h after HPX (lanes 3-6), resulted in the generation of faster migrating CRE/protein complexes. Figure 28B showed that the changes in the composition of the shifted complexes seen in Figure 28A were coincident with the transient decrease in CRE-binding activity of individual proteins. This was especially marked in the case of the 47 kDa CREB factor whose DNA-binding activity could not be detected at 3 and 6 h after HPX (lanes 3 and 4), but was restored by 12 h after HPX (lane 5). The other CREB proteins retained some DNA-binding activity during that period of time, which might account for the changes in the gel shift seen in panel A.

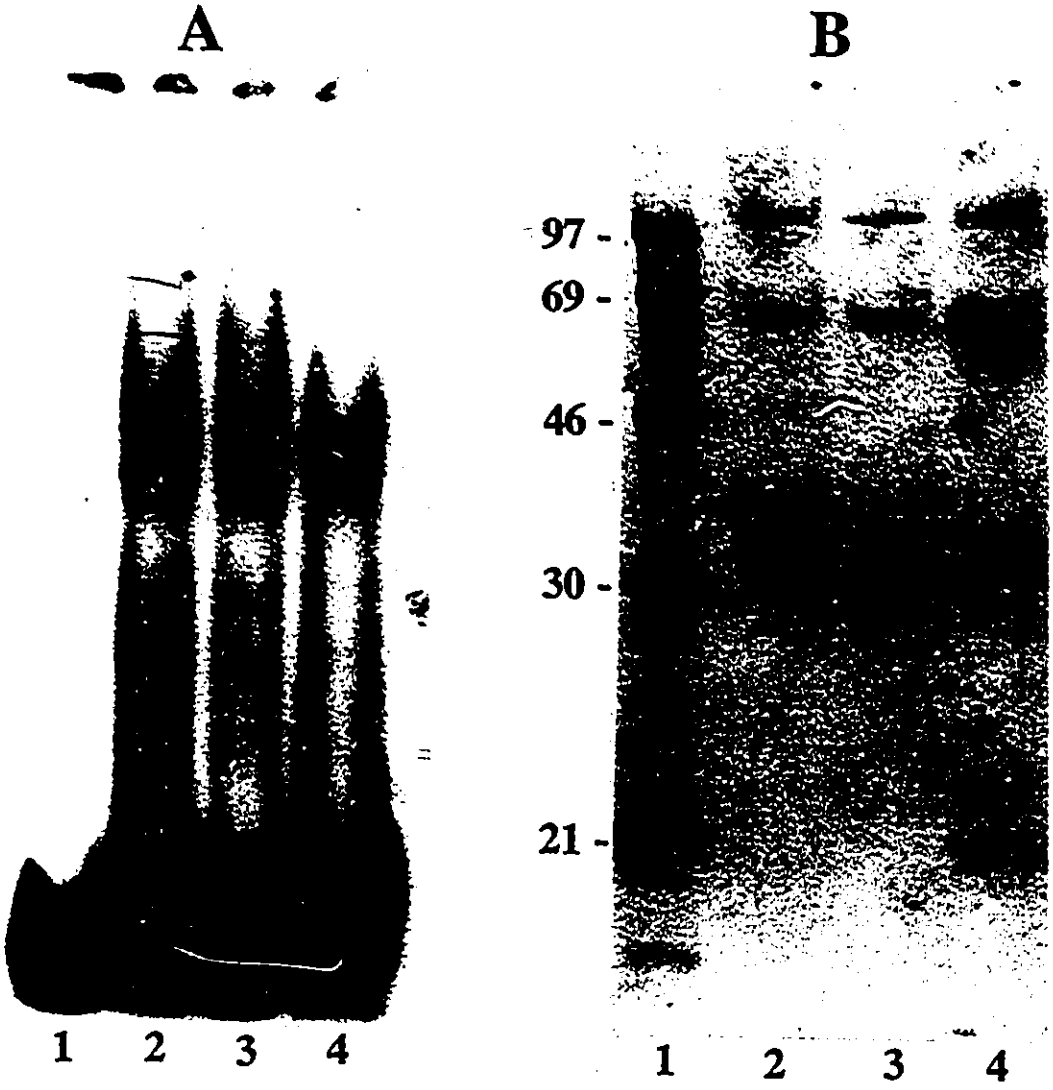
Analysis of SRE-Binding Proteins Following Partial Hepatectomy

In contrast to the CRE-binding proteins there was no change in the DNA-binding properties of SRE-specific proteins during liver regeneration as analyzed by the gel shift assay and by Southwestern blotting (Fig. 29A and B). As assessed by these methods, the SRE-binding activities were essentially the same in quiescent liver (lane 2, panels A and B), as in regenerating liver at 30 min after HPX (lane 3, panels A and B), or in hepatoma tissue (lane 4, panels A and B). As seen in Figure 13, the solid Morris hepatoma also contained the 20 kDa presumptive SRF proteolytic fragment which retained DNA-binding properties (lane 4, panel B). Yet the expression of the c-*fos* gene in these tissues is very different with expression being low in the quiescent liver tissue, highly induced at 30 min after HPX, and constitutively high in hepatoma tissue (Fig. 27, and see below). These results are consistent with earlier reports which document identical SRE-binding activities in cells both before and during induction of the c-*fos* gene (249).

Figure 29. Analysis of SRE-binding proteins in rat liver following partial hepatectomy

Panel A: Gel shift analysis with the c-*fos* SRE sequence.
10 μg of nuclear proteins from normal liver (lane 2), liver 30 min after HPX (lane 3), and Morris hepatoma 5123tc solid tumour (lane 4), were incubated with 0.5 ng of ^{32}P-labeled SRE probe in the presence of 2 μg of poly (dI-dC) as described in Fig. 2 and in the Methods section. Free SRE probe is shown in lane 1. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel and visualized by autoradiography with an overnight exposure time.

Panel B: Southwestern blot with the c-*fos* SRE sequence.
100 μg of nuclear proteins from normal rat liver (lane 2), liver 30 min after HPX (lane 3), and Morris hepatoma 5123tc solid tumour (lane 4), were resolved by 10% SDS-PAGE, transferred onto a nitrocellulose filter and hybridized with a ^{32}P-labeled c-*fos* SRE probe. Amersham ^{14}C-labeled molecular size markers are shown in lane 1 and are indicated in kilodaltons. SRE-binding proteins were visualized by autoradiography with two days exposure.


A
B
97 -
69 -
46 -
30 -
21 -
1 2 3 4
1 2 3 4

Effect of Dephosphorylation on CRE-binding Proteins Following Partial Hepatectomy

The transient loss of CRE-binding activity in regenerating rat liver was observed up to 9 h after HPX (Fig. 28). Amongst the CRE-binding proteins, the 47 kDa protein was the most affected and its DNA-binding activity was not detected at 3 and 6 h after HPX. As shown in Figure 12, the DNA-binding properties of this protein depended on its state of phosphorylation. In order to determine whether the loss of DNA-binding was a result of its phosphorylation, nuclear proteins prepared from regenerating liver at 3 and 6 h after HPX were dephosphorylated by alkaline phosphatase and then analyzed for CRE-binding by Southwestern blotting (Fig. 30). Dephosphorylation of nuclear proteins from control livers resulted in a decrease in CRE-binding activity (lane 1, panels A and B, see also Fig. 12). When nuclear proteins from HPX liver were dephosphorylated, however, the CRE-binding activity of the 47 kDa protein could only be partially restored at 3 h after HPX (lane 2, panels A and B), and not at all at 6 h after HPX (lane 3, panels A and B). These results indicated that the transient loss of the DNA-binding activity of the 47 kDa protein in regenerating liver, following partial hepatectomy, was not due to its modification by phosphorylation.

Induction of c-*fos* Expression by cAMP

In an attempt to determine the functional significance of the observed transient changes in c-*fos* promoter binding activity, rats subjected to HPX were also treated with cAMP to establish whether the ability of liver cells to induce gene expression in response to cAMP was also compromised. The results are presented in Figure 31. Dibutryl-cAMP was injected intraperitoneally into hepatectomized rats between 3 and

Figure 30. The effect of dephosphorylation on CRE-binding activity in rat liver following partial hepatectomy

Panel A: Southwestern blot with the somatostatin CRE sequence.Nuclear proteins were analyzed for CRE-binding activity by Southwestern blotting exactly as described in Fig. 26, panel B.
Lane 1 - nuclear proteins from control intact rat liver, lane 2 - nuclear proteins from rat liver 3 h after HPX, lane 3 - nuclear proteins from rat liver 6 h after HPX.
Panel B: Southwestern blot with the somatostatin CRE sequence.Nuclear proteins were analyzed for CRE-binding activity after dephosphorylation with 2 units/10 μg protein of alkaline phosphatase immobilized on agarose beads.

Incubations were carried out at 37°C for 30 min and the beads were then removed by centrifugation. The dephosphorylated proteins were analyzed exactly as in panel A.
Lane 1 - nuclear proteins from control intact rat liver, lane 2 - nuclear proteins from rat liver 3 h after HPX, lane 3 - nuclear proteins from rat liver 6 h after HPX.

Autoradiograms were exposed for two days and molecular sizes of CRE-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated in kilodaltons.

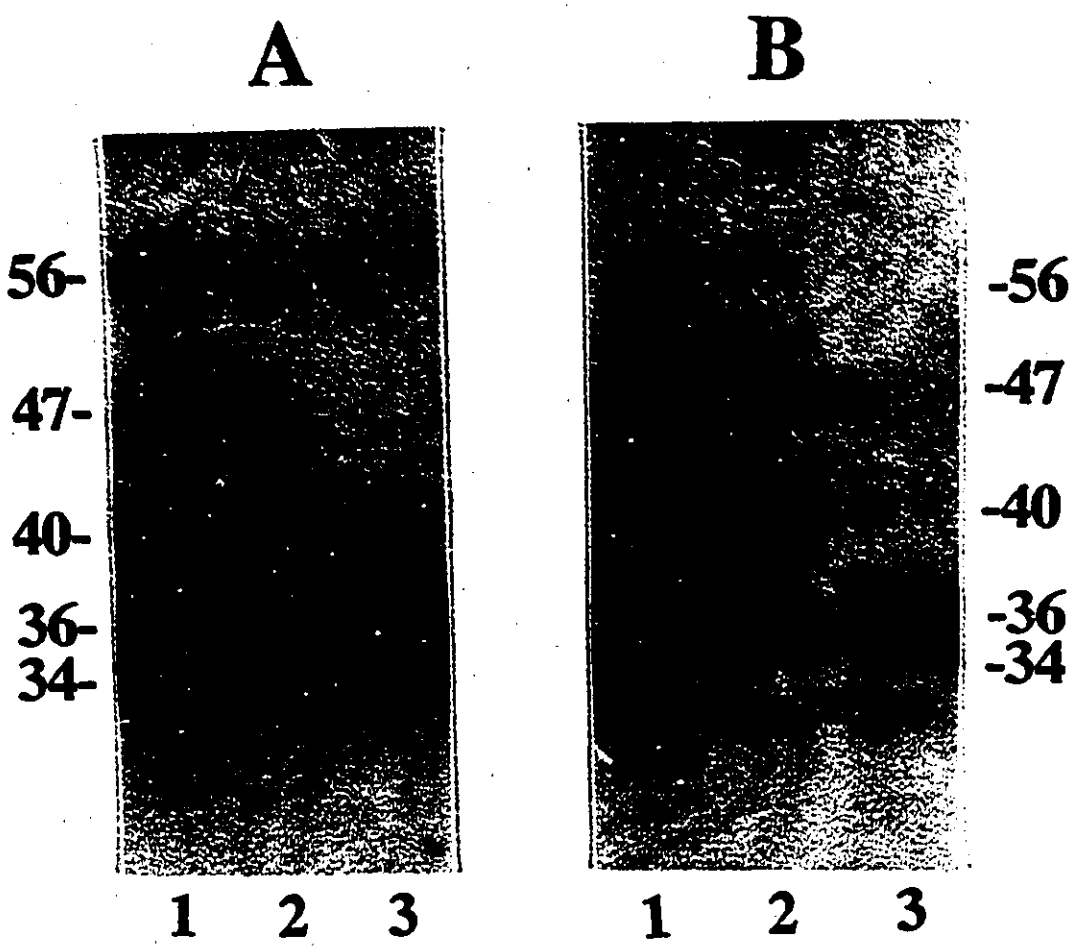
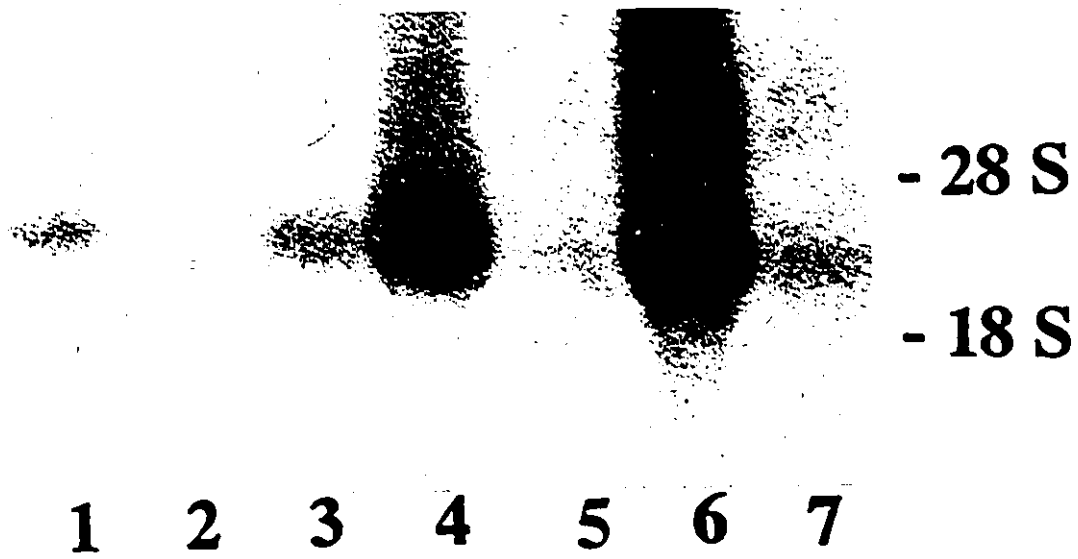

A
B
56-
47-
40-
36-
34-
-56
-47
-40
-36
-34
1 2 3
1 2 3

Figure 31. Induction of c-*fos* expression by cAMP in rat liver

Rats were subjected to either HPX or sham operated as described in the Methods section. At the indicated times following the operation, 0.2 mg/100 g body weight of dibutryl-cAMP and 0.2 mg/100 g body weight of theophylline was injected intraperitoneally. The animals were sacrificed and livers removed at the appropriate times. Total cellular RNA was purified and poly(A)+ RNA selected as described in the Methods section.
1 μg of mRNA was analyzed by Northern blotting for the expression of the c-*fos* gene using a 2.2 kbp restriction fragment of the c-*fos* cDNA.

Lane 1 - mRNA from rat liver at 6 h after HPX, lane 2 - mRNA from rat liver at 6 h after HPX with dibutyrl-cAMP injected at 3 h after HPX, lane 3 - mRNA from rat liver 9 h after HPX, lane 4 - mRNA from rat liver at 9 h after HPX with dibutryl-cAMP injected twice at 3 and 6 h after HPX, lane 5 - mRNA from sham operated rat at 12 h after surgery, lane 6 - mRNA from rat liver at 12 h after HPX with dibutryl-cAMP injected twice at 3 and 7.5 h after HPX, lane 7 - mRNA from sham operated rat at 12 h with dibutryl-cAMP injected twice at 3 and 7.5 h after surgery.

The positions of the 28S and 18S ribosomal RNA subunits are indicated at the right.


- 28 S
- 18 S
1 2 3 4 5 6 7

7.5 h after surgery spanning the period of reduced CRE-binding activity. A single injection of cAMP at 3 h after HPX had no effect on the level of c-*fos* expression analyzed 3 h later (i.e. at 6 h after HPX, compare lanes 1 and 2). Repeated injection of cAMP at 3 and 6 h, or at 3 and 7.5 h after HPX significantly induced c-*fos* expression when analyzed 3 h after the final injection (lane 4), or 6 h after the final injection (lane 6). This phenomenon was specific for proliferating cells since the same treatment with cAMP of sham operated rats had no effect on the status of c-*fos* gene expression (lanes 5 and 7). These results clearly established that the c-*fos* gene could not respond to cAMP in the absence of nuclear CRE-binding activity (3 to 6 h after HPX). Only after the recovery of nuclear CRE-binding was the gene induced by cAMP (9 to 12 h after HPX).

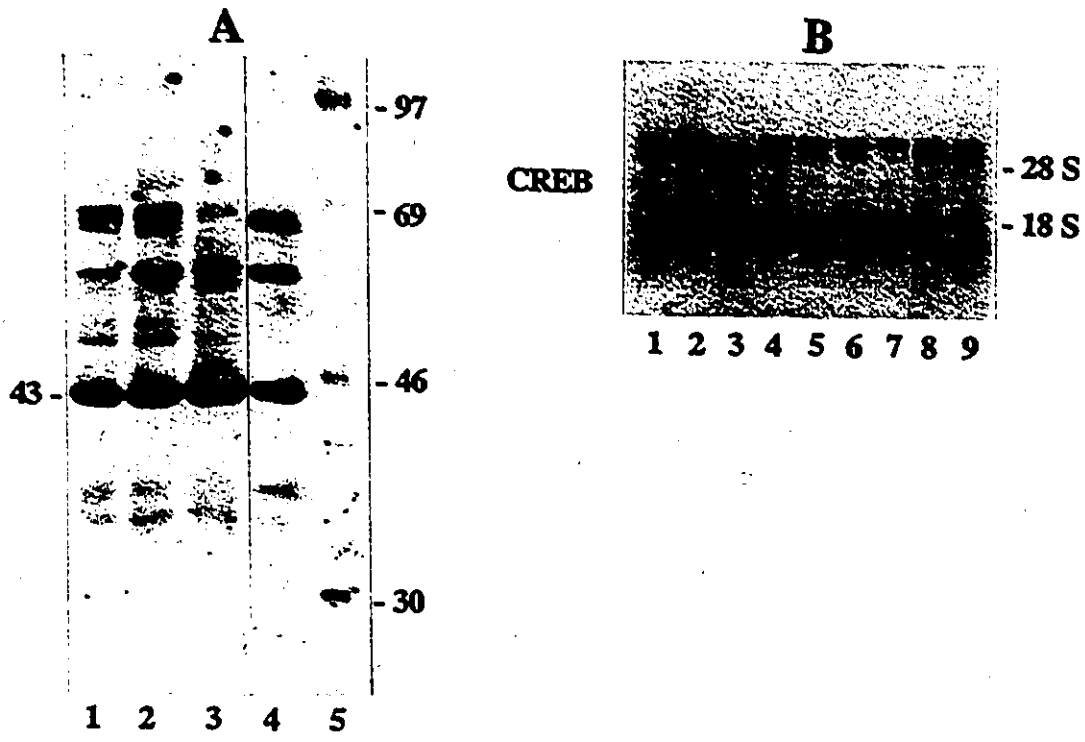
Analysis of CREB mRNA and Protein Level Following Partial Hepatectomy

While the CRE-binding activity of the 47 kDa CREB factor was significantly perturbed during the early stages of liver regeneration it was of interest to establish whether any changes in other CREB proteins were also induced by partial hepatectomy in proliferating liver cells. Protein levels were assessed by Western blotting of nuclear proteins from liver at various times after HPX with the same polyclonal antibody raised against the CREB protein described in Figure 14. Figure 32A showed that, by Western blotting, the level of CREB protein remained essentially unchanged during the first 24 h following HPX. Similarly, the level of CREB mRNA, as assessed by Northern blotting (Fig. 32B), did not change either. However, liver tissue expressed multiple mRNA species of CREB which is consistent with the observations of others (290,292), and represented different isoforms of the protein produced by alternative splicing of the

Figure 32. Analysis of CREB-341/327 mRNA and protein levels in rat liver following partial hepatectomy

Panel A: Western blot with peptide-derived rabbit polyclonal anti-CREB-341/327 serum.
100 μg of nuclear proteins prepared from HPX rats as described in the Methods section and as in Fig. 25 were resolved by 8.5% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (100 mA, 16 h). The membrane was then blocked followed by incubation with anti-CREB antiserum at a dilution of 5 μl/ 10 ml as described in Fig.13. Polypeptides recognized by the antiserum were detected by ^{125}I-labeled Protein A and then visualized by autoradiography with an exposure time of five days.
Lane 1 - nuclear proteins from control intact rat liver, lanes 2 to 4 - nuclear proteins from rat livers 3, 6, 24 h after HPX, lane 5 - ^{14}C-labeled molecular size markers. The calculated molecular size of the major polypeptide recognized by the antiserum is indicated on the left in kilodaltons.

Panel B: Northern blot of rat liver poly (A)+ RNA hybridized with a 0.6 kbp AvaII/Stu I restriction fragment of the CREB 341 cDNA.
Total cellular RNA was prepared and poly(A)+ was selected as described in the Methods section and in the legend to Fig. 25. Northern blotting was performed as described in Fig. 25. Expression of CREB mRNA was then detected by autoradiography with an exposure time of two days.
Lane 1 - mRNA from control intact rat liver, lane 2 - mRNA from rat liver 0.5 h after a sham operation, lanes 3 to 5 - mRNA from rat liver 0.5, 1, 2 h after HPX respectively, lane 6 - mRNA from rat liver 3 h after a sham operation, lanes 7 to 9 - mRNA from rat livers 3, 4, 6 h after HPX.
The positions of the 28S and 18S ribosomal RNA subunits are indicated at the right


A
97
69
43
46
30
1 2 3 4 5
B
CREB
28 S
18 S
1 2 3 4 5 6 7 8 9

gene. There did not appear to be any difference in the levels of CREB expression in control liver, sham operated or HPX liver suggesting that the constant level of CREB protein detected by Western blotting is a result of constitutive CREB mRNA synthesis. The absence of the larger mRNA species from the sample prepared 0.5 h after HPX was not reproducible and is an experimental error. It is clear that the changes in the 47 kDa protein's CRE-binding activity did not coincide with changes in either the level of the CREB protein or its mRNA, providing further evidence that the two proteins are not only distinct, but also appear to be differentially regulated during liver regeneration.

Comparison of CRE-binding Protein Profiles and CREB mRNA Expression Between Rat Liver and Morris Hepatoma 5123tc Cells in Culture

Since the CRE-binding activities of Morris hepatoma 5123tc cells were similar to, but not identical with, those seen in normal liver (see Figures 11 and 19), a comparison between their CRE-binding activities in Southwestern blots and CREB expression patterns in Northern blots was performed to establish whether the differences could be accounted for by altered CREB gene expression. Figure 33A showed the differences in CRE-binding activities between the Morris hepatoma cells (lane 2), and normal rat liver (lane 3). It is important to note that these samples were probed for CRE-binding with the somatostatin CRE in order to emphasize the existence of multiple CRE-binding proteins in these samples. The Southwestern blotting profiles were compared to the expression of the CREB gene by Northern blotting (Fig. 33B). Clearly the different patterns of CRE-binding activities correlated with different mRNA species present in

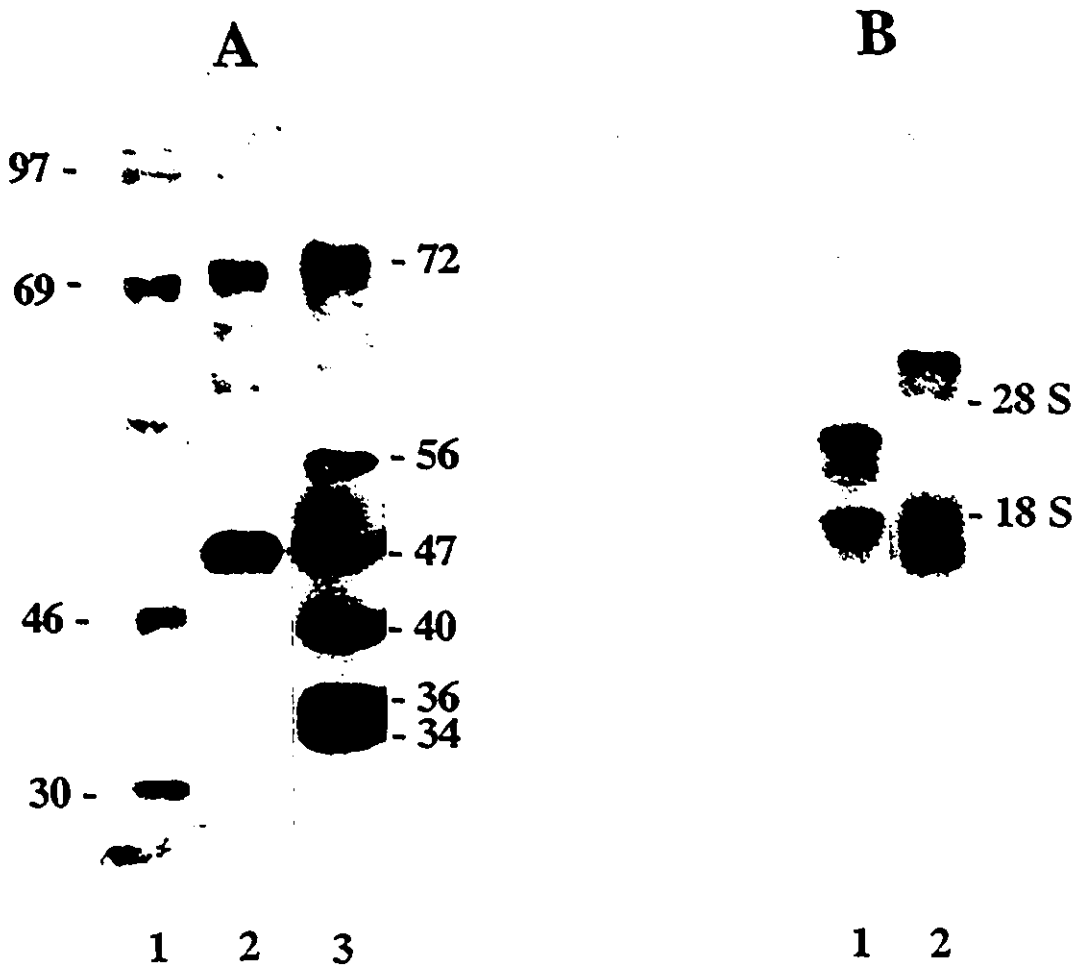
these cells. The appearance of mRNA isoforms in the Morris hepatoma cells which were distinct from those recognized in the liver tissue may indicate the presence of novel CREB isoforms in the hepatoma cells, however this remains to be confirmed.

Together, the results presented in this section demonstrated that c-*fos* expression was transiently induced both in serum stimulated Morris hepatoma cells and following HPX, and that, in both cases this transient expression correlated with a transient loss of CRE-binding activity, particularly of the 47 kDa CREB factor. This property together with its absence from solid Morris hepatoma tissue suggested that deregulation of the DNA-binding property of this 47 kDa CREB factor could be an important early event associated with aberrant c-*fos* transcription and cellular transformation. This possibility was further studied by examining both CRE and SRE-binding activities during early and later stages of chemically induced hepatocarcinogenesis.

Figure 33. The profiles of CRE-binding proteins and CREB mRNA in normal rat liver and Morris hepatoma 5123tc cells

Panel A: Southwestern blot with the somatostatin CRE sequence. 100 μg of nuclear proteins from Morris hepatoma 5123tc cells in culture (lane 2) or normal rat liver (lane 3) were analyzed for CRE-binding activity by Southwestern blotting as described in the legend to Fig. 11 including the renaturation step. ^{14}C-labeled molecular size markers are shown in lane 1. The Southwestern blot was exposed for two days and molecular sizes of CRE-binding proteins were calculated from a polynomial standard curve drawn through the positions of the markers and are indicated in kilodaltons.

Panel B: Northern blot of poly(A)+ RNA hybridized with a ^{32}P-labeled 0.6 kbp AvaII/StuI restriction fragment of the CREB-341 cDNA. The RNA was isolated from Morris hepatoma 5123tc cells in culture (lane 1), or from normal rat liver (lane 2), resolved on an agarose/formaldehyde gel, transferred onto a nylon filter and blotted as described in the Methods section and in Fig. 18. CREB expression was visualized by autoradiography with a two day exposure and the positions of the 28 S and 18 S ribosomal RNA species are indicated on the right.


A
B
97 -
69 -
46 -
30 -
- 72
- 56
- 47
- 40
- 36
- 34
- 28 S
- 18 S
1 2 3
1 2

Part 3: Analysis of c-*fos* Promoter-Binding Proteins During Chemically Induced Hepatocarcinogenesis: The Resistant Hepatocyte Model

It is well established that chemically induced hepatocarcinogenesis proceeds via a multi-step mechanism involving the stages of initiation, promotion and progression (reviewed in 327). Although the molecular mechanisms involved in the sequence of events leading to carcinoma formation are not fully understood, it is clear that phenotypic changes seen during experimental hepatocarcinogenesis are accompanied by both biochemical and genetic alterations. Several well defined aberrations in enzyme activities involved in carbohydrate metabolism have been reported in neoplastic nodules (328). Key enzymes in the glycolytic pathway show increased activity while those of the gluconeogenic pathway demonstrate reduced activity in both preneoplastic and neoplastic hepatocytes (328,329). Accompanying the enzyme perturbations in carbohydrate metabolism are changes in the balance of the xenobiotic metabolic enzymes (reviewed in 329).

A common feature of neoplastic cells is their hyperproliferative capacity and the ability to flourish under conditions which do not favour normal cell growth. A number of investigators have probed the expression of the family of immediate early genes during both normal hepatic cell growth and experimentally induced hepatocarcinogenesis and found aberrant patterns of expression during carcinogenesis (321,324,330-333). For example, high levels of c-*fos* expression have been reported in chemically induced hepatocarcinogenesis beginning as early as 8 days after carcinogen administration and persisting for a period of at least 70 weeks (324). In the same study, increased c-*fos* expression was also observed in hepatoma cells in culture. These results combined

with the documented role of both c-*fos* and c-*jun* in proliferation (reviewed in 119), suggest the possibility that altered expression of these genes may play a significant role in cellular transformation.

Although it is clear that the expression of this gene is both induced and inappropriately regulated in neoplastic nodules and in hepatomas, the basis for this lack of tight genetic control has not been demonstrated. Changes in the DNA-binding properties of the 47 kDa CRE-binding protein were observed following serum stimulation of Morris hepatoma 5123tc cells and following HPX in normal rat hepatocytes. Furthermore, this protein was completely absent from the solid Morris hepatoma 5123tc (334). The following studies were undertaken to determine at which point during the process of hepatocarcinogenesis this specific loss occurs and to assess the possible contribution changes in DNA-binding activities make towards the aberrant expression of the c-*fos* gene. To analyze changes in DNA-binding protein activities during initiation and progression the Solt and Farber model of chemically induced hepatocarcinogenesis was employed (300). This model was chosen because earlier studies show that the distinctive sequential pattern of hepatic morphological changes parallel the multi-step nature of hepatocarcinogenesis (335). Since multiple regulatory sequence elements govern c-*fos* expression, interactions of transcription factors with the c-*fos* serum response element (SRE), cAMP response element (CRE), and to some extent the *sis*-conditioned medium response element (SCMRE), were examined in an attempt to understand the nature of aberrant c-*fos* expression during hepatocarcinogenesis.

Magnetic Resonance Imaging of Rats During Chemically Induced Hepatocarcinogenesis

The progression of hepatocarcinogenesis was followed by the noninvasive procedure of *in situ* whole animal magnetic resonance imaging (MRI). MRI was used to asses the status of individual rats at various stages without the need for sacrifice in order to perform a histopathological examination of the liver. The results of these MRI studies are summarized in Figure 34. The image shown in panel A is that of an untreated control animal. Images taken from various slices through the same plane did not reveal any apparent abnormalities in the control liver. Panels B and C showed the MRI of two individual rats 4 months after carcinogen administration. The livers of both of these rats contained nodular lesions which were easily visible and appeared as light, spherical areas. At the time of imaging, in this case, the smallest nodules measured less than 1 cm^2. The progression of the lesions was examined 6 months after carcinogen administration and the results of 2 individual rats are presented in panels D and E. Both rats were shown to harbour hepatic lesions which had increased both in size and in number compared to the images taken after 4 months. In these images, the kidneys are clearly visible and show no abnormalities indicating that while the lesions had grown, they did not metastasize to the kidneys. The lungs were also scanned for lesions by MRI since this is the primary site of metastasis, but showed no abnormalities at this stage of hepatocarcinogenesis. Rats were also analyzed at 9 months after the carcinogenic regimen and the MRI revealed a massive hepatic lesion in addition to multiple smaller ones (panel F). Despite the presence of these lesions comprising approximately 30% of the liver mass, the animal did not appear to be in any distress.

Figure 34. Magnetic resonance imaging of rat liver during chemically induced hepatocarcinogenesis

Hepatocarcinogenesis was initiated with a single intraperitoneal injection of diethylnitrosamine (DENA), at a dose of 20mg/100g body weight in 0.9% sterile saline. Two weeks after DENA injection, rats were fed by gavage with 20mg/kg body weight of 2-acetoaminofluorine (2-AAF), as described in the Methods section. Following the last day of 2-AAF administration the rats were subjected to 2/3 hepatectomy under halothane anesthesia (299).
Rats were anesthetized and placed in a shielded gradient coil and place in a magnetic field of 4.7 tesla for imaging as described in the Methods section. Total image acquisition time was approximately 30 min. Images were obtained in coronal section.

Panel A: MRI of a control intact rat liver.

Panel B: MRI of a rat liver 4 months after initiation of hepatocarcinogenesis.

Panel C: MRI of a rat liver 4 months after initiation of hepatocarcinogenesis.

Panel D: MRI of a rat liver 6 months after initiation of hepatocarcinogenesis.

Panel E: MRI of a rat liver 6 months after initiation of hepatocarcinogenesis.

Panel F: MRI of a rat liver 9 months after initiation of hepatocarcinogenesis.


A
B
C
D
F

The use of MRI provided a means of screening experimental animals for hepatic lesions and following the progression of lesion growth.

Histological Analysis of Rat Liver Sections During Chemically Induced Hepatocarcinogenesis

Experimental animals were sacrificed for histological and biochemical analyses of their livers at different stages of carcinogenesis. Histological examination confirmed the well characterized morphological changes seen in previous studies at different stages of the Solt and Farber model of hepatocarcinogenesis (335). Figure 35 showed histological staining of liver sections with neutral red which, under these conditions, revealed regions of basophilia indicative of active RNA synthesis. Panel A showed the characteristic homogeneous neutral red staining pattern seen in control rat liver. The neutral red staining pattern of rat livers 1 week after HPX displayed basophilia (panel B), consistent with the proliferative activity of hepatocytes. However, there was a clear difference in the neutral red staining pattern of liver sections collected from animals 1 week after both carcinogen treatment and proliferative stimulus (panel C). A pronounced basophilia was seen in the livers of animals at this stage of hepatocarcinogenesis suggesting an enhanced level of gene expression in these livers. Four weeks after the carcinogenic regimen the extensive basophilia had subsided, but a number of lighter staining nodule-like lesions appeared as shown in panel D. The surrounding liver tissue maintained a normal morphology indicating that the nodular regions had been altered as a result of the carcinogen treatment. As seen on MRI, during the progression of carcinogenesis over a period of 6-9 months some of the hepatic nodules persisted and numbered from 2-4 nodules/liver.

Figure 35. Histology of rat liver sections following hepatocarcinogenesis

Rat liver sections were obtained from rats treated with a regimen inducing hepatocarcinogenesis as described in the Methods section and in Fig. 31. Liver slices were placed in Carnoy's fixative, paraffin embedded and sectioned. The sections were then stained with neutral red as described in the Methods section.

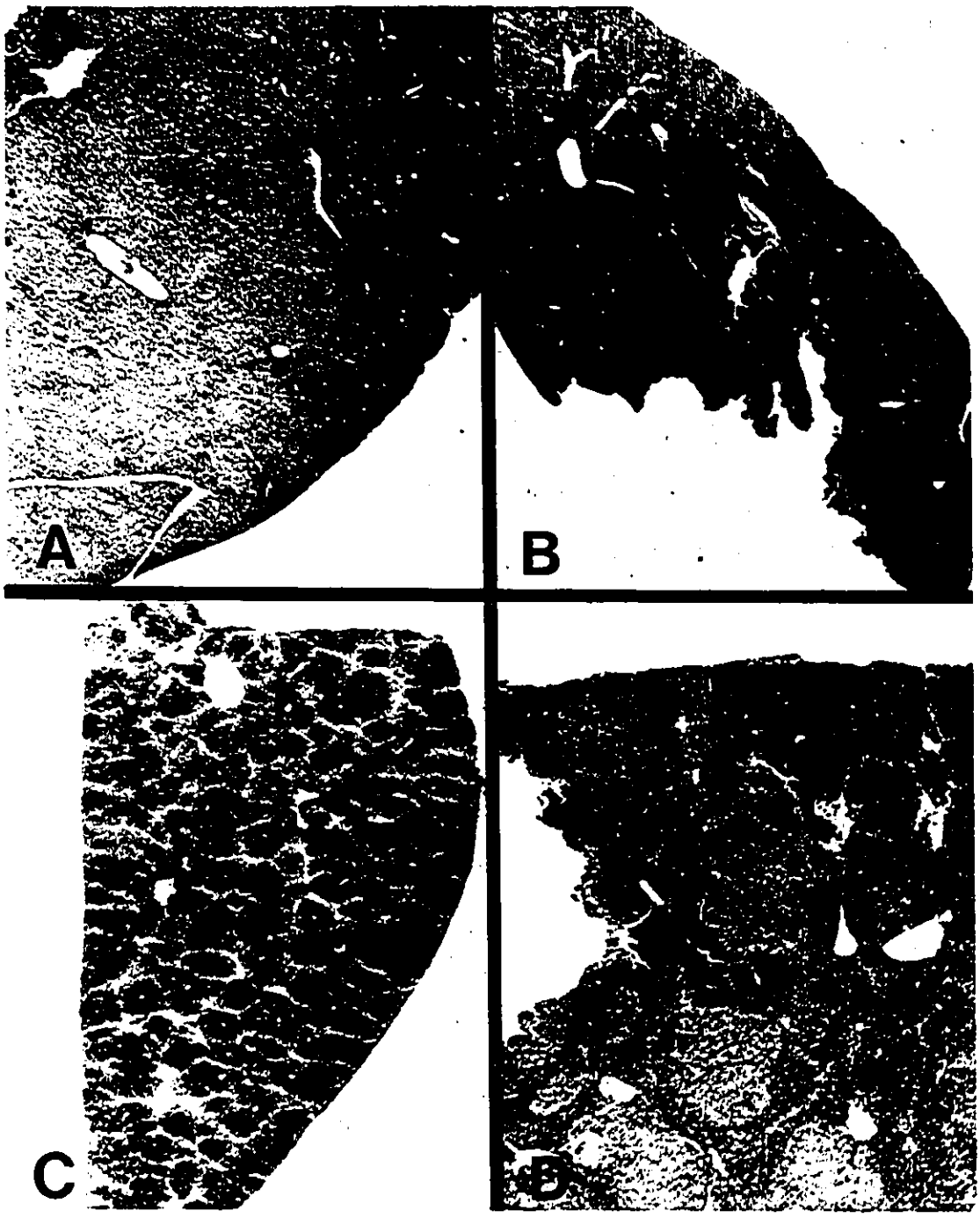
Panel A: Control normal rat liver section.

Panel B: Rat liver section one week after HPX.

Panel C: Rat liver section one week after HPX and carcinogen treatment.

Panel D: Rat liver section four weeks after HPX and carcinogen treatment.

Stained liver sections were examined by light microscopy and are shown at a magnification of 50 X.


A
B
C
B

Further histological examination of experimental liver samples was performed using hematoxylin and eosin staining to highlight cytoplasmic and nuclear morphology (Fig. 36). As early as 3 days after the carcinogenic treatment there were clear morphological alterations consisting of darker staining nuclei and a patchy cytoplasmic appearance (panel B), as compared to an even staining pattern in the cytoplasm and nuclei seen in control liver sections (panel A). By 1 week following the experimental regimen (panel C), even more dramatic cellular alterations and abnormal tissue architecture were apparent. However, most of the early cellular alterations have been shown to be transient with normal liver morphology and function restored at later times.

Despite this tissue remodeling, a few nodules remained and have the capacity to develop into an hepatocellular carcinoma. Histological examination of a nodule persisting for 9 months was performed and compared to the fast growing and malignant solid Morris hepatoma 5123tc to establish whether it had acquired the properties of an hepatocellular carcinoma (panels D and E). Histologically, the nodule had the typical "ground glass" appearance which has been observed in a number of prior studies (336). Several morphological features of the Morris hepatoma were common to those seen in the nodule. Both had a lightly staining cytoplasm and enlarged nuclei with centrally located prominent nucleoli and multiple mitotic figures. Despite their similarities, the morphology of the nodule was still distinct from that of the malignant tumour.

Analysis of Gene Expression During Chemically Induced Hepatocarcinogenesis

Histological examination of experimental livers indicated that morphological abnormalities occurred during early stages in the Solt and Farber model of

Figure 36. Histology of rat liver sections following hepatocarcinogenesis

Rats were treated with chemical carcinogens as described in the Methods section and in Fig. 34. Liver sections were fixed in Carnoy's fix for 4 h and then transferred to 90% ethanol. Tissue sections were processed for histology as described in the Methods section and stained with HPS (hematoxylin/ Phloxine B/ Alcohol saffron de Gatinais).

Panel A: Stained section from a control normal rat liver.

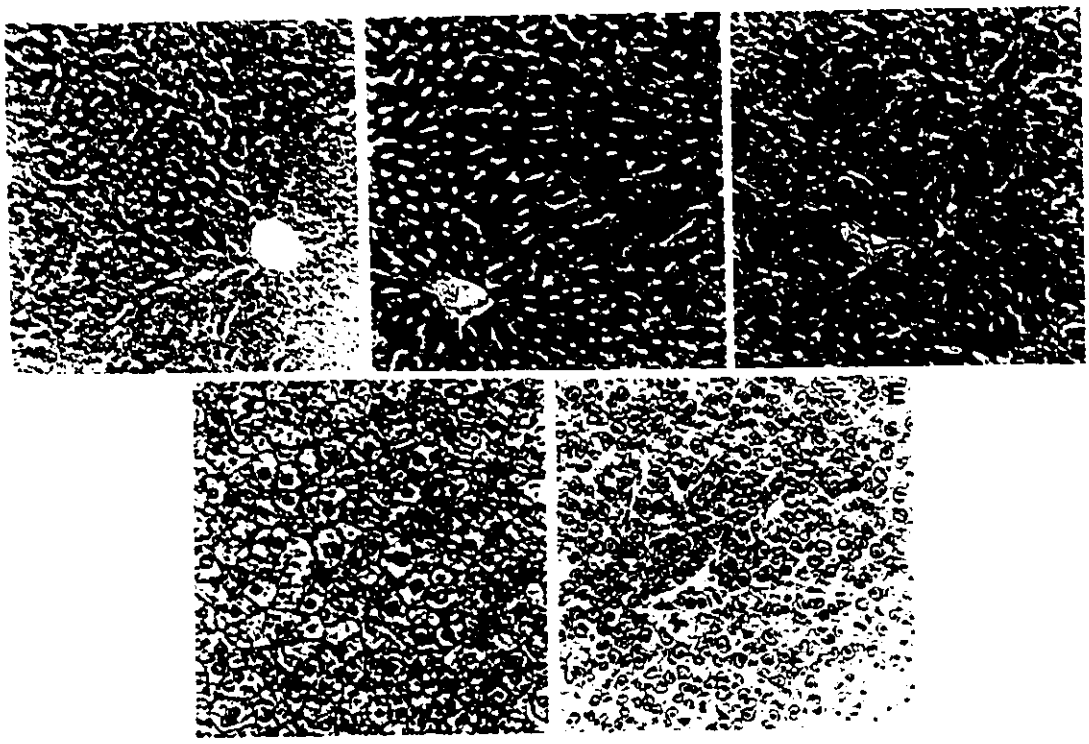
Panel B: Stained section of rat liver 3 days after HPX and carcinogen treatment.

Panel C: Stained section of rat liver 7 days after HPX and carcinogen treatment.

Panel D: Stained section of a nodule developed in a rat liver 9 months after the experimental regimen of carcinogenesis.

Panel E: Stained section of a solid Morris hepatoma 5123tc.

The stained sections were examined by light microscopy. Panels A-C are shown at a magnification of 500 X. Panels D and E are shown at a magnification of 1250 X



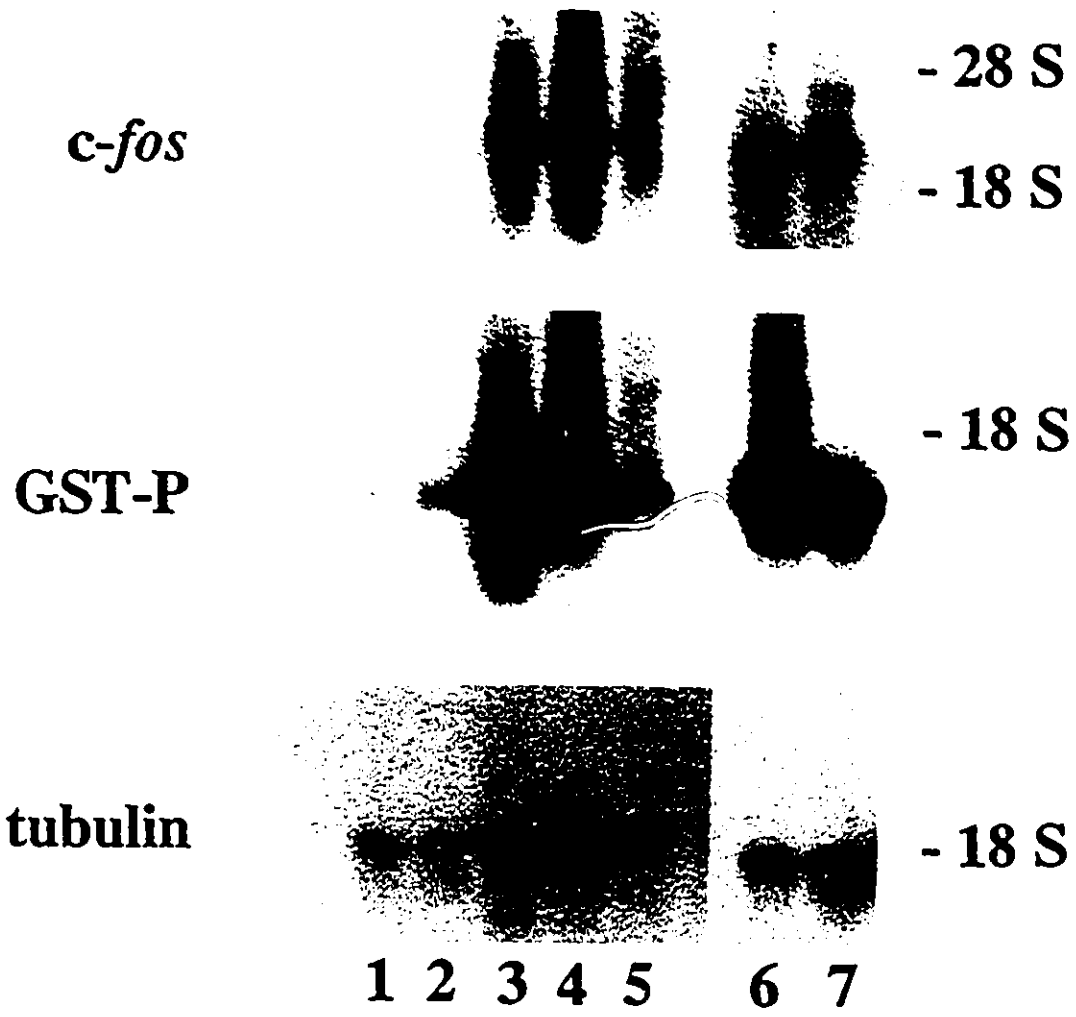
hepatocarcinogenesis which might have resulted from aberrant gene expression. It was of interest to examine the changes in c-*fos* gene expression which were reported to occur under similar experimental conditions in earlier studies (324,332). It was particularly important to establish the role that aberrant regulation of this gene might have played in the etiology of nodule formation and development. The results shown in Figure 37 confirmed that c-*fos* expression was increased in the livers of experimentally treated rats as early as 1 week after HPX and remained elevated for at least 3 weeks after HPX. Furthermore, in a nodule isolated 9 months after the experimental treatment, c-*fos* expression was also elevated (lane 6), as it was in a solid Morris hepatoma tumour (lane 7), in comparison with intact liver (lane 1), or liver taken from a rat 1 week after feeding with carcinogen alone (lane 2). The prolonged elevation in c-*fos* expression clearly represented a departure from the normally tight regulation of this gene and may be, at least in part, responsible for the abnormal phenotype observed histologically.

As mentioned above, earlier studies showed that during hepatocarcinogenesis there was an increase in the expression of genes coding for the xenobiotic metabolizing enzymes, particularly of the glutathione-S-transferase gene and that the isozymic variant expressed is the placental type (GST-P). The same liver RNA samples were probed for the expression of this gene since the synthesis of this isozyme is a characteristic feature of hepatocarcinogenesis. It can be seen clearly that GST-P expression was strongly induced by 1 week after hepatectomy and remained elevated throughout the hyperplasia and into the nodule formation stage. GST-P levels remained highly induced both in the nodule isolated 9 months after treatment and in the Morris hepatoma 5123tc.

Figure 37. Gene expression during chemically induced hepatocarcinogenesis

Livers from rats treated with the carcinogenic regimen described in the Methods section were removed and immediately placed in liquid nitrogen and stored at -80°C until all samples were collected. Total cellular RNA was isolated and poly(A)+ RNA selected according to the procedure described in the Methods section and in Fig. 25. The blot was hybridized with a ^{32}P-labeled probe consisting of a 2.2 kb restriction fragment of a c-*fos* cDNA. c-*fos* expression was visualized by autoradiography with an exposure time of two days. The c-*fos* probe was removed by boiling in distilled water for two minutes and then rehybridized with a radiolabeled probe consisting of a 460 nucleotide restriction fragment of part of the coding sequence of a GST-P cDNA clone. The expression of GST-P was visualized by autoradiography with an exposure time of six h. The filter was stripped again following the same procedure and rehybridized with a radiolabeled probe consisting of a restriction fragment of an α-tubulin cDNA clone and detected by autoradiography with an exposure time of 12 h.

Lane 1 - mRNA from control rat liver, lane 2 - mRNA from rat liver one week after carcinogen treatment, but not hepatectomized, lanes 3 to 5 - mRNA from rat livers 1, 2, 3 weeks after both carcinogen treatment and HPX, lane 6 - mRNA from a nodule developed in a rat liver 9 months after carcinogen and HPX treatments, lane 7 - mRNA from a solid Morris hepatoma 5123tc tumour.


c-fos
- 28 S
- 18 S
GST-P
- 18 S
tubulin
- 18 S
1 2 3 4 5 6 7

Comparatively low expression levels of GST-P were observed in the control liver samples. As a control of RNA loading, the same filter was probed for tubulin expression.

Analysis of c-*fos* Promoter-Binding Proteins During Chemically Induced Hepatocarcinogenesis

Since aberrant expression of the c-*fos* gene during hepatocarcinogenesis was observed it was of interest to determine the underlying mechanisms responsible for this deregulated expression. Therefore, the interactions occurring at the regulatory sites during elevated c-*fos* expression in the model of chemically induced hepatocarcinogenesis were studied in an attempt to define any changes. Figure 38 showed the Southwestern blotting pattern of nuclear proteins from control and treated rat livers probed with the c-*fos* SRE. Consistent with results already presented, there were three predominant proteins of molecular weight 112,67 and 36 kDa labeled by the SRE probe. Control and experimentally treated liver samples showed little difference in SRE-binding protein profiles between 1-3 weeks after the experimental regimen. It is interesting to note that on the Southwestern blot a SRE-binding protein of approximately 72 kDa bound the SRE probe and may have represented the same protein identified in the protein complex binding to the SRE by gel shift elution and SRE affinity chromatography (Figs.5 and 6). While the Southwestern blot analysis indicated that the SRE-binding protein profile was unchanged, there was a reduction in SRE complex formation in the gel shift assay seen at 1 week after HPX specifically in experimental animals (Fig. 38). This change was transient and SRE complexes formed with nuclear proteins from animals at 3 weeks after the experimental regimen remained essentially

Figure 38. Analysis of c-*fos* SRE-binding proteins by Southwestern blotting during chemically induced hepatocarcinogenesis

Liver samples were collected at different times after the experimental treatment. Nuclei were isolated according to the Triton X-100 washing procedure and nuclear proteins extracted as detailed in the Methods section.
100 μg of nuclear proteins were resolved by 8.5% SDS-PAGE and then electrotransferred onto a nitrocellulose filter (100 mA/ 16 h) without prior washing in buffer containing 4 M urea. The blot was blocked and then a ^{32}P-labeled oligonucleotide probe representing the c-*fos* SRE was incubated with the filter. SRE-binding proteins were visualized by autoradiography with an exposure time of 2 days.

Lanes 1, 4, 7 - SRE-binding proteins from rat liver 1, 2, 3 weeks after HPX alone, lanes 2, 5, 8 - SRE-binding proteins from rat liver 1, 2, 3 weeks after treatment with carcinogens alone, lanes 3, 6, 9 - SRE-binding proteins from rat liver 1, 2, 3 weeks after both carcinogen and HPX treatments.
The sizes of the main SRE-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated on the left in kilodaltons.

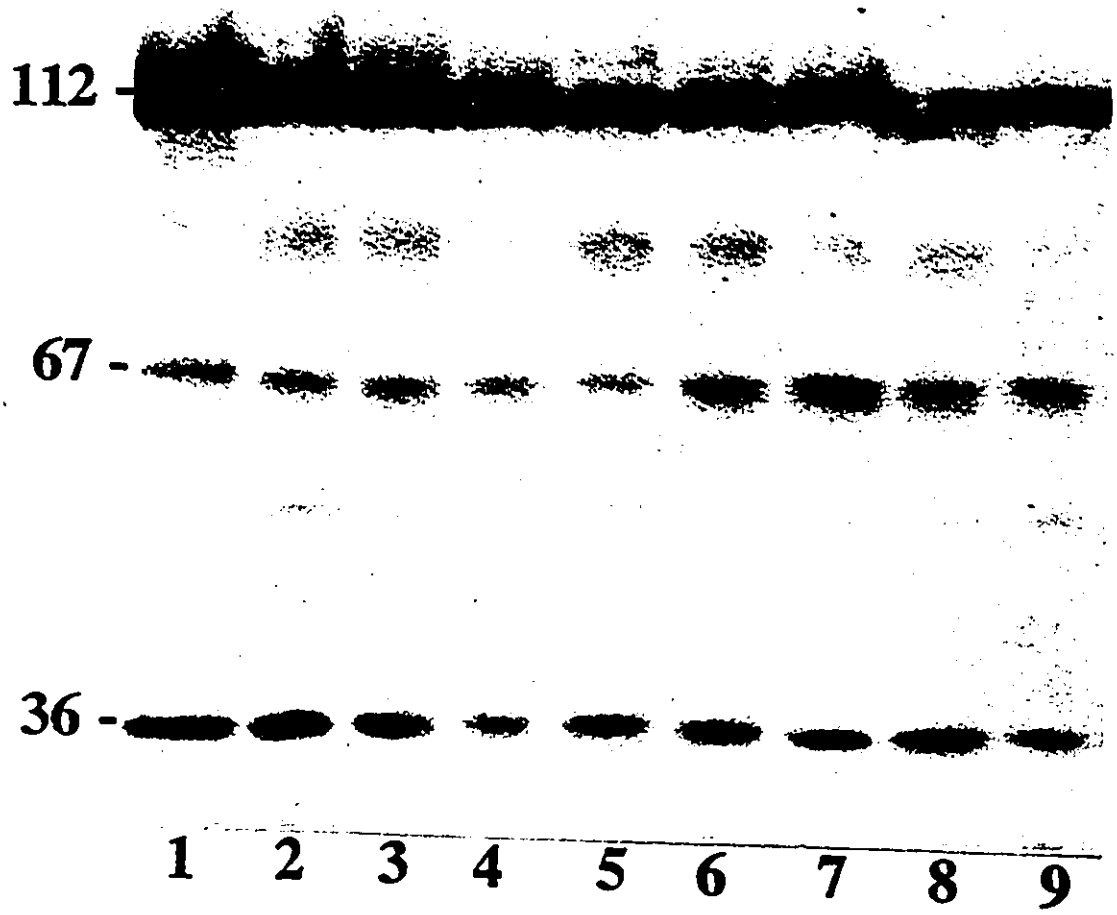
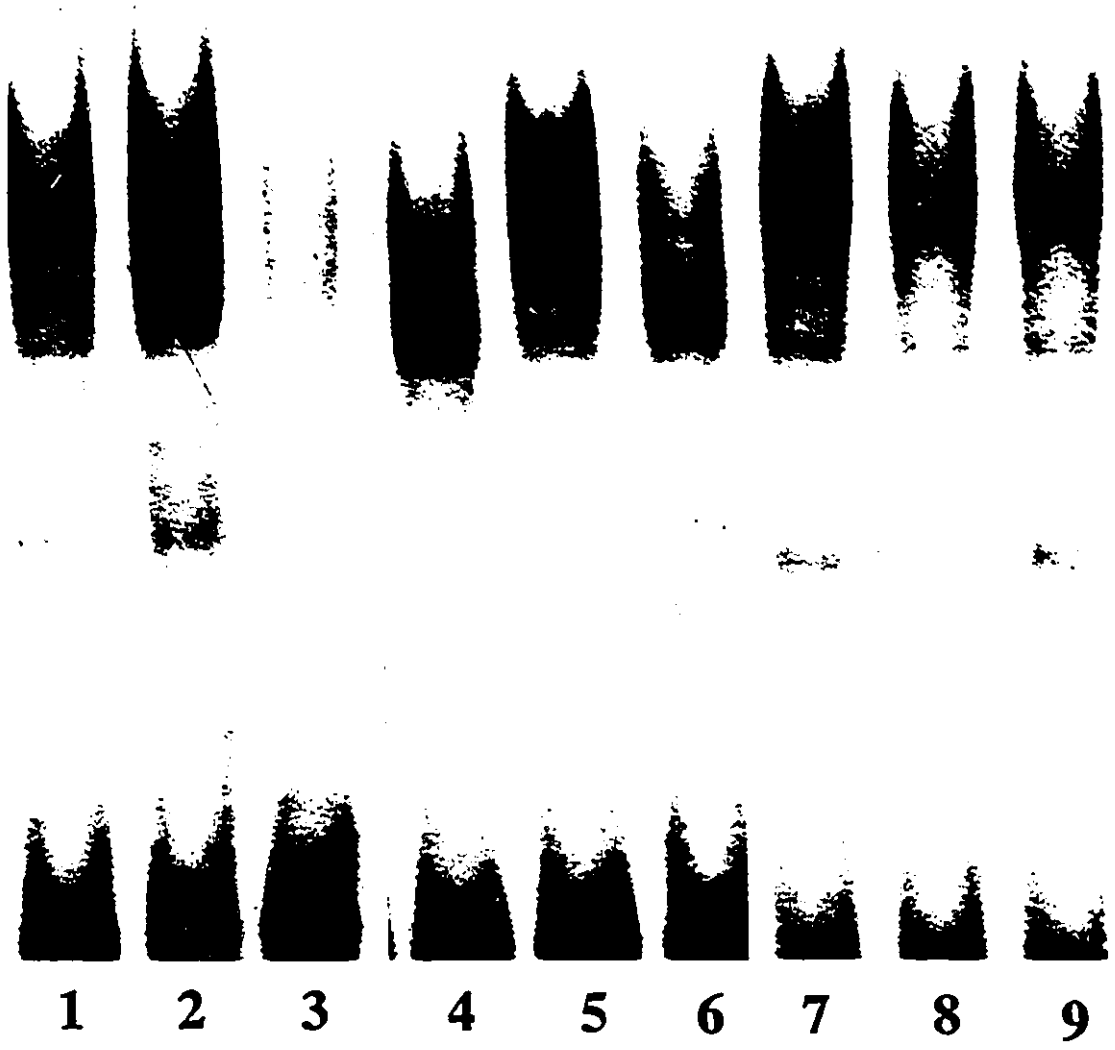

112
67
36
1
2
3
4
5
6
7
8
9

Figure 39. Gel shift analysis of c-*fos* SRE-binding proteins during chemically induced hepatocarcinogenesis

10 μg of nuclear proteins prepared as described in Fig. 38 were incubated at room temperature with a ^{32}P-labeled c-*fos* SRE probe in the presence of 2 μg of poly(dI-dC) as competitor DNA for 30 min. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel as detailed in the Methods section and were then visualized by autoradiography with an overnight exposure time.

Lanes 1, 4, 7 - SRE-binding proteins from rat livers 1, 2, 3 weeks after HPX alone, lanes 2, 5, 8 - SRE-binding proteins from rat livers 1, 2, 3 weeks after treatment with carcinogens alone, lanes 3, 6, 9 - SRE-binding proteins from rat livers 1, 2, 3 weeks after both carcinogen and HPX treatments.


1 2 3 4 5 6 7 8 9

indistinguishable from control samples. These results emphasized that although no apparent changes in SRE-binding proteins were evident by the Southwestern blot, the more sensitive gel shift assay was able to detect a transient decrease in complex formation.

Western blotting with an antibody directed against SRF indicated that the level of this protein remained unchanged during the experimental regimen (Fig. 40). The SRF antibody also recognized a protein of 45 kDa which has not been characterized so far, and whose level also remained constant. Therefore, a transient decrease in SRE complex formation seen in experimental animals was not due to the absence of SRF protein.

As was previously shown during proliferation of normal hepatocytes (Fig. 28), the most dramatic changes were found in c-*fos* CRE-binding activities during hepatocarcinogenesis (Fig. 41). In all of the experimentally treated animals up to 4 weeks after treatment, there was a dramatic reduction in the CRE-binding activity of the 47 kDa CREB factor. This loss of DNA-binding activity over a prolonged period of time correlated with increased c-*fos* expression similar to that seen during normal cell proliferation. There were also changes in the CRE-binding activity of the 56 and 72 kDa CRE-binding proteins occurring in experimental animals (lanes 3, 6, 9, 11).

Dramatic changes in CRE-binding protein activities in experimentally treated animals seen in Southwestern blots were also evident in gel shift assays (Fig. 42). Nuclear proteins from experimental animals analyzed up to 3 weeks after treatments did not interact with the c-*fos* CRE in this assay.

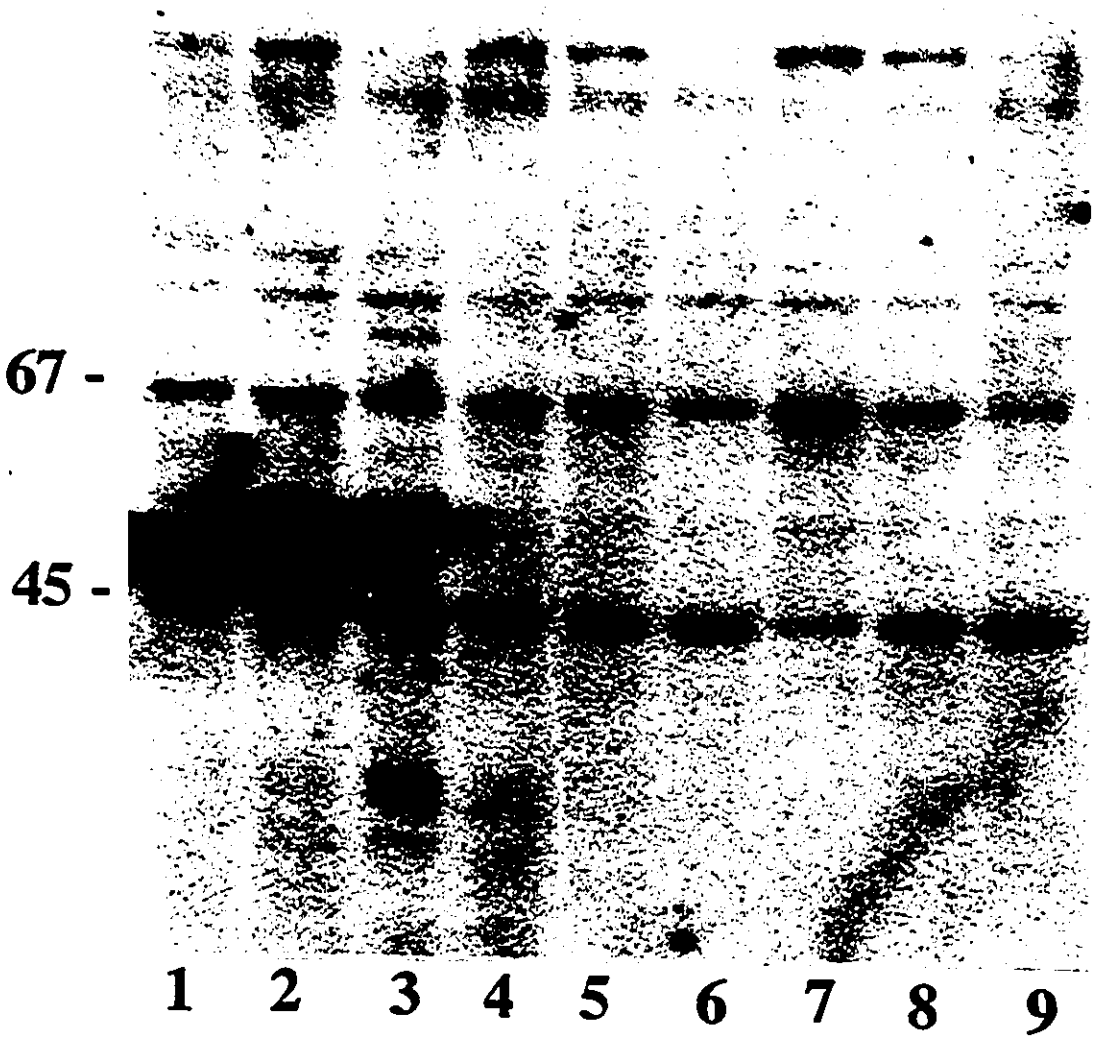

67 -
45 -
1 2 3 4 5 6 7 8 9

Figure 40. Western blot analysis of SRF protein during hepatocarcinogenesis

150 μg of nuclear proteins were resolved by 8.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane (100 mA/ 16 h) and the membrane was blocked in a buffer containing BSA as described in the Methods section and in Fig. 13. The filter was incubated with a peptide-derived rabbit polyclonal antibody raised against SRF at a dilution of 10 μl/ 10 ml, and then antibody - antigen complexes detected using ^{125}I-labeled protein A and visualized by autoradiography with a 9 day exposure time.

Lanes 1, 4, 7 - nuclear proteins from rat liver samples 1, 2, 3 weeks after HPX, lanes 2, 5, 8 - nuclear proteins from rat liver samples 1, 2, 3 weeks after carcinogen treatment alone, lanes 3, 6, 9 - nuclear proteins from liver samples 1, 2, 3 weeks after carcinogen feeding and HPX.

The molecular sizes of the major polypeptides recognized by the antiserum were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated on the left in kilodaltons.

Figure 41. Analysis of c-*fos* CRE-binding proteins by Southwestern blotting during hepatocarcinogenesis

Rat liver nuclear proteins were obtained at various times after treatments exactly as described in Fig. 34. Southwestern blotting included the gel renaturation step in 4M urea as described in the Methods section. The filter was then incubated with a ^{32}P-labeled c-*fos* CRE probe and then CRE-binding proteins were detected by autoradiography with an exposure time of 4 days.

Lanes 1, 4, 7, 10 - nuclear proteins from rat livers 1, 2, 3, 4 weeks after HPX alone, lanes 2, 5, 8, 11 - nuclear proteins from rat livers 1, 2, 3, 4 weeks after treatment with carcinogens alone, lanes 3, 6, 9, 12 - nuclear proteins from rat livers 1, 2, 3, 4 weeks after both carcinogens and HPX treatments.

The sizes of the main CRE-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated on the left in kilodaltons.

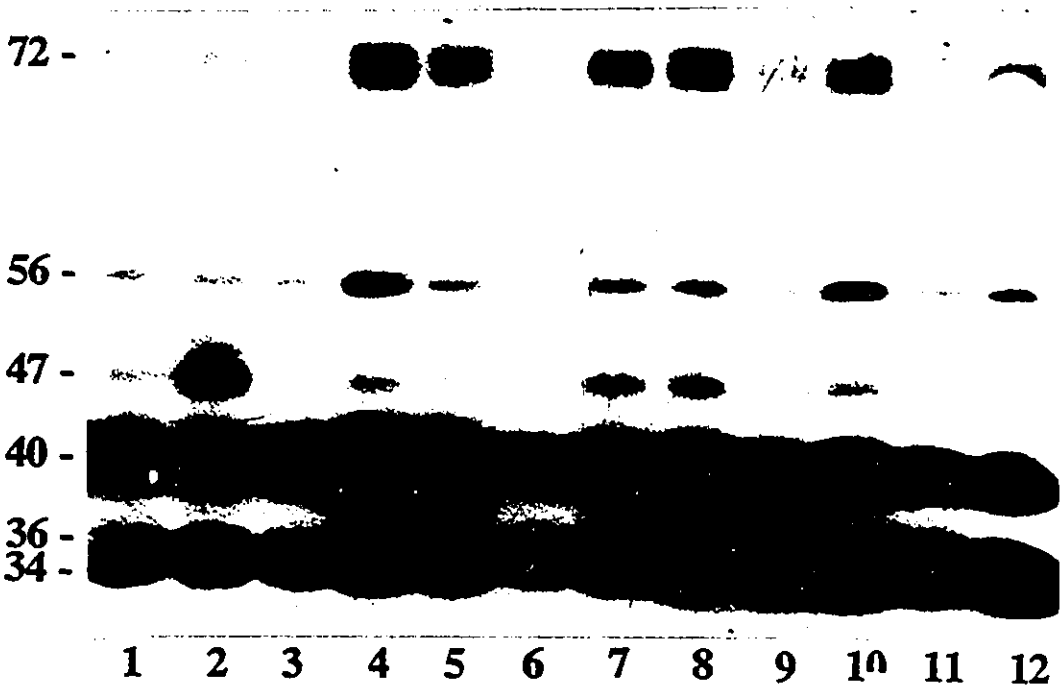

72 -
56 -
47 -
40 -
36 -
34 -
1 2 3 4 5 6 7 8 9 10 11 12

Figure 42. Gel shift analysis of c-*fos* CRE-binding proteins during chemically induced hepatocarcinogenesis

Nuclear proteins were prepared as described in Fig. 38. 10 μg of nuclear proteins were incubated at room temperature with a ^{32}P-labeled c-*fos* CRE probe in the presence of 2 μg of poly(dI-dC). DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel as detailed in the Methods section and were then visualized by autoradiography with an overnight exposure time.

Lanes 1, 4, 7 - nuclear proteins from rat livers 1, 2, 3 weeks after HPX alone, lanes 2, 5, 8 - nuclear proteins from rat livers 1, 2, 3 weeks after treatment with carcinogens alone, lanes 3, 6, 9 - nuclear proteins from rat livers 1, 2, 3 weeks after both carcinogens and HPX treatments.

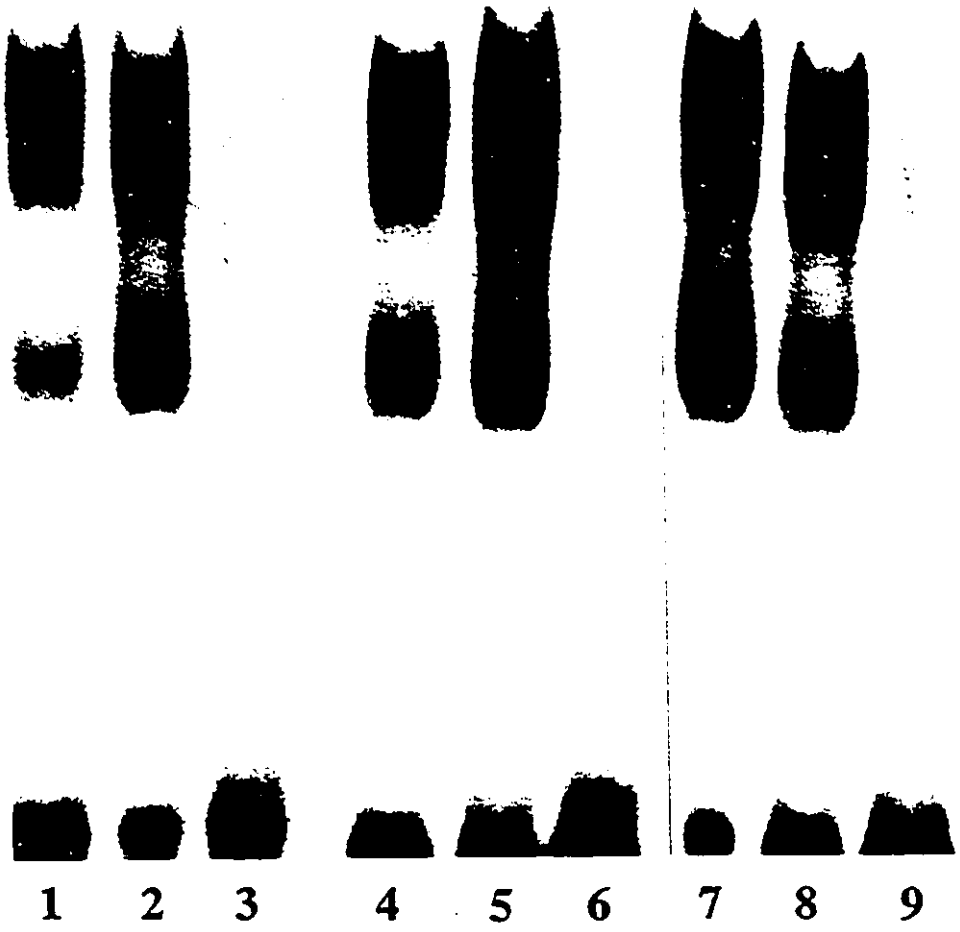

1 2 3 4 5 6 7 8 9

Figure 43. Western blot analysis of CREB-341/327 protein during hepatocarcinogenesis

Nuclear protein extracts prepared as described in Fig. 38 were resolved by 8.5% SDS-PAGE, transferred to a nitrocellulose filter and immunoblotted with a rabbit polyclonal anti-CREB serum as described in the Methods section and in Fig. 14 . Proteins recognized by the antiserum were detected by ^{125}I-labeled protein A and visualized by autoradiography with a 5 day exposure time.

Lanes 1, 4, 7 - nuclear proteins from rat livers 1, 2, 3 weeks after HPX alone, lanes 2, 5, 8 - nuclear proteins from rat livers 1, 2, 3 weeks after treatment with carcinogens alone, lanes 3, 6, 9 - nuclear proteins from rat livers 1, 2, 3 weeks after both carcinogen and HPX treatments.
The molecular size of the predominant polypeptide recognized by the antiserum was calculated and is indicated on the left in kilodaltons.

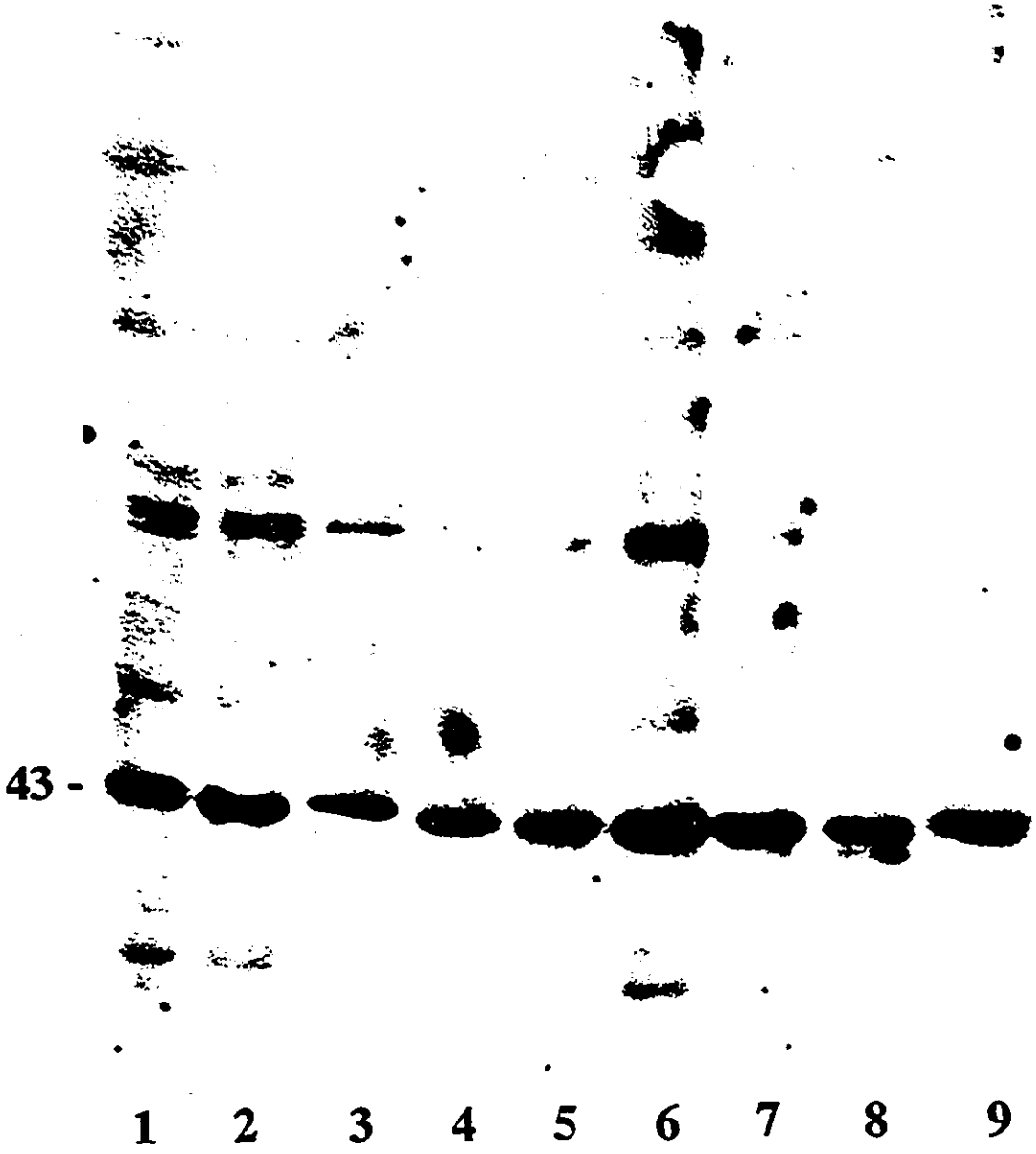
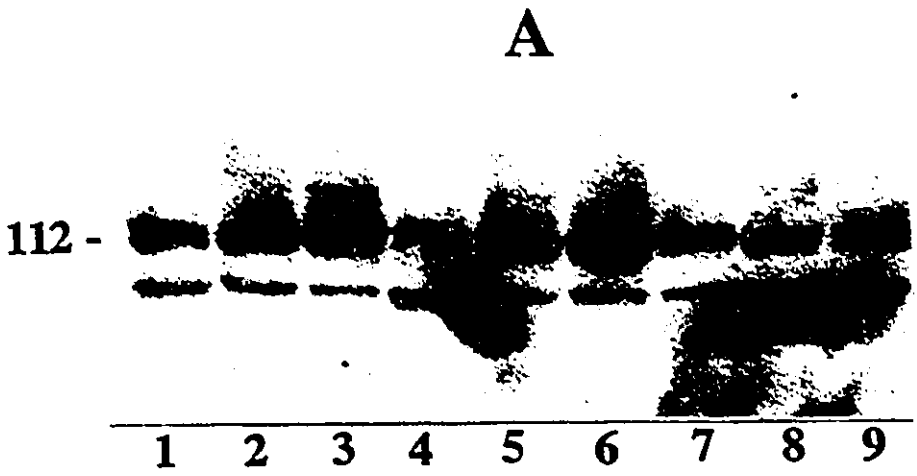

43 -
1
2
3
4
5
6
7
8
9

Figure 44. Analysis of c-*fos* SCMRE-binding proteins in rat liver following chemically induced hepatocarcinogenesis

Panel A: Southwestern blot with c-*fos* SCMRE sequence. Nuclear proteins from animals treated with the regimen of carcinogens were prepared exactly as described in the Methods section and in the legend to Fig. 38.
100 μg of nuclear proteins were resolved by 8.5% SDS-PAGE and then directly electrotransferred onto a nitrocellulose filter (100 mA/ 16 h). The filter was then blocked and incubated with a ^{32}P-labeled oligonucleotide probe representing the c-*fos* SCMRE. SCMRE-binding proteins were visualized by autoradiography with an exposure time of 2 days.

Lanes 1, 4, 7 - SCMRE-binding proteins from rat liver 1, 2, 3 weeks after HPX alone, lanes 2, 5, 8 - SCMRE-binding proteins from rat liver 1, 2, 3 weeks after treatment with carcinogens alone, lanes 3, 6, 9 - SCMRE-binding proteins from rat liver 1, 2, 3 weeks after both carcinogen and HPX treatments.
The calculated molecular size of the main SCMRE-binding protein was calculated from a polynomial standard curve drawn through the positions of co-electrophoresed ^{14}C-labeled markers and is indicated on the left in kilodaltons

Panel B: Gel shift analysis with the c-*fos* SCMRE sequence. 10 μg of nuclear proteins prepared as described in panel A were incubated at room temperature with a ^{32}P-labeled c-*fos* SCMRE probe in the presence of 2 μg of poly(dI-dC) for 30 min. DNA-protein complexes were visualized by autoradiography with an overnight exposure time.
Lane 1 - nuclear proteins from rat liver one week after HPX alone, lane 2 - nuclear proteins from rat liver one week after treatment with the carcinogenic regimen alone, lane 3 - nuclear proteins from rat liver one week after both the carcinogenic regimen and HPX treatments.


A
112 -
1 2 3 4 5 6 7 8 9
B
1 2 3

during the early stages was maintained in the preneoplastic and in the fully transformed hepatocytes. Notably, the 47 kDa CRE-binding activity was completely absent in these two tissues, consistent with previously published data (334). While the SRE-binding activity of the 112 kDa protein was not altered in the nodule or in the hepatoma, there was a decrease in the SRE-binding activity of the 67 kDa SRF in the nodule (panel B). Conversely, the 36 kDa SRE-binding protein was present at control levels in the nodule, but was decreased in the hepatoma. These results suggest that while no significant alterations in SRE-binding activity were observed during the early stages of hepatocarcinogenesis, the perturbations seen at later stages may contribute to the constitutively elevated expression of c-*fos* observed in isolated nodules and in hepatomas.

Clearly, elevated expression of the c-*fos* gene correlated with a decrease in CRE-binding proteins at all stages examined. Furthermore, the most consistent change seen during elevated expression of the c-*fos* gene at all stages was the loss of CRE-binding activity of the 47 kDa CREB factor.

Since changes in the DNA-binding property of the 47 kDa CREB protein correlated with the transcriptional activity of the c-*fos* gene, it was important to compare the levels of CREB expression with profiles of CRE-binding proteins in cells and tissues with different levels of c-*fos* expression levels such as in quiescent and proliferating liver cells, hepatic nodules, malignant hepatoma, and hepatoma cells in culture (Fig. 46). The profiles of CRE-binding proteins identified by Southwestern blotting are shown in panel A. The corresponding Northern blot of CREB expression is shown in panel B. Normal and proliferating liver cells expressed the same mRNA species of the

Figure 45. The patterns of c-*fos* CRE-binding and SRE-binding proteins in a hepatic nodule and in 5123tc Morris hepatoma tumour

Panel A: Southwestern blot with c-*fos* CRE sequence. Nuclei were isolated by the Triton X-100 method and nuclear proteins extracted as described in the Methods section.
100 μg of proteins were resolved by 8.5% SDS-PAGE, renatured in a buffer containing 4 M urea and electrotransferred onto a nitrocellulose filter (100 mA/ 16 h). The filter was blocked and then incubated with a ^{32}P-labeled c-*fos* CRE probe. The CRE-binding proteins were visualized by autoradiography with an exposure time of 2 days.

Lane 1 - nuclear proteins from normal rat liver, lane 2 - nuclear proteins extracted from a liver nodule developed 9 months after the carcinogenic regimen, lane 3 - nuclear proteins from a solid Morris hepatoma 5123tc tumour.

Panel B: Southwestern blot with c-*fos* SRE sequence. The same protein samples described in panel A were resolved by 8.5% SDS-PAGE and then directly electrotransferred onto nitrocellulose. The filter was blocked and then incubated with a ^{32}P-labeled c-*fos* SRE probe. SRE-binding proteins were visualized by autoradiography with an exposure time of 2 days.

Lane 1 - nuclear proteins from normal rat liver, lane 2 - nuclear proteins extracted from a liver nodule developed 9 months after the carcinogenic regimen, lane 3 - nuclear proteins from a solid Morris hepatoma 5123tc tumour.

The molecular sizes of the CRE-binding and SRE-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated in kilodaltons.

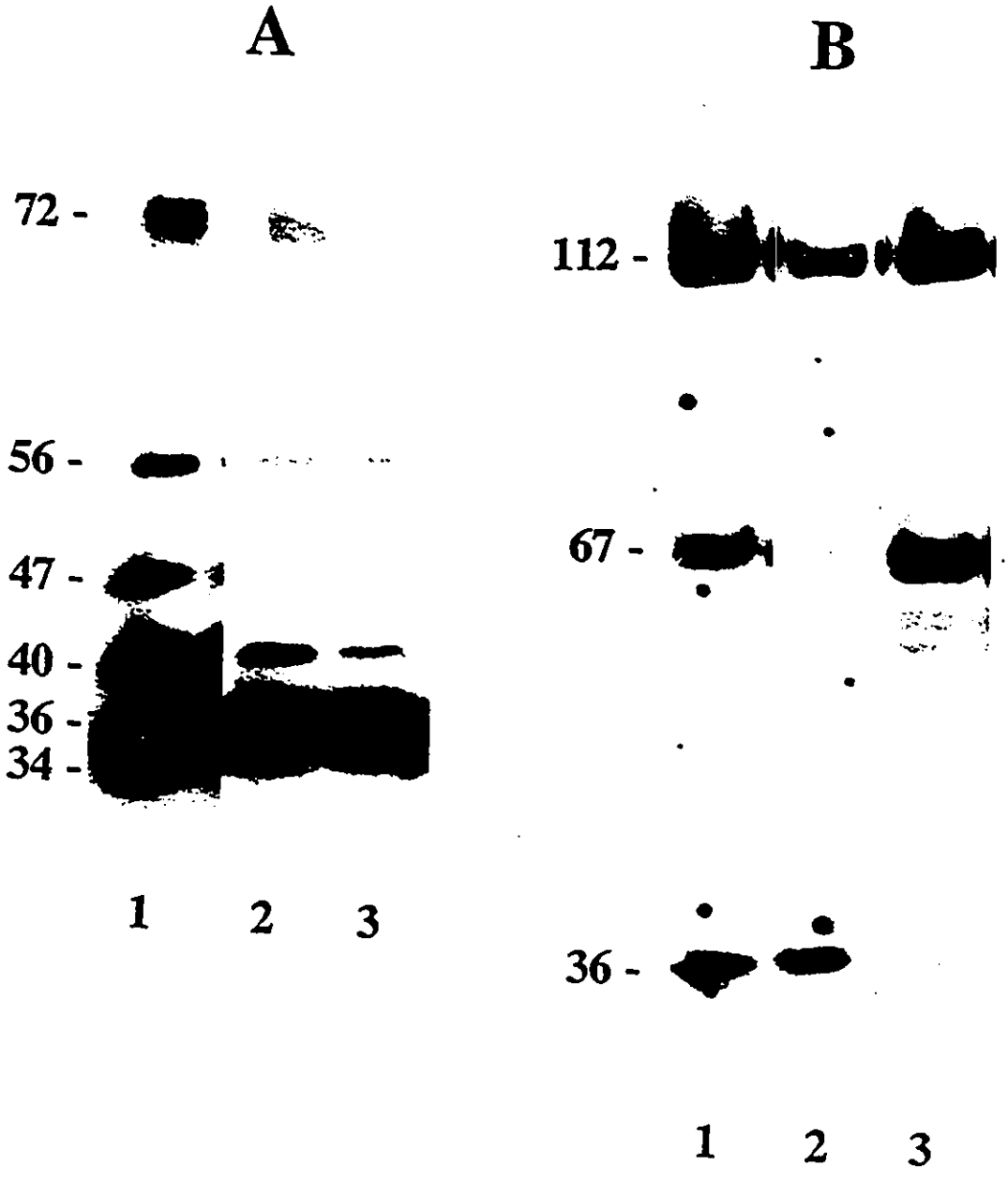

A
B
72 -
56 -
47 -
40 -
36 -
34 -
1
2
3
112 -
67 -
36 -
1
2
3

Figure 46. Comparison of CRE-binding protein patterns and CREB mRNA expression in different rat tissues

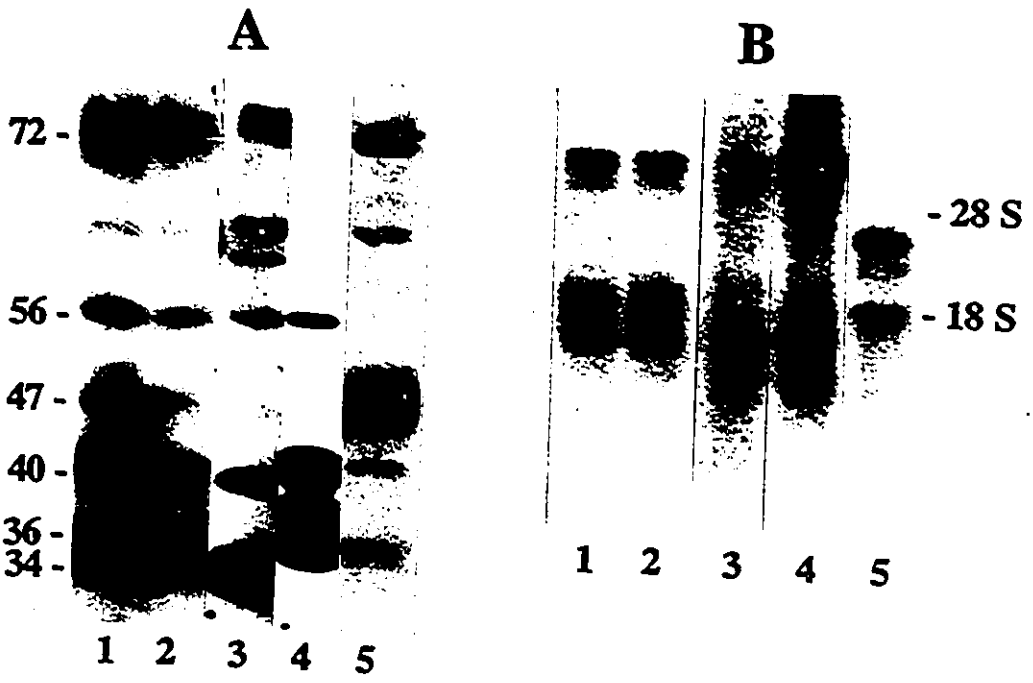
Panel A: Southwestern blot with somatostatin CRE sequence.
100 μg of nuclear proteins from different rat tissues were resolved by 8.5% SDS-PAGE, renatured in 4 M urea and electrotransfered. The filter-bound proteins were incubated with a radiolabeled somatostatin CRE probe and visualized by autoradiography with a 2 day exposure time.

Lane 1 - nuclear proteins from normal rat liver, lane 2 - nuclear proteins from rat liver 1.5 h after HPX, lane 3 - nuclear proteins from a hepatic nodule developed 9 months after carcinogenic regimen, lane 4 - nuclear proteins from a Morris hepatoma 5123tc solid tumour, lane 5 - nuclear proteins from Morris hepatoma 5123tc cultured cells.

The molecular sizes of the CRE-binding proteins were calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers and are indicated in kilodaltons.

Panel B: Northern blot of poly(A)+ RNA hybridized with a 0.6 kbp AvaII/StuI restriction fragment of a CREB-341 cDNA.
mRNA was prepared from the same tissues described in panel A and was resolved on an agarose/formaldehyde gel and then transferred to a nylon membrane for Northern blotting as described in the Methods section and in Fig. 18. Expression of the CREB gene was visualized by autoradiography with a 3 day exposure.

Lane 1 - nuclear proteins from normal rat liver, lane 2 - nuclear proteins from rat liver 1.5 h after HPX, lane 3 - nuclear proteins from a hepatic nodule developed 9 months after carcinogenic regimen, lane 4 - nuclear proteins from a Morris hepatoma 5123tc solid tumour, lane 5 - nuclear proteins from Morris hepatoma 5123tc cultured cells. The positions of the 28 S and 18 S ribosomal RNA species are indicated.


A
72 -
56 -
47 -
40 -
36 -
34 -
1 2 3 4 5
B
- 28 S
- 18 S
1 2 3 4 5

CREB gene (panel B, lanes 1,2), despite the fact that proliferating liver cells at 1 h after HPX had a decrease in CRE-binding activity, particularly of the 47 kDa CREB factor (panel A, lanes 1, 2). Similarly, the hepatic nodule and tumour (panel B, lanes 3, 4), expressed similar species of CREB mRNA , however, neither of these tissues contained the 47 kDa CREB factor (panel A, lanes 3, 4). Nuclear protein extracts prepared from Morris hepatoma 5123tc cells, however, displayed a different pattern of CRE-binding activities than that displayed in the tumour that these cells originated from (compare lanes 4, 5, panel A). These cells displayed a high level of the 47 kDa and 72 kDa CRE-binding proteins. The pattern of CREB mRNA in these cells was also different from that seen in other tissues (lane 5, panel B). These results indicated that under conditions where c-*fos* expression is elevated and where there was no 47 kDa CREB factor, there was no difference in the expression of the CREB gene. The only different pattern of CREB expression was seen in Morris hepatoma cells in culture where the 47 kDa CREB was present, but the activities of other CRE-binding proteins were either decreased or absent. Together these results lend further support to those results presented in Figure 15 and define the 47 kDa CREB factor as unique and distinct from CREB.

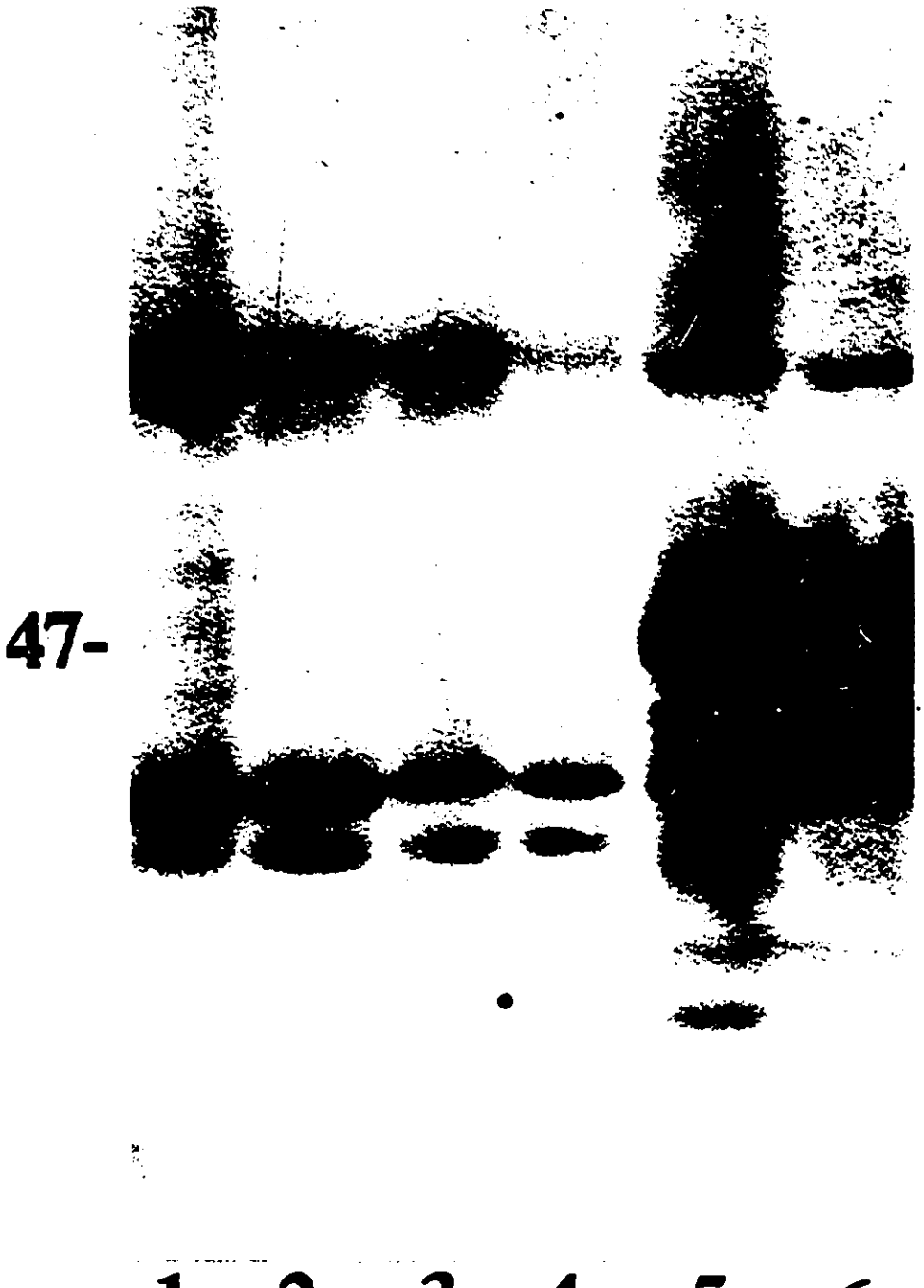
Alterations in the DNA-Binding Property of the 47 kDa CREB Factor During *in vitro* and *in vivo* Growth

It was surprising that the DNA-binding activity of the 47 kDa CREB factor, which was absent in nuclear protein extracts prepared from solid hepatoma tissue (for example, see Fig. 17), was present in the same cells placed in culture conditions (Fig. 47). The data shown in Figure 47 demonstrated that the presence or absence of the 47

Figure 47. Alterations in the DNA-binding property of the 47 kDa CREB factor during *in vitro* and *in vivo* growth of 5123tc Morris hepatoma cells.

Morris hepatoma 5123tc cells were injected bilaterally into the inguinal region of male Buffalo rats (10^6 cells/ site), and tumours harvested 3-4 weeks later. Tumour samples were used either to establish primary cultures or were transplanted to the next host rat.
For propogation, 1 g of tumour tissue was minced in 10 ml of sterile PBS containing 0.2 ml of penbritin and 1 ml injected/ rat.
For primary cultures, 1 g of tumour tissue was minced in 10 ml RPMI medium supplemented with 10 % fetal bovine serum and 20 μg/ ml gentamicin. After 24 h the medium was changed and the adherent cells were propagated in culture.
The CRE-binding activities were analyzed both from tumour and cell culture samples by Southwestern blotting as described in the Methods section.

Lane 1 - CRE-binding proteins from a solid tumour harvested 3 weeks after injection of an established 5123tc hepatoma cell culture, lane 2 - CRE-binding proteins from a tumour harvested 4 weeks after injection of primary hepatoma cells established from a previously harvested tumour, lane 3 - CRE-binding proteins from a tumour generated by direct transplantation without tissue culture passage, lane 4 - CRE-binding proteins from a tumour generated by a second consecutive direct transplantation, lane 5 - CRE-binding proteins from the established 5123tc cell culture used to generate the tumour described in lane 1, lane 6 - CRE-binding proteins from a primary hepatoma cell culture derived from the tumour described in lane 1 and used to generate the tumours described in lanes 2-4. The position of the 47 kDa CREB factor is indicated.


47-
1 2 3 4 5 6

kDa CREB protein in transformed hepatoma cells was a reversible phenomenon. These 5123tc cells, when placed in culture and under the control of serum growth factors, were capable of generating the 47 kDa CRE-binding activity (lanes 5, 6). The same cells, when injected into a host animal, produced a solid tumour which did not express this CRE-binding activity (lanes 1-4). These features were produced regardless of repeated passages between tumour and culture conditions. These results indicated that the differences between *in vivo* and *in vitro* expression of DNA-binding activity of the 47 kDa protein may reflect the different cellular environments encountered by the cells in the presence of serum growth factors. However, the 5123tc hepatoma cells retained the ability to regulate the changes in the CRE-binding activity of the 47 kDa protein in a cell cycle-dependent manner as described earlier (Fig. 18).

Changes in CRE-Binding Proteins and the Pattern of c-*fos* Expression in Normal, Preneoplastic and Neoplastic Liver Cells

A comparative analysis of CRE-binding proteins and c-*fos* gene expression under physiological and pathological conditions is presented in Figure 48. It can be seen (panel A), that the transient loss of DNA-binding activity of the 47 kDa CREB factor following HPX clearly coincided with transient induction of c-*fos* expression. Similarly, during carcinogenic progression of preneoplastic hepatocytes (panel B), there was also a loss of the DNA-binding property of the 47 kDa CRE-binding protein. Under these conditions, however, the loss of this activity was not transient, but rather extended for a period of at least three weeks. At the same time the expression of c-*fos* was also elevated and persisted during the same time period. Finally, in fully transformed hepatocytes of the solid Morris hepatomas 5123tc and 5123D (panel C), the

Figure 48. Changes in CRE-binding proteins c-*fos* expression patterns in normal, preneoplastic and neoplastic liver cells

Panel A: Southwestern blot of nuclear proteins from normal rat liver with the c-*fos*
CRE sequence and corresponding Northern blot of total cellular mRNA hybridized with a 2.2 kbp restriction fragment from a c-*fos* cDNA.

Lane C - control intact rat liver, lane 3 - rat liver 3 h after HPX, lane 6 - rat liver 6 h after HPX, lane 9 - rat liver 9 h after HPX.

Panel B: Southwestern blot of nuclear proteins from preneoplastic livers during experimental hepatocarcinogenesis with the c-*fos* CRE sequence and a corresponding Northern blot of total cellular mRNA hybridized with a 2.2 kbp restriction fragment from a c-*fos* cDNA.

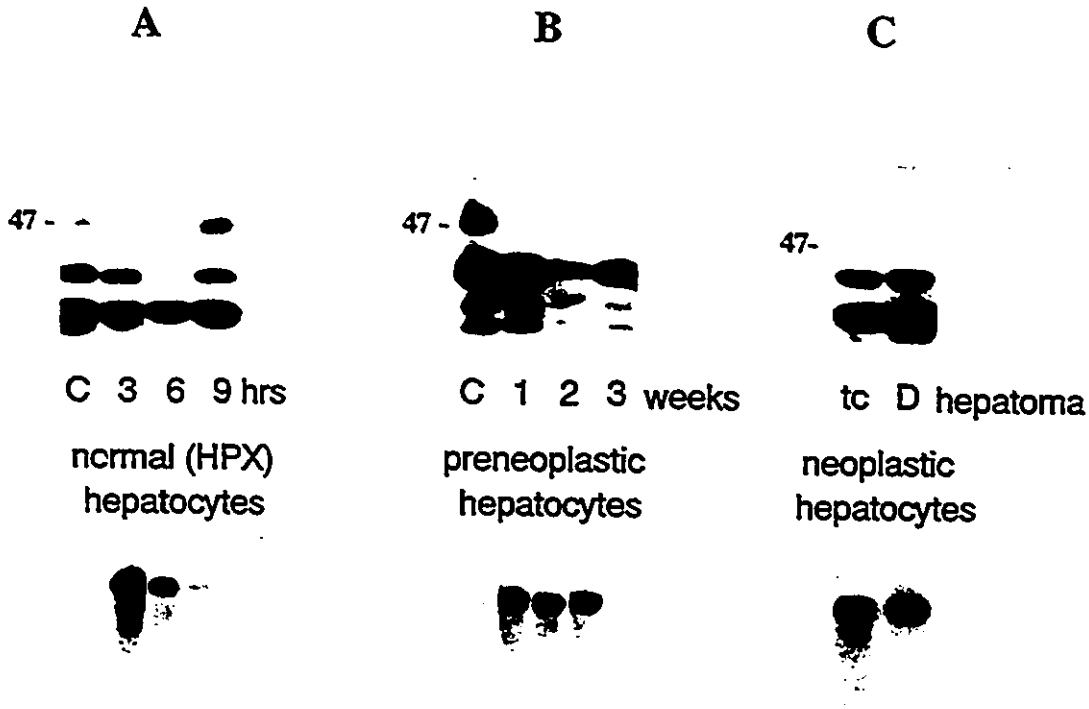
Lane C - control intact rat liver, lane 1 - rat liver 1 week after carcinogen and HPX treatments, lane 2 - rat liver 2 weeks after carcinogen and HPX treatments, lane 3 - rat liver 3 weeks after carcinogen and HPX treatments.

Panel C: Southwestern blot of nuclear proteins from malignant hepatoma tumours with the c-*fos* CRE sequence and corresponding Northern blot of total cellular mRNA hybridized with a 2.2 kbp restriction fragment of a c-*fos* cDNA.

Lane tc - Morris hepatoma 5123tc solid tumour, lane D - Morris hepatoma 5123D solid tumour.

100 μg of nuclear proteins were used for Southwestern blotting. The proteins were resolved by 8.5% SDS-PAGE, renatured in 4 M urea, electrotransferred onto a nitrocellulose filter (100 mA/ 16 h), and incubated with a radiolabeled c-*fos* CRE probe. The CRE-binding proteins were visualized by autoradiography with a 2 day exposure. The position of the 47 kDa CREB factor is indicated.

Approximately 1 μg of poly(A)+ RNA from each sample was loaded and the blot was hybridized with a radiolabeled c-*fos* cDNA probe. Expression of the c-*fos* gene was detected by autoradiography with an overnight exposure.


A
B
C
47 -
47 -
47-
C 3 6 9 hrs
normal (HPX)
hepatocytes
C 1 2 3 weeks
preneoplastic
hepatocytes
tc D hepatoma
neoplastic
hepatocytes

activity of the 47 kDa CREB factor could not be detected. In both of these hepatomas, c-*fos* gene expression was constitutively high. Together these results indicated that the temporary loss of 47 kDa CRE-binding activity correlated with transient c-*fos* expression while a prolonged or constitutive loss of this activity correlated with elevated expression of the gene.

Part 4: *In vitro* transcription directed by c-*fos* regulatory elements

To establish a relationship between the DNA-binding activity at individual regulatory elements within the promoter of the c-*fos* gene, and the transcriptional activation of the gene, an *in vitro* transcriptional analysis was performed. For these studies it was important to determine the transcriptional potential of isolated regulatory elements which could then be related to the DNA-binding activities detected in nuclear proteins under different physiological states. To achieve this, transcriptional templates were generated by placing different c-*fos* regulatory elements upstream of a TATA box and followed by a cassette of 400 nucleotides which, upon transcription, result in a transcript devoid of guanine residues. This provides a convenient system to assess transcription from a defined promoter which will proceed in the absence of GTP and result in a transcript of defined length.

Construction of Recombinant Plasmids Harbouring c-*fos* Regulatory Elements

Generation of transcription templates containing different c-*fos* regulatory elements ligated upstream of the TATA box and G-free cassette is demonstrated in Figure 49. Plasmids containing the incorporated regulatory sequences were purified (panel A), and analyzed by Southern blotting for the presence of the regulatory sequences (panel B). The use of radiolabeled oligonucleotides representing individual

Figure 49. Construction of recombinant plasmids harbouring c-*fos* regulatory elements

Recombinant plasmids consisting of individual c-*fos* regulatory elements ligated upstream of the 400 nucleotide G-free cassette were constructed as described in the Methods section. *E. coli* strain HB101 transformed with the plasmids were grown overnight in LB medium containing 50 μg/ml ampicillin and the plasmids then purified by the procedure described in the Methods section.

Panel A: Ethidium bromide stained 0.8% agarose gel of purified plasmid preparations.
Approximately 1 μg of purified plasmid was electrophoresed through an agarose gel in TAE buffer (10 mM Tris-HCl, pH 7.5, 1 mM sodium acetate, 1 mM EDTA). After electrophoresis, the gel was stained with 5 μg/ml ethidium bromide in distilled water and then photographed under transilluminator.

Lane 1 - SRE-containing G-free cassette, lane 2 - CRE-containing G-free cassette, lane 3 - SCMRE-containing G-free cassette.

Panel B: Southern blot analysis of recombinant plasmids.
Approximately 1 μg of each plasmid was electrophoresed through an 0.8% agarose gel as described in panel A and then transferred by capillary action onto a nylon memebrane as described in the Methods section. The filter was then hybridized with a radiolabeled oligonucleotide and then, after washing the filter, the hybridized probe was visualized by autoradiography with an overnight exposure.

Lane 1 - SRE-containing G-free cassette hybridized to the SRE probe, lane 2 - CRE-containing G-free cassette hybridized to the CRE probe, lane 3 - SCMRE-containing G-free cassette hybridized to the SCMRE probe.

A

1 2 3

B

1 2 3

regulatory elements were used as probes and confirmed that the plasmid constructs, indeed, contained the c-*fos* SRE (lane 1), CRE (lane 2), and SCMRE (lane 3).

***In vitro* Transcription from c-*fos* Regulatory Elements**

Transcription promoted by nuclear proteins from normal rat liver was first examined since it was found to contain a full complement of DNA-binding proteins (Fig. 50). As shown in panel A, nuclear proteins from normal rat liver were able to stimulate transcription only from the template containing the c-*fos* SRE sequence giving rise to a 400 nucleotide G-free transcript (lane 4). In contrast transcriptional templates harbouring either the CRE or SCMRE sequences did not function under these conditions (lanes 2,3). There was no transcription from the vector plasmid which did not contain regulatory sequences (lane 1). To demonstrate that isolated nuclear proteins were able to support *in vitro* transcription from a constitutive promoter, a plasmid containing the adenovirus major late promoter upstream of a 200 nucleotide G-free cassette was used as an internal control. Additional experiments were performed to titrate the amount of nuclear proteins needed for efficient *in vitro* transcription (panel B). In this experiment increasing amounts of nuclear proteins were used to generate transcripts *in vitro*.

It can be seen that with increasing amounts of nuclear proteins, there was increased transcription, which appeared to be saturable by 80 μg of proteins. Interestingly, the internal reference plasmid did not show the same saturation kinetics which may have indicated that this template had a higher affinity for the transcriptional apparatus.

Figure 50. ***In vitro*** **transcription assay with nuclear proteins from normal rat liver**

Nuclear protein extracts for the *in vitro* transcription assay were prepared from normal rat liver according to the procedure described in the Methods section. 60 μg of nuclear proteins were incubated with 800 ng of the appropriate plasmid construct and 400 ng of the plasmid containing the adenovirus major latepromoter (pAd ML) under the conditions described in the Methods section. Transcripts were isolated by phenol/chloroform extraction and ethanol precipitation and then analyzed by separation on a 4% polyacrylamide gel containing 6 M urea followed by autoradiography with an overnight exposure.

Panel A: *In vitro* transcription assay with different c-*fos* promoter regulatory sequences.

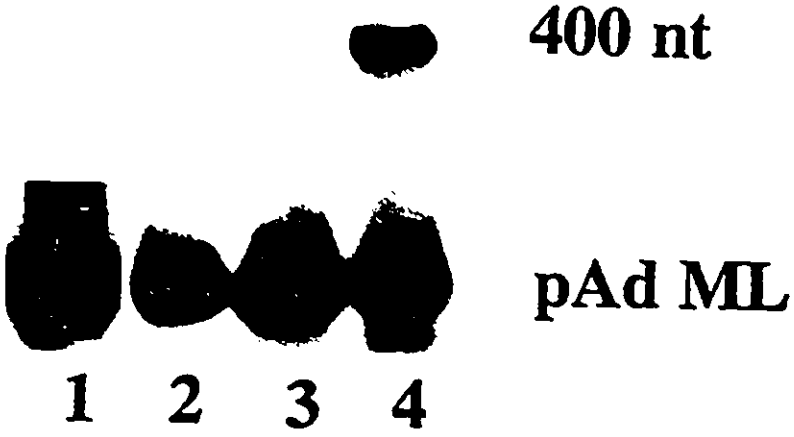
Lane 1 - control G-free cassette plasmid containing no oligonucleotide insert, lane 2 - CRE-containing G-free cassette, lane 3 - SCMRE-containing G-free cassette, lane 4 - SRE-containing G-free cassette.

The positions of transcripts corresponding to the 400 nt G-free cassette and the Ad ML internal control are indicated.

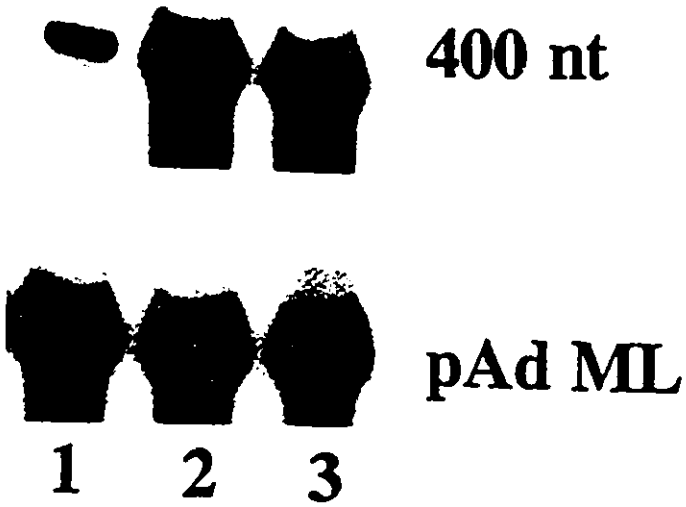
Panel B: *In vitro* transcription assay with c-*fos* SRE regulatory sequence. The assay was performed using increasing amounts of nuclear proteins prepared from normal rat liver. Following electrophoresis the gel was dried and exposed for two days.

Lane 1 - 20 μg of nuclear proteins, lane 2 - 40 μg of nuclear proteins, lane 3 - 60 μg of nuclear proteins.
The positions of transcripts corresponding to the 400 nt G-free cassette and the Ad ML internal control are indicated.

A



B



In vitro Transcription using Nuclear Extracts from Normal and Proliferating Rat Livers

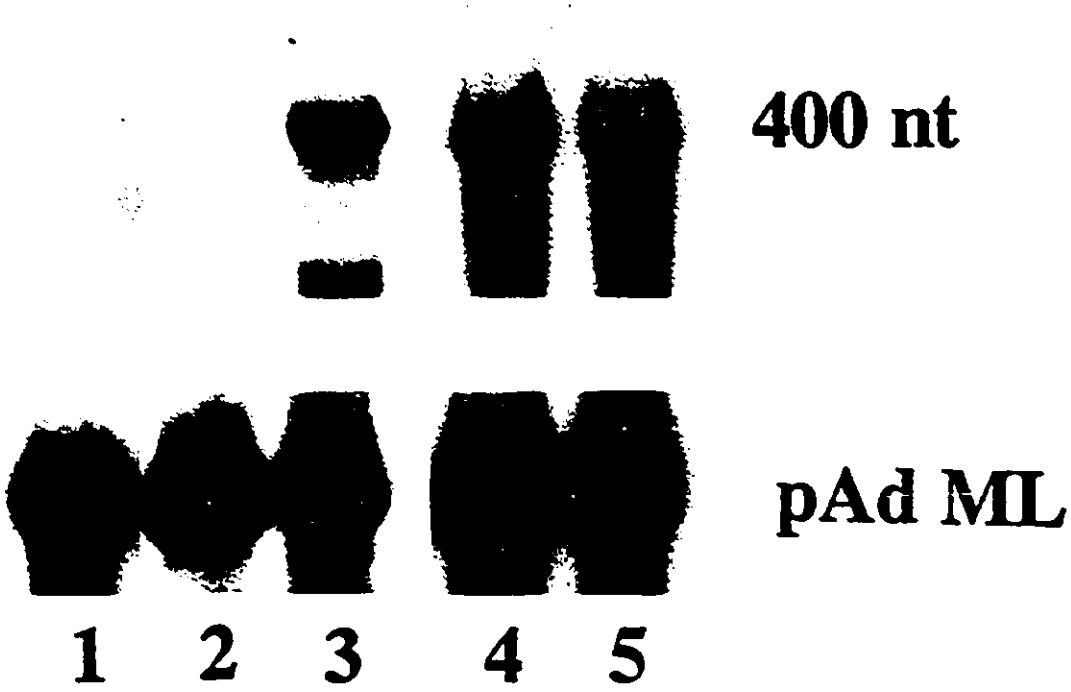
While normal rat liver contained the full complement of DNA-binding proteins, only the SRE template was able to promote transcription. This suggested that in spite of the ability of protein to bind to the CRE and SCMRE sequences these templates were transcriptionally incompetent in quiescent cells. Since during early times after HPX there was a transient downregulation of c-*fos* CRE-binding activity which correlated with elevated expression of the gene, it was important to assess the *in vitro* transcriptional potential of nuclear proteins under these conditions. Figure 51 showed the results of *in vitro* transcription reactions using nuclear proteins prepared from rat livers 30 minutes after HPX. Nuclear proteins from HPX livers did not stimulate transcription from the control plasmid which did not contain any subcloned regulatory element sequences (lane 1). Nuclear protein extracts prepared from normal rat liver and HPX liver did; however they show a marked difference in their ability to stimulate transcription from templates containing the CRE (lanes 2,3). Whereas there was no transcription promoted by nuclear proteins from quiescent cells (lane 2), the proteins prepared from proliferating cells were able to generate the 400 nucleotide transcript (lane 3). There was no difference in the ability of nuclear proteins from quiescent or proliferating cells to drive transcription from the SRE-containing template (lanes 4, 5). These results established that the transcriptional potential of the c-*fos* CRE was activated coincident with the downregulation of DNA-binding activity of the 47 kDa CREB. Furthermore, this indicated a functional significance of the transient decrease in CRE-binding activities seen in proliferating cells, particularly with respect to the transcriptional activation of the gene.

Figure 51. ***In vitro*** **transcription assay of c-*fos* promoter elements using nuclear proteins from normal and proliferating rat livers**

80 μg of nuclear proteins were incubated with 400 ng of control pAd ML plasmid and 800 ng of the plasmid containing the appropriate regulatory element as described in the Methods section.

Lane 1 - control G-free plasmid without c-*fos* sequences incubated with nuclear proteins from rat liver 30 min after HPX, lane 2 - CRE-containing G-free plasmid incubated with nuclear proteins from normal rat liver, lane 3 - CRE-containing G-free plasmid incubated with nuclear proteins from rat liver 30 min after HPX, lane 4 - SRE-containing G-free plasmid incubated with nuclear proteins from normal rat liver, lane 5 - SRE-containing G-free plasmid incubated with nuclear proteins from rat liver 30 min after HPX.

The positions of transcripts corresponding to the 400 nt G-free cassette and the Ad ML internal control are indicated. Transcripts were visualized by autoradiography with an overnight exposure.


400 nt
pAd ML
1 2 3 4 5

SUMMARY

1. Liver cells contained nuclear proteins capable of specific interaction with c-*fos* regulatory elements. These proteins are listed in Table 2 together with their identities where known.

2. Complexes formed with individual regulatory elements consisted of multiple protein factors. Several of these factors have not been previously identified and this work represents the first documented evidence for the existence of such a multiprotein complex interacting with the c-*fos* SRE. These results have been reported in *Mol. Cell. Biol.* **11**:2752-2759 (1991).

3. The DNA-binding properties of several proteins interacting with the SRE and CRE were affected by phosphorylation. Furthermore, their DNA-binding activities were differentially affected by phosphorylation. Notably a 47 kDa CRE-binding protein displayed affinity for DNA in the dephosphorylated form. This property is unique among the CREB family of transcription factors.

4. Complexes contained proteins capable of interacting with all three regulatory elements. The identification of the 112 and 36 kDa multielement binding proteins represents an original finding and may contribute to the understanding of regulatory element communication.

5. During liver regeneration, an increase in c-fos expression correlated with a decrease in the DNA-binding activities of CRE, but not SRE-binding proteins. This is the first demonstration of a correlation between c-*fos* expression and the down regulation of CRE-binding activity.

6. In the Solt/Farber model of chemically induced hepatocarcinogenesis, elevated c-fos expression persisted for at least 3 weeks following the experimental regime.

This also correlated with a decrease in DNA-binding activity of CREB proteins, in particular the 47 kDa factor.

7. c-*fos* expression remained high in preneoplastic liver nodules and in malignant hepatomas. Neither of these tissues contained a 47 kDa CRE-binding activity. This finding was reported in *Cancer Res.*51:528-535 (1991).

8. Normal liver cells contained a unique CRE-binding protein of M_r 47 kDa which was distinct from the CREB 327/341 family. This protein was not immunoprecipited by the CREB antibody and it displayed DNA-binding activity in the dephosphorylated form. The 47 kDa CRE-binding protein has not been previously identified and is the first CRE-specific binding protein which binds preferentially in the dephosphorylated form. This data has been published in *J.Biol.Chem.*268:19581-19585 (1993).

9. The DNA-binding activity of the 47 kDa CREB factor decreased during normal proliferation and transformation. A decrease in the CRE-binding activity of the 47 kDa factor, or its loss from hepatoma tissue, correlated with increased c-*fos* gene expression. Based on these observations it is proposed that the 47 kDa CREB could function as a CRE-specific constitutive repressor.

10. *In vitro* transcription driven by the individual regulatory elements suggested that there was a transcriptional block at the c-*fos* CRE in quiescent cells and that this block was relieved in proliferating cells. This gives further support to the hypothesis that the 47 kDa CREB might participate in this mechanism of transcriptional repression.

Table 2. Summary of DNA-binding Proteins Studied

Regulatory Element	Binding protein (kDa)	Identity
CRE	36	delta CREB
	40	alfa CREB
	47	novel
	56	CRE-BP1 (?)
	72	novel
	112	novel
SRE	36	novel
	45	novel
	62	$p62^{TCF}$,$p62^{DBF}$
	67	SRF
	72	novel
	112	novel
SCMRE	36	novel
	112	novel

DISCUSSION

The studies were designed, in part, to characterize the interactions of nuclear proteins with three distinct regulatory sequences of the c-*fos* promoter: The serum response element (SRE), the cAMP response element (CRE), and the *sis* conditioned medium response element (SCMRE). The SRE element plays a central functional role in the c-*fos* gene promoter. It is required for the full transcriptional response of individual regulatory sequences (i.e. CRE and SCMRE elements), it mediates responsiveness from various signal transduction pathways and it is also the site for c-*fos* gene repression. To achieve this, a set of functional interactions, mediated by multiple protein complexes, must occur at these regulatory elements. Such protein complexes must be capable of interacting with individual elements, provide communication between the elements and respond to different signalling pathways in order to regulate the expression of the gene.

Several techniques shown previously to be appropriate for studying DNA-binding proteins were employed in this study. These included the gel shift assay, sequence-specific DNA affinity chromatography and Southwestern blotting (253,313,334). The Southwestern blotting technique, despite its apparent limitations, has proved to be a useful tool in analyzing the DNA-binding activities of transcription factors. In applying this technique, however, it is important to remember that due to the denaturation and separation processes that occur during SDS-PAGE, identification is restricted to those proteins which retain their DNA binding properties upon renatura-

tion and can bind to regulatory elements either as monomers or possibly homodimers. Therefore, such information will permit one to assess only selected aspects of the interactions between proteins and regulatory elements.

Combined application of different methodologies was used in this study to show that multiple proteins present in nuclear extracts prepared from normal rat livers were capable of specific interaction with each of the regulatory elements.

Six different proteins of molecular weight 112, 67, 62, 45, and 36 kDa were purified by the SRE sequence-specific affinity chromatography. The presence of the same complement of SRE-binding proteins was observed when proteins bound to the SRE complex were eluted and examined by SDS-PAGE. Amongst these proteins the 112, 67 and 36 kDa factors displayed a direct DNA-binding activity, with the 67 kDa protein demonstrating the highest affinity and specificity for the SRE sequence. The presence of such a large number of protein factors interacting with the element implied a multiplicity of interactions both at the DNA sequence and/or at the protein/protein level. Indeed, later studies have confirmed the results presented here, and also identified a complex of four or five different proteins, depending on the nuclear protein extract used, able to bind to the c-*fos* SRE (253,255). Additionally, distinct SRE-binding proteins have also been seen in neonatal murine tissues (252).

Three distinct nuclear proteins were previously reported to interact with the SRE. Their estimated molecular weights are 67 and 62 kDa and they are known as the $p67^{SRF}$, $p62^{TCF}$ and $p62^{DBF}$ (222,237,256). Of the two p62 SRE-associated proteins only the $p62^{DBF}$ was shown to have DNA-binding properties (248). Since no protein of that molecular weight was found here to bind directly to the SRE, it is probable that the

62 kDa protein purifying in the SRE multiprotein complex represented $p62^{TCF}$. Consistent with the properties of $p62^{TCF}$ its presence in the SRE complex must have resulted from indirect SRE-binding through protein/protein interaction with $p67^{SRF}$. Such interaction of these two factors was shown to be required for serum inducibility at least in NIH 3T3 cells (250). In BALB/c 3T3 cells mutations which prevent the interaction between $p67^{SRF}$ and $p62^{TCF}$ result in the inability of the SRE to respond to agents which activate PKC. In the same cell system the growth factor responsiveness is maintained suggesting that, in these cells, $p62^{TCF}$ is a target for PKC activation (254). Although the precise role of $p62^{TCF}$ is not known, it has been shown to have a synergistic effect with SRF on complex formation at the SRE (258).

The best characterized SRE-binding factor is the 67 kDa SRF protein. A direct SRE-binding protein of the same molecular weight was identified here in normal rat liver nuclei. Western blotting with an anti-SRF serum revealed that the same nuclear protein extract which contained the SRE-binding p67 factor also contained a 67 kDa protein recognized by the antibody. Based on these results it is possible to suggest that 67 kDa SRE-binding protein which was identified here was the SRF factor. Further evidence for the identity of these two proteins is provided by the effects of post translational modification on their DNA-binding properties. The functional significance of protein phosphorylation in the regulation of c-*fos* transcription is at present unclear, but initial studies showed that SRF can be phosphorylated *in vivo* in HeLa or A431 cells treated with EGF which results in its increased DNA-binding activity (239). Furthermore, phosphatase treatment of crude SRF preparations also resulted in a decrease in DNA-binding activity as determined by gel shift assays. It has also been shown that

recombinant SRF can be phosphorylated by casein kinase II leading to an enhanced affinity of the SRF for the SRE through a conformational change in the DNA-binding domain of the SRF factor (240,241). In contrast, the results presented here showed that the affinity of the 67 kDa protein for the SRE was unaffected by dephosphorylation, but that protein complex formation at the element was abolished upon dephosphorylation of liver nuclear extracts. These results have been confirmed by an observation that the affinity of SRF for the SRE is not affected by the phosphorylation status, but that the rate of exchange between SRF and the SRE is increased upon phosphorylation of SRF with casein kinase II (242). Recent results have also shown that phosphorylation of SRF does not change upon growth factor stimulation and that a SRF mutant binds constitutively to the SRE independent of its phosphorylation status (243). This is consistent with the fact that SRE binding activity does not change upon serum or growth factor stimulation of cells and may indicate that SRF phosphorylation is involved in regulating protein interactions between SRF and the general transcriptional machinery or other SRE-binding proteins. Based upon these properties, it can be concluded that the 67 kDa SRE-binding protein purified from normal rat liver nuclear extracts and present in the SRE multiprotein complex was indeed the SRF factor.

The other proteins, the 112 and 36 kDa, which were shown to be associated with the SRE complex have not been previously described and their presence in these complexes resulted from interaction with direct SRE-binding proteins.

Therefore, nuclei of normal liver cells contained multiple protein components capable of interacting with the c-*fos* SRE. They are likely to be involved in transducing the signals from diverse pathways which converge at this regulatory element. Clearly,

the ability of the SRE to respond to different stimuli from different pathways is likely to involve a complex set of DNA/protein and protein/protein interactions which are probably modified by post-translational modifications and which differ depending upon cellular context. It is possible that subsets of proteins within the SRE multiprotein complex respond to distinct signal transduction pathways consistent with the results of Graham and Gilman (254), who showed that distinct SRE-binding proteins respond to different signals. The involvement of multiple proteins interacting at the SRE has been predicted on the basis of DNA footprinting studies (249). The results of these studies also show that there were proteins in the complex which, although they do not leave a footprint, may be equally important in controlling the expression of the c-*fos* gene, and presumably other genes, *in vivo*. Modulation of transcription may involve the modification of individual components of the complex which may, in turn, alter the protein/protein interactions within the complex. Such complexes would thus be able to multiplex complex second messenger signals into an integrated response by the gene.

Multiple proteins were also retained by both c-*fos* and somatostatin CRE affinity columns and they were shown to bind to the CRE sequences on Southwestern blots. Their molecular weights ranged from 112 to 34 kDa. They most likely represented members of CREB/CREM/ATF families. Indeed, over the last few years a number of different protein factors interacting with the CRE sequence have been identified. In fact, several distinct cDNA clones encoding CREB proteins have been isolated so far, such as CREB-327 (delta) and CREB-341 (alfa) (167,168), CRE-BP1 (171,288), CRE-BP2 (286), CREM (288), CREB-2 (289), and a family of activating transcription factors (ATF) (187, 290). Some of these proteins, such as the CREB or CREM isoforms

were derived from alternative splicing of the transcript from the same gene, whereas others are different gene products.

Most of the CRE-binding proteins identified here had molecular weights similar to the known CREB factors, therefore it was important to determine their identity. There was a 43 kDa protein which was purified from normal liver cells extracts by the CRE-affinity and Mono Q columns. This protein was shown to be phosphorylated *in vitro* by cAMP-dependent protein kinase, suggesting that it was the CREB-341 factor. Western blotting using anti-CREB-341/327 serum confirmed that the rat liver nuclear extract contained a protein of 43 kDa which was recognized by the antiserum. A comparison of both the Southwestern and Western blot patterns, as well as immunoprecipitation studies, showed that the 40 and 36-34 kDa proteins were most likely the members of the extensive CREB family (290). These CREB proteins retained their DNA-binding properties after 1D and 2D electrophoretic separation, therefore, they all must be capable of binding to the DNA as monomers or homodimers. The 34, 36 and 40 kDa and possibly the 56 kDa protein bands were different isoforms of the CREB family since they reacted with the CREB-341/327 antibody.

The 36 kDa band actually consisted of three CRE-binding activities with slightly different pIs that could be resolved by 2D SDS-PAGE. A group of proteins of similar molecular weight and phosphorylation-dependent DNA-binding activity has also been reported in HeLa cells (288). It has also been shown previously (253, 334) and confirmed in the present study that the DNA-binding property of the 36 kDa protein band was dependent on its state of phosphorylation. Therefore, the three protein spots seen in Figure 15 most likely represent the same CREB-327 (delta) factor phosphorylated to

different degrees. The existence of multiple phosphorylated subdomains within the structure of CREB-327 protein has been demonstrated by Lee *et al.* (294). These authors have shown that phosphorylation of serine-119 by protein kinase A is essential, but not sufficient to achieve full transactivating potential of CREB-327. To become a productive transactivator, phosphorylation of additional serines located both N terminal and C terminal from serine-119 in the CREB-327 structure is required. Results here showed that phosphorylation of the CREB-327 might also play a role in determining promoter-specific recognition since these delta CREB isoforms displayed some specificity towards the CREs of different genes, such as c-*fos* and somatostatin.

The 40 kDa band was equivalent to CREB-341 (283), by the criteria of the overlapping protein patterns on the Southwestern and Western blots as well as immunoprecipitation by the CREB antibody. Its direct DNA interaction was also phosphorylation dependent. In contrast to CREB-327, only a single form of this protein could be identified after 2D separation. However, under the electrophoretic conditions used for the Southwestern blotting this protein migrated with a molecular weight slightly lower than on the Western blots. The molecular weight of CREB-341, predicted from the amino acid sequence, is only 37 kDa although in some publications (possibly as a result of different electrophoretic conditions), the CREB-341 protein appears to have a molecular weight between 43 and 48 kDa on SDS-PAGE (165,283,292).

The 47 kDa CRE-binding protein which was previously identified in rat liver (334), appeared to be a unique factor. The protein did not cross-react with the CREB-327/341 antibody. Furthermore, its tissue distribution did not overlap with those of the CREB family. For example, liver tumors and placental tissue which did

not contain the 47 kDa DNA-binding protein contained a 40-43 kDa protein labeled by the CREB antibody (334). An additional difference between the CREB-341/327 family and the 47 kDa factor was revealed by 2D separation. The latter protein could not be detected on the 2D Southwestern blots after electrophoretic separation in the pH range 4.0 - 8.0, suggesting that its isoelectric point was outside this range. The isoelectric points of the CREB isoforms as calculated using the University of Wisconsin GCG software package (337), are 5.18 for CREB-341, 5.03 for CREB-327 and 4.67 for CREB-2. The pI values for both CREB-327 (the 36 kDa band), and CREB-341 (the 40 kDa band), obtained in this study are in agreement with these values.

Most significantly, the unique 47 kDa CRE-binding factor was capable of interaction with DNA after dephosphorylation. In fact, phosphorylation of nuclear protein extracts by the catalytic subunits of protein kinase A completely abolished the DNA-binding activity of this factor highlighting its uniqueness. It has been shown recently (313), that this novel 47 kDa CREB factor binds directly to the octameric core element (TGACGTCA) regardless of the sequences flanking the element and is unable to distinguish between the promoters of cAMP-inducible and non-inducible genes. On the other hand, it did not bind at all to the closely related AP-1 element. This implies an important physiological role for this factor in distinguishing between cAMP and TPA-inducible pathways.

Northern blot analysis of CREB gene expression and Southwestern blot analysis of CRE-binding proteins revealed that even the same cell system, placed under different environmental conditions (i.e. Morris Hepatoma cells in solid tumors and in cell culture conditions), expressed different subsets of CREB mRNAs and generated a dis-

tinct pattern of CRE-binding proteins capable of interacting with the enhancers of cAMP-inducible genes. This clearly showed the complexity of transcriptional control which exists within a single cell which contains a mechanism(s) to generate transcriptional competence depending on environmental conditions.

Using Southwestern blotting, two proteins were shown to interact directly with the SCMRE. Their molecular weights were estimated to be 112 and 36 kDa respectively. The identity of these proteins are at present not known, however the 36 kDa protein was similar to the lower molecular weight band interacting with the CRE sequence. Since the c-*fos*-SCMRE contains a fragment of the CRE core element, a sequence which reads TGACG, it is possible that as few as 5 nucleotides of sequence is sufficient to support a weak interaction with some of the CREB factors (226, 230).

Generally, little is known about protein factors interacting with SCMRE element. However, the presence of an inducible binding factor to this site has been reported in A431 cells in response to EGF treatment with a kinetics correlating with the transcriptional activity of the c-*fos* gene (230). This suggests that the SCMRE may also respond to different growth factor stimuli (249). A protein of molecular weight 91 kDa (p91), has been implicated in EGF-mediated gene induction (277), but none of the proteins identified here was equivalent in molecular weight to that factor.

Thus, all of the c-*fos* regulatory elements examined could interact with multiple proteins suggesting that the combined activities of these proteins are necessary for the accurate regulation of the gene in response to the diverse set of stimuli to which it responds.

It was also found that similar protein factors interacted with more than one regulatory element of c-*fos* gene promoter. For example, the 36 and 112 kDa proteins could bind to all three elements, whereas others , such as the 72 kDa factor bound to the CRE and SRE elements. Interestingly, it had been documented t. . maximal transactivation from the SRE sequence requires the presence of the CRE (221). Similarly induction of the c-*fos* gene by PDGF requires not only the SCMRE but also the sequences located between positions -100 and -57 which include the CRE element (224). This indicates that cooperative interactions between promoter elements are likely to be involved in transcriptional activation (275).

Further evidence for element interaction is demonstrated by the ability of the SRE to respond to a signal generated by PDGF, but also requiring sequences between position -222 and -100 (275,278). In one study, mutation of the SRF binding site within the SRE in a transfected promoter construct significantly decreased the PDGF response suggesting that the SRE is necessary for full PDGF inducibility (225). Additionally, the presence of the SCMRE and the SRE are additive in their response to PDGF (275). It has also been shown that cooperative interactions between the SRE and downstream *cis*-acting sequences also occur during the induction of c-*fos* expression in response to TPA and PDGF in NIH 3T3 cells, but that the SRE alone is sufficient to activate transcription in response to TPA in HeLa cells (278). These results are interpreted as evidence for sequence cooperation in regulation of the c-*fos* gene in fibroblasts which is relaxed in HeLa cells. Additionally, early studies suggested that the negative regulation of Fos on its own promoter might be mediated by the c-*fos* TRE (115), but studies described above have now attributed this function to the core SRE sequence. Also,

while the SRE is necessary for the induction of c-*fos* by TPA, the TRE is required for full TPA responsiveness suggesting further potential for cooperative interaction between promoter elements (278).

Such cooperative responses of the promoter regulatory elements may be accounted for by the interaction of regulatory sequences through a protein(s) able to interact with more than one regulatory sequence. The protein complexes identified here may serve to coordinate and integrate the response of the gene at multiple regulatory elements in the manner reviewed by Ptashne (338), and Dynan (339). It is not surprising, therefore, to find that the same proteins may be shared by different complexes to coordinate overall gene activity. As mentioned above, similar proteins were seen binding to all three elements and cross competition analyses in the gel shift assays confirmed such a cross binding of the factors. It was apparent that proteins interacting with the SRE had some affinity for the CRE sequence, but interestingly, proteins bound to the CRE were stable to competition by the SRE suggesting that CRE-bound proteins had a higher affinity for this sequence than for the SRE. It is possible that the relative affinities of proteins for different regulatory elements may be functionally important in transcriptional regulation. The stability of the CRE-bound protein complex to competition by other regulatory sequences may reflect the important role that the CRE plays in regulating the basal level expression of the gene (221,226).

Thus, it was established that normal quiescent liver cells contained an array of c-*fos* promoter-binding proteins with multiple factors capable of binding to each of the three elements studied. These factors bound to the promoter in the absence of c-*fos* gene expression clearly demonstrating the enormous complexity of the regulation of this

gene. Further studies were designed to assess changes in the DNA-binding properties of c-*fos* promoter-binding complexes during the period of transient induction of the gene. It is well established that c-*fos* gene is transiently expressed following growth factor stimulation of cultured cells (11,55,56,86). Such cell systems represent a convenient model to study cell cycle related changes in gene expression. The cell system employed here consisted of Morris hepatoma 5123tc cells. The cells were synchronized by growth factor deprivation and stimulated to re-enter the cycle by serum re-addition. There was a transient induction in c-*fos* expression in these cells shortly after serum readdition typical of responses of immediate early genes (79).

The properties of c-*fos* promoter-binding proteins were also examined during the transient expression of the gene *in vivo*, following partial hepatectomy. Partial hepatectomy stimulates quiescent liver cells to enter and progress through the cell cycle in a synchronous, controlled fashion, accompanied by correlative alterations in the expression of specific genes including c-*fos*. Therefore, this provided a useful system to test the behavior of c-*fos* promoter-binding proteins during transient expression of the gene *in vivo*.

Analysis of c-*fos* promoter-binding complexes, during the period of transient gene expression, revealed a transient loss of DNA-binding activities of both the CRE and SRE-binding proteins. These decreases observed in both experimental models (i.e. rat hepatocytes proliferating *in vivo* in response to partial hepatectomy and in cultured hepatoma cells *in vitro*) , were transient and coincided with the elevated expression of the gene.

Of the CRE-binding proteins, the DNA-binding property of the 47 kDa protein was the most affected. In fact, there was a transient loss of DNA-binding associated with this nuclear factor which again, was observed in both cells types. This cell cycle related change in the property the 47 kDa protein could not be accounted for by changes in the expression of protein encoded by the CREB gene. The expression of messages encoded by the CREB gene did not change in stimulated cells. However, each cell system expressed different set of CREB mRNAs. The loss of this activity could not be accounted for by a generalized loss of cellular protein as a result of enhanced protein turnover or decreased synthesis as judged from experiments with radiolabeled proteins. Furthermore, the DNA-binding properties of the CRE-binding proteins could neither be restored by *in vitro* phosphorylation nor dephosphorylation of nuclear proteins. These types of experiments were also performed in both experimental systems. This observation is of particular significance since, as discussed earlier, in quiescent cells interaction of the CRE-specific factors, and notably the 47 kDa protein, with the DNA was modified by *in vitro* phosphorylation/dephosphorylation.

A role of *in vivo* phosphorylation in mediating the DNA-binding activity of the 47 kDa nuclear factor was further examined following inhibition of either of the two major signal transduction systems, a receptor mediated tyrosine kinase(s) and cAMP-dependent protein kinase(s) pathways. It was found that treatment of synchronized Morris hepatoma 5123tc cells with an inhibitor of tyrosine kinase activity resulted in a low DNA-binding activity the 47 kDa protein which did not change further. At the same time in the absence of tyrosine kinase(s) activity, the cells did not complete a cell cycle. In a parallel experiment it was shown that inhibition of cAMP- dependent

protein kinase during growth factor stimulation of the cells led to a constitutively high level of DNA-binding associated with this protein which did not change during stimulation. This treatment of the cells also resulted in retardation of the cell cycle.

All of these results suggested that the transient down-regulation of DNA-binding property of the 47 kDa protein factor was a proliferation specific, cell cycle related phenomenon. They also demonstrated that the 47 kDa CREB protein could be a target for multiple signal transduction pathways, and may play a fundamental role in regulating both c-*fos* expression and cell cycle progression. Since previously described experiments showed that *in vitro* phosphorylation/dephosphorylation was unable to restore CRE complex formation during early stages of the cell cycle in both growth factor stimulated cells in culture and in rat hepatocytes *in vivo*, it is possible to suggest that either a different type of post translational modification is involved in modulation of the DNA-binding activities of these transcription factors or they became targets of cell cycle-specific proteolysis similar to that described for cell cycle specific cyclins (340).

To determine the functional significance of this transient cell cycle specific down-regulation of c-*fos* promoter binding activities an attempt was made to induce the gene by agents such as TPA and cAMP during periods of both low and constitutive DNA-binding activity of nuclear transcription factors. The results obtained with two different cultured cell lines, 5123tc Morris Hepatoma and BALB/c 3T3 mouse fibroblast stimulated by serum and followed by TPA, showed that the cells lost their ability to increase the expression of c-*fos* gene when TPA was added during periods of the down-regulated DNA-binding activity. Similarly, injection of dibutryl cAMP into hepatectomized rats during the G_1 transit of liver cells, under conditions of low CRE-

binding activity including the complete lack of the 47 kDa CRE-binding, was unable to induce c-*fos* expression in this tissue. The gene could be induced in regenerating livers by cAMP injections only when the CRE-binding activities return to their basal level. Significantly, cAMP had no effect on the c-*fos* gene in control, non-proliferating liver cells. These results clearly underscored the importance of the CREB proteins in directing accurate gene regulation and that their down-regulation was a specific cell cycle event relevant to the cells ability to induce and regulate the c-*fos* gene expression.

These results prompted the examination of c-*fos* promoter-binding proteins during stages in cellular transformation from pre-neoplasia to neoplasia such as those observed in chemically induced carcinogenesis. It has been reported that under those conditions the c-*fos* gene is constitutively expressed (324,332). The resistant hepatocyte model of carcinogenesis was chosen for these studies since defined stages of neoplastic progression can be observed following treatments of animals with the carcinogenic regimen (300,328,335). Therefore, the significance of any changes in the activities of the c-*fos* promoter-binding proteins could be assessed with respect to the aberrant expression of the gene. It was demonstrated in earlier studies that fully malignant Morris hepatoma tumors lost the ability to express the 47 kDa CRE-binding protein (334). Therefore, it was important to evaluate the interactions of DNA-binding proteins with the c-*fos* promoter during the early stages of hepatocarcinogenesis to determine whether altered activities of DNA-binding proteins could account for the increase in c-*fos* expression. It was also essential to establish whether the loss of 47 kDa CREB protein represented an early event in tumorigenesis or occurred after tumor

formation.

Magnetic resonance imaging and histopathological examinations of experimentally treated rats confirmed that carcinogenic progression occurred giving rise, ultimately, to several hepatic lesions. Although pathological examination of hepatic nodules determined that the lesions were not malignant, it was clear that they constituted severely altered and potentially aggressive and invasive hepatocytes.

The expression of the c-*fos* gene was elevated during the early stages of carcinogenesis and remained at the high level for a prolonged period after the carcinogenic regimen, which clearly represented a departure from the normal transient expression of the gene demonstrated for many cell types (56,86). This elevated expression of the c-*fos* gene was observed for at least three weeks after the experimental regimen and persisted in both nodules and a solid hepatoma. Analysis of c-*fos* promoter-binding proteins in the same samples showed that the loss of the 47 kDa CRE-binding activity occurred as early as one week after partial hepatectomy and also persisted into both nodule and tumor formation paralleling the increase in c-*fos* expression. As this loss of DNA-binding activity appeared to be an early occurrence associated with hepatocarcinogenesis it may represent a fundamental event with respect to the aberrant expression of the c-*fos* gene associated with cellular transformation.

In contrast to the dramatic changes in the behavior of the CRE-binding proteins during hepatocarcinogenesis, the changes in the SRE-binding factors were much more subtle. There were no changes in their pattern on Southwestern blots with only a transient change being observed by gel shift assay. Since it is generally accepted that the binding activity of SRF is constitutive (249), activation of the gene through the SRE

may be mediated by either post translational modification or by changes in protein/protein interactions at the element, both of which may not be detected by the Southwestern analysis. Indeed, experiments with the gel shift assay demonstrated a reduction in complex formation with nuclear proteins obtained from experimentally treated animals. Similarly, there were no detectable changes in SCMRE-binding proteins. However, since all three regulatory elements are required for the full transcriptional responsiveness of the gene, any changes in protein binding occurring at one element will most certainly affect the function of the gene promoter.

Protooncogene activation in the multistep process of carcinogenesis may be the result of either a mutational event, increased transcriptional activity, or both. These studies showed that increased expression of the c-*fos* gene correlated with altered DNA-binding activities of both CRE and SRE-binding proteins and/or their ability to form complexes at their respective binding sites. The earliest change in DNA-binding activity that was observed was the loss of activity of a 47 kDa CRE-binding protein. The loss of DNA-binding activity began 1 week after the experimental regimen and persisted in both liver nodules and in Morris hepatoma. The positive correlation between changes in the DNA-binding properties of c-*fos* promoter-binding proteins and the increased expression of the gene suggests that altered transcriptional activity is involved in activation of the gene during experimentally induced hepatocarcinogenesis. It is possible that the loss of 47 kDa CRE-binding activity constitutes a fundamental event associated with deregulated c-*fos* expression and, subsequently, increased hepatocyte proliferation.

The analysis of c-*fos* promoter-binding proteins was also performed in an hepatic nodule 9 months after experimental treatment as well as in the solid Morris hepatoma 5123tc, both of which represented later stages in neoplasia. In both of these tissues constitutive expression of the c-*fos* gene correlated with alterations in promoter-binding activities. The specific change that highlighted this correlation in both tissues was a loss of the 47 kDa CRE-binding activity.

When different rat tissues were probed for CRE-binding activity by Southwestern blotting, it was found that the 47 kDa CREB protein was also absent from placental tissue. Significantly, it was previously reported that placental cells have constitutively high expression of c-*fos* gene (83).

Therefore, in all cell systems tested elevated expression of the c-*fos* gene correlated with the lack of the 47 kDa CRE-binding protein. This change was transient and cell cycle specific in cells responding to extracellular stimuli by rapid and transient induction of the gene. On the other hand, the cells which had high constitutive expression of the gene either due to neoplastic transformation or as a specific tissue function, completely lacked this protein. As described earlier, this 47 kDa CREB protein was distinct from the previously characterized CREB proteins both immunologically and by its DNA-binding protein properties, and as such represents a unique member of the CREB family of CRE-binding proteins.

The data presented here and supported by data published elsewhere (313), clearly emphasizes the distinct nature of the 47 kDa protein among an expanding family of CREB factors. This protein could be a constitutive repressor which in normal cells occupies the CRE sequence and represses CRE-containing genes. Upon transcriptional

activation, modification of the protein by phosphorylation (possibly by protein kinase A), leads to the loss of its DNA-binding activity, allowing other positive transcription factors, either sequence specific CREB proteins or general ones, to increase the rate of gene transcription. This hypothesis was tested in *in vitro* transcription assays driven by individual regulatory elements within the c-*fos* promoter. The expression of c-*fos* in normal liver was very low as expected from a population of essentially quiescent cells. Nuclear protein extracts prepared from normal liver did not support *in vitro* transcription from templates containing the c-*fos* CRE or SCMRE sequences. However, normal liver nuclear proteins stimulated transcription from the template containing the c-*fos* SRE. Nuclear proteins obtained from proliferating liver cells, on the other hand, supported transcription from both the CRE and SRE regulatory elements. Since the expression of the gene does not occur in quiescent liver cells, there must exist a block to the transcription promoted by the SRE at a different promoter location. Normal liver contained its full complement of CRE-binding activities which included the 47 kDa CREB protein. Therefore, it is possible that the putative transcriptional repressor may function through blocking SRE-promoted transcription as well as silencing the CRE in quiescent cells.

CONCLUSIONS

1. Liver cells contained multiple protein factors capable of interacting with the regulatory elements of the c-*fos* gene promoter either through direct DNA binding or by protein/protein interactions.

2. A subset of these proteins was able to interact with multiple regulatory elements and might be able to direct functional communication between them. These multiple factors could be targeted by different signalling pathways.

3. In quiescent liver cells only protein factors interacting with the c-*fos* SRE were able to stimulate *in vitro* transcription. Therefore, a transcriptional block must be exerted by other factor(s) and/or other element(s).

4. Elevated expression of the gene clearly correlated with a decrease in protein binding to the c-*fos* CRE both in normal and in transformed cells. This decrease was consistent with either a transient or permanent loss of DNA-binding activity of the 47 kDa CREB protein.

5. The 47 kDa protein was highly CRE-specific , but was distinct from the CREB-327/341 family. It displayed DNA-binding properties only in the dephosphorylated form and, as such, is a perfect candidate for a CRE-specific constitutive repressor responsible for the transcriptional block in quiescent cells.

All of the findings presented in this thesis are presented in the following model of the of c-*fos* gene regulation (Figs. 52-54).

Figure 52. Regulation of c-*fos* gene expression: The uninduced state

The interaction of proteins with c-*fos* SRE and CRE elements is depicted by colored elipses representing $p67^{SRF}$ (purple), and p47 CREB (red).

REGULATION OF c-fos GENE EXPRESSION

1. UNINDUCED STATE

GENE IS REPRESSED BY p67 BINDING TO SRE AND 47 kDa CREB BINDING TO CRE.

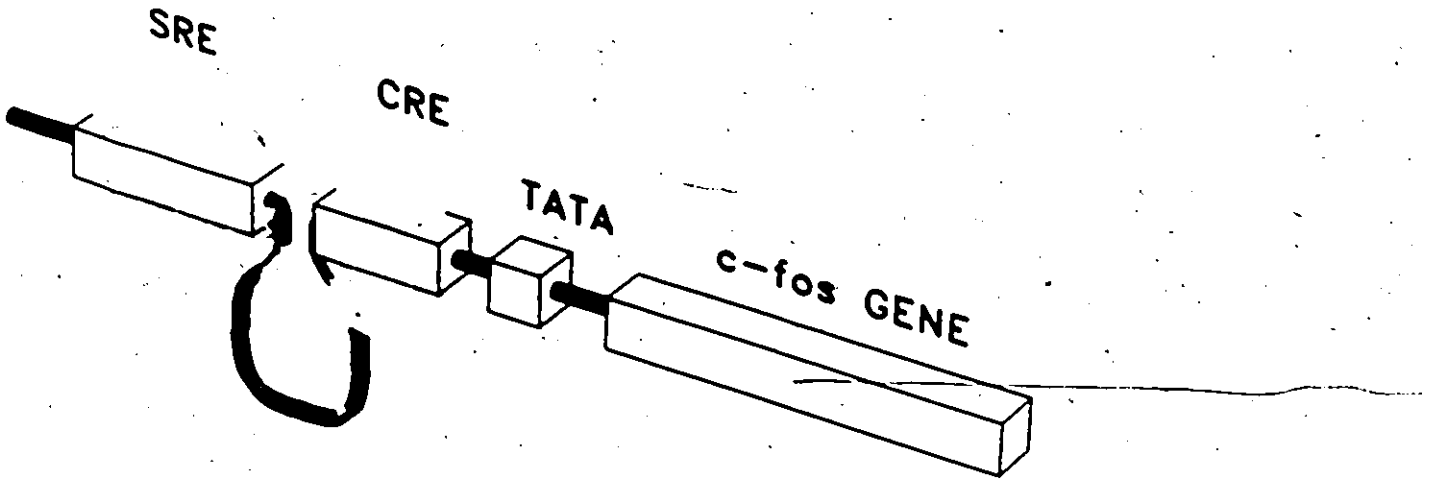


Figure 53. Regulation of c-*fos* gene expression: The induction by phosphorylation

The interaction of proteins with c-*fos* SRE and CRE is depicted as described in the legend to Figure 52. Additional proteins are represented by $p62^{TCF}$ (blue), p112 (green), other CREBs (yellow) and the Fos protein (white).

REGULATION OF c-fos GENE EXPRESSION

2. STIMULATION OF TRANSCRIPTION BY PHOSPHORYLATION OF TRANSCRIPTION FACTORS

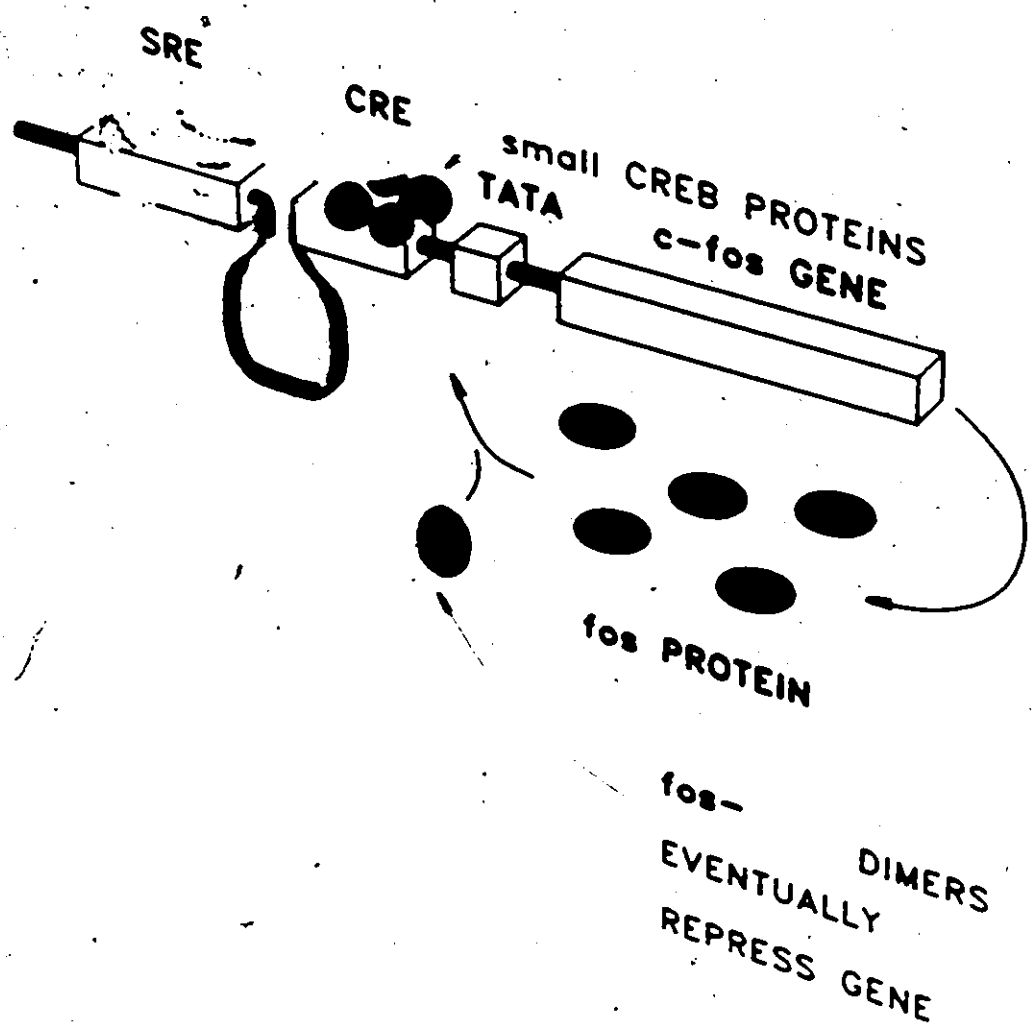
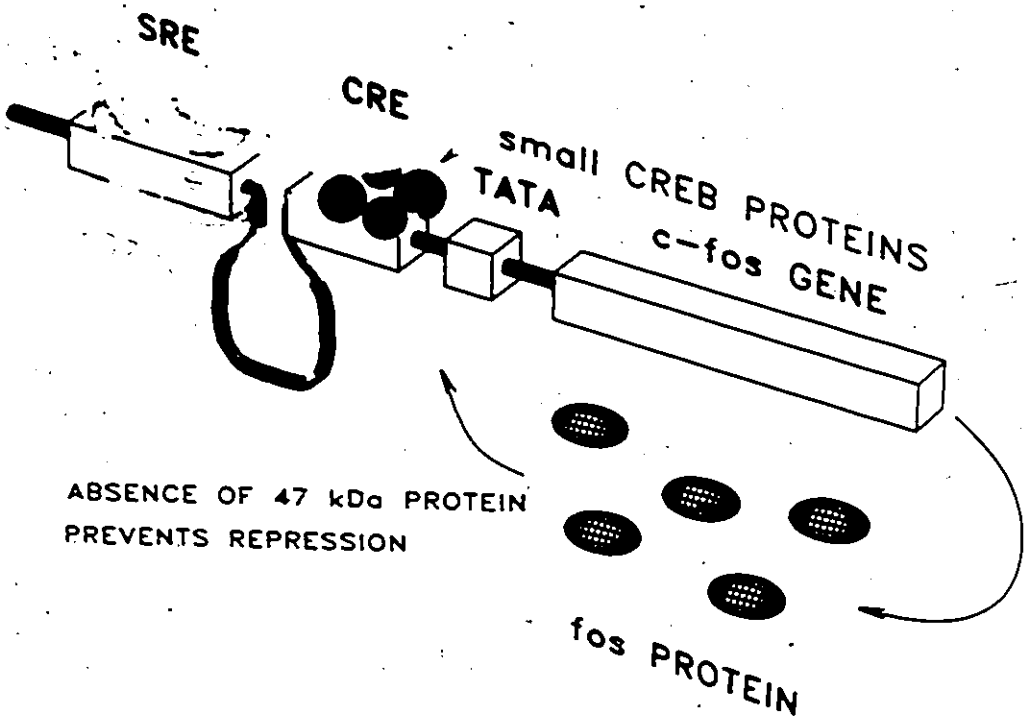


Figure 54. Regulation of c-*fos* expression: Constitutive expression

The interaction of proteins with the c-*fos* SRE and CRE are as described in Figures 52 and 53.

REGULATION OF c-fos GENE EXPRESSION

3. CONSTITUTIVE EXPRESSION IN TRANSFORMED CELLS



Model for the regulation of c-*fos* expression by multiple protein complexes

There is constitutive binding of proteins to the regulatory elements of the uninduced c-*fos* gene promoter in quiescent cells. This includes $p67^{SRF}$ as well as unphosphorylated p47 CREB whose high affinity for the core CRE might prevent protein/protein association between complexes within the promoter resulting in a transcriptional block (Fig. 52).

Stimulus-mediated protein phosphorylation leads to changes in the properties of transcription factors. This would result in the loss of DNA-binding activity of p47 CREB and increase CRE-binding of other CREBs. Similarly, a protein complex could be formed at the SRE, i.e. between $p67^{SRF}$ and $p62^{TCF}$. Phosphorylation of transcription factors might also promote further interactions between protein complexes associated with different elements, i.e. the SRE and CRE. The existence of proteins capable of association with multiple c-*fos* regulatory elements (112 and 36 kDa proteins), suggest their role in orchestrating a coordinated response of the promoter as a whole. Down regulation of the gene expression might be mediated through an interaction of newly synthesized Fos protein with the p47 CREB and/or some other factor(s) which results in the restoration of CRE-binding of p47 CREB and repression of trascriptional activity (Fig. 53).

Constitutive expression of the c-*fos* gene which occurs in the absence of the p47 CREB, as seen in solid hepatomas, clearly indicates that this protein might play an integral role in such a feedback regulatory mechanism (Fig. 54).

REFERENCES

1. Rous, P. (1911). Transmission of a malignant new growth by means of a cell-free filtrate. *J.Am.Med.Assoc.* **56**: 198.

2. Bishop, J.M. (1983). Cellular oncogenes and retroviruses. *Ann.Rev.Biochem.* **52**: 301-354.

3. Eckhart, W. (1972). Oncogenic viruses. *Ann.Rev.Biochem.* **41**: 503-516.

4. Martin, G.S. (1970). Rous sarcoma virus: a function required for the maintenance of the transformed state. *Nature* **227**: 1021-1023.

5. Fried, M. (1965). Cell transformation ability of a temperature-sensitive mutant of polyoma virus. *Proc.Natl.Acad.Sci.USA* **53**: 486-491.

6. Stehelin, D., Varmus, H.E., Bishop, J.M. and Vogt, P.K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* **260**: 170-173.

7. Spector, D.H., Varmus, H.E. and Bishop, J.M. (1987). Nucleotide sequences related to the transforming gene of avian sarcoma virus are present in DNA of uninfected vertebrates. *Proc.Natl.Acad.Sci.USA* **75**: 4102-4106.

8. Bishop, J.M. (1985). Viral oncogenes. *Cell* **42**: 23-38.

9. Bishop, J.M. (1981). Enemies within: the genesis of retrovirus oncogenes. *Cell* **23**: 5-6.

10. Hartwell, L. (1992). Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* **71**: 543-546.

11. Metz, R., Gorham, J., Siegfried, Z., Leonard, D., Gizang-Ginsberg, E., Thompson, M.A., Lawe, D., Kouzarides, T., Vosatka, R., MacGregor, D.,

Jamal, S., Greenberg, M.E. and Ziff, E.B. (1988). Gene regulation by growth factors. *Cold Spring Harbor Symp.Quant.Biol.* LIII: 727-737.

12. Pardee, A.B., Dubrow, R., Hamlin, J.L. and Kletzien, R.F. (1978). Animal cell cycle. *Ann.Rev.Biochem.* 47: 715-750.

13. Temin, H.M. (1970). Control of multiplication of uninfected rat cells and rat cells converted by Murine Sarcoma Virus. *J.Cell.Physiol.* 75: 107-120.

14. Johnsson, A., Heldin, C.-H., Wasteson, A., Westermark, B., Deuel, T.F., Huang, J.S., Seeburg, P.H., Gray, A., Ullrich, A., Scrace, G., Stroobant, P. and Waterfield, M.D. (1984). The c-*sis* gene encodes a precursor of the B chain of platelet-derived growth factor. *EMBO J.* 3: 921-928.

15. Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.N. (1983). Simian sarcoma virus *onc* gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. *Science* 221: 275-277.

16. Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Wastermark, B., Heldin, C.-H., Huang, J.S. and Deuel, T.F. (1983). Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. *Nature* 304: 35-39.

17. Cochran, B.H., Reffel, A.C. and Stiles, C.D. (1983). Molecular cloning of gene sequences regulated by platelet-derived growth factor. *Cell* 33: 939-947.

18. Heldin, C.-H. and Westermark, B. (1984). Growth factors: Mechanism of action and relation to oncogenes. *Cell* 37: 9-20.

19. Marquardt, H., Hunkapiller, M.W., Hood, L.E., Twardzik, D.R.,

De Larco, J.E., Stephenson, J.R. and Todaro, G.J. (1983). Transforming growth factors produced by retrovirus-transformed rodent fibroblasts and human melanoma cells: amino acid sequence homology with epidermal growth factor. *Proc.Natl.Acad.Sci.USA* **80**: 4684-4688.

20. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1984). Close similarity of epidermal growth factor receptor and v-*erb-B* oncogene protein sequences. *Nature* **307**: 521-527.

21. Kris, R., Lax, I., Gullick, W., Waterfield, M., Ullrich, A. and Schlessinger, J. (1985). Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF receptor and v-*erbB* protein. *Cell* **40**: 610-625.

22. Privalsky, M.L., Ralston, R. and Bishop, J.M. (1984). The glycoprotein encoded by the retroviral oncogene v-*erb-B* is structurally related to tyrosine specific protein kinases. *Proc.Natl.Acad.Sci.USA* **81**: 704-707.

23. Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D. and Seeburg, P.H. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* **309**: 418-425.

24. Yamamoto, T., Nishida, T., Miyajima, N., Kawai, S., Ooi, T. and Toyoshima, K. (1983). The *erbB* gene of avian erythroblastosis virus is a member of the *src* gene family. *Cell* **35**: 71-78.

25. Sherr, C.J., Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, .T. and Stanley, E.R. (1985). The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* **41**: 65-676.

26. Wheeler, E.F., Rettenmier, C.W., Look, A.T., Sherr, C.J. (1986). The v-*fms* oncogene induces factor independence and tumorigenicity in a CSF-1 dependent macrophage cell line. *Nature* **234**: 377-380.

27. Hunter, T. and Cooper, J.A. (1985). Protein-tyrosine kinases. *Ann.Rev.Biochem.* **54**: 897-930.

28. Collett, M.S. and Erikson, R.L. (1978). Protein kinase activity associated with the avian sarcoma virus *src* gene product. *Proc.Natl.Acad.Sci.USA* **75**: 2021-2024.

29. Takeya, T. and Hanafusa, H. (1983). Structure and sequence of the cellular gene homologous to the RSV *src* gene and the mechanism for generating the transforming virus. *Cell* **32**: 881-890.

30. Konopka, J.B., Watanabe, S.M. and Witte, O.N. (1984). An alteration of the human c-*abl* protein in K562 leukemia cells unmasks associated tyrosine kinase activity. *Cell* **37**: 1035-1042.

31. Moelling, K., Heimann, B., Beimling, P., Rapp, U.R., and Sanders, T. (1984). Serine and threonine-specific protein kinase activities of purified *gag-mil* and *gag-raf* proteins. *Nature***312**: 558-561.

32. Kloetzer, W.S., Maxwell, S.A. and Arlinghaus, R.B. (1983). $P85^{gag\text{-}mos}$ encoded by ts110 Moloney murine sarcoma virus has an associated protein kinase activity. *Proc.Natl.Acad.Sci.USA* **80**: 412-416.

33. Bonner, T.I., Kerby, S.B., Sutrave, P., Gunnell, M.A., Mark, G. and Rapp, U.R. (1985). Structure and biological activity of human homologs of the *raf/mil* oncogene. *Mol.Cell.Biol.* 5: 1400-1407.

34. Rapp, U.R., Cleveland, J.L., Bonner, T.I. and Storm, S.M. (1988). The *raf* oncogenes. In: *The oncogene handbook* pp. 213-253, Reddy, E.P., Skalka, A.M. and Curran, T. (eds.).

35. Morrison, D.K., Kaplan, D.R., Rapp, U. and Roberts, T.M. (1988). Signal transduction from membrane to cytoplasm:Growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. *Proc.Natl.Acad.Sci.USA* **85**: 8855-8859.

36. Gilman, A.G. (1984). G proteins and dual control of adenylate cyclase. *Cell* **36**: 577-579.

37. Gilman, A.G. (1987). G proteins: transducers of receptor-generated signals. *Ann.Rev.Biochem.* **56**: 615-649.

38. Whitfield, J.F., Durkin, J.P., Franks, D.J., Kleine, L.P., Raptis, L., Rixon, R.H., Sikorska, M. and Walker, P.R. (1987). Calcium, cyclic AMP and protein kinase C - partners in mitogenesis. *Cancer Metast.Rev.* 5: 205-250.

39. Gibbs, J.B., Sigal, I.S., Poe, M. and Scolnick, E.M. (1984). Intrinsic GTPase activity distinguishes normal and oncogenic *ras*p21 molecules. *Proc.Natl.Acad.Sci.USA* **81**: 5704-5708.

40. Gibbs, J.B., Sigal, I.S. and Scolnick, E.M. (1985). Biochemical properties of normal and oncogenic *ras p21*. *TIBS* **10**: 350-353.

41. Kolch, W., Heidecker, G., Lloyd, P. and Rapp, U.R. (1991). Raf-1 protein

kinase is required for growth of induced NIH/3T3 cells. *Nature* **349**: 426-428.

42. Van Aelst, L., Barr, M., Marcus, S., Polverino, A. and Wigler, M. (1993). Complex formation between RAS and RAF and other protein kinases. *Proc.Natl.Acad.Sci.USA* **90**: 6213-6217.

43. Crews, C.M. and Erikson, R.L. (1992). Purification of a murine protein-tyrosine/threonine kinase that phosphorylates and activates the *Erk-1* gene product: relationship to the fissation yeast *byr1* gene product. *Proc.Natl.Acad.Sci.USA* **89**: 8205-8209.

44. Crews, C.M. and Erikson, R.L. (1993). Extracellular signals and reversible protein phosphorylation: what to Mek of it all. *Cell* **74**: 215-217.

45. Schonthal, A., Gebel, S., Stein, B., Ponta, H., Rahmsdorf, H.J. and Herrlich, P. (1988). Nuclear oncoproteins determine the genetic program in response to external stimuli. *Cold Spring Harbor Symp.Quant.Biol.* **LIII**: 779-787.

46. Cochran, B.H., Zullo, J., Verma, I.M. and Stiles, C.D. (1984). Expression of the c-*fos* gene and of an *fos*-related gene is stimulated by platelet-derived growth factor. *Science* **226**: 1080-1082.

47. Ryder, K. and Nathans, D. (1988). Induction of protooncogene c-*jun* by serum growth factors. *Proc.Natl.Acad.Sci.USA* **85**: 8464-8467.

48. Lau, L.F. and Nathans, D. (1987). Expression of a set of growth-related immediate early genes in BALB/C 3T3 cells: coordinate regulation with c-*fos* or c-*myc*. *Proc.Natl.Acad.Sci.USA* **84**: 1182-1186.

49. Jacob, F. and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis

of proteins. *J.Mol.Biol.* 3: 318-356.

50. Mitchell, P.J. and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 245: 371-378.

51. Johnson, P.F. and McKnight, S.L. (1989). Eukaryotic transcriptional regulatory proteins. *Ann.Rev.Biochem.* 58: 799-839.

52. Sukhatme, V.P., Kartha, S., Toback, F.G., Taub, R., Hoover, R.G. and Tsai-Morris, C.-H. (1987). A novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. *Oncogene Res.* 1: 343-355.

53. Pertovaara, L., Sistonen, L., Bos, T.J., Vogt, P.K., Keski-oja, J. and Alitalo, K. (1989). Enhanced *jun* gene expression is an early genomic response to transforming growth factor ß stimulation. *Mol.Cell.Biol.* 9: 1255-1262.

54. Dean, M., Levine, R.A., Ran, W., Kindy, M.S., Sonenshein, G.E. and Campisi, J. (1986). Regulation of c-*myc* transcription and mRNA abundance by serum growth factors and cell contact. *J.Biol.Chem.* 261: 9161-9166.

55. Greenberg, M.E. and Ziff, E.B. (1984). Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. *Nature* 311: 433-438.

56. Greenberg, M.E., Hermanowski, A.L. ad Ziff, E.B. (1986). Effect of protein synthesis inhibitors on growth factor activation of c-*fos*, c-*myc*, and actin gene transcription. *Mol.Cell.Biol.* 6: 1050-1057.

57. Yamamoto, K.R. (1976). Steroid receptors: elements for modulation of eukaryotic transcription. *Ann.Rev.Biochem.* 45: 721-746.

58. Verma, I.M., Visvader, J., Lamph, W.W., DeTogni, P., Barber, J. and Sassone-Corsi, P. (1989). Regulation of *fos* gene: a paradigm for nuclear oncogenes. In: *Gene transfer and gene therapy* pp.129-149.

59. Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. and Goodman, H.M. (1986). A cyclic AMP- and phorbol ester-inducible DNA element. *Nature* **323**: 353-356.

60. Kerr, L.D., Inoue, J., and Verma, I.M. (1992). Signal transduction: the nuclear target. *Curr. Opin. Cell Biol.* **4**: 496-501.

61. Morello, D., Lavenu, A. and Babinet, C. (1990). Differential regulation and expression of *jun*, c-*fos* and c-*myc* proto-oncogenes during mouse liver egeneration and after inhibition of protein synthesis. *Oncogene* **5**: 1511-1519.

62. Treisman, R. (1985). Transient accumulation of c-*fos* RNA following serum stimulation requires a conserved 5' element and c-*fos* 3' sequences. *Cell* **42**: 889-902.

63. Bohmann, D. and Tjian, R. (1989). Biochemical analysis of transcriptional activation by Jun: differential activity of c- and v-Jun. *Cell* **59**: 709-711.

64. Martland, H.S. (1931). The occurence of malignancy in radioactive persons. *Am.J.Cancer* **XV**: 2435-2516.

65. Curran, T. (1988). The *fos* oncogene. In: *The oncogene handbook* pp. 307-325, Reddy, E.P., Skalka, A.M. and Curran, T. (eds.).

66. Finkel, M.P., Biskis, B.O. and Jinkins, P.B. (1966). Virus induction of osteosarcomas in mice. *Science* **151**: 698-701.

67. Curran, T., Peters, G., Van Beveren, G., Teich, N.M. and Verma, I.M.

(1982). FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. *J. Virol.* **44**: 674-682.

68. Curran, T. and Teich, N.M. (1982). Candidate product of the FBJ murine osteosarcoma virus oncogene: characterization of a 59,000-dalton phosphoprotein. *J. Virol.* **42**: 114-122.

69. Curran,T., MacConnell, W.P., van Straaten, F. and Verma, I.M. (1983). Structure of the FBJ murine osteosarcoma virus genome: molecular cloning of its associated helper virus and the cellular homolog of the v-*fos* gene from mouse and human cells. *Mol. Cell. Biol.* **3**: 914-921.

70. van Sraaten, F., Muller, R., Curran, T., van Beveren, C. and Verma, I.M. (1983). Complete nucleotide sequence of a human c-*onc* gene: deduced amino acid sequence of the human c-*fos* protein. *Proc. Natl. Acad. Sci. USA* **80**: 3183-3187.

71. Van Beveren, C., Enami, S., Curran, T. and Verma, I.M. (1984). FBR murine osteosarcoma virus. *Virology* **135**: 229-243.

72. Van Beveren, C., van Straaten, F., Curran, T., Muller, R. and Verma, I.M. (1983). Analysis of FBJ-MuSV provirus and c-*fos* (mouse) gene reveals that viral and cellular *fos* gene products have different carboxy termini. *Cell* **32**: 1241-1255.

73. Barker, P.E., Rabin, M., Watson, M., Breg, W.R., Ruddle, F.H. and Verma, I.M. (1984). Human c-*fos* oncogene mapped within chromosomal region 14q21-q31. *Proc. Natl. Acad. Sci. USA* **81**: 5826-5830.

74. Miller, A.D., Curran, T. and Verma, I.M. (1984). c-Fos protein can induce

cellular transformation: a novel mechanism of activation of a cellular oncogene. *Cell* **36**: 51-60.

75. Meijlink, F., Curran, T., Miller, A.D. and Verma, I.M. (1985). Removal of a 67-base-pair sequence in the noncoding region of protooncogene *fos* converts it to a transforming gene. *Proc.Natl.Acad.Sci.USA* **82**: 4987-4991.

76. Whitfield, J.F. (1990). *Calcium, cell cycles, and cancer*. CRC Press,Inc.

77. Waechter, D.E., Avignolo, C., Freund, E., Riggenbach, C.M., Mercer, W.E., McGruire, P.M. and Baserga, R. (1984). Microinjection of RNA polymerase II corrects the temperature-sensitive defect of tsAF8 cells. *Mol.Cell.Biochem.* **60**: 77-82.

78. Lau, L.F. and Nathans, D. (1985). Identification of a set of genes expressed during the G_0/G_1 transition of cultured mouse cells. *EMBO J.* **4**: 3145-3151.

79. Almendral, J.M., Sommer, D., MacDonald-Bravo, H., Burckhardt, J., Perera, J. and Bravo, R. (1988). Complexity of the early genetic response to growth factors in mouse fibroblasts. *Mol.Cell.Biol.* **8**: 2140-2148.

80. Irving, S.G., June, C.H., Zipfel, P.F., Siebenlist, U. and Kelly, K. (1989). Mitogen-induced genes are subject to multiple pathways of regulation in the initial stages of T-cell activation. *Mol.Cell.Biol.* **9**: 1034-1040.

81. Herschman, H.R. (1991). Primary response genes induced by growth factors and tumor promoters. *Ann.Rev.Biochem.* **60**: 281-319.

82. Sassone-Corsi, P., Lamph, W.W. and Verma, I.M. (1988). Regulation of proto-oncogene *fos*: a paradigm for early response genes. *Cold Spring Harbor Symp.Quant.Biol.* **LIII**: 749-760.

83. Muller, R., Verma, I.M., and Adamson, E.D. (1983). Expression ofc-*onc* genes:c-*fos* transcripts accumulate to high levels during development of mouse placenta,yolk sac and amnion. *EMBO J.* 2: 679-684.

84. Muller, R., Curran, T., Muller, D. and Guilbert, L. (1985). Induction of c-*fos* during myelomonocytic differentiation and macrophage proliferation. *Nature* **314**: 546-548.

85. Mitchell, R.L., Zokas, L., Schreiber, R.D. and Verma, I.M. (1985). Rapid induction of the expression of proto-oncogene *fos* during human monocytic differentiation. *Cell* **40**: 209-217.

86. Greenberg, M.E., Greene, L.A. and Ziff, E.B. (1985). Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *J.Biol.Chem.* **260**: 14101-14110.

87. Hass, R., Brach, M., Kharbanda, S., Giese, G., Traub, P. and Kufe, D. (1991). Inhibition of phorbol ester-induced monocytic differentiation by dexamethasone is associated with down-regulation of c-*fos* and c-*jun* (AP-1). *J.Cell.Physiol.* **149**: 125-131.

88. Muller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984). Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. *Nature* **312**: 716-720.

89. Kruijer, W., Cooper, J.A., Hunter, T. and Verma, I.M. (1984). Platelet-derived growth factor induces rapid but transient expression of the c-*fos* gene and protein. *Nature* **312**: 711-716.

90. Riabowol, K.T., Vosatka, R.J., Ziff, E.B., Lamb, N.J. and Feramisco, J.R.

(1988). Microinjection of *fos*-specific antibodies blocks DNA synthesis in fibroblast cells. *Mol.Cell.Biol.* 8: 1670-1676.

91. Nishikura, K. and Murray, J.M. (1987). Antisense RNA of proto-oncogene *c-fos* blocks renewed growth of quiescent 3T3 cells. *Mol.Cell.Biol.* 7: 639-649.

92. Holt, J.T., Venkat Gopal, T., Moulton, A.D. and Nienhuis A.W. (1986). Inducibile production of *c-fos* antisense RNA inhibits 3T3 cell proliferation. *Proc.Natl.Acad.Sci.USA* 83: 4794-4798.

93. Bravo, R., Burckhardt, J., Curran, T. and Muller, R. (1986). Expression of c-*fos* in NIH3T3 cells is very low but inducible throughout the cell cycle. *EMBO J.* 5: 695-700.

94. Ruther, U., Garber, C., Komitowski, D., Muller, R. and Wagner, E.F. (1987). Deregulated c-*fos* expression interferes with normal bone development in transgenic mice. *Nature* 325: 412-416.

95. Ruther, U. and Wagner, E.F. (1989). The specific consequences of c-*fos* expression in transgenic mice. *Prog.Nuc.Acid Res.Mol.Biol.* 36: 235-245.

96. Curran, T., Miller, D., Zokas, L. and Verma, I.M. (1984). Viral and cellular Fos proteins: a comparative analysis. *Cell* 36: 259-268.

97. Curran, T. and Morgan, J.I. (1986). Barium modulates c-*fos* expression and post-translational modification. *Proc.Natl.Acad.Sci.USA* 83: 8521-8524.

98. Barber, J.R. and Verma, I.M. (1987). Modification of Fos proteins: phosphorylation of c-Fos, but not v-Fos, is stimulated by 12-tetradecanoyl-phorbol-13-acetate and serum. *Mol.Cell.Biol.* 7: 2201-2211.

99. Curran, T., Gordon, M.B., Rubino, K.L. and Sambucetti, L.C. (1987).

Isolation and characterization of the c-*fos*(rat) cDNA and analysis of post-translational modification *in vitro*. *Oncogene* 2: 79-84.

100. Ofir, R., Dwarki, V.J., Rashid, D. and Verma, I.M. (1990). Phosphorylation of the C terminus of Fos protein is required for transcriptional transrepression of the c-*fos* promoter. *Nature* **348**: 80-82.

101. Kingston, R.E., Baldwin, A.S. and Sharp, P.A. (1985). Transcription control by oncogenes. *Cell* **41**: 3-5.

102. Bravo, R., Neuberg, M., Burckhardt, J., Almendral, J., Wallich, R. and Muller, R. (1987). Involvement of common and cell type-specific pathways in c-*fos* gene control: stable induction by cAMP in macrophages. *Cell* **48**: 251-260.

103. Curran, T., Van Beveren, C., Ling, N. and Verma, I.M. (1985). Viral and cellular *fos* proteins are complexed with a 39,000-dalton cellular protein. *Mol.Cell.Biol.* 5: 167-172.

104. Sambucetti, L.C. and Curran, T. (1986). The Fos protein complex is associated with DNA in isolated nuclei and binds to DNA cellulose. *Science* **234**: 1417-1419.

105. Setoyama, C., Frunzio, R., Liau, G., Mudryj, M. and de Crombrugghe, B. (1986). Transcriptional activation encoded by the v-*fos* gene. *Proc.Natl.Acad.Sci.USA* **83**: 3213-3217.

106. Distel, R.J., Ro, H.-S., Rosen, B.S., Groves, D.L. and Spiegelman, B.M. (1987). Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct partecipation of c-*fos*. *Cell* **49**: 835-844.

107. Rauscher III, F.J., Sambucetti, L.C., Curran, T., Distel, R.J. and Spiegelman, B.M. (1988). Common DNA binding site for Fos protein complexes and transcription factor AP-1. *Cell* 52: 471-480.

108. Maki, Y., Bos, T.J., Davis, C., Starbuck, M. and Vogt, P.K. (1987). Avian sarcoma virus 17 carries the *jun* oncogene. *Proc.Natl.Acad.Sci.USA* 84: 2848-2852.

109. Vogt, P.K., Bos, T.J. and Doolittle, R.F. (1987). Homology between the DNA-binding domain of the GCN4 regulatory protein of the yeast and the carboxyl-terminal region of a protein coded for by the oncogene *jun*. *Proc.Natl.Acad.Sci.USA* 84: 3316-3319.

110. Hope, I.A. and Struhl, K. (1986). Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* 46: 885-894.

111. Lee, W., Haslinger, A., Karin, M. and Tjian, R. (1987). Activation of tran scription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature* 325: 368-372.

112. Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K. and Tjian, R. (1987). Human proto-oncogene c-*jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* 238: 1386-1392.

113. Angel, P., Allegretto, E.A., Okino, S.T., Hattori, K., Boyle, W.J., Hunter, T. and Karin, M. (1988). Oncogene *jun* encodes a sequence-specific *trans*-activator similar to AP-1. *Nature* 332: 166-171.

114. Bos, T.J., Bohmann, D., Tsuchie, H., Tjian, R. and Vogt, P.K. (1988). v-*jun*

encodes a nuclear protein with enhancer binding properties of AP1. *Cell* 52: 705-712.

115. Franza, Jr., B.R., Rauscher III, F.J., Josephs, S.F. and Curran, T. (1988). The Fos complex and Fos-related antigen recognize sequence elements that contain AP-1 binding sites. *Science* 239: 1150-1153.

116. Rauscher III, F.J., Cohen, D.R., Curran, T., Bos, T.J., Vogt, P.K., Bohmann, D., Tjian, R. and Franza, Jr., B.R. (1988). Fos-associated protein p39 is the product of the *jun* proto-oncogene. *Science* 240: 1010-1016.

117. Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T. and Karin, M. (1988). The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* 54: 541-552.

118. Sassone-Corsi, P., Lamph, W.W., Kamps, M. and Verma, I.M. (1988). Fos-associated cellular p39 is related to nuclear transcription factor AP-1. *Cell* 54: 553-560.

119. Angel, P. and Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochimica et Biophysica Acta* 1072: 129-157.

120. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987). Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* 49: 729-739.

121. Lee, P., Mitchell, P. and Tjian, R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* 49: 741-752.

122. Bohmann, D., Admon, A., Turner, D.R. and Tjian, R. (1988). Transcriptional regulation by the AP-1 family of enhancer-binding proteins: a nuclear target for signal transduction. *Cold Spring Harbor Symp.Quant.Biol.*LIII: 695-700.

123. Ryseck, R.-P. and Bravo, R. (1991). c-Jun, JunB and JunD differ in their binding affinities to AP-1 and CRE consensus sequences: effect of Fos proteins. *Oncogene* 6: 533-542.

124. Cohen, D.R. and Curran, T. (1988). *fra*-1: a serum inducible, cellular immediate-early gene that encodes a Fos-related antigen. *Mol.Cell.Biol.* 8: 2063-2069.

125. Matsui,M., Tokuhara, M., Konuma,Y., Nomura, N. and Ishizaki, R. (1990). Isolation of human *fos*-related genes and their expression during monocyte-macrophage differentiation. *Oncogene* 5: 249-255.

126. Nishina, H., Sato, H., Suzuki, T., Sato, M. and Iba, H. (1990). Isolation and characterization of *fra*-2, an additional member of the *fos* gene family. *Proc.Natl.Acad.Sci.USA* 87: 3619-3623.

127. Zerial, M., Toschi, L., Ryseck, R.-P., Schuermann, M., Muller, R. and Bravo, R. (1989). The product of a novel growth factor activated gene, FosB, interacts with Jun proteins enhancing their DNA binding activity. *EMBO J.* 8: 805-813.

128. Cohen, D.R., Ferreira, P.C.P., Gentz, R., Franza, R.,Jr. and Curran, T. (1988). The product of a *fos*-related gene, *fra*-1, binds cooperatively to Jun: transcription factor AP-1 is comprised of multiple protein complexes. *Genes Dev.* 173: 173-184.

129. Schuermann, M., Jooss, K. and Muller, R. (1991).*fos*B is a transforming gene encoding a transcriptional activator. *Oncogene* **6**: 567-576.

130. Lazo, P.S., Dorfma, K., Noguchi, T., Mattei, M.-G. and Bravo, R. (1992). Structure and mapping of the *fos*B gene. FosB downregulates the activity of the *fos*B promoter. *Nucl.Acids Res.* **20**: 343-350.

131. Nakabeppu, Y. and Nathans, D. (1991). A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. *Cell* **64**: 751-759.

132. Yen, J., Wisdom, R.M., Tratner, I. and Verma, I.M. (1991). An alternative spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins. *Proc.Natl.Acad.Sci.USA* **88**: 5077-5081.

133. Dobrzanski, P., Noguchi, T., Kovary, K., Rizzo, C.A., Lazo, P.S. and Bravo,R. (1991). Both products of the *fos*B gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. *Mol.Cell.Biol.* **11**: 5470-5478.

134. Nakabeppu, Y., Ryder, K. and Nathans, D. (1988). DNA binding activities of three murine Jun proteins: Stimulation by Fos. *Cell* **55**: 907-915

135. Lamph, W.W., Wamsley, P., Sassone-Corsi, P. and Verma, I.M. (1988). Induction of proto-oncogene *jun/AP-1* by serum and TPA. *Nature* **334**: 629-631.

136. Ryseck, R.-P., Hirai, S.I., Yaniv, M. and Bravo, R. (1988). Transcriptional activation of c-*jun* during the G_0/G_1 transition in mouse fibroblast. *Nature* **334**: 535-537.

137. Simmons, D.L., Levy, D.B., Yannoni, Y. and Erikson R.L. (1989). Identification of a phorbol ester-repressible v-*src*-inducible gene.

Proc.Natl.Acad.Sci.USA 86:1178-1182.

138. Hattori, K., Angel, P., Le Beau, M.M. and Karin, M. (1988). Structure and chromosomal localization of the functional intronless human *jun* protooncogene. *Proc.Natl.Acad.Sci.USA* 85: 9148-9152.

139. Angel, P., Hattori, K., Smeal, T. and Karin, M. (1988). The *jun* proto-oncogene is positively autoregulated by its product, jun/AP-1. *Cell* 55: 875-885.

140. Ryder, K., Lau, L.F. and Nathans, D. (1988). A gene activated by growth factors is related to the oncogene v-*jun*. *Proc.Natl.Acad.Sci.USA* 85: 1487-1491.

141. Chiu, R., Angel, P. and Karin, M. (1989). Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. *Cell* 59: 979-986.

142. Hirai, S.-I., Ryseck, R.-P., Mechta, F., Bravo, R. and Yaniv, M. (1989). Characterization of *jun*D: a new member of the *jun* proto-oncogene family. *EMBO J.* 8: 1433-1439.

143. Ryder, K., Lanahan, A., Perez-Albuerne, E. and Nathans, D. (1989). *Jun-D*: a third member of the *jun* gene family. *Proc.Natl.Acad.Sci.USA* 86: 1500-1503.

144. Hartl, M., Hutchins, J.T. and Vogt, P.K. (1991). The chicken *junD* gene and its product. *Oncogene* 6: 1623-1631.

145. Berger, I. and Shaul, Y. (1991). Structure and function of human *jun*-D. *Oncogene* 6: 561-566.

146. Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. and Goodman, R.H. (1986). Identification of a cyclic-AMP-responsive element

within the rat somatostatin gene. *Proc.Natl.Acad.Sci.USA* 83: 6682-6686.

147. Kovary. K. and Bravo, R. (1991). Expression of different Jun and Fos proteins during the G_0-to-G_1 transition in mouse fibroblasts: *in vitro* and *in vivo* associations. *Mol.Cell.Biol.* 11: 2451-2459.

148. Sonnenberg, J.L., Macgregor-Leon, P.F., Curran, T. and Morgan, J.I. (1989). Dynamic alterations occur in the levels and composition of transcription factor AP-1 complexes after seizure. *Neuron* 3: 359-365.

149. Kovary, K. and Bravo, R. (1992). Existence of different Fos/Jun complexes during the G_0-to-G_1 transition and during exponential growth in mouse fibroblast: differential role of Fos proteins. *Mol.Cell.Biol.* 12: 5015-5023.

150. Castellazzi, M., Spyrou, G., La Vista, N., Dangie, J.-P., Piu, F., Yaniv, M. and Brun, G. (1991). Overexpression of c-*jun*, *jun*B, or *jun*D affects cell growth differently. *Proc.Natl.Acad.Sci.USA* 88: 8890-8894.

151. Landshulz, W.H., Johnson, P.F., and McKnight, S.L. (1988). The leucine zipper:A hypothetical structure common to a new class of DNA binding proteins. *Science* 240: 1759-1764.

152. Landschulz, W.H., Johnson, P.F., Graves, B.J., and McKnight, S.L. (1988). Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.* 2: 786-800.

153. O'Shea E.K., Rutkowski, R., Kim, P.S. (1989). Evidence that the leucine zipper is a coiled coil. *Science* 243: 538-542.

154. O'Shea, E.K., Rutkowski, R., and Kim, P.S. (1989). Evidence that the leucine zipper is a coiled coil. *Science* 243: 538-543.

155. Nakabeppu,Y. and Nathans, D. (1989). The basic region of Fos mediates specific DNA binding. *EMBO J.* **12**: 3833-3841.

156. Gentz, R., Rauscher III, F.J., Abate, C. and Curran, T. (1989). Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. *Science* **243**: 1695-1699.

157. Turner, R. and Tjian, R. (1989). Leucine repears and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. *Science* **243**: 1689-1694.

158. Vinson, C.R., Sigler, P.B. and McKnight, S.L. (1989). Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* **246**: 911-916.

159. Sassone-Corsi, P., Ransone, L.J., Lamph, W.W. and Verma, I.M. (1988). Direct interaction between Fos and Jun nuclear oncoproteins: role of the "leucine zipper" domain. *Nature* **336**: 692-695.

160. Kouzarides, T. and Ziff, E. (1988). The role of the leucine zipper in the Fos-Jun interaction. *Nature* **336**: 646-651.

161. Halazonetis, T.D., Georgopoulos, K., Greenberg, M.E. and Leder, P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* **55**: 917-924.

162. O'Shea, E.K., Rutkowski, R. and Kim, P.S. (1992). Mechanism of specificity in the Fos-Jun oncoprotein heterodimer. *Cell* **68**: 699-708.

163. Ransone, L.J., Wamsley, P., Morley, K.L. and Verma, I.M. (1990). Domain swapping reveals the modular nature of Fos, Jun, and CREB proteins. *Mol.Cell.Biol.* **10**: 4565-4573.

164. Roesler, W.J., Vanderbark, G.R. and Hanson, R.W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. *J.Biol.Chem.* 263: 9063-9066.

165. Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H. and Montminy, M.R. (1988) Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* 334: 494-498.

166. Hai, T., Liu, F., Coukos, W.J. and Green, M.R. (1989). Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* 3: 2083-2090.

167. Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L. and Habener, J.F. (1988). Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. *Science* 242: 1430-1433.

168. Gonzalez, G.A., Yamamoto, K.K., Fischer, W.H., Karr, D., Menzel, P., Biggs III, W., Vale, W.W. and Montminy, M.R. (1989). A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature* 337: 749-752.

169. Sassone-Corsi, P., Ransone, L.J. and Verma, I.M. (1990). Cross-talk in signal transduction: TPA-inducible factor Jun/AP-1 activates cAMP-responsive elements. *Oncogene* 5: 427-431.

170. Rauscher III, F.J., Voulalas, P.J., Franza, Jr., B.R. and Curran, T. (1988). Fos and Jun bind cooperatively to the AP-1 site: reconstitution *in vitro*. *Genes Dev.* 2: 1687-1699.

171. Maekawa, T., Sakura, H., Kanei-Ishii, C., Sudo, T., Yoshimura, T., Fujisawa, J-i., Yoshida, M. and Ishii, S. (1989). Leucine zipper structure of the

protein CRE-BP1 binding to the cyclic AMP response element in brain. *EMBO J.* 8: 2023-2028.

172. Macgregor, P.F., Abate, C. and Curran, T. (1990). Direct cloning of leucine zipper proteins: Jun binds cooperatively to the CRE with CRE-BP1. *Oncogene* 5: 451-458.

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

174. Smeal,T., Angel, P., Meek, J. and Karin, M. (1989). Different requirements for formation of Jun:Jun and Jun:Fos complexes. *Genes Dev.* 3: 2091-2100.

175. Hoeffler, J.P., Deutsch, P.J., Lin, J. and Habener, J.F. (1989). Distinct adenosine 3', 5'- monophosphate and phorbol ester-responsive signal transduction pathways converge at the level of transcriptional activation by the interactions of DNA-binding proteins. *Mol.Endocrinol.* 3: 868-880.

176. Kruijer, W., Schubert, D. and Verma, I.M. (1985). Induction of the proto-oncogene *fos* by nerve growth factor. *Proc.Natl.Acad.Sci.USA* 82: 7330-7334.

177. Bravo, R., Neuberg, M., Burckhardt, J., Almendral, J., Wallich, R. and Muller, R. (1987). Involvement of common and cell type-specific pathways in c-*fos* gene control: stable induction by cAMP in macrophages. *Cell* 48: 251-260.

178. de Groot, R.P. and Sassone-Corsi, P. (1992). Activation of Jun/AP-1 by protein kinase A. *Oncogene* 7: 2281-2286.

179. Yoshimasa, T., Sibley, D.R., Bouvier, M., Lefkowitz, R.J. and Caron, M.G. (1987). Cross-talk between cellular signalling pathways suggested by phorbol-

ester-induced adenylate cyclase phosphorylation.

180. Schule, R., Rangarajan, P., Yang, N., Kliewer, S., Ransone, L.J., Bolado, J., Verma, I.M. and Evans, R.M. (1991). Retinoic acid is a negative regulator of AP-1-responsive genes. *Proc.Natl.Acad.Sci.USA* **88**: 6092-6096.

181. Riabowol, K., Schiff, J. and Gilman, M.Z. (1992). Transcription factor AP-1 activity is required for initiation of DNA synthesis and is lost during cellular aging. *Proc.Natl.Acad.Sci.USA* **89**: 157-161.

182. Seshadri, T. and Campisi, J. (1990). Repression of c-*fos* transcription and an altered genetic program in senescent human fibroblasts. *Science* **247**: 205-209.

183. Irving, J., Feng, J.,Wistrom, C., Pikaart, M. and Villeponteau, B. (1992). An altered repertoire of *fos/jun* (AP-1) at the onset of replicative senescence. *Exp.Cell Res.* **202**: 161-166

184. Lucibello, F.C., Brusselbach, S., Sewing, A. and Muller, R. (1993). Suppression of the growth factor-mediated induction of c-*fos* and down modulation of AP-1-binding activity are not required for cellular senescence. *Oncogene* **8**: 1667-1672.

185. Rose, D.W., McCabe, G., Feramisco, J.R. and Adler, M. (1992). Expression of c-*fos* and AP-1 activity in senescent human fibroblasts is not sufficient for DNA synthesis. *J.Cell Biol.* **119**: 1405-1411.

186. Hunter, T. and Karin, M. (1992). The regulation of transcription by phosphorylation. *Cell* **70**: 375-387.

187. Hai, T., Liu, F., Allegretto, E.A., Karin, M. and Green, M.R. (1988). A family of immunologically related transcription factors that includes multiple

forms of ATF and AP-1. *Genes Dev.* 2: 1216-1226.

188. Sharrocks, A.D., Gille, H. and Shaw, P.E. (1993). Identification of amino acids essential for DNA binding and dimerization in $p67^{SRF}$: implications for a novel DNA-binding motif. *Mol.Cell.Biol.* 13: 123-132.

189. Smeal, T., Binetruy, B., Mercola, D., Grover-Bardwick, A., Heidecker, G., Rapp, U.R. and Karin, M. (1992). Oncoprotein-mediated signalling cascade stimulates c-Jun activity by phosphorylation of serines 63 and 73. *Mol.Cell.Biol.* 12: 3507-3513.

190. Smeal, T., Binetruy, B., Mercola, D.A., Birrer, M. and Karin, M. (1991). Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature* 354: 494-496.

191. Binetruy, B., Smeal, T., and Karin, M. (1991). Ha-Ras augments c-*jun* activity and stimulates phosphorylation of its activation domain. *Nature* 351: 122-127.

192. Baker, S.J., Kerppola, T.K., Luk, D., Vandemberg, M.T., Marshak, D.R., Curran, T. and Abate, C. (1992). Jun is phosphorylated by several protein kinases at the same sites that are modified in serum-stimulated fibroblasts. *Mol.Cell.Biol.* 12: 4694-4705.

193. Alvarez, E., Northwood, I.C., Gonzalez, F.A., Latour, D.A., Seth, A., Abate, C., Curran, T. and Davis, J.R. (1991). Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. *J.Biol.Chem.* 266: 15277-15285.

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

670-674.

195. Boyle, W.J., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M. and Hunter, T. (1991). Activation of protein kinase C decreases phosphorylation of c-*jun* at sites that negatively regulate its DNA-binding activity. *Cell* **64**: 573-584.

196. Abate, C., Baker, S.J., Lees-Miller, S.P., Anderson, C.W., Marshak, D.R. and Curran, T. (1993). Dimerization and DNA binding alter phosphorylation of Fos and Jun. *Proc.Natl.Acad.Sci.USA* **90**: 6766-6770.

197. Auwerx, J. and Sassone-Corsi, P. (1991). IP-1: a dominant inhibitor of Fos/Jun whose activity is modulated by phosphorylation. *Cell* **64**: 983-993.

198. Auwerx, J. and Sassone-Corsi, P. (1992). AP-1 (Fos-Jun) regulation by IP-1: effect of signal transduction pathways and cell growth. *Oncogene* 7: 2271-2280.

199. Miner, J.N., Diamond, M.I. and Yamamoto, K.R. (1991). Joints in the regulatory lattice: composite regulation by steroid receptor-AP1 complexes. *Cell Growth Diff.* 2: 525-530.

200. Yang-yen, H.-F., Chambard, J.-C., Sun, Y.-L., Smeal, T., Schmidt, T.J., Drouin,J. and Karin, M. (1990). Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* **62**: 1205-1215.

201. Jonat, C., Rahmsdorf, H.J., Park, K.-K., Cato, A.C.B., Gebel, S., Ponta, H. and Herrlich, P. (1990). Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* **62**: 1189-1204.

202. Schule, R., Rangarajan, P., Kliewer, S., Ransone, L.J., Bolado, J., Yang, N., Verma, I.M. and Evans, R.M. (1990). Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* **62**: 1217-1226.

203. Beato, M. (1989). Gene regulation by steroid hormones. *Cell* **56**: 335-344.

204. Chandler, V.L., Maler, B.A. and Yamamoto, K.R. (1983). DNA sequences bound specifically by glucocorticoid receptor *in vitro* render a heterologous promoter hormone responsive *in vivo*. *Cell* **33**: 489-499.

205. Pabo, C.O. and Sauer, R.T. (1992). Transcription factors: structural families and principles of DNA recognition. *Annu.Rev.Biochem.* **61**: 1053-1095.

206. Mordacq, J.C. and Linzer, D.I.H. (1989). Co-localization of elements required for phorbol ester stimulation and glucocorticoid repression of proliferin gene expression. *Genes Dev.* **3**: 760-769.

207. Diamond, M.I., Miner, J.N., Yoshinaga, S.K. and Yamamoto K.R. (1990). Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* **249**: 1266-1272.

208. Zhang, H. and Young, A.P. (1991). A single upstream glucocorticoid response element juxtaposed to an AP1/ATF/CRE-like site renders the chicken glutamine synthetase gene hormonally inducibile in transfected retina. *J.Biol.Chem.* **266**: 24332-24338.

209. Lucibello, F.C., Slater, E.P., Jooss, K.U., Beato, M. and Muller, R. (1990). Mutual transrepression of Fos and the glucocorticoid receptor: involvement of a functional domain in Fos which is absent in FosB. *EMBO J.* **9**: 2827-2834.

210. Kerppola, T.K., Luk, D. and Curran, T. (1993). Fos is a preferential target of

glucocorticoid receptor inhibition of AP-1 activity in vitro. *Mol.Cell.Biol.* **13**: 3782-3791.

211. Abate, C., Patel, L., Rauscher III, F.J. and Curran, T. (1990). Redox regulation of Fos and Jun DNA-binding activity *in vitro*. *Science* **244**: 1157-1161.

212. Abate, C., Luk, D. and Curran, T. (1990). A ubiquitous nuclear protein stimulates the DNA-binding activity of Fos and Jun indirectly. *Cell Growth Diff.* **1**: 455-462.

213. Xanthoudakis, S. and Curran, T. (1992). Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO J.* **11**: 653-665.

214. Okuno, H., Akahori, A., Sato, H., Xanthoudakis, S., Curran, T. and Iba, H. (1993). Escape from redox regulation enhances the transforming activity of Fos. *Oncogene* **8**: 695-701.

215. Renz, M., Neuberg, M., Kurz, C., Bravo, R. and Muller, R. (1985). Regulation of c-*fos* transcription in mouse fibroblasts: identification of DNase I-hypersensitive sites and regulatory upstream sequences. *EMBO J.* **4**: 3711-3716.

216. Elgin, S.C.R. (1984). Anatomy of hypersensitive sites. *Nature* **309**: 213-214.

217. Feng, J. and Villeponteau, B. (1990). Serum stimulation of the c-*fos* enhancer induces reversible changes in c-*fos* chromatin structure. *Mol.Cell.Biol.* **10**: 1126-1133.

218. Stewart, A.F., Herrera, R.E. and Nordheim, A. (1990). Rapid induction of c-*fos* transcription reveals quantitative linkage of RNA polymerase II and DNA

topoisomerase I enzyme activities. *Cell* **60**: 141-149.

219. Wellington,C.L., Greenberg, M.E. and Belasco, J.G. (1993). The destabilizing elements in the coding region of c-*fos* mRNA are recognized as RNA. *Mol.Cell.Biol.* **13**: 5034-5042.

220. Wilson, T. and Treisman, R. (1988). Fos C-terminal mutations block down-regulation of c-*fos* transcription following serum stimulation. *EMBO J.* **7**: 4193-4202.

221. Gilman, M.Z., Wilson, R.N. and Weinberg, R.A. (1986). Multiple protein-binding sites in the 5'-flanking region regulate c-*fos* expression. *Mol.Cell.Biol.* **6**: 4305-4316.

222. Treisman, R. (1986). Identification of a protein-binding site that mediatestranscriptional response of the c-*fos* gene to serum factors. *Cell* **46**: 567-574.

223. Fisch, T.M., Prywes, R. and Roeder, R.G. (1987). c-*fos* sequences necessary for basal expression and induction by epidermal growth factor, 12-0-tetradecanoyl phorbol-13-acetate, and the calcium ionophore. *Mol.Cell.Biol.* **7**: 3490-3502.

224. Stumpo, D.J., Stewart, T.N., Gilman, M.Z. and Blackshear, P.J. (1988). Identification of the c-*fos* sequences involved in induction by insulin and phorbol esters. *J.Biol.Chem.* **263**: 1611-1614.

225. Gilman, M.Z. (1988). The c-*fos* serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. *Genes Dev.* **2**: 394-402.

226. Berkowitz, L.A., Riabowol, K.T. and Gilman, M.Z. (1989). Multiple sequence

elements of a single functional class are required for cyclic AMP responsiveness of the mouse c-*fos* promoter. *Mol.Cell.Biol.* 9: 4272-4281.

227. Fisch, T.M., Prywes, R., Simon, M.C. and Roeder, R.G. (1989). Multiple sequence elements in the c-*fos* promoter mediate induction by cAMP. *Genes Dev.* 3: 198-211.

228. Wagner, B.J., Hayes, T.E., Hoban, C.J. and Cochran, B.H. (1990). The SIF binding element confers *sis*/PDGF inducibility onto the c-*fos* promoter. *EMBO J.* 9: 4477-4484.

229. Runkel, L., Shaw, P.E., Herrera, R.E., Hipskind, R.A. and Nordheim, A. (1991). Multiple basal promoter elements determine the level of human c-*fos* transcription. *Mol.Cell.Biol.* 11: 1270-1280.

230. Hayes, T.E., Kitchen, A.M. and Cochran, R.H. (1987). Inducible binding of a factor to the c-*fos* regulatory region. *Proc.Natl.Acad.Sci.USA* 84: 1272-1276.

231. Greenberg, M.E., Siegfried, Z. and Ziff, E.B. (1987). Mutation of the c-*fos* gene dyad symmetry element inhibits serum inducibility of transcription *in vivo* and the nuclear regulatory factor binding *in vitro*. *Mol.Cell.Biol.* 7: 1217-1225.

232. Treisman, R. (1990). The SRE: a growth factor responsive transcriptional regulator. *Semin.Cancer Biol.* 1: 47-58.

233. Mohun, T., Garrett, N. and Treisman, R. (1987). *Xenopus* cytoskeletal actin and human c-*fos* gene promoters share a conserved protein-binding site. *EMBO J.* 6: 667-673.

234. Minty, A. and Kedes, L. (1986). Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an

evolutionarily conserved repeated motif. *Mol.Cell.Biol.* **6**: 2125-2136.

235. Treisman, R. (1987). Identification and purification of a polypeptide that binds to the c-*fos* serum response element. *EMBO J.* **6**: 2711-2717.

236. Prywes, R. and Roeder, R.G. (1987). Purification of the c-*fos* enhancer-binding protein. *Mol.Cell.Biol.* **7**: 3482-3489.

237. Schroter, H., Shaw, P.E. and Nordheim, A. (1987). Purification of intercalator-released p67, a polypeptide that interacts specifically with the c-*fos* serum response element. *Nucl.Acids Res.* **15**: 10145-10158.

238. Norman, C., Runswick, M., Pollock, R. and Treisman, R. (1988). Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-*fos* serum response element. *Cell* **55**: 989-1003.

239. Prywes, R., Dutta, A., Cromlish, J.A. and Roeder, R.G. (1988). Phosphorylation of serum response factor, a factor that binds to the serum response element of the c-*fos* enhancer. *Proc.Natl.Acad.Sci.USA* **85**: 7206-7210.

240. Manak, J.R., de Bisschop, N., Kris, R.M. and Prywes, R. (1990). Casein kinase II enhances the DNA binding activity of serum response factor. *Genes Dev.* **4**: 955-967.

241. Manak, J.R. and Prywes, R. (1991). Mutation of serum response factor phosphorylation sites and the mechanism by which its DNA-binding activity is increased by casein kinase II. *Mol.Cell.Biol.* **11**: 3652-3659.

242. Marais, R.M., Hsuan, J.J., McGuigan, C., Wynne, J. and Treisman, R. (1992). Casein kinase II phosphorylation increases the rate of serum response

factor-binding site exchange. *EMBO J.* **11**: 97-105.

243. Manak, J.R. and Prywes, R. (1993). Phosphorylation of serum response factor by casein kinase II: evidence against a role in growth factor regulation of *fos* expression. *Oncogene* **8**: 703-711.

244. Boxer, L.M., Prywes, R., Roeder, R.G. and Kedes, L. (1989). The sarcomeric actin CArG-binding factor is indistinguishable from the c-*fos* serum response element. *Mol. Cell Biol.* **9**: 515-522.

245. Walsh, K. (1989). Cross-binding of factors to functionally different promoter elements in c-*fos* and skeletal actin genes. *Mol. Cell. Biol.* **9**: 2191-2201.

246. Walsh, K. and Schimmel, P. (1987). Two nuclear factors compete for the skeletal muscle actin promoter. *J. Biol. Chem.* **262**: 9429-9432.

247. Gustafson, T.A. and Kedes, L. (1989). Identification of multiple proteins that interact with functional regions of the human cardiac-actin promoter. *Mol. Cell. Biol.* **9**: 3269-3283.

248. Ryan, W.A., Franza, Jr., B.R. and Gilman, M.Z. (1989). Two distinct cellular phosphoproteins binds to the c-*fos* serum response element. *EMBO J.* **8**: 1785-1792.

249. Herrera, R.E., Shaw, P.E. and Nordheim, A. (1989). Occupation of the c-*fos* serum response element *in vivo* by a multi-protein complex is unaltered by growth factor induction. *Nature* **340**: 68-70.

250. Shaw, P.E., Schroter, H. and Nordheim, A. (1989). The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-*fos* promoter. *Cell* **56**: 563-572.

251. Shaw,P.E., Frasch, S. and Nordheim, A. (1989). Repression of c-*fos* transcription is mediated through $p67^{SRF}$ bound to the SRE. *EMBO J.* **8**: 2567-2574.

252. Levi, B.-Z., Kasik, J.W., Burke, P.A., Prywes, R., Roeder, R.G., Appella, E. and Ozato, K. (1989). Neonatal induction of a nuclear protein that binds to the c-*fos* enhancer. *Proc.Natl.Acad.Sci.USA* **86**: 2262-2266.

253. de Belle, I., Walker, P.R., Smith, I.C.P. and Sikorska, M. (1991). Identification of a multiprotein complex interacting with the c-*fos* serum response element. *Mol.Cell.Biol.* **11**: 2752-2759.

254. Graham, R. and Gilman, M. (1991). Distinct protein targets for signals acting at the c-*fos* serum response element. *Science* **251**: 189-192.

255. Radtke, J., Dooley, S., Blin, N. and Unteregger, G. (1991). A set of 4 nuclear proteins binds to a DNA sequence within the *fos* promoter region. *Mol.Biol.Reports* **15**: 87-92.

256. Attar, R.M. and Gilman, M.Z. (1992). Expression cloning of a novel zinc finger protein that binds to the c-*fos* serum response element. *Mol.Cell Biol.* **12**: 2432-2443.

257. Dalton, S. and Treisman, R. (1992). Characterization of SAP-1, a protein recruited by serum response factor to the c-*fos* serum response element. *Cell* **68**: 597-612.

258. Schroter, H., Mueller, C.G.F., Meese, K. and Nordheim, A. (1990). Synergism in ternary complex formation between the dimeric glycoprotein $p67^{SRF}$, polypeptide $p62^{TCF}$ and the c-*fos* serum response element. *EMBO J.* **9**:

1123-1130.

259. Mueller, C.G.F. and Nordheim, A. (1991). A protein domain conserved between yeast MCM1 and human SRF directs ternary complex formation. *EMBO J.* **10**: 4219-4229.

260. Dolan, J.W. and Fields, S. (1991). Cell-type-specific transcription in yeast. *Biochim.Biophys.Acta* **1088**: 155-169.

261. Grueneberg, D.A., Natesan, S., Alexandre, C. and Gilman, M.Z. (1992). Human and *Drosophila* homeodomain proteins that enhance the DNA-binding activity of serum response factor. *Science* **257**: 1089-1095.

262. Prywes, R. and Roeder, R.G. (1986). Inducible binding of a factor to the c-*fos* enhancer. *Cell* **47**: 777-784.

263. Norman, C. and Treisman, R. (1988). Analysis of serum response element function *in vitro*. *Cold Spring Harbor Symp.Quant.Biol.* **LIII**: 719-726.

264. Gille, H., Sharrocks, A.D. and Shaw, P.E. (1992). Phosphorylation of transcription factor $p62^{TCF}$ by MAP kinase stimulates ternary complex formation at c-*fos* promoter. *Nature* **358**: 414-417.

265. Zinck, R., Hipskind, R.A., Pingoud, V. and Nordheim, A. (1993). c-*fos* transcriptional activation and repression correlate temporally with the phosphorylation status of TCF. *EMBO J.* **12**: 2377-2387.

266. Hipskind, R.A., Rao, V.N., Mueller, C.G.F., Reddy, E.S.P. and Nordheim, A. (1991). Ets-related protein Elk-1 is homologous to the c-*fos* regulatory factor $p62^{TCF}$. *Nature* **354**: 531-534.

267. Wasylyk, B., Wasylyk, C., Flores, P., Begue, A., Leprince, D. and

Stehelin, D. (1990). The c-*ets* proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. *Nature* **346**: 191-193.

268. Wasylyk, B., Hahn, S.L. and Giovane, A. (1993). The Ets family of transcription factors. *Eur.J.Biochem.* **211**: 7-18.

269. Gualberto, A., lePage, D., Pons, G., Mader, S.L., Park, K., Atchison, M.L. and Walsh, K. (1990). Functional antagonism between YY1 and the serum response factor. *Mol.Cell.Biol.* **12**: 4209-4214.

270. Sassone-Corsi, P. and Verma, I.M. (1987). Modulation of c-*fos* gene transcription by negative and positive cellular factors. *Nature* **326**: 507-510.

271. Konig, H., Ponta, H., Rahmsdorf, U., Buscher, M., Schonthal, A., Rahmsdorf, H.J. and Herrlich, P. (1989). Autoregulation of *fos*: the dyad symmetry element as the major target of repression. *EMBO J.* **8**: 2559-2566

272. Subramaniam, M., Schmidt, L.J., Crutchfield III, C.E. and Getz, M.J. (1989). Negative regulation of serum-responsive enhancer elements. *Nature* **340**: 64-66.

273. Rivera, V.M., Sheng, M. and Greenberg, M.E. (1990). The inner core of the serum response element mediates both the rapid induction and subsequent repression of c-*fos* transcription following serum stimulation. *Genes Dev.* **4**: 255-268.

274. Gius, D., Cao, X., Rauscher III, F.J., Cohen, D.R., Curran, T. and Sukhatme, V.P. (1990). Transcriptional activation and repression by Fos are independent functions: the C terminus represses immediate-early gene expression via CArG elements. *Mol.Cell.Biol.* **10**: 4243-4255.

275. Wagner, B.J., Hayes, T.E., Hoban, C.J. and Cochran, B.H. (1990). The SIF binding element confers *sis*/PDGF inducibility onto the c-*fos* promoter. *EMBO J.* **9**: 4477-4484.

276. Sadowski, H.B. and Gilman, M.Z. (1993). Cell-free activation of a DNA-binding protein by epidermal growth factor. *Nature* **362**: 79-83.

277. Fu, X.-Y. and Zhang, J.-J. (1993). Transcription factor p91 interacts with the epidermal growth factor receptor and mediates activation of the c-*fos* gene promoter. *Cell* **74**: 1135-1145.

278. Siegfried, Z. and Ziff, E.B. (1989). Transcription activation by serum, PDGF, and TPA through the c-*fos* DSE: Cell type specific requirements for induction. *Oncogene* **4**: 3-11.

279. Habener, J. (1990). Cyclic AMP response element binding proteins: A cornucopia of transcription factors. *Mol. Endocrinol.* **4**: 1087-1094.

280. Lucas, P.C., and Granner, D.K. (1992). Hormone response domains in gene transcription. *Ann. Rev. Biochem.* **61**: 1131-1173.

281. Deutsch, P.J., Hoeffler, J.P., Jameson, J.L., Lin, J.C. and Habener, J.F. (1988). Structural determinants for transcriptional activation by cAMP-respon sive elements. *J.Biol. Chem.* **263**: 18466-18472.

282. Deutsch, P.J., Hoeffler, J.P., Jameson, J.L. and Habener, J.F. (1988). Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements that bind distinct proteins. *Proc. Natl. Acad. Sci. U.S.A.* **85**: 7922-7926.

283. Montminy, M.R., and Bilezikjian, L.M. (1987). Binding of a nuclear protein to

the cyclic-AMP response element of the somatostatin gene. *Nature* **328**: 175-178.

284. Powers, A.C., Tadeschi, F., Wright, K.E., Chan, J.S., and Habener, J.F. (1989). Somatostatin gene expression in pancreatic islet cells is directed by cell-specific DNA control elements and DNA-binding proteins. *J.Biol.Chem>* **264**: 10048-10056.

285. Short, M.L., Manohar, C.F., Furtado, M.R., Ghadge, G.D., Wolinsky, S.M., Thimmapaya, B., and Jungmann, R.A. (1991). Nucleotide and derived amino-acid sequences of the CRE-binding proteins from rat C6 glioma and HeLa cells. *Nucleic Acids Res.* **19**: 4920.

286. Ivashkiv, L.B., Liou, H., Kara, C.J., Lamph, W.W., Verma, I. M., and Glimcher, L.H. (1990). mXBP/CRE-BP2 and c-Jun form a complex ehich binds to the cyclic AMP, but not to the 12-o-tetradecanoylphorbol-13-acetate response element. *Mol. Cell. Biol.* **10**: 1609-1621.

287. Kara, C.J., Liou, H., Ivaskiv, L.B., and Glimcher, L.H. (1990). A cDNA for a human cyclic AMP response element binding protein which is distinct from CREB and expressed preferentially in brain. *Mol. Cell. Biol.* **10**: 1347-1357

288. Foulkes, N.S., Borelli, E., and Sassone-Corsi, P. (1991). CREM gene: Use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* **64**: 739-749.

289. Karpinski, B.A., Morle, G.D., Huggenvik, J., Uhler, M.D., and Leiden, J.M. (1992). Molecular cloning of human CREB-2: An ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response

element. *Proc. Natl. Acad. Sci. U.S.A.* **89**: 4820-4824.

290. Merino, A., Buckbinder, L., Mermelstein, F.H., and Reinberg, D. (1989). Phosphorylation of cellular proteins regulates their binding to the cAMP response element. *J. Biol. Chem.* **264**: 21266-21276.

291. Ruppert, S., Cole, T.J., Boshart, M., Schmid, E. and Schutz, G. (1992). Multiple mRNA isoforms of the transcriptional activator protein CREB: generation by alternative splicing and specific expression in primary spermatocytes. *EMBO J.* **11**:1503-1512.

292. Jungmann, R.A., Wang, X., Milkowski, D.M. and Short, M.L. (1992). Glucocorticoid induction of CRE-binding protein isoform mRNAs in rat C6 glioma cells. *Nucleic Acids Res.* **20**: 825-829.

293. Gonzalez, G.A., and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**: 675-680.

294. Lee C.Q., Yun, Y., Hoeffler, J.P., and Habener, J.F. (1990). Cyclic AMP-responsive transcriptional activation of CREB-327 involves interdependent phosphorylated subdomains. *EMBO J.* **9**: 4455-4465.

295. Sheng, M., Thompson, M.A., and Greenberg, M.E. (1991). CREB: A Ca-regulated transcription factor phosphorylated by calmodulin dependent kinases. *Science* **252**: 1427-1430.

296. Masquilier, D. and Sassone-Corsi, P. (1992). Transcriptional cross talk: Nuclear factors CREM and CREB bind to AP-1 sites and inhibit activation by Jun. *J. Biol. Chem.* **267**: 22460-22466.

297. Robbins,P.D., Horowitz,J.M., and Mulligan, R.C. (1990). Negative regulation of human c-*fos* expression by the retinoblastoma gene product. *Nat.***346**:668-671.

298. Kim,S.-J., Lee,H.-D., Robbins,P.D., Busam, K., Sporn, M.B., and Roberts, A.B. (1991). Regulation of transforming growth factor ß1 gene expression by the product of the retinoblastoma susceptibility gene. *Proc. Natl. Acad. Sci. U.S.A.***8**:3052-3056.

299. Rixon, R.H., Walker, P.R., and Whitfield, J.F. (1977). Fibrinolysis and liver regeration. *J. Cell. Physiol.* **92**: 13-22.

300. Solt, D. and Farber, E. (1976). New principle for the analysis of chemical carcinogenesis. *Nature* **263**: 701-703.

301. MacManus, J.P. (1979). Occurrence of a low molecular weight calcium-binding protein in neoplastic liver. *Cancer Res.* **39**: 3000-3005.

302. Morris, H.P. (1965). Studies on the development, biochemistry and biology of experimental hepatomas. *Adv. Cancer Res.* **9**: 227-302.

303. L.G. Luna (1968). Haematoxylin-phloxine and saffron staining. In: Manual of Histologic staining methods of the Armed Forces Institute of Pathology.
3rd edition, pp. 39-40.

304. Sikorska, M., MacManus, J.P., Walker, P.R., and Whitfield, J.F. (1980). The protein kinases of rat liver nuclei. *Biochem. Bipphys. Res. Commun.* **93**: 1196-1203.

305. Filipski, J.,and Kohn, K.W. (1982). Elipticine-induced protein-associated DNA breaks in isolated L1210 nuclei. *Biochim. Biophys. Acta* **698**: 280-286.

306. Maire, P., Wuarin, J., and Schibler, U. (1989). The role of cis-acting promoter

elements in tissue-specific albumin gene expression. *Science* **244**: 343-346.

307. Dignam, J.D., Lebowitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**: 1475-1489.

308. Kadonaga, J.T., and Tjian, R. (1986). Affinity purification of sequence-specific DNA-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* **83**: 5889-5893.

309. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

310. Silva, C.M., Tully, C.B., Petch, L.A., Jewell, C.M., and Cidlowski, J.A. (1987). Application of a protein-blotting procedure to the study of human glucocorticoid receptor interactions with DNA. *Proc. Natl. Acad. Sci. U.S.A.* **84**: 1744-1748.

311. Garner, M.M., and Revzin, A. (1981). A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. *Nucleic Acids Res.* **9**: 3047-3060.

312. Fried, M. and Crothers, D.M. (1981). Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* **9**: 6505-6525.

313. Kwast-Welfeld, J., de Belle, I., Walker, P.R., Whitfield, J.F., and Sikorska, M. (1993). Identificatiion of a new cAMP responsc element binding factor by Southwestern blotting. *J. Biol. Chem.* **268**: 19581-19585.

314. Chirgwin, J.J., Przbyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979).

Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294-

315. Ausubel, F.M, Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G., and Struhl, K. (Eds.). Current Protocols in Molecular Biology (1987)., J. Wiley & Sons.

316. Jameson, B.A., and Wolf, H. (1988). The antigenic index: a novel algorithm for predicting antigenic determinants. *Comput. Appl. Biosci.* **4**: 181-186.

317. Houghten, R.A. (1985). General method for the rapid solid-phase synthesis of large numbers of peptides:specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. U.S.A.* **82**: 680-685.

318. Houghten, R.A., Bray, M.B., DeGraw, S.T.,and Kirby, C.J. (1986). Simplified procedure for carrying out simultaneous multiple hydrogen fluoride cleavages of protected peptide resins. *Int. J. Peptide Protein Res.* **27**: 673-678.

319. MacManus, J.P., and Brewer, L.M. (1987). Isolation, localization and properties of the oncodevelopmental calcium-binding protein oncomodulin. *Meth. Enzymol.* **139**: 156-168.

320. O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J.Biol.Chem.* **250**: 4007-4021.

321. Mohn, K.L., Laz, T.M., Melby, A.E. and Taub, R. (1990). Immediate-early gene expression differs between regenerating liver, insulin-stimulated H-35 cells, and mitogen-stimulated Balb/c 3T3 cells. *J.Biol.Chem.* **265**: 21914-21921.

322. Kruijer, W., Skelly, H., Botteri, F., van der Putten, H., Barber, J.R., Verma, I.M. and Leffert, H.L. (1986). Proto-oncogene expression in

regenerating rat liver is stimulated in cultures of primary adult rat hepatocytes. *J.Biol.Chem.* **261**: 7929-7933.

323. Thompson, N.L., Mead, J.E., Braun, L., Goyette, M., Shank, P.R. and Fausto, N. (1986). Sequential protooncogene expression during rat liver regeneration.*Cancer Res.* **46**: 3111-3117.

324. Corral, M., Defer, N., Paris, B., Raymondjean, M., Corcos, D., Tichonicky, L., Kruh, J., Glaise, D., Kneip, B. and Gugen-Guillouzo, C. (1986). Isolation and characterization of complementary DNA clones for genes overexpressed in chemically induced rat hepatomas. *Cancer Res.* **46**: 5119-5124.

325. Gillen, M.F., Banville, D., Rutledge, R.G., Narang, S., Seligy, V.L., Whitfield, J.F., and MacManus, J.P. (1987). A complete complementary DNA for the oncodevelopmental calcium-binding protein, oncomodulin. *J.Biol.Chem.* **262**: 5308-5312.

326. Verrier, B., Muller, D., Bravo, R. and Muller, R. (1986). Wounding a fibroblast monolayer results in the rapid induction of the c-*fos* proto-oncogene. *EMBO J.* 5: 913-917.

327. Saeter, G., and Seglen, P.O. (1990). Cell biology of hepatocarcinogenesis. *Crit. Rev. Oncogenesis* **1**: 437-466.

328. Epstein, S., Ito, N., Merkow, L., and Farber, E. (1967). Cellular analysis of liver carcinogenesis:the induction of large hyperplastic nodules i. the liver with 2-fluorenylacetamide or ethionine and some aspects of their morphology and glycogen metabolism. *Cancer Res.* **27**: 1702-1711.

329. Farber, E. (1986). Some emerging general principles in the pathogenesis of

hepatocellular carcinomas. *Cancer Surveys* 5: 695-717.

330. Goyette, M., Petropoulos, C.J., Shank, P.R., and Fausto, N. (1984). Regulated transcription of c-Ki-*ras* and c-*myc* during compensatory growth of rat liver. *Mol. Cell. Biol.* 4: 1493-1498.

331. Corcos, D., Defer, N., Raymondjean, M., Paris, B., Corral, M., Tichonicky, L., Kruh, J., Glaise, D., Saulnier, A., and Gugen-Guillouzo, C. (1984). Correlated increase of the expression of the c-*ras* genes in chemically induced hepatocarcinomas. *Biochem. Biophys. Res. Commun.* 122: 259-264.

332. Cote, G.J., Lastra, B.A., Cook, J.R., Huang, D., and Chiu, J. (1985). Oncogene expression in rat hepatomas and during hepatocarcinogenesis. *Cancer Letters* 26: 121-127.

333. Sakai, M., Okuda, A., Hatayama, I., Sato, K., Nishi, S., and Muramatsu, M. (1989). Structure and expression of the rat c-*jun* messenger RNA: tissue distribution and increase during chemical hepatocarcinogenesis. *Cancer Res.* 49: 5633-5637.

334. Kwast-Welfeld,J., de Belle, I., Walker, P.R., Isaacs, R.J., Whitfield, J.F., and Sikorska, M. (1991). Changes in cyclic adenosine monophosphate responsive element binding proteins in rat hepatomas. *Cancer Res.* 51: 528-535.

335. Kitagawa, T. (1976). Sequential phenotypic changes in hyperplastic areas during hepatocarcinogenesis in the rat. *Cancer Res.* 36: 2534-2539.

336. Scheurer, P.J.(1988). Pathologic types of hepatic tumours. PP. 15-36. In *Liver Cell Carcioma*, Bannasch, Keppler, and Weber Eds. Kluwer Academic Publishers.

337. Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of

sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**: 387-395.

338. Ptashne, M. (1986). Gene regulation by proteins acting nearby and at a distance. *Nature* **322**: 697-701.

339. Dynan, W.S. (1989). Modularity in promoters and enhancers. *Cell* **58**: 1-4.

340. Hunt, T. (1991). Cyclins and their partners:from a simple idea to complicated reality. *Semin. Cell Biol.* **2**: 213-222.

VITA

Date and Place of Birth:	November 5th, 1961, Montreal, Canada
Nationality:	Canadian
Social Insurance Number:	470 077 116
Languages (spoken and written):	English and French

Education:

1988-1994	Ph.D. Department of Biology, University of Ottawa, Ottawa, Canada
1983-1986	M.Sc. Department of Biochemistry, Queen's University, Kingston, Canada
1980-1983	B.Sc.(Hons.) Department of Biochemistry, Queen's University, Kingston, Canada.

Scholarships:

1988-1993 Graduate Scholarship funded by National Research Council of Canada

PUBLICATIONS:

Refereed papers:

1. Isolation and characterization of tropomyosin kinase from chicken embryo.
   I. de Belle and A. S. Mak
   Biochimica et Biophysica Acta **925**, 17-26 (1987).

2. Regulation of the synthesis of DNA polymerase α in regenerating liver by calcium and 1,25-dihydroxyvitamin D_3.
   M. Sikorska, I. de Belle, J.F. Whitfield, and P.R. Walker
   Biochem. Cell Biol. **67**, 345-351 (1989).

3. Changes in cyclic Adenosine monophosphate-responsive element binding proteins in rat hepatomas.
   J. Kwast-Welfeld, I. de Belle, P.R. Walker, R.J. Isaacs, J.F. Whitfield, and M. Sikorska
   Cancer Res. **51**, 528-535 (1991).

4. Identification of a multiprotein complex interacting with the c-*fos* serum response element.
I. de Belle, P.R. Walker, I.C.P. Smith, and M. Sikorska
Mol. Cell Biol. **11**, 2752-2759 (1991).

5. Identification of a new cAMP response element-binding factor by Southwestern blotting.
J. Kwast-Welfeld, I. de Belle, P. R. Walker, J. F. Whitfield and M. Sikorska
J. Biol. Chem. **268**, 19581-19585 (1993).

Manuscripts in preparation:

6. Analysis of DNA-binding proteins interacting with the c-*fos* gene promoter during chemically induced hepatocarcinogenesis.
I. de Belle, R.H. Rixon, J.K. Saunders, P.R. Walker, and M. Sikorska
Cancer Res. (submitted).

7. Infrared spectroscopy of rat liver tissue during chemically induced hepatocarcinogenesis
P.T.T. Wong, I. de Belle, S. Commons, J.K. Saunders, and M. Sikorska
Cancer Res. (submitted).

8. Changes in the DNA-binding properties of constitutive transcription factors during liver regeneration.
J. Kwast-Welfeld, I. de Belle, P.R. Walker, T. Youdale, and M. Sikorska
Hepatology (submitted).

9. Evidence for degradation of constitutive transcription factors during apoptosis in rat thymocytes.
I. de Belle, L. Testolin, V.M. Weaver, P.R. Walker, U. Armato and M. Sikorska.
Cell Death and Differentiation (manuscipt in preparation).

10. Cell-cycle related changes in c-*fos* promoter binding proteins.
I. de Belle, L. Testolin, P.R. Walker, U. Armato and M. Sikorska
(manuscript in preparation).

11. Electon microscopic, spectroscopic and biochemical analysis of Rosenthal Fibers.
B. Lach, M. Sikorska, P. Rippstein, P.T.T. Wong, and I. de Belle
(manuscript in preparation).

ABSTRACTS

1. Isolation of proteins binding to the regulatory regions of the c-*fos* gene from liver and hepatoma.
M. Sikorska, I. de Belle, and P.R. Walker
4th International Congress of Cell Biology, Montreal, Quebec 1988.

2. Oligonucleotide affinity purification of transcription factors from liver and hepatomas.
M. Sikorska, I. de Belle, and P.R. Walker
XVI International Congress of Genetics, Toronto, Ontario 1988.

3. Identification of novel transcription factors that bind to the c-*fos* serum responsive element.
I. de Belle, M. Sikorska, and P.R. Walker
Falk Symposium, Basel, Switzerland 1989.

4. Identification of a multiprotein complex binding to the c-*fos* serum responsive element.
I. de Belle, M. Sikorska, I.C.P. Smith, and P.R. Walker
29th Annual Meeting of the American Society for Cell Biology,
Houston, Texas 1989.

5. Nuclear factors from normal and tumour tissues interacting with the CRE and SRE elements of the c-*fos* gene.
J. Kwast-Welfeld, I. de Belle, P.R. Walker, R.H. Rixon, J.F. Whitfield, and M. Sikorska
30th Annual meeting of the American Society for Cell Biology,
San Diego, California 1990.

6. Mammalian cells contain a 47 kDa nuclear factor which may function as a CRE-specific constitutive repressor.
J. Kwast-Welfeld, I. de Belle, P.R. Walker, and M. Sikorska
31st Annual meeting of the American Society for Cell Biology, Boston, Mass. 1991.

7. Changes in cAMP-responsive element binding proteins in rat hepatomas.
J. Kwast-Welfeld, I. de Belle, P.R. Walker, R.J. Isaacs, J.F. Whitfield, and M. Sikorska
Winternational Symposium, Ste. Foy, Quebec 1991.

8. MRI-MRS studies of chemically induced lesions in rat liver.
Sikorska, M., de Belle, I., Kozlowski, P., Butler, K., and Saunders, J.K.
17th L.H. Gray Conference. University of Kent, Canterbury , UK, April, 1992.

9. Regulation of a 47 kDa CREB repressor during the cell cycle of rat Hepatoma cells.
de Belle, I., Kwast-Welfeld, J., Adcharamoorthy, Y., Walker, P. R., and Sikorska, M.
CFBS, Victoria, BC, June 1992.

10. Cell-cycle related changes in c-*fos* promoter binding proteins.
de Belle, I., Kwast-Welfeld, J., Testolin, L., Walker, P. R., and Sikorska, M.
84th Annual Meeting of the American Association for Cancer Research, Orlando, FL, May, 1993.

11. Evidence for degradation of constitutive transcription factors during apoptosis in rat thymocytes.
36th Annual Meeting of Canadian Federation for Biological Sciences, Windsor, ON, June, 1993.

12. Nuclear matrix stability and chromatin destruction during apoptosis.
Weaver, M. V., de Belle, I., Raymond, Y., Walker, P. R.,m and Sikorska, M.
36th Annual Meeting of Canadian Federation for Biological Sciences, Windsor, ON, June 1993

13. Electon microscopic, spectroscopic and biochemical analysis of Rosenthal Fibers.
B. Lach, M. Sikorska, P. Rippstein, P.T.T. Wong, and I. de Belle
XIIth International Congress of Neuropathology
Toronto, ON, September, 1994