Transcriptional Regulation of Chicken Apolipoprotein A-I Gene Expression

Wen Lin Kumfert
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TRANSCRIPTIONAL REGULATION OF CHICKEN APOLIPOPROTEIN A-I GENE EXPRESSION

by

Wen Lin Kumfert
B.S. August 1988, Wuhan University, Wuhan, China

A Dissertation Submitted to the Faculty of Eastern Virginia Medical School and Old Dominion University in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

EASTERN VIRGINIA MEDICAL SCHOOL
December 2000

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ABSTRACT

TRANSCRIPTIONAL REGULATION OF THE CHICKEN APOLIPOPROTEIN A-I GENE EXPRESSION

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Eastern Virginia Medical School, 2000
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Deregulation of a set of critical cellular genes has long been speculated as a cause of the oncogenic transformation induced by v-Jun oncoprotein. In the past several years, extensive efforts have been made to identify such genes. Several target genes have been identified that are specifically associated with the v-Jun induced transformation phenotype in chicken embryo fibroblasts (CEF).

We have undertaken the objective to identify and characterize the genes that become deregulated in response to, or as a consequence of, Jun-induced transformation. By exploiting the difference in oncogenic potential between v-Jun and c-Jun in CEF, the chicken apolipoprotein A-I (apoA-I) gene has been identified in our laboratory as one of the target genes whose expression is repressed in response to v-Jun overexpression in CEF. The overall objective of this study is to investigate the underlying molecular mechanisms by which the apoA-I gene expression is regulated, especially by the v-Jun oncoprotein.

In this study, we mapped the v-Jun responsive elements within nucleotides -311 to +19 upstream of the apoA-I transcription start site. Biochemical analysis of functional domains of v-Jun indicates that DNA binding specificity of v-Jun and its ability to heterodimerize with diverse partners are absolutely required for repression of apoA-I transcription. Further, the sequences between amino acid residues 108 to 128 in the amino terminus of v-Jun proteins that contains the acidic region III of its transactivation
domain are important for its repressor activity. In addition, an enhancer located between -6.8 kb to -6.0 kb upstream of the transcription start site of the apoA-I gene was identified and characterized. The enhancer is capable of stimulating transcription from the apoA-I promoter in a distance- and orientation-independent manner and is CEF-specific. Sequence information of the enhancer revealed that it is 791-bp long and contains many putative binding sites for known transcription factors. Deletion analysis of the enhancer activity indicates that there are many modules, both positive and negative, located within the 791-bp enhancer region. Nuclear transcription factors Sp1, C/EBP, HNF-3, and an unknown factor were found that might interact with the cis-acting elements of the enhancer.
To my husband, Gary
ACKNOWLEDGEMENTS

It has been a long journey. Without all the help and kindness I have received from so many people along the way, I would not make it.

First and foremost, I want to thank my committee, Dr. Kerry, Dr. Somers, Dr. Stenberg, Dr. Wasilenco, and my advisor Dr. Bos for the enormous time, patience, and guidance they bestowed on me through these years.

Specially, I want to thank my advisor, Dr. Timothy Bos. If I have learned anything in conducting research, I owe it to him. He has been a gentle mentor, an excellent teacher. I am very lucky indeed to have him as my advisor.

Also, I want to thank Dr. Osgood, who is kind enough to serve as an outside observer for my dissertation defense, and who is always so kind and encouraging and keeps a special interest in my study and work through the years. My thanks also to Dr. Buescher, who offered me the first opportunity to start my study here.

I want to thank my colleagues in Dr. Bos' laboratory, Melissa, Janet, Lynn, Johnny, and Joe. They made my study and work in the lab a pleasant and memorable experience.

My gratitude also goes to my parents, who think education is the beginning and the end; to Marion, who brought me here from China.

My deepest gratitude goes to Joann and Don. They have been my role model and inspiration in life. They provide a shelter for me, they feed me, encourage me, and even proofread my draft. I am deeply in debt to their boundless love.
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CHAPTER 1
INTRODUCTION

BACKGROUND

Jun: transcription factor and oncoprotein

Oncogene v-jun, and its cellular counterpart, c-jun, code for a major component of the transcription factor complex activator protein 1 (AP-1). AP-1 was originally defined as a DNA-binding activity recognizing the TPA (12-O-tetradecanophorbol-13-acetate) response element (TRE) and responsible for transcriptional induction of a number of genes in response to activation of protein kinase C (PKC). After molecular cloning revealed that Jun is a major component of AP-1, Jun has been shown to bind specific DNA sequences and transactivate transcription of many genes. Ever since its identification, the role of Jun in transcriptional regulation and in oncogenesis has been the subjects of intensive study. Significant progress has been made in understanding the complexity of Jun regulation.

Cloning of v-Jun, c-Jun, and related proteins

The viral oncogene v-jun was originally isolated as the transforming gene from avian sarcoma virus 17 (ASV 17), a replication defective retrovirus isolated from a spontaneous chicken fibrosarcoma (1). Infection with ASV 17 produces sarcomas in chickens and transforms cultured chick embryo fibroblasts (CEF) into neoplastic cells (2). ASV 17 contains a 930-bp non-viral, cell-derived insert, the jun encoding sequence, that is flanked by gag and env sequences. At the time of its description, no cross-
hybridization with known oncogene sequences was found, therefore the gene was given a new name, \textit{jun}, abbreviated from the Japanese \textit{ju-nana}, meaning "17" (1). The presence of \textit{v-jun} homologous sequences in DNA from all vertebrate species examined, suggested that \textit{v-jun} is derived from a cellular proto-oncogene \textit{c-jun}. In ASV 17 the \textit{v-jun} sequences are fused 3' to \textit{gag} sequences resulting in the expression of a 65 kDa gag-Jun fusion protein. The gag sequences were shown to be dispensable for transformation, because a \textit{v-jun} sequence inserted into a non-transforming avian retrovirus vector, RCAS, is transforming both in intact animals and in cell cultures (3). Computer analysis indicated that the predicted v-Jun protein contains a region of 60 amino acids adjacent to its C-terminus that is 44% identical to the DNA-binding domain of the yeast transcription factor GCN4 (1). The optimal DNA binding sequence of GCN4 is essentially identical to that of the mammalian transcription factor AP-1 (activating protein-1) (4,5). These findings suggested a possible relationship between Jun and AP-1. Indeed, antibodies directed against different regions of the viral Jun protein strongly reacted with a 39 Kda (p39) peptide in an AP-1 preparation purified by affinity chromatography (6-9). Bacterially expressed partially purified human c-Jun proteins containing the C-terminal DNA binding domain protect the same nucleotides as does purified AP-1 in footprinting assays. In addition, overexpression of v-Jun or c-Jun leads to transactivation of promoters containing AP-1 binding sites (7,10-12). These key discoveries quickly led to the identification of Jun as a component of transcription factor complex AP-1.

Shortly after the cloning of \textit{c-jun} from human, murine, and avian cells (7,9,13), two other closely related proteins were cloned by hybridization of cDNA libraries using probes derived from the \textit{c-jun} carboxyl-terminus. These two proteins, Jun B (14) and Jun
D (15,16), are highly homologous to the carboxyl terminus of c-Jun, bind to the same DNA binding site and transactivate promoters containing AP-1 sites (14,16). Their amino-terminal domains are different from that of c-Jun.

**Biochemical Functions of Jun**

The Jun family proteins have three well characterized structural domains. The amino terminus contains transactivation domains. The carboxyl terminus contains a highly conserved basic DNA binding domain and an adjacent leucine zipper motif. These structural domains are responsible for dimerization, specific DNA binding, and transcriptional activation/repression (10,12). Fig. 1 shows the structural organization of Jun family proteins in relation to the oncoprotein v-Jun.

**Dimerization** is a prerequisite for Jun to function. The Jun proteins belong to the bZIP family of transcription factors. BZIP transcription factors share a common structural motif termed the “leucine zipper”. The “leucine zipper” motif was first identified in transcription factor C/EBP (17,18). In transcription factor C/EBP and other bZIP proteins, there is a basic domain that mediates binding of the protein to the major groove of DNA while an immediately adjacent amphipathic domain with 4 to 5 leucines residues paced at 7 amino acid intervals is responsible for the homo- or heterodimerization between bZIP proteins. The leucine zipper region forms an extensive α-helix in which every seventh amino acid is a leucine. Due to this arrangement, the leucine side chains protrude from one side of the α helix and form a hydrophobic surface. Interdigitation of the leucine side chains facilitates dimerization between two helices (18). This periodic array was also noted in the sequences of the Fos, Jun and GCN4 proteins (18). The leucine zipper is required and sufficient for dimerization of Jun.
protein which, in turn, is a prerequisite for DNA binding. However, not all bZIP proteins dimerize with each other. C-Fos dimerizes with the various Jun proteins but not with the bZIP proteins GCN4, or other Fos protein (11). C-Jun molecules do not heterodimerize with GCN4. C-Jun can also form specific heterodimers with the CREB/ATF family of transcription factors (19-21). Formation of different complexes depends on the relative abundance of each monomer at that particular time. The monomers are differentially regulated under various cellular conditions, and different complexes display different DNA binding affinities to related but not identical enhancer sequences. In addition, each complex can display different transactivation capabilities. The regulatory specificity of Jun is dependent on its dimerization partners. That Jun proteins have the ability to form homodimers and a large number of heterodimers with different partners expands the array of target genes that are regulated by Jun.

**DNA binding specificity** - Specific DNA binding activity of Jun is mediated by a basic domain immediately adjacent to the leucine zipper (Fig. 1). The amino acid sequences of the basic domain are highly conserved among all of the Jun and Fos proteins (10). The basic domain is also conserved to a lesser degree in the various CREB and ATF proteins that interact with a sequence similar to the TRE (11). Target recognition specificity is determined by the monomeric constitution of the dimer. The different Jun proteins c-Jun, Jun B and Jun D bind as homo- and heterodimers both to 7-bp TREs (TGACTCA) and to 8-bp cAMP-responsive element (CREs) (TGACGTCA) (22,23). All members of the Fos family (including c-Fos, Fos B, Fra-1, and Fra-2) stimulate binding of the Jun proteins to the TRE-like sequences (12), such as those present in human collagenase I, stromelysin, and CD44 genes (5). Heterodimers formed
FIG. 1. **Structural organization of Jun proteins.** Jun proteins contain three major structural domains. The acidic domains (filled boxes) in the amino-terminal half of the proteins are important for transcriptional activation. The basic domain (hatched box) in the carboxyl-terminal half of the proteins is responsible for specific DNA binding. The adjacent leucine zipper domain (crosshatched box) is responsible for dimerization with other bZIP proteins. The dotted box in c-Jun indicates the glutamine- and proline-rich region.
between c-Jun and members of the CREB/ATF family bind preferentially to CRE-like sequences (19-21,24,25). Studies have shown that a new group of conserved coactivators increase the specificity of AP-1 transcription factors (26). One of these proteins, JAB-1 (Jun-activation-domain-binding protein 1), interacts with c-Jun and Jun D, but not with Jun B or v-Jun. As a result, JAB1 selectively potentiates transactivation by only c-Jun or Jun D through specifically stabilizing complexes of c-Jun or Jun D with AP-1 sites and does not affect binding of either Jun B or v-Jun (26).

Transcriptional regulatory activity – Apart from the basic domain and the leucine zipper motif which are important for DNA binding and dimerization, structure-function analysis of the c-Jun protein has identified a potent transactivation domain located within the c-Jun amino-terminus (A1 domain) and a less pronounced region adjacent to the basic domain (A2 domain) (27). The A1 activation domain of c-Jun consists of three independent acidic regions important for transcriptional activation; I (amino acids 6-12), II (amino acids 68-81), and III (amino acids 105 to 120) (Fig. 1) (28,29). Regions II and III contain sequences that are also found in the c-fos protein and act cooperatively and heterologously to activate transcription (30). Dimerization and DNA binding are prerequisites for direct activation of transcription from AP-1 responsive promoters. Studies have shown that members of the Jun family of transcriptional activators interact with general transcription factors, TBP (TATA-binding protein) and TFIIB (31). TBP binds to both the amino-terminal activation domain and carboxyl-terminal bZIP regions of c-Jun, whereas TFIIB binds to only the c-Jun bZIP domain. This interaction requires the dimerization of the Jun protein (31). In addition, studies have also shown that c-Jun proteins interact with CBP (cAMP-responsive-element
binding protein binding protein), a coactivator that binds to components of the basal transcriptional machinery on one hand and multiple transcription factors on another (32,33). Deletion analysis of c-Jun indicates that the CBP binding site is within the amino-terminal activation. Loss of binding to CBP in vitro correlates with severely reduced transactivation capacity in vivo (33).

Different dimeric complexes have different transactivation capabilities. One example is c-Jun and Jun B. Both c-Jun homodimers and Jun B homodimers bind to TRE-like sites, and both are capable of efficiently transactivating genes containing multiple TREs in their promoters. However, only c-Jun homodimers are capable of stimulating transcription from promoters containing single TRE. Under certain cellular conditions, elevated levels of Jun B may lead to the preferential formation of c-Jun/Jun B heterodimers which have low binding affinity to single TRE, and this may in turn lead to repression of genes controlled by single TRE (34-36).

In addition to direct regulation of transcription from promoters containing AP-1 responsive sites, Jun can also indirectly regulate transcription from non AP-1 containing promoters. In this case, the activity of a gene may be controlled not only by direct binding of a transcription factor to its promoter but also by protein-protein interactions between transcription factors, in which only one factor is directly associated with the DNA. Jun has been shown to physically interact with non leucine zipper transcription factors that recognize completely different consensus DNA sequences, including glucocorticoid receptor (GR) (37,38), NFkB (39), Myo D1 (40), Jif (41), and E2A (42). Jun modulates the expression of genes that are responsive to these transcription factors by either interfering with their DNA binding ability or altering their transcriptional
regulatory properties. Overexpression of c-Jun prevents the glucocorticoid-induced activation of genes carrying a functional glucocorticoid response element (GRE). Conversely, GR is able to repress AP-1-mediated transcriptional activation. Direct protein-protein interaction is responsible for this mutual inhibition. Mutational analysis reveals that the ligand-binding and DNA binding domain of GR are required for repression of Jun/AP-1 activity, and the leucine zipper of c-Jun is required for repression of GR activity. While bacterially expressed c-Jun inhibits GR-GRE complex formation, GR disturbs AP-1 activity without disturbing the binding of AP-1 to its binding site (37,43). Overexpression of c-Jun (or c-Fos) enhances the activity of NFκB-dependent genes, and this enhancement depends on the integrity of the NFκB-binding site. Conversely, overexpression of NFκB activates AP-1-mediated transcription. This mutual transactivation is due to enhanced binding of the transcription factors to either the NFκB or AP-1 binding site. The leucine zipper and basic region of c-Jun or c-Fos are responsible for the interaction with NFκB (39). Similarly, c-Jun interacts negatively with the muscle specific helix-loop-helix protein Myo D and inhibits myogenic differentiation (40). Jif interacts with the leucine zipper of c-Jun and inhibits the DNA binding and transactivation potential of c-Jun in vivo (41). Taken together, through transcription factor “cross-talk”, both the repertoire and complexity of Jun transcription regulation are increased. Jun can directly regulate the transcription of genes that contain AP-1 sites and indirectly modulate the transcription of genes that do not contain AP-1 consensus sequences.
**Regulation of Jun activity**

Regulation of Jun activity is at transcriptional and posttranslational levels. Almost all agents known to influence the fate of cells in culture induce the transcription of the *c-jun* message. These include a number of different growth factors such as platelet derived growth factor (PDGF) (44,45), epidermal growth factor (EGF) (46), tumor necrosis factor α (TNFα) (47), a variety of steroid hormones (48), interleukins (49,50), tumor promoters, chemical mutagens, ethanol, asbestos, morphines, peroxisome proliferators, tamoxifen (51) and certain oxidants and antioxidants (11,12). Many of these agents also stimulate transcription of the *c-fos* gene. The genes *c-jun* and *jun B* seem to be controlled in a coordinate manner, while *jun D* appears relatively insensitive to most agents controlling cellular proliferation and differentiation (12). Activation of *c-jun* transcription by these agents is mainly mediated through specific DNA recognition elements in the *c-jun* promoter. Under certain circumstances, c-Jun itself will positively autoregulate its own transcription (52). The positive autoregulation of Jun is likely to be responsible for signal amplification and conversion of transient extracellular signals into a long lasting transcriptional response.

The activity of Jun is regulated by posttranslational modifications. Posttranslational modification regulates Jun activity by two means: regulation of binding of the protein to the promoter element and, when bound, regulation of its transactivation activity. Two processes positively or negatively affect the DNA binding and therefore the transactivation activity of Jun; one is phosphorylation, the other is reduction-oxidation.
Two-dimensional peptide maps and phosphoamino acid analysis revealed that serine 243, serine 249 and threonine 239 located directly upstream of the basic DNA binding region of the c-Jun protein were phosphorylated in vivo (53). Glycogen synthase kinase-3 (GSK-3) was shown to phosphorylate the two serine and one threonine residues of bacterially expressed c-Jun in vitro (53). A c-Jun protein in which serine 243 has been replaced with phenylalanine or alanine, mutations which interfere with phosphorylation at all other sites, binds better to DNA and is a much stronger transactivator in vivo than the wild type protein (53). Other studies have shown that phosphorylation at carboxyl-terminal residue serine 243 of human c-Jun (which corresponds to chicken serine 226 and serine 216 in v-Jun) (see Fig. 2) is correlated with low DNA binding ability of the c-Jun protein (53). Treatment with TPA or other stimulatory agents dephosphorylates this serine site and increases DNA binding activity of AP-1 (11). In the oncogenic v-Jun protein, this serine (serine 216) is replaced by a phenylalanine, one of only two amino acid changes in the carboxyl-terminal domain of the protein which distinguish c-Jun from v-Jun (11,12) (see Fig. 2).

The target for redox regulation is cysteine 269 (which corresponds to cysteine 252 in the chicken c-Jun and cysteine 242 in v-Jun, see Fig. 2). Reduction of this residue by chemical oxidizing agents or by a ubiquitous nuclear redox factor (Ref-1) stimulates Jun DNA binding activity in vitro, whereas oxidation or chemical modification of the cysteine has an inhibitory effect on DNA binding activity (54,55). Very recent studies show that Ref-1 mediates the activation of c-Jun in HeLa and NIH 3T3 cells in response to heat shock and modulates the AP-1 stress response in the rat conceptus (56,57).
FIG. 2. **Structural differences between chicken c-Jun and v-Jun.** There are several structural differences between chicken c-Jun and v-Jun. v-Jun contains a 220 amino acid gag encoded leader, a deletion of 27 amino acid region (delta region) in the amino-terminus, and two non-conservative point mutations in the carboxyl terminus. The 220 aa Gag leader sequences are dispensible for transformation. The truncated v-Jun, VJ-1, is used in this study as transforming oncoprotein v-Jun.
Interestingly, in the oncogenic v-Jun protein, this cysteine residue is mutated to serine. These two mutations render v-Jun non-susceptible to negative regulation (Fig. 2).

The activity of Jun is also regulated by phosphorylation in the amino-terminal transactivation domain. Phosphorylation of the two serine residues in the transactivation domain of c-Jun, serine 63 and serine 73, increases the transactivation activity of c-Jun (58,59). This phosphorylation is mediated by Jun N-terminal kinases JNK (or stress-activated kinases, SARK) (60). Studies showed that both phosphorylated serine 63 and serine 73 are required for coactivator CBP induced stimulation \textit{in vivo} (33) and CBP binding \textit{in vitro} (61). Serine 63 and serine 73 are located near a region in the N-terminus of c-Jun protein called the delta region which is believed to serve as a docking site for JNK (62) (Fig. 2). With delta region deleted, v-Jun does not bind to the JNKs/SARKs, and the serines in the protein corresponding to serine 63 and serine 73 are not phosphorylated (63). The mechanism of transactivation of v-Jun remains elusive. It is suggested that JNK/SARK acts primarily as a positive regulator of c-Jun transactivation \textit{in situ}, and that loss of the docking site for JNK/SARK physically uncouples v-Jun from this control (62).

In addition to the modulation of its activity by phosphorylation or redox, the Jun activity is also subject to regulation by a variety of protein-protein interactions with both bZIP and non leucine zipper proteins.

Recently, studies revealed that the activity of Jun proteins is also regulated by ubiquitin-dependent proteolytic process (64). Ubiquitin-dependent degradation is an important pathway of non-lysosomal protein breakdown. It is responsible for the removal of naturally short-lived, as well as aberrant, such as misfolded or damaged
proteins. This process is mechanistically well understood. It involves the covalent attachment of ubiquitin, an evolutionary highly conserved 76-amino acid polypeptide which is abundantly present in all eukaryotic cells, to the ε-amino group of one or more lysine side chains of target proteins. Consecutive rounds of additional ubiquitination on previously attached ubiquitin moieties lead to the formation of branched protein species consisting of the substrate protein bearing multi-ubiquitin chains. Proteins that are modified in this manner are recognized by the proteasome, a large multi-catalytic protein complex that catalyzes the majority of protein breakdown in both the cytosol and the nucleus (65). At the proteasome the targeted protein is rapidly degraded in an ATP-dependent reaction. The selectivity of ubiquitin-dependent protein degradation is conferred by specific interactions between cis-acting degradation signals on the substrate proteins and trans-acting factors of the ubiquitination machinery. Ubiquitination signals often consist of a recognition site and a ubiquitin attachment site (64). The relative short half-life of c-Jun, measured at 60 to 90 min in fibroblasts, was found to be, at least in part, mediated by ubiquitin-dependent protein degradation (66). The delta domain of c-Jun is essential for ubiquitination (66). C-Jun is targeted for ubiquitination by its association with inactive JNK through its docking site-delta domain-for JNK. Phosphorylation of c-Jun on Ser 73 by activated JNK protects c-Jun from ubiquitination, thus prolonging its half-life (67-69). V-Jun has a longer half-live than c-Jun (66). The 27-amino acid delta domain that serves as a docking site for JNK is deleted in v-Jun, thus enabling v-Jun to escape regulation by degradation via the ubiquitin-proteasome pathway (64,66).
Transforming activity of Jun

AP-1 transcription factors regulate many cellular processes in response to a variety of extracellular signals (for review, see (10-12,70)). There is ample evidence that the activity of AP-1 components, especially that of c-Jun, is essential for the proliferation and differentiation of some cell types. Recent studies suggested that Jun is also involved in induction of apoptosis (70). Addition of retinoic acid to non-differentiated F9 embryonal carcinoma (EC) cells leads to induction of c-jun mRNA and triggers the differentiation of these cells into a primitive epithelial cell-type. Expression of both c-jun and c-fos is rapidly increased in many cell types in response to mitogens such as serum, EGF, TGFα, PDGF. Growth factors and cytokines (e.g., EGF, PDGF, TNFα, IL-1) exert their function by binding to specific cell surface receptors. Receptor binding triggers tightly coordinated multistep signal transduction cascades to the nucleus. In this cascade, nuclear transcription factors like c-Jun serve as terminal acceptors and convert these transient signals into long-term changes in gene expression. Such changes in cellular gene expression culminate in the initiation of DNA synthesis and eventually result in cell proliferation, differentiation or apoptosis. It is conceivable then that aberrant function of any component involved in these cascades may have dramatic consequences, leading to loss of growth control and neoplastic transformation.

Although the role of Jun proteins in transcriptional regulation has been extensively studied in recent years, the oncogenic properties of Jun are still poorly understood. Overexpression of v-Jun in CEFs results in strong oncogenic cell transformation and the transformed cells are tumorigenic when injected into birds (10,11). Overexpression of c-Jun is only weakly transforming in CEFs and the
transformed CEFs are not tumorigenic (10,11). While overexpression of mammalian c-Jun is sufficient to induce neoplastic transformation in an immortalized line of rat fibroblasts, Rat-1a (71), transformation of primary cultures of rat embryo fibroblasts (REF) by c-Jun or v-Jun requires the cooperation with an activated Ha-ras (36,71).

The differences in oncogenic potential between v-Jun and c-Jun in CEF have been exploited extensively to elucidate the mechanisms by which v-Jun manifest its oncogenicity. A comparison of the structure of v-Jun with its cellular counterpart, c-Jun, revealed a number of differences (Fig. 2). In the carboxyl-terminal DNA binding domain, v-Jun contains two non-conservative point mutations. In the amino-terminal half of v-Jun, a 27 amino acid region termed delta region was deleted. In addition, v-Jun also contains a truncated long AU rich 3' untranslated region (3). These structural differences are collectively responsible for the enhanced v-Jun oncogenicity (3).

Identification of chicken apolipoprotein A-I as a target of Jun regulation

Deregulation of a set of crucial cellular genes has long been speculated as a cause of oncogenesis by v-Jun. Extensive efforts have been made to identify such genes. Several target genes have been identified that are especially associated with the v-Jun induced transformation phenotype in avian fibroblasts, including JTAP-1 (72), bjk (73), and heparin-binding epidermal growth factor-like growth factor (74). JTAP-1, bjk, and heparin-binding epidermal growth factor-like growth factor are all upregulated in response to v-Jun overexpression in CEF.

In our laboratory, we have undertaken the objective of identifying target genes that are associated with the v-Jun induced transformation phenotype. Using a subtractive hybridization approach to exploit the differences in oncogenic potential between v-Jun
and c-Jun in CEF, we have identified a target gene, chicken apolipoprotein A-I (apoA-I), that appears to be specifically associated with v-Jun induced transformation in CEF (75).

We generated a subtractive cDNA library and identified a number of different clones. One of the clones, JTAP-4, whose mRNA level was 5 to 10-times lower in v-Jun-transformed CEF as compared to that in RCAS vector infected CEF or c-Jun-infected CEF. After repeating Northern blot analysis on RNA isolated from more than five different sets of RCAS VJ-1 (v-Jun), RCAS CJ-3 (c-Jun) or RCAS vector infected CEF using the clone JTAP-4 as a probe, we confirmed that JTAP-4 was a negative target of v-Jun overexpression. Two independent JTAP-4 cDNA isolates were sequenced and the sequence information revealed that JTAP-4 is identical to the chicken apolipoprotein A-I (75). The chicken apoA-I gene transcription was repressed 5- to 10-fold in response to v-Jun overexpression (Fig. 3). Treatment with actinomycin D demonstrated that, although the overall levels of apoA-I mRNA are lower in v-Jun-transformed CEF, the relative stability of the mRNA is identical to that in normal CEF. These results suggest that the decreased steady state apoA-I mRNA levels observed in Northern blot analysis is more likely due to a decrease in the apoA-I gene transcription (75). While overexpression of oncoproteins c/v-Myc and v-Src also resulted in reduction in the apoA-I mRNA levels, overexpression of other oncoproteins including c-Fos, v-Ha-Ras, c-Src and c-Ski either had no effect on the apoA-I expression or even resulted in a modest increase (75). These results indicate that repression of apoA-I gene expression is not strictly correlated with oncogenic transformation because overexpression of c-Fos, v-Ha-Ras also resulted in a transformed phenotype (75).
FIG. 3. The chicken apoA-I gene is a negative target of v-Jun overexpression. Chicken embryo fibroblasts (CEF) were infected with either vector (R=RCAS)), RCAS CJ-3 (C=c-Jun), or RCAS VJ-1 (V=v-Jun). Total cellular RNA and proteins were isolated. (A). Immunoblot of CEF infected with RCAS, RCAS CJ-3, or RCAS VJ-1 probed with an antibody against Jun (Oncogene Science AP-1 Ab-1). (B). Northern Blot of the same infected CEF with the chicken apoA-I as a probe. Ethidium bromide stained ribosomal RNA is shown below the Northern Blot to demonstrate equal RNA loading (Hadman & Bos. (1998).Oncogene 16:655-660).
Chicken apolipoprotein A-I: function and regulation

ApoA-I is the major protein constituent of serum high-density lipoprotein (HDL). HDL plays a major role in protection against atherosclerosis through its involvement in the reverse cholesterol transport mechanism in which cholesterol is transported from extrahepatic tissues to the liver for processing to bile salts (76-78). Plasma HDL levels are determined by the level of apoA-I gene transcription (79). In mammals, the apoA-I gene is predominantly expressed in the liver and intestine. Expression of the mammalian apoA-I gene is regulated by the interplay of multiple transcription factors that bind to the apoA-I gene regulatory region. In human liver cells, expression of the apoA-I gene is conferred by a hepatocyte-specific enhancer, located within nucleotides -220 to -110 upstream of the apoA-I gene transcription start site (80). The enhancer activity depends on synergistic interaction between transcription factors bound to three distinctive sites within the enhancer: A (-214 to -192), B (-169 to -146), and C (-134 to -119) (80,81) (see Fig. 4 and 5). Site A and C bind many members of nuclear receptor superfamilies including the liver-enriched hepatocyte nuclear factor 4 (HNF-4) (82-84), retinoid X receptor α (85), and apolipoprotein A-I regulatory protein-1 (ARP-1) (86,87). Site B binds the hepatocyte-enriched factors HNF-3β (81,88) (see Fig. 4 and 5). Synergy between these factors during enhancer activation determines the tissue-specific apoA-I gene expression.

The apoA-I expression is regulated by various developmental, hormonal, dietary, pharmacological and physiological factors. Physical exercise (89-91), alcohol consumption (92), and being female (93) are well known factors that have a beneficial effect on the abundance of apoA-I protein. Pharmacological agents such as fibrates,
phenytoin and phenobarbital also modulate the concentrations of this protein (94-96). A host of natural hormones, including thyroid hormones (87,97,98), glucocorticoid (99), sex steroids (100,101), and retinoic acids (102,103), were also reported to influence apoA-I transcription (see Fig. 5). In addition, we identified oncoprotein v-Jun as a factor that negatively regulates the apoA-I expression in CEF (75). Some factors, such as the orphan nuclear receptor ARP-1, a member of nuclear receptor superfamily, regulate the apoA-I gene expression by binding to its promoter region directly. ARP-1 was shown to repress human apoA-I expression through binding directly to site A of the apoA-I gene promoter region in human hepatoma HepG2 cells (82,87). Recent studies showed that transcription of the human and rat apoA-I genes declines with age (104). There were age-dependent changes of binding activity to two proximal sites, B and C. Decreased site B binding activity correlated with lower mRNA levels encoding the activator, HNF-3β. The age-dependent change in the pattern of binding to site C was due to a switch from the activator, HNF-4, to the repressor, ARP-1 (104). In both cases, ARP-1 regulates the apoA-I transcription by binding to the apoA-I promoter cis-elements directly. In addition to ARP-1, other members of the nuclear receptor superfamily including thyroid receptors, retinoic acid receptor-α, and HNF-4, also bind to the apoA-I promoter directly (105,106).

Other factors modulate the apoA-I gene indirectly, such as the glucocorticoids (99,107). Glucocorticoids were shown to increase apoA-I gene expression in both in vivo and in vitro models (108). Recent studies showed that in the presence of dexamethasone, the rat apoA-I promoter activity was stimulated three fold (99). Deletion of site B (-170 to -145) from the promoter completely abolished the stimulatory effects of glucocorticoids (99). But site B does not contain a consensus glucocorticoid receptor

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FIG. 4. Comparison of the chicken apoA-I promoter region with that of the human apoA-I gene. Positions of site A, B, C, CCAAT box, and TATA box were boxed. The numbers above the sequences are corresponding to the chicken apoA-I promoter (Lamon-Fava., et al. (1992) J. Lipid Res. 33: 831-842).
FIG. 5. **Schematic representation of the human apoA-I promoter.** The cis-acting elements are shown by both geometric shapes and the most 5' nucleotide for each site. List below each site (A, B, C, D, or TATA) indicates the hormones and transcription factors that act at these sites. The +/- signs indicate whether the hormones or transcription factors have a positive (+) or negative (-) effect on the site (Hargrove, et al., J. Mol. Endocrinology (1999) 22, 103-111).
motif and does not bind to bacterially expressed glucocorticoid receptor when used as a probe. However, the GR antagonist, RU486, completely blocks the increase in apoA-I mRNA levels following treatment with dexamethasone (107). These results suggest that GR acts via an indirect mechanism (99). Such a mechanism could involve GR activation of a factor that, in turn, binds to site B. Studies have demonstrated that HNF-3β binds to the site B in human apoA-I gene promoter and increases transcription of the gene (81,88), and that glucocorticoids induce site B binding activities (99). It is possible then that the stimulatory effect of glucocorticoids on expression of the apoA-I gene may act through increased abundance or activity of HNF-3β.

Similarly, sex steroid estrogen also stimulates the apoA-I transcription through an indirect mechanism (109). The actions of the hormone on the apoA-I gene transcription involve estrogen receptor, but the apoA-I gene promoter does not contain a binding motif for the nuclear estrogen receptor. Studies showed that estradiol potentiates the synergistic interactions between two transcription factors, HNF-4 and HNF-3β that bind to the cis-acting elements in the apoA-I promoter, site A and site B (81,88,110). The synergism between HNF-4 and HNF-3β is believed to be mediated by factor(s) that influence protein-protein interactions between these two proteins. Co-repressor, RIP140, is thought to be such a factor, and the activity of this factor is modulated by estradiol (101). It is suggested that estrodiol-activated endoplasmic reticulum somehow sequesters RIP140 protein, leading to the relief of repression and thus increased levels of apoA-I gene transcription. Another sex hormone, androgen, acts in a fashion opposite to that of estrogen. Studies suggest that androgens act by antagonizing the actions of estrogen, and that androgens have no direct effect on the apoA-I transcription (111).
The pattern of tissue expression of the apoA-I gene in avian species is very different from that in mammals. Although the liver and the intestine are major sites of chicken apoA-I mRNA synthesis, apoA-I gene expression also occurs in most other chicken tissues (112-117). The chicken apoA-I gene spans 1.5 kb and contains 4 exons and 3 introns, closely resembling the mammalian gene (118,119). While certain sequences in the 5’ upstream region of chicken apoA-I gene are highly homologous to the mammalian gene site C and the 5’ half of site B, the chicken gene lacks sequences corresponding to the mammalian gene site A and the remaining 3’ half of site B (Fig. 4).

Most apolipoproteins have originated from duplication of an ancestor apolipoprotein gene and subsequent modification of the duplicated copy. ApoA-I, C-III, and A-IV genes are closely arranged in a gene cluster in both birds and mammals. It is possible that the sites A, B, and C organization of the human apoA-I gene promoter may have arisen during evolution as the duplication of the half site B and site C, which is still the structure of the chicken gene (120,121). The broader tissue expression of the chicken apoA-I also resembles that of mammalian apoE, which is absent in avian species. It has been suggested that, in birds, apoA-I may be the functional homologue of mammalian apoE (122). Mammalian apoE also plays an important role in cholesterol transport. In addition, recent studies have shown that mammalian apoE plays a critical role in neurobiology. The apoE4 allele is the major susceptibility gene related to the occurrence and early age of onset of Alzheimer’s disease (123). Finally, unlike human apoA-I, which aggregates in the absence of lipid, chicken apoA-I is monomeric in the lipid-free state (124). This physical characteristic makes chicken apoA-I a good model to further characterize the apoA-I protein and to study the mechanism by which it functions in
cholesterol transport. In recent years, extensive studies have been done to define the cis- and trans-acting factors involved in regulation of hepatocyte and intestinal specific expression of the mammalian apoA-I gene, but little is known regarding the factors and cis-acting elements that regulate the chicken apoA-I gene expression. Our preliminary studies suggest that v-Jun may play an important negative regulatory role in regulation of the chicken apoA-I gene expression.

SIGNIFICANCE

Atherosclerotic cardiovascular disease is the leading cause of death in industrial societies. The important role of high-density lipoprotein (HDL) and its major protein component, apolipoprotein A-I (apoA-I), as being anti-atherogenic has been well established. There is sufficient evidence to support a causal association between plasma HDL levels and the subsequent development of coronary heart disease. The observation that plasma HDL levels are directly correlated with the liver apoA-I mRNA levels suggests that factors affecting apoA-I gene expression play an important role in atherosclerosis susceptibility. Recent studies in transgenic mice and rabbits for human apoA-I showed that overexpression of the apoA-I protein increases serum HDL cholesterol concentration and reduces high-cholesterol-diet-induced atherogenesis (125). The results suggest that up-regulation of apoA-I synthesis may afford a new approach for the prevention or therapy of atherosclerotic disease. The understanding of how the chicken apoA-I gene is regulated in CEF and the differences in regulation between mammalian and avian species regarding the differential expression of the apoA-I gene is valuable in developing strategies to increase human apoA-I gene expression.
Derangement of cholesterol homeostasis and apolipoprotein synthesis has been associated with certain types of cancer. Correlative reports have identified decreased levels of HDL in patients suffering from acute lymphocytic leukemia (ALL) (126,127). HDL levels were restored to normal levels with successful chemotherapy.

Apolipoprotein D (Apo D) is a protein component of the human plasma lipid transport system which is present in benign and malignant human breast tissues. It was reported that expression of the apoD is elevated in the breast cancer specimens (128). Studies have shown that there was a positive association of Apo D content in male breast tumors with favorable outcome and that apo D expression was a significant independent indicator of relapse-free survival in male breast cancer (129). Recent studies suggest a possible relationship of the apoE4 gene to both breast cancer and prostate cancer (130,131). It is thought that apoE4 keeps serum triglycerides elevated by reducing their clearance and that triglycerides themselves appear to be a risk factor for breast cancer (130). A link between atherosclerosis and tumor angiogenesis involving apolipoprotein (a) was also suggested (132). In addition, the elevated apoA-I mRNA levels were used to detect homogenous colon cancer dissemination in a clinical study and was found to be highly specific and sensitive (133). It appears that deregulation of apolipoprotein expression may be a consequence of certain types of oncogenic activity.

**SPECIFIC AIMS**

We have identified v-Jun as one of the factors that affect chicken apoA-I gene expression. Overexpression of oncoprotein v-Jun in CEF represses chicken apoA-I mRNA level by 10-fold. The decrease of apoA-I steady state mRNA level is due to a decrease at the transcriptional level (75).
During the process of initial deletion analysis, we also observed consistently elevated CAT activity in the plasmid containing -6800 bp upstream of the apoA-I transcription start site, but not in the plasmid containing -6000 bp upstream of the transcription start site. We speculated that there is an enhancer located within the 800 bp DNA fragment spanning from -6800 bp to -6000 bp upstream of the apoA-I transcription start site.

The overall objective of this study is to provide some insight into the underlying mechanisms by which the apoA-I gene transcription is regulated, especially by the v-Jun oncoprotein.

Aim 1: To map the cis-acting elements in the chicken apoA-I promoter region that mediate the repressor activity of v-Jun

Regulation of gene expression depends on binding of transcription factors to gene promoter elements. The v-Jun oncoprotein regulates target gene expression through both direct DNA binding and through transcription factor “cross-talk”. As a first step to understanding the underlying mechanism by which apoA-I gene expression is repressed by v-Jun, the cis-acting elements in the apoA-I promoter region that mediate the repressor activity of v-Jun were mapped.

Aim 2: To determine if v-Jun represses the apoA-I gene transcription through binding to the apoA-I promoter directly

As a transcription factor, v-Jun can regulate target gene expression either directly by binding to DNA or indirectly by protein-protein interactions. To distinguish between these possibilities, a series of gel shift assays and supershift assays were performed.
**Aim 3: To determine the biochemical domains of v-Jun required for the repression of the apoA-I gene expression**

V-Jun has three well-characterized functional domains: basic DNA binding, leucine zipper dimerization, and transactivation. How each domain contributes to repression of the apoA-I gene was assessed by a structure-and-function approach.

**Aim 4: To characterize an enhancer element in the chicken apoA-I gene 5’-flanking region**

An 800-bp enhancer located within nucleotides –6800 bp to –6000 bp upstream of the apoA-I gene transcription start site was identified. The functional properties and species specificity of the enhancer were investigated. The cis-acting elements within the enhancer that mediates binding of nuclear factors were characterized.
CHAPTER II
MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTIONS

Culture of primary CEF was performed on 10-day-old embryo as previously described (3). In subsequent passages, CEFs were maintained in cloning media 1X F10 (GIBCO) containing 4% deactivated chicken serum (GIBCO), 10% donor calf serum (Atlantic Biological), 1% L-glutamine, 1% penicillin/streptomycin, 1% MEM vitamin solution (all from GIBCO) and 0.4% DMSO (Sigma). Hep G2 cells, NIH3T3 cells and MCF7 cells were grown in MEM culture medium supplemented with 10% fetal bovine serum (Atlantic Biological).

For CEFs that were infected with RCAS vector, RCAS VJ-1 or RCAS v-Jun mutants retroviruses, transient transfections were carried out by the DMSO shock method described in (3). Cells were seeded at a density of $3.0 \times 10^6$ in 100-mm plates 24 hours prior to transfection. Five micrograms of plasmid DNA was used. Half microgram of CMV-β-galactosidase was cotransfected in some experiments as an internal transfection efficiency control. Cells were harvested 96 hours later and cellular proteins were extracted using three cycles of freeze-thaw at $-70^\circ C$ and $37^\circ C$, respectively. Protein assays were performed using Bio-Rad method (75). Two hundred to six-hundred micrograms of protein were used in CAT assays.

For CEFs that are used for the enhancer identification and characterization, transient transfections were carried out by FuGene 6 method (Roche) according to manufacturer's instructions. Cells were seeded at a density of $4.0 \times 10^5$ to $5.0 \times 10^5$ in 2-mm 6-well plates 24 hours prior to transfection. 2.5 μg to 2.9 μg of plasmid DNA were
used along with 0.5 ug to 0.1 ug CMV-β-galactosidase as an internal transfection efficiency control. Cells were harvested 48 hours later and cellular protein was extracted using three cycles of freeze-thaw at -70°C and 37°C, respectively. Protein assays were performed using Bio-Rad method (75). 50ug to 100ug of protein were used in CAT assays.

PLASMIDS CONSTRUCTIONS

The constructions of RCAS VJ-1, RCAS VJ-3, RCAS VJδNco, and RCAS VJGLZ have been described previously (3,134). The constructions of RCAS VJ/C/EBP and RCAS VJGLZ/C/EBP are described (Bos, et al., in press). Plasmids, p-6800/+1.CAT, p-311/19.CAT, and p-60/19.CAT, consist of nucleotides -6800 to +1, -311 to +19, and -60 to +19, respectively, of the chicken apoA-I gene 5’-flanking region fused to a chloramphenicol acetyltransferase (CAT) gene were reported previously (75). Plasmid p-194/19.CAT was made using a PCR amplified product obtained with forward primer: CGGGGACGTCCTGTTTGCTGAGG (the apoA-I promoter -194 to -172), reverse primer: CACTGCTCGTCCCGTGTG (positions +19 to +2). Plasmid p-311/263.CAT was used as template. The PCR product was first cloned into pGEM-T (Promega) and then was cut with Spe I, blunt-ended with Klenow, cut again with Not I. The final product was gel-purified and cloned into pCAT/Blue cut with Hind III, blunt-ended with Klenow and then cut with Not I. The apoA-I promoter CAT plasmids with the 800 bp homologous enhancer p(-6800 to -6000)-311/19.CAT, p(-6800 to -6000)-194/19.CAT and p(-6800 to -6000)-60/19.CAT were constructed by first digesting the corresponding plasmids p-311/19.CAT, p-194/19.CAT, and p-60/19.CAT with Asp718 and Not I. The Asp 718-Not I DNA fragments containing the apoA-I promoter regions...
and the CAT gene were ligated with Bam HI-Not I fragment containing the 800 bp enhancer from p-6800/+1.CAT (75).

All the plasmids used to identify and characterize the enhancer were generated by blunt-end ligation to obtain the constructs containing inserts in both orientations. To construct p (-6800 to -6010)-311/19.CAT, 800-bp Asp718 and BamHI fragment was excised from p-6800/+1.CAT, blunt-ended with Klenow (Promega) and inserted upstream in both orientations into p-311/19.CAT digested with Asp718. To construct p (-6800 to -6000) Bluescript SK (+), the same 800-bp fragment was inserted into pBluescript SK (+) (Stratagene) digested with Asp718 and BamHI. This 800-bp Asp718 and BamH I fragment of plasmid -6800/+1.CAT was also used as an intermediary step to generate most of the 5’- and 3- deletions of the full length enhancer. To construct plasmids p (-6800 to -6135)-311/19.CAT and p (-6135 to -6010)-311/19.CAT, the 800-bp fragment was digested with HinF I, blunt-ended with Promega Klenow and the resulting two fragments (669-bp and 128-bp long) were inserted upstream in both orientations into p-311/19.CAT. To construct plasmids p (-6800 to -6428)-311/19.CAT and p (-6428 to -6010).CAT, the blunt-ended 800-bp fragment was digested with Sac I, 3’-blunt-ended with Klenow and the resulting two fragments (550-bp and 241-bp long) were inserted upstream in both orientations into p-311/19.CAT. To construct plasmids p (-6800 to -6557)-311/19.CAT and p (-6557 to -6406)-311/19.CAT, the 800-bp fragment was digested with Ban I and Ava I, blunt-ended with Klenow, and the resulting two fragments (247-bp and 155-bp long, respectively) were inserted upstream in both orientations into p-311/19.CAT. To construct plasmids p (-6800 to -6629)-311/19.CAT and p (-6629 to -6010)-311/19.CAT, the blunt-ended 800-bp fragment was digested with

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Pvu II and the resulting two fragments (171-bp and 620-bp long) were inserted upstream in both orientations into p-311/19.CAT. To construct plasmid p (-6629 to -6406)-311/19.CAT, the 800-bp fragment was digested with Pvu II and Ava I, the resulting 223-bp fragment was then blunt-ended and inserted upstream in both orientations into p-311/19.CAT. Plasmid p (-6800 to -6406)-311/19.CAT was constructed by digesting p-6800/+1.CAT with Asp718 and Ava I, the 395-bp fragment was blunt-ended and then inserted upstream in both orientations into p-311/19.CAT. Plasmid p (-6406 to -6010)-311/19.CAT was constructed by digesting plasmid -6800/+1.CAT with Ava I and BamHI and the resulting 396-bp fragment was blunt-ended and inserted upstream in both orientations into p-311/19.CAT.

To generate plasmids p (-6800 to -6010) CAT-Promoter, the 800-bp Asp718-BamHI fragment excised from p (-6800 to -6010) Bluescript was blunt-ended and inserted downstream of the SV40 promoter in both orientations into pCAT-Promoter (Promega) digested with BamHI and blunt-ended. To generate plasmid p (-6800 to -6010) SV2CAT, plasmid p-6800/+1.CAT was first digested with BamHI, blunt-ended, and then was digested with Xho I, the resulting 800-bp fragment was inserted upstream in forward orientation into pSV2CAT (135,136) that was digested with SalI first, blunted-ended, and then digested with Xho I.

Plasmid containing the human apoA-I promoter 1.3kb (positions -1064 to +234), p1.3kbhAl.CAT, was first constructed by PCR and served as an intermediate plasmid for subsequent construction of plasmids containing human apoA-I minimal promoter with or without the 800-bp enhancer sequences. To construct plasmid p1.3kbhAl.CAT, genomic DNA was extracted from human breast cancer MCF7 cells and 100ng was used as
template in PCR reaction to amplify 1.3-kb fragment using primers corresponding to –1064 to –1042 (forward: gatgggtgctcaaacaccaacgg) and +234 to +215 (reverse: ctgaagggccgtgpprgacc). The resulting 1.3-kb PCR products were eluted out of a low melting agarose gel and ligated into vector pGEM-Teasy (Promega). The 1.3-kb fragment was excised from pGEM-Teasy with EcoR I digestion, blunt-ended with Klenow and then subcloned into vector pCAT-Basic (Promega) which was digested with Xba I, blunt-ended with Klenow and dephosphorylated with calf intestinal alkaline phosphatase (GIBCO). To construct plasmid containing human apoA-I minimal promoter, p-41/234hAI.CAT-Basic, p1.3kbbhAI.CAT-Basic was digested with Pst I and the 4.6-kb DNA fragment containing the vector pCAT-Basic and human apoA-I minimal promoter (positions –41 to +234) was excised and self-ligated. Plasmid, p (-6800 to –6000)-41/234hAI.CAT-Basic was constructed by inserting the 800-bp fragment excised from p (-6800 to –6000) Bluescript SK (+) with Asp718 and BamH I digestion and blunt-ended with Klenow downstream in both orientations into the vector p-41/234hAI.CAT-Basic digested with Hind III, blunt-ended with Klenow and dephosphorylated with calf intestinal phosphatase. Some of the constructs were confirmed by sequencing and all the constructs were confirmed by restriction enzyme digestions.

**PREPARATION OF NUCLEAR EXTRACT FROM CEF**

Crude nuclear extract was isolated from chicken embryo fibroblasts as described (134). Briefly, cells were collected, incubated in hypotonic solution buffer A (10mM HEPES, pH 7.9, 1.5mM MgCl₂, 10mM KCl, 0.5% CA-630, 0.5mM DTT, 1mM PMSF, 2µg/ml Aprotinin, 0.5µg/ml Leupeptin and 1µg/ml Pepstatin) at 4°C for 10 min, mixing gently every minute. Nuclei were sedimented at 1,000x g for 10 min, resuspended in
extraction buffer, buffer C (20mM HEPES, pH 7.9, 25% Glycerol, 0.42M NaCl, 1.5mM MgCl₂, 0.2mM EDTA, 1mM PMSF, 0.5mM DTT, 1ug/ml Aprotinin, 0.5ug/ml Leupeptin, and 1ug/ml Pepstatin) at 4°C for 30 min with gentle mixing. Insoluble debris was removed by centrifugation at 4°C at 10,000 x g for 30 min. The supernatant was dialyzed against buffer D (20mM HEPES, pH 7.9, 20% Glycerol, 0.1M KCl, 0.2mM EDTA, 0.5mM PMSF, and 0.5mM DTT) at 4°C for 4 hours with one change at 2 hours. Samples were stored at -70°C in 30ul aliquots. Protein concentration was determined by the Bio-Rad method (75).

SYNTHESIS OF OLIGONUCLEOTIDES

The oligonucleotides that are labeled as probes were all synthesized commercially (GIBCO). All of the oligonucleotide sequences described all from 5' to 3'. Six pairs of oligonucleotides were synthesized to assemble the 332-bp apoA-I promoter region.

Oligonucleotides -311 to -244, forward: ggtaccaaccggcgccggctctgcgccaatccccgggggctgcga, reverse: tccggccccgccccctgcagccccggggattggcgcagagc.

Oligonucleotides -251 to -192, forward: gggccggaatttgggtcacccgcagcgtaaacatctcctc; reverse: cccggatgcgcggtgcagagcttgttcagcag. Oligonucleotides -211 to -142, forward: ggccaacgccgcatccccggggagctcctttgctgaggctgaggctctgcaaag; reverse: gagccggccaaggacgcggctttgcagcagctcagcaaacaggagctcc.

Oligonucleotides -156 to -85, forward: ggctctcccgggctgcgcacgatccttgactctacgcgccacatcg; reverse: tcccggcgcgggcgtagttggccgcgtaggtccagcagcatgtggccgac. Oligonucleotides -91 to -26, forward: gcccggagtattctttgggtgcggctgcgctgttatctctgctgcgggaac; reverse: tgcaggaggggcagttccccgcaggagataagccagcgcagcagcag. Oligonucleotides -41 to +21, forward:
tgccctcctgcataaatagcggcggcggggaaccgcgggct; reverse:
cgcactgtcgtcctccgttgagcccgcgtcccccgcggg.

Four pairs of oligonucleotides were selectively synthesized from the apoA-I enhancer. Oligo I, forward: tgtatgctcaggacgagtctctggaaagca (positions -6150 to -6121), reverse: gggagctggcaagatgcgtgctttccagag (positions -6103 to -6132). Oligo II, forward: ggtatgaccaggacagttttggggttgggtt (positions -6178 to -6149), reverse: ctctgctctgcacacagttttttttttt. Oligo III, forward: tggaaagggccggcaggctctcgggcggcc (positions -6416 to -6387), reverse: aggcctcctgactgcaggggccgcccac (positions -6367 to -6396). Oligo IV, forward: cgggtcggcggcggctctggcgaaggg (positions -6506 to -6477), reverse: gcggcagggctcctgctcccttggccccc (positions -6457 to -6493). C/EBP, HNF-3, SP-1, HNF-4 and AP-1 specific oligos are listed in Table II.

**GEL MOBILITY SHIFT ASSAYS**

Overlapping single-stranded oligonucleotides were annealed at 48°C for 10 min and allowed to cool gradually to room temperature. The resulting double-stranded oligonucleotides were labeled by fill-in with Klenow DNA polymerase I incorporating the appropriate [α-32P]dNTPs. The probe -311 to +21 of the apoA-I promoter region was PCR-generated and cloned into pGEM-T vector (Promega), subsequently excised, gel-purified, and 5'-end labeled with [α-32P]dNTPs. Gel mobility shift assays were performed as previously described (134) with modifications. Briefly, 16 µg of nuclear extract were preincubated in the binding buffer (20mM HEPES, pH 8.0, 35% glycerol, 0.2mM EDTA, 40mM NaCl, 8mM MgCl2, 40mMDTT, 4mM spermidine) with 1 µg non-specific competitor poly(dI-dC) on ice for 15 min. The reaction mixture was then
incubated with 1 µl of $^{32}$P-labeled probe for 15 min on ice. Competitors were added with probes in competition gel shift assays. Samples were run on 6% acrylamide gels at 25 mA for 2 to 3 h. Gels were dried and exposed to x-ray films (FUJI). Fifty to one-hundred-fold excess of unlabeled oligonucleotides was added as competitor. The following antibodies were used in supershift assays: Ab-1, rabbit polyclonal antibody raised against c-Jun DNA binding domain residues 247 to 263 (Santa Cruz); Ab-2, rabbit polyclonal antibody raised against c-Jun DNA binding domain residues 209 to 225 (Oncogene); Ab-3, rabbit polyclonal antibody raised against c-Jun amino terminal domain residues 91 to 105 (Santa Cruz). The antibodies were either added 30 min before the probe or added after the probe for 30 min on ice.

**SEQUENCING**

Sequence analysis of the 800-bp fragment was obtained using an automatic sequencer (Pharmacia LKB. A.L.F.). The sequencing reactions were performed using the AutoRead™ Kit (Pharmacia) according to the manufacturer’s instructions. Five micrograms of plasmid p (-6800 to -6000) Bluescript SK (+) were used as template.
CHAPTER III
RESULTS

MAPPING OF THE V-JUN RESPONSIVE ELEMENTS IN THE APOA-I
PROMOTER REGION

The role of v-Jun in transcriptional regulation has been extensively examined in recent years. First, Jun can recognize and bind to specific DNA sequences, such as AP-1- or CRE-like elements. Once bound, Jun can regulate target gene expression from nearby promoters through complex formation with its many dimerization partners. Second, Jun proteins can regulate, positively or negatively, many target genes that do not contain AP-1- or CRE-like sites in their promoter region through protein-protein interaction with non-leucine zipper transcription factors. Repression of target gene expression in response to v-Jun overexpression can be mediated by several molecular mechanisms (Fig. 6). The simplest mechanism involves competition for a common DNA binding site, for instance, v-Jun may exclude the binding of an activator by virtue of recognizing the same, overlapping, or adjacent binding site. This passive repression may be augmented by active repression once v-Jun is promoter bound (Fig. 6a). Yet, based on published sequence information (118,119), there is no consensus AP-1 site within the chicken promoter region. Therefore, if v-Jun binds to the apoA-I promoter directly, it would recognize a fairly divergent AP-1 or CREB-like site. A second mechanism involves protein-protein interactions. V-Jun may repress either basal or activated transcription by competing for binding of activators or competing with an activator for a limiting common coregulator or general transcription factor (Fig. 6b and 6c). This would result in depleting of these factors, a phenomenon called squelching. A third mechanism involves
promoter modification (Fig. 6d). For example, overexpression of v-Jun may upregulate methyltransferase resulting in methylation of the apoA-I promoter, which is extremely GC rich. CpG methylation has long been associated with repressed gene transcription (137-141). A fourth mechanism involves secondary gene expression regulation (see Fig. 6e and 6f). V-Jun may repress target gene expression by either upregulating a repressor or downregulating an activator. A fifth mechanism may involve reduction of transactivation potential of an activator by v-Jun. These mechanisms are not exclusive. V-Jun may employ more than one mechanism to repress target gene transcription.

A large part of gene regulation is governed by sequence-specific transcription factors, which bind to cis-acting elements located within the promoter region of responsive genes. As a first step to investigate the molecular mechanism by which Jun functions as a repressor on apoA-I gene expression, the cis-acting element(s) in the apoA-I promoter region that mediate the repression by v-Jun were mapped.

Initial deletion analysis

We have shown previously that overexpression of v-Jun in CEFs represses apoA-I gene transcription (Fig. 3) (75). To determine whether v-Jun regulates apoA-I at the level of promoter activity, a genomic clone of apoA-I from a chicken EMBL3 genomic library (Clontech) was isolated. A genomic DNA fragment that extends to −6800 bp has been fused to the gene for chloramphenicol acetyltransferase (CAT), and its activity has been assayed in CEF overexpressing v-Jun, or RCAS vector alone. Activity of the promoter-reporter construct decreased 10-fold in CEF overexpressing v-Jun as compared to that in control CEF infected with RCAS vector alone (Fig. 8, lane 1, both panels). In initial studies to map the negative regulatory element(s) responsive to v-Jun, a series of 5′-
deletion mutations of the chicken apoA-I gene fused to the CAT gene were generated, and the resulting plasmids were transiently transfected into CEF either infected with RCAS VJ-1 or RCAS vector alone (Fig. 7 and 8). Repression of the apoA-I promoter activity by v-Jun was determined by measuring the reporter gene CAT activities. The CAT activities of the plasmids containing -6000 bp to -311 bp upstream of the apoA-I gene transcription start site were at or below basal level even in control CEF infected with RCAS vector alone, suggesting that the apoA-I promoter is subject to complex positive and negative regulatory controls. Deletion to -311 bp upstream of the apoA-I transcription start site resulted in the reappearance of promoter activity in control CEF, but the promoter activity decreased dramatically in CEF infected with RCAS VJ-1 (Fig. 8, lane 6, both panels). Taken together, these results indicate that the v-Jun responsive element(s) is located within nucleotides -311 to +19 upstream of the apoA-I gene transcription start site.

Further mapping of the v-Jun responsive elements in the apoA-I promoter region

Initial deletion analysis located the v-Jun responsive elements in the chicken apoA-I promoter within nucleotides -311 to +19 upstream of the apoA-I gene transcription start site. Further deletion to -193 and -60 (Fig. 9 and Fig. 10, lane 1, 2, 3) resulted in almost total loss of promoter activity even in RCAS infected CEF. Because promoter activation was weak, it was difficult to determine any inhibitory effect by v-Jun. For this reason, a strategy to increase apoA-I promoter activity was developed.

During the process of the initial deletion analysis, we observed consistently elevated CAT activity in the plasmid containing -6800 bp upstream of the apoA-I transcription start site, but not in the plasmid containing -6000 bp upstream of the gene.
FIG. 7. Schematic representation of 5'-deletion mutations of the apoA-I promoter-reporter constructs. A series of 5'-deletions of the apoA-I promoter spanning from -6800 to -311 nucleic acids upstream of the gene transcription start site were fused to the chloramphenicol acetyltransferase (CAT) gene.
FIG. 8. Initial deletion analysis of the apoA-I promoter activity in response to infection with RCAS vector, or RCAS VJ-1 (v-Jun). A series of 5'-deletions of the apoA-I promoter were fused to the CAT gene and were transiently transfected into CEF either infected with RCAS vector or RCAS VJ-1. The cells were harvested 96 hours later and CAT activities were measured to determine the apoA-I promoter activity. Percent acetylation is shown numerically above each lane. Lane 1, -6800 CAT; lane 2, -6000 CAT; lane 3, -5000 CAT; lane 4, -3300 CAT; lane 5, -2500 CAT; lane 6, -311 CAT.
transcription site (Fig. 8). We speculated that there is an enhancer located within the 800 bp DNA fragment spanning from \(-6800\) bp to \(-6000\) bp upstream of the gene transcription start site.

This enhancer was inserted upstream in the forward orientation into three plasmids containing various 5’-deletions of the apoA-I promoter fused to the CAT gene (Fig. 9). The resulting plasmids were transiently transfected into CEFs either infected with retroviral vector RCAS or with RCAS VJ-1. The promoter activity was determined by measuring CAT reporter activity. Figure 9 shows the schematic representations of the apoA-I promoter reporter constructs used in transient transfection experiments. Insertion of the 800-bp homologous enhancer increased the activity of all three apoA-I promoter-reporters dramatically compared to the corresponding ones without the homologous enhancer (Fig. 10 and 11). Consistent with our previous results, the activated transcriptional activity of the construct containing the 330-bp apoA-I promoter region (positions \(-311\) to +19) is much higher than that of the constructs with further 5’-deletions (positions \(-193\) to +19 and \(-60\) to +19 respectively), suggesting the presence of a strong activating sequence within the \(-311\) to \(-193\) promoter region (Fig. 10, RCAS panel, lane 5, 6, 7). Repression of apoA-I promoter activity by v-Jun was observed in all the apoA-I promoter reporters containing nucleotides \(-311\) to +19, -193 to +19 and \(-60\) to +19 respectively (Fig. 10 and 11), suggesting that the v-Jun responsive element may lie within the first 60 nucleotides. However, the decrease in promoter activity is much more pronounced in the construct containing the \(-311\) to +19 promoter region (Fig. 11, lane 5, 6, 7). Deletion of the promoter from \(-311\) to \(-193\) reduced the magnitude of decrease in promoter activity, suggesting that the strong activating sequence located
FIG. 9. Further mapping of v-Jun responsive elements in the apoA-I promoter region — schematic representation of 5'-deletion mutations of the apoA-I promoter-reporter constructs with the enhancer element. The 800-bp enhancer element was inserted upstream in a forward orientation into three 5'-deletions of the chicken apoA-I promoter-reporter plasmids. An internal deletion construct was made with nucleotides -311 to -60 of the apoA-I promoter region deleted.
FIG. 10. Further mapping of v-Jun responsive elements in the apoA-I promoter region — autoradiograph of CAT assay of apoA-I promoter activity. 5 ug of the apoA-I promoter-reporter constructs without or with the enhancer element and the internal deletion constructs were transiently transfected into CEF either infected with RCAS vector (RCAS) or RCAS VJ-1 (v-Jun). The infected cells were harvested 96 hours later and CAT activity was assayed. 1. p-311.CAT; 2. p-193.CAT; 3. p-60.CAT; 4. p (-6800 to -311)-60.CAT; 5. p (-6800 to -6000)-311.CAT; 6. p (-6800 to -6000)-193.CAT; 7. p (-6800 to -6000) -60.CAT.
FIG. 11. **Further mapping of v-Jun responsive elements in the apoA-I promoter region** – quantitation of apoA-I promoter activity in CEF in response to infection of RCAS vector or RCAS V-1. 5 µg of a series of 5'-deletions of apoA-I promoter-reporter constructs without or with the 800-bp homologous enhancer and an internal deletion plasmid were transiently transfected into CEF either infected with RCAS vector (RCAS) or RCAS VJ-1 (v-Jun). The infected cells were harvested 96 hours later and CAT activity was examined. 1 µg of CMV-β-Galactosidase was cotransfected with the apoA-I promoter-reporter constructs in some experiments as internal transfection efficiency control. Results are represented as percentage of CAT acetylation. Data represents the average of at least three independent transfection experiments. Lanes are the same as that in the legends of Figure 10.
within the -311 to -193 promoter region might be involved in mediating apoA-I transcriptional repression by v-Jun as well. We reasoned that if the v-Jun responsive element lies within the first 60 nucleotides, deletion of the -311 to -60 promoter region would not affect decrease in promoter activity; otherwise, if the v-Jun responsive element lies somewhere within the -311 to -60 promoter region, deletion of this fragment would abolish v-Jun responsiveness of the promoter. To that end, an internal deletion was made. This internal deletion contains -6800-bp 5'-flanking region of the apoA-I with the promoter region -311 to -60 deleted. Deletion of nucleotides within -311 to -60 of the apoA-I promoter reduced promoter activity to below basal level, and no repression by v-Jun could be observed (Fig. 10, lane 4 in both panels).

Even though both CEF infected with vector and CEF infected with RCAS VJ-1 come from the same source of primary CEF, they were maintained separately after retroviral infection. The long infection cycle lasts for 10 to 14 days. It might be possible that v-Jun-induced transformation in CEF affects the ability of these cells to take up plasmid DNA in transient transfection assays. To determine whether transient transfection efficiency in CEF infected with vector is the same as that in CEF infected with RCAS VJ-1, plasmid CMV-β-Galactosidase was used to cotransfect CEFs along with the apoA-I promoter reporter constructs as an internal transfection efficiency control. Meanwhile, β-Gal staining experiments were also performed in control CEF infected with RCAS vector and CEF infected with RCAS VJ-1. Results from β-Galactosidase assays demonstrated that β-Galactosidase activity is similar in both control CEF and CEF overexpressing v-Jun. In addition, β-Gal staining results shown in Figure 12 indicate that there are similar numbers, if not more, of CEF which were stained blue in
FIG. 12. β-Gal staining of chicken embryo fibroblasts to determine transfection efficiency. Plasmid CMV-β-Galactosidase was used to cotransfect with the apoA-I promoter-reporter constructs into CEF either infected with RCAS vector or RCAS VJ-1. After 96 hours of infection, β-Gal staining experiments were performed on RCAS CEF or v-Jun CEF to determine the transient transfection efficiency of these cells.
CEF infected with RCAS VJ-1 compared to control CEF infected with RCAS vector. These results suggest that indeed, the decrease of apoA-I promoter activity in response to v-Jun overexpression is due to repression of apoA-I transcription by v-Jun. Taken together, these results indicate that there might be more than one responsive element scattered within nucleotides -311 to +19 of the apoA-I promoter. Repression of apoA-I transcription by v-Jun may be through interactions among trans-acting nuclear factors bound to these different cis-acting elements.

**The homologous enhancer does not mediate repression by v-Jun**

In the experiments above, we added a homologous enhancer to the apoA-I promoter-reporter constructs to increase promoter activity so that we could view repression by v-Jun. In order to make sure that this enhancer element was not contributing to v-Jun repression, we inserted it downstream in a forward orientation into the plasmid pCAT-promoter (Promega) (Fig. 13 and 14). pCAT-promoter constructs with or without the homologous enhancer were transiently transfected into CEF either infected with RCAS VJ-1 or RCAS vector alone. The CAT activities of the plasmids were assayed. For both plasmids, even though the plasmid with the enhancer has higher activity than the one without, their respective CAT activities were similar in either control CEF or CEF overexpressing v-Jun, suggesting that the activities of neither plasmid is affected by v-Jun overexpression. These results indicate that the enhancer does not mediate repression and the repression of apoA-I transcription by v-Jun is mediated through the apoA-I promoter region.
FIG. 13. The homologous enhancer does not mediate repression by v-Jun. The homologous enhancer was inserted downstream in a forward orientation into pCAT-promoter (Promega). The plasmids with or without the enhancer were transiently transfected into CEF either infected with vector (RCAS) or RCAS VJ-1 (v-Jun). The infected cells were harvested 96 hours later and CAT activity was assayed. (A), Schematic representation of pCAT-promoter without or with the chicken homologous enhancer. (B), Autoradiography of CAT assay.
FIG. 14. The homologous enhancer does not mediate repression by v-Jun – quantitation of CAT assay. 5 µg of pCAT-promoter plasmids with or without enhancer were transiently transfected into CEF either infected with RCAS vector (RCAS) or RCAS V-1 (v-Jun). The infected cells were harvested 96 hours later and CAT activity was assayed. Results are presented as percentage of CAT acetylation. The lanes in this figure are the same as that in the legend to Figure 13.
V-JUN REPRESSIONS APOA-I TRANSCRIPTION THROUGH A MECHANISM THAT DOES NOT INVOLVE DIRECT BINDING TO THE APOA-I PROMOTER

The deletion analysis described above suggested that the v-Jun responsive elements lie somewhere within the -311 to +19 promoter fragment, possibly within the first 60 nucleotides. Because our most dramatic effect was observed with the -311 to +19 promoter fragment, we next analyzed the protein-DNA interactions that occur on this region in the presence or absence of v-Jun overexpression. To do this, a series of gel mobility shift assays and supershift assays were performed.

No difference in DNA binding between control CEF or CEF overexpressing v-Jun when the apoA-I promoter region −311 to 19 was used as probe

The different promoter response to v-Jun overexpression could be due to a difference in binding to the apoA-I promoter or to a difference in transcriptional activation. To determine if there is any difference in DNA binding between nuclear proteins from CEFs either infected with RCAS vector or RCAS VJ-1, gel mobility shift assays were performed using the −311 to +19 promoter fragment as labeled probe. The nuclear protein binding was analyzed on a 6% acrylamide gel (Fig. 15). A wide complex was formed and the binding is specific, as the complex was completely competed by 100-fold excess of unlabeled probe but not by 100-fold excess of non-specific oligonucleotides. Yet there is no difference in the binding pattern between nuclear extract from RCAS CEF and RCAS VJ-1 CEF. These results suggest that there are nuclear factors in both RCAS-VJ-1 CEF and control RCAS CEF that specifically bind to the apoA-I promoter. The 330-bp fragment contains many potential transcription factor binding sites, as shown in figure 17. We see a wide smear that indicates binding by a
FIG. 15. Gel mobility shift analysis of the apoA-I promoter – 311 to +19. The apoA-I promoter –311 to +19 fragment was labeled as probe. 1 µg of labeled probe was used to incubate with 5 µg of nuclear extracts from CEFs either infected with RCAS vector or RCAS VJ-1 for 15 min on ice. 100-fold excess of unlabeled probe was added as specific competitor. 100-fold excess of unlabeled non-specific oligonucleotides was added as non-specific competitors (NC). The reaction mixtures were analyzed on 6% acrylamide gel. NS oligo: non-specific similar length oligonucleotides.
number of different transcription factors. With the probe of this size, we were not able to resolve any difference in DNA binding between RCAS VJ-1 CEF and control RCAS CEF under our experimental conditions.

**V-Jun does not bind to the apoA-I promoter**

To determine if v-Jun oncoprotein binds to the apoA-I promoter directly or if v-Jun is even present in the protein-DNA complexes formed between nuclear factors and the apoA-I promoter, supershift assays with anti-Jun antibody were performed. The rabbit polyclonal anti-Jun antibody is raised against c-Jun amino terminal residues 91 to 105. The -311 to +19 apoA-I promoter fragment was labeled as probe to incubate with nuclear extracts from RCAS VJ-1 CEF or control RCAS CEF. The anti-Jun antibody was added 15 minutes after the probe was added. The antibody was not able to supershift the specific binding in either RCAS VJ-1 CEF or control RCAS CEF (Fig. 16A). To determine the ability of the anti-Jun antibodies to supershift the specific binding where Jun is involved, supershift assays were performed using AP-1 consensus oligonucleotides as labeled probe to incubate with nuclear extracts from both RCAS-VJ-1 CEF and control RCAS CEF (Fig. 16B). AP-1 consensus probe elicited a strong protein-DNA binding. The binding is specific, as the binding was completely abolished by 100-fold excess unlabeled probe but not by 100-fold excess non-specific oligonucleotides. Addition of the specific anti-Jun antibody supershifted the AP-1 specific binding, suggesting that under our supershift experimental condition the specific anti-Jun antibody is able to supershift the protein-DNA complex when v-Jun is involved. Taken together, these results indicate that v-Jun neither binds to the apoA-I promoter directly nor is present in
FIG. 16. Supershift assays of the apoA-I promoter with anti-Jun antibody. The -311 to +19 apoA-I promoter fragments and AP-1 specific oligonucleotides were labeled as probes. 1 µg of labeled probes was used to incubate with 5 µg (A) or 16 µg (B) of nuclear extracts from CEF either infected with RCAS VJ-1 or RCAS vector for 15 min on ice. 2 µl rabbit polyclonal anti-Jun antibodies were added. The reaction mixtures were run on 6% acrylamide gel. A, Supershifts of the apoA-I promoter probe with anti-Jun antibody. B, Supershifts of AP-1 consensus oligo probe with anti-Jun antibody. Anti-Jun antibody: rabbit polyclonal anti-Jun antibody corresponding to Jun protein amino terminus (residues 91 to 105). NONE/SC: no antibody, only 100-fold excess of non-specific oligonucleotides as non-specific competitor.
the protein-DNA complexes, and that v-Jun modulates apoA-I gene expression through an indirect mechanism.

**Putative binding sites for known transcription factors within the apoA-I promoter region**

DNA sequences of the chicken apoA-I promoter nucleotides -311 to +21 upstream of its transcription start site have been previously reported (118,119). Computer analysis of the DNA sequences revealed that there are many putative binding sites for known transcription factors located within this promoter region (Fig. 17). Yet, surprisingly, no AP-1 consensus site was found in this region. Among these putative binding sites, EGR-1, HNF-5, Sp1, CTCF, GCF, and AP-2 sites are of particular interest. Early growth response factor 1 (EGR-1), which is transiently expressed in various pathophysiologic states of the liver, was found to activate the apoA-I expression and overcome ARP-1-mediated repression in HepG2 cells (142). It is also involved in the induction of human apoA-I gene expression by the experimental nephritic syndrome in transgenic mice (143). HNF-5 is a liver-enriched nuclear factor and its binding sites are found to be located close to those of other liver-specific factors (144). Sp1 is a ubiquitous transcription factor frequently associated with promoter activation. AP-2 has been shown to be either a transcriptional repressor (145) or an activator (146). Some studies have shown that CpG methylation inhibits proenkephalin gene expression by blocking AP-2 binding (139). CTCF was first identified as a zinc-finger nuclear factor binding to CCCTC-motif within oncogene c-myc promoter (147). Its amino acid sequences are highly conserved among species, with chicken and human CTCF sharing 93% homology (148). It was found to repress transcription of several genes, including
FIG. 17. **DNA sequences of the chicken apoA-I gene promoter.** Potential binding sites for transcription factors are shown. CAAT box and TATA box are underlined. EGR-1: early growth response factor 1; GATA-1: erythroid transcription factor. GCF: epidermal growth factor negative regulator. MTF-1: metallothionein (MT) factor 1; CTCF: CCCTC-binding factor.
c-myc (148) and lysozyme (149). Recently, studies have shown that CTCF binds to chicken beta-globin 42 bp insulator for enhancer blocking activity in human cells and it is suggested that directional enhancer blocking by CTCF is a conserved component of gene regulation in vertebrate (150). GC factor (GCF) is reported to be a transcriptional regulator that binds to specific GC-rich sequences in the epidermal growth factor (EGF) receptor gene promoter and represses its transcription (151,152). Studies also showed that GCF represses transcription of other growth factor/receptor genes (153,154).

**Multiple nuclear factors from CEF bind to the overlapping oligonucleotides from the apoA-I promoter but the protein-DNA interactions are not affected by v-Jun overexpression**

The apoA-I promoter region contains clusters of putative binding sites for many transcription factors. We speculated that there are many nuclear proteins from CEF nuclear extracts binding to the apoA-I promoter thus masking any difference in DNA binding in response to v-Jun overexpression when the long DNA fragment of the 330-bp apoA-I promoter was used as a probe. To further investigate DNA binding properties of nuclear proteins from RCAS VJ-1 CEF compared to that from control CEF, six overlapping oligonucleotides encompassing the 330-bp apoA-I promoter region were synthesized and individually used in gel mobility shift analysis. Fig. 18A shows schematic representations of these oligonucleotides. This approach was designed to examine thoroughly the DNA binding properties of nuclear factors in CEF to the apoA-I promoter and unveil differences in DNA binding, if there is any, between CEF infected with RCAS-VJ-1 and control CEF infected with vector RCAS.
FIG. 18. Gel mobility shift analysis of oligonucleotides -311 to -244 of the apoA-I promoter. Six oligonucleotides were made to encompass the 332-bp long chicken apoA-I gene promoter region (positions -311 to +21). 1ml of each labeled oligo was used as probe to incubate with 16 mg of nuclear extracts from CEFs either infected with RCAS VJ-1 or RCAS vector for 15 min on ice. 100-fold excess unlabeled oligonucleotides were added as competitors. A, Schematic representation of oligonucleotides encompasses the apoA-I promoter region -311 to +21. B, Gel mobility shifts of oligonucleotides -311 to -244.
When oligonucleotides −311 to −244 were used as labeled probes in gel shift assays, an intense complex was formed (Fig. 18B). The binding is specific as it was completely competed by 100-fold excess unlabeled probe. The −311 to +19 promoter fragment was also able to abolish the band completely. AP-1 specific competitor was not able to compete for the band, again suggesting that transcription factor complex AP-1 is not involved in binding to this fragment. No differences in DNA binding are observed between RCAS-VJ-1 CEF and control RCAS CEF.

When oligonucleotides −251 to −192 were labeled and used as probes, one intense complex was formed in both RCAS-VJ-1 CEF and control CEF (Fig. 19A). The binding is specific as the complex was completely competed by 100-fold excess unlabeled probe. AP-1 specific oligonucleotides were not able to compete for the binding in either RCAS CEF or VJ-1 CEF. Again, no differences in DNA binding between control CEF and CEF overexpressing v-Jun were observed.

When oligonucleotides −211 to −142 and oligonucleotides −41 to +21 were labeled as probes, one specific complex was formed in each gel shift assay (Fig. 19B and Fig. 19D). AP-1 specific oligonucleotides were not able to compete for the specific binding and no differences in DNA binding between RCAS VJ-1 CEF and control RCAS CEF were observed. When oligonucleotides −91 to −26 were labeled as probe, no distinctive band was formed and no differences in DNA binding between RCAS-VJ-1 CEF and control RCAS CEF were observed (Fig. 19C). Taken together, these results suggest that there are multiple nuclear factors from CEF that bind to the apoA-I promoter, but the protein-DNA interactions are not affected by v-Jun overexpression.
FIG. 19. Gel mobility shift analysis of apoA-I promoter oligonucleotides -251 to -192, -211 to -142, -91 to -26, and -41 to +21. 1 µl of labeled oligonucleotides were used as probes to incubate with 16 µg of nuclear extract from CEFs either infected with VJ-1 or RCAS vector for 15 min on ice. 100-fold excess of unlabeled nucleotides were added as competitors.  

A, Gel shifts of oligonucleotides -251 to -192.  
B, Gel shifts of oligonucleotides of -211 to -142.  
C, Gel shifts of oligonucleotides -91 to -26.  
D, Gel shifts of oligonucleotides -41 to +21.  
NS OLIGO: non-specific oligonucleotides.
AP-1 specific oligonucleotides compete for the nuclear factors that bind to the apoA-I oligonucleotides -156 to -85

More gel shift assays were performed with the apoA-I oligonucleotides -156 to -85 as labeled probe. This fragment contains putative binding sites for EGF negative regulator GCF, early growth response factor 1 (EGR-1), a region that is homologous to mammalian apoA-I enhancer element C (site C), and a CAAT box (shown in Fig. 20A). In the gel shift assay shown in Fig. 20B, a weak complex was formed in both control CEF and CEF overexpressing v-Jun. The binding is specific, as the complex was completely competed by 100-fold excess unlabeled probe. AP-1 specific oligonucleotides were able to compete for the specific binding completely in RCAS VJ-1 CEF, and to a lesser degree, in control RCAS CEF, suggesting that AP-1 specific oligonucleotides somehow interfere the protein-DNA interaction between the nuclear factor and the apoA-I promoter oligonucleotides -156 to -85 in CEF overexpressing v-Jun. HNF-3 specific oligonucleotides, Sp1 specific oligonucleotides and similar length non-specific oligonucleotides were not able to compete for the specific binding. These results suggest two possibilities. First, the nuclear factors in VJ-1 CEF that bind to the apoA-I promoter oligonucleotides also recognizes the AP-1 consensus site, even though there is no AP-1 consensus binding site within the apoA-I promoter fragment. Second, v-Jun interferes with the binding of the nuclear factor to the apoA-I promoter -156 to -85.

BOTH DNA BINDING SPECIFICITY AND HETERODIMERIZATION OF V-JUN CONTRIBUTE TO ITS REPRESSOR ACTIVITY

To further elucidate the molecular mechanisms by which v-Jun oncoprotein represses apoA-I gene transcription, a structure-and-function approach was developed to
FIG. 20. **Gel mobility shift analysis of the apoA-I promoter oligonucleotides -156 to -85.** 1 mg of labeled oligonucleotides -156 to -85 were used as probes to incubate with 16 mg of nuclear extracts from RCAS v-Jun CEF or control CEF for 15 min on ice. 100-fold excess of unlabeled oligonucleotides was added as competitors. The reaction mixtures were run on 6% acrylamide gel. A, DNA sequences of oligonucleotides -156 to -85. Some putative binding sites for known transcription factors, CAAT box and TATA box are shown. Site C: a homologous region to human apoA-I enhancer element C. B, Gel shifts of oligonucleotides -156 to -85. NS oligo: non-specific oligonucleotides. HNF-4, AP-1 and Sp1 oligonucleotides: oligonucleotides contain known or consensus binding sites for these transcription factors, respectively.
investigate systematically the biochemical requirement for repression of apoA-I transcription by v-Jun.

The functional and structural domains of v-Jun have been well characterized. The structural domains include: (1) A leucine zipper motif located at the carboxyl terminus, (2) A basic DNA binding domain immediately adjacent to the leucine zipper domain at carboxyl terminus, and (3) A transactivation domain located at the amino-terminus of the protein (see Fig. 1 and 2). Three well-defined biochemical functions of Jun are assigned to the structural domains: the ability to dimerize to the leucine zipper domain; the ability to bind to a specific DNA sequence to the basic DNA binding domain; and the ability to activate or repress a responsive gene to the transactivation domain.

Our previous results indicate that v-Jun does not bind to the apoA-I promoter directly and that v-Jun is not even present in the protein-DNA complexes formed between CEF nuclear factors and the apoA-I promoter. These results suggest that v-Jun represses apoA-I transcription through an indirect mechanism. Several possible indirect mechanisms include protein-protein interactions, promoter modification, secondary gene expression regulation, and reduction of transactivation potential of an activator (Fig. 6).

The protein-protein interaction mechanism involves competition for binding of activators or competition with activators for a limiting common coregulator or general transcription factors by v-Jun. Methylation of the apoA-I promoter by v-Jun overexpression may result from upregulation of methyltransferase, which indicates that the DNA binding and transactivation ability of v-Jun would be necessary for repression. Similarly, the DNA binding specificity and transactivation activity of v-Jun will be absolutely necessary for upregulation of a repressor or downregulation of an activator of
apoA-I transcription by v-Jun through secondary gene regulation mechanisms. To sort out some of these possibilities, a panel of v-Jun mutants was selected.

A schematic representation of various v-Jun mutants is shown in Figure 21. The transactivation domain of v-Jun encompasses the major part of the amino-terminus. To determine the role played by the v-Jun transactivation domain in its repressor activity, two amino-terminal mutants were selected. Mutant VJ-3 has a portion of its amino-terminal transactivation domain (108 amino acids) deleted. This deleted region contains acidic region I and II that are important for Jun transactivation activity. Mutant VJΔNco has 128 amino acids of the v-Jun amino terminus deleted. This region contains all three independent acidic regions I, II, and III that are important for Jun transactivation.

Dimerization is a prerequisite for Jun to function. Dimerization is mediated through a leucine zipper region near the carboxyl terminus. Jun can form dimers with Fos, ATF/CREB family members as well as with other members of the Jun family. Previous studies have shown that the Jun homodimer is sufficient to induce cell transformation and that heterodimerization with Fos- or Fos-related proteins is not necessary (134). To examine the ability of v-Jun to repress apoA-I transcription in CEF without the interaction with other AP-1 family members, mutant VJGLZ was selected. The leucine zipper dimerization domain of this mutant has been replaced with that of the yeast transcription factor GCN4 (134). GCN4 is a bZIP protein that can only homodimerize (134). The resulting chimeric protein VJGLZ retains all of the DNA binding and transcriptional activation domains of v-Jun but is incapable of forming heterodimers with other bZIP proteins but itself.
FIG. 21. Structure-and-function analysis of v-Jun functional domain required for its repressor activity-schematic representation of v-Jun and various v-Jun mutants. Retroviruses containing wild-type V-Jun and various v-Jun mutants along with control vector RCAS were used to infect CEFs.
Jun is a nuclear protein with specific DNA binding activity. This DNA binding specificity is mediated by the basic domain adjacent to the leucine zipper region in the carboxyl-terminus of the protein. To determine how the specific DNA binding ability of v-Jun contributes to its repressor activity, an available mutant with altered DNA binding specificity was selected. Mutant VJ/C/EBP has its DNA binding specificity altered from the Jun DNA binding consensus sequences (TGACTCA) to that of liver-enriched transcription factor C/EBP (ATTGCAGAAT) (Bos, et al., in preparation). The rest of the protein is kept intact and its biological functions as well as its altered DNA binding specificity have been tested (Bos, et al., in preparation). Finally, a double mutant, VJGLZ/C/EBP, which has both altered DNA binding specificity and limited dimerization ability, was also selected (Bos, et al., in preparation).

Retroviruses carrying these mutated v-Jun sequences as well as wild-type VJ-1 alone with control RCAS vector were used to infect primary CEFs. The infected CEFs were harvested 13 days after infection. Total RNA was isolated and northern blot analysis was performed to examine the endogenous apoA-I mRNA expression levels (Fig. 22). Consistent with our previous results, overexpression of wild-type oncoprotein VJ-1 represses apoA-I transcription 5- to 10-fold in CEF as compared to that in control CEF (Fig. 22, lane 1 and 2). Overexpression of mutant VJGLZ resulted in 50% reduction of apoA-I transcription as compared to control CEF, suggesting that heterodimerization of v-Jun with other AP-1 family members contributes to the repression, but is not required (Fig. 22, lane 1 and 3). Overexpression of mutant VJ/C/EBP did not have any effect on apoA-I gene transcription, suggesting that mutant VJ/C/EBP lost its repressor activity and that the DNA binding specificity of v-Jun is absolutely required for its
repressor activity (Fig. 22, lane 4). Overexpression of double mutant VJGLZ/C/EBP did not repress apoA-I transcription when compared to that of the control. Actually, it even had a slightly stimulatory effect on apoA-I gene transcription (Fig. 22, lane 5). This result suggests that the DNA binding specificity of v-Jun and its heterodimerization ability with other AP-1 family members contribute synergistically to repression of apoA-I transcription by v-Jun. Overexpression of mutant VJ-3, which has 108 amino acids at the amino terminus that contain acidic region I and II deleted, represses apoA-I gene transcription to a similar level as wild type VJ-1 does, suggesting that acidic region I and II of v-Jun transactivation domain are not required for repressor activity of v-Jun. Overexpression of mutant VJ5Nco, which has 128 amino acids at the amino terminus domain that contains all three acidic regions deleted, did not have significant effect on apoA-I gene transcription, suggesting that independent acidic region III of v-Jun transactivation domain located between amino acid residues 105 to 120 is important for repression. To confirm that all v-Jun mutants were properly expressed, total cellular proteins of infected CEFs were isolated and Western blot analysis probed with various anti-Jun antibodies were performed (Fig. 23). Taken together, these results indicate that both v-Jun DNA binding specificity and its heterodimerization ability with other AP-1 family members are required for repression of apoA-I transcription, while DNA binding specificity of v-Jun is crucial for the function, heterodimerization plays a minor role. Furthermore, the independent acidic region III of v-Jun transactivation domain is important for its repressor activity.
FIG. 22. Structure-and-function analysis of v-Jun functional domain required for its repressor activity. Northern blot analysis of the endogenous apoA-I mRNA levels in response to infection with v-Jun and various v-Jun mutants. Retroviruses containing wild-type VJ-1 and various v-Jun mutants along with control vector RCAS were used to infect CEFs. 13 days after infection, total cellular RNA was isolated and Northern blot analysis with the chicken apoA-I gene as probe was performed. Lane 1, RCAS vector; lane 2, VJ-1; lane 3, VJGLZ; lane 4, VJ/C/EBP; lane 5, VJGLZ/C/EBP; lane 6, VJ-3; lane 7, VJ8Nco.
FIG. 23. Structure-and-function analysis of v-Jun functional domain required for its repressor activity-Western blot analysis of v-Jun and various v-Jun mutants protein expression. Retroviruses containing wild-type VJ-1 and various v-Jun mutants along with RCAS vector were used to infect CEF. 13 days after infection, cell lysates were harvested and Western blot analysis were performed to determine the expression levels of v-Jun and v-Jun mutants. The same blot was first probed with anti-Jun antibody Ab-1, then reprobed with anti-Jun antibody Ab-2. Ab-1 is rabbit polyclonal antibody raised against c-Jun DNA binding domain (residues 209 to 225) (Oncogene Science). Ab-2 is rabbit polyclonal antibody raised against c-Jun amino terminus (residues 91 to 105) (Santa Cruz).
IDENTIFICATION AND CHARACTERIZATION OF AN ENHANCER IN THE APOA-I 5'-FLANKING REGION

Presence of Enhancer Sequences in the ApoA-I 5'-Flanking Region

In our previous work, we generated a series of 5'-deletion mutations of the chicken apoA-I gene fused to the chloramphenicol acetyltransferase (CAT) gene and transfected these resulting plasmids into primary chicken embryo fibroblasts (CEFs). We observed consistently elevated CAT activity in the plasmid containing 6.8 kb of DNA upstream of the chicken apoA-I gene but not in the plasmid containing 6.0 kb of DNA upstream of the gene (see Fig. 8). We speculated that there is an enhancer located within the 800 bp DNA fragment spanning from −6.8 kb to −6.0 kb upstream of the gene. We have actually used this region as an enhancer of apoA-I promoter activity in figure 9 and figure 10 and found it to function in a distance-independent manner. Because this region is so far upstream, it has not been previously characterized.

To further characterize this enhancer sequence in the 5'-flanking region of the chicken apoA-I gene, we inserted the 800-bp DNA fragment upstream in both forward and reverse orientations into the plasmid containing 330 bp (positions -311 to +19) of the chicken apoA-I gene promoter region (Fig. 24). More than 9 to 10-fold increase of the chicken apoA-I promoter transcriptional activity was observed when the fragment was inserted in the reverse orientation, and nearly a 9-fold increase was observed when the fragment was inserted in the forward orientation (Fig. 24A and 24B). These results demonstrate that the 800-bp DNA fragment exhibits true enhancer activity as defined by distance- and orientation-independent stimulation of promoter activity.
FIG. 24. Enhancer activity in the chicken apoA-I gene. Fragment spanning -6.8 kb to -6.0 kb upstream of the transcription start site of the apoA-I gene was assayed for enhancer activity. The fragment was inserted in both orientations upstream into the -311 to +19 fragment of the apoA-I promoter, which was fused to the CAT gene. (A), left, schematic representations of the constructs. Right, results of transient transfection experiments with CEF. CEFs were transfected with equal amounts of the indicated plasmids together with CMV-b-galactosidase as a control for transfection efficiency, and CAT activity was measured in cell lysates after 48 h. Results are expressed as a -fold increase relative to the control promoter p-311/19.CAT and represent the mean and S.E. of at least three independent transfections. (B), Autoradiograph of one of representative CAT assays.
The enhancer sequence is capable of stimulating transcriptional activity of heterologous promoters

The previous experiments demonstrate that the 800-bp enhancer is capable of stimulating transcription from the homologous chicken apoA-I promoter in a distance- and orientation-dependent manner in CEF. Next, to determine if the enhancer is also capable of stimulating transcriptional activity of heterologous promoters, the 800-bp fragment was inserted in both orientations downstream of the SV40 early promoter of pCAT-promoter plasmid (Promega) and upstream in both orientations of the human apoA-I gene minimal promoter (positions -41 to +234) (Fig. 25). The enhancer activity was examined by measuring CAT activities after transiently transfecting the resulting plasmids into CEF. The fragment enhanced transcriptional activity of the SV40 early promoter modestly but reproducibly by nearly 2-fold when inserted in the forward orientation, but did not have any effect when inserted in the reverse orientation (Fig. 25A). Interestingly, opposite results were obtained for the human apoA-I minimal promoter. The fragment did not have any noticeable effect on transcriptional activity of the minimal promoter when inserted in the forward orientation, but enhanced the transcription of the human apoA-I minimal promoter nearly 2-fold when inserted in the reverse orientation (Fig. 25B). These results show that the enhancer sequence is capable of stimulating transcription mediated by both homologous and heterologous promoters. The differences in magnitude of stimulation of transcriptional activity by the enhancer between the chicken apoA-I promoter and heterologous promoters may be due to the high basal activity of SV40 promoter and the human minimal apoA-I promoter. Another
FIG. 25. Enhancer activity mediated through heterologous promoters in CEF. The 800 bp enhancer was inserted in both orientations either downstream of the SV40 promoter or upstream of the human apoA-I minimal promoter (positions -41 to +234) to determine its effect on transcriptional activities of heterologous promoters. Transfection experiments were carried out under the same conditions as those presented in Fig. 24. (A), the enhancer downstream of the SV40 promoter. (B), the enhancer is upstream of the human apoA-I minimal promoter.
possibility is that there may be some specific interactions between the factors bound to the enhancer and the factors bound to the chicken promoter.

The enhancer appears to be cell type-specific

To determine if the enhancer element is tissue- or cell-specific, the constructs containing either the chicken apoA-I promoter, the human apoA-I minimal promoter, or the SV40 promoter, with or without the 800 bp enhancer, were transiently transfected into either human hepatic carcinoma HepG2 cells, in which the apoA-I is predominantly expressed, or mouse fibroblast NIH 3T3 cells, in which the apoA-I gene is not expressed. In neither the HepG2 cells nor the NIH 3T3 cells was the enhancer able to stimulate transcription from any of the promoters tested (Fig. 26). In fact, the enhancer exhibited an inhibitory effect on all the promoters in an orientation-independent manner in HepG2 cells. The inhibitory effect in HepG2 cells was overridden by the strong SV40 enhancer, as our data show that the constructs containing both SV40 promoter and SV40 enhancer with or without the 800-bp enhancer had similar CAT activities. In NIH 3T3 cells, the activities of both the chicken apoA-I promoter and the human apoA-I minimal promoter were inhibited by the enhancer in an orientation-independent manner. For the SV40 promoter, the inhibitory effect of the enhancer was observed only when the 800 bp fragment was inserted in the reverse orientation but not when it was inserted in the forward orientation. These results indicate that the activity of the chicken apoA-I enhancer is clearly cell type-specific. The inhibition of transcriptional activities of the chicken apoA-I promoter and the human apoA-I minimal promoter suggests that both cis- as well as trans- factors may be involved in the regulation of the enhancer activity.
FIG. 26. The cell specificity of the enhancer. The plasmid constructs containing the chicken apoA-I promoters (A), the SV40 promoter (B), the human apoA-I minimal promoter (C), or the SV40 promoter with the SV40 enhancer (D), with or without the 800 bp enhancer were transiently transfected into either HepG2 cells or mouse fibroblast NIH 3T3 cells. The transfection experiments were carried out under the same conditions described in Fig. 24. Results are represented as percentage relative to each individual control plasmid (those without the enhancer) respectively and represent the mean and S.E. of at least two independent transfections. * These experiments were only done once.
Sequence Information of the Enhancer

To further characterize the enhancer, we cloned the 800bp DNA fragment into the pBluescript SK(+) plasmid (Invitrogen) and sequenced it using an automatic sequencer. The full-length enhancer is 791 bp long (Fig. 27). Computer analysis of the full-length enhancer revealed that there are many potential binding sites for known nuclear transcription factors. Some liver-enriched nuclear factors such as HNF-3, HNF-4, CAAT/enhancer binding protein (C/EBP) and the ubiquitous Sp-1 transcription factor are of particular interest. Liver-enriched nuclear factors HNF-3, HNF-4, and C/EBP have been shown to regulate human apolipoprotein gene expression (81,83,88,155). The direct protein-protein interaction between self-associated ubiquitous transcription factor Sp1 and the resulting DNA looping mechanism is known to account for many enhancer functions (135,136). It was previously observed that the distantly located Sp-1 site functions in synergism with the promoter-proximal Sp-1 site to strongly activate transcription in vivo. Using conventional and scanning transmission electron microscopy, these studies demonstrate that this synergism is likely to be a direct consequence of interactions between remote and local Sp1, the remote Sp1 translocated to the promoter by a DNA loop. Scanning transmission electron microscopy shows that Sp1 initially forms a tetramer and subsequently assembles multiple tetramers stacked in register at the DNA loop juncture (135).

Localization of the Enhancer Activity

To further delineate the activating elements in the full length enhancer of the chicken apoA-I gene, a series of deletions of the 791 bp enhancer were made. These are shown in Figure 28. One set of deletions extends from the 5’- to the 3’-end of the
FIG. 27. **The nucleotide sequence of the 800 bp enhancer.** The 800 bp enhancer was cloned into plasmid pBluescript SK(+) and was sequenced. The full-length enhancer is 791 bp long. Computer analysis revealed that there are many potential binding sites for known transcription factors. Underlined are four oligonucleotides designed to cover some important potential binding sites and were used as probes in the subsequent gel mobility shift assays. Some potential sites are shown.
enhancer (1 to 5). A second set of deletions extends from the 3' to the 5'-end of the enhancer (6 to 9). Two internal fragments were also made (10 and 11). Each of the fragments was inserted in both orientations upstream of the chicken apoA-I promoter (positions -311 to +19). The resulting plasmids were transiently transfected into CEFs. The results are presented in Figure 28 as CAT activities of these deletion constructs relative to that of the plasmid with full-length enhancer, whose activity is set at 100%. The basal level CAT activity, the transcriptional activity by the promoter in the absence of enhancer stimulation, is 10% of the full-length enhancer.

Deletion of the sequences between -6135 to -6010 relative to the apoA-I transcription start site (designated 1), which contains a consensus C/EBP site, a AP-2 site, two TCF sites, a Est-1 site, a Myb site, and two GATA-1 sites, had reduced the relative CAT activity in either orientation more than 50% as compared with the full length enhancer. But the deleted fragment alone (designated 9) had barely detectable CAT activity in the reverse orientation and had no change in the forward orientation. This indicates that transcription factor C/EBP or other transcription factors located in this region may play an important role for the enhancer activity, but by itself is not sufficient. When the 791-bp fragment was further truncated to position -6250 (designated 2), the relative CAT activity was slightly increased in the forward orientation but did not change in the reverse orientation. The relative CAT activity of the corresponding 5'-deletion containing sequence -6010 to -6250 (designated 8) increased 70% when inserted in the forward orientation and did not change in the reverse orientation. This indicates that the sequence between -6250 to -6135, which contains an HNF-3-like site, a GATA-1 site, and an AP-2 site may contribute positively to the enhancer function in an orientation-
dependent manner. Further truncations to positions –6406 (designated 3), –6557 (designated 4), and –6629 (designated 5) in the series of 3’-deletions did not have any significant effect on relative CAT activity in CEFs compared to the previous deletion, regardless of orientation. In contrast, elongation of the corresponding 5’-deletions had a significant effect on relative CAT activity. Extending the sequence from –6250 to –6406 (compare 5’-deletions 7 and 8) not only fully restored CAT activity when compared to the full-length enhancer, but the relative CAT activity of 5’-deletion 7 is 70% higher than that of the full-length enhancer when inserted in the reverse orientation. The extension did not have any effect on CAT activity when inserted in the forward orientation. These results suggest that the sequences between –6250 to –6406, which contain a consensus Sp1 site, contain all necessary positive elements for the enhancer function in the reverse orientation but not in the forward orientation. Further elongation of the 5’-deletion to position –6629 (designated 6) resulted in even stronger relative CAT activity when inserted in the forward orientation, which is nearly 150% higher than that of the full-length enhancer. When inserted in the reverse orientation, the relative CAT activity of deletion 6 is only about 50% of that of the full-length enhancer. These data suggest that the sequences between –6629 and –6010 contain all necessary activating elements that are crucial for the full-length enhancer activity in the forward orientation but not in the reverse orientation. The data also suggest that the sequences between –6629 to –6406, which contain putative binding sites for transcription factors HNF-4, NF-IL6, AP-2, SIF, GATA-1, and E2A, may play a crucial role for the enhancer stimulation. To determine how this fragment contributes to the enhancer function, a fragment containing only the sequence between –6629 and –6406 was made and inserted in either orientation upstream
of the chicken apoA-I promoter p-311/19.CAT. Surprisingly, the relative CAT activity of the resulting construct is below basal level regardless of orientation when it was transiently transfected into CEFs. This suggests that the sequences between −6629 and −6406 by itself have no stimulatory effect on transcriptional activity of the chicken apoA-I promoter. Further truncation of the 5’-end to position -6557 restored 100% of full-length enhancer activity when inserted in the reverse orientation and about 80% of the full-length enhancer activity when inserted in the forward orientation. This result clearly suggests that there are strong negative regulatory sequences located within the sequences between −6629 to −6406 and that regulation of the enhancer function is complex, involving many positive as well as negative modules of the cis-acting elements, and many trans-acting transcription factors bound to these cis-acting elements.

**Analysis of protein-DNA interaction: possible involvement of the liver-enriched Transcription factor C/EBP**

To determine which nuclear transcription factors may be involved in regulation of the enhancer function, a series of gel mobility gel shift assays were performed. Four oligonucleotides (designated I to IV) (Fig. 27) were designed as probes based on deletion analysis of the enhancer experiments. These oligonucleotide probes contain putative binding sites for some liver-enriched nuclear factors and some ubiquitous transcription factors that are known to be involved either in mammalian apoA-I gene regulation or are important for enhancer function. Table I summarizes the oligonucleotide sequences and similarities of these sequences to the consensus recognition sequence for various transcription factors. To identify and confirm the transcription factors that bind to the oligonucleotide probes, double-stranded oligonucleotides that represent known strong
FIG. 28. **Deletion analysis of the enhancer activity.** Relative CAT activity represents CAT gene expression for at least three independent transfection experiments. The transfection experiments were carried out under the same conditions as described in Fig. 24. All activities are reported relative to the activity of the full 791-bp enhancer (100%) and S.E. At the top are shown potential binding sites for known transcription factors, based on computer analysis. These sites all represent similarities to known binding sites, though some deviate from consensus sequences. The sites provide a basis for selecting deletions and designing further experiments, but do not represent identification of actual sites. F=Forward orientation. R=Reverse orientation.
TABLE I

Similarities of the sequences of four oligonucleotides in the enhancer of the chicken apoA-I gene to the consensus binding sites for various known transcription factors.

Consensus sequences for vertebrate-encoded transcription factors [174] were used to create a database. The full-length enhancer 791-bp was computer-analyzed. Among more than 50 sites identified, only most significant sequence similarities are shown.

<table>
<thead>
<tr>
<th>Source of sequence (Ref)</th>
<th>Comparative sequence</th>
<th>Oligo</th>
<th>Apo A-I location</th>
<th>Transcription factor</th>
<th>Bases matched</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I Consensus [174]</td>
<td>GTGGGAAAG</td>
<td>I</td>
<td>-6130 to -6123</td>
<td>C/EBP</td>
<td>8/8</td>
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<td></td>
<td>GTGG/AT/AT/AT/AG</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ApoA-I Consensus [174]</td>
<td>CTGGAAAA</td>
<td>I</td>
<td>-6130 to -6125</td>
<td>H-APF-1</td>
<td>7/7</td>
</tr>
<tr>
<td></td>
<td>CTGGRAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I Consensus [174]</td>
<td>TGATGT</td>
<td>I</td>
<td>-6149 to -6134</td>
<td>GATA-1</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>T/AGATAR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I Consensus [174]</td>
<td>TTTGGGTTTTGT</td>
<td>II</td>
<td>-6161 to -6151</td>
<td>HNF-3</td>
<td>9/11</td>
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<td></td>
<td>TATTGAC/TTTA/TG</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ApoA-I Consensus [174]</td>
<td>GGGCGG</td>
<td>III</td>
<td>-6394 to -6389</td>
<td>Sp-1</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>GGGCGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I Consensus [174]</td>
<td>GCGGCCC</td>
<td>III</td>
<td>-6392 to -6386</td>
<td>GCF</td>
<td>7/7</td>
</tr>
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<td></td>
<td>C/GCGC/GC/GC/GC/GC</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>ApoA-I Consensus [174]</td>
<td>GGCAAGGGAAGGC</td>
<td>IV</td>
<td>-6485 to -6473</td>
<td>HNF-4</td>
<td>9/12</td>
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<td></td>
<td>G/TGCA/TAA/GGT/GC/TCAC/T</td>
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<tr>
<td>ApoA-I Consensus [174]</td>
<td>CCCCCGTCC</td>
<td>IV</td>
<td>-6492 to -6485</td>
<td>AP-2</td>
<td>7/8</td>
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<tr>
<td></td>
<td>CCCA/CNG/CG/CG/C</td>
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<tr>
<td>ApoA-I Consensus [174]</td>
<td>TGCGCTC</td>
<td>IV</td>
<td>-6468 to -6462</td>
<td>MEP-1/MTF-1</td>
<td>7/7</td>
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<tr>
<td></td>
<td>TGCRNC</td>
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<td></td>
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</table>

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### TABLE II

*Specific oligonucleotide competitors [173]*

Double-stranded oligonucleotides were constructed to represent known strong binding sites for specific individual transcription factors (upper case) with an additional
4-base sticky end (lower case).

<table>
<thead>
<tr>
<th>Factors</th>
<th>Oligonucleotide</th>
<th>Gene and Location</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBP</td>
<td>tcgtTTTTCCATCTTACTCAACATCCTCC AAGGTAGAATGAGTTGTAGGAGGtgct</td>
<td>Murine transthyretin enhancer – 1890 to – 1866</td>
<td>[167, 168]</td>
</tr>
<tr>
<td>HNF-3</td>
<td>tcgtAGTGACTAAGTCAATAATCAGAAT ACTGATTCAGTTATTAGTCTTAGTCTcgt</td>
<td>Murine transthyretin promoter – 111 to –85</td>
<td>[166]</td>
</tr>
<tr>
<td>SP1</td>
<td>tcgtGGGGCGGGGCG CCCGCCCCGtgct</td>
<td>Consensus</td>
<td>[175]</td>
</tr>
<tr>
<td>HNF-4</td>
<td>tcgtCCCTAGGCAAGGTTCATATGGCC GGATCGCTTCCAAAGTATACCGGtgct</td>
<td>Murine transthyretin promoter – 156 to –138</td>
<td>[176]</td>
</tr>
<tr>
<td>AP-1</td>
<td>ACCCAGGGATCCTCTAGAATGACTCATCGG TACTGAGTAGGTACGGACGTCCGTACGTTC</td>
<td>Consensus</td>
<td>[3]</td>
</tr>
</tbody>
</table>

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binding sites for individual transcription factors Sp1, HNF-3, HNF-4, and C/EBP were also synthesized as specific competitors. Table II summarizes the sequences and references for these specific competitors.

Radiolabeled double-stranded nucleotides, Oligo I, containing a putative binding site for liver-enriched transcription factor C/EBP (Fig. 29A and Table I) were incubated with the nuclear extracts from CEFs, and nuclear protein binding was analyzed by gel shift assays (Fig. 29B). Two distinct DNA-protein complexes were formed. The binding is specific, as the two bands were competed by 100-fold excess of unlabeled nucleotides, but not by unrelated nucleotides that are about the same length (Fig. 29, lane 1,2 and 3). The binding was completely abolished by specific competition from unlabeled C/EBP specific oligonucleotides (Fig. 29B, lane 4 and Table II), suggesting that Oligo I and specific C/EBP oligonucleotides compete for the same nuclear factor binding. A reciprocal gel shift assay was performed using the specific C/EBP competitors as radiolabeled probes. A single, distinct complex was formed (Fig. 29C). The binding is specific as the band was completely competed by unlabeled C/EBP nucleotides. However, the band was not competed by Oligo I (Fig. 29C, lane 4), suggesting that the specific C/EBP Oligonucleotide has higher affinity for C/EBP than Oligo I.

**Binding of Liver-enriched Transcription Factor HNF-3 to the Enhancer Element**

Gel shift assays were also performed with Oligo II, which contains an HNF-3 consensus site and a GATA-1 site (Fig. 30A and Table I). Incubation of radiolabeled Oligo II with the nuclear extracts from CEF resulted in two protein-DNA complexes. All the complexes are specific as they were completely competed by 100-fold excess unlabeled probes. Complex 1 is completely competed by unlabeled HNF-3 specific
FIG. 29. Gel mobility shift analysis of the enhancer fragment oligo I. The labeled double-stranded oligonucleotides used for nuclear factor binding as well as competitors are described under "Materials and Methods". All competition experiments were performed by adding 100-fold excess of unlabeled double-stranded competitors. A, oligo I probe of the enhancer. The sequences and putative binding sites for transcription factors are illustrated. B, Gel shifts of Oligo I. C, Reciprocal gel shifts of specific oligo C/EBP.

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FIG. 30. Gel mobility shift analysis of the enhancer fragment oligo II. The labeled double-stranded oligonucleotides used for nuclear factor binding as well as the competitors are described under “Materials and Methods”. All competition experiments were performed by adding 100-fold excess of unlabeled double-stranded oligonucleotides. A, Oligo II probe of the enhancer. The sequences and putative transcription factor binding sites are illustrated. B, Gel shifts of Oligo II probe. C, Reciprocal gel shifts of specific HNF-3 oligo.
competitor, suggesting that Oligo II and HNF-3 specific oligonucleotides compete for the binding of the same nuclear factor. The lower band, complex 2, was completely competed by Oligo I as well as C/EBP specific competitor (Fig. 30B, lane 4 and 5), suggesting that C/EBP family proteins may be involved in the complex. Interestingly, when Oligo I was used as a competitor, complex 1 was intensified while complex 2 was competed, suggesting that possibly some protein-protein interactions were interrupted and a certain balance was shifted. C/EBP specific competitors were able to compete for both complex 1 and complex 2, suggesting that there are some interactions between C/EBP family proteins and HNF-3 family proteins.

Reciprocally, when the HNF-3 specific competitor was used as radiolabeled probe, two specific protein-DNA complexes were formed (Fig. 30C, lane 1 and 2). Although both complexes were competed by Oligo II, competition was greater for the upper complex (Fig. 30C, lane 3). C/EBP specific competitors were able to compete for the two complexes to a similar degree as Oligo II, further confirming the interactions between C/EBP family proteins and HNF-3 family proteins (Fig. 30C, lane 5). Again, it is not surprising that as a weaker binding site for C/EBP, Oligo I did not compete with the strong HNF-3 specific binding oligonucleotides (Fig. 30C, lane 4). Together, these results suggest that HNF-3 family proteins may bind to the enhancer and interact with the C/EBP family proteins.

**Binding of the Transcription Factor Sp1 to the Enhancer Element**

The results from the deletion analysis experiments show that the sequences between –6406 to –6250 are crucial for the full-length enhancer activity. Within this DNA fragment, there are a Sp1 binding site, a GCF site and a NF-IL6 site. Oligo III was
designed to cover the Sp1 and GCF sites (Fig. 31A). Gel shift assays were performed using Oligo III as labeled probes. Three protein-DNA complexes were formed (Fig. 31B). The binding is specific as all the complexes were competed by 100-fold excess unlabeled probe (Fig. 31B, lane 2). Complex 2 was completely competed by 18-bp long Sp1 specific competitors, suggesting that Oligo III and Sp1 specific oligonucleotides compete for the same nuclear factor binding (Fig. 31B, lane 3). Transcription factor AP-1 specific competitor was able to partially compete for the lower band, complex 3 (Fig. 31B, lane 4). Interestingly, the DNA fragment of chicken apoA-I gene 330-bp promoter region (positions -311 to +19) was able to completely abolish formation of complex 1 and complex 2 (Fig. 31B, lane 5), suggesting that the chicken apoA-I promoter region contains sequences recognized by the same nuclear factors, possibly Sp1.

To further confirm the binding of transcription factor Sp1 to the enhancer, reciprocal gel shift assays were performed using labeled Sp1 specific competitors as probes (Fig. 31C). One major complex and two minor ones were formed. All complexes are specific as they were all competed by 100-fold excess unlabeled probes (Fig. 31C, lane 1 and 2). Oligo III completely abolished the formation of all the complexes; strongly suggesting that Sp-1 does bind to Oligo III (Fig. 31C, lane 3). The DNA fragment from the chicken apoA-I promoter region was able to compete partially for all the specific protein-DNA complexes whereas AP-1 specific competitors did not have any effect (Fig. 31C, lane 4 and 5). Overall, these results suggest that ubiquitous transcription factor Sp1 binds to both the enhancer and the promoter of chicken apoA-I gene, and that Sp1-Sp1 interaction may play a role in transcriptional stimulation of the chicken proximal promoter by the distal enhancer.
FIG. 31. Gel mobility shift analysis of the enhancer fragment oligo III. The labeled double-stranded oligonucleotide used for nuclear factor binding as well as competitors are described under "Materials and Methods". All competition experiments were performed by adding 100-fold excess of unlabeled double-stranded oligonucleotides. A, Oligo III probe of the enhancer. The sequences and putative transcription factor binding sites are illustrated. B, Gel shifts of Oligo III. C, Reciprocal gel shifts of specific Sp-1 oligo.
Binding of an unknown nuclear factor to Oligo IV

More gel shift assays were performed with Oligo IV as labeled probes. Oligo IV contains a liver-enriched nuclear factor HNF-4-like site, an AP-2 site and a Metallothionein (MT) Factor 1 (MTF-1) site (Fig. 32A). One protein-DNA complex was formed (Fig. 32B, lane 1). The binding is specific as the complex was completely competed for by unlabeled probes (Fig. 32B, lane 2). HNF-4 and AP-1 specific competitors were not able to compete for the specific binding (Fig. 32, lane 3 and 4), suggesting that neither is involved in the protein-DNA complex. This result was further confirmed by reciprocal gel shift assays. No binding was observed when the HNF-4 specific oligonucleotides were used as labeled probes (Fig. 32C). Together, these results suggest that an unknown nuclear factor, possibly AP-2 or MTF-1, binds to Oligo IV.
FIG. 32. Gel mobility shift analysis of the enhancer fragment oligo IV. The labeled double-stranded oligonucleotides used for nuclear factor binding as well as competitors are described under "Materials and Methods". All competition experiments were performed by adding 100-fold excess of unlabeled double-stranded oligonucleotides. A, Oligo IV probe of the enhancer. The sequences and putative transcription factor binding sites are illustrated. B, Gel shifts of Oligo IV. C, Reciprocal gel shifts of specific HNF-4 oligo.
CHAPTER IV
DISCUSSION

We have previously reported the identification of the chicken apoA-I gene as a negative target of v-Jun overexpression. In this study, we further investigated the possible underlying mechanisms by which v-Jun represses apoA-I gene expression. Our data presented here demonstrate that v-Jun does not bind to the apoA-I promoter directly and suggests that v-Jun regulates apoA-I gene expression through an indirect mechanism. In addition, we reported the identification of an enhancer located between -6.8 kb to -6.0 kb upstream of the transcription start site of the chicken apoA-I gene that stimulates transcription from the apoA-I promoter in a distance- and orientation-independent manner and thus exhibits enhancer characteristics. We described the characterization of nuclear transcription factors Sp1, C/EBP, HNF-3, and an unknown factor that interact with the cis-acting elements of the enhancer. We also investigated the functional properties and species specificity of the enhancer.

TRANSCRIPTIONAL REGULATION BY V-JUN

The role of v-Jun in transcriptional regulation has been extensively examined in recent years. As a major component of the transcription factor complex AP-1, the Jun protein can recognize and bind to specific DNA sequences and regulate target gene expression from nearby promoters in the form of a complex with its many dimerization partners. In addition, the Jun proteins can also regulate many target genes that do not contain AP-1- or CRE-like sites in their promoter region through protein-protein interaction with non-leucine zipper transcription factors.
Our data presented in this study show that v-Jun does not bind to the apoA-I promoter directly. Therefore the first repression model (see Fig. 6) involving direct DNA binding to the apoA-I promoter is unlikely. Our data favor the indirect models.

PROTEIN-PROTEIN INTERACTIONS

Protein-protein interaction is a common indirect mechanism found in the regulation of many genes. Studies have shown that Jun represses glucocorticoid-responsive genes and myogenesis through direct physical protein-protein interactions with glucocorticoid receptor and Myo D (37,40). V-Jun may repress either basal or activated transcription by competing for binding of activators or competing with an activator for a limiting common coregulator or general transcription factors and deplete these factors. Our deletion analysis mapped the multiple v-Jun responsive elements to the apoA-I promoter –311 to +19 upstream of the gene transcription start site, indicating that there may be multiple trans-acting factors that may interact with v-Jun. The apoA-I promoter region mediates binding of many transcription factors including HNF-4, HNF-3β, ARP-1, EGR-1, and C/EBP as well as the actions of many hormones and other factors.

The orphan nuclear receptor hepatocyte nuclear factor 4 (HNF-4) is required for development and maintenance of the liver phenotype. HNF-4 activates several hepatocyte-specific genes, including the gene encoding apolipoprotein AI (apoA-I). The apoAI gene is activated by HNF-4 through a nuclear receptor binding element (site A) located in its liver-specific enhancer. Studies have shown that HNF-4 activates apoA-I transcription by facilitating assembly of a preinitiation complex intermediate consisting of TBP, the TATA box-binding protein component of TFIID and TFIID, via direct
physical interactions with TFIIB (110). Members of the Jun family of transcriptional activators were also reported to interact with both TBP and TFIIB in vitro. TBP binds to both the N-terminal activation domain and C-terminal bZIP regions of c-Jun, whereas TFIIB binds to only the c-Jun bZIP domain. This interaction requires the dimerization of the Jun protein. The ability of the N-terminal activation domains of c-Jun, JunB, JunD and v-Jun to interact with TBP in vitro correlates with their transcriptional activity in vivo. It is suggested that the Jun family of activator proteins may activate transcription by interacting with the general transcription factors TBP and TFIIB (31). Our results in this study did show that the ability of v-Jun to form heterodimers and the acidic region III of v-Jun transactivation domain are required for repression of apoA-I transcription. It is possible then that v-Jun competes for general transcriptional factors with activators of apoA-I transcription such as HNF-4 and depletes these factors thus resulting in reduced transcription of the apoA-I gene.

METHYLATION OF THE APOA-I PROMOTER

Methylation is an epigenetic mechanism for modulation of gene expression in vertebrates. The process is mediated by an enzymatic methyl transfer reaction at cytosine located 5' to guanine (CpG dinucleotides) residues in the unmethylated nascent DNA strand across from methylated CpG dinucleotides. Acquisition of DNA methylation at a previously unmethylated site can not be accomplished by maintenance methylation and requires de novo methylation (137,156). DNA cytosine-5 methyltransferase is the only methyltransferase (Mtase) that has been identified to date in mammalian as well as in avian cells (157). Abnormal patterns of DNA methylation are a consistent molecular feature of immortalized and neoplastic cells (137). It has been demonstrated that

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overexpression of murine DNA MTase is transforming for NIH 3T3 cells (158) and that cellular levels of DNA MTase increases 20-fold in SV40-transformed human fibroblasts and the DNA methylation accounts for the inhibition of collagen VI expression (141).

CpG methylation has long been associated with repressed gene transcription (137-141). Recent studies showed that the tissue inhibitor of metalloproteinases-3 (TIMP-3) gene is specifically downregulated in neoplastic cells of the mouse JB6 progression model. On the basis of 5-azacytidine reversal, the mechanism for this down-regulation appears to involve changes in the methylation state of the TIMP-3 promoter. Expression of antisense methyltransferase resulted in reactivation of the endogenous TIMP-3 gene (159).

Overexpression of v-Jun may upregulate methyltransferase resulting in methylation of the apoA-I promoter, which is extremely GC rich. Although we have not specifically examined the methylation status of the apoA-I promoter in v-Jun transformed cells, the upregulation of the DNA MTase remains a possibility. Several mechanisms have been proposed to explain how CpG methylation affects gene expression. One is that methylation at a specific site repels the transcription factors that would normally bind there. Repression of proenkephalin gene expression by methylation through repelling the binding of the transcription factor AP-2 (139) is one example. Another mechanism is that methylation attracts proteins that themselves mediate repression (160-162). However, in either case, the final effect has to be achieved through changes in DNA binding by different transcription factors. Our results show that there is no difference in DNA binding of the apoA-I promoter between nuclear proteins from CEF infected with RCAS VJ-1 and control CEF infected with vector RCAS alone. If v-Jun represses apoA-
I transcription through methylation of the apoA-I promoter, the mechanism will be quite different.

SECONDARY GENE EXPRESSION REGULATION

Jun is a ubiquitous transcription factor and regulates expression of many genes. The observation that the DNA binding specificity of v-Jun and the acidic region III of its transactivation are absolutely required clearly favor the secondary gene regulation mechanism. A simple secondary gene regulation event is to upregulate a repressor or downregulate an activator of apoA-I gene transcription. Recent studies showed that G10BP, a serum-inducible factor, represses transcription of the fibronectin gene through binding to the G-rich sequences in the promoter excluding Sp1 from binding to these sequences (163). Further studies revealed that the G10BP-1 gene is a target of Myc and Jun/Fos and that these factors repress fibronectin gene expression through induction of G10BP-1 during G1-to-S phase progression (163,164).

The observation that v-Jun can simultaneously activate and repress different genes in CEF illustrates the role of promoter-specific factors in regulating the activities of v-Jun (72,75). The apoA-I promoter region mediates the binding of many transcription factors and the actions of many hormones (see Fig. 5 and Fig. 17). Studies have shown that EGR-1 activates the apoA-I gene promoter and overcomes orphan nuclear receptor ARP-1-mediated repression of the promoter in hepatoblastoma HepG2 cells (142). EGR-1 was also involved in the full induction of human apoA-I gene expression by the experimental nephrotic syndrome in transgenic mice (143). EGR-1 is an immediately-early gene whose expression is found, in many cases, to be regulated similar to that of c-Jun. Overexpression of v-Jun is known to repress c-Jun expression (165). Other candidate
genes that may be regulated by v-Jun are GC factor (GCF), CTCF, and AP-2. GCF was first identified as a transcription factor that binds to the GC rich promoter region of EGF receptor and represses its transcription (151). Later studies have shown that GCF represses the transcription of several other growth factor/receptor genes, including insulin-like growth factor receptor (153,154). CTCF was first identified as a zinc-finger nuclear factor binding to a CCCTC-motif within oncogene the c-myc promoter (147). Its amino acid sequences are highly conserved among species, with chicken and human CTCF sharing 93% homology (148). It was found to repress transcription of several genes, including c-myc (148) and lysozyme (149). Recently, studies have shown that CTCF binds to the chicken beta-globin 42 bp insulator for enhancer blocking activity in human cells and it is suggested that directional enhancer blocking by CTCF is a conserved component of gene regulation in vertebrates (150). AP-2 has been reported as both an activator and repressor of transcription (145,146). More recently, studies reported that expression of the apoA-I gene declines with age both in humans and in rats. The age-dependent changes of DNA binding activity to two proximal sites, B and C of the apoA-I promoter, are responsible for the decline in apoA-I expression. Decreased site B binding activity correlated with lower mRNA levels encoding the activator, HNF-3β. The age-dependent change in the pattern of binding to site C was due to a switch from the activator, HNF-4, to the repressor, ARP-1 (104). It is reasonable to speculate that overexpression of v-Jun interferes with the cellular balance of apoA-I regulators which may affect apoA-I transcription without a pronounced change in DNA binding activity. Treatment with the protein synthesis inhibitor cycloheximide may be needed to determine whether a labile protein is necessary for repression by v-Jun.
REDUCTION IN TRANSCRIPTION POTENTIAL BY V-JUN

In this study, even though extensive efforts were made to unveil differences in DNA binding of the apoA-I promoter between nuclear extracts from CEF infected with RCAS-VJ-1 or control CEF, we did not detect any difference in DNA binding under our experimental conditions. Though many nuclear factors from CEF bind to the apoA-I promoter specifically, supershift assays demonstrated that v-Jun is not even present in the protein-DNA complexes. Yet, the DNA binding specificity of v-Jun, its ability to heterodimerize, and the acidic region III of its transactivation domain are required for repression of apoA-I transcription by v-Jun. Taken together, these results prompts us to speculate that v-Jun may repress apoA-I transcription by reducing the transactivation potential of an activator.

Studies have shown that c-Jun inhibits insulin gene transcription in pancreatic beta cells by reducing the transactivation potential of the E2A proteins present in the ICE activator complex (42). The pancreatic beta-cell-type-specific transcription of the insulin gene is principally controlled by trans-acting factors which influence insulin control element (ICE)-mediated expression. The ICE activator is composed, in part, of the basic helix-loop-helix proteins E12, E47, and E2-5 encoded by the E2A gene. It was found that ICE activation in beta cells was repressed in vivo by c-Jun. C-Jun was shown to specifically repress the transactivation potential of the E2A proteins. Repression of E2A was mediated by the basic leucine zipper domain of c-Jun, which is also the essential domain of this protein necessary for controlling ICE activator-stimulated expression in vivo. It is possible that v-Jun affects the transactivation potential of an activator without affecting its DNA binding ability.
These mechanisms described above are not exclusive. V-Jun may employ more than one mechanism to repress target gene transcription.

In summary, in this study, the v-Jun responsive elements were mapped within nucleotides −311 to +19 upstream of the apoA-I transcription start site. Within this fragment, there are multiple elements which may mediate the repressor activity of v-Jun. Gel mobility shift assays and supershift assays demonstrated that v-Jun does not bind to the apoA-I promoter directly. Biochemical analysis of functional domains of v-Jun indicates that DNA binding specificity of v-Jun is absolutely required for repression of the apoA-I transcription, and that its ability to heterodimerize with diverse partners also plays a role. In addition, the sequences between amino acid residues 108 to 128 in the amino terminus of v-Jun proteins that contain the acidic region III of its transactivation domain are important for its repressor activity. Taken together, these results clearly suggest the mechanism by which v-Jun represses apoA-I transcription does not involve direct DNA binding of v-Jun to the apoA-I promoter but occurs by an indirect mechanism.

THE ENHANCER CONSISTS OF MANY MODULES, BOTH POSITIVE AND NEGATIVE

Transcription of the chicken apoA-I gene is readily detectable in chicken embryo fibroblasts, yet the apoA-I basal promoter is extremely weak when tested in transient transfection assays. Optimal transcription of the chicken apoA-I gene, like that of many genes, might depend on the presence of one or more enhancer regions which stimulate transcription from the proximal promoter in a distance- and orientation-independent manner. In this study we reported the identification of an enhancer located between −6.8
kb to −6.0 kb upstream of the transcription start site of the chicken apoA-I gene that stimulates transcription from the apoA-I promoter in a distance- and orientation-independent manner and thus exhibits enhancer characteristics. The characterization of nuclear transcription factors Sp1, C/EBP, HNF-3, and an unknown factor that interacts with the cis-acting elements of the enhancer, the functional enhancer properties, and its species specificity were also investigated.

The 800 bp full-length enhancer was first identified by transient transfection assays using a series of deletion mutations of the chicken apoA-I gene 5'-flanking region (75). Insertion of this fragment upstream in both orientations of the chicken apoA-I promoter (positions −311 to +19) resulted in 9- to 12-fold increases in transcriptional activity. The complexity of the enhancer regulation was suggested by deletion analysis of the enhancer.

While the 791-bp full-length enhancer is orientation-independent, two deletion fragments, deletion 6 and deletion 7, were not (see Fig. 28), both exhibiting higher than full-length enhancer activity in one orientation, but not the other. Deletion 11, a 152-bp internal fragment spanning −6557 to −6406, could confer 100% of the full-length enhancer activity in the reverse orientation and 80% of the full-length enhancer activity in the forward orientation. A 73-bp long 5'-extension from −6557 to −6629 completely abolished the enhancer activity in both orientations. Meanwhile, 3'-deletions from −6135 to −6010 (126-bp) reduced the enhancer activity to 30% of the full-length enhancer, but by itself, it only showed 35% enhancing activity in the forward orientation and basal level in the reverse orientation. Taken together, these results suggest that the enhancer is composed of many domains, both positive and negative. Interactions among nuclear
factors bound to different enhancer modules contribute to the complexity of the enhancer regulation.

**CELL TYPE SPECIFICITY OF THE ENHANCER**

The enhancer is strictly cell type-specific. It modestly stimulates transcriptional activity mediated by heterologous SV40 promoter and the human apoA-I minimal promoter in CEFs. But when human hepatoma HepG2 cells or mouse fibroblasts NIH3T3 were transfected with plasmids containing either the homologous chicken apoA-I promoter or heterologous promoters, the enhancer exhibits a pronounced inhibitory effect. The SV40 enhancer is able to override the inhibition in both HepG2 and NIH3T3 cells when inserted in the same plasmid construct as the chicken apoA-I enhancer. In human breast carcinoma MCF7 cells, the enhancer has no effect on all the promoters tested (data not shown). Two interesting questions arise. What cis-acting and trans-acting factors contribute to the expression of the apoA-I gene in CEF? What differences of trans-acting factors exist between CEFs and HepG2 or NIH3T3 cells to make the enhancer stimulatory in one cell type and inhibitory in others? The control of differentiated gene expression lies mainly at the level of transcriptional initiation. The mammalian apoA-I gene has a powerful liver-specific enhancer located within –220 to –110 nucleotides upstream of the transcription start site. Our study here shows that chicken apoA-I gene has a strong cell-specific enhancer located within –6800 to –6000 nucleotides upstream of the transcription start site. Cell-specific DNA elements apparently mediate their effects on transcription through interaction with trans-acting nuclear factors. It has long been demonstrated that liver-specific (or liver-enriched) transcription factors exist and participate in the activation of more than one gene in liver
One scheme of explanation for differential regulation of the chicken apoA-I gene expression is that the gene has multiple activating sites that are simultaneously required for activity, and that some of the liver-enriched factors are active in CEF but are not in mammalian cell types. Results from our study certainly support this possibility. We showed that there are liver-enriched nuclear factors such as C/EBP and HNF-3 in CEF nuclear extracts, and that the chicken apoA-I enhancer contains binding sites for these factors. One obvious difference between CEF and HepG2 cells is that CEFs do not contain the liver-enriched nuclear transcription factor HNF-4. It is possible that HNF-4 is responsible for the inhibitory effect exhibited by the enhancer in HepG2 cells. However, this does not explain the inhibition by the enhancer in NIH3T3 cells that do not have high level of endogenous HNF-4 (106,169).

The cell type specificity as well as the function of the enhancer is mediated through interactions between its cis-acting elements and nuclear transcription factors. Computer analysis of the full-length enhancer revealed that there are many putative binding sites for transcription factors. Based on the computer analysis, we designed four oligonucleotides as probes in gel mobility shift assays. These four probes contain binding sites for ubiquitous transcription factor Spl and liver-enriched transcription factors C/EBP, HNF-3 and HNF-4, respectively. Gel shift results from our study suggests that Spl, C/EBP, HNF-3, and an unknown factor from CEF nuclear extracts all bind to the enhancer, while HNF-4 is not likely to be present at high levels in CEF.

**TRANS-ACTING NUCLEAR FACTORS**

Ubiquitous transcription factor Spl is a constitutive activator of housekeeping genes and acts by binding to GC boxes in their proximal promoter regions. It has also
been found to play an important role in the activity of several enhancers, including the apoE upstream regulatory element (170), the immunoglobulin-κ-3 enhancer (171), the apo(a)-plasminogen intergenic region enhancer (172), and α-fetoprotein enhancer (173). Sp1-Sp1 binding between proximal promoter and distal enhancer is a known mechanism for long distance promoter stimulation (135,136). In these studies, binding of human transcription factor Sp1 to 10-base-pair G+C-rich elements ("GC boxes") located at -100 and +1700 relative to the RNA start site was examined to investigate the physical basis of long-range enhancer effects between distal and proximal elements in an eukaryotic promoter. It was previously observed that the distantly located Sp-1 site functions in synergism with the promoter-proximal Sp-1 site to strongly activate transcription in vivo. Conventional and scanning transmission electron microscopy demonstrate that this synergism is likely to be a direct consequence of interactions between remote and local Sp1, the remote Sp1 translocated to the promoter by a DNA loop. Scanning transmission electron microscopy shows that Sp1 initially forms a tetramer and subsequently assembles multiple tetramers stacked in register at the DNA loop juncture. This finding not only provides the physical basis for loop formation but also defines a biological process leading to strongly increased concentration of activator protein at the promoter.

In our gel mobility shift assays in which Oligo III of the enhancer that contains a putative Sp-1 binding site was used as a labeled probe, we demonstrated that the Sp1 specific oligo was able to compete for the specific binding. Reciprocally, when the Sp1 specific oligo was used as a labeled probe, Oligo III was able to compete for the specific binding as well. When the chicken apoA-I promoter DNA fragment (positions -311 to +19) was used as a competitor in both binding experiments, the apoA-I promoter region
was able to compete for the specific binding in both gel shifts. We hypothesize that interactions between Sp1-Sp1 might play a pivotal role in regulation of the enhancer function. To determine if Sp1-Sp1 interactions are at least part of the underlying molecular mechanisms responsible for the enhancer function, a series of mutagenesis experiments would be needed.

The gel mobility shift assays using oligonucleotides containing a putative HNF-3 site as the labeled probes showed formation of two specific DNA-protein complexes. Specific HNF-3 oligonucleotides were able to compete for the weaker upper band, whereas Oligo I, which contains a putative C/EBP binding site, was able to compete for the lower band. Specific C/EBP oligonucleotides were able to compete for both the weaker upper and the stronger lower bands. Reciprocal experiments using labeled specific HNF-3 oligo as probe showed formation of two specific complexes migrating together to a position similar to that of the weaker upper band of Oligo II. Both Oligo II and specific C/EBP oligo were able to compete almost completely with the upper band and partially with the lower band. There are three isoforms of HNF-3: HNF-3α, HNF-3β, HNF-3γ. Most likely, the two bands are formed by two HNF-3 isoforms. Oligo II contains a weak HNF-3 binding site and probably binds to one of the three HNF-3 isoforms. To determine which HNF-3 isoform is in the DNA-protein complexes, supershift assay experiments using specific antibody against each of the HNF-3 isoforms must be done.

Gel shift experiments using Oligo I that contains a putative C/EBP site as probe and subsequent reciprocal experiments using specific C/EBP oligo as labeled probe suggest that Oligo I contains a weak C/EBP binding site and that C/EBP family proteins

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bind to the enhancer. A preliminary supershift assay using rabbit polyclonal antibodies against C/EBPα failed to shift the specific binding, suggesting that it may not be C/EBPα but other family members that are involved in the DNA-protein complex formation (data not shown).

When Oligo IV that contains a putative HNF-4 binding site was used as labeled probe, a specific complex formation was observed but was not competed for by 100-fold excess unlabeled specific HNF-4 oligo. When specific HNF-4 oligo was used as labeled probe in reciprocal gel shift experiments, no binding was observed at all. These results suggest that there is either no, or low level of endogenous HNF-4 nuclear factor in CEF. The specific binding in Oligo IV gel shifts is formed by an unknown nuclear factor in CEF. Since oligo IV also contains a binding site for AP-2 and a binding site for GATA-1, these two factors are obvious candidates.

Results from gel shift experiments using Oligo II and specific HNF-3 oligo as labeled probes also suggest that there are some interactions between HNF-3 and C/EBP. The 791-bp full-length enhancer contains many putative binding sites for transcription factors. Some of these sites (including HNF-3 site and C/EBP site that are 20 bp apart) cluster together. It is not surprising that there are some interactions between them. Preliminary competition gel shift experiments using either Oligo I or specific C/EBP oligo as labeled probes and 100-fold excess unlabeled Oligo II and specific HNF-3 as competitors were performed. Results from these experiments show that both Oligo II and specific HNF-3 oligo were able to completely abolish the specific binding in gel shifts of specific C/EBP oligo. Of the two DNA-protein complexes formed in Oligo I gel shift experiments, Oligo II was able to compete for both bindings, whereas specific HNF-3
oligo was only able to compete for the weaker upper band, but not the stronger lower band (data not shown). For all gel shift experiments, the ultimate confirmation may come from gel shift experiments using purified C/EBP, HNF-3 and Sp-1 or supershift assays using specific antibodies against these factors.

In summary, we have identified a strong enhancer in the 5’-flanking region of the chicken apoA-I gene that could be important for the optimal expression of the apoA-I gene \textit{in vivo}. We have begun to elucidate the trans-acting factors that mediate the enhancer function. The results reported here indicate the complexity of regulation of the apoA-I enhancer. The enhancer is composed of many modular positive and negative elements, all of which are necessary for the enhancer function. The specificity and activity of the enhancer rely on binding sites for many nuclear factors including ubiquitous transcription factor Sp1, liver-enriched nuclear factors HNF-3 and C/EBP, and an unknown nuclear factor. The understanding of how chicken apoA-I gene expression is regulated in CEF and the differences between mammalian and avian species regarding the differential expression of the apoA-I gene may be valuable to develop strategies to increase human apoA-I target gene expression.
CHAPTER V
CONCLUSIONS

1. The v-Jun responsive elements in the chicken apoA-I 5'-flanking region have been mapped to -311 to +19 upstream of the apoA-I gene transcription start site. It appears that there are multiple cis-acting elements that may mediate repression of the apoA-I transcription by v-Jun.

2. Gel shift assays and supershift assays demonstrated that neither v-Jun binds to the apoA-I promoter directly nor is it involved in the specific protein-DNA interactions between nuclear factors from chicken embryo fibroblasts and the apoA-I promoter.

3. Biochemical functional analysis indicates that the DNA binding specificity of v-Jun oncoprotein is absolutely required for repression of apoA-I gene expression. The ability of v-Jun to heterodimerize with diverse partners also contributes to its repressor activity. In addition, the amino acid residues 108 to 128 located in the carboxyl terminus of v-Jun that contain the independent acidic region III of its transactivation domain are also important for its repressor activity.

4. 791-bp enhancer in the apoA-I 5'-flanking region spanning nucleotides -6800 bp to -6000 bp upstream of the transcription start site was identified and characterized. The enhancer is capable of stimulating transcription from both homologous as well as heterologous promoters. It is strictly CEF cell-specific. The enhancer consists of many positive and negative modular elements, all of which are necessary for enhancer function. Sequence information of the enhancer revealed that there are many putative binding sites for known transcription factors. Sp1, C/EBP, HNF-3, and an unknown nuclear factor were found to interact with the cis-acting elements of the enhancer. Interactions among

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nuclear factors bound to different cis-acting elements may contribute to the complexity of enhancer regulation.
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VITA

Wen Lin Kumfert was born and raised in Anshan, Liaoning Province, People's Republic of China. After graduating from high school in 1984, she enrolled in Wuhan University. In 1988, she earned a Bachelor's degree in Science with a major in Cell Biology. After receiving her undergraduate degree, Wen traveled to Guangzhou, the capital of Canton Province in southern China. There, she worked first as a journalist in a short-lived liberal newspaper, and then as an assistant lecturer in Sun Yat-San University of Medical Sciences where she taught Medical Biology and Cell Biology laboratory courses.

After four and a half years, Wen relocated to Norfolk, Virginia where she was accepted as a graduate student in the Biomedical Sciences Program, a joint program between Old Dominion University and Eastern Virginia Medical School. For three years, she worked in Dr. Buescher's laboratory in the Center for Pediatric Research of Eastern Virginia Medical School and completed all course requirements for a doctorate degree. She spent another three and half years in Dr. Bos' laboratory in the Department of Microbiology and Molecular Cell Biology of Eastern Virginia Medical School. There she completed her dissertation work.

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