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IDENTIFICATION AND CHARACTERIZATION OF GENES ASSOCIATED WITH *V-JUN* INDUCED CELL TRANSFORMATION

by

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B. S. May 1980, Fordham University

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF GENES ASSOCIATED WITH V-JUN INDUCED CELL TRANSFORMATION

Martin Hadman
Eastern Virginia Medical School, 1995
Advisor: Dr. Timothy J. Bos

The *v-jun* oncogene was initially identified as the causative agent for fibrosarcomas in chickens. Studies show that overexpression of v-Jun proteins transforms chicken embryo fibroblasts (CEF) *in vitro*, and forms tumors in chickens *in vivo*. The mechanisms for this are not clearly defined. Conceivably, overexpression of an unregulated transcription factor would cause cell transformation by illicit regulation of its target genes. In support of this, we show that *in vivo* v-Jun complexes exhibit differential binding to *in vitro* generated AP-1 and 'AP-1 like' target sequences, suggesting that the pattern of target gene expression is altered during cell transformation. With this in mind, we set out to identify genes associated with v-Jun induced cell transformation. We have isolated several clones by subtractive hybridization, and a modified differential display procedure. One of these is clone 4, showing strong sequence homology, both at nucleotide and amino acid level, to cysteine thiol proteases. Northern blot analysis shows that the steady state levels of clone 4 mRNA are 3 to 7 times higher in v-Jun transformed CEF (VJ-1), when compared to c-Jun overexpressing CEF (CJ-3), or normal CEF infected with vector sequences only (RCAS).

Another is clone 15-15, showing strong sequence identity to the chicken Apolipoprotein A1 (ApoA1) gene. Northern blot analysis demonstrates that the steady state levels of ApoA1 mRNA in RCAS is 3 to 10 times higher than in VJ-1 cells, indicating that v-Jun might repress this gene by transcriptional mechanisms. To investigate this possibility, we generated several ApoA1 reporter CAT constructs containing 5' deletions in the promotor, and tested them in VJ-1 and RCAS cells. Our findings suggest that three potential cis-acting sequences could regulate this promotor in normal RCAS CEF. Quite remarkably, none of these constructs were transcriptionally active in VJ-1 cells. DNA binding

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studies utilizing one of the potential cis-acting regions, suggests that a specific factor is present in normal nuclear extracts, but absent from v-Jun transformed extracts. This observation suggests that this specific factor may be a positive activator protein. In addition, actinomycin D studies demonstrate that the ApoA1 mRNA has a long half-life of up to 20 hours. We therefore propose that ApoA1 is positively regulated by at least three cis-acting sequences, and maintained at high steady state levels in normal CEF. Several possible mechanisms exist to explain ApoA1 repression in normal RCAS and VJ-1 cells. One possibility is the direct repressor mechanism, whereby a silencer region directly inhibits ApoA1 expression in normal cells. In VJ-1 cells however, a squelching mechanism could predominate. In this case, overexpressed v-Jun proteins would sequester and inactivate potential factors that positively regulate ApoA1 transcription, leading to repression.

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Dedication

I dedicate this dissertation
to my daughter, Kaitlin Theresa Hadman,
to my wife, Theresa Louis Christie,
to my parents, and to my brothers and sisters,
who all encouraged me in achieving
this monumental goal.

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Acknowledgement

I have enjoyed working with Dr. Timothy J. Bos, who introduced me to the exciting field of transcription factors. I would like to thank the members of my committee, the faculty members and graduate students at Eastern Virginia Medical School and Old Dominion University. This dissertation would not have been possible without exceptional help from Linn Gabos, Bao-Ling Adam, and Cara Haley.

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Chapter I. INTRODUCTION

Discovery of the *v-jun* oncogene.

Avian sarcoma virus (ASV-17) was isolated from a spontaneous sarcoma in adult chickens. Retroviral isolates of ASV-17 form polyclonal tumors when injected into chickens, *in vivo*, and transform chicken embryo fibroblasts cultures (CEF), *in vitro*. These transformed fibroblasts have a characteristic fusiform shape organized in parallel arrays when grown in liquid medium, and form anchorage independent colonies in soft agar. Interestingly, *in vitro* transformed CEF are not immortalized by ASV-17; instead they become highly vacuolated after 25 to 30 cell doublings, and die. In contrast, cultures derived from ASV-17 induced tumors have a longer lifespan, indicating that additional genetic modifications are responsible for cell immortalization (reviewed in ref 35).

Further studies show that ASV-17 is a defective retrovirus requiring non-defective helper virus to replicate efficiently, and produce infectious progeny. Characterization of the ASV-17 retroviral genome revealed a 0.93 kb insert showing strong homology to a cellular *c-jun* gene, and to the DNA binding domain of a yeast transcription factor, GCN4. When either the cellular gene (*c-jun*) or the ASV-17 derived insert (*v-jun*) was cloned into a retroviral expression vector, RCAS, and overexpressed in CEF, phenotypic properties similar to ASV-17 induced cell transformation occurred *in vitro*. This finding demonstrates that the factor responsible for transformation was acquired from the cell by ASV-17, and establishes that the *jun* gene is a true oncogene (35).

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Discovery of c-jun.

Seminal studies propelling *jun* into forefront of oncogene research began with the characterization of a transcription factor complex from HeLa cell extracts that specifically recognized the SV40 enhancer element and the human metallothionein IIA (hMIIA) promotor. This transcription factor complex, called AP-1 fraction, (activator protein-1) binds the sequence, TGA(C/G)TCA. This sequence, later called the AP-1 DNA binding site, is present in regulatory regions of several viral and cellular genes. Interestingly, this site is homologous to the TRE site (TPA responsive element) responsible in the regulation of cellular genes stimulated by cell treatment with phorbol esters. Definitive proof that Jun protein is a component of the AP-1 fraction comes from immunoblot assays showing that antibodies to v-Jun peptides, PEP1 and PEP2, recognize a 39 kd polypeptide in purified AP-1 preparations. Sequencing of AP-1 tryptic digests showed strong similarity to predicted Jun amino acid sequences. Moreover, bacterial expressed Jun proteins are able to bind the AP-1 site. Further studies showed that the AP-1 fraction consists of other proteins involved in recognizing the AP-1 binding site: these include the well characterized oncogene, c-Fos, other Fos related proteins, and Jun related proteins. Further characterization of Jun and Fos proteins demonstrated that they form homodimers and function as important cellular transcription factors. These observations provided incentive to investigate the role of Jun and other AP-1 factors in gene regulation and cellular function (35).

Role of c-Jun in cellular events.

The *c-jun* gene is expressed at basal levels in most tissues, and is rapidly induced in response to serum and extracellular growth factors (29, 35). For this reason, c-Jun activity has been implicated in several growth related events

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including: cell growth and proliferation (66), differentiation (14, 93-96), embryonic development (65), wound healing (35), tissue regeneration (35), and UV induced cell damage (68). Interestingly, a recent study demonstrates a role for Rb (Retinoblastoma susceptibility gene product) in c-Jun regulation, and further suggests that these transcription factors could regulate cell growth (98). It is therefore possible that Rb/Jun targets might include cell cycle regulators. Indeed, the expression of *c-jun* as an immediate early gene (29, 35, 66, 67) correlates well with induction of cell growth, implying that it could potentially effect downstream targets to regulate the entrance or exit from the cell cycle.

c-Jun structure.

The *c-jun* gene is highly conserved in several species, and present in non-vertebrates like Drosophila, and in vertebrate species of chickens, mouse, rat, and humans. The human *c-jun* gene, located on chromosome 1p3.1-3.2, is a single copy gene, as determined by stringent Southern blot analysis of genomic restriction fragments with a jun probe. Interestingly, the 984 bp coding region does not contain any introns. Other important characteristics include a long GC rich 5' untranslated region, and an AT rich 3' region, common in growth factor genes and proto-oncogenes. The promotor region contains several transcription factor binding sites, including an AP-1, SP-1 and CTF sites, and two variant TATA-like boxes. Two major transcripts, 2.7 and 3.4 kb, are produced from multiple transcription initiation sites and two poly A addition signals. Although several ATG start sites exist, further studies suggests that the various c-*jun* transcripts all code for the same Jun protein (29, 35).

The *c-jun* gene codes for a 39 kd protein. Functional domains include the transcriptional activation region, the basic region, and leucine zipper, (figure 1).

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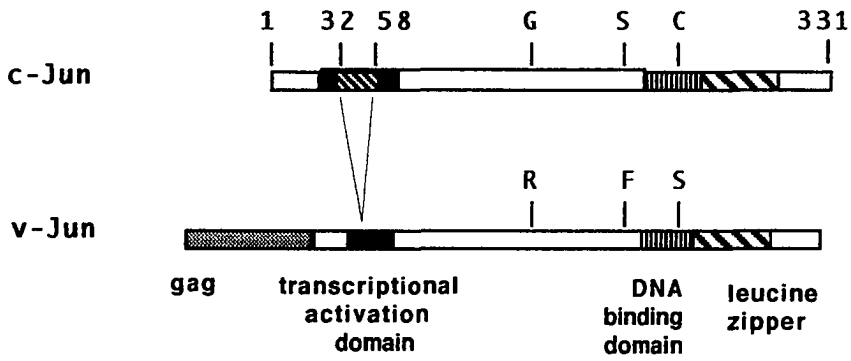

Figure 1. Structural differences between c-Jun and v-Jun.
The figure above shows the functional domains on the c-Jun protein. Note that v-Jun contains several structural alterations, including a gag region acquired from the transforming retrovirus, a 27 amino acid deletion in the transactivation domain (called the delta region), and three point mutations at the COOH region. The numbers above denote positions of amino acid residues; G: glycine; S: serine; C: cysteine; R: arginine; F: phenylalanine.

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The COOH terminal half of the Jun protein contains the basic region and leucine zipper motif, responsible for dimerization and subsequent DNA binding to the AP-1 site. The leucine zipper consists of five leucine residues which are equally spaced by seven amino acid residues. Structural analysis suggests that this arrangement forms an alpha helical structure with the leucine residues protruding from the sides and aligning in a straight line, favorable for dimerization with other proteins with similar conserved structures. These proteins include the yeast activator protein, GCN4, the fos family of proteins, c-Fos, Fra-2, and FosB, and Jun family of proteins, JunB and JunD. Dimerization with c-Jun is specific since other proteins containing leucine zipper motifs, like c-Myc, do not form heterodimers with either Jun or Fos proteins. Moreover, CREB II, which belongs to the CREB (cAMP responsive element binding) family of proteins, can form heterodimers with c-Jun but not c-Fos. This observation suggests that dimerization specificity is maintained by the structural organization of the leucine zipper conserved among Jun, Fos and CREB family of proteins. Other regulatory regions outside the conserved leucine zipper domain might regulate dimerization, since neither c-Fos nor Fra-1 form homodimers; other CREB members form homo- and heterodimers among their family members, but not with c-Jun proteins (29, 35).

Immediately upstream from the leucine zipper region is the basic domain, or DNA binding domain. This region, also conserved in Jun, Fos, and CREB, families, consists of long stretches of positively charged amino acids. Site directed mutagenesis suggest that the basic region is responsible for DNA binding and not dimerization, although other domains outside the Jun basic domain could contribute to the DNA binding affinity or specificity to target sequences. This observation was verified by several domain swapping

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experiments. Since dimerization precedes DNA binding, it is possible that dimerization induces conformational changes favorable to DNA binding specificity (29, 35). These observations imply that dimerization partners, or their availability, influence DNA binding and the overall regulation of target gene expression.

The transcriptional activation domain (TAD) of c-Jun, located in the amino terminal half, consists of negatively charged amino acids, including proline and glutamine. This region of acidic amino acids, conserved in the Jun family of proteins, can confer an activator protein of new sequence specificity when fused to a heterologous DNA binding domain. A cluster of proteins with a molecular mass around 52 to 55 kDa (p52/55) specifically recognize the Jun TAD, and could potentially regulate the transcriptional activation of Jun target genes (64).

More importantly, v-Jun has a 27 amino acid deletion, called the delta region, that maps to the transactivation domain of c-Jun. While this deletion does not appear to affect the overall transactivation potential of v-jun, it reduces its dimerization and DNA binding properties (29). Moreover, the delta region could regulate significant post-translational modifications of c-jun, since a peptide encoding this region reduces the ability of JNK (Jun N-terminal kinase) to efficiently phosphorylate the Jun protein *in vitro* (102). Evidence suggests that kinase phosphorylation at ser 63/73 affects the dimerization and DNA binding properties of Jun proteins (58). The relevance of these observations to c-Jun regulation and cell transformation will be discussed later.

Regulation of c-Jun activity.

c-Jun activity is stringently regulated during the normal cell process, as

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aberrant Jun activity leads to cell transformation *in vitro,* and tumorigenesis *in vivo.* Cellular mechanisms regulating c-Jun can be divided into: (a) factors that enhance its expression at the transcriptional and translational level, including the factors that regulate its mRNA and protein stability; (b) signal transduction mechanisms that effect post-translational modifications, which in turn regulate its overall function including dimerization and transactivation; and, (c) other poorly defined regulatory mechanisms, including the role of potential stabilizing factors, and control of transport to the nucleus (75 -78).

Signal transduction events regulating c-Jun.

Binding of extracellular factors to cell surface receptors initiates multiple signal transduction cascades. Studies indicate that some of these signaling events regulate Jun related responses by activating basal levels of preexisting c-Jun proteins present in resting G_0 cells (6, 39). These activated Jun proteins form Jun heterodimers, and positively autoregulate *c-jun* gene transcription, and coordinate the expression of Jun target genes necessary to effect cellular responses. Northern blot analyses indicate a 30 fold increase in steady state levels of *c-jun* mRNA 30 minutes after serum treatment, accompanied by increased Jun heterodimers formation, and binding to AP-1 sites. Expression of corresponding target genes, such as collagenase, occurs 8 to 12 hours later, depending on the cell type. In this way, amplification of an initial transient extracellular signal to achieve the desired cellular response, is mediated by activated Jun heterodimers (29, 35).

Several extracellular factors and stimuli inducing *c-jun* gene expression, c-Jun activation, as well as Jun target gene expression, are shown in table 1. Some factors, such as TNF-alpha, and TGF-beta, induce prolonged *c-jun* transcription

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Table 1. Listing of factors known to affect *c-jun* gene expression.

stimuli	comments	reference
serum	transient increase of c-jun mRNA in most cells, including fibroblasts and mouse 3T3 cells	29, 35
NGF	decreased c-jun transcription in PC-12 cells; response is differentiation dependent	90, 91
EGF	stimulation of c-jun mRNA in NIH/3T3, rat fibroblasts	79, 59
TGF-B	stimulation of c-jun mRNA in adult rat hepatocytes and A549 human lung adenocarcinoma;	80, 85
TNF	prolonged activation of c-jun mRNA in human fetal fibroblasts, monocytes and granulocytes;	81, 82
IL-1 and IL-2	enhanced c-jun mRNA expression in T-cells;	86..
IL-3	no c-jun mRNA detected in 32D hemato poetic cells but increases noted in FDCP-1 cells;	66 ..
IL-6, oncostatin M	rapid and transient c-jun mRNA increase in human fibroblasts;	83..
endothelin isopeptides	induction of c-jun mRNA and activity in mesangial cells;	84..
GM-CSF	c-jun expression and AP-1 enhancer activity observed in U-937 cells;	72..
jun/fos	positive auto-regulation in HeLa, HEPG2, F9	29, 35
CREB	phosphorylated CREB activates c-jun gene transcription in NIH 3T3;	88..
retinoic acid	strong c-jun mRNA expression in undifferentiated EC stem cells	89..
NF-jun	transcription factor affects c-jun transcription in human myeloid leukemia cells	87..
Rb	Rb activates c-jun transcription thru the SP-1 binding site	98..
progesterone	down regulation of c-Jun in avian oviduct	108..
FSH/ follicle stimulating hormone	FSH inhibits c-jun in Rat Steroli cells	99..

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rates, and higher steady state levels of *c-jun* message. Further evidence suggests that the cell type involved, the duration of cell treatment, and the convergence of dual signals are responsible, as will be discussed later. The first signal pathway identified to induce *c-jun* transcription and activity is the protein kinase C (PKC) pathway, as cell treatment with phorbol ester, such as TPA, both increase *c-jun* mRNA steady state levels and c-Jun activity, as evidenced by binding to TPA responsive elements (TRE/ AP-1 sites) (29,35).

A listing of kinases and phosphatases known to regulate c-Jun activity are shown in table 2. Extensive studies show that c-Jun activity is regulated by post-translational modifications involving phosphorylation and dephosphorylation at several potential serine and threonine sites on the c-Jun protein. In resting cells, latent or inactive c-Jun is phosphorylated at the C-terminal ser 243 residue, located just upstream from the DNA binding site. PKC activation induces a rapid dephosphorylation at this site, associated with increased binding to AP-1 sites (56). Studies also show that resting cells have a dephosphorylated ser 63/ 73 residue at the N-terminal half of c-Jun, thereby restricting its activity. MAP kinases (Mitogen Activated Protein-serine Kinases) induced by mitogens, phorbol esters, and an activated ras oncogene, specifically phosphorylate these sites, resulting in an increase in c-Jun transactivation activity (7, 73). This suggests that a balanced phosphorylation/ dephosphorylation mechanism regulates c-Jun activity. However, these observations contrast with another study demonstrating that phosphorylation of Jun at either ser 63 /73, or ser-246, does not significantly alter c-Jun dimerization, DNA binding, or *in vitro* transactivation, and strongly suggest that these sites may not be critical for c-Jun regulation (57). Although the discrepancy may be in the use of different cell types and extracellular stimuli utilized in each study, it is clear that the regulation

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Table 2. Kinases and phosphatases affecting c-Jun activity.

enzyme	phosphorylation site affected	comments	reference
PKC / TPA	c-Jun is not a direct substrate; purified c-Jun is not phosphorylated in vitro; phosphorylation and dephosphorylation of potential ser and thr sites noted in HeLa and fibroblasts cells;	cell treatment with TPA increases jun/fos heterodimerization and binding to AP-1/ TRE sites	29, 35, 56
casein kinase II	phosphorylation at thr-231 and ser-249	phosphorylation negatively affects DNA binding and AP-1 activity	97..
GSK-3; glycogen synthetase kinase-3	phosphorylation at thr-239, ser-243, ser-249; in vitro	decrease in DNA binding, in vitro;	56..
Ha-Ras / MAP kinase and cdc/p34 kinase	phosphorylation at ser 63/73; dephosphorylation at thr-239, ser-243, ser-249	phosphorylation/ dephosphorylation mechanisms activate c-Jun;	7, 57, 73
protein phosphatase 2A (PP2A)	sites not determined; variations in phosphorylation demonstrated by changes in electrophoretic mobility;	loss of PP2A activity is associated with deletion in the delta region;	70, 71
JNK / SAPK (Jun N terminal kinase/ Stress activated protein kinase)	phosphorylation at ser 63/ 73, both in vitro and in vivo;	JNK/ SAPK kinase activity is regulated by delta region;	58, 92, 101, 102,

of c-Jun activity is complex, requiring other post-translational regulatory events, and stabilizing factors. Indeed, other post-translational modifications include glycosylation (35), and a reduction/ oxidation pathway, as will be discussed later.

Intensive research committed to elucidating the active participants involved in extracellular signaling have established that MAP kinases are important in growth factor responses and c-Jun protein activation. (39, 74). Studies report a UV induced signaling pathway involving Ras, Raf and MAP kinases in the regulation of c-Jun activity. The terminal effector in the cascade directly responsible for c-Jun phosphorylation at ser-63/ 73 residues belongs to a family of related MAP kinases (7, 8), known as the JNK-1 kinase (jun -N-terminal kinase) (58), or Stress Activated Protein Kinase (SAPK) (101). *In vitro* studies demonstrate a direct binding between a purified JNK to c-Jun, and implicate the importance of delta domain. *In vivo* studies confirm that c-Jun phosphorylation enhances DNA binding and transactivation of AP-1 sites. The UV induced pathway mentioned above is related to a growth factor signaling pathway, in that both pathways utilize Ras, Raf, and MAP kinases (68). They differ in that the latter pathway involves extracellular-signal-regulated-kinases (ERKs) (74). These pathways are distinct since agents that activate the former, such as UV and stress, only weakly activate the ERK pathway (39, 74). Moreover, studies show that the existence of a Ras signaling pathway that activates c-Jun, but does not involve ERKs (103). Although extracellular growth factors or receptor tyrosine kinases have been established in the regulation of c-Jun activity, the immediate effectors acting on c-Jun have yet to been identified. For instance, the phosphatase(s) directly involved in activating c-Jun by dephosphorylation at ser 246, or at other potential C-terminal sites, have yet to

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be determined. It is conceivable that this putative phosphatase is regulated by the ERKs, since PKC, an important constituent of the tyrosine kinase pathway, induces dephosphorylation at this site (56).

Extensive studies demonstrate that other signal pathways may be involved in c-Jun regulation. For instance, a PKC-independent pathway induced by the EGF receptor has been proposed to activate c-*jun* transcription (59). In this study, EGF or serum stimulation was able to induce *c-jun* gene transcription in PKC depleted NIH /3T3 cells. Another study investigated the role of diacyl-glycerol and Ca^{2+} in the regulation of c-Jun activity in U-937 cells (60). When cells are treated with either low doses of diacyl-glycerol, or agents increasing intracellular Ca^{2+}, *c-jun* gene transcription was observed without changes in c-Jun activation or AP-1 enhancer activity. However, when these agents act in synergy, AP-1 enhancer activity and expression of U-937 differentiation markers were noted. This last study emphasizes that regulation of *c-jun* gene transcription and AP-1 enhancer activity could be under separate control. It also suggests that dual converging pathways serve to regulate c-Jun activity. In support of this, studies demonstrate that activation of JNK-1 in T-cells, is dependent on Ca^{+2} mobilization and PKC activation (92).

Another interesting study reports that a factor isolated and characterized from HeLa cell extracts, the Ref-1 gene product, facilitates AP-1 DNA binding activity. (61, 62). Reduction of a conserved cys-272 residue on c-Jun protein, with either DTT, thioredoxin, or a purified Ref-1 gene product, increased DNA binding of Jun/Fos heterodimers to AP-1 sequences, *in vitro*. In contrast, chemical oxidation or modification of critical residues from cysteine to serine, inhibits DNA binding. Interestingly, the Ref-1 gene product has apurinic/

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apyrimidinic (AP) endonuclease activity involved in DNA repair. The redox and DNA repair properties of Ref-1 can be distinguished biochemically. The reports further suggest that a redox signaling pathway could regulate c-Jun activity during periods of oxidative stress, or severe DNA damage. In support of this, a redox signaling pathway involving nitric oxide (NO) proposes that transcription factors involving CREB, NF-KB, c-Jun and c-Fos are likely NO targets (69). It remains to be shown how these events relate to or differ from the UV and stress induced signaling events known to regulate c-Jun.

Dimerization regulates c-Jun activity.

Another important aspect in the regulation of c-Jun activity is dimerization. Since this critical event is a prerequisite for DNA binding, it follows that factors regulating dimerization could ultimately regulate c-Jun activity. As described earlier, c-Jun specifically interacts with other protein family members that have an identical or conserved leucine zipper structure. Since other leucine zipper containing proteins do not interact with c-Jun, an important aspect in maintaining dimerization specificity is the structure of the leucine zipper itself.

But how does dimerization regulate c-Jun activity? While c-Jun can form homodimers, it binds weakly to AP-1 sites, *in vitro*, as compared with Jun/Fos heterodimers, suggesting that Jun dimer partners influence the binding specificity and affinity to AP-1 sites. Indeed, studies show that the composition of Jun homodimers and heterodimers change rapidly during cell stimulation (29). During resting G_0 states, c-Jun homodimers predominate. Immediately after stimulation, the composition changes to c-Jun/c-Fos heterodimers, followed by c-Jun homodimers after c-Fos levels decay. Heterodimerization with JunD and

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JunB also occurs, but play a minor role. Further studies suggest that while Jun homodimers are responsible for maintenance of basal cellular function, Jun heterodimers play a role in initiating the induction response. More importantly, it demonstrates that the availability of Jun dimer partners at any given time point could determine Jun target specificity, and consequent regulation of Jun target genes. Interestingly, c-Jun can interact with other transcription factors that do not involve the leucine zipper. These include the steroid family of transcription factors, myoD, NF-KB, and NF/AT complex. This observation further supports the assumption that the promiscuous behavior of c-Jun endows it with new DNA binding specificity, which could be responsible for various cellular responses.

Negative regulation of c-Jun activity.

An important requirement in the maintenance of appropriate cellular function is the immediate inactivation of c-Jun activity after extracellular stimulation. Negative regulation can be achieved, in part, by decreasing c-Jun protein levels. One mechanism is the transcriptional attenuation of *c-jun* gene transcription that occurs within 30 minutes of extracellular stimuli. Although the exact mechanism is unclear, studies suggest that the immediate decay of c-Fos proteins contribute to low levels of Jun/Fos heterodimers, which in turn reduce *c-jun* gene transcription, and diminish the activation of AP-1 target genes. The *c-jun* message also has inherent destabilizing properties: the 3' end has the sequence, AUUUA, which contributes to early mRNA degradation. Moreover, the message also has an unusually long GC-rich 5'-untranslated region that reduces its translational efficiency. Kinetic studies show that although TPA treatment induces a 15 fold increase in *c-jun* mRNA levels, only a 3 - 4 fold increase in protein synthesis is observed. Finally, the c-Jun protein has PEST

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regions (proline, glutamic acid, serine, threonine) known to enhance protein degradation. Recently, the delta region of c-Jun has been linked to ubiquitin mediated degradation (63). These destabilizing factors, in combination with necessary post-translational requirements for dimerization and DNA binding, restrict c-Jun activity to critical events in the cell cycle. It follows then, that deregulated c-Jun activity leads to cell transformation.

Overexpression of v-Jun induces cell transformation in CEF.

Extensive studies from our laboratory and others report that deregulated overexpression of *v-jun* induces a rapid and efficient cell transformation of CEF *in vitro* (3, 4). The fibroblasts appear transformed by two important criteria: loss of contact inhibition and anchorage independence. In addition, these phenotypic properties closely resemble ASV-17 induced tumors in chickens (3, 35, 37). In contrast, overexpression of *c-jun* transforms CEF weakly when compared to *v-jun* (10-25 fold less) (4, 32), and fails to form tumors in chickens (30). Intriguingly, Jun transformed CEF are not immortalized; instead, they become vacuolated after 25 to 30 cell doublings, and die. Possible differences exist between mammalian and avian systems: the former requires a cooperating Ha-Ras oncogene for efficient cell transformation (35).

The mechanisms involved in the Jun induced cell transformation of CEF are not clearly defined. A working model proposes that the structural alterations in v-Jun affect its functional properties, as exhibited by an increase in its transformation potential. These points will be discussed below.

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Structural alterations in Jun protein affect its functional properties.

The structural differences between c-Jun and v-Jun are shown in figure 1. v-Jun has a *gag* leader sequence obtained from the transforming retrovirus, a 27 amino acid deletion at the amino terminus that maps to a transactivation domain, and contains three point mutations at the carboxyl terminus (35). Studies show that deletion of the 27 amino acid region (called the delta domain) is critical to v-Jun's transformation potential (4, 5, 70). The delta domain contains regulatory sequence information influencing Jun stability (63), DNA binding, dimerization (58), *trans*-activating properties, as well as its translocation to the nucleus (75). In addition, loss of the delta region affects the ability of specific kinases, such as JNK/ SAPK, to efficiently phosphorylate Jun proteins *in vitro* and *in vivo* (39). Deletion also increases v-Jun's half-life by reducing ubiquitin mediated degradation (63). Consequently, post-translational modifications responsible for appropriate stringent control are lost, contributing to a stable, unregulated v-Jun protein. Although c-Jun possesses no structural defect, weak transformation still occurs since expression from the retroviral LTR, maintains high levels of c-Jun, thus prolonging its activity (4). Taken together, the differences in transformation potential between c-Jun and v-Jun, can be explained, in part, by their structural differences, namely loss of the delta region in v-Jun.

Dimerization affects Jun transforming ability.

During normal physiological conditions, the half-life of potential Jun heterodimer combinations is low, and restricted to the G_0 to G_1 transition phase *in vivo* (67). Studies estimate that dimerization among the Jun and Fos family members alone contribute to 15 potential dimer combinations with varying affinities and specificities to AP-1 sequences *in vitro* (22). Although the estimated

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number of complexes appear high, and may increase since Jun interacts with other non-leucine zipper factors, the predicted number of complexes at any given time point is actually lower since Jun proteins are inherently unstable and turnover rapidly. However, when stable v-Jun proteins are overexpressed, the half-life and repertoire of potential dimer combinations along the cell cycle would increase dramatically. It follows that these new complexes would illicitly regulate their target genes, and ultimately contribute to cell transformation.

Changes in v-Jun functional properties could induce transformation by inappropriate regulation of target genes.

The mechanisms involved in cell transformation have received much attention in the scientific community (1). One model proposes that oncogenic transformation might result from a change in the pattern of target gene expression mediated by changes in DNA binding or transactivation of target genes (2). A similar situation could occur for v-Jun induced cell transformation. Conceivably, overexpressed v-Jun would lead to stable interactions with other transcription factors, thereby increasing the repertoire of potential transcription regulators. Such interactions could occur both at the protein and DNA level. Consequently, cell transformation would arise from the illicit regulation of AP-1 and AP-1 related target genes.

Although there is no evidence that v-Jun behaves this way, studies show that its cellular homolog does. For example, unregulated c-Jun proteins can inhibit estrogen receptor activity in human breast cancer derived cells (13). c-Jun proteins can also interact with a variety of transcription factors both at the DNA level and the protein level (10-15, 18-20, 23, 51). For instance, the master regulatory protein, myoD, interacts with Jun proteins during myocyte cell

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differentiation (14, 15). Jun/AP-1 proteins also interact with the thyroid hormone receptor (18), and the glucocorticoid receptor complex (11, 12). Interactions with other factors alter the pattern of target gene expression and overall cellular response. For instance, NF-KB p65 and Jun/AP-1 protein interactions produce potentiated biological responses (10); myoD and Jun interactions affect myogenic differentiation (14). Finally, some of these interactions are regulated by extracellular signals: Dimerization between the NF-AT p/c and c-Jun proteins are dependent on calcium and PKC signals, respectively. The resulting NF/AT complex is required to activate critical target genes. Activation of these factors by PKC or calcium alone, stimulates a different set of target genes (39).

Problem and hypothesis

The mechanisms involved in Jun induced cell transformation are not completely understood. One model proposes that the structural changes in v-Jun contribute to its altered functional properties (4). We test the hypothesis that such changes influence the pattern of target gene expression (2). Consequently, several target genes would be activated or repressed inappropriately. Cell transformation would result from a change in the balance between potential oncogenic and tumor suppressor target genes (2). This dissertation therefore aims to support the hypothesis by identifying potential genes regulated by v-Jun, and to implicate them in cell transformation.

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Objectives

A detailed listing of the objectives are as follows:

AIM 1: Determine v-Jun target specificity *in vitro*.

- construct *in vitro* AP-1 target sequence mutants;
- characterize the DNA binding specificity of each mutant AP-1 target sequence in VJ-1, CJ-3, and RCAS nuclear extracts;
- determine the transactivation potential of each *in vitro* AP-1 fragment in VJ-1, CJ-3 and RCAS cells;

AIM 2: Isolate *in vivo* target genes associated with v-Jun induced cell transformation by:

- subtractive hybridization:
  - differential screening of subtracted library;
  - identification of differentially expressed clones;
  - Northern blot analysis to confirm differential nature;
- differential display:
  - improve the technique;
  - identification of differentially expressed clones;
  - Northern blot analysis to confirm differential nature;
  - partial DNA sequence analysis;
  - GENBANK database searches;

AIM 3: Characterize clone 4, a gene up-regulated in v-Jun transformed CEF;

- DNA sequence analysis;
- GENBANK and SWISS-PROT database searches;
- generation of full length cDNA clone by 5' RACE;

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AIM 4: Characterize clone 15-15, a gene repressed in v-Jun transformed CEF;

- evaluate the stability of *apo-A1* mRNA;
- identify the Jun responsive element on the *apo-A1* promoter;
- characterize the Jun responsive element by promotor deletion analysis and DNA transactivation studies;
- assess the DNA binding properties of the putative responsive element;
- speculate on a mechanism for the transcriptional regulation of the chicken *apo-A1* gene in v-Jun transformed CEF;

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Chapter II. MATERIALS and METHODS

Table 3. Primers used in this study

primer	sequence shown 5' to 3'
ApoA1 primers	
ApoA1-primer 1	CACTGCTCGTCCCGTGTGAG
ApoA1-primer 2	CCTCCGTCCACTTGGCAGAGAAC
ApoA1-primer 3	GGAGAGGAGATTAAGGAGGGGTCC
ApoA1-primer 4	CTCACACGGGACGAGCAGTG
ApoA1-primer 5	CGGGGAGCTCCTGTTTGCTGAGG
ApoA1-primer 6	GCTCTCCTGCCGCTGCTCCG
T7 primer	TAATACGACTCACTATAGGG
SP6 primer	GGATTTAGGTGACACTATAG
SP6-T primer	GATTTAGGTGACACTATAGAATACT-11
differential display primers	
DD-17 5'-primer	CTGCTCTCA
DD-18 5' primer	CTTGATTGCC
DD-20 5' primer	CTGATCCATG
DD-A 3' primer	T12-VA V=A,G, or C
DD-G 3' primer	T12-VG V=A,G, or C
DD-T3' primer	T12-VT V=A,G, or C
DD-C 3' primer	T12-VC V=A,G, or C
clone 4 primers	
clone 4 primer B	TACATACAGCTCTCATCCTGCCCGATGTAG
clone 4 nested primer	GTCCGGCCCTTCAGCTGCC
anchor primer	CACGAATTCACTATCGATTCTGGAACCTTCAGAGG
reverse anchor primer	CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG
AP-1 primers	
	GGGTCTAGAATGACNCATCGGATCCTGCAGGA
	GGGTCTAGAATGACTNATCGGATCCTGCAGGA
	GGGTCTAGAATGACTCNTCGGATCCTGCAGGA
	GGGTCTAGAATGANTTATCGGATCCTGCAGGA
	GGGTCTAGAATGACNTATCGGATCCTGCAGGA

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Transformation of chicken embryo fibroblasts

Primary chicken embryo fibroblasts (CEF) were isolated from 9-11 day old chicken embryos and transformed by infection with a replication competent retroviral vector, RCAS, designed to overexpress either v-Jun or c-Jun (4, 38). CEF infected with vector sequences alone do not transform and serve as normal control. Jun overexpression is verified by Western blot analysis. These cells are used to isolate nuclear extracts for DNA binding assays, to isolate RNA for Northern blot analysis, the differential display and subtractive hybridization approach; CEF were also used for DNA transfection assays.

Generation of mutant AP-1 fragments and reporter CAT constructs.

Sixteen in vitro generated mutant AP-1 sequences containing variations in the consensus AP-1 site, were constructed by primer extension of two overlapping oligonucleotides. Five different oligonucleotides (table 1) containing a degenerate nucleotide at a single position were allowed to anneal, and were then extended with Klenow to generate double stranded sequences. After restriction digestion, sequences were cloned into pGEM4 vector and amplified in appropriate hosts. Individual clones were isolated and verified by sequencing. The overall strategy is described in figure 2. The resulting sequences are shown in table 4. These sequences are used in a DNA binding assay described below, and were cloned upstream from the human metallothionein promotor of a reporter CAT construct, pMCAT III, for DNA trans-activation studies.

DNA binding assays

DNA binding assays are a modification from Nakabeppu (43). Briefly, 1 to 10 ug of nuclear extracts in a reaction buffer containing 1 ug/ uL poly dI-dC poly dI-dC, 10 mM HEPES pH 8.0, 4 mM $MgCl_2$ 17.5% glycerol, 0.1 mM EDTA,

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20 mM NaCl, 2 mM DTT, and 2 mM spermidine are pre-incubated on ice for 15 minutes, followed by incubation with 50,000 cpms of labeled probe. The resulting complexes formed are separated on a 4-6 percent (59:1) acrylamide/ bis -acrylamide /TBE gel, dried, and exposed to autoradiographic film. Competition shift assays used a 40 to 100 fold excess of unlabeled homologous oligonucleotide or specific antibody. Quantitation of binding intensity is determined by densitometric scanning.

DNA transactivation

Sequences of interest were either cloned upstream from the human metallothionein promotor at the BglII site of the pMCAT 3 construct, or into the multiple cloning site of the pCAT/Blue construct, which contains the CAT gene in a pBluescript SK (+)vector. These reporter CAT constructs were transiently transfected into either v-Jun transformed, c-Jun overexpression or normal CEF by a DMSO-polybrene procedure (40). Relative transcriptional activation from these sequences are evaluated by a CAT assay (45); quantitation of CAT protein expression was done on a phospho-imager (Molecular Dynamics). CAT expression was normalized to B-galactosidase activity from the construct, pCH110.

Northern blot analysis

Total RNA was isolated from transformed or normal CEF by an acid-phenol extraction procedure (41). Briefly, CEF are lysed in guanidine-isothio-cyanate solution, and extracted with acid phenol and chloroform. The aqueous phase containing RNA was precipitated with isopropanol, and rinsed with ethanol. 10 to 15 ug of total RNA was then separated on a 1 per cent agarose/ formaldehyde gel, transferred to nylon membrane, UV-crosslinked at 150

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mJoules/ cm^2, and hybridized to a labeled probe at 42 °C for 3 to 4 hours. Excess probe was removed by several 20 minute stringent washes in 0.1 X SDS /0.1 %SSC at 42 ° to 60 °C, and exposed to X-ray film for autoradiography (53).

Differential screening of a subtractive cDNA library

Enrichment of unique sequences from a subtracted VJ-1 cDNA library were accomplished by a Reverse Northern procedure (42). Briefly, 5 ug of mRNA from v-Jun transformed CEF (VJ-1), c-Jun overexpressing (CJ-3), and normal CEF (RCAS) were each extended by reverse transcription using 20 pmoles of SP6 Oligo T primer, in 1X RT buffer (BRL) containing 400 mM dNTP, 100 mM DTT, 5 ng/mL Actinomycin D, and 300 units of MoMuRT, at 37 °C for 45 minutes. The cDNA sequences were converted into double stranded sequences with Klenow and random hexamers (53). The resulting sequences contained an SP6 site at one end allowing generation of large amounts of labeled anti-sense RNA using SP6 RNA polymerase (42, 53). 3,000 colonies from the subtracted VJ-1 library were poked out into LB plates in quadruplicate. Colony lifts were prepared from three sets; the nylon filters were UV-crosslinked at 150 mJoules (BioRad UV-crosslinker) and pre-hybridized for 5 hours at 65 °C (53). Each set of labeled anti-sense transcripts were then hybridized to individual colony lifts from the subtracted library overnight at 65 °C, washed in stringent conditions, and exposed for autoradiography (53). Unique colonies hybridizing to VJ-1 labeled transcripts but not to CJ-3 and RCAS were identified, and isolated from the unhybridized set.

Construction of ApoA1 promotor CAT plasmids.

All promotor fragments used in the study utilized the pCAT/ Blue construct generated by inserting the HinDIII/ BamHI CAT gene fragment from

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pSV2CAT plasmid, into the HinDIII/ BamHI site of pBluescript SK (+) phagemid. The -193/20 fragment was generated by PCR amplification using ApoA1 primer 5 and 1. The -193/20 fragment was generated by digesting the 5/1 PCR fragment with SacII which removes 20 bases of exon 1. The -300/263 fragment was generated by isolating the 563 bp KpnI/PstI fragment from the p7.5 ApoA1/Blue construct, which contains the 7.5 kb genomic sequence cloned into pBluescript. The -300/43 fragment was generated by PCR amplifying the p-300/263 ApoA1 CAT construct with ApoA1-primer 6 and a T7 primer which hybridizes to polylinker sequences. The -300/20 fragment was generated by PCR amplifying the p-300/263 ApoA1 construct with ApoA1 primer 1 and T7 primer. The -3.3/43 construct was generated by inserting the 3.0 kb Asp718 fragment from p7.5 ApoA1/ Blue into the unique Asp718 site of p-300/43 ApoA1 CAT construct. The -3.3/20 construct was generated by inserting the 3.0 kb Asp718 fragment into p-300/20 ApoA1 CAT construct. The p-300/0 fragment was isolated from a KpnI/ Sac II digestion of p7.5 ApoA1/Blue. The 3.3/0 ApoA1 construct was generated by inserting the 3.0 kb KpnI/ KpnI fragment from p7.5 ApoA1/Blue into the KpnI site of p-300/0 ApoA1-CAT. The p-6.8/0 ApoA1- construct was generated by ligating the 7.6 kb HinDIII/ XbaI fragment from p7.5 ApoA1/Blue with 4.1 kb HinDIII/ XbaI fragment from p-3.3/0 ApoA1 CAT construct.

Differential display

The modified differential display procedure is described in reference 46. Briefly, the reverse transcriptase step was accomplished by annealing 0.2 ug of mRNA with 250 pmoles of unlabeled 3' primer (Table 1) at 65 °C for 5 minutes, followed by extension at 37 °C for 1 to 1.5 hours. The reaction was terminated at 90 °C for 3 minutes. The RT reaction buffer contained 10 mM DTT, 0.5 mM

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dNTP, 12.5 ng/uL Actinomycin D and 300 units of Moloney murine Reverse transcriptase (BRL Gibco). After the RT reaction, the RNA template was removed with 1 ug/mL RNAse A digestion. Excess primers, unincorporated dNTP and degraded RNA were removed by size exclusion on a Chromaspin-10 column. One fourth of the resulting single stranded cDNA sequences were then PCR amplified with 20 pmoles 5'-primer and 20 pmoles of end labeled 3'-primer, for 35 to 40 cycles at 94° C/ 45 seconds, 40 ° to 42 °C / 2 minutes, and 70 °C/1 minute. The 1X PCR buffer (Promega) includes 200 uM dNTP, 1 mM $MgCl_2$ and 5 units of sequencing grade Taq DNA polymerase (Promega). The PCR products were separated on a 5% denaturing poly-acrylamide /urea gel, and exposed for autoradiography. Selected bands were excised, PCR amplified, and sub-cloned into a PCR cloning vector, pGEM-T (Promega). Differential expression was verified by Northern blot analysis. Selected clones were sequenced by a dideoxy method using a USB sequencing kit, according to manufacturer's suggestions.

Construction of cDNA libraries and subtractive hybridization

Briefly, three plasmid cDNA libraries were prepared from mRNA isolated from v-Jun transforming (VJ-1), c-Jun overexpressing (CJ-3), and from normal CEF (RCAS). To obtain sequences unique to v-Jun transformed cells, a VJ-1 minus RCAS subtraction was performed. Briefly, single stranded sequences were obtained from RCAS cDNA libraries by phage rescue utilizing dNTP-biotin incorporation. These single stranded sequences were then extensively hybridized with double stranded plasmid sequences from VJ-1 cDNA library, and separated on a Strep-Avidin column. Common sequences are retained in the column, while unique sequences elute out, and were packaged for infection to appropriate hosts. Similar strategies to isolate sequences unique to c-Jun

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overexpressing cells were performed. Plasmid and subtracted libraries were prepared by Dr. Timothy J. Bos.

DNA Sequence Analysis

DNA sequence analysis was performed by the dideoxy sequencing method utilizing the USB Sequenase Kit, according to manufacturer's recommendations.

Table 4. AP-1 and 'AP-1 like' sequences used in study.

sequence	description
TGACTCA	consensus AP-1
TGACATCA	chicken jun promotor
TGACGTCA	CREB
TGACTAA	SV40 promotor
TGACTCG	
TGACTCT	
TGACTGA	
TGACTTA	
TGACTCC	
TGACCCA	
TGACCTCA	
TGACGCA	
TGACACA	
TGATTTCA	
TGAGTTCA	
TGACTTCA	

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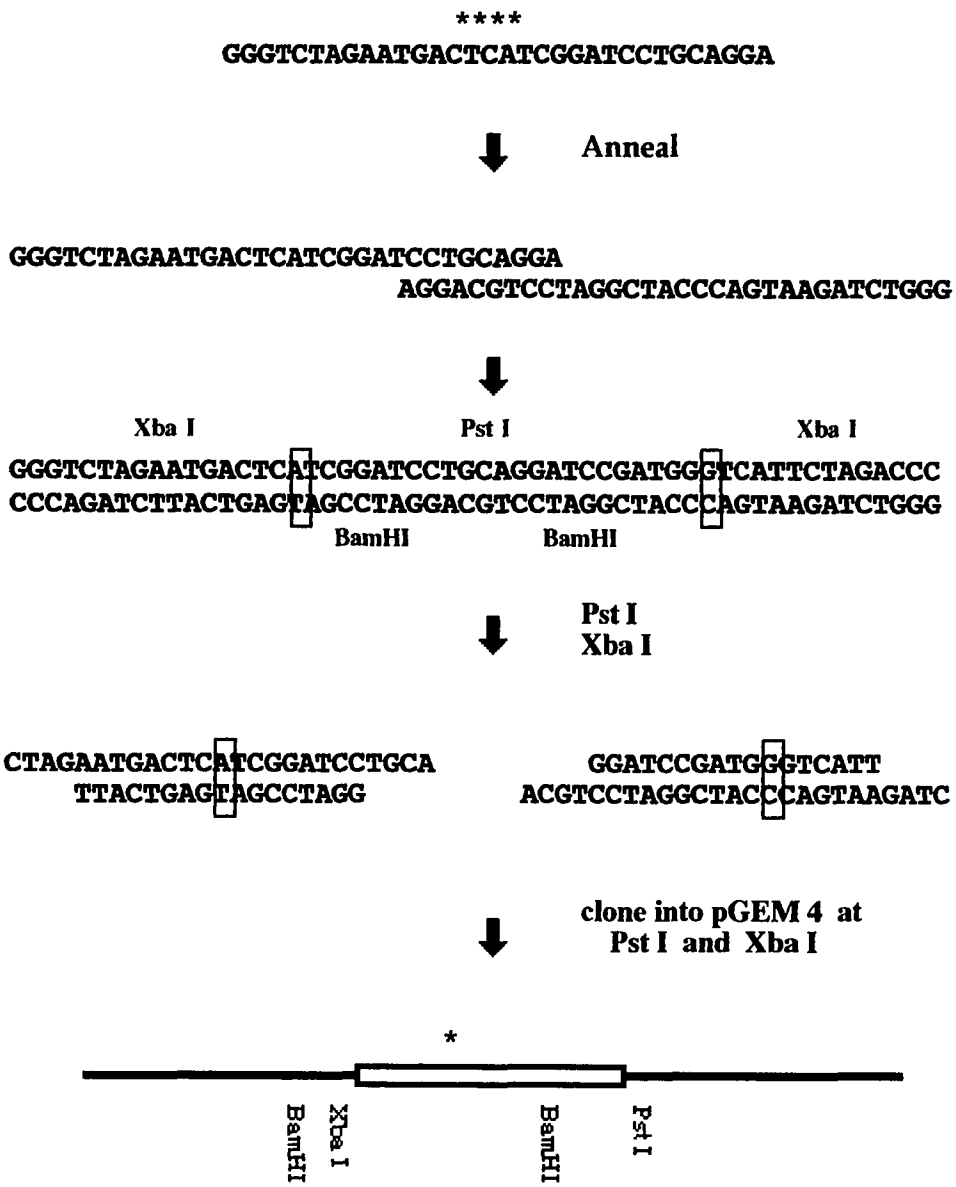

Figure 2 Strategy for generating 16 different AP-1 mutant sequences. Five different oligonucleotides containing a degenerate nucleotide at a single position were allowed to anneal, and then extended with Klenow to generate double stranded sequences. After restriction digestion, sequences were cloned into pGEM4 vector and amplified in appropriate hosts, Individual clones were isolated and verified by DNA sequencing. The (*) indicates residues in the primer that contained all four nucleotides. Individual primers contained only one degenerate nucleotide.

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Chapter III. RESULTS

AIM 1: Determine v-Jun target specificity *in vitro.*

As mentioned earlier, overexpression of Jun proteins could potentially increase the repertoire of possible dimer combinations, and consequently alter the pattern of target gene expression. To evaluate this possibility, the DNA binding properties of overexpressed Jun complexes formed *in vivo* against *in vitro* generated AP-1 and 'AP-1 related' sequences were assessed. We wished to compare our findings with previous studies demonstrating variations in DNA binding using *in vitro* translated Jun proteins to *in vitro* generated AP-1 sequences (22, 52). We also wished to determine the trans-activation potential of these AP-1 sequences in the context of v-Jun transformed conditions and normal conditions, and correlate them to DNA binding. We expect to find qualitative and quantitative differences in target recognition and transcriptional activation between v-Jun and c-Jun proteins during overexpression. These studies not only establish experimental conditions necessary to identify *in vivo* target genes but will give us an idea on potential regulatory mechanisms involved in v-Jun induced transformation.

DNA binding to consensus AP-1 and related AP-1 sequences.

To investigate the DNA binding properties of *in vivo* Jun complexes, nuclear extracts from v-Jun transformed, c-Jun overexpressing, and normal CEF, were isolated and used in a gel shift assay to the consensus AP-1 and to the various AP-1 like sequences. These nuclear extracts represent possible Jun dimer complexes formed *in vivo* under those conditions. Figure 3 shows that the consensus AP-1 sequence, TGACTCA, is recognized by two distinct complexes

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Figure 3 DNA binding to consensus AP-1 and related AP-1 sequences.
Competition shift assays of related AP-1 sequences against the consensus AP-1 sequence to *in vivo* Jun complexes present in v-Jun transformed (VJ-1), c-Jun overexpressing (CJ-3), and normal CEF infected with retroviral sequences only (RCAS), were assessed. A 40 to 50 Molar excess of competitor fragments were used. Arrows show position of complex 1 and 2. lane positions are:

lane	1	probe alone TGACTCA
lane	2	no competition
lane	3	TGACTCA
lane	4	TGACTCG
lane	5	TGACTCT
lane	6	TGACTCC
lane	7	TGACTTA
lane	8	TGACTAA
lane	9	TGACTGA
lane	10	TGACCCA
lane	11	TGACGCA
lane	12	polylinker
lane	13	TGACACA
lane	14	TGATTTCA
lane	15	TGAGTTCA
lane	16	TGACTTCA
lane	17	TGACCTCA
lane	18	TGACATCA
lane	19	TGACGTCA
lane	20	polylinker

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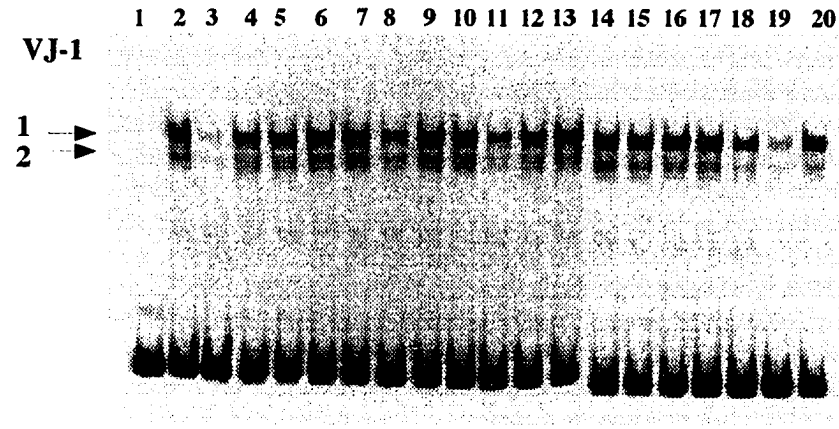

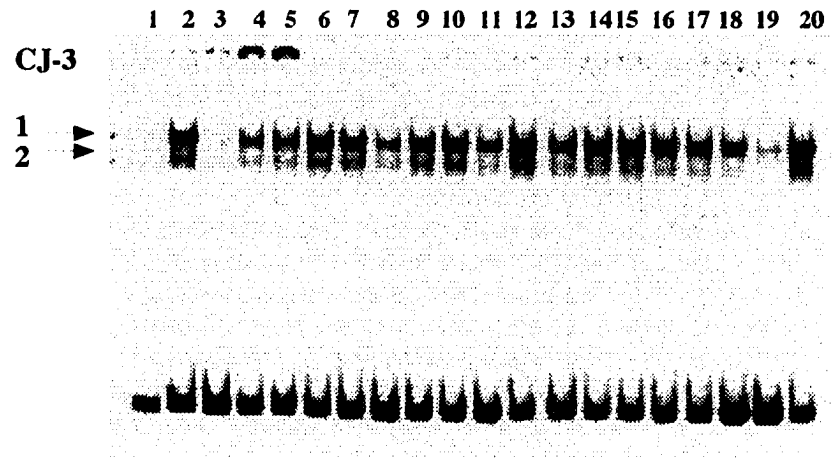

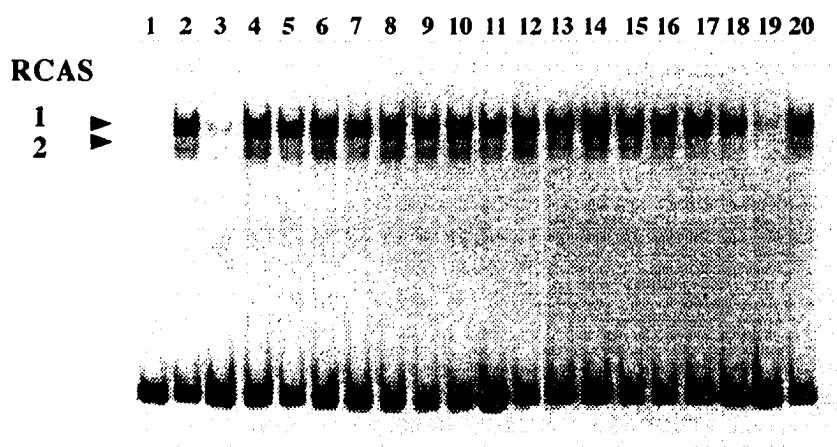

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present in all three cell types. Interestingly, no significant differences in band intensity were noted among the three conditions even when Western blot analysis show that Jun proteins are greatly overexpressed in CEF transformed with either c-Jun or v-Jun. (Figure 4). This finding suggests that only small amounts of Jun proteins are required for DNA binding to the consensus AP-1 site, or that much of the overexpressed protein may be inactive.

To determine if other AP-1 related sequences are preferentially recognized over the consensus AP-1 sequence, sixteen different but related AP-1 sites (figure 2; table 4) were used in competition shift assays (figure 3). The study shows marked differences in the ability of each nuclear extract to preferentially recognize the consensus AP-1 site. Table 5 shows the statistical analysis of relative DNA binding among the different AP-1 sequences, between both complexes, and among cell types. Interestingly, the variations occur in the lower band. While most of the sequences did not compete with the consensus AP-1 site, five of the sixteen AP-1 like sequences show dramatic differences. This finding suggests that complexes formed during v-Jun overexpression preferentially recognize a different subset of AP-1 related promotor sequences, as compared to the complexes when c-Jun is overexpressed, or to Jun complexes during normal conditions. Such differences in substrate specificity indicate that Jun overexpression increases the repertoire of available heterocomplexes, and consequently affect DNA binding or DNA trans-activation of Jun target genes.

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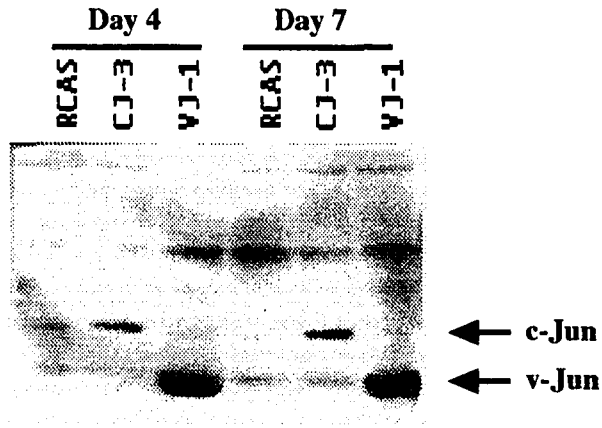

Figure 4 Western blot of overexpressed Jun proteins.
Protein from v-Jun transformed CEF (VJ-1), c-Jun overexpressing CEF (CJ-3), and normal CEF infected with vector sequences alone (RCAS) were assayed for Jun expression using a Jun specific antibody, PEP-1. Expression was monitored 4-7 days after infection.

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Table 5 Statistical analysis of DNA binding to AP-1 and AP-1 like sequences.

Relative competition by AP-1 and 'AP-1 like' sequences for binding to specific *in vivo* complexes.

	VJ-1		CJ-3		RCAS	
	complex 1	complex 2	complex 1	complex 2	complex 1	complex 2
TGACTCA	86 +/- 08	82 +/- 04	87 +/-09	89 +/-06	86 +/-12	93 +/-04
TGACTCG	20 +/-19	18 +/-15	34 +/-25	47 +/-11	6+/-08	28 +/-12
TGACTCT	23 +/-16	31 +/-18	28 +/-24	36 +/-22	24 +/-12	60 +/-17
TGACTCC	26 +/-16	10 +/-14	18 +/-07	14 +/-09	9 +/-12	3 +/-04
TGACTTA	5 +/-09	11 +/-10	09 +/-05	15 +/-19	30 +/-13	57 +/-11
TGACTAA	47 +/-14	37 +/-23	56 +/-08	70 +/-14	35 +/-38	50 +/-36
TGAGCTCA	9 +/-13	13 +/-18	21 +/-07	3 +/-04	10 +/-14	3 +/-04
TGACCCA	9 +/-13	0 +/-00	12 +/-07	0 +/-00	7 +/-09	26 +/-16
TGACGCA	61 +/-09	60 +/-17	64 +/-16	70 +/-14	48 +/-30	63 +/-29
TGACACA	41 +/-21	26 +/-19	54 +/-23	66 +/-06	43 +/-26	45 +/-07
TGATTCA	7 +/-11	0 +/-00	15 +/-19	26 +/-03	10 +/-17	32 +/-11
TGAGTTCA	12 +/-10	14 +/-17	7 +/-09	34 +/-16	9 +/-08	57 +/-09
TGACTTCA	12 +/-17	13 +/-15	7 +/-12	68 +/-13	15 +/-11	76 +/-05
TGACCTCA	17 +/-17	14 +/-13	13 +/-15	58 +/-03	18 +/-23	68 +/-05
TGACATCA	48 +/- 22	51 +/-25	50 +/-28	83 +/-07	43 +/-41	82 +/-11
TGACGTCA	80 +/-17	70 +/-28	82 +/-18	96 +/-03	83 +/-11	100 +/-07

Significant differences between complex 1 and complex 2 within each cell type are denoted by boxes. Differences calculated by ANOVA with P = 0.05.

Variations in target recognition by individual complexes between cell

	complex 1			complex 2		
	v-Jun	**c-Jun**	**RCAS**	**v-Jun**	**c-Jun**	**RCAS**
TGACTTA	5 +/-09	19 +/-05	30 +/-13	11 +/-10	15 +/-19	57 +/-11
TGACACA				26 +/-19	66 +/-06	45 +/-07
TGATTTCA				00 +/-00	26 +/-03	32 +/-11
TGAGTTCA				14 +/-17	34 +/-16	57 +/-09
TGACTTCA				13 +/-15	68 +/-13	76 +/-05
TGACCTCA				13 +/-15	58 +/-03	68 +/-05
TGACATCA				51 +/-25	83 +/-07	82 +/-11

Significant differences were determined by ANOVA with P = 0.05.

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Identification of *in vivo* complexes that recognize the AP-1 site.

Having demonstrated variability in DNA target recognition among Jun complexes to different AP-1 related sequences, antibody shift experiments were performed to identify the complexes present in both bands. Figure 5 shows that a Jun specific antibody reduces the band intensity of both complexes in all three cell conditions. This finding suggests that Jun proteins form at least two complexes with other nuclear factors resulting in different molecular weight aggregates. It is interesting to note that *in vitro* generated c-Jun and v-Jun homodimers bind DNA very weakly, and require other dimer partners to efficiently demonstrate DNA binding. Most likely, Jun proteins require other nuclear factors to efficiently trans-activate AP-1 target sequences. Efforts to identify other Jun partners were also performed. Anti-fra-2 antibodies cause a super-shift in all three nuclear extracts (figure 5). It is possible that one or both complexes contain Fra-2 proteins.

DNA transactivation studies.

Having demonstrated that overexpression of either v-Jun or c-Jun complexes alters the DNA binding pattern to different AP-1 target sequences, we wished to determine if such differences correlate with transcriptional activation or repression of these target sequences in the various cell conditions. To accomplish this, reporter CAT constructs containing the consensus AP-1 sequences were first tested in a DNA trans-activation study. While noticeable transcriptional activity occurs from the consensus AP-1 site, no significant differences in DNA trans-activation were noted in all three cell conditions (figure 6). This finding is consistent with the DNA binding data from the consensus AP-1 sequence, and indicates that no preferential transcriptional activity occurs during v-Jun or c-Jun overexpression, or during normal conditions.

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Figure 5 Antibody Shift Competition assay
Nuclear extracts from v-Jun transformed CEF (VJ-1), c-Jun overexpressing (CJ-3), and normal CEF infected with vector sequences only (RCAS) were used against the consensus AP-1 site. Lane 1: probe alone: TCACTCA; lane 2: no competition; antibodies used were: anti-Jun polyclonal (3); anti-Jun PEP1 (4); anti-CREB (5); anti-Fra-2 (6); anti-Fos B (7); anti-Jun B (8); anti-Fos (9); goat anti-rabbit (10). *In vitro* translated v-Jun/ c-Fos proteins were also used against consensus AP-1 site; no competition (A); anti-Jun polyclonal (B); anti-Jun PEP1 (C); and anti-c-Fos (D). Arrows show locations of complex 1 and 2; Dark spot on lane 6 shows supershift using anti-Fra-2 antibodies.

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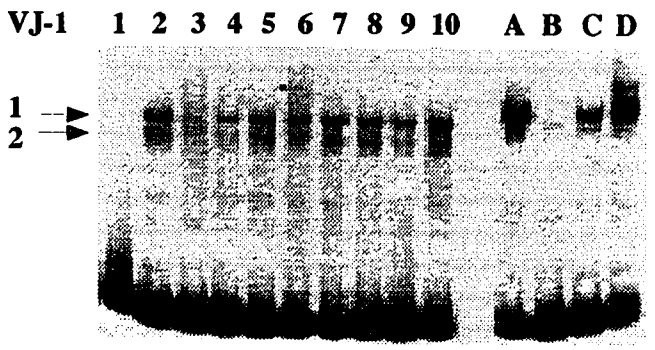

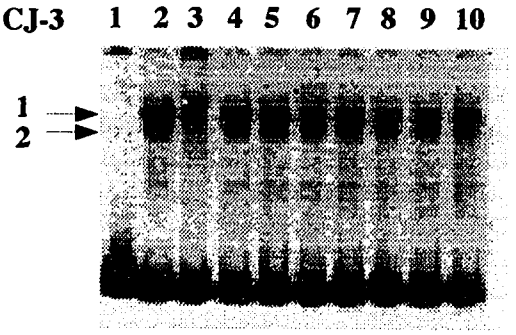

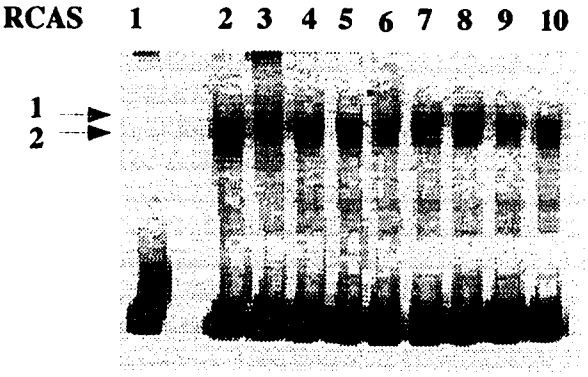

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Figure 6 Transcriptional activation of AP-1 and AP-1 like sequences.

Reporter-CAT constructs containing AP-1 and AP-1 like sequences were transfected into v-Jun transformed (VJ-1, c-Jun overexpressing (CJ-3) and Normal CEF infected with retroviral sequences only (RCAS), to investigate their transcriptional responses. This figure shows the results from the CAT assay. All AP-1 sequences were cloned pMCAT-3 construct, which contains the human metallothionine promotor; jun-CAT construct contains the jun promotor; lane descriptions are:

lane	1	TGACTCA
lane	2	TGACTTA
lane	3	TGACTGA
lane	4	TGACTCT
lane	5	TGACTCC
lane	6	TGACGCA
lane	7	TGACACA
lane	8	TGACTCG
lane	9	TGACTAA
lane	10	pMCAT-3
lane	11	jun-CAT
lane	12	TGACCTCA
lane	13	TGACTTCA
lane	14	TGAGTTCA
lane	15	TGATTTCA
lane	16	TGACTCA
lane	17	TGACCCA
lane	18	TGACATCA
lane	19	TGACGTCA
lane	20	pMCAT-3

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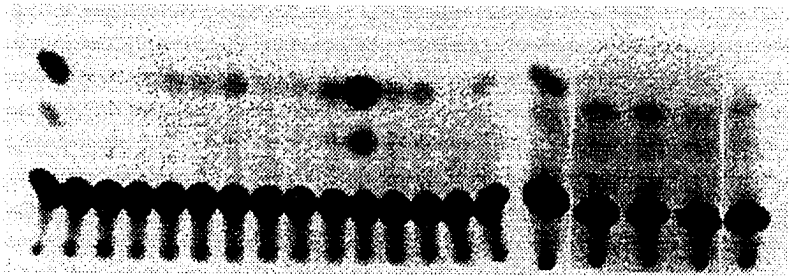
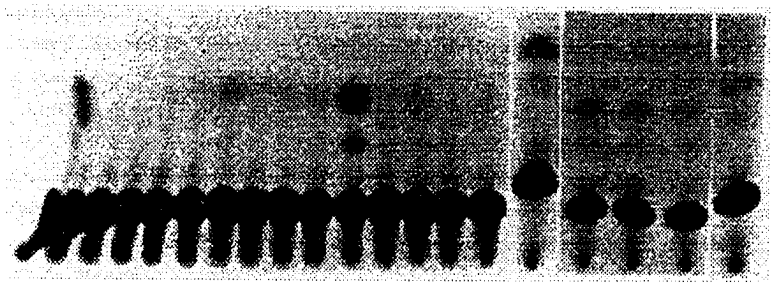
VJ-1



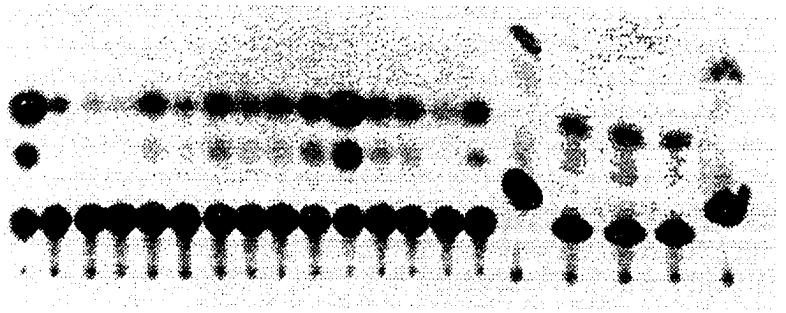
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

CJ-3



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

RCAS



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

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When the mutant AP-1 sequences were tested for transcriptional activity in the 3 cell conditions, the responses produced were below background levels (figure 6). While these observations are difficult to interpret since the flanking sequences close to the AP-1 site greatly affect its transcriptional potential (22), it is conceivable that a majority of the overexpressed Jun complexes are inactive, or that DNA binding to these mutant AP-1 sequences results in transcriptional repression.

In summary, we show that overexpression of v-Jun proteins results in a change in substrate specificity *in vitro*. It is conceivable then that a corresponding change in the pattern of target gene expression occurs *in vivo*. For this reason, we chose to identify potential target genes induced during v-Jun transformation.

AIM 2: Isolate *in vivo* target genes associated with v-Jun induced cell transformation.

Two approaches to identify genes induced during v-Jun transformation are differential display and subtractive hybridization. Both strategies utilized mRNA obtained from v-Jun and c-Jun transformed CEF, as well as from normal CEF. mRNA from c-Jun expressing cells are included to characterize transformation associated genes from the weakly transforming c-Jun gene. CEF are chosen in this study since they are effectively transformed by the v-Jun oncogene without cooperation from other cytoplasmic oncogenes. This is significant since other cooperating oncogenes could potentially activate another distinct set of target genes complicating analysis (36).

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Three potential target genes were isolated by subtractive hybridization.

Using the subtractive hybridization approach, three potential target genes were isolated. Clone 4 is a 700 bp clone showing consistent over-expression in v-Jun transformed CEF over normal CEF. Northern blot analysis from 5 separate RNA preparations show a 3 to 7 fold increase in steady state mRNA levels (figure 7). DNA sequence analysis and GENBANK database searches show strong homology to a cysteine thiol protease. (see further details in Aim 3.)

Clone 943 is a 2.2 kb clone that appears to be differentially expressed in v-Jun transformed CEF by Northern blot analysis (figure 7). Recent experiments however, show variability in the level of gene expression, perhaps due to different cell growth conditions, or different stages in the progression of cell transformation. Further studies to address this are under investigation. Partial DNA sequence information for clone 943 using SP6 and T7 primers is shown in figure 8. GENBANK database searches do not show homology to any known genes.

Clone 15-15 and 14-67 are two of several clones obtained by differential screening of a subtracted v-Jun cDNA library. Northern blot data from 5 different RNA preparations show a consistent 3 to 10 fold higher level of clone 15-15 expression in normal CEF over v-Jun transformed CEF (Figure 7). DNA sequence analysis show that both clone 15-15 and 14-67 are identical. GENBANK database searches show strong identity to a previously isolated chicken Apolipoprotein A1 gene. (see further details in Aim 4.)

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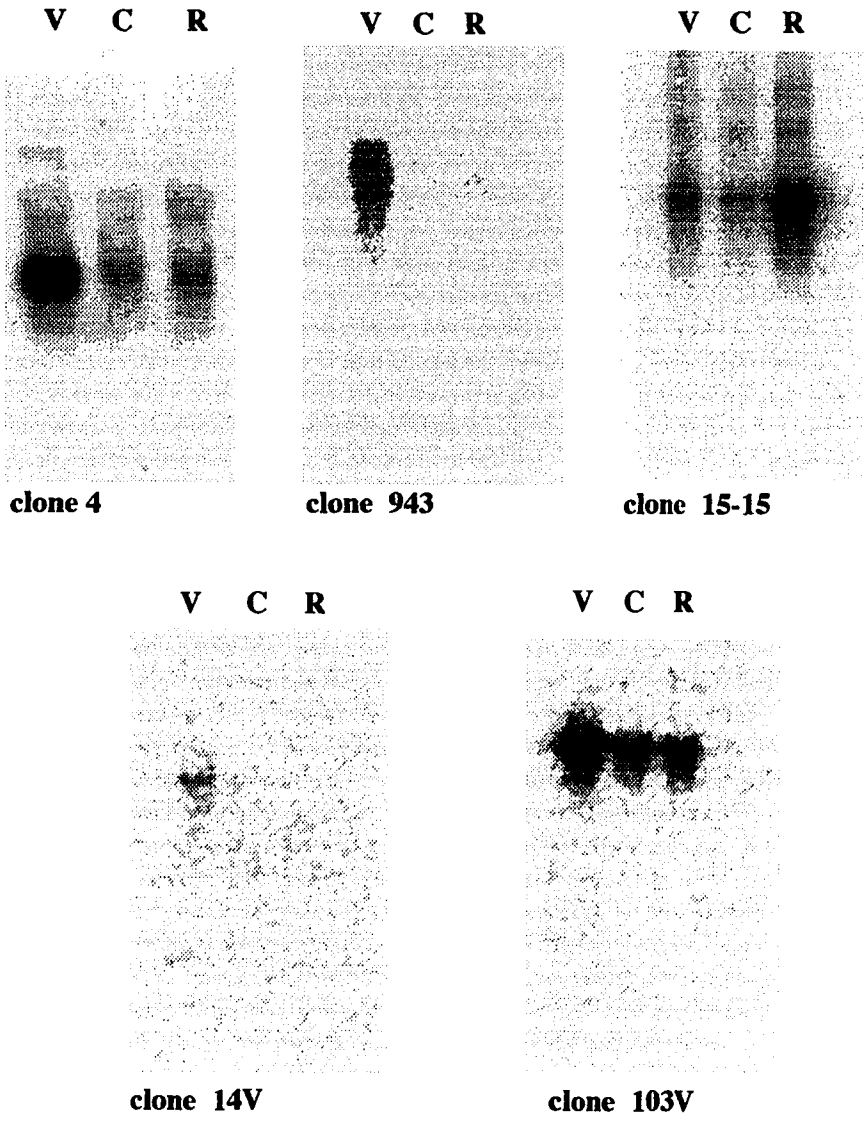

Figure 7 Northern blot analysis of v-Jun target genes
Northern blot analysis showing differentially expressed clones 943 and 14V; overexpressed clones 4 and 103 V; and a repressed clone 15-15; total RNA was isolated from v-Jun transformed CEF (V); from c-Jun overexpressing cells (C); and from normal CEF infected with vector sequences only (R).

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```
5'---TGAGTGTTTCCTGAATAAATGGACACAGAAATAGAAGGATTGAATATTG
     TACCCAATTGGTTTTGAATTTAACCTATTTGCTAGGCTTTTACAGACAG
     ATCAGGACAAACAGCACTTAAATAAATCTACAGASTGCAAAACAATAAT
     GVAAMMAGTGAGCAGAGATAGCTTACYTSGHYGATGAATGMACCATSCA
     CMCTTTTGCGACGTTTCATGTAATTGCTTTTCCTCTCCGGTCATTAAGA
     ACMGAAATGTTCCAACTGGCTTTAGGTATGAGATTTTTATTGTTATTTT
     AAATTGCCAGTTGTTGGRGATCAGATATTGAGTGCMGTCAGCACTGGAG
     TTCCTCTCTGCTTTTWTGTWGATAAACAAATATATCTWGATATCCTTGT
     AGTATGCTCTTATCTGTTGGAGTTGCACAGCAGTGGTGTGATGCTGCGT
     GCTCCTGCCCTAAACATACAGAACAAGTGCTCTGCACTCCCCAGAGGGC
     TGCAATGGGAAGGGACTTAATTGTACTGGGTGTCTTCTGGGTTACATCC
     CTCTGTTTTTCACTCCTGCTTTGTGGATCGAATTTYCTAAGTAGAAAAA
     CAGGGGAAAGGAGCAGGTGACTCTTGCTGGGAAWTGTAGCAGAGAATAC
     TTATTTCTANYTCTWGATYTAAGTWCAMTYTGATTACTYHDB?AGAGTT
     TGGATACAYBCATGCAAATTAAAGAWTTAAACTAAATCTGATAAACTGT
     CTGTGTGATTGTAGGCTCTGGSCARGGGTGAGGGGGAAATACCTTTAAC
     CTAGAAAGCTTGAYRSMTWKYTWSAGTATTCTAT----------------
     ------------//----------------------------------
     TCTGAATGAAAGGATCACAGCAACAAMCTCACAAGTGTATTTTATCCTG
     CAGCTGGTAATATTTGGGACMAAGGTCTAAGGTGCTGACTTTACCAAAA
     ATGGTAGACAATGATAGATACCAGCAAMWTRDAGGCAGCTT?GAAGAGA
     ATTTCATATGRACTGGCAGCGCTAAACGTGTRGAAAATWTATAAATCST
     TTRRGSARGAAWTTAAACTCTTTTAAAATGAGGGAAATAAAACTGTTTT
     CTCATGAAACATTACAACCACTTGGCCTTTCTGTTCCCTTTGGTGCAGA
     GCTGTGTTGCTTAGGAGGGGCTCCACTGGCTCACTCATTGAAAGGCCCA
     GTGTTCCTGAAGTACATTGCCACTGATGTCAATGAGAGCAGAAGCAAGT
     CAGAAACGATGCAAAGAGAAAGTTAAGCAAAGTTGTGAAGAGCTCAGCT
     TCTGCCAACAGAACAACAAACAATCTGGGTTGTGTTAAACTCATGTCTG
     GTGSCTTATTTCTGCSWACATTACTAGATGAAACATCWTCMARGTGGCT
     TAAGATGCAAAGTTTTCATTTCTTTATGGTCTACAGCTGATAAGAGCAT
     ACCTTTAGATAATACTGTTTTCAGCCMTGGTTGCTCCBTAWTTTCWAAT
     BCATGTTCCTCTYCYTCCCCACAAAGGACCAGCAACACTTTGGCATTTT
     TCCTGTTDTCCCACCAAATBGTGTTCCCATTTTCCAATTTGTGTGCCAA
     ATTGAAATGACAATTCTATNAAATAAAACCTCTGAAAAAATAAAAAAAA
     AAAAAAAAAAAAAAA---3'
```

Figure 8. Partial sequence for clone 943.
DNA alphabet: A=Adenosine; **C**=Cytidine; **G** =Guanosine; **T** =Thymidine; **R** =A or G; **Y** =C or T; **N** =G, A, T, or C; **B** =C, G, or Y, not A; **D** =A, G or T, not C; **H**,=A, C, or T not G; **V** =A, C, or G not T; **K**=G or C; **M** =A or C; **S** =G or C; **W** =A or T;

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Differential display

As an alternative to the conventional subtractive hybridization strategy, the differential display approach (25-27), was exploited to identify genes associated with v-Jun induced cell transformation. The flow diagram in figure 9, highlights the important aspects of this technique. In this approach, mRNA or total RNA is extended by a reverse transcriptase reaction (RT) utilizing 1 of 4 types of 12 to 14-mer 3'-primers (table 1). These 3' primers have sequences complementary to the polyA tail of mRNA, and terminate with a degenerate penultimate base and specific base. These features are designed to accommodate all 4 possible 3' end combinations of the poly-A tail, to anchor specifically at the poly-A tail sequence, and to extend towards the 5' end of the mRNA sequence. The resulting single stranded cDNA fragments are then labeled by PCR (polymerase chain reaction) amplification utilizing the same 3' RT primer, and a 5' randomly generated 9 to 12-mer oligonucleotides (table 1). Both primers have identical annealing temperature. Labeling of PCR fragments is accomplished by either ^{32}P-dNTP incorporation, or by 3'-kinased primer incorporation. This process can be repeated using mRNA from several populations or cell conditions. The resulting RT-PCR products from several cell population are separated side by side on a denaturing poly-acrylamide gel able to resolve up to one base pair difference among sequences, and exposed for autoradiography. Since each band represents a potential individual gene sequence amplified by a distinct PCR primer set, unique sequences present in one cell treatment can be distinguished from sequences common among cell treatments based on their length and location on the gel. Due to the randomness of the 5' primer, the procedure can be repeated several times with different 5' and 3' primer combinations. Following autoradiography, the differentially expressed bands are excised, eluted, PCR reamplified, and cloned into a PCR vector. This

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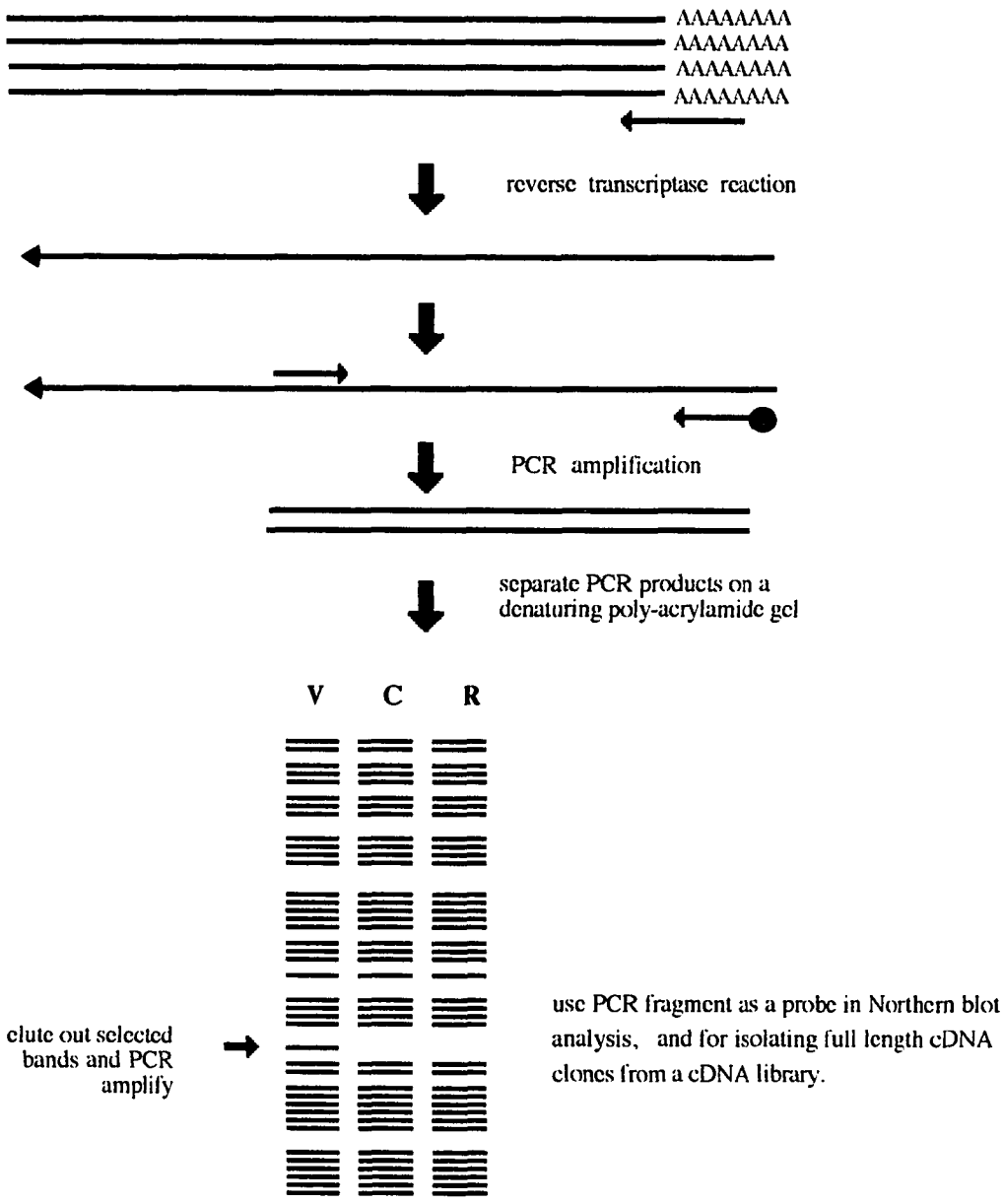

Figure 9 Differential display strategy.
This figure shows the flow diagram for the differential display strategy. In this modified procedure, a kinase end labeled primer (shown as a dark spot) is utilized for labeling fragments in the PCR step. This modification greatly enhances the banding pattern and decreases the chance of isolating false positives. (reference 46).

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cloning step guarantees that characterized sequences are generated from a single clone. The sequences will be used as a probe in a Northern blot assay to confirm differential expression. Positive sequences can then be used to select a full length clone from a cDNA library. This strategy has proven successful in identifying differentially expressed clones (24).

Advantages

The differential display method has several advantages over conventional subtractive hybridization. For instance, sequences from several cell populations or treatments can be compared and identified simultaneously, whereas only two comparisons are possible with the latter. Furthermore, both activated and repressed gene sequences can be identified by the differential display method concurrently, while the later will require several subtractive strategies. A major determinant in the success of the differential display approach depends on the fortunate selection of the 5' primer. Using comparisons among normal, metastatic and tumorigenic cell biopsies, others have identified several genes including a potential tumor suppressor, the alpha integrin gene (24).

Limitations

A potential problem arising from the differential display strategy is random misincorporations and mispriming in the PCR step resulting in an anomalous banding pattern. Optimal PCR conditions require specific 18 to 25 bp primer sequences with high annealing temperatures of 60 to 65 °C, a 20 to 200 uM dNTP concentration, and a minimum number of amplification cycles: 25 - 30 cycles. However, the PCR parameters described in differential display involve: [1] a low annealing temperature of short 10-12 mer PCR primers at 40 °C in asymmetric proportions, [2] a low dNTP concentration of 2 to 4 uM, and [3] a

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high cycle number of 40 cycles. While short primers are necessary to reduce the number of 5' primers required to accommodate all possible primer pair combinations, these parameters contribute to non-specific template amplification and further complicate the high error rate and infidelity of Taq DNA polymerase. The low dNTP concentration containing ^{32}P dATP unfortunately generates incomplete extension products. In our experience, utilizing only one random 5' primer without a 3' primer can generate extension products and PCR amplification fragments that contribute to a high background or false differential banding pattern. Furthermore, DNA sequence analysis of one differentially selected band consisted of several comigrated sequences having identical molecular weight or number of base pairs. In a recent report on the differential display technique, Pardee acknowledges that only 20% of the "differential bands" characterized are authentic differentially expressed sequences (27).

Modifications

To overcome the limitations of the differential display strategy, several modifications were implemented. First, to optimize the reverse transcriptase reaction, mRNA was selected as a starting template, since potential hairpin loop extension could occur from tRNA or rRNA present in total RNA. Actinomycin D was included to prevent hairpin loop extension from mRNA secondary structure. After the RT reaction, the resulting products were treated with RNAse to remove the RNA template, and size selected by column chromatography to obtain longer cDNA extension products, and remove unincorporated nucleotides and primers. Second, the labeling procedure in the PCR step was improved by utilizing a 3'-end labeled primer and a non-labeled 5'-arbitrary primer. Since the 3'-primer is in the same sense-orientation as the

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cDNA template, labeled extension can only occur from a 5' extension sequence, after the second amplification cycle. In this way, a majority of PCR amplified sequences labeled occur from 3'-primer incorporation. This strategy has several advantages over the recommended ^{32}P-dATP incorporation. Labeling by 3'-primer extension eliminates detection of PCR products generated from two 5'-primers in opposing orientations, and detection of false 5'-primer extensions. Third, PCR conditions were optimized. For example, a DNA Taq polymerase without 5'-phosphatase activity was selected. Nucleotide and primer concentrations were adjusted to prevent random misincorporations obtained from low dNTP concentrations.

Figure 10 shows the improvements from labeling by 3'-primer extension. Notice that the banding pattern in lane 1 using both 5' and 3' primers with ^{32}P-dNTP incorporation and has an identical pattern to lane 2 using only a 5' primer. These observations suggests that ^{32}P-dNTP labeling can lead to false priming events in the PCR reaction, and possible isolation of several false positives. In contrast, the modifications described above results in a "cleaner" banding pattern (figure 10; lane 9). These modifications have been reported (46).

Two potential target genes were isolated by the differential display strategy.

Using the modified differential display strategy, two potential target genes were isolated from three cell conditions: mRNA from v-Jun transforming, c-Jun overexpressing, and normal CEF. Clone 14V and 103V are approximately 250 base pair fragments that shows weak differential expression in v-Jun transformed cells (figure 7). Partial DNA sequence analysis is shown in figure 11. GENBANK database searches do not show homology to any known genes. Clone 4 and 15-15 were chosen for further investigation in AIM 3 and AIM 4.

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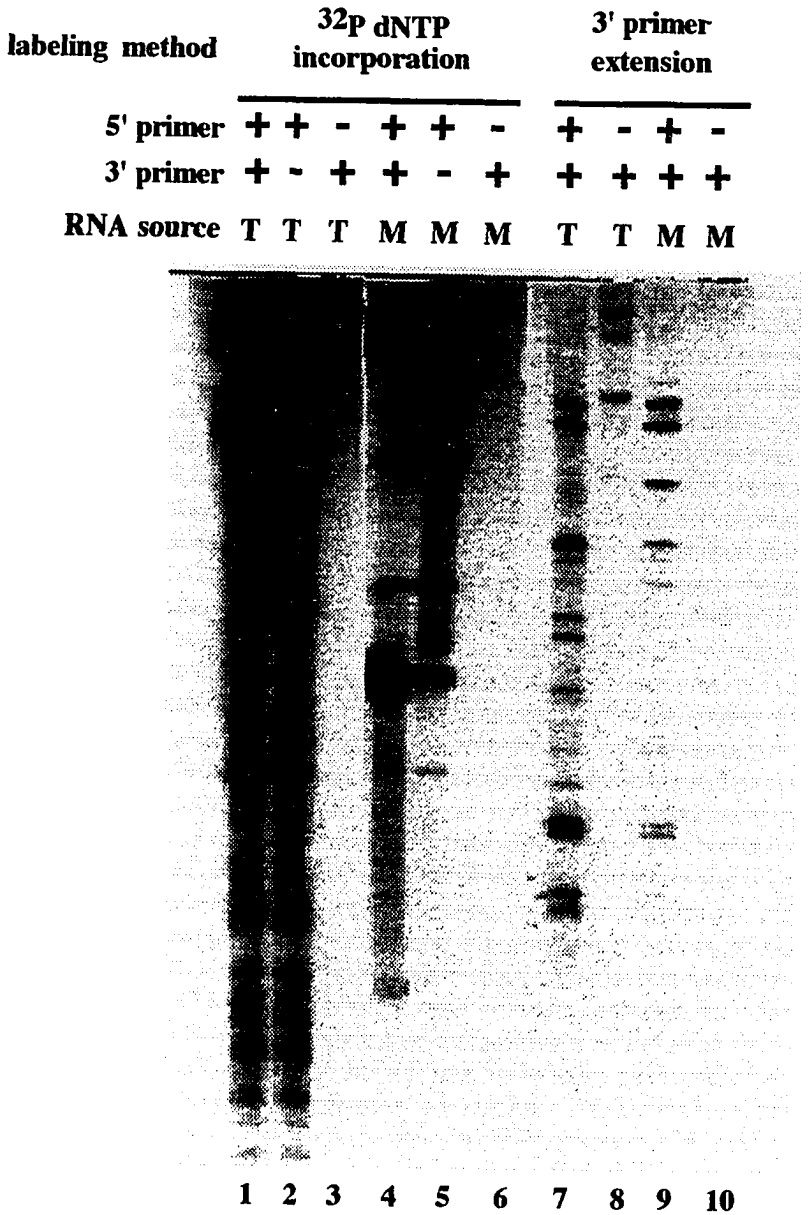

Figure 10. Differential display improvements showing the advantages of using a 3' end labeled primer. T=total RNA; M=mRNA;

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clone 14V

```
5'---TTTAACACTATGATCTAAGGTATAGATAAATTTGTCCGATG
     TGGTTGGTTTTGGGGCACTGAGTCCCGCCCCAGATTGTTTA
     AGGTGAGAGGAGAGGCATGT---------//----------
     TAATATCTCTAAGACATACTGATGCTCTGCTCTACTAAAAT
     CTGGCGGACTAGTGCCAAAACCAACCACATAGGACAAAATT
     TATCTATAACCTTGAGATACATGAGTGTGGAAAAAAAAAAA
     AAAAGATTGG---3'
```

clone 103V

```
5'---TGAGGGCAAAAGAATCTTCCAGAGCATCAGTTCTCAAATG
     AAAGGGAACTTCACACTCCAGAGGTAGCAGAATGTTTTGA
     TGAATATCTATGTAGATTCAAAAGAGAAGTCAGAACTCTG
     ACATTAGAGAAGTAGAA-----//------GAGTGAAAGA
     AGAAGCTAGAACTCTGAACAATATAAAGAAGAAAGTAAGA
     ATTCTTCTTCCGGCCAGAAAGAAANAGAACTGGCTCAAAG
     AAAAANAAGAAGAGGAATAAAAATAGT---3'
```

Figure 11 Partial DNA sequence for clone 14V-5 and 103V-5. This figure shows partial DNA sequence information for two sequences isolated by differential display. The 5' and 3' orientations are based on locations poly A rich regions. More DNA sequence analysis is essential to confirm these sequences.

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AIM 3: Characterize clone 4, a gene up-regulated in v-Jun transformed CEF.

Clone 4 belongs to a family of related cathepsin proteases.

Clone 4 was identified by subtractive hybridization. Northern blot analysis demonstrates its differential expression in v-Jun transformed CEF (figure 7). Further screening of the v-Jun subtracted cDNA library with a clone 4 probe, identified two longer cDNAs: 4-572 and 4-768. Figure 12 shows overlapping DNA sequence information aligning the new clones with Exo III digested fragments of clone 4.

The consensus DNA sequence for clone 4 is shown in figure 12. GENBANK database searches reveal strong nucleotide sequence homology between clone 4 and *Homarus Americanus* (American Lobster) cysteine-thiol protease. SWISS-PROT database searches using all six reading frames show significant homology to 34 known protease sequences (table 6). Amino acid alignment between clone 4 and cathepsin L proteins from different species show significant homology to the active site of these thiol proteases (figure 13). Altogether, these observations strongly suggest that clone 4 belongs to a family of related cysteine-thiol proteases. This finding is significant since a known c-Jun target gene, stromelysin, is a known protease. Interestingly, clone 4 is novel, and shows no DNA or amino acid sequence identity to any known chicken cathepsin thiol protease.

A closer analysis of the clone 4 cDNA sequence reveals that the 5' end of the gene is missing, since a good open reading frame is not available. Moreover, protein sequence alignment of clone 4 with Cathepsin-like proteins suggests that a portion of the cysteine thiol active site is missing from the NH3

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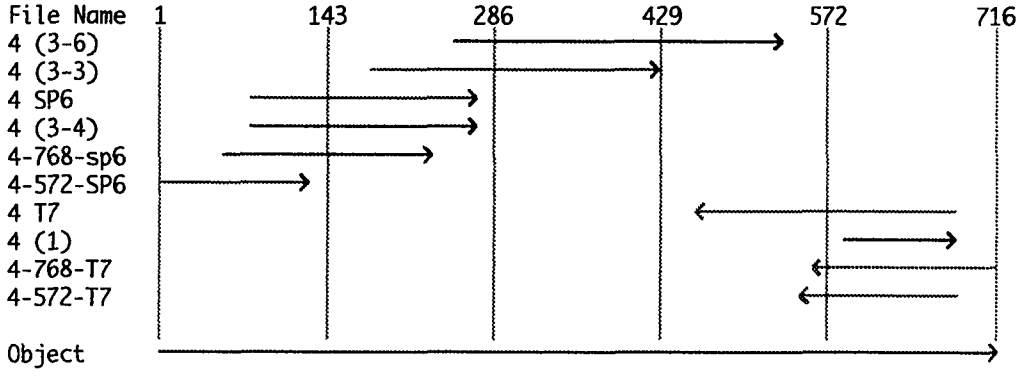


```
GCGGCTCCTG-TTGGGCGTTC-AGCTCAGTGG-GGGCTCTGGA--40
GGGGCAGCTG-AAGGGCCGGA-CGGGGAAACT-GCTGTCCCTC--80
AGCCCCCAGA-ATTTGGTGGA-CTGCGTCTCC-AACAACAACG--120
GCTGCGGGGG-GGGTTATATG-ACCAACGCCT-TCGAATACGT--160
CCGCCTTGAA-CCGCGGCATC-GACTCGGAGG-AYSYGTACCC--200
CTACATCGGG-CAGGATGAGA-GCTRTATGTA-CAGCCCCACC--240
GGAAGKCGGC-CARATCGGCK-ACGGMKATCC-GRGAGATCCC--280
CGAAGCAACG-AGAAGGCTCT-GAAGCGCGCG-GTGGCCCGGA--320
TTCGCCGGGT-CTCGGTGGGC-ATCGATGCAG-TCTGCCCTCC--360
TTCCAGTTCT-ACAGCCGCGG-GGTGTACTAC-GACACGAGCT--400
GCAACCCGGA-GAACATCAAC-CATGCGGTGT-GGCGGTGGGG--440
TACGGCGCAC-AGAAGGGCAC-CAAGWCACTG-SATCATCAAG--480
AACAGCTGGG-CACGAGTGGC-AATAAGGCTA-CGTGCTGCTG--520
ACCGCATATG-ACAAGCTGCG-CATGCACCTG-CCAGCTTCCC--560
CAAGATGTGW-GCTCTGGAGG-TGCCAACGTC-CGTCTGCAGG--600
AGTGGGGTTG-GGGGGCTGSA-ACCCCCCCCC-CCCCCCRRRT--640
ATCACATCTC-TGAGTCCWWW-GGGGGGATGC-GGAGAACGAT--680
GGGATTTTGT-TCTTCAAATA-AAAGCAGTGG-GGGAGA     --716
```

Figure 12. Partial DNA sequence for clone 4.
Shown above is the sequence alignment of clone 4 with two longer cDNAs clones: 4-572 and 4-768, obtained by further screening the v-Jun subtracted library The consensus sequence is shown below.
DNA alphabet: A=Adenosine; C=Cytidine; G=Guanosine; T=Thymidine; R=A or G; Y=C or T; N=G, A, T, or C; B=C, G, or Y, not A; D=A, G or T, not C; H,=A, C, or T not G; V =A, C, or G not T; K=G or C; M =A or C; S =G or C; W =A or T.

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Table 6 SWISS-PROT database search for clone 4

Listing of entries in the SWISS-PROT database showing strongest homology to clone 4 amino acid sequence. Note that all entries belong to a family of proteases from various species.

entry	name
ALEU HORVU	THIOL PROTEASE ALEURAIN PRECURSOR
CATB BOVIN	CATHEPSIN B
CATB HUMAN	CATHEPSIN B PRECURSOR
CATB MOUSE	CATHEPSIN B PRECURSOR
CATH HUMAN	CATHEPSIN H PRECURSOR
CATH RAT	CATHEPSIN H PRECURSOR
CATL CHICK	CATHEPSIN L
CATL HUMAN	CATHEPSIN L PRECURSOR
CATL MOUSE	CATHEPSIN L PRECURSOR
CATL RAT	CATHEPSIN L PRECURSOR
CATS BOVIN	CATHEPSIN S
CATS HUMAN	CATHEPSIN S PRECURSOR
CATS RAT	CATHEPSIN S PRECURSOR
CYS1 DICCI	CYSTEINE PROTEINASE PRECURSOR
CAS1 HOMAM	DYGESTIVE CYSTEINE PROTEINASE 1
CYS1 HORVU	CYSTEINE PROTEINASE EP-B 1 PRECURSOR
CYS2 DICDI	CYSTEINE PROTEINASE 2 PRECURSOR
CYS2 HOMAM	DIGESTIVE CYSTEINE PROTEINASE 2 PRECURSOR
CYS3 HOMAM	DIGESTIVE CYSTEINE PROTEINASE 3 PRECURSOR
CYS4 BRANA	CYSTEINE PROTEINASE COT 44 PRECURSOR
CYSL LYCES	LOW TEMP INDUCED CYSTEINE PRECURSOR
CYSP PEA	CYSTEINE PROTEINASE 15A PRECURSOR
CYSP PLAFA	THROPHOZOITE CYSTEINE PROTEINAS PRECURSOR
CYSP THEPA	CYSTEINE PROTEINASE PRECURSOR
EUMI EURMA	MITE GROUP 1 ALLERGENIC PROTEIN
LCPA LEIME	CYSTEINE PROTEINASE PRECURSOR
MMAL DERPT	MAJOR MITE FECAL ALLERGENIC PRECURSOR
ORYA ORYSA	ORYZAIN ALPHA CHAIN PRECURSOR
ORYC ORYSA	ORYZAIN GAMMA CHAIN PRECURSOR
P34 SOYBN	P34 PROBABLE THIOL PROTEASE PRECURSOR
PAP2 CARPA	CHYMOPAPAIN PAPAYA
PAP3 CARPA	CARICAIN PAPAYA PRECURSOR
PAP4 CARPA	PAPAYA PROTEINASE IV
PAPA CARPA	PAPAIN PRECURSOR

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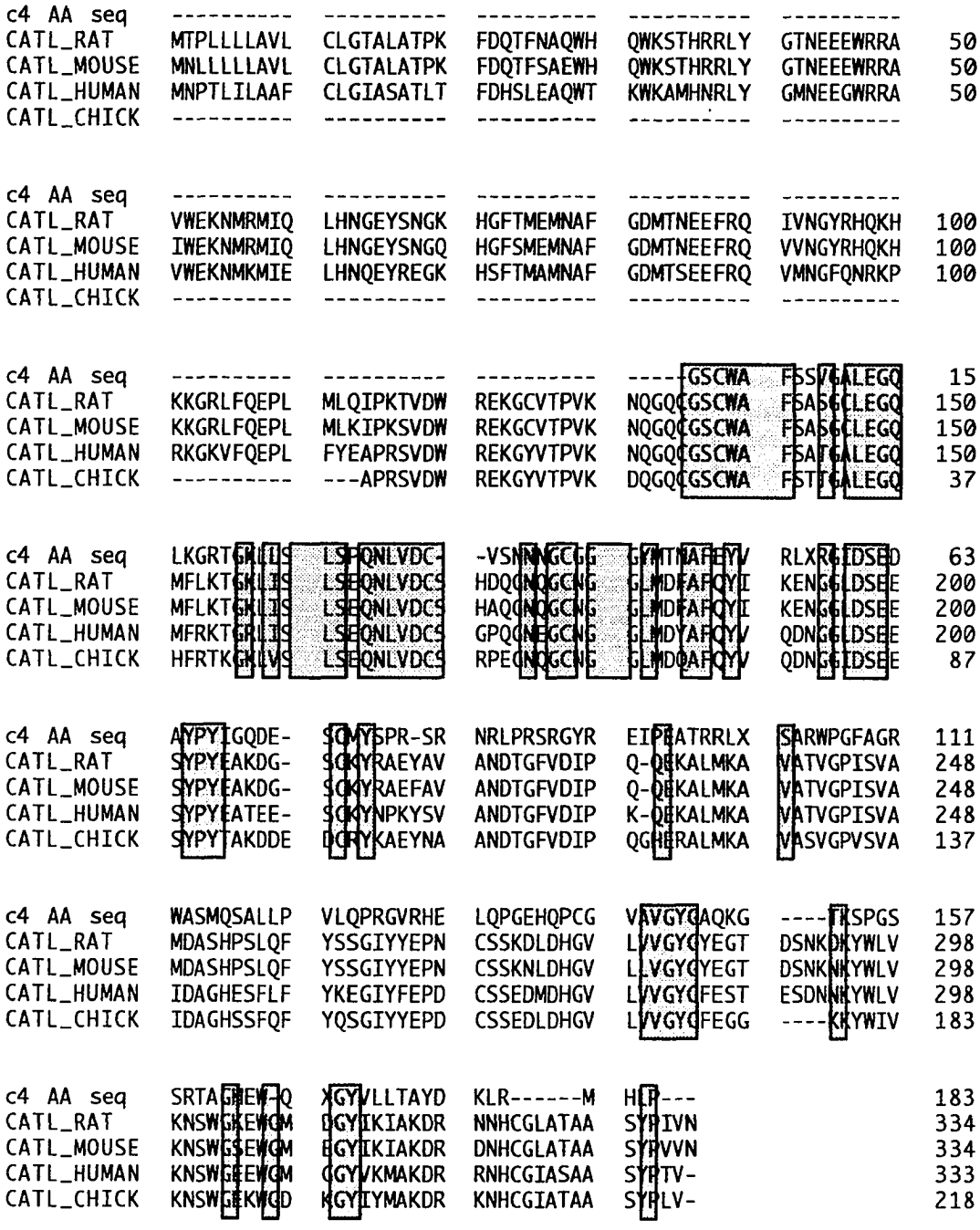

Figure 13. Alignment of clone 4 to several Cathepsin L proteases.
Shaded areas are regions of strongest homology between clone 4 and family of Cathepsin L proteases. Dark line show the cysteine thiol protease active site having the consensus sequence: QXXX[G/E]XCWXX[STAG].

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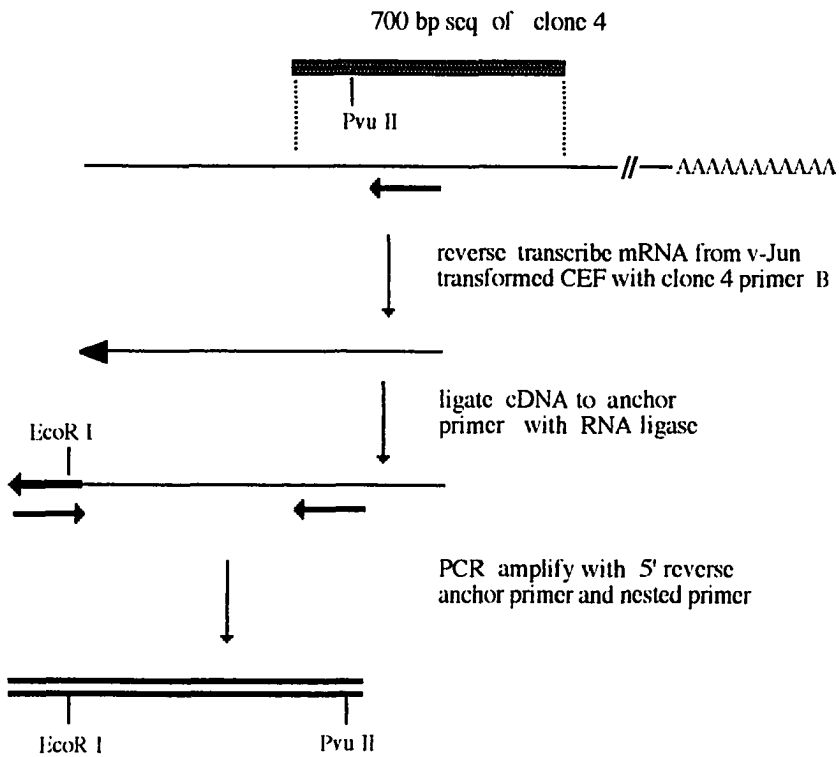

Figure 14. 5'-RACE strategy used to determine the 5' end of clone 4. RACE Rapid Amplification of Cloning Ends: mRNA isolated from v-Jun transformed CEF is reverse transcribed with primer B which is complementary to the 5' terminal region of clone 4. The resulting fragment is then ligated to an anchor sequence with RNA ligase, and PCR amplified with a nested primer complementary to sequences upstream from primer B, and a reverse anchor primer. The resulting PCR fragment can be verified by DNA sequencing and restriction analysis. The convenient restriction sites in the anchor sequence and PCR fragment can be used to generated a full length sequence.

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terminal region (figure 13). To obtain the 5' end of the gene, a 5' RACE (Rapid Amplification of Cloning Ends) strategy was attempted (figure 14). A full length clone can easily be constructed utilizing convenient restriction sites.

AIM 4: Characterize clone 15-15.

Clone 15-15 was isolated by differential screening a subtracted v-Jun cDNA library and shown to be preferentially expressed in normal CEF. DNA sequencing and GENBANK database searches show strong sequence identity to the chicken *apolipoprotein A-1* gene. Clone 15-15 was therefore chosen for study since its identity is known, and the regulatory mechanisms in mammalian systems were well characterized.

Previous studies

Apolipoprotein A1 (Apo-A1) is the major protein constituent of high density lipoproteins (HDL). Early studies suggest that these proteins are responsible for overall cholesterol metabolism, transport and distribution, and play an important role in reducing coronary heart disease (47 and references cited).

The *apo-A1* gene is highly conserved in several species; the rat, human and chicken genes show strong sequence homology in the 5' regulatory region and coding areas. While mammalian *apo-A1* expression is restricted to the liver and intestine, avian species express it in all tissues, although predominantly in liver and intestine. Appreciable amounts are detected in kidney, ovary/testes, brain, lung, skeletal and heart muscle. Furthermore, males express higher

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levels of Apo-A1 protein in comparison to females. These findings suggest that different regulatory mechanisms exist among species and within different tissue environments (47).

The regulation of the *apo-A1* gene at the transcriptional level has received much attention. The mammalian *apo-A1* regulatory region contains four responsive elements (site A, B, C and D) (figure 15). Transcription factors that recognize site A include a placental transcription factor (ARP-1) and the retinoic acid receptor (RXRa), which decrease and increase *apo-A1*, respectively (47, 48). In rat hepatocytes, a member of the thyroid/ steroid receptor super-family (HNF-4), positively regulates *apo-A1* through site C (50). Site B is recognized by several factors. Studies conclude that transcriptional regulation of the mammalian *Apo-A1* gene is determined by an interaction among several transcription factors both at the protein level and at DNA binding sites.

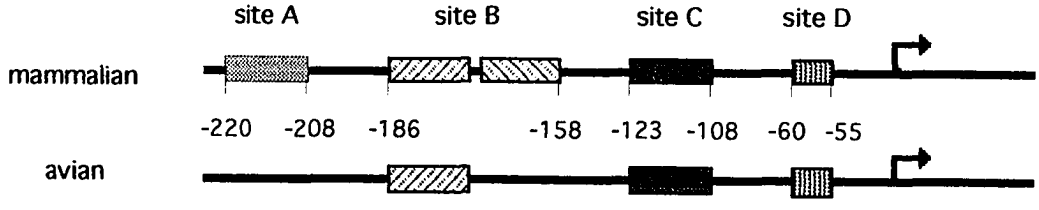

Figure 15. Structure of the ApoA1 regulatory region.
This figure shows a comparison between the mammalian and avian upstream regulatory elements, highlighting the regions of significant homology.

In contrast to the mammalian regulatory region, the chicken sequences show no homology to site A, have homology to only half of site B, and strong homology to sites C and D. Functional analysis of the chicken *apo-A1* regulatory region identifies site D (-60 to -54) as the positive enhancer element in several

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transformed cell lines, including human hepatoma (HEP-G2), human colon carcinoma (Caco2), human cervical epithelial (HeLa), mouse embryonal fibroblasts (NIH/3T3), and RSV transformed quail myoblasts (QMLA29) (47). Although site D resembles a GC rich SP-1 binding site, SP-1 or other factors have not been demonstrated to recognize this site. Surprisingly, these studies also show that constructs containing upstream sequences beyond -300 nucleotides are weakly *trans*-activated in these cell lines. In addition, avian sequences corresponding to mammalian enhancer elements A, B, and C are transcriptionally inactive (47, 48)). This finding contrasts with another report showing *apo-A1* to be repressed in quail myoblasts transformed with a temperature sensitive Rous Sarcoma Virus (RSV) at the permissive temperature, but expressed during non-permissive temperatures (49). Clearly, different regulatory mechanisms exist between mammalian and avian species (47).

ApoA1 regulation.

Northern blot analysis in our laboratory suggest that the steady state levels of *apo-A1* mRNA are approximately 3 to 10 fold higher in normal CEF, as compared to v-Jun transformed CEF (figure 7). There are several possibilities. One explanation is that transcriptional repression occurs in v-Jun transformed CEF; that is, the transcription rates are slower in v-Jun transformed CEF, as compared with normal CEF. Another possibility could be that the turnover rates in both cell conditions are different. Accumulation of a stable *apo-A1* mRNA could occur in normal CEF. Alternatively, overexpressed v-Jun proteins could contribute to unstable *apo-A1* mRNA in v-Jun transformed cells. With these questions in mind, we decided to determine how v-Jun directly or indirectly influences chicken *apo-A1* gene expression in transformed CEF, as compared to normal CEF.

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ApoA1 mRNA is very stable.

Actinomycin D treatments were used to assess the half-life, and message stability of *apo-A1* mRNA during normal and transformed conditions. This drug blocks transcription by inhibiting RNA polymerase II activity, allowing an evaluation of mRNA stability during transcriptional repression. A rapid decay rate strongly indicates message instability, while a slow decay rate suggests that high message stability contributes to accumulation.

v-Jun transformed (VJ-1) and normal CEF infected with vector sequences only (RCAS) were treated with 5 ug/mL of Act D at different time points. Total RNA was isolated, and the steady state levels of *apo-A1* mRNA were estimated by Northern blotting. Although the levels of *apo-A1* message are clearly higher in normal CEF (figure 16), the decay rates in both v-Jun transformed and normal CEF are identical (Figure 17). This gradual decrease for up to 20 hours indicates that *apo-A1* mRNA is very stable in both cell conditions (t1/2 = 15 to 20 hours), and reveals that the differences in steady state levels observed in the Northern blots can not be explained by mRNA stability. More importantly, the data suggests the v-Jun overexpression does not contribute to *apo-A1* message instability. It is therefore conceivable that the variations in *apo-A1* message seen in Northern blot analysis are due to accumulation of a stable message from an upstream regulatory sequence, or that transcriptional mechanisms are responsible. With these considerations, we set out to identify potential regulatory sequences by promotor deletion analysis, and verify them by DNA binding studies.

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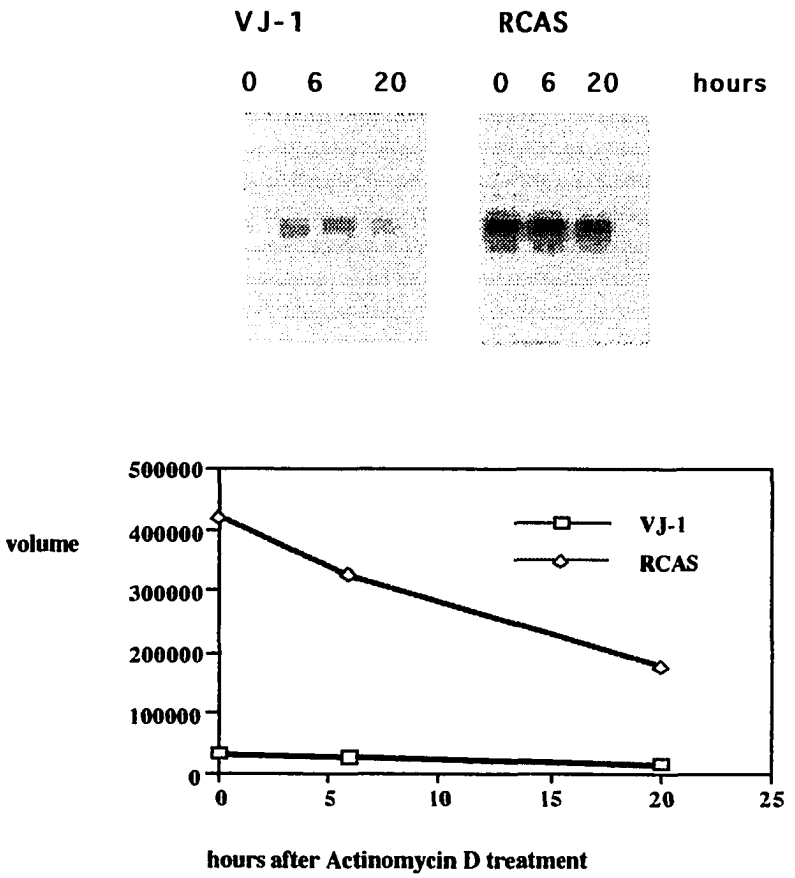

Figure 16. Actinomycin D studies.
Top figure shows Northern blot analysis of total RNA from v-Jun transformed CEF (VJ-1) and normal CEF infected with retroviral sequences only (RCAS) treated with 5 ug/mL Actinomycin D at indicated time points. Bottom figure shows quantitation of mRNA levels at indicated time points, demonstrating higher steady state levels of *apo-AI* message in RCAS than in VJ-1.

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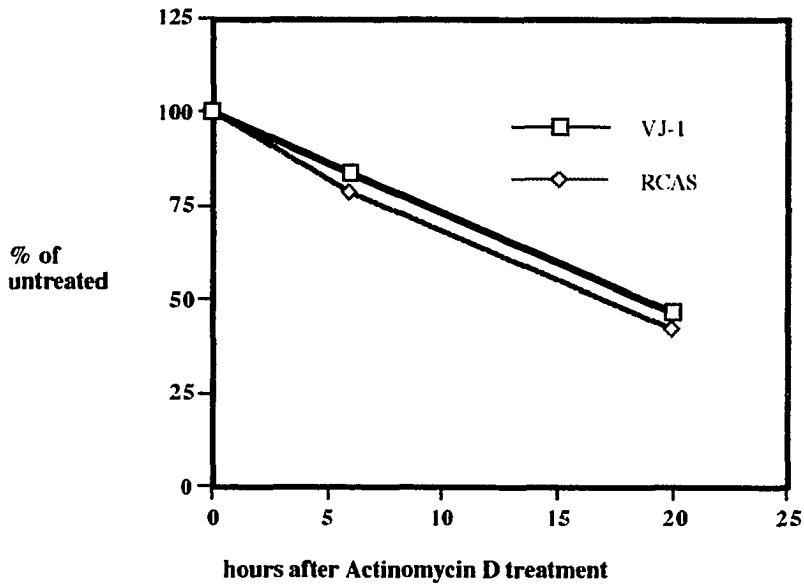

Figure 17 Estimation of *apo-A1* mRNA half life.
Quantitation of *apo-A1* mRNA steady state levels in v-Jun transformed CEF (VJ-1), and Normal CEF treated with retroviral sequences only (RCAS) after Actinomycin D treatment. Study shows identical decay rates in both VJ-1 and RCAS CEF. Estimated mRNA half life is approximately 20 hours.

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Isolation of an *apo-A1* genomic clone.

To determine if transcriptional control mechanisms influence *apo-A1* gene expression during normal and transformed conditions, we first isolated the upstream regulatory region of *apo-A1* from a genomic clone. The 13 kb genomic clone was isolated by screening a lambda chicken genomic library with a 700 bp clone 15-15 sequence as a probe. Restriction analysis, Southern blotting and partial DNA sequencing verify the authenticity of the genomic clone, as reported previously. Figure 18 below shows the genomic map of the *apo-A1* gene and sequences corresponding to clone 15-15.

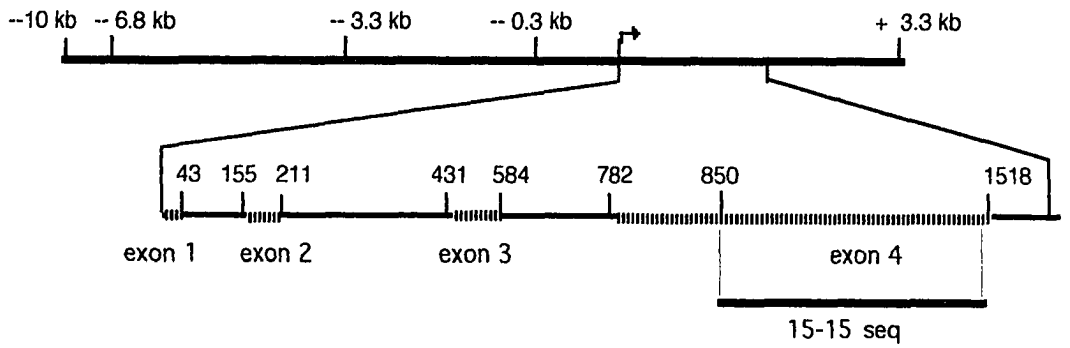

Figure 18. Structure of the ApoA1 genomic clone. This figure shows the *apo-A1* regulatory region in relation to its coding region and sequences corresponding to clone 15-15.

Convinced that an authentic genomic clone was isolated, we set out to search for potential activator or repressor elements by promotor deletion analysis.

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Promotor deletion analysis.

To identify potential *cis*-acting sequences on the *apo-A1* regulatory region, several reporter CAT constructs containing 5' deletions were transiently transfected into v-Jun transformed CEF (VJ-1), and normal CEF infected with retroviral sequences only (RCAS). A summary of reporter CAT constructs and corresponding responses are shown in figure 19; the CAT assay is shown in figure 20.

Studies show three potential *cis*-acting regulatory elements on the *apo-A1* gene. A strong activator region is present between -6800 to -3300 nucleotides. Deletion of this region to generate the p-3300/0 construct, reduces the level of expression from 11 fold to background levels in normal cells. Another responsive site may be located within 20 bases of exon 1. Exclusion of this site reduces the level of expression by approximately 1 to 2.5 fold [compare p-193/20 to p-193/0; p-3300/20 to p-3300/0 and p-300/20 to p-300/0]. However, the remaining half of exon 1 (bases 20 to 43) could hold repressive regions. Addition of these sequences appears to reduce expression by 1.4 to 4 fold; [compare p-300/43 to p-300/20; and p-3300/43 to p-3300/20]. Finally, a potential repressive region could be located within sequences -3300 to 300. Deletion of this sequence appears to increase expression by 1.5 fold [compare p-3300/0 to p-300/0]. Interestingly, none of these promotor CAT constructs are responsive in VJ-1 CEF. This findings agree with Northern blot data showing high steady state levels of the *apo-A1* message in normal CEF, and strongly suggests that transcriptional mechanisms regulate the *apo-A1* promotor during normal and transformed conditions.

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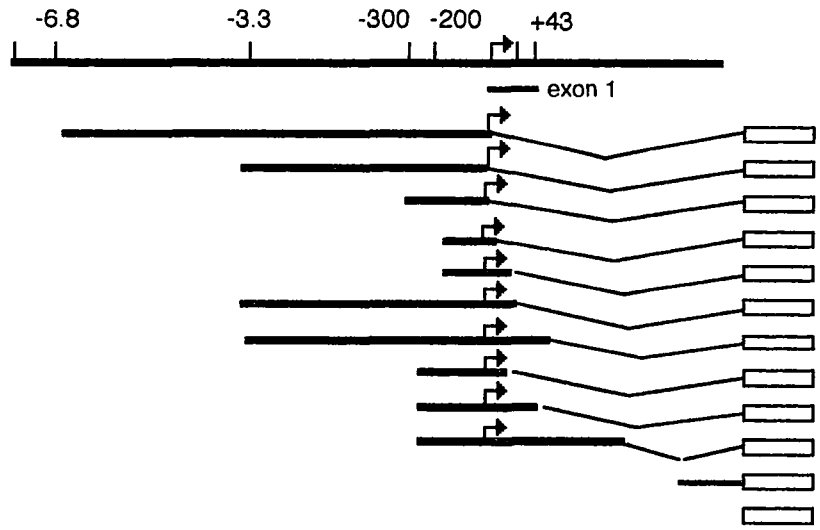

	fold over bkgnd	
construct	VJ-1	RCAS
p-6800/0	1.0	11.6
p-3300/0	1.0	0.9
p-300/0	1.1	1.5
p-193/0	1.1	1.4
p-193/20	1.1	1.7
p-3300/20	1.2	1.4
p-3300/43	0.8	1.0
p-300/20	1.0	3.9
p-300/43	1.0	0.9
p-300/263		
pSV2CAT/blue	20.6	24.4
pCAT/blue	1.0	1.0

Figure 19 Summary of ApoA1 promotor deletion analysis.
Several ApoA1 reporter CAT constructs containing 5' deletions in the promotor were transfected into VJ-1 and RCAS CEF. Summary of DNA trans-activation studies are shown. Fold over background is calculated as the per cent acetylation of each construct relative to the negative control: pCAT/Blue. pCAT/Blue is a promotor-less reporter CAT construct; positive control: pSV2CAT/ Blue with SV40 enhancer and promotor sequences.

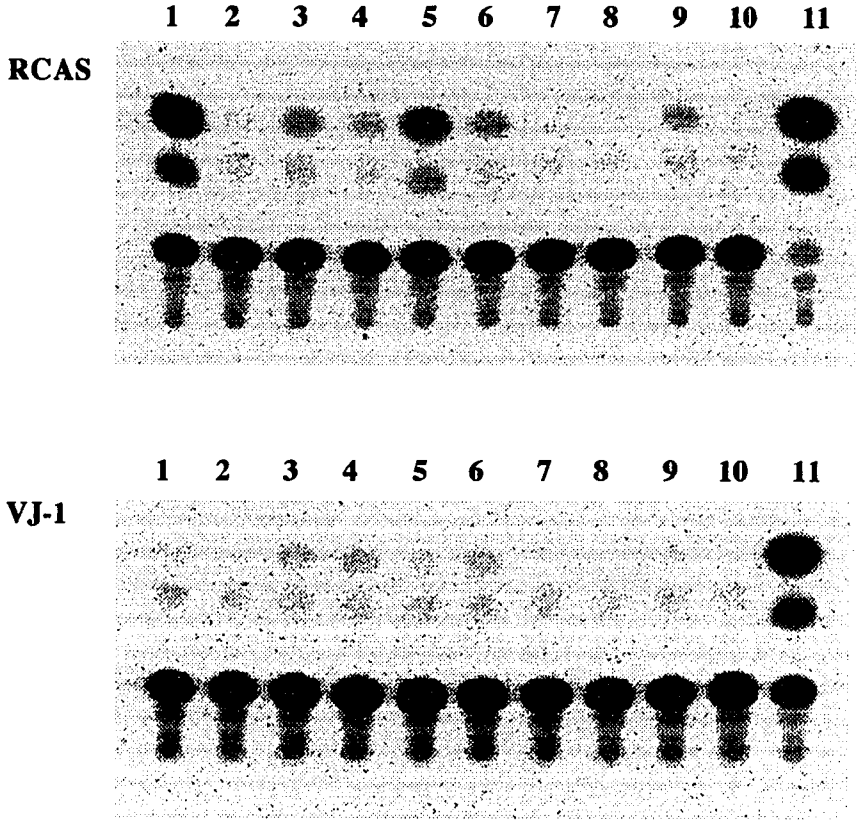

Figure 20. CAT assay of *apo-A1* promotor CAT constructs.
Responses of *apo-A1* reporter constructs in VJ-1 and RCAS cells. Lane designations are: p-6800/0 (1); p-3300/0 (2); p-300/0 (3); p-3300/20 (4); p-300/20 (5); p-193/20 (6); p-3300/43 (7); p-300/43 (8); p-193/0 (9); negative control used is pCAT/ Blue, a promotor less CAT construct (10); positive control: pSV2CAT/ Blue containing the SV40 promotor and enhancer elements(11).

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Figure 21. Possible models for eukaryotic gene repression.
Several models for eukaryotic gene repression are illustrated (reference 44). In model 1, positive transcription is initiated by the formation of a transcriptional activation complex (TAC) at the transcriptional start site. This complex consists of several large heterocomplexes including RNA polymerase II, TATA binding proteins and co-factors. A positive *cis*-acting sequence or enhancer region aids in the formation and stability of the TAC. In general, repression can occur by disrupting or preventing the formation of the TAC. In the competition models, negative factors compete for binding to either the TAC (competition #2), or to the activator sequence (direct competition #3). In the direct repression model (#5), a repressor protein binds a functional repressor sequence, or silencer region, distinct from the activator sequence. Quenching (#4) is accomplished by protein-protein interactions between the activator and repressor protein; these larger heterocomplexes recognize adjacent or overlapping *cis*-acting DNA sequences to effect repression. This is different from the squelching mechanism (#6) which does not require DNA binding; repression occurs when overexpression of another factor sequesters the functional activator protein, thus, preventing DNA binding and transcriptional activation. It is important to remember that these are simplistic models, and that transcriptional repression could involve multiple combinations of these models depending on the overall context of the promotor, the availability of transcription factors, and the physiological cell conditions. Nevertheless, all of these models could potentially repress *apo-AI* in v-Jun transformed CEF.
Legend: A: activator protein; R: repressor protein; B: overexpressed factors; clear circles: transcriptional activator complex; arrows indicate the transcriptional start site; shaded areas represent regulatory elements.

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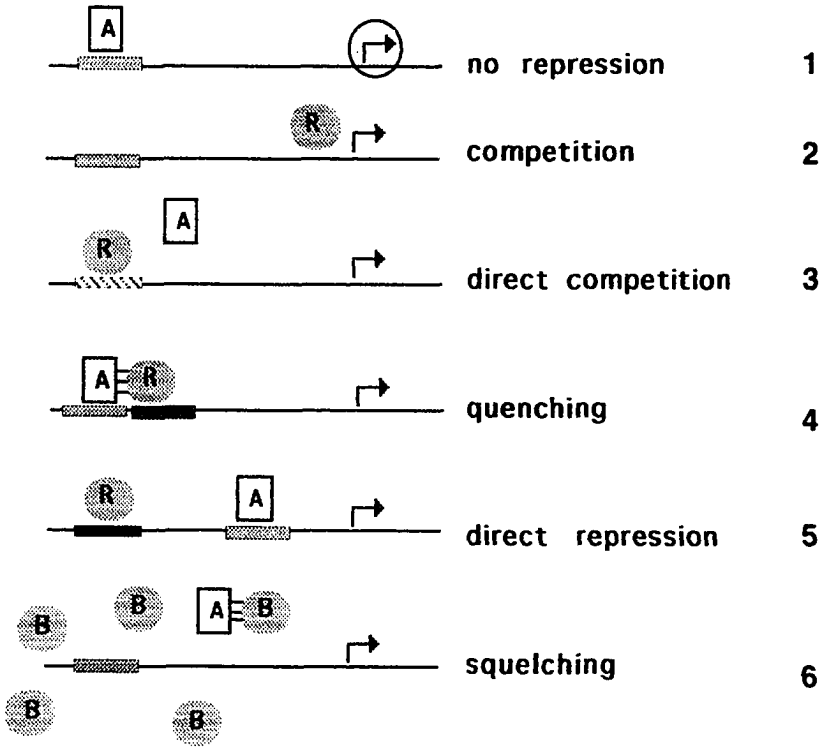

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Putative responsive element identifies a single distinct complex present in normal nuclear extracts only.

Promotor deletion analysis of the *apo-A1* regulatory region indicates that several *cis*-acting elements could regulate its expression. We focused our attention on the putative responsive element located in the -193 to +20 region. Studies indicate that this region has a 1.7 fold level of activity over background in normal CEF, and is 1.5 fold times higher in normal CEF over VJ-1 cells. Although this may not be significant, sequence analyses reveals that this region contains three potential factor binding sites that could regulate *apo-A1* gene expression (47) (see figure 15). Since three of the possible repression models described in figure 21 require DNA binding to effect repression, we wanted to determine if any repressor heterocomplexes present in normal or transformed CEF would recognize sites on the -193/20 promotor region.

Gel shift analysis using a labeled 213 bp fragment shows a single distinct complex present in normal CEF, but absent in v-Jun transformed CEF (fig. 22). The intensity of the shifted band appears to increases proportionately with increasing concentration of nuclear extracts. A 100 fold excess of homologous unlabeled competitor specifically competes with the shifted band, while a non-specific competitor, pGEM 4 plasmid, does not (figure 23). This finding supports the existence of a specific factor in normal cells that could act as a positive activator protein. In addition, specific complexes that recognizing the 213 bp fragment were not detected in v-Jun transformed CEF, suggesting that DNA binding may not be required to repress the p-193/20 reporter CAT construct in v-Jun transformed CEF. This observation suggests that a squelching mechanism may be involved in the repression of the p-193/20 *apo-A1* -CAT construct.

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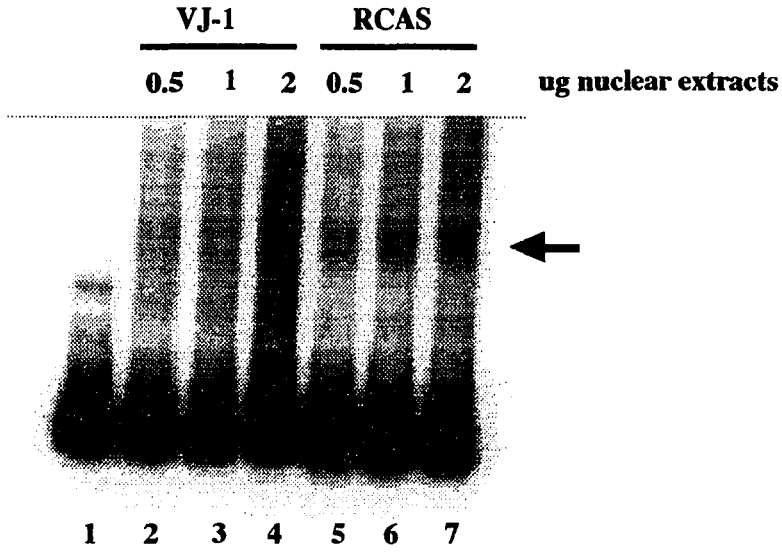

Figure 22. DNA binding assay using 213 bp ApoA1 promotor fragment
Gel shift analysis using the 213 bp *apo-A1* promotor fragment encompassing 193 nucleotides upstream from the transcriptional start site and 20 bp of exon 1 identifies a distinct band present in nuclear extracts from normal CEF (lane 5-7), but barely detectable in v-Jun transformed CEF (lane 2-4). Intensity of the shifted band appears to increase proportionately with increasing concentrations of nuclear extracts. lane 1, probe alone;

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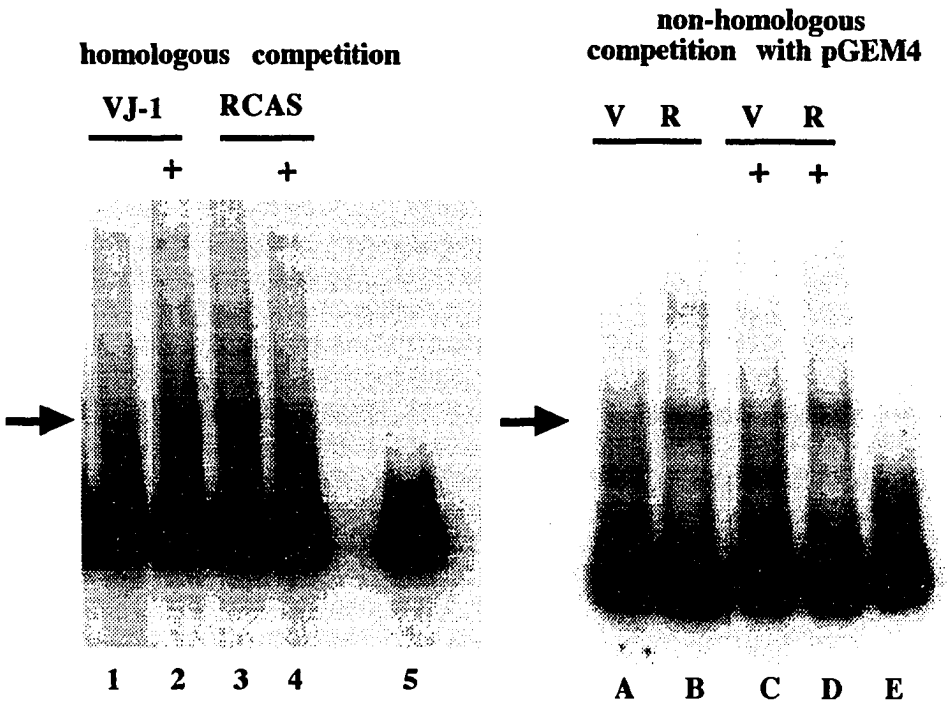

Figure 23. Competition shift analysis .
Nuclear extracts from v-Jun transformed CEF (lane 1, 2, A, C) and normal CEF infected with vector sequences only (lane 3, 4, B, D) were used in a gel shift assay with the 213 bp fragment A 100 fold excess of unlabeled homologous competitor specifically competes with the labeled probe in normal extracts (lane 4), while a non-specific sequence does not (lane D) (at arrows). This suggests the existence of a specific factor in normal CEF, not found in v-Jun transformed CEF. (lane 5, E: probe only).

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Antibody shift assays suggest that Jun proteins are absent in heterocomplexes that recognize the 213 bp fragment.

One of the possible models to explain *apo-A1* repression in v-Jun transformed CEF is the squelching model. In this scenario, overexpressed v-Jun proteins bind to the positive activator proteins and sequester them from binding to its cognate sequence, hence preventing transcription. To determine if v-Jun proteins are involved in such a mechanism, Jun antibodies were included in VJ-1 nuclear extracts to possibly neutralize binding to the positive activator protein. However, anti-Jun antibodies failed to restore DNA binding to the 213 bp sequence (lane 3; fig. 24). A possible explanation is that the antibodies were unable to neutralize overexpressed v-Jun proteins due to large proportional differences, or that the physical interactions between v-Jun and the activator protein were stronger. It would be interesting to see if large amounts of bacterial expressed v-Jun proteins could squelch DNA binding in normal nuclear extracts.

Antibody shift assays were also performed to identify other components of the specific complex in normal nuclear extracts. Other antibodies including anti-Jun, anti-c-myc, and anti-ras, did not cause a supershift or decrease in band intensity, indicating that their cognate proteins may be absent from these complexes (figure 24). More intensive tests are necessary to verify this.

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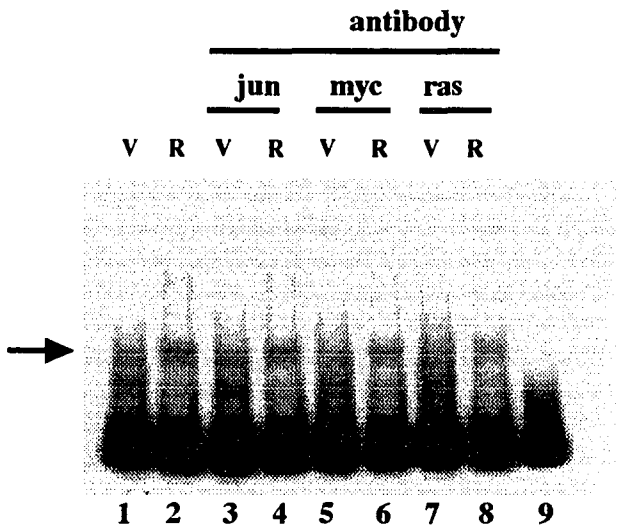

Figure 24 Antibody gel shift assay
Antibody competition shift assays using several antibodies, including anti-Jun fail to cause a super-shift or a decrease in band intensity in normal nuclear extracts (lane 4) suggesting that Jun proteins may be absent from complexes that recognize the 213 bp fragment . lane 1,2: no antibody; lane 3,4: anti-c-Jun; lane 5,6: anti-c-myc; lane 7,8: anti-ras; lane 9: probe alone; VJ-1: nuclear extracts from v-Jun transformed CEF; RCAS: nuclear extracts from normal CEF infected with vector sequences only;

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Chapter IV. DISCUSSION

The molecular mechanisms responsible for v-Jun induced cell transformation are not clearly understood (1, 2). An interesting possibility is that v-Jun influences the pattern of target gene expression by inappropriately activating or repressing its target genes (2). v-Jun behaves this way due to the structural changes at the protein level that ultimately alter its functional properties (4). For instance, its stability and dimerization aspects could change. Consequently, overexpression would increase the repertoire of available Jun heterocomplexes through an interaction with other nuclear factors. In support of this, we demonstrate that *in vivo* Jun complexes exhibit differential binding to *in vitro* generated AP-1 target sequences (34). These observations have important implications concerning v-Jun induced cell transformation.

In vivo Jun complexes exhibit differential binding to *in vitro* generated AP-1 target sequences.

To analyze the DNA binding properties of Jun complexes formed during overexpression, nuclear extracts from normal, c-Jun overexpressing and v-Jun transformed CEF were used in a gel shift assay against AP-1 and related AP-1 sequences. Using mutant AP-1 sequences against the consensus AP-1 site in a competition assay, we were able to demonstrate that Jun complexes formed during overexpression preferentially recognize distinct subsets of AP-1 related sequences. This finding suggests that the in vitro substrate specificity may be altered during Jun overexpression, relative to the normal condition. DNA transactivation studies using these AP-1 related sequences show that their transcriptional responses were below basal levels in all three nuclear extracts,

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indicating that overexpressed Jun complexes could contribute to transcriptional repression.

We also investigated the DNA binding properties of Jun complexes against the consensus AP-1 site. We expected to find more shifted bands, or more Jun heterocomplexes formed during overexpression, relative to the normal condition. Instead, our study shows that two distinct Jun heterocomplexes recognize the consensus AP-1 site in all three cell conditions. Furthermore, the DNA binding intensities in all three conditions were identical. This finding is consistent with DNA trans-activation data showing the consensus AP-1 sequence to be equally responsive in all three cell conditions, with no significant differences. Altogether these finding suggest that although Jun proteins are indeed overexpressed, either very low levels of activated Jun are required to bind the consensus AP-1 sequence, or that a majority of Jun complexes are inactive and do not bind DNA.

Our DNA binding data agrees well with another study investigating the DNA binding affinities of *in vitro* translated Jun and Fos family members to several *in vitro* generated AP-1 and CREB target sequences. (22). This study reports that various homodimer and heterodimer combinations of Jun proteins demonstrate different binding affinities to various AP-1 sequences. For example, Jun heterodimers bind AP-1 and related sequences with higher affinity, when compared to Jun homodimers. Moreover, Jun heterodimerization with various Fos proteins significantly changes the stability and half-life of protein/DNA complexes, suggesting that Jun dimer partners and their availability influence binding to AP-1 sites.

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Identification of genes associated with v-Jun induced cell transformation

Having demonstrated that *in vivo* Jun complexes formed during cell transformation recognize an altered pattern of *in vitro* target sequences, we wished to determine if a similar situation occurs *in vivo*. We began with the identification of potential target genes regulated by v-Jun. Utilizing the subtractive hybridization and differential display approach, we isolated five differentially expressed target genes. *Clone 4* is a novel cathepsin-like gene that belongs to a family of related cysteine thiol proteases. This finding is significant since proteases have long been implicated in cell transformation processes, and in metastasis of tumor cells. Furthermore, proteases, such as *stromelysin* and *collagenase*, are c-Jun target genes. A second gene, the *apolipoprotein A-1* gene, is repressed in v-Jun transformed CEF.

Positive regulation of ApoA1 expression in normal CEF.

The mechanisms regulating chicken *apo-A1* expression in normal CEF are not clearly defined. Differences exist among cell and tissue types, and between mammalian and avian systems. Promotor deletion analysis in our laboratory has identified three potential *cis*-acting regions on the *apo-A1* promotor. Two of these appear to be enhancer regions located at -6.8 kb to -3.3 kb. and -193 to +20.

We chose to characterize the possible activator sequence located between -193 to +20 since this region contained several transcription factor binding sites (47, 48, 50). Our DNA binding studies show that this 213 bp fragment recognizes a single distinct factor present in normal RCAS nuclear extracts, but not in VJ-1 extracts. In addition, antibody shift assays suggest that Jun proteins are absent from these complexes (figure 24). These findings agree with our Northern blot

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data establishing that the steady state levels of *apo-A1* mRNA are considerably higher in normal cells, as compared to v-Jun transformed CEF (figure 7), and further suggests that positive transcriptional mechanisms regulate *apo-A1* gene expression. This finding also agrees with previous studies demonstrating by DNA *trans*-activation studies, by DNA binding studies, and by DNA foot printing analysis that a short segment of the chicken *apo-A1* promotor encompassing -60 to -54 nucleotides indeed regulates this gene in several transformed cell lines (47).

We therefore propose that normal CEF regulate *apo-A1* expression in a positive way by at least two activator sequences. One located between -6800 and -3300 nucleotides, and another between -193 to +20. Since Actinomycin D treatments show that mRNA is relatively stable with a half-life of up to 20 hours, it is possible that the high steady state levels seen in Northern blot analysis is due to accumulation of stable message expressed from a constitutive promotor. More likely, the activator sequence located between -6800 to -3300 would predominantly regulate this gene.

Negative regulation of ApoA1 gene expression in normal CEF.

Several potential mechanisms could repress *apo-A1* expression in normal CEF. Promotor deletion analysis suggest that potential repressor sites could be located between -3.3 kb to -0.3 kb, and on sequences encompassing half of exon 1 (+20 to +43). For example, expression from the p-3300/0 construct is 1.5 times weaker than the p-300/0 construct. Deletion of the +20 to +43 region appears to reduce transcriptional responses. For instance, the p-300/43 construct is 4 times lower than the p-300/20 construct. In addition, a 1.4 fold reduction is observed between p-3300/20 and p-3300/43. While such discrepancies suggest that more

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defined deletions are necessary to delineate these elements, it is clear that negative regulation of *apo-A1* transcription occurs in normal CEF. Several possibilities exist (figure 21). Repression could occur by the direct competition model (#3), the quenching model (#4), or the direct repression model (#5). More DNA sequence information, DNA binding studies and DNA transactivation experiments are necessary to determine which of these mechanisms predominate.

Negative regulation of ApoA1 expression in v-Jun transformed CEF.

The *apo-A1* gene is one of the target genes isolated that appears to be repressed in v-Jun transformed CEF. Northern blot data show that the steady state levels of *apo-A1* mRNA are 3 to 10 times lower in v-Jun transformed CEF, as compared to normal CEF (figure 7). Promotor deletion analysis demonstrates that all of the reporter CAT constructs containing 5' deletions are repressed in v-Jun transformed CEF. Altogether, the observations suggest that v-Jun overexpression negatively influences *apo-A1* gene expression. Possible repression mechanisms are discussed below.

Repression by DNA binding.

It is conceivable that v-Jun proteins act as repressors and inhibit *apo-A1* expression by binding directly on the *apo-A1* promotor. v-Jun could exert its repressive action as a heterodimer involving other binding partners. There are three possibilities: In the quenching model, v-Jun heterodimers would recognize adjacent or overlapping sites on the promotor. In the direct competition model, v-Jun heterodimers would compete with an activator protein for a common regulatory site. In the direct repression model, v-Jun heterodimers would bind to a silencer region on the promotor. Alternatively, v-

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Jun might not bind the *apo-A1* promotor, but indirectly influence *apo-A1* by inducing the expression of a functional repressor protein. Further proof of which models are responsible depends on demonstrable DNA binding of v-Jun complexes to distinct promotor regions, and identification of repressor regions on the ApoA1 promotor by DNA transactivation studies.

Repression not involving DNA binding.

In an attempt to demonstrate that v-Jun overexpression influences *apo-A1* expression, we investigated the regulation of the p-193/20 reporter construct in a DNA transactivation study. We chose this region since it contains several transcription factor binding sites. Our preliminary analysis suggest that expression of this construct is 1.7 times lower in v-Jun transformed CEF, as compared to normal CEF. Although this may not be significant, DNA binding studies suggest that no apparent "repressor protein" recognizes this region, suggesting that DNA binding may not be required for repression of this sequence. Interestingly, these observations agree well with a squelching model of repression. As mentioned earlier, this model depends on the active sequestering of potential regulators that effect repression by preventing DNA binding, and eliminating positive transactivation of its target genes. More work is necessary to verify this.

Implications of study on v-Jun induced cell transformation.

The results of this study have important implications concerning the mechanisms involved in v-Jun induced cell transformation. Overexpression of v-Jun proteins can result in two related possibilities that ultimately lead to cell transformation: an increase in the repertoire of potential transcription factors, and squelching of target genes. Each possibility will be discussed below.

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One possibility is that the repertoire of possible transcription factors is increased. This can occur since Jun proteins can interact with a variety of transcription factors, both at the DNA level and through protein-protein interactions (10-15, 18-21, 23, 51). The increased repertoire can consist of both activator and repressor complexes which could influence gene expression by either transcriptional activation or repression. The new repertoire would recognize variations in AP-1 sequences, or bind to new non-AP-1 sites. Since this illicit event is not stringently controlled, cell transformation can develop. These assumptions are supported by the observations in the study. For instance, overexpression of v-Jun proteins leads to a change in substrate specificity *in vitro*, suggesting a change in the pattern of target gene expression *in vivo*. Indeed, our study has identified two genes showing altered patterns of gene expression, although the role of these genes in cell transformation has yet to be established.

The second possibility of v-Jun overexpression is a squelching phenomena (54, 55). In this situation, v-Jun actively sequesters other transcription factors rendering them as inactive complexes. As a result, target genes of the sequestered factor are repressed. If such genes are important in the regulation of cell proliferation, cell transformation could occur. Evidence suggests that overexpressed c-Jun proteins could indeed interact with members of the steroid receptor family of transcription factors, and squelch their target genes (11, 12). Again, these assumptions reflect the observations of the study. For example, no change in DNA binding or DNA transactivation occurs using the consensus AP-1 sequence. DNA transactivation using mutant AP-1 sequences produced responses that were below basal levels, suggesting that a majority of the overexpressed Jun complexes may be inactive. Indeed, the v-Jun induced

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repression of ApoA1 during cell transformation may occur by a squelching mechanism.

Intuitively, neither possibility would predominate to effect cell transformation. Rather, a combination events would occur; the outcome would ultimately depend on promotor structure, or the organization of enhancer and repressor sites along the upstream regulatory region. Studies show that c-Jun proteins can interact with other nuclear factors resulting in DNA binding to new non-AP-1 sites. These members include the steroid hormone receptor family, myoD, and NF-KB (10-15, 18-21, 23, 51). Studies suggest that the promiscuous behavior of c-Jun proteins may contribute to changes in the pattern of target gene expression, and could ultimately be responsible for a variety of cellular responses. It is therefore possible that overexpressed v-Jun could behave similarly. If this is so, these newly formed Jun-heterocomplexes with longer half-lives could interact with both activator and repressor factors serving to either positively or negatively affect their target genes. This could drastically alter the pattern of target gene expression. In support of this, the dissertation project has identified two target genes showing altered patterns of gene expression: *clone 4*, which probably functions as a protease, is overexpressed in v-Jun transformed CEF, while *apo-A1*, responsible for overall cholesterol metabolism, is repressed. Indeed, more deregulated genes are anticipated. Potential candidates include genes coding for positive and negative regulators and effectors whose aberrant expression would change the balance in favor of cell transformation. Potential repressed targets include tumor suppressor genes, negative cell cycle regulatory genes, or anti-apoptosis genes; activated targets would include oncogenes, positive cell cycle regulators, growth factors and their receptors, or positive signal transducers.

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Undoubtedly, interactions occurring from overexpression are not the only explanation for v-Jun induced cell transformation. Clearly, more than one mechanism could be responsible since cell transformation is a complex process requiring multiple independent but related steps. For example, v-Jun homodimers alone are sufficient to transform CEF (33). A major c-Jun partner, c-Fos, is not required. Overexpression of a chimeric v-Jun construct (VJ-GLZ) that only forms homodimers through the GCN4 leucine zipper, weakly transforms CEF. Interestingly, the phenotypic characteristics of VJ-GLZ transformed CEF differ from v-Jun transformed CEF: the former are arranged in random focal arrays as compared to circular swirls observed in v-Jun transformed CEF. Their kinetic properties are different too; the transformation potential of VJ-GLZ homodimers resembles c-Jun: weak and inefficient. Rapid transformation obviously requires other parameters, perhaps partners that do not involve dimerization.

Although this last study suggests that v-Jun induced cell transformation in CEF does not require dimerization with c-Fos or other nuclear factors, and may accomplish this without increasing the repertoire of Jun heterocomplexes, it does not argue against it. It is possible that potential interactions occur with other nuclear factors that do not involve the Jun leucine zipper.

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CHAPTER V. FUTURE DIRECTIONS

The dissertation project tests the hypothesis that deregulated expression of v-Jun contributes to cell transformation in CEF by influencing the pattern of target gene expression. It predicts that several downstream target genes would be activated or repressed inappropriately. In support of this, two target genes were isolated showing altered patterns of gene expression For this reason, identification of other target genes is necessary, if not mandatory.

Importance of clone 4.

An important concern is the transcriptional regulation of clone 4 during normal and transformed conditions. Although a differential pattern of clone 4 gene expression has been demonstrated, it is not certain if it is a direct or indirect primary gene target of v-Jun, or a secondary target expressed as a consequence of cell transformation. Likewise, if clone 4 is a primary target, can basal levels of c-Jun regulate this protease during normal conditions? To address this question, investigations on its regulation at the transcriptional level are necessary, as well as the identification of AP-1, or essential non-AP-1 binding sites on the promotor. Furthermore, the regulatory factors governing its functional aspects at the protein level should not be ignored. Answers to these questions relate to its possible involvement in cell transformation, as will be discussed below.

Several approaches to correlate a role for clone 4 in cell transformation exist. One strategy is to determine the functional aspects of clone 4 during normal conditions, and investigate how its deregulated overexpression may contribute to cell transformation. Since extensive nucleotide and amino acid

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sequence analysis indicates that this gene could belong to a family of related cathepsin-like proteases, several important questions arise, including: what is its chromosomal location, and is this a site of known chromosomal aberration?, What is its tissue distribution? Where is its predominant cellular location, or sites of action? Since most proteases are initially produced in an inactive proenzyme form, what are the requirements for optimal activity? What are its direct substrates? Important answers to the functional aspects clone 4 will provide us with clues on how its deregulation may contribute to cell transformation in CEF. For instance, well known proteases like stromelysin and cathepsin, have been implicated in tumor progression and metastasis (104, 105). Interestingly, stromelysin is a Jun target gene. Its direct substrates are extracellular matrix components. It would be interesting to see if clone 4 belongs to the cathepsin family of proteases possessing similar functional properties, since cathepsins are a prognostic tumor marker for breast cancer (105).

Another strategy to establish a role for clone 4 in transformation is to determine its requirements in v-Jun induced transformation. For instance, will overexpression of clone 4 be sufficient to transform CEF? Rapid transformation occurring independent of v-Jun activation suggests a strong correlation, while weak to no transformation will indicate that clone 4 may be required, but not sufficient. In a similar way, if clone 4 is a downstream effector of v-Jun, then blocking clone 4 expression, or activity during v-Jun overexpression, should reduce v-Jun's transformation potential. Possible sites of inhibition could be accomplished at the transcriptional or translational level (with antisense RNA), or at protein level (with clone 4 antibodies). Answers to these questions would also confirm if clone 4 is a primary or secondary target of v-Jun.

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Importance of Apo lipoprotein A1

The transcriptional control mechanisms regulating *apo-A1* expression during normal and transformed conditions have not yet been resolved completely. In addition to the mechanisms proposed earlier, two studies suggest that other factors may be involved. First, studies show that the steroid family of transcription factors cooperate best with Jun proteins to regulate gene expression. Quite remarkably, the *apo-A1* promotor contains several potential steroid binding sites located between -193 to +20 (see figure 15). It would be interesting to see whether these factors act exclusively, or in concert with Jun proteins to regulate *Apo-A1* gene expression.

Second, it is possible that cholesterol could directly affect *apo-A1* transcription. Recently, low levels of membrane cholesterol have been shown to regulate transcription by stimulating the cleavage and post-translational activation of an ER- membrane bound transcription factor, SREBP-1 (sterol regulatory element binding protein -1) (reviewed in reference 106). Intriguingly, SREBP-1 and -2 belongs to a family of bHLH-ZIP (basic-helix-loop-helix leucine zipper) transcription factors responsible for the regulation of low density lipoprotein (LDL) receptor through the SRE (sterol regulatory element). These observations raise several possibilities on the role of Jun proteins and *apo-A1* regulation. Since Apolipoprotein-A1 is a constituent of HDL (high density lipoproteins) involved in regulating cholesterol mobilization, it is conceivable that concentrations of cholesterol could in turn regulate *apo-A1* expression through SREBP-1. Although the SREBP leucine zipper does not resemble the Jun leucine zipper, it is conceivable that overexpression of v-Jun would form SREBP/ Jun heterocomplexes to repress *apo-A1* transcription by a squelching mechanism. It would be interesting to see if a SRE site exists on the *apo-A1*

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promotor. This would suggest that cholesterol or SREBP may regulate *apo-A1* similar to the LDL receptor during normal conditions, and that this site may be squelched during v-Jun overexpression. Answers to these questions can provide clues on how deregulated expression may contribute to cell transformation.

Another interesting inquiry is a possible role for apolipoproteins in cell transformation, *in vitro*, or tumorigenesis *in vivo*. An indication that this may be so comes from studies with ApoE demonstrating its ability to regulate neuronal growth *in vitro* (107). This study shows that ApoE3 increases neurite outgrowth in neurons *in vitro*, while an ApoE isoform ApoE4, has the opposite effect. These ApoE mediated cellular responses are believed to occur through low density lipoprotein (LDL) receptor interactions, since blocking with specific antibodies, affects ApoE mediated response. However, it is not certain if these growth related events are mediated by extracellular receptor signaling pathways, or from internalized ApoE /LDL receptor complexes, and whether the responses are specific to neural cells only. Nevertheless, other studies show that HDL (high density lipoprotein) and LDL mediated receptor binding stimulates signaling events involving phosphoinositide catabolism and Ca^{+2} mobilization in a number of cell types, including smooth muscle cells (111). In kidney mesangial cells, LDL receptor activation induces a number of growth related genes, including c-Fos and c-Jun transcription (109). Since Apo-A and Apo-E share similar regulatory roles, namely the mobilization of plasma cholesterol, it is conceivable that HDL containing Apo-A1 lipoproteins would behave in a similar manner to affect cellular growth responses, such that deregulated overexpression would cause transformation. Future studies to establish such a role should therefore include a possible autocrine pathway where secreted ApoA1 would influence cellular growth responses, perhaps

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through the HDL recrptor.

Finally, understanding the transcriptional mechanism regulating *clone 4* and *apo-A1*, as well as their requirements in cell transformation, would give us an idea on how future Jun target genes might be regulated. One could expect the isolation of other deregulated Jun targets, the identification of new transcription factors interacting with Jun, how these factors cooperate to affect transcription, and why activation should occur instead of repression. This information would help us undertake strategies to correct for Jun related disorders, which can be investigated by gene therapy.

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