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Differential Regulation of the alpha-Fetoprotein and Albumin Genes by c-Ha-ras, c-jun, and c-fos in Human Hepatoma Cells

by

Denise E. Lawless

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The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Differential Regulation of the alpha-Fetoprotein and Albumin Genes By c-Ha-ras, c-jun, and c-fos in Human Hepatoma Cells" submitted by Denise Lawless in partial fulfillment of the requirements for the degree of Master of Science.

Supervisor, Dr. T. Tamaoki
Department of Medical Biochemistry

Dr. M. Hollenberg
Department of Pharmacology

Dr. N. Wong
Department of Medical
Biochemistry/Medicine

Dr. S.L. Wong
Department of Biological Sciences

February 1, 1991
Date

ABSTRACT

Using transient transfection, the effects of three c-Ha-*ras* gene mutants; *ras* $^{Arg-61}$, *ras* $^{Leu-61}$, *ras*^{X}, and the effects of c-*jun* and c-*fos* on alpha-fetoprotein (AFP) and albumin genes in a human hepatoma cell line, HuH-7, were analyzed. Cotransfection experiments using plasmids with different lengths of AFP 5'-flanking regulatory sequences linked to a chloramenphenicol acetlytransferase reporter gene and expression plasmids, indicated that *ras*, *jun* , and *fos* repressed AFP promoter activity. The Arg and Leu mutants of the *ras* gene, but not *ras*^{X}, repressed the activity of the 1 kb 5'-flanking region of the AFP gene. A 98 bp sequence was sufficient for repression by *jun* and *fos*. A different effect was observed for the albumin promoter which was not affected by the *ras* mutants and stimulated by *jun* and *fos*. The combined results obtained from cotransfection studies and gel mobility shift analysis indicated that AT-rich sequences (AT motif) of the AFP promoter, which bind to a hepatoma specific nuclear factor AFP1, may be required but not sufficient for repression by *ras*, *jun*, and *fos* of AFP promoter activity. That *ras*, *jun*, and *fos* suppressed the AFP gene suggests a role for signal transduction cascades in regulating its expression.

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DEDICATION

I dedicate this work to my mother and father who will always be a great source of strength and inspiration for all things.

It is also dedicated to the cause of unicorns everywhere...

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ABBREVIATIONS

AFP	alpha-fetoprotein
ATP	adenosine triphosphate
bp	base pairs
C/EBP	CCAAT/enhancer binding protein
CAT	chloramphenicol acetlytransferase
cDNA	complementary DNA
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
EDTA	ethylene diamine tetracetate
EGF	epidermal growth factor
fmol	femtomole
GTP	guanosine 5'-triphosphate
HBV	hepatitis B virus
HEPES	N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid
HRE	hormone responsive element
kb	kilobases or kilobase pairs
kD	kilodaltons
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
PDGF	platelet derived growth factor
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SV40	simian virus 40
µg	microgram
µl	microlitre

CHAPTER 1 INTRODUCTION

1.1 Transcriptional regulation of eukaryotic gene expression

In higher eukaryotes, there are many potential levels at which gene expression can be controlled. Gene expression can be regulated in a gene specific way at any one of several sequential steps; 1/ activation of gene structure (reviewed in Gross and Garrard, 1988), 2/ specific initiation of transcription on open chromatin (reviewed in Wasylyk, 1988), and at post-transcriptional steps 3/ processing of transcript and transport to the cytoplasm (reviewed in Leff et al., 1986), 4/ regulation of mRNA stability (reviewed in Shapiro et al., 1989) and, 5/ translation of mRNA (reviewed in Fink, 1986).

1.1.1 General transcriptional regulation

Recently, much of the research conducted in gene expression has focused on initiation of transcription. However, this step can be controlled at the level of chromatin structure, which determines the accessibility of genes to the transcriptional apparatus. Genomes are compartmentalized into active and inactive chromatin (Weintraub, 1985). Aquisition of an active chromatin structure is the first step required for gene expression. In chromatin, 'nuclease hypersensitive sites' (nucleosome free regions) are thought to represent 'open windows' that allow enhanced access of cis-acting DNA sequences, required for transcription and transcriptional control, to transcription factors. If a cis-acting sequence is working to affect a chromosomal process, it will be associated with a hypersensitive site. Hypersensitivity is necessary but not sufficient for the underlying DNA sequence to exert its function. It is the first step in committing a cis-acting sequence to be functionally active in chromatin (Gross and Garrard, 1988).

1.1.2 Specific transcriptional regulation

1.1.2.1 Transcription initiation

A great deal is known about transcription initiation and the proteins involved, (reviewed in Wingender, 1989, and Saltzman and Weinmann, 1989) .It appears to be a major level at which gene expression is controlled.

RNA polymerase II is a complex multi-subunit enzyme which polymerizes ribonucleotides into RNA from a DNA template. This enzyme depends on several transcription factors to recognize the genes to be transcribed. Studies with adenovirus 2 major late promoter has revealed that at least five general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, and TFIIF) (Matsui et al., 1980, and Flores et al., 1988) are required for correct transcription initiation. TFIIA is the first to bind to the template, followed by TFIID binding to the TATA box, such that together the proteins form a preinitiation complex. When RNA pol II binds to this complex, TFIIB, TFIIE, and TFIIF rapidly associate with the other proteins to form the initiation complex. Initiation is complete once the first dinucleotide is formed from hydrolysis of ATP.

1.1.2.2 Transcription regulatory elements

Most eukaryotic regulatory regions display a modular structure (reviewed in Dynan, 1989) consisting of a number of control elements that act together. Thus, the rate of initiation of transcription can be modulated by a diversity of DNA-protein and protein-protein interactions of trans-acting factors binding at either upstream sequences, sequences in close proximity to the transcription initiation site, or downstream from the cap site. The transcription factors binding at these sites are ubiquitous or tissue-specific. It is the complexity of the cis-regulatory regions and their modular organization that allows transcription to be regulated by diverse signals. Consequently, this dictates the specific transcriptional patterns of a gene.

i) Promoters

Detailed molecular genetic analysis of a number of different promoters (reviewed by Dynan and Tijan, 1985, and McKnight and Tijan, 1986) have provided a fundamental basis for the current understanding of how promoters are organized. Promoters consist of discrete functional modules, composed of approximately 7-20 base pairs (bp) of DNA, and containing at least one recognition site for transcriptional activator proteins. Each promoter has at least one module which functions to direct RNA polymerase II to the start site to ensure accurate initiation. For most genes, the 'TATA' box, of consensus sequence TATA(A/T)A(A/T), located about 30 bp upstream of the transcription start site (Goldberg, 1979), serves this purpose.

Additional upstream promoter elements located in the region between -30 and -110 bp of the start site, bind to transcription factors which regulate the frequency of transcriptional initiation. Such elements have also been found downstream of the start site (references within Smale and Baltimore, 1989).

ii) Enhancers

Enhancers are cis-acting elements which increase transcription from a homologous or heteologous promoter in an orientation and distance-independent manner (reviewed in Khoury and Gruss, 1983). Regions of DNA with enhancer activity are also organized in a modular fashion like promoters (Dynan, 1989), with the basic unit of enhancer structure represented by a short sequence of DNA, termed enhanson, corresponding to individual binding sites for trans-acting proteins (Ondek et al., 1988). Enhancer activity is generated by a single enhanson, or in combination with a tandemly repeated copy or a different enhanson (Fromental et al., 1988).

1.1.2.3. Transcription Factors

Control of transcription involves the interaction of transcription factors with the complex arrangement of cis-acting elements which make up the promoter and other regulatory regions of the gene. Recently, there has been rapid progress in the identification, characterization and cloning of sequence-specific DNA -binding proteins. It is becoming increasingly clear that these trans-acting factors regulate interactions with other proteins to bring about their biological effects. Even more complexity is realized by the findings that the same protein can either trans-activate or trans-repress, depending on the context in which it binds to a particular regulatory element and its interactions with other regulatory proteins through protein-protein interactions. From the vast amount of data acccumulated about DNA-binding proteins, several structural motifs have been identified besides the common helix-turn-helix motif (Pabo and Sauer, 1984), including the zinc finger and the leucine zipper motifs.

The zinc finger motif was first identified in Xenopus laevis transcription factor TFIIIA (Miller et al., 1989). Potential metal binding fingers is a common structure in many DNA binding proteins (for review see Berg,1986) including factors likely to influence polymerase II transcription. At least two classes of zinc finger proteins have been characterised, according to the number and position of the cysteine and histidine residues available for zinc coordination (Evans and Hollenberg, 1988). Pairs of cysteines and histidines separated by a loop of 12 amino acids, comprise the basic structural unit for the C_2H_2 class. The TFIIIA factor, which is required for the efficient transcription of the 5S ribosomal RNA gene, and the Sp1 transcritpion factor, which recognizes the sequence GGGCGG in the 21 bp repeat elements of the SV40 early promoter (Dylan and Tijan, 1983; Kadonaga et al., 1987), belong to this class. TFIIIA has several 'finger'-like projections, each one binding in the major groove of the helix and interacting with about five nucleotides (Foirall et al., 1986). The C_X class of zinc finger proteins have a variable

number of conserved cysteines available for metal chealation instead of a paired cysteine and paired histidine arrangement. The stroid hormone receptors belong to this family.

The leucine zipper motif was originally described for the CCAAT /enhancer binding protein (C/EBP) and is also found in the Jun, Fos and ATF/CREB families of nuclear proteins (Landschulz er al, 1988 ; Jones, 1990). The motif consists of a periodic repetition of leucine residues which allows for dimerization between proteins containing this structural motif. The leucine regions can adopt a coiled-coil structure, stabilized by additional hydrophobic residues located between the leucines, which can interdigitate with the leucine side chain of a second polypeptide to form a stable noncovalent linkage. The zipper dimerization region is adjacent to a region of basic residues. Both these regions are required for DNA binding.

Proteins from these families can bind as homo- or hetero-dimers. Dimerization allows for diverse combinations of heterodimeric formations between proteins, resulting in complexes which exhibit different binding or activation potential. The Jun and Fos proteins exemplify this diversity in regulation due to different dimerization patterns. While Jun homodimers can bind to the AP1 binding site, Jun-Fos heterodimers bind to the same site with higher affinity, due to the heterodimer complex being much more stable than the homodimer complex (Chui et al., 1988; Ransone et al., 1989).

1.1.2.4. Models of positive and negative regulation of transcription.

Expression of a particular gene is complexed by the ability of more than one protein or protein complex to bind to a regulatory element or influence binding at that element. Such factors as the abundance of regulatory proteins, their distibution in the cell and their interactions with other proteins contribute to their biological effect. The consequences of such regulatory proteins can range from efficient activation to complete inhibition. As such, the transcritpional pattern of a cell involves a complex interplay of positive and negative effects.

Models for how eukaryotic transcriptional activators work have been primarily derived from extensive analysis of gene activation by bacterial repressors(for example, lac and cro repressors) and yeast activators (reviewed in Ptashne, 1988; Ptashne and Gann,1990). The general model proposes that an activator binds DNA and interacts with a target component of the transcriptional machinery and aids in the formation of the initiation complex near the transcription start site. Currently, activators are classified into two categories; 'universal' activators and those whose ability to function is restricted by cell type. Universal activators function in a wide spectrum of cell types because they possess both a DNA-binding surface and an acidic activating region, whereas the other class of activators contains only one of these regions and therefore only functions in cells which provide the missing function by an auxiliary protein. It is believed that both classes of activators mediate their effect by interacting with TFIID. The universal activators can interact directly, while others require intermediary proteins providing acidic activating regions.

It is possible for activator proteins to exert an inhibitory effect on transcription through a sequestration phenomenon termed squelching (Ptashne, 1988). This is based on the possibility that the putative interaction between the activator and the target protein might take place while both are off the DNA. If the target protein is present in limiting amounts, its interaction with the activator would sequester target protein, consequently making it unavailable for transcription. Thus, a protein bearing a strong activating region and present at artificially high concentrations, could repress transcription from both promoters lacking the activator binding sites and those containing the binding sites.

An increasing number of studies have demonstrated that transcriptional activation and repression can be mediated by a single protein factor. Two basic mechanisms appear to be involved; one in which negative regulation is independent of the DNA-binding domain, and another in which the DNA-binding function of the protein is required. However, the consequences of both these usually involve the inactivation or displacement of transcription

factors required for basal promoter activity, either through protein-protein interactions, or through competition in binding.

Gene regulation by steroid hormone receptors offers several examples which fall into these categories. Steroid hormone receptors belong to a family of ligand-regulated nuclear receptors which interact with specific DNA elements, referred to as hormone responsive elements (HRE), to activate or inhibit transcription of target genes (reviewed in Beato, 1988)

The studies of Rosenfeld and associates on inhibition of rat prolactin gene expression by thyroid and estrogen receptors, have shown that separate functional domains of the protein are required for activation and inhibition (Adler et al., 1988). Negative control by the thyroid hormone receptor does not require the DNA-binding domain but appears to involve receptor C'-terminal protein-protein interactions which prevent it from acting as a transcriptional activator (Holloway et al., 1990). They speculate that the receptor interactions inhibit Pit-1(a tissue specific transcription factor) - dependent stimulation of prolactin gene expression.

In many steroid hormone regulated genes, inhibition of transcription results from the receptor binding at adjacent or overlapping DNA recognition sites of positive transcription factors in the promoter. In the pro-opiomelanocortin (POMC) gene, purified glucocorticoid receptor binds to a promoter sequence overlapping a putative CCAAT motif and cAMP-responsive element (Drouin et al., 1987). A similar steric occlusion of basal promoter elements is possible in the interleukin-6 (IL-6) promoter. Ray et al. (1990) showed, using DNase I footprint analysis, that a purified DNA-binding fragment of the glucocorticoid receptor bound across a TATA box and RNA start site (Inr motif) of the IL-6 promoter.

1.2 Oncogenes as regulators of gene expression

Since the detection of oncogenes as parts of the genome of acutely transforming retroviruses and as being responsible for transformation of cells in culture in gene transfer experiments (reviewed in Bishop, 1987), over 40 different oncogenes have been characterized from both cellular and tumor virus genomes. The actions of this myriad of genes falls into four basic biochemical mechanisms: metabolic regulation by proteins that bind GTP (Hurley et al., 1984), like the regulatory G protein family (Gilman, 1987); protein phosphorylation, with either tyrosine or serine and threonine as the phosphorylation site (Hunter and Cooper, 1985); control of gene expression by regulating transcription (Kingston et al., 1985); and participation in DNA replication (Strudzinski et al., 1986).

An interesting aspect of oncogene action is the interconnection of the function of oncogene proteins products located towards the periphery of the cell: the extracellular space, the plasma membrane, and the cytosol, where growth factors, growth factor receptors, GTPases, and protein kinases that transduce external signals reside, and nuclear oncogenes coding for transcription factors, which convert these extracellular stimuli into changes of gene activity and subsequent phenotypic changes. Also, there appears to be a hierarchy of components of signal transduction pathways (reviewed in Herrlich and Ponta, 1989). External signals mediated by growth factors/growth factor receptors (for example the c-*sis* oncogene encoding a chain of platelet derived growth factor (PDGF), and c-*erb* encoding the epidermal growth factor receptor) are passed on by membrane associated oncogenes, the *ras* family, to cytoplasmic oncogenes. Finally, in the nucleus, transcription of oncogenes such as *fos*, *jun*, *myc*, and *myb* is turned on, and their protein products are involved in regulation of specific sets of genes. Thus, analyzing the responsiveness of a particular gene to oncogenes in signaling pathways provides a means to examine the mechanisms controlling the expression of the particular gene.

1.3 Human alpha-fetoprotein (AFP) gene

AFP is a major serum protein synthesized predominantly in the fetal liver and yolk sac, with low levels being expressed in the kidney and pancreas (Nahon et al., 1988). It functions similarly to albumin, another major serum protein to which it is evolutionarily related, in maintaining normal osmolarity and binding to various biological molecules in the blood (reviewed in Uriel, 1977; Berde et al.,1979; Peters, 1985).

1.3.1 Protein Structure.

The amino acid sequence of human AFP has been deduced from cDNA clones and revealed 19 amino acids in the signal sequence and 590 amino acids in mature AFP, with the protein having a molecular weight of 70 kD and a potential site for N-glycosylation. AFP has fifteen regularly spaced disulphide bridges arranged in three groups of loops or triplicate domains (Morinaga et al., 1983), similar to the three domain structure of albumin (Brown,1976).

AFP, albumin and the vitamin D binding protein belong to a family of closely related serum proteins. AFP and albumin share a 39% homology in amino acid sequence (Morinaga et al., 1983) and there is also significant conservation of primary structure between AFP and the vitamin D binding protein.

1.3.2 Gene Structure

Analysis of genomic clones (Sakai et al., 1985) revealed that the AFP gene is about 20 kb long and contains 15 exons and 14 introns. A high degree of similarity exists between the human AFP gene and albumin gene, which contains 15 exons of similar size and 14 introns (Lawn et al., 1981).

The AFP gene is mapped to the long arm of chromosome 4 at q11-22 and is in linkage with the albumin gene (Harper and Dugaiczyk, 1983). The AFP and albumin genes are tandemly arranged in both humans (Urano et al.,1984) and rodents (Ingram et al.,

1981) and are believed to be derived from a common ancestral gene. The AFP and albumin genes cover a region of at least 60 kb, with the 5' end of the AFP gene separated from the 3' end of the albumin gene by about 14 kb (Urano et al. 1985).

1.3.3 Regulation of AFP expression

AFP is of particular interest since it belongs to a group of 'oncodevelopmental proteins' that are present in high levels in fetal and neoplastic tissue. Moreover, expression of the AFP gene provides a good model to study regulated gene activity in development.since the AFP and albumin genes are differentially expressed throughout liver growth. In the fetal liver, both AFP and albumin genes are activated at the same time. At birth, the AFP gene undergoes a rapid transcriptional decline and becomes hardly detectable in adult life, whereas albumin transcription increases and is maintained at high levels (Tilghman and Belayew, 1982; Belanger et al., 1983). Thus, while the AFP and albumin genes are structurally and functionally related, they differ in that only the AFP gene is developmentally regulated. (Tamaoki and Fausto, 1984; Tilghman , 1985).The mechanisms involved in regulating AFP expression throughout development are not known. In the mouse AFP gene, postnatal repression is mediated within 1 kb of the AFP start site. Repression is independent of the AFP enhancers and does not require cooperation or interaction of the AFP enhancers with its promoter (Camper and Tilghman, 1989).

AFP expression is controlled by several hormones during development, particularly steroid hormones (Belanger et al.,1985). The human AFP gene is upregulated by dexamethasone (Nakabayashi et al.,1989). In the rat gene however, AFP transcription is repressed by both glucocorticoid and progesterone involving a competition mechanism in which the hormone receptor probably displaces an AFP gene activator whose recognition sequence in the AFP promoter overlaps its binding site (Guertin et al.,1988; Tureotte et al., 1990).

High AFP synthesis is associated with liver regeneration (reviewed in Sell et al, 1975; Tamaoki and Fausto,1984). As well, AFP expression is often significantly elevated in association with hepatocarcinogenesis (Atryzek et al., 1980 Otsuru et al., 1988) and therfore can serve as an oncofetal marker. However, AFP expression is variable in human and rat hepatomas (Stillman and Sell, 1979), making the study of AFP regulation clinically relevant.

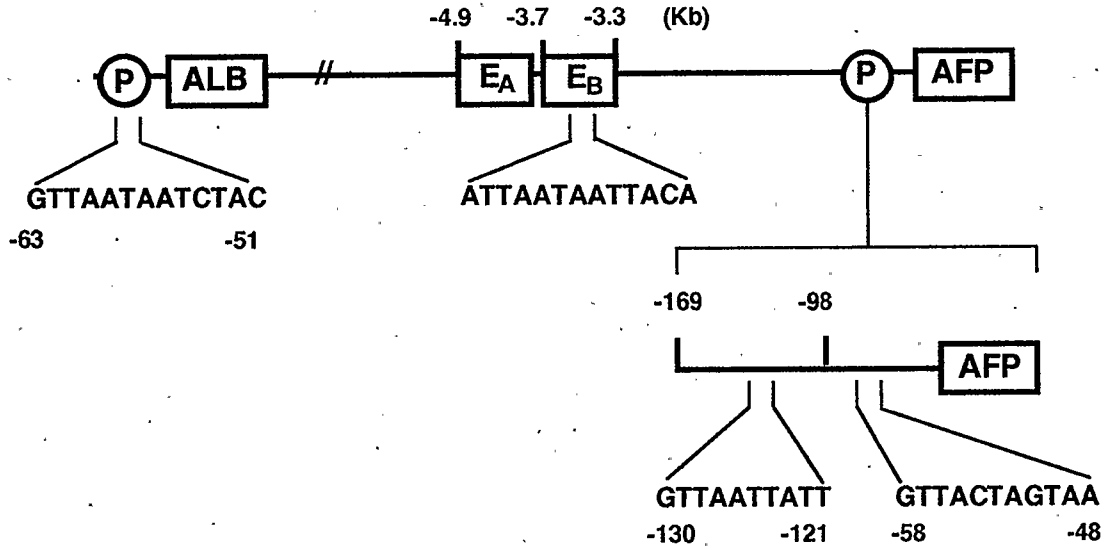
1.3.4 Regulatory elements of the human AFP gene

Transient expression studies in tissue culture cells have identified cis-acting sequences important for AFP transcription. The regulatory elements have included promoter, enhancer, and glucocorticoid responsive elements (Watanabe et al., 1987; Sawadaishi et al.,1988 and Nakabayashi et al., 1989). Equivalent regulatory regions have been identified in the rat (Guertin et al,. 1988; Muglia and Roffman-Dens, 1986) and mouse (Godbout et al., 1986, 1988)

In the human AFP gene, the cell specificity of the AFP enhancer and promoter elements has been characterized by using an AFP producing hepatoma cell line, Huh-7 (Nakabayashi et al., 1984) and a non-AFP producing cell line (HeLa). Both transient gene transfer and an *in vitro* transcription system have been employed for these analyses. AT-rich sequences present in both the promoter and enhancer have been found to be important for cell specific expression of the AFP gene.

Enhancer activity is found in the 400 bp region between -3.7 and -3.3 kb of the AFP start site (Watanabe et al, 1987). This region, referred to as domain B, contains a 54-bp sequence with a core AT rich element (AT motif). Another enhancer region, domain A, located between -4.9 and -3.7 kb of the start site has been characterized (H. Nakabayashi and T. Tamaoki, unpublished results) (see Fig. 1). While both domains alone exhibit typical enhancer effects, maximum activation of the AFP gene is obtained with both domains A and B together.

Fig. 1 Schematic map of important cis-acting elements of the AFP and albumin genes. AT-rich sequences which bind the hepatoma-specific AFP1 nuclear factor are indicated. Promoter and enhancer regions are indicated by P and E, respectively.


-4.9
-3.7
-3.3
(Kb)
P
ALB
E_A
E_B
P
AFP
GTTAATAATCTAC
-63
-51
ATTAATAATTACA
-169
-98
AFP
GTTAATTATT
-130
-121
GTTACTAGTAA
-58
-48

A HuH-7 nuclear protein, termed AFP1, binds to the AT rich region, TGATTAATAATTACA, of the domain B 'core' element and confers cell specific transcriptional enhancement to this region (Sawadaishi et al., 1988). The AFP and albumin promoters contain similar AT rich sequences, $^{-130}$GTTAATTATT^{-121} and $^{-63}$GTTAATAATCTAC^{-51}, respectively, which bind AFP1 with high affinity (Y. Miyao, J. Chan and T. Tamaoki, unpublished data). The 98-bp AFP core promoter region, that is, containing only the CCAAT motif and TATA box and no proximal AT motif, contains another similar AT motif, termed 'most' proximal AT motif: $^{-58}$GTTACTAGTAA^{-48}. In an *in vitro* transcripton assay, a fragment from -98 to +17 containing the most proximal AT motif, supports 70% of AFP transcription compared to 100% basal promoter activity from a -169 to +29 fragment containing the proximal AT motif (K. Tjong and T. Tamaioki, unpublished data). It is likely that AFP1 is a common transcription factor that regulates both the AFP and albumin genes though the mechanism by which it does so remain to be elucidated.

1.4 Aims of this study

The AFP and albumin genes are differentially expressed in the developing liver. While much information is available on the cis- and trans- acting regulatory elements of the AFP and albumin genes, the mechanisms involved in the differential expression of these genes remains to be elucidated. One possible mechanism is that AFP transcription is responsive to growth signaling pathways during development, whereas the albumin gene is not.

Recent studies addressing the differential expression of the AFP and albumin genes demonstrated that c-Ha-*ras* oncogene negatively regulates the AFP gene but not the albumin gene in human hepatoma cells (Nakao et al., 1990). The *ras* gene family codes for a membrane associated protein, p21, that shares biochemical and biological properties with the GTP-binding regulatory G proteins (Gilman, 1987). They are believed to act as

transducers of extracellular signals across the plasma membrane for a variety of growth factor receptors and other associated molecules (Barbacid, 1987; Lu et al., 1989; Kaplan et al., 1990). Thus, this study supports the hypothesis that signaling pathways are involved in the differential regulation of the AFP gene since it implicates a link between G-protein mediated signal transduction and AFP expression.

The *ras* proteins are controlled by GTP and GDP. They exist in equilibrium between an active GTP-bound form and an inactive GDP-bound form which is regulated by their intrinsic GTPase activity (Trahey et al., 1987). For the normal *ras* gene, the mitogenic signal is transient. However mutated oncogenic *ras* proteins exhibit a reduced GTPase activity (Gibbs et al., 1984) such that the protein remains in an active form which presumably can trigger a constitutive mitogenic signal.

Nakao et al reported that in a transient transfection system, the normal c-Ha-*ras*, *ras*$^{\text{Gly-12}}$, and an oncogenic mutant, *ras*$^{\text{Val-12}}$, suppressed AFP expression in a human hepatoma cell line. Oncogenic *ras*$^{\text{Val-12}}$ was found to have a greater suppressive effect on AFP expression than the normal form of the gene. This is in agreement with the oncogenic form of the *ras* gene being capable of mediating an enhanced effect compared with the normal form. Thus, the suppression of AFP expression by *ras* is a specific phenomenon and appears to be due to the biochemical properties of the *ras* protein.

In this study it was tested whether transient transfections of three other mutant *ras* genes had the same effect on AFP expression, in order to substantiate the finding that the AFP gene is responsive to extracellular signaling systems. *ras* mutants were used in this analysis since the mutanat form of the gene has been found to have a greater effect on AFP expression than the normal gene.

Several groups have postulated the involvement of the nuclear oncogenes *jun* and *fos* in *ras* mediated deregulation of gene expression The *jun* and *fos* nuclear oncogene products are thought to play an important role in gene regulation through their involvement in cell proliferation and differentiation. They form a heterodimer complex which regulates

transcription from promoters containing AP-1 binding sites (or TRE: TPA responsive element) (Chiu et al., 1988). DNA transfer studies indicate that *ras* stimulates *jun* and *fos* transcription in different cell lines, suggesting that *jun* and *fos* are involved in ras-mediated gene regulation (Sistonen et al., 1989: Fukumoyo et al., 1990; Sassono-Corsi et al., 1989). Therefore, whether *jun* and *fos* cooperate in *ras* mediated suppression of the AFP gene was also analyzed. Thus, this study examines the involvement of signal transduction cascades in regulating the differential expression of AFP and albumin.

CHAPTER 2 MATERIALS AND METHODS

2.1 Reagents

Acrylamide and ammonium persulfate were from Bio-Rad. Restriction endonucleases were purchased from Bethesda Research Laboratories (BRL), Boehringer Mannheim Biochemicals, Pharmacia, or New England Biolabs. Cesium chloride (Ultra pure, Optical Grade) was from BRL. Urea and Sucrose (Ultra Pure, Enzyme Grade) were from BRL.

2.2 Cell line

The AFP producing human hepatoma cell line, HuH-7, parental, established from a human hepatocellular carcinoma was used (Nakabayashi et al., 1982).

2.2.1 Culture Medium

IS-RPMI, a chemically defined synthetic medium (Nakabayashi et al., 1984) supplemented with 100 units of penicillin, 100 μg of sreptomycin (Gobco) was used for maintenance of HuH-7 cells.

2.2.2 Culture conditions

HuH-7 cells were grown as monolayer cultures in 150 cm^2 flasks (Corning). Upon reaching confluence, the cells were subcultured according to Nakabayashi et al. (1982). The medium was removed and the cells were rinsed once with phosphate-buffered saline (PBS), and incubated with 10 ml PBS containing 0.01% EDTA at 37° for 5 min. This solution was then replaced with a 10 ml solution of 0.1% trypsin and 0.005% EDTA. After incubation at 37° for 10 min, 10 ml of 1% serum-containing medium was added and the detached cells were dispersed into single cells by gently pipetting and collected by centrifugation at 250 *g* for 5 min. The cell pellet was resuspended in growth medium and a

viable cell count was determined by erythrosin B exclusion in a hemocytometer.Cells were plated at a density of 1 X 10^7 cells for the 150 cm^2 flasks and 5 X 10^6 for the 75 cm^2.flasks. Cells were incubated at 37° under 5% CO_2 in air and replaced with fresh medium every two days.

2.3 Growth experiments

Huh-7 cells grown in IS-RPM1 were harvested by trypsin-EDTA treatment (as described in section 2.2.2) and seeded at 1.67 X 10^6 cells per 25 cm^2 flask with 10 ml medium. The following day the cells were transfected (see section 2.6.1) with 6 μg DNA per flask. After a 48hr incubation period the cells were harvested, and the number of cells were counted with a hemocytometer.

2.4 RNA analysis.

2.4.1 RNA isolation

Total cellular RNA was isolated from Huh-7 cells by either a guanidinium isothiocyanate/CsCl step gradient method (Ausubel et al., 1987), or a rapid RNA preparation method according to Schreiber et al , 1989 and Sough, 1988 with the following exceptions. The cells were first washed with 10 ml PBS, and then incubated with 10 ml PBS / 2mM EDTA at 37°C for 5 mins. The sides of the flask were hit to loosen the cells completely. They were gently pipetted and added to 50 ml centrifugation tubes and washed twice with 10 ml PBS. The washed pellet was then resuspended in 1 ml PBS.

2.4.2 Northern blot analysis

RNA was fractionated in 1.1% agarose gel with 6.6% w/v formaldehyde (Ausubel et al., 1987). 1μl of EtBr (400μg/ml) was added to the RNA samples prior to heating (Rosen and Villa-Komaroff, 1990) to allow for visualization of the RNA samples prior to

transfer. After electrophoresis, RNA was transferred onto Nytran membranes (Schleicher and Schull) and baked at 80°C.

The following fragments were random primer labeled (Einberg and Vogelstein) and used as probes for northern hybridization: a 1.3 kb Hpa II fragment from the plasmid pHAF-7 (Morinaga et al , 1983), a 727 bp Pst I - Hind III fragment from the plasmid phalb-7 (Urano et al , 1984), a 2.0 kb Bam HI - Hind III fragment from a pHßAPr-1 plasmid derivative and a 2.1 Kb Eco RI fragment from a pcDL-SRalpha296 plasmid (Takebe et al. ,1988) derivative (M.Sakai , unpublished data). These were used as AFP, albumin, rat c-*jun*, human c-*fos* cDNA probes respectively.

Prehybridization was carried out at 42°C in 6XSSPE (SSPE: 0.18M NaCl, 10mM NaH_2PO_4 , 1mM EDTA pH7.4) , 5X Denhardt's solution (0.1% each of ficoll, polyvinylpyrollidone and bovine serum albumin), 0.5% sodium dodecyl sulphate (SDS), 100 µg/ml denatured salmon sperm DNA and 50% formamide. Hybridization was carried out in 6XSSPE, 0.5% SDS , 100 µg/ml denatured salmon sperm DNA, 50% formamide and 10^6 cpm/ml of ^{32}P-labeled DNA probe at 42°C for 18hrs with constant agitation. The filters were washed twice at room temperature in 1XSSPE and 0.1% SDS for 15 min each, twice at 65°C in 0.5XSSPE and 0.1%SDS for 15mins each, followed by a brief wash in 0.1XSSPE at room temperature. The membrane was air-dried and exposed to Kodak X-Omat AR film at -70°C.

In preparation for rehybridization of the same filter to a different probe, probe removal was carried out at 75°C for 2h in 1mM Tris (pH8.0), 1mM EDTA (pH8.0) and 0.1XDenhardt's solution, followed by a brief rinse in 0.1XSSPE at room temperature.

2.5 Plasmids

2.5.1. Expression plasmids

The coding sequences of the c-Ha-*ras* mutants, ras^{Val-12} (G to T transversions in codon 12 to encode valine instead of glycine), ras^{Arg-61} and ras^{Leu-61} (A to G transition,

or A to T transversion of codon 61 to code for arginine or leucine instead of glutamine, respectively) and ras^x (regions coding for Lys-5 to Ser-17 and Val-109 to Ala-21 were exchanged) were chemically synthesized (Miura et al , 1986). They were inserted into pRSVΔras, derived from pRSVras (Borman et al , 1983) to yield pRSV-rv^{12}, pRSV-ra^{61}, pRSV-$r1^{61}$ (Kamiya et al , 1989) and pRSV-rx (Miura et al , 1988), respectively. A 2.0 kb rat c-*jun* cDNA was inserted to pHßAPr-1 to yield pßA-*jun*, and a 2.1 kb human *fos* cDNA was inserted into pcDL-SRα296 to yield pSR-*fos* (Sakai et al , 1989).

2.5.2 CAT fusion plasmids

Construction of the chloramphenicol acetyltransferase (CAT) plasmids used in this study has been previously described (Watanabe et al., 1987; Sawadaishi et al., 1988; Nakabayashi et al., 1989; Nakao et al., 1989). pBR-CAT contains the CAT gene and the SV40 poyadenylation signal.

pAF5.1-CAT, pAF1.0-CAT, pAF0.17-CAT, and pAF0.1-CAT contain 5.1 kb, 1.0kb, 169 bp, and 98 bp of the AFP 5'-flanking sequence linked to the CAT gene in pBR-CAT. pSVAF2.4-CAT, pSVAF0.4-CAT, pSVAF0.3(R)d-CAT, and pSVAF0.05CAT contain 2.4 kb of the full AFP enhancer (-5.3 to -2.9 kb), 408 bp of the domain B AFP enhancer region (-3.7 to -3.3 kb), 318 bp of the domain A AFP enhancer in reverse orientation (-4.0 to -3.7), and the 54 bp AFP1 binding site fragment of the domain B AFP enhancer region, respectively, inserted at the 5' end of the CAT gene in pSV1'-CAT. pAF5.1Δ1-CAT, pAF5.1Δ2-CAT, and pAF5.1Δ2.7-CAT, contain the full enhancer region (-5.1 to -2.9 kb) and 169 bp of the AFP promoter with internal deletions between the positions -2.9 and -1.8 kb, -2.9 and -0.95 kb, and -2.9 kb and -169 bp, respectively (H. Nakabayashi and T. Tamaoki, unpublished data). pHB139 contains 139 bp of the HBV pre-S1 5'-flanking sequence inserted into pBR'-CAT. pHBAF103-CAT contains 103 bp of the HBV pre-S1 promoter (-139 to -37 bp) linked upstream of the AFP core promoter (-98

to +29) in pAF0.1-CAT. pALB0.1-CAT contains 90 bp of the albumin 5'-flanking sequence (-90 to +17) inserted into pBR-CAT.

2.5.3. Plasmid construction

To construct pßAΔ-*jun*, the BamHI-HindIII 2.0 kb *jun* cDNA insert was isolated from a pßA-*jun*. The ends were filled in by the Klenow fragment of DNA polymerase I, and blunt-end ligated. To construct pSRΔ-*fos*, the Eco-RI 2.1 kb *fos* cDNA insert was isolated from pSR-*fos*, the ends were filled in by Klenow and blunt-end ligated together.

2.5.4 Plasmid preparation

Plasmids for transfection assays were prepared according to Norgard , 1981. They were purified by cesium chloride-ethidium bromide equilibrium gradient centrifugation, followed by 15% (w/v) polyethylene glycol precipitation to remove small RNA (Watanabe et al., 1987).

2.6 Transient cell transfection and CAT assay

HuH-7 cells were transfected with 20 µg of plasmid DNA per 75 cm ^{2} flask (inoculated 2 days before with 5 X 10^6 cells) by the calcium phosphate precipitation method (Graham and van der Eb, 1973) followed by glycerol shock (15% glycerol, for 30 s) (Parker and Stark, 1979).

For all cotransfection experiments, an equal ration of CAT plasmid and expression/carrier DNA was transfected. One can expect the ratio of the mixed plasmids to be maintained as the ratio of DNA transfected since a major strength of calcium phosphate transfection is that the cells take up a representative sampling of the various plasmids in the precipitate (Ausubel et al., 1987). The transfection efficiency of HuH-7 cells by *ras* expression vectors is very high, 50%, relative to other hepatoma and non-hepatoma cell lines (Nakao et al., 1990). This was determined by using flow cytometry to measure the

percentage of cells labeled with a monoclonal anti-p21 antibody after transfection. Conceivably, transfection with different *ras* mutants or oncogenes results in a similar efficiency of transfection. Expression of both the normal *ras* and its oncogenic form has been confirmed by immunoprecipitation of the proteins following transfection. Both protein forms were expressed to a 20-fold higher level relative to control cells (Nakao et al., 1990).

After 48 h incubation, the cells were harvested by scraping and lysed by 5 cycles of freezing and thawing in 100 µl of 0.25M Tris-HCl (pH7.5). Cell lysates were heated at 60 °C for 10 min to inactivate deacytylase activity (Crabb and Dixon, 1987). The supernatant was collected and assayed for CAT activity as previously described (Gorman et al., 1982; Watanabe et al., 1987). CAT activity is represented by the percent conversion of the chloramphenicol to its acetylated forms. The percentage of [^{14}C] chloramphenicol acetylated is estimated by cutting out the thin-layer chromatography spots corresponding to the substrate and the acetylated derivitives and measuring their associated radioactivity by scintillation counting.

2.7 Gel mobility shift assay

Nuclear extracts were prepared from HuH-7 cells according to the methods of Wu, 1984; 1985. Binding reactions were carried out as previously described (Sawadaishi et al., 1988) with the exception that 5 fmol of end-labeled DNA was used and incubated at room temperature. Free and bound complexes were separated on a 4% polyacrylamide gel at room temperature.

CHAPTER 3 RESULTS

3.1 The effects of transfected c-Ha-*ras* mutants on the AFP and albumin genes

The effect of *ras* on the AFP and albumin genes was studies by transiently transfecting various c-Ha-*ras* mutants into a human hepatoma cell line and examining subsequent alterations in AFP and albumin expression at the mRNA level, at an *in vivo* transcriptional level, and by examining the DNA binding properties of proteins known to be important in transcription of the AFP and albumin genes. The effect of three *ras* mutants: *ras*$^{\text{Arg-61}}$, *ras*$^{\text{Leu-61}}$, and *ras*$^{\text{X}}$ on AFP and albumin expression were studied. The effect of the normal c-Ha-*ras* gene, *ras*$^{\text{Gly-12}}$, has been analyzed by Nakao et al., 1990. The normal *ras* gene had the same effect as the *ras* mutant *ras*$^{\text{Val-12}}$, although the mutant had a more enhanced effect.

3.1.1 The effect of *ras* on AFP and albumin mRNA levels

To determine the effect of *ras* mutants on AFP and albumin mRNA levels, total RNA was isolated from transfected HuH-7 cells and analyzed by Northern hybridization. As shown in Fig. 2, transfection of either *ras*$^{\text{X}}$, *ras*$^{\text{Arg-61}}$, or *ras*$^{\text{Leu-61}}$ caused no significant change in either AFP or albumin mRNA levels as compared to RNA preparations from cells transfected with the control vector (expression vector without insert).

3.1.2 *ras* represses the activity of the AFP 5'-flanking sequence

To determine whether *ras* affects the regulation of AFP transcription, mutant *ras* expression vectors and AFP fusion plasmids carrying different lengths of AFP 5'-flanking sequences linked to a CAT reporter gene, were transiently cotransfected into the hepatoma cell line HuH-7 by the calcium phosphate precipitation method. Results from such

Fig. 2 Northern blot analysis of AFP and albumin mRNA from *ras* - transfected HuH-7 human hepatoma cell line. Total RNA was fractionated by denaturing agarose/formaldehyde gels, and transferred to a nylon filter. Lanes contained 20 μg of RNA from HuH-7 cells transfected with either the pRSV-Δneo (C-control), pRSV-rx (x), pRSV-ra^{61} (a), or pRSV-rl^{61} (l) expression vectors. The filter was subsequently hybridized with ^{32}P-labeled AFP (A) or albumin (B) cDNA probe.

A. AFP mRNA

c x a l

1 2 3 4

B. Albumin mRNA

c x a l

1 2 3 4

experiments showed that transfection of *ras*$^{Arg-61}$ and *ras*$^{Leu-61}$ significantly reduced the transcriptional activity mediated by the AFP 5' upstream region but that *ras*^{X} had no effect on the activity of AFP-CAT fusion plasmids.

CAT expression from pAF5.1-CAT and pAF1.0-CAT, containing 5.1 or 1.0 -kb of AFP 5' upstream sequence, respectively, was repressed by transfected *ras*$^{Arg-61}$ and *ras*$^{Leu-61}$ by greater than 5-fold in all cases, but was not affected by *ras*^{X} (Fig. 3). The degree of repression mediated by the *ras*$^{Arg-61}$ and *ras*$^{Leu-61}$ mutants was not significantly different (for pAF5.1-CAT: 7.9-fold and 11.5-fold, for pAF1.0-CAT: 6.8-fold and 5.3-fold) (Table 1).

3.1.2.1 Effect of *ras* on AFP enhancer activity

To study the effect of *ras* on the AFP enhancer, cotransfections were carried out using chimeric AFP plasmids in which AFP enhancer sequences are linked to a simian virus 40 (SV40) early promoter region in pSV1'-CAT, which contains only 30% of one SV40 72-bp repeat and the TATA box (Watanabe et al., 1987). Cotransfection of pSV1'-CAT and *ras* expression vectors serves as a negative control since CAT expression from pSV1-'CAT is unaffected by *ras* (results not shown). *ras*$^{Leu-61}$ was able to repress CAT expression from pSVAF2.4-CAT (containing the 2.4-kb full AFP enhancer) and both *ras*$^{Arg-61}$ and *ras*$^{Leu-61}$ had a greater suppressive effect on the 408-bp domain B alone, contained within pSVAF0.4-CAT (Table 1). However, the *ras* mutants did not affect CAT expression from pSVAF0.05-CAT, containing the 54-bp 'core' element of domain B. While the *ras* mutants suppress the activity of domain B, the full enhancer activity contained within pSV2.4-CAT is not greatly suppressed by both the Arg and Leu mutants. Thus, the AFP enhancers do not appear to be major mediators of the negative effect of *ras* on AFP transcription.

Fig. 3 Effect of *ras* transfection on AFP 5'-flanking activity. HuH-7 cells were cotransfected with 10 μg of pAF5.1-CAT or pAF1.0-CAT and either 10 μg of the pRSV-Δneo (C), pRSV-rx (X), pRSV-ra^{61} (A), or pRSV-rl^{61} (L), expression vectors, as indicated. pRSV-Δneo is the expression vector without the insert. CAT assays were performed after 48 h using heat-treated cell extracts, containing either 25 μg or 100 μg of protein, incubated for either 1 h or 3 h for pAF5.1-CAT and pAF1.0-CAT, respectively. Cm, chloramphenicol; 1-Ac, chloramphenicol; 3-Ac, 3-acetate chloramphenicol.

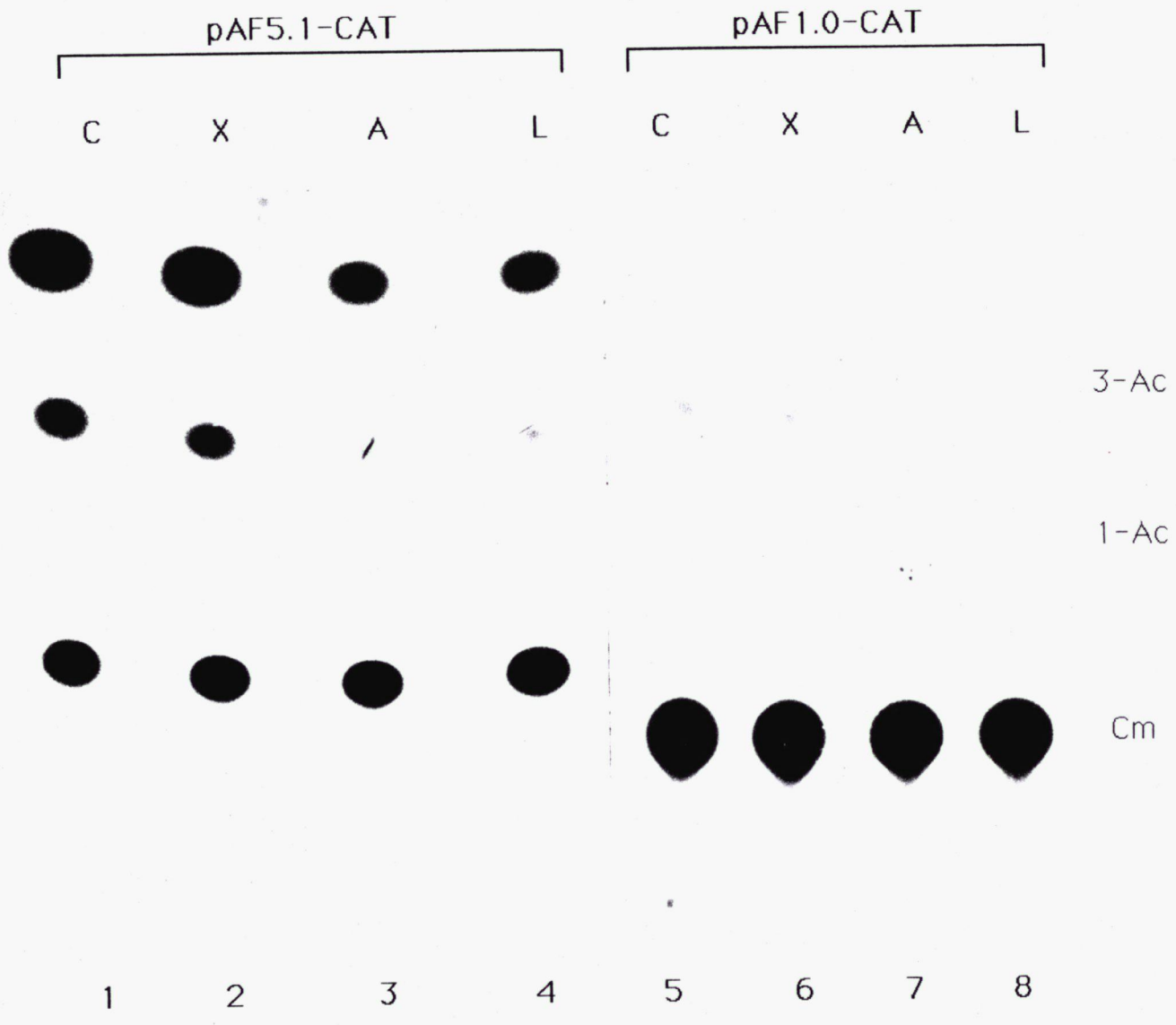

pAF5.1-CAT
pAF1.0-CAT
C
X
A
L
C
X
A
L
3-Ac
1-Ac
Cm
1
2
3
4
5
6
7
8

Table 1

Repression of AFP 5'- flanking activity by ras mutants in HuH-7 cells

	Relative CAT activity Average fold reduction in CAT activity by ras mutants relative to control		
CAT construct	ras^{X}	$ras^{Arg\text{-}61}$	$ras^{Leu\text{-}61}$
pBR-CAT plasmids			
pAF5.1-CAT	1.6 ± 0.7	7.9 ±3.7*	11.5 ± 3.8*
pAF1.0-CAT	1.5 ± 0.7	6.8 ± 2.8*[1]	5.3 ± 2.3*
pSV1'CAT plasmids			
pSVAF2.4-CAT	1.0 ± 0.1	2.3 ± 0.5	2.7 ± 0.3*
pSVAF0.4-CAT	1.3 ± 0.1	4.2 ± 0.8*	5.2 ± 1.5*[1]
pSVAF0.05-CAT	1.4 ± 0.6	1.3 ± 0.3	1.1 ± 0.1

CAT results (percent conversion) represent the fold reduction in CAT activity of cells cotransfected with the fusion plasmid and expression vector over control cells transfected with the CAT plasmid and the expression vector without the insert. Results marked with an asterisk are significantly different from results of the control transfection for each respective CAT construct.

*$p < 0.05$, using Student's t-test, comparing means of relative fold reduction

*[1]$p < 0.01$, using Student's t-test, comparing means of relative fold reduction

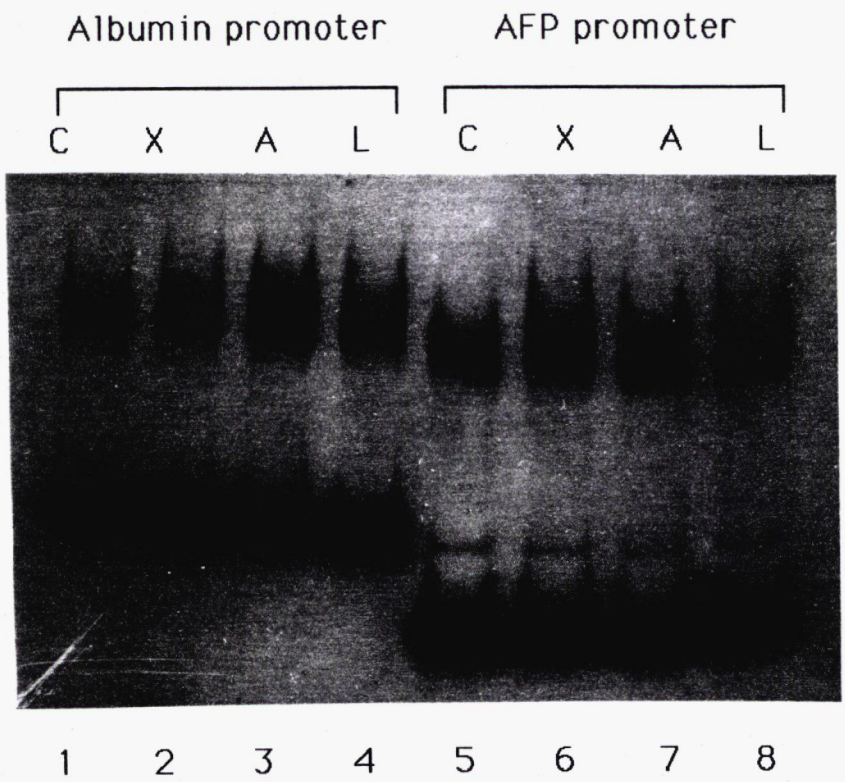
3.1.3 Changes in the binding of HuH-7 nuclear proteins to the A+T-rich sequences of the AFP and albumin genes as a result of *ras* transfection

Gel mobility shift assay was used to examine whether *ras* transfection alters the interaction of HuH-7 nuclear proteins with the AT motif. Nuclear extracts were prepared from *ras*-transfected HuH-7 cells and incubated with AT motif containing AFP and albumin end-labeled probes.

With a 72-bp AFP promoter probe, nuclear extracts from $ras^{\text{Arg-61}}$, $ras^{\text{Leu-61}}$, and ras^{X}-transfected cells formed a complex band which was broader than that formed with control nuclear extracts preparations (Fig. 4). The same phenomenon was seen using ras^{X}, and $ras^{\text{Arg-61}}$-transfected cells and a 54-bp domain B enhancer probe (results not shown). This suggests a structural modification of a nuclear HuH-7 protein(s) which binds AT rich elements. Consequently, the formation of several complexes of different electrophoretic mobility would form a smeared band.

However, the change in the binding of HuH-7 nuclear proteins to the AFP AT motif resulting from *ras* transfection is not unique to the AFP A+T-rich sequences. The same broadening of the complex formation results when a 108-bp AT motif containing albumin probe is used in gel shift analysis with nuclear extracts prepared from ras^{X}, $ras^{\text{Arg-61}}$, and $ras^{\text{Leu-61}}$-transfected HuH-7 cells. Thus, the change in the binding of HuH-7 nuclear proteins to the AFP1 sites as a result of *ras* transfection is not solely responsible for mediating suppression by *ras* of AFP expression.

Fig. 4 Effect of *ras* transfection on binding of nuclear proteins to the AT motif of the albumin and AFP promoter. The 108-bp albumin promter fragment (-91 to + 17 bp); lanes 1-4, or the 72-bp AFP promoter fragment (-169 to -98 bp); lanes 5-8, were end labeled with ^{32}P, incubated with nuclear extracts (2 μg of protein) from HuH-7 cells transfected with either pRSV-Δneo (C), pRSV-rx (X), pRSV-ra^{61} (A), or pRSV-rl^{61} (L) as indicated, in the presence of poly(dIdC)·poly(dIdC), and electrophoresed on 4% acrylamide gels.


Albumin promoter
AFP promoter
C X A L
C X A L
1 2 3 4 5 6 7 8

3.2 Differential effects of transfected c-*jun* and c-*fos* on the AFP and albumin genes

The effects of c-*jun* and c-*fos* on AFP and albumin expression were studied following the same approach as that used for the *ras* study. HuH-7 hepatoma cells were used in a transient transfection system to analyze the effect of *jun* and *fos* transfection on the AFP and albumin genes at the mRNA level, at an *in vivo* transcriptional level, and by examining the binding patterns of nuclear proteins to the AT motif.

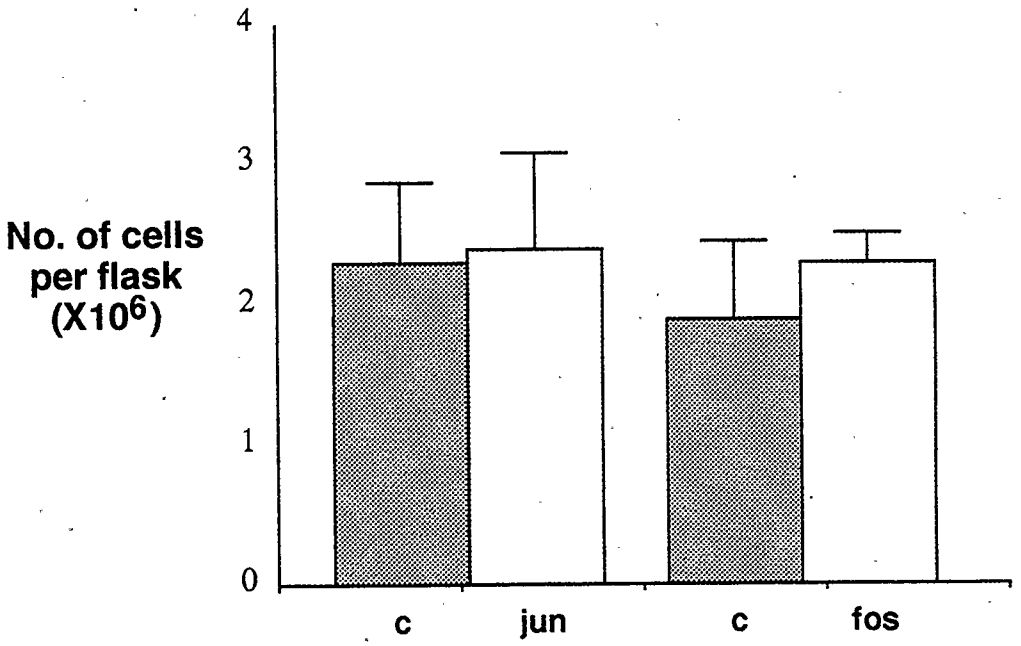
3.2.1 *jun* and *fos* transfection does not affect HuH-7 cell growth

Whether overexpression of the c-*jun*and c-*fos* oncogenes affected HuH-7 growth rate was examined in order to determine whether or not the changes in AFP and albumin expression resulting from *jun* and *fos* transfection were non-specific. The effect of *jun* and *fos* transfection on HuH-7 cell growth was quantitated by numerical estimation of growth based on viable cell count. The growth experiments were performed 48h after transfection. No significant change in the total number of cells between control and *jun* or *fos*-transfected flasks was observed (Fig. 5).

3.2.2 No apparent change in the AFP and albumin mRNA levels after *jun* and *fos* transfection

To determine the effect of *jun* and *fos* on AFP and albumin mRNA levels, total RNA was isolated from transfected HuH-7 cells for Northern analysis. The same nylon membrane was used for hybridization to AFP, albumin, *jun*, and *fos* probes. The amount of RNA transferred to each lane was normalized by photographing the EtBr stained gel prior to transfer and by hybridizing the blot to a control ß-actin probe.

Fig. 5 Effect of *jun* and *fos* transfection on HuH-7 cell growth. HuH-7 cells were seeded at 1.67 X 10^6 cells per 25 cm^2 flask and the following day transfected with 6.7 µg expression plasmid DNA per flask. After 48 h incubation, the cells were harvested and a viable cell count was taken of triplicate flasks. The control expression plasmids (C) were pßA-Δjun and pSR-Δfos for *jun* (pßA-jun) and *fos* (pSR-fos) transfections, respectively.


No. of cells per flask (X10^6)
4
3
2
1
0
c
jun
c
fos

RNA was prepared from a total of ten separate transfection experiments using two different methods. Analysis of the hybridization patterns shows that there was no significant difference in AFP mRNA levels between cells transfected with *jun*, or *fos*, and RNA levels from cells transfected with control plasmids (vector wothout insert). *jun* and *fos* also had no affect on albumin mRNA levels (Fig. 6, panels A and B).

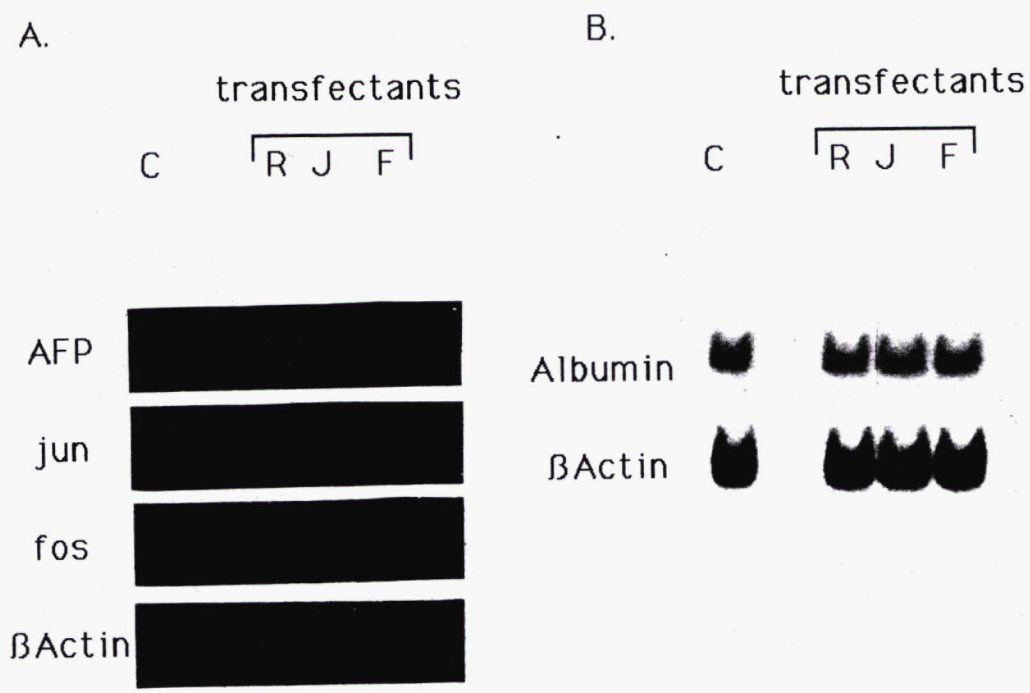
3.2.2.1 Analysis of *jun* and *fos* mRNA levels in *ras*, *jun*, and *fos*-transfected cells

The levels of *jun* and *fos* mRNA were analyzed in transfected HuH-7 cells to confirm that transfection of *jun* and *fos* expression vectors resulted in overexpression of these genes, and to test whether *ras* transfection had any effect on the endogenous *jun* and *fos* mRNA levels. When the blots were hybridized to a *jun* probe, a high signal was detected for *jun*-transfected cells compared to control cells, and no change in endogenous *jun* levels as a result of *ras* or *fos* transfection was apparent. Likewise, using a *fos* probe, no endogenous *fos* mRNA was detected in RNA preparations from control, *ras* or *jun*-transfected cells, but a high signal representing the *fos* overexpression of the *fos*-transfected cells was (Fig. 6, panel A).

3.2.3 Suppression of AFP 5'-flanking sequence activity by *jun* and *fos*

5.1-kb of the 5'-flanking region of the AFP gene was analyzed for its involvement in *jun* and *fos* repression using a transient transfection assay. HuH-7 cells were cotransfected with either a c-*jun* or a c-*fos* expression vector, or both, and an AFP-CAT fusion gene which would serve as a reporter of transcriptional activity of the AFP 5' upstream sequences.

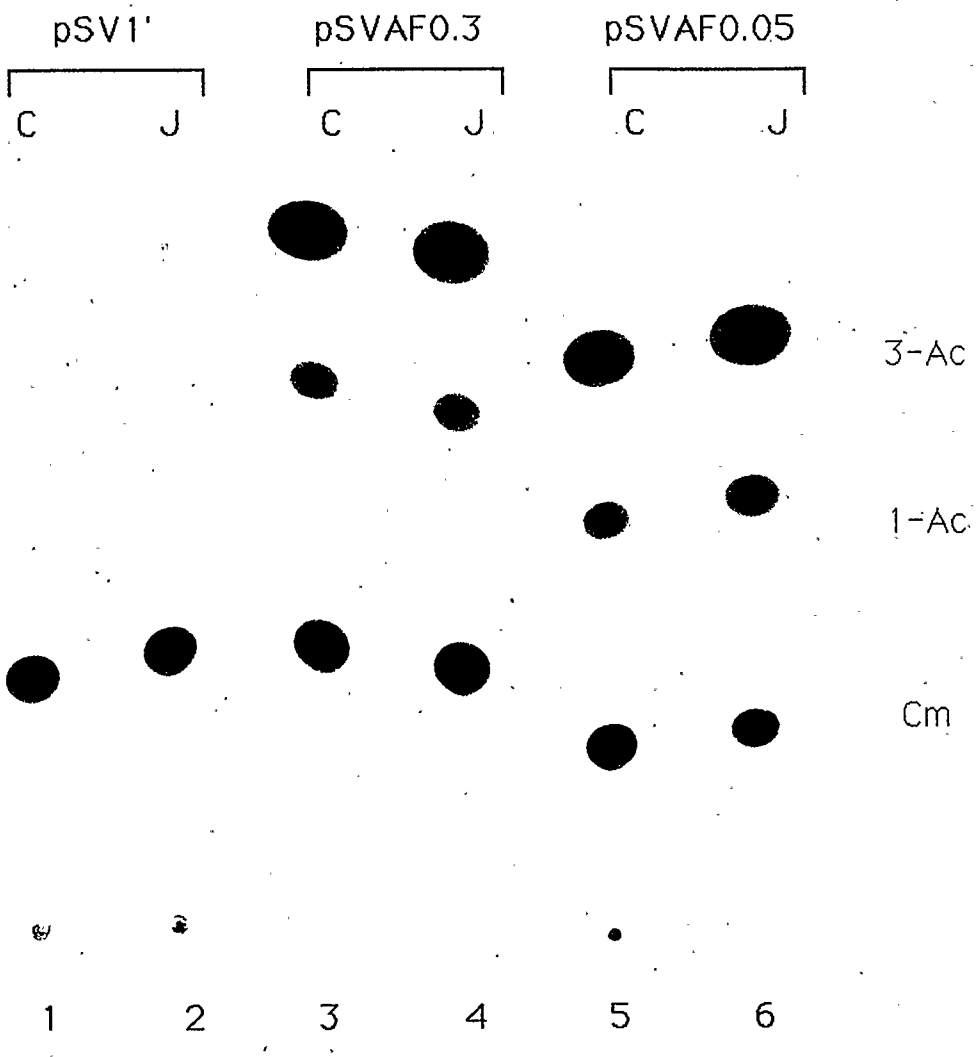
Fig. 6 Northern blot analysis of AFP, albumin, *jun and fos* mRNA levels in transfected HuH-7 human hepatoma cell line. Total RNA was fractionated by denaturing agarose/formaldehyde gels and transferred to a nylon filter. Lanes contained either 10 μg (A) or 20 μg (B) of RNA from HuH-7 cells transfected with either the pβA-Δj (C-control), *ras* (R), *jun* (J), or *fos* (F) expression vectors (pRSV-rv12, pβA-j, and pSR-f, respectively). The filter was subsequently hybridized with ^{32}P-labeled AFP, albumin, *jun* , *fos* , and as a control, ß-actin cDNA probes, as indicated.


A.
transfectants
C
R J F
AFP
jun
fos
ßActin
B.
transfectants
C
R J F
Albumin
ßActin

3.2.3.1 *jun* does not suppress AFP enhancer activity

The AFP domains A and B are contained within the AFP 5.1-kb region and it was tested whether *jun* could mediate a negative effect through these specific regions. This was done in cotransfections using chimeric AFP enhancer plasmids under the control of the SV40 early promoter region in pSV1'-CAT. jun was found to have no effect on CAT expression from pSV1'-CAT. Two constructs were used for analyzing the effect of *jun* on the AFP enhancers; pSVAF0.3$(R)_d$-CAT, and pSV0.05AF-CAT, carrying a 300-bp region of domain A, and a 54-bp 'core' region of domain B, respectively. Cotransfection results showed that CAT expression driven by either of these enhancer regions were not affected by *jun* (Fig. 7). Thus, *jun* does not appear to affect AFP enhancer activity.

Fig. 7 Effect of jun transfection on AFP enhancer activity. HuH-7 cells were cotransfected with pSV1'-CAT, pSVAF0.3$(R)_d$-CAT, or pSVAF0.05-CAT, and either the pβA-Δjun (C-control) or pβA-jun (J) expression vectors. CAT assays were performed after 48 h using heat-treated cell extracts under the following conditions; 50 μg protein and 1 h incubation, 25 μg protein and 30 min. incubation, 25 μg protein and 2h incubation, for pSV1'-CAT, pSVAF0.3$(R)_d$-CAT, and pSVAF0.05-CAT, respectively. Cm: chloramphenicol, 1-Ac: 1-acetate chloramphenicol, 3-Ac: 3-acetate chloramphenicol.


pSV1'
pSVAF0.3
pSVAF0.05
C
J
C
J
C
J
3-Ac
1-Ac
Cm
1
2
3
4
5
6

3.2.3.2 Repression by *jun* is primarily mediated through the AFP promoter

jun transfection significantly reduced CAT expression from pAF5.1-CAT and pAF1.0-CAT (containing 5.1-kb and 1.0-kb of AFP 5'-flanking sequence, respectively) by a similar degree (Fig. 9, and Fig. 10; lanes 1 and 2, and Table 2). This suggests that repression by *jun* is primarily mediated through the 1-kb AFP 5'-flanking region However, it was tested whether any region between the AFP enhancer region (from -4.9 to -3.3) and 170-bp upstream of the AFP start site were important for repression by *jun*. A series of internal deletion mutants of the 5.1-kb 5'-flanking region were tested in cotransfection experiments for this purpose. The constructs contain the full enhancer region (within -5.1 to -2.9) and 170-bp of the AFP promoter (Fig. 8). Cotransfections of *jun* with pAF5.1[Δ1]-CAT(-2.9/-1.8), pAF5.1[Δ2]-CAT(-2.9/-0.95), and pAF5.1[Δ2.7]-CAT(-2.9/-0.169) resulted in a similar decrease in CAT expression as for the *jun* and pAF5.1-CAT cotransfection result (Fig. 9 and Table 2). This indicates that repression by *jun* is primarily mediated through a 170-bp promoter sequence since i) *jun* did not affect AFP enhancer activity and ii) no region between -2.9 and -0.17-kb was found to affect the degree of suppression by *jun*.

Fig. 8 Schematic map of AFP pAF5.1-CAT deletion mutants. Sequences of the AFP 5.1-kb 5'-flanking region with the indicated deletions (stippled boxes) were inserted at the HindIII site of pBR-CAT to produce the AFP-CAT fusion plasmids.

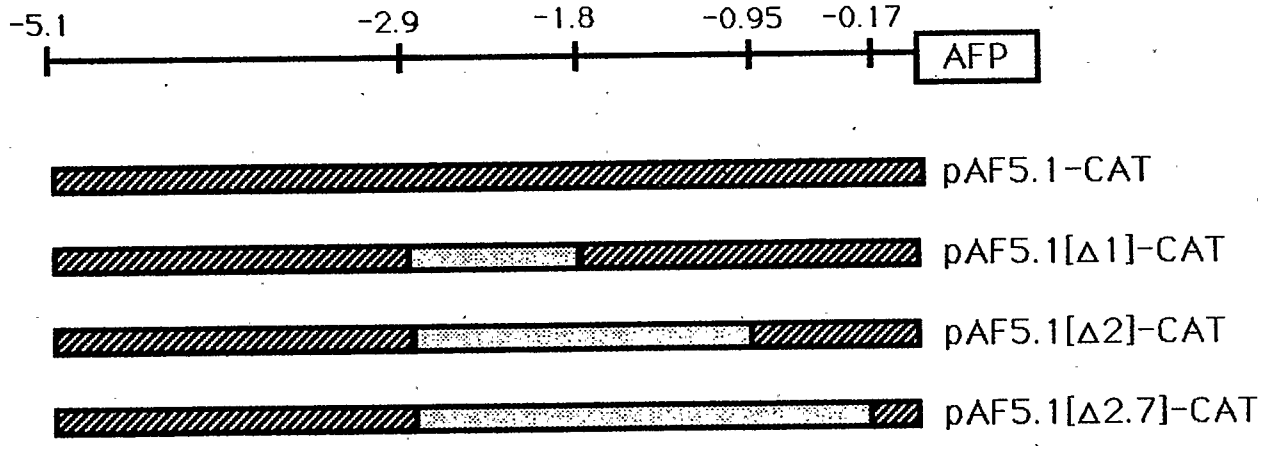
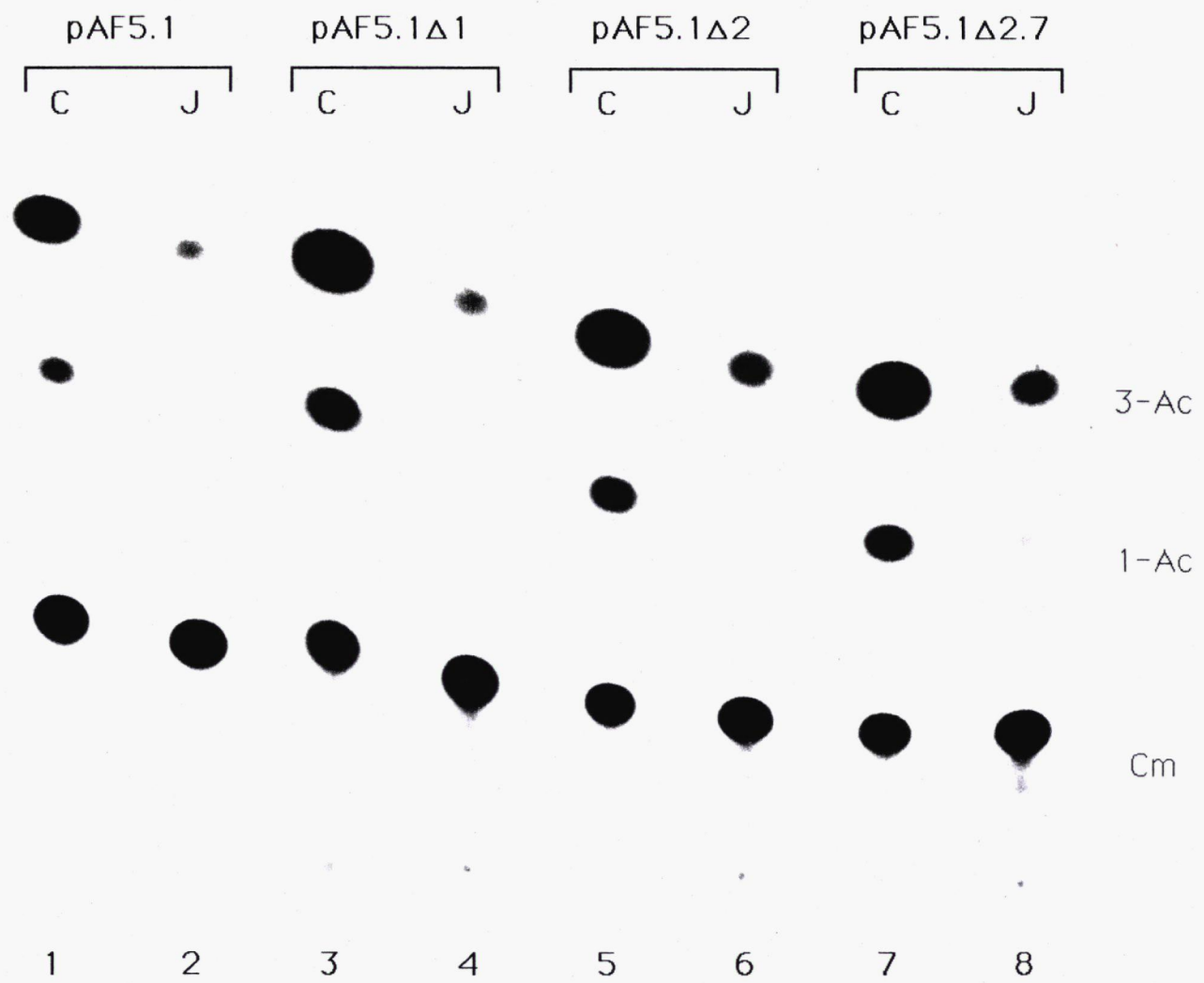

-5.1
-2.9
-1.8
-0.95
-0.17
AFP
pAF5.1-CAT
pAF5.1[Δ1]-CAT
pAF5.1[Δ2]-CAT
pAF5.1[Δ2.7]-CAT

Fig. 9 Effect of *jun* transfection on AFP 5'-flanking activity. HuH-7 cells were cotransfected with pAF5.1-CAT, pAF5.1Δ1-CAT, pAF5.1Δ2-CAT, or pAF5.1Δ2.7-CAT, and either the pβA-Δj (C-control), or pβA-j (J) expression vectors, as indicated. CAT assays were performed using heat-treated cell extracts, containing 25 μg of protein incubated for 30 min. Cm: chloramphenicol, 1-Ac: 1-acetate chloramphenicol, 3-Ac: 3-acetate chloramphenicol.


pAF5.1
pAF5.1Δ1
pAF5.1Δ2
pAF5.1Δ2.7
C
J
C
J
C
J
C
J
3-Ac
1-Ac
Cm
1
2
3
4
5
6
7
8

3.2.3.3 Delineation of the region within the AFP promoter necessary for *jun* and *fos* mediated repression

To delineate the region of the 1-kb upstream AFP sequence conferring repression by *jun*, the CAT activity driven by shorter lengths (169-bp, and 98-bp; constructs pAF0.17-CAT, and pAF0.1-CAT, respectively) were analyzed for their responsiveness to *jun* transfection (Fig.10). The activity of pAF0.17-CAT was reduced about 4-fold by *jun* (Table 2). CAT expression from pAF0.1-CAT, carrying the 98-bp 'core' AFP promoter, was too low for accurately quantitating the effect of *jun* on its activity

To more adequately analyze the effect of *jun* on the AFP 'core' promoter, a chimeric CAT plasmid, pAFHB103-CAT, in which a 103-bp fragment (-139 to -37) of the HBV pre-S1 promoter was linked upstream to the AFP core promoter in pAF0.1-CAT (Nakao et al., 1989), was tested in co-transfection experiments with *jun* and *fos*.(Fig. 10, and Fig. 11). The 98-bp AFP promoter contains both a 'CCAAT' motif and TATA box but lacks the proximal AT motif, such that it only supports very low CAT expression in HuH-7 cells. The addition of the pre-S1 proximal AT box, which is functionally similar to the AFP promoter AT motif, results in a 5-fold increase of CAT expression in pAFHB103-CAT compared to pAF0.1-CAT (Nakao et al., 1989). HBV pre-S1 promoter activity was not affected in cotransfection experiments of *jun* with pHB139-CAT (-139 to +9), containing 148-bp of this promoter (Fig. 10). Thus, the pAFHB103-CAT construct provides a means to quantitate the effect of *jun* and *fos* transfection on the 98-bp AFP promoter activity.

Cotransfection experiments with pAFHB103-CAT showed that the AFP core promoter activity was repressed approximately 3-fold by *jun* transfection and to a lesser degree of 1.5-fold by *fos*. Simultaneously transfecting both *jun* and *fos* resulted in a 2.5-fold reduction in CAT expression (Fig. 12). *fos* repressed the activity of the AFP 1-kb region by 4.5-fold but as in the case of the 'core' promoter, the degree of repression mediated by *jun* and *fos* together was not additive (Fig. 12).

Fig. 10 Effect of *jun* transfection on AFP and pre-S1 HBV promoter activity. HuH-7 cells were cotransfected with 10 μg of pAF1.0-CAT, pAF0.17-CAT, pHB139-CAT, or pHBAF103-CAT, and either 10 μg of the pβA-Δjun (C-control), or pβA-jun (J) expression vectors, as indicated. CAT activity was assayed by using cell extracts containing 50 μg of protein and incubating for 3 h.

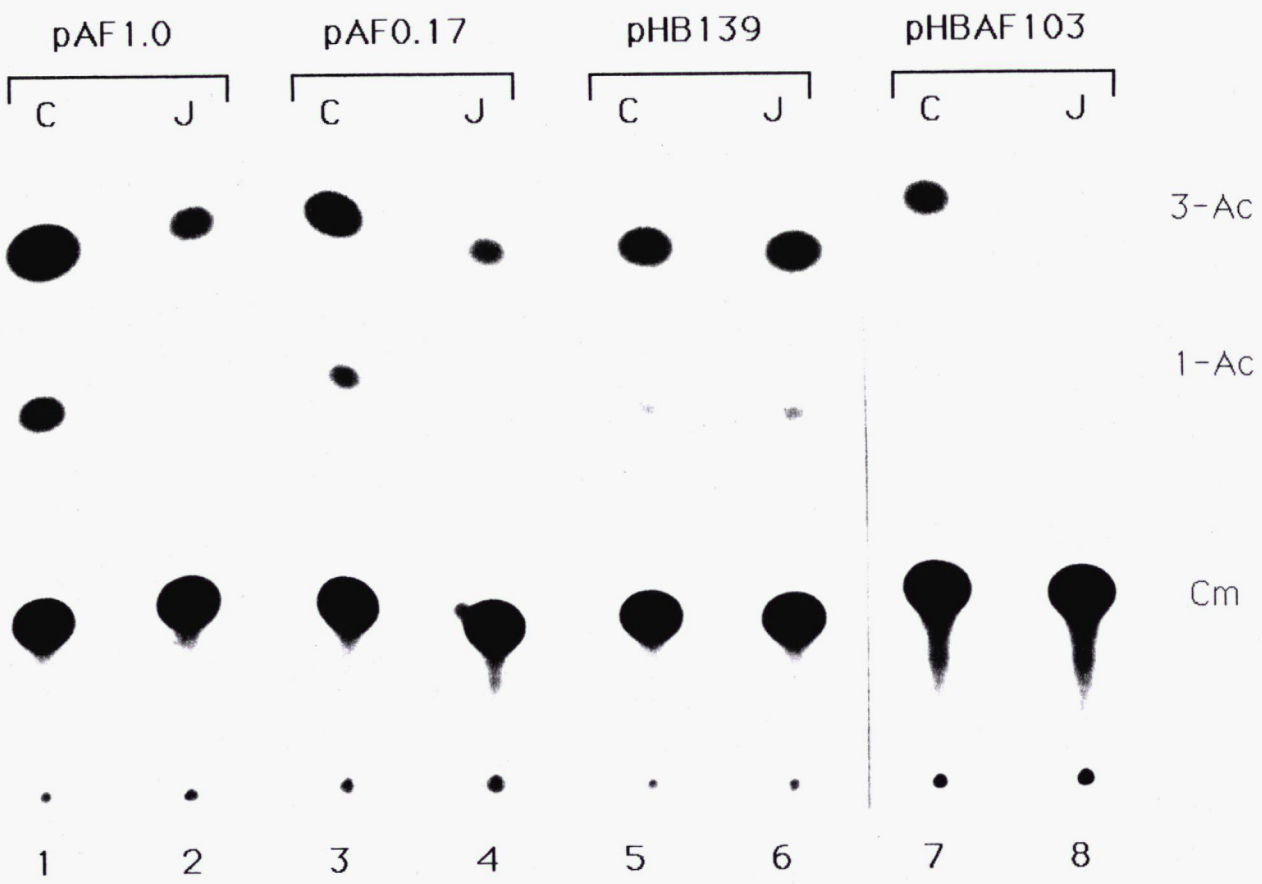

pAF1.0
pAF0.17
pHB139
pHBAF103
C
J
C
J
C
J
C
J
3-Ac
1-Ac
Cm
1
2
3
4
5
6
7
8

Table 2

Repression of AFP 5'- flanking activity by jun in HuH-7 cells

Relative CAT activity Average fold reduction in CAT activity by jun relative to control	
CAT construct	jun
pAF5.1-CAT	8.8 ± 2.0
pAF5.1Δ1-CAT	12.0 ± 1.1
pAF5.1Δ2-CAT	10.1 ± 2.8
pAF5.1Δ2.7-CAT	8.4 ± 1.9
pAF1.0-CAT	8.6 ± 3.9
pAF0.17-CAT	3.6 ± 0.2
pAFHB103-CAT	3.2 ± 0.8

CAT results (percent conversion) represent the fold reduction in CAT activity of cells cotransfected with the fusion plasmid and expression vector over control cells transfected with the CAT plasmid and the expression vector without the insert.

3.2.4 *jun* and *fos* activate albumin promoter activity

Albumin promoter activity was significantly stimulated by both *jun* and *fos* (Fig. 11). CAT expression driven by 90-bp of albumin 5'-flanking sequence (pALB0.1-CAT) was stimulated 3.7-fold. *fos* transfection had less of an effect on the albumin 90-bp promoter activity (1.6-fold induction), but there was an additive activation (5.7-fold) of the albumin promoter when *jun* and *fos* were both cotransfected with pALB0.1-CAT (Fig. 12).

3.2.5 The effect of *jun* and *fos* on HuH-7 nuclear proteins which bind to A+T-rich sequences of the AFP gene

Gel mobility shift assay was used to examine whether *jun* or *fos* transfection alters the interaction of HuH-7 nuclear proteins with the AFP AT motif. Nuclear extracts prepared from *jun* and *fos*-transfected cells were incubated with either an AT motif containing 30-bp AFP promoter probe, or a 15-bp probe encompassing the AFP enhancer AT box. Results obtained from testing nuclear extracts prepared from five separate transfection experiments showed no significant difference in the binding activity of extracts prepared from transfected cells and extracts from control cells (Fig. 13).

Fig. 11 Effect of *jun* and *fos* transfection on albumin and AFP promoter activity. HuH-7 cells were cotransfected with 10 μg of pALB0.1-CAT, or pHBAF103-CAT, and either 10 μg of the pβA-Δjun (C-control), or 5 μg of the *jun* (J) or *fos* (F) expression vectors (pβA-Δjun, pSR-fos respectively), or both *jun* and *fos* together (J+F), as indicated. The total amount of DNA was adjusted to 20 μg using pβA-Δjun as carrier DNA for lanes 2, 3, 6, and 7. CAT activity was assayed by using cell extracts containing 75 μg of protein and incubating for 3 h.

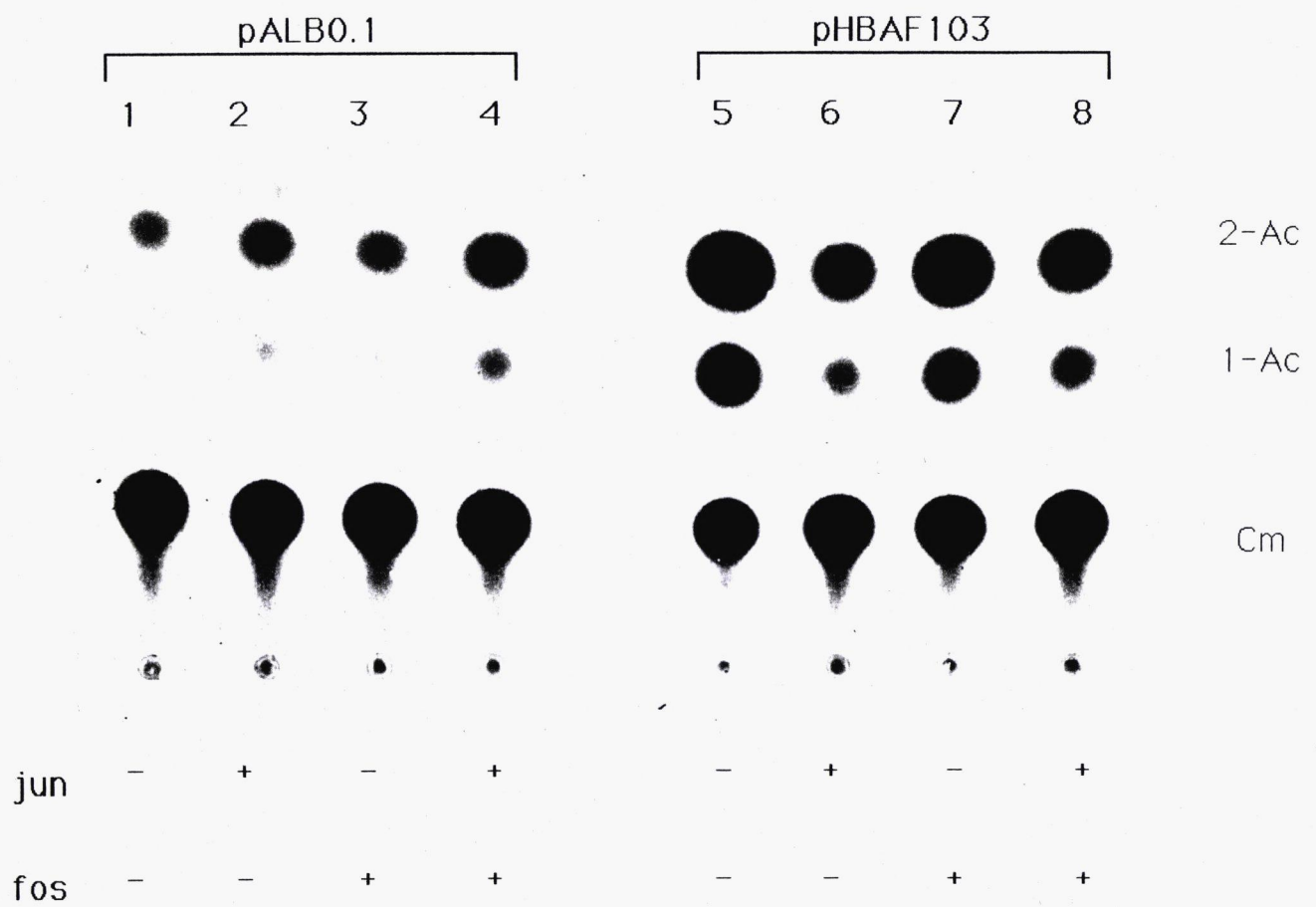
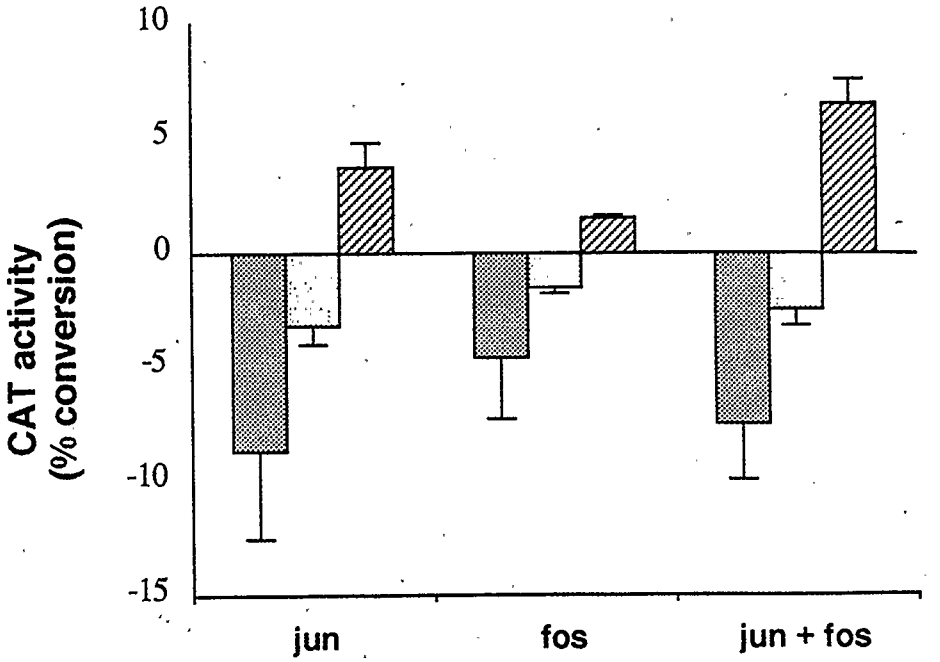

pALB0.1
pHBAF103
1
2
3
4
5
6
7
8
2-Ac
1-Ac
Cm
jun
fos
–
+
–
+
–
+
–
+
–
–
+
+
–
–
+
+

Fig. 12 Differential effect of *jun* and *fos* on AFP and albumin promoter activity. A. HuH-7 cells were cotransfected with the CAT fusion construct and expression plasmids as described in Fig. 8 and Fig. 9. CAT results (percent conversion) are graphed as the fold induction or inhibition in CAT activity of cells cotransfected with the fusion plasmid and expression vector, over control cells (transfected with the CAT plasmid and expression vector without insert). A value of 1 therefore represents no inhibition, and values less than 1 indicate a repressive effect of the oncogene. Results are expressed as the mean ±(s.d.) of at least three individual experiments. B. Schematic diagram (not drawn to scale) of AFP, and HBV pre-S1, fusion CAT genes. Open and thatched bars represent AFP and HBV pre-S1 promoter sequences respectively. pAFHB103-CAT contains both the pre-S1 proximal AT motif-containing region (-139 to -37) and the AFP core promoter (-98 to +29). Open and thatched ovals represent the AFP AT motifs and HBV-pre-S1 proximal AT motif, respectively.

A.


10
5
0
-5
-10
-15
CAT activity
(% conversion)
jun
fos
jun + fos
pAF1.0-CAT
pHBAF103-CAT
pALB0.1-CAT

B.

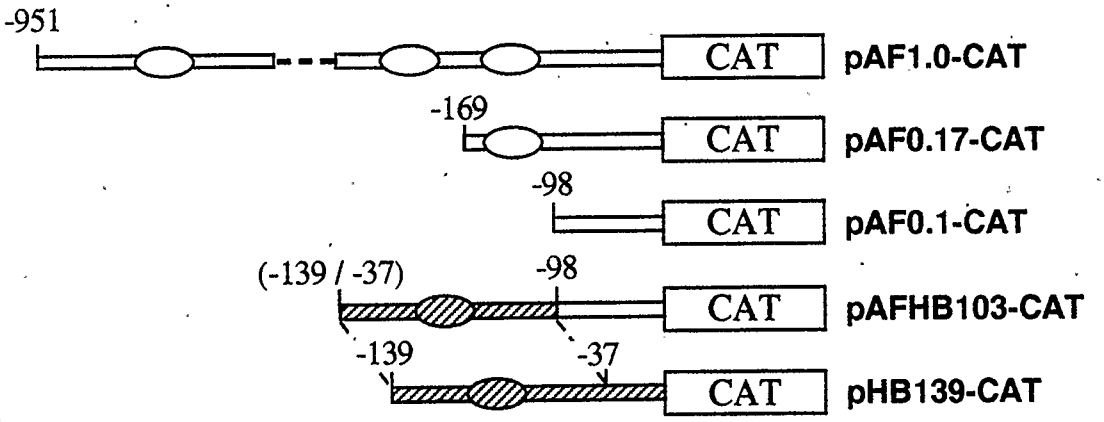
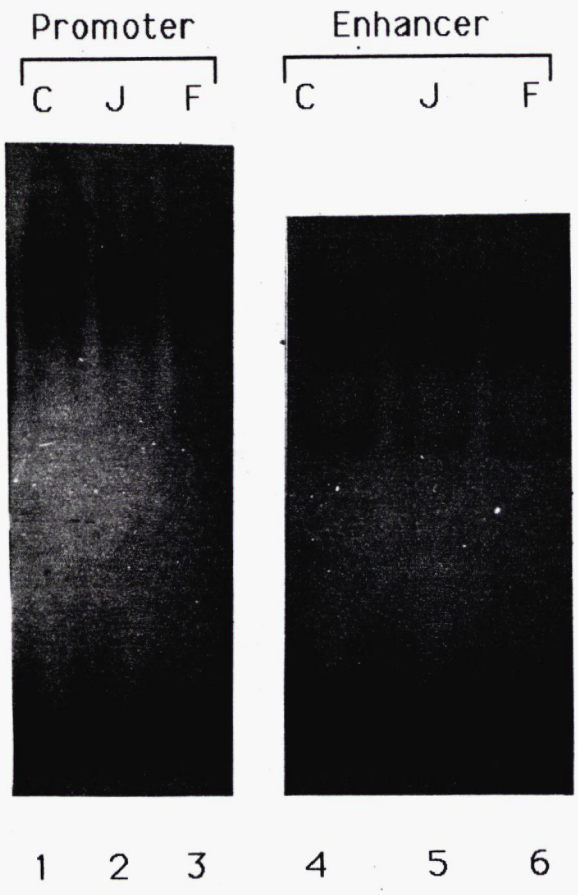

-951
CAT
pAF1.0-CAT
-169
CAT
pAF0.17-CAT
-98
CAT
pAF0.1-CAT
(-139 / -37)
-98
CAT
pAFHB103-CAT
-139
-37
CAT
pHB139-CAT

Fig. 13 Effect of *jun* and *fos* transfection on binding of nuclear proteins to the AT motif of the AFP promoter and enhancer. The 30-bp AFP promoter fragment (-139 to -110); lanes 1-3, or the 15-bp AFP1 binding site AFP enhancer fragment ; lanes 4-6, were end-labeled with ^{32}P , incubated with 6 μg nuclear extract form HuH-7 cells transfected with either pβA-Δjun (C), pβA-jun (J), or pSR-fos (F), as indicated, in the presence of poly(dIdC)·poly(dIdC) and electrophoresed on 4% polyacrylamide gels.


Promoter
Enhancer
C J F
C J F
1 2 3 4 5 6

CHAPTER 4 DISCUSSION

4.1 The effects of *ras*, *jun*, and *fos* transfection on AFP and albumin expression are not due to general effects on HuH-7 cell growth

There is substantial evidence implicating proto-oncogenes in the control of cell growth and differentiation or as mediators of intracellular signaling systems (Varmus, 1984, Bishop, 1987). *ras* genes have been implicated in growth control (Barbacid, 1987) and are responsive to a variety of agents mediating growth signals such as serum, and growth factor receptors of EGF, PDGF-ß, and insulin (Imler et al., 1988; Lu et al., 1989; Kaplan et al., 1990). c-Ha-*ras* can stimulate expression of the c-*jun* (Sistonen et al., 1989) and c-*fos* (Schonthal et al., 1988) genes which belong to the group of nuclear oncogenes which are believed to act as specific transactivators and regulators of RNA and DNA synthesis (Kingston et al., 1985).

Overexpression of the c-Ha-*ras*, c-*jun*, and c-*fos* oncogenes in HuH-7 cells through transfection may consequently affect the growth rate of the cultured cells. Therefore, it is important to determine whether this occurs since hepatocyte growth state affects AFP expression (Nakabayashi et al., 1985). Transfection of HuH-7 cells with c-Ha-*ras* or treatment with dexamethasone did not significantly change the total number of cells after 48 hours when compared to control cells (Nakao et al., 1990, Nakabayashi et al., 1989). Growth experiments showed there was no significant change in HuH-7 cell growth, as estimated by viable cell count, 48 hours after the cells were transfected with *jun* or *fos*. Thus, the changes in AFP and albumin expression elicited by *ras*, *jun*, and *fos* are not due to the general effect of these oncogenes on HuH-7 cell growth.

4.2 *ras*, *jun*, and *fos* down-regulate the AFP gene

4.2.1 *ras* mutants differ in their effect on the AFP 5'-flanking activity

Results of cotransfection experiments showed that both the *ras*$^{\text{Arg-61}}$ and *ras*$^{\text{Leu-61}}$ mutants significantly repressed transcription stimulatory activity of the AFP 5'-flanking sequence, but that *ras*^{X} had no effect. It is interesting to consider the different effects mediated by the *ras* mutants in terms of the biochemical properties of the proteins they code for. The *ras* genes can be converted to an activated (oncogenic) form, as a result of a single point mutation within its coding region (Taparowski et al., 1982), which is associated with a reduced intrinsic GTPase activity (Gibbs et al., 1984). Substitution of Gln^{61} to Arg^{61} or Leu^{61} yields *ras* oncogenes (Der et al., 1986) and the synthetic mutants coded by the *ras*$^{\text{Arg-61}}$ and *ras*$^{\text{Leu-61}}$ expression vectors are able to transform NIH-3T3 cells (Kamiya et al., 1989). Both genes code for p21 proteins with greatly reduced GTPase activity, but no change in the base specificity in nucleotide binding (Miura et al., 1987). *ras*^{X} codes for a mutant protein in which the domains of guanine binding and phosphate binding are exchanged. This has altered the biochemical properties of the protein such that it has no GTPase activity, and while it still binds GTP, it shows a reduction in the base specificity in nucleotide binding (Miura et al., 1988). This suggests that the exchange of the two domains has resulted in altering the complex formation between GTP and the catalytic site. Thus, it appears that p21^{X} does not maintain the biochemical properties required for acting as a signal transducing G protein. Therefore, it is not surprising that *ras*^{X} has no effect on AFP expression, unlike *ras*$^{\text{Arg-61}}$ and *ras*$^{\text{Leu-61}}$.

4.2.2 Repression of the AFP gene by *ras* is mediated primarily through a AFP 1-kb upstream sequence

Results from cotransfection experiments using the AFP enhancer constructs fused to a heterologous promoter suggest that the AFP enhancer domains are not highly sensitive to suppression by *ras*$^{\text{Arg-61}}$ and *ras*$^{\text{Leu-61}}$. Both mutants were able to repress the activity

of the 408-bp domain B fragment. However, activity of the 54-bp 'core' element of domain B was not repressed. This region of domain B is the AFP1 binding site which is important for the cell-specific transcriptional enhancement of this sequnce. Only *ras*$^{\text{Leu-61}}$ was able to suppress the activity of the 2.4-kb full AFP enhancer (domain A and domain B), but to a lesser extent than the mutants affecting domain B alone. This suggests that domain A activity is not significantly repressed by the *ras* mutants. In addition, domain B activity is repressed by *ras*$^{\text{Arg-61}}$ and *ras*$^{\text{Leu-61}}$, but not through the element thought to be primarily responsible for confering the region's enhancer-like function.

Both *ras*$^{\text{Arg-61}}$ and *ras*$^{\text{Leu-61}}$ significantly repressed the activity of the 5.1-kb and 1.0-kb AFP 5'-flanking sequnces. The 5.1-kb.5'-flanking sequence in pAF5.1-CAT contains the full AFP enhancer, whereas the 1-kb sequence in pAF1.0-CAT contains the promoter region only. CAT expression from pAF5.1-CAT was reduced by a greater degree than that of pAF1.0-CAT by *ras*$^{\text{Leu-61}}$ only (11.5-fold compared to 5.3-fold). However, considering that the *ras*$^{\text{Arg-61}}$ and *ras*$^{\text{Leu-61}}$ mutants do not significantly affect the AFP enhancer regions, it appears that the suppression of AFP 5'-flanking activity by the *ras* mutants is primarily mediated through the 1-kb promoter containing sequence.

4.2.3 A 98-bp upstream AFP promoter region is sufficient for repression by *jun* and *fos*

jun was found to repress the activity of the 5.1-kb and 1.0-kb AFP 5'-sequences by a similar degree. Neither the enhancer activity of the 300-bp region of domain A or the 54-bp region of domain B was affected by *jun* transfection. In addition, *jun* was able to suppress the activity of the internal deletion mutants to the same extent that it suppressed activity of the 5.1-kb and 1.0-kb sequences. There was also no significant degree of variation in the extent of repression of the different deletion mutants, indicating that there is no region between -2.9 and -0.17 of the AFP gene required for repression by *jun*.

Analysis of CAT expression driven by 1-kb of AFP upstream sequence and shorter lengths of the promoter indicate that the minimal sequence required for suppression by *jun* is a 98-bp upstream promoter region. Upon examining this sequence, no elements known to be responsive to *jun* or *fos* are apparent.

jun and *fos* have been found to trans-activate AP-1 dependent transcription (Lucibello et al., 1988, Schonthal et al., 1988). Fos protein has also been found to trans-repress the c-*fos* promoter (Sassone-Corsi et al., 1988), and other immediate-early genes; *Egr*-1, and *Egr*-2 (Gius et al., 1990) through a core consensus element, $CC(A/T)_6GG$ or CArG box of the SRE element (Treisman, 1986). The mechanism involved is distinct from trans-activation (Lucibello et al., 1989). c-Jun has also been found to down-regulate the c-*fos* promoter (Schonthal et al., 1989), as well as enhance trans-repression by Fos (Lucibello et al., 1989), although Gius et al. claim that trans-repression by Fos through CArG-like elements is independent of Jun and does not involve the leucine zipper and DNA binding domain of Fos. However, it is agreed that the mechanism of repression involves indirect action on the CArG element. It has been postulated that Fos and Jun, acting together or independently, interact by an unknown mechanism on the same or different target proteins. The formation of such protein complexes could modify pre-existing transcriptional complexes interacting with the serum responsive element (SRE), such as binding of the serum responsive factor (SRF) to the SRE, which is important for the function of the SRE (Norman et al., 1988). As well, evidence exists for the binding of the Fos protein to be involved in TGF-B1 inhibition of transin/stomelysin gene expression (Kerr et al., 1990).

The mechanism involved in repressing AFP promoter activity by *jun* and *fos* is not known. The absence of elements known to mediate trans-regulation by *jun* or *fos* suggest that *jun* and *fos* have an indirect effect on AFP promoter activity. Trans-repression by Fos is presumed to be the result of the Fos protein, alone or complexed with Jun or another protein, interacting with a target protein(s) in a manner that affects proteins important in the

transcriptional activity of the promoter. Conceivably, overexpression of c-*jun* and c-*fos* in HuH-7 cells could affect normal transcription of the AFP gene through an interference or squelching mechanism. The 98-bp AFP promoter sequence contains both a CCAAT motif and TATA box, as well as the 'most proximal' AT motif of the AFP gene. Protein complexes at these sites may be affected in some way, by protein-protein interactions induced by *jun* and *fos* expression, such that AFP transcription is repressed.

Repression of the activity of the 1-kb AFP 5'-flanking region by *jun* was three times greater compared to repression of the 98-bp core promoter activity(8.6-fold and 3.2-fold reduction, respectively). The same is true of suppression by *fos* of these regions (4.5-fold compared to 1.5-fold). This suggests that sequences between -0.98 and -1.0 are involved in repression by *jun* and *fos*. The 1-kb upstream sequence supports a higher degree of transcriptional activity than the 98-bp core promoter in HuH-7 cells (Tjong et al., unpublished results), primarily due to the presence of the proximal AT motif. *jun* and *fos* may be indirectly affecting the protein complexes of the AFP proximal AT motif, although according to the gel shift studies, *jun* transfection has not been found to affect binding of AFP1 to the AT motif. In addition, CAT expression from pAF0.17-CAT, which contains the proximal AT motif, and pHBAF103-CAT, which does not, is repressed by *jun* to the same extent. Therefore, the reason why *jun* repression of the AFP 1-kb activity is greater than that of the 98-bp and 170-bp regions is unclear. The explanation may lie in the ability to quantitate inhibition of CAT activity of particular fusion constructs in transient transfections. There is significantly higher CAT expression from pAF1.0-CAT than from pHBAF103-CAT, and therefore it is easier to detect repression in activity through CAT analysis of the former construct.

4.3 A 90-bp upstream albumin promoter region is sufficient for *jun*/*fos* mediated activition.

Albumin promoter activity was trans-activiated by *jun*, and this effect was enhanced by *fos*. Examination of the 90-bp albumin promoter sequnce reveals the presence of an element, $^{-71}$TGAGTCT^{-65}, that bears a high degree of homology to the TRE consensus sequnce TGA(G/C)T(C/A) (Angel et al., 1987, Lee et al., 1987). Conceivably, the synergistic activation of *jun* and *fos* on albumin promoter activity is mediated through the putative TRE element by the mechanism involved in trans-activiation of TRE containing promoters, such as in the metallothionein (Angel et al., 1987) and collagenase (Schonthal et al., 1988) genes.

Activation of the albumin promoter by *jun* and *fos* was a suprising result since the *ras* mutants had no effect on the albumin promoter. The *ras* protein has been found to influence transcription from regulatory elements containing AP-1 binding sites (Wasylyk et al., 1987) and a *ras* responsive element (RRE) identified in the polyoma virus enhancer was found to have the same sequnce as the TRE (Imler et al., 1988). However, findings by Owen et al. (1990) indicate that transcriptional activiation by *ras* of the TGF-ß1 gene through a TRE-like element is not solely dependent on c-*jun* and c-*fos*. Therefore, one might question why *ras* transfection did not activiate albumin promoter activity. Activated *ras* genes can rapidly increase expression of the c-*jun* (Sistonen et al., 1989) and c-*fos* (Schonthal et al., 1988) genes which would result in activation of genes that contain AP-1 binding sites. However, the activation of *jun* and *fos* by *ras* is transient, lasting only a few hours (Stacey et al., 1987). Northern analysis of *ras*-transfected HuH-7 cells is consistent with the transient activiation of *jun* and *fos*, since endogenous levels of neither c-*jun* or c-*fos* were altered 48 h after transfection with *ras*. Thus, it appears that in HuH-7 cells, transient transfection of *ras* is not sufficient to activate albumin expression through a putative AP-1 site present in its promoter.

It was also surprising that the presence of a TRE element had not been documented in the well characterized albumin rat promoter (Early et al., 1980). However, the corresponding sequence in the rat albumin promoter is TTAGTGT, which lacks homology

to the TRE at the nucleotides important for TRE function (Angel et al., 1987) and for Fos-Jun binding to the AP-1 consensus sequence (Risse et al., 1989).

4.4 Northern analysis does not detect the transcriptional effect of *ras*, *jun*, and *fos* on the AFP and albumin genes

There were no changes in AFP or albumin mRNA levels as a result of *ras*, *jun*, or *fos* transfection, according to Northern analysis. mRNA levels analyzed in Northern hybridization do not only represent the rate of transcription, but a balance between the rate of nuclear RNA synthesis, processing and transport to the cytoplasm, and rates of cytoplasmic mRNA degradation. The degradation rate of a mRNA is important in controlling the speed with which the changes in the transcription rate of a gene are reflected in changes in its cytoplasmic mRNA level. As such, molecules exhibiting a long half-life respond more slowly to changes in transcription as compared to molecules possessing a quicker turnover rate (Kafatos, 1972). AFP and albumin mRNA both exhibit a very high half-life, between 60-120 hours for mouse yolk sac AFP mRNA (Andrews et al., 1982) and 40 hours for rat AFP mRNA in hepatoma cells (Innis and Imler, 1979). Transiently transfecting HuH-7 cells with either *ras*, *jun*, or *fos*, and analyzing total RNA preparations by Northern hybridization 48 hours after transfection, does not detect the changes in AFP/albumin transcription resulting from transfection. It appears that a more specific assay for transcriptional activity , such as nuclear run-off assay, is required for the detection of transcriptional changes to the AFP and albumin genes resulting from *ras*, *jun*, and *fos* transfection.

4.5 Role of A+T-rich sequnces in repression of the AFP gene

The study by Nakao et al. (1990) suggests that *ras* transfection leads to a structural modification of the HuH-7 nuclear proteins complexing with A+T-rich sequences. Thus, the broadening of the retarded band would be due to the formation of several complexes of

different electrophoretic mobility. The modification induced by *ras* transfection appeared to be phosphorylation since the broadened band was reversed by phosphate treatment. However, they conclude that this effect alone is not responsible for the specific repression of AFP promoter activity since a similar effect was observed for the albumin and HBV-pre-S1 promoters containing homologous AT rich elements, and whose activity is not suppressed by *ras* transfection. Still, the importance of DNA-protein interactions at the AFP AT rich elements with respect to suppression of the AFP gene remained to be discerned. Thus, the possibility that the supressive action of the *ras* mutants, *jun*, and *fos* is targetted towards HuH-7 nuclear proteins that interact with the AFP1 binding sites (AT motif) was tested using nuclear extracts prepared from transfected cells and AT motif containing probes in gel mobility shift assay.

Nuclear extracts prepared from HuH-7 cells transfected with either of the three *ras* mutants formed a broadened complex band with both AFP promoter and enhancer fragments encompassing an AT motif.The same broadening phenomenon was observed in gel shift analysis using *ras*-transfected nuclear extracts and an albumin promoter fragment. Thus, the change in binding of HuH-7 nuclear proteins to the A+T-rich sequnces is not unique to the AFP AT motif. Since i) nuclear extracts prepared from ras^X-transfected cells induce a change in the AFP AT motif/HuH-7 nuclear protein complex, but ras^X has no effect on AFP enhancer or promoter activity, and ii) ras^X, $ras^{Leu\text{-}61}$, and $ras^{Arg\text{-}61}$ transfection induce a change in the protein binding pattern of nuclear proteins with the AT motif of the albumin promoter, but it has no affect on albumin promoter activity, it appears that the changes in the binding pattern of HuH-7 nuclear proteins with the AT motif resulting from transfection of the *ras* mutants is not sufficient to mediate repression of the AFP gene by *ras*.

Results from gel shift analysis using HuH-7 nuclear extract from *jun* or *fos*-transfected cells and AFP promter or enhancer AT-motif containing fragments differed from the *ras* mutants experiments. No broadening of the complexed band, or any other

differences, were observed with the transfected nuclear extracts. This suggests that *ras*, *jun*, or *fos* transfection does not repress AFP expression solely through altering the interaction of nuclear proteins to the A+T-rich sequnces of the AFP promoter or enhancer.

CAT analysis of the cotransfection experiments indicate that the minimal region required for repression by *jun* is a 98-bp 5'-flanking region. The activity of this region is also repressed by *ras* $^{Val-12}$ (K.Nakao, personal communication). This sequence does not contain the proximal AT motif, again supporting the observation that repression of AFP promoter activity is not targetted towards HuH-7 nuclear proteins that interact with the AFP AT motifs. However, the 'most proximal' AT motif is located within this 98-bp region, at -58 to -45, in between the CCAAT motif and TATA box. The most proximal AT motif, contained within the construct pAF0.1-CAT (-98 to +29), supports 70% of AFP basal promoter activity from pAF0.17-CAT (-169 to +29) in an *in vitro* transcription system (K. Tjong and T. Tamaoki, unbublished data). CAT expression from pAF0.17-CAT and pHBAF103-CAT is repressed by *jun* to the same degree. If *jun* independently affected binding at both the proximal and most proximal AT motif, the two constructs should be suppressed by different degrees. If however, only the function of the most proximal AT motif were affected, the same degree of repression should be seen since the activity of the proximal AT motif is believed to be dependent on the most proximal element. Cotransfection results are compatible with the latter situation. However, it is difficult to clearly distinguish which elements are important in repression using these CAT constructs. Recently available are AFP promoter mutants which code for mutations in either the proximal or most proximal AT elements. These constructs would be helpful in determining the precise role of the different AT elements in repression by *ras*, *jun*, or *fos* of the AFP gene.

Therefore, from my results, the involvement of the AFP most proximal AT motif in repression by *ras*, *jun*, or *fos* cannot be ruled out, though it appears that protein

interactions at the AFP domain B enhancer AT motif and the promoter proximal AT motif are not crucial for repression of the AFP gene.

4.6 Differential regulation by *ras*, *jun*, and *fos* of the AFP and albumin genes

AFP promoter activity was repressed by *ras*, *jun*, and *fos*, whereas albumin promoter activity was activated by *jun* and *fos* and unaffected by *ras*. It appears that *jun* and *fos* mediate repression of the AFP gene through a mechanism similar to that involved in repression of the AFP gene by *ras*. This suggests that *jun* and *fos* cooperate in the *ras*-mediated suppression of AFP. For all three oncogenes, the promoter sequences are sufficient for directing repression of AFP. This is compatible with the study by S. Camper and S. Tilghman (1989) indicating that elements within 1-kb of the 5'-flanking region of the mouse AFP gene are sufficient for postnatal repression of the gene in transgenic mice.

The mechanisms of inhibition and activation by *jun* and *fos* appear to be different. The activation of *jun* and *fos* of the albumin promoter is presumably mediated through a TRE-dependent mechanism. It appears that repression by *jun* and *fos* of AFP promoter activity results from indirect interference of the protein interactions required for basal promoter activity of the AFP gene. That apparently different mechanisms are involved in activation and repression by *jun* and *fos* of the albumin and AFP genes is consistent with experimental findings indicating that AP-1 dependent trans-activation and repression by *jun* and *fos* involve distinct mechanisms (Lucibello et al., 1989, Gius et al., 1990).

This study shows the responsiveness of the AFP gene to three oncogenes known to be involved in signal transduction. The *ras* genes code for membrane bound transducing proteins and *jun* and *fos* encode nuclear trans-acting factors. All three oncogenes have been shown to cooperate in deregulation of several genes. That the AFP gene was repressed and the albumin gene was unaffected or activated by *ras*, *jun*, or *fos*, supports the hypothesis

that signal transduction cascades are involved in the differential regulation of the AFP and albumin genes throughout development.

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