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TRANSCRIPTIONAL REGULATION OF THE HUMAN APOLIPOPROTEIN GENES

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1. ABSTRACT

This review provides experiments and putative mechanisms which underlie the transcription of the human apolipoprotein genes *in vitro* and *in vivo*. Summarized below are the key findings for individual genes and gene clusters.

ApoA-II

1- The –911/+29 promoter is sufficient to direct expression of a reporter gene exclusively in the liver and thus represents a liver-specific promoter.

2- Important factors for the activity of this promoter are hormone nuclear receptors and the ubiquitous factor USF.

3- SREBP-1 and SREBP-2 bind to five and four sites respectively and transactivate the apoA-II promoter. Their role in the *in vivo* transcription of the apoA-II gene has not been established.

ApoB

1- Regulatory sequence extending 5 Kb upstream and 1.5 Kb downstream of the apoB promoter are sufficient to direct hepatic expression of the apoB gene. The intestinal expression of the apoB gene requires in addition a 315 bp intestinal enhancer located 56 Kb upstream of the apoB gene.

2- Important factors for apoB gene transcription appear to be C/EBP, HNF-3, HNF-4 and other nuclear receptors which bind both on the proximal promoter and the intestinal enhancer.

ApoE/ApoCI/ApoCIV/ApoCII Cluster

1- The expression of the genes of the apoE/apoCI/apoCII/apoE cluster are controlled by two homologous hepatic control regions designated HCR-1 and HCR-2 of approximately 600 bp located 15 and 27 Kb 3′ of the apoE gene. Either region is sufficient to direct gene expression *in vivo*, although HCR-1 appears to have a dominant effect on apoE and apoCI and HCR-2 has a dominant effect on apoCIV and apoCII gene expression.

2- Two other homologous regulatory regions designated ME-1 and ME-2 located 3.3 and 15.9 Kb downstream of the apoE gene can direct independently the expression of the apoE gene in macrophages and adipocytes.

3- Important factors for apoE gene regulation appear to be SP1 on the proximal promoter, and possibly HNF-3, C/EBP and hormone nuclear receptors on the enhancers.

4- Important factors for apoCII gene transcription appear to be HNF-4 and RXR-alpha/T3R-beta which binds to a thyroid response element of the proximal promoter.

ApoA-I/ApoCIII/ApoA-IV Gene Cluster

1- The transcription of the apoA-I/apoCIII/apoA-IV gene cluster is controlled by a common enhancer located 590 to 790 nucleotides upstream of the apoCIII gene.

2- Important factors for the activity of the enhancer are SP1, HNF-4 and possibly other nuclear receptors. Important factors for the activity of the proximal promoters are HNF-4, and possibly other nuclear receptors.

3- The HNF-4 binding site of the apoCIII enhancer is required for the intestinal expression of apoA-I and apoCIII gene and enhances synergistically the hepatic transcription of the two genes and possibly of apoA-IV *in vivo*. The three SP1 sites of the enhancer are also required for the intestinal expression of apoA-I and apoCIII genes *in vivo* and for the enhancement of the hepatic transcription.

4- Pro-inflammatory cytokines such as TNF-alpha and IL-1 repress, and TGF-beta stimulates the apoCIII promoter activity. The TGF-beta pathway activates SMAD3/4 proteins which interact with HNF-4 bound to the apoCIII promoter and enhancer and increase its activity.

5- It appears that other factors activated by different signaling pathways (NF-kappa-B, Jun and others) interact with HNF-4 bound to the enhancer and thus repress the activity of apoCIII promoter.

Understanding the transcriptional regulatory mechanism of the apolipoprotein genes may allow, in the long run, selective increase of anti-atherogenic lipoproteins and thus reduce the risk of cardiovascular disease.

2. INTRODUCTION

The transport of lipids in plasma and their homeostasis in cells and tissues is achieved with the help of lipoprotein particles. The transport and homeostasis of lipids requires the concerted action of several proteins including apolipoproteins, plasma enzymes, and transfer proteins and lipoprotein receptors. These proteins participate in the biogenesis and catabolism of three major classes of lipoproteins: the chylomicrons, the group of VLDL/IDL/LDL and the group of HDL lipoproteins shown in figure 1A-C.

Biogenesis of chylomicrons occurs in the intestine, and of VLDL/LDL and HDL in the liver. The lipoproteins are altered in plasma by the action of enzymes and transfer proteins and are subsequently recognized by cell receptors. Under physiological conditions, existing homeostatic mechanisms in the body strive to maintain physiological concentration of the various classes of lipoproteins and their derivatives in plasma. When the function or the regulation of synthesis of one or more proteins of the lipoprotein system is altered, then the concentration or the function(s) of one of the lipoproteins may be altered. These changes may affect the levels of cholesterol and/or triglycerides in plasma and in some instances cause atherosclerosis and other complications. This review is going to focus on the regulation of expression of the human apolipoprotein genes. The roles of key apolipoproteins in the pathway of biogenesis, and catabolism of the different lipoprotein classes are shown in figure 1A-C.

Our ability to understand better the role of apolipoproteins in the homeostasis of cholesterol and other lipids and their contribution to atherogenesis was assessed earlier by the study of human patients. This knowledge has been greatly enhanced during the last 10 years by the generation of animal models where one or more

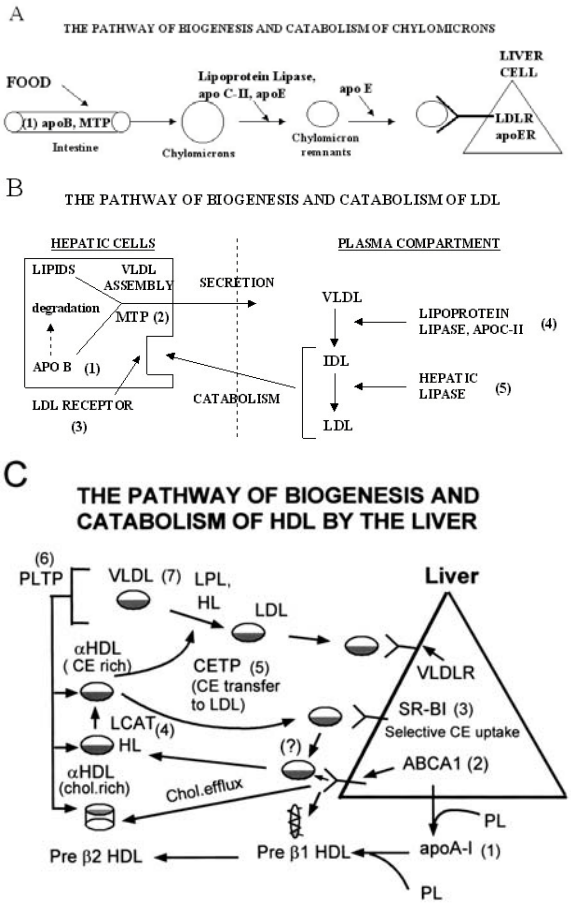


Figure 1A-C. Schematic representation of the pathways of biogenesis and catabolism of chylomicrons (**Panel A**) VLDL/IDL (**Panel B**) and HDL (**Panel C**) depicting the role of key apoproteins (apoE, apoB, apoA-I, apoCII) in cholesterol and triglyceride homeostasis. Numbers 1 to 6 indicate different proteins participating in these pathways.

apolipoprotein(s) has been altered with the addition or subtraction of the corresponding genes. The combined knowledge from the study of human patients and animal models is summarized in table 1. These studies showed that the plasma levels of apoB and apoA-I are correlated directly with plasma LDL and HDL levels (1,2). Existing biochemical and genetic data suggest that increased plasma apoA-I and decreased plasma apoB levels could decrease the LDL/HDL ratio and thus protect humans against atherosclerosis (3). Overexpression of apoA-II leads to high triglyceride levels and abnormal composition of HDL, and predisposes to atherosclerosis (4,5). Thus, reduction in plasma apoA-II levels could have some protective role against atherosclerosis (4,5). ApoE expression may affect positively or negatively the catabolism of chylomicrons, depending on the plasma apoE levels (6,7), whereas lack of apoE predisposes to atherosclerosis (8). Overexpression of apoCIII as well as apoCI, apoE and apoCII, or diminished expression of apoCII, are associated with hypertriglyceridemia by interfering with the hydrolysis of triglycerides of chylomicrons and VLDL (9). Finally, overexpression of apoA-IV in mice increases HDL levels and protects from atherosclerosis, most likely by assuming some of the beneficial functions of HDL (10).

The plasma levels of the apolipoproteins could in principle be altered by altering selectively the level of gene transcription. A rational approach towards this objective would require a thorough understanding how eukaryotic genes are regulated at the level of transcription.

3. GENERAL INTRODUCTION ON GENE TRANSCRIPTION

The transcription of eukaryotic genes is a complex biological event involving numerous proteins including RNA polymerase II, the proteins of the basal transcription initiation complex, a variety of promoter- and enhancer-specific transcription factors, and requires an ATP-dependent activation step (11-22). The regulation of transcription is responsible for the tissue-specific gene expression as well as gene expression during differentiation, development and in response to intracellular and extracellular stimuli such as hormones and metabolites.

Numerous studies have established that there exists a precise array of regulatory elements in each promoter/enhancer which are occupied by transcription factors. It has been proposed that this promoter/enhancer-specific arrangement of factors permits the formation of stereo specific DNA protein complexes. These complexes may directly or indirectly interact with the basal transcription complex, thus leading to the transcriptional activation of the target gene (23,24).

3.1. Liver-specific and ubiquitous transcription factors implicated in apolipoprotein gene regulation

The major sites of apolipoprotein synthesis are the liver and the small intestine. The synthesis of several liver specific proteins is regulated at the transcriptional level (25). Four major families of liver specific proteins have been isolated and have been designated HNF-1, HNF-3 and HNF-4 and C/EBP. The features of the transcription factors relevant to this review are shown in figure 2A and are reviewed below.

3.1.1. HNF-4

Hepatocyte nuclear factor-4 (HNF-4) was initially purified from rat liver nuclear extracts and cloned (26,27). The HNF-4 mRNA is primarily expressed in liver, kidney and intestine (27). Structural analysis showed extensive similarity between HNF-4 and other members of the steroid/thyroid hormone receptor superfamily and was classified as an orphan nuclear receptor (28) (figure 2A). A report that HNF-4 binds to, and is activated by, omega 3 C18 and C20 fatty acyl CoA derivatives has not been confirmed, and thus HNF-4 is still considered an orphan nuclear receptor (29). HNF-4 binds DNA as a homodimer or heterodimer with other HNF-4 isoforms which most likely arise from differential splicing (29-31). An HNF-4 homologue has been identified in *Drosophila* HNF-4(D). A *Drosophila* mutant that lacks HNF-4(D) fails to develop tissues where HNF-4(D) is expressed (32). Similarly, inactivation of the HNF-4 gene in mice is associated with embryonic lethality suggesting a crucial role of HNF-4 in development (33).

Table 1. Role of the apolipoproteins in lipid homeostasis and in atherogenesis: lessons from animal models and human patients

Protein affected	Susceptibility to atherosclerosis	Lipoprotein profile
ApoA-I transgenic	Resistant	HDL↑
ApoA-I knockout		HDL ↓
ApoA-II transgenic	Susceptible	HDL ↑ abnormal composition
ApoA-IV transgenics	Resistant	HDL↑
ApoB transgenic	Susceptible	LDL↑
ApoB transgenic x LDL receptor knockout	Susceptible – used as models to study development of atherosclerosis	LDL ↑
ApoCI transgenics		Mild hypertriglyceridemia
ApoCII or ApoCIII Transgenics (overexpression of apoCIII or apoCII)		Severe hypertriglyceridemia
ApoCII deficiency		Severe hypertriglyceridemia
ApoE transgenic	Resistant	VLDL ↓ LDL ↓
ApoE transgenic (overexpression of apoE)		Hypertriglyceridemia
ApoE knockout	Very susceptible	8- to 25-fold increase in plasma cholesterol. Accumulation of lipoprotein remnants

3.1.2. ARP-1

ARP-1 (COUPTFII) and EAR-3 (COUPTFI) are orphan nuclear receptors with sequence homology and common DNA binding specificity with HNF-4 (34) (figure 2A). Both factors have been implicated in embryonic development (35,36) and neuronal cell fate determination (37). When ARP-1 binds to the same sites as HNF-4 it inhibits transcription (38). However, in promoters where HNF-4 binds to unique sites, APR-1 has the capacity to potentiate the activity of HNF-4 via protein-protein interactions (39,40).

3.1.3. Ligand-dependent hormone nuclear receptors (retinoic acid receptor (RAR), retinoid X receptor (RXR), thyroid hormone receptor-beta (T_3R-beta), peroxisome proliferator-activated receptor (PPAR)

Nuclear hormone receptors represent a superfamily of transcriptional factors that are inducible by small lipophilic hormones such as retinoids, steroids, thyroid hormone, peroxisome proliferators and vitamin D and regulate developmental processes, cell differentiation, homeostasis and reproduction (41). The ligand for RXR-alpha-beta-gamma family members is 9-cis retinoic acid (42,43) whereas both 9-cis and all-trans retinoic acid are ligands for RAR-alpha-beta-gamma . Nuclear receptors dimerize and interact with hormone response elements (HREs) which contain 2 inverted or direct AGG/TTCA repeats (44-46). The structure of nuclear receptors is highly conserved throughout evolution. The most conserved DNA binding domain (C) contains two zinc fingers (47). A more distal element (D-box) has been shown to be important for determining the spacing between the half sites (48). The less conserved carboxy-terminal domain (E) contains several short hydrophobic heptad repeats (49) and is required for ligand binding, dimerization and ligand-dependent transcriptional regulation (figure 2A). Ligand for thyroid hormone receptors (TRs) is triiodothyronine (T3). TRs bind as monomers or homodimers (44). The binding of RAR and TR to HREs can be greatly enhanced by heterodimer formation with the RXR (50-52). Mutations in RXR-alpha or inactivation of the RXR-alpha gene in mice is associated with embryonic lethality (53,54). PPAR is activated by peroxisome proliferators such as fibrates and various fatty acids and may play a role in the regulation of genes in response to nutrients (55,56). It has been shown that 15-deoxy delta-12,14 prostaglandin J2 is a ligand for PPAR-gamma (57). Inactivation of PPAR-alpha results in viable mice that are refractory to the action of peroxisome proliferators (58). Inactivation of the RXR-beta results in sterile males (59). The homo- or heterodimers of nuclear receptors behave differently on different HREs, thus allowing high specificity and flexibility which can be modified further by the binding of their ligand. These properties explain partially the pleiotropic effects of steroid/retinoid hormones in biological systems. Hormone nuclear receptors are found in all the apolipoprotein promoters and appear to play a pivotal role in apolipoprotein gene regulation (figure 3A,C,D,F,H-K,N-P).

3.1.4. HNF-3/forkhead protein of transcription factors

HNF-3 was initially purified from hepatic nuclear extracts and cloned (60). Three homologous family members alpha, beta, gamma were identified which also shared homology with the *Drosophila* forkhead protein (61,62) (figure 2A). The consensus HNF-3 binding site is VAWTRTTKRYTY (where V = G or C or A, W = A or T, R =A or G, Y = C or T) (62). X-ray crystallography has shown the HNF-4 recognizes the DNA via a winged helix motif (63). HNF-3 alpha and beta and gamma are expressed during embryonic development (64,65). All three isoforms are expressed by the liver and HepG2 cells (60) and have been shown to participate in the regulation of liver-specific genes (66-68) including apoB (figure 3C,F).

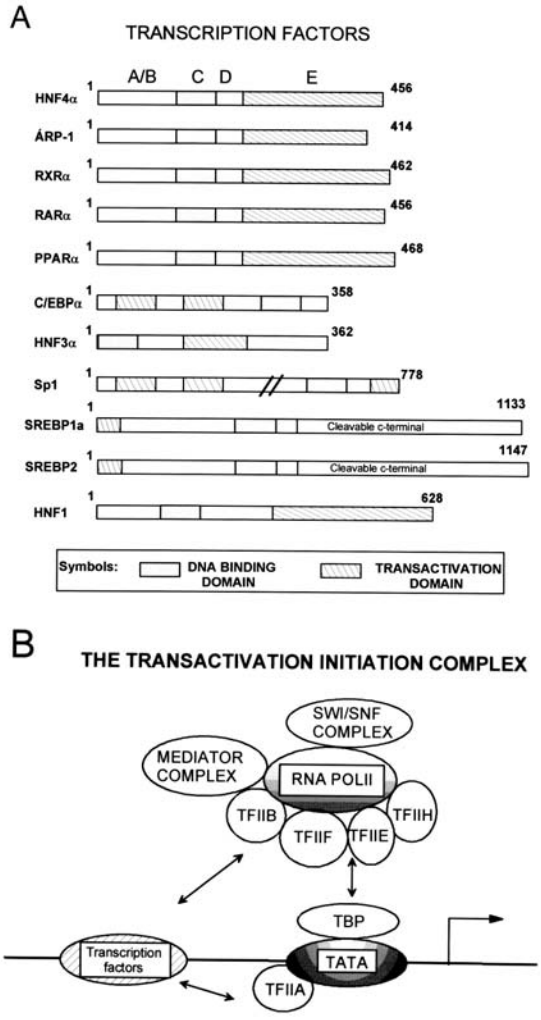


Figure 2A,B. Panel A: Features of transcription factors which bind to the apolipoprotein promoters. Panel B: Simplified version of the proteins of the basal transcription complex. Arrows indicate participation of the activation domains of transcription factors in the recruitment of the proteins of the basal transcription to the TATA box and/or initiator elements.

3.1.5. C/EBP

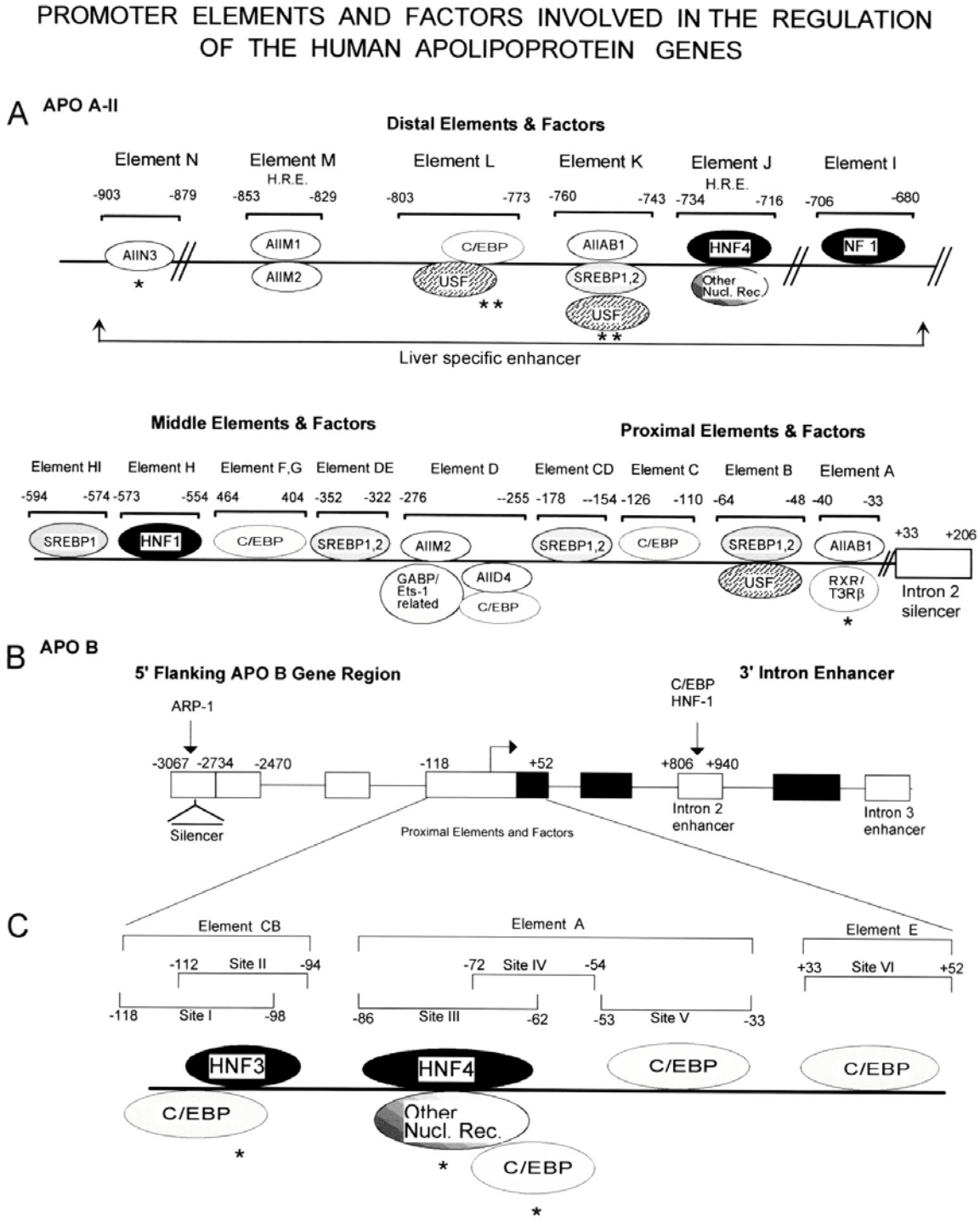
The CCAAT enhancer binding protein (or C/EBP) is a heat stable protein that was initially identified in rat liver nuclear extracts and cloned from an expression library (69,70). This protein consists of a carboxy terminal leucine zipper dimerization motif followed by a positively charged DNA binding domain known as bZip (71) followed by transcription activation domains (figure 2A). The consensus binding site is RTTGCGYAAY (where Y=C or T and R=A or G) (72). Several members of the C/EBP family of transcription factors exist, designated C/EBP beta, gamma, epsilon, delta, n- and CHOP (73-76). C/EBP-beta is found to regulate acute phase genes and is predominantly expressed in liver and in the myelomonocytic lineage of the hematopoietic system (77-79). Inactivation of the C/EBP-beta gene did not affect viability but affected the inflammatory response and native immunity functions (80). Both C/EBP-alpha and beta have isoforms of different sizes which arise from the utilization of different AUG codons (70,81). C/EBP-delta is expressed in adipocytes and granulocytes and plays a role in the acute phase response in many organs, including liver (82). C/EBP-alpha is expressed abundantly in liver, fat and intestine and its expression level increases during differentiation of 3T3 422A preadipocytes to adipocyte cells (83). C/EBP-alpha knockout mice have normal liver development but expression of glycogen synthase and other liver specific genes is affected (84,85). Although C/EBP-alpha, beta and delta are expressed in similar tissues, both tissue culture and animal studies suggest temporal expression during adipocyte and myelogenic differentiation as well as liver regeneration (73-76,86). C/EBP binding sites exist in the promoter and/or enhancer elements of a large number of liver specific genes including the albumin gene, and the apolipoproteins apoA-I, apoA-II, apoB, apoE and apoCIII genes (69,72,87-89) (figure 3A,C,F,I,J,N,O).

3.1.6. SP1

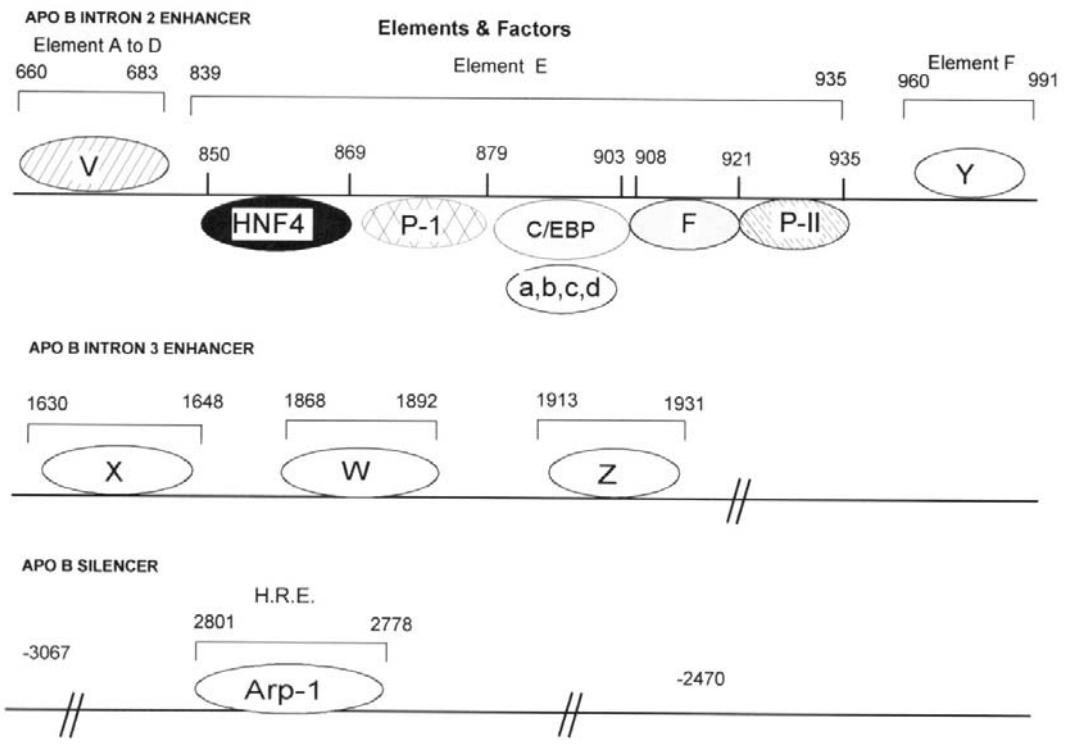
SP1 is a ubiquitous transcription factor which activates several cellular and viral promoters that contain the consensus binding site 5′ G(T)G(A)GGC(A)GG(T)G(A)-3′ (90). SP1 has been purified in two forms as 95 and 106 kD polypeptides from HeLa cells (91) and the full-length cDNA has been obtained (92) (figure 2A). SP1 can act *in vivo* from enhancer sites that are distal to the promoter and exhibit synergistic interaction with proximal promoter sites through self-association, joining distant DNA segments (93). Synergistic activation by SP1 may also occur through multimerization of this factor on a single binding site (94). Different activation domains of the SP1 molecule are responsible for the distinct functions, and the formation of protein-protein complexes between SP1 and other factors may direct synergistic activation of a given promoter (95). Three other SP1 related genes, SP2, SP3 and SP4, have been identified (96). Inactivation of the SP4 gene by homologous recombination showed that the majority of the deficient mice die a few days after birth and males which survive do not breed. Inactivation of SP1 causes embryonic lethality (97,98). *SP1 binds to three sites, F, H and I, on the apoCIII enhancer as well as on the proximal apoE promoter (*figure 3H,N*).*

3.1.7. SREBP-1 and SREBP-2

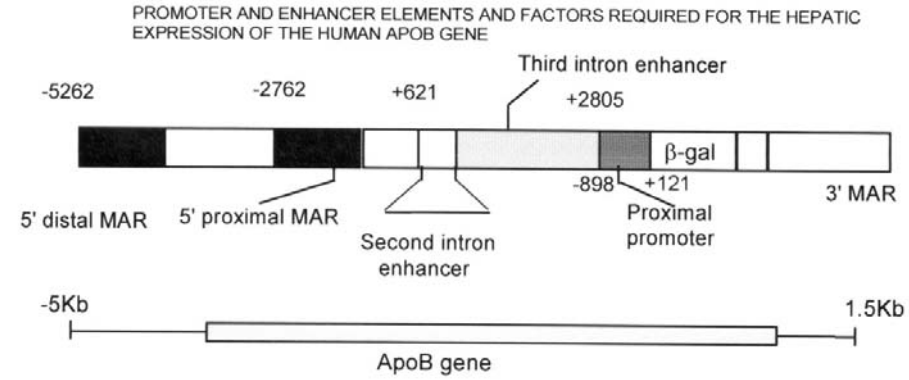
Sterol regulatory element binding proteins-1 and 2 (SREBP-1 and SREBP-2) are members of the membrane-bound basic helix-loop-helix transcription factors that control cellular cholesterol homeostasis (99,100) (figure 2A). SREBP-1 has two isoforms, designated SREBP-1a and SREBP-1c. SREBP-2 has 47% overall homology and 71% homology with the bHLHZip motif of SREBP-1a (99). The SREBP-1 protein was also cloned independently by screening of an adipocyte expression library with E-box elements as probes (101). The protein was named ADD1 for adipocyte differentiation-dependent protein 1 on the basis of its induction after differentiation of the 3T3L1 preadipocytes. It has been proposed, on the basis of the high levels of expression of SREBP-1 in liver and fat tissues, that SREBP family members may regulate not only cholesterol-responsive genes (102,103), but also genes involved in fatty acid biosynthesis and metabolism in adipocytes (104-107). All members of the family have a highly acidic N-terminal activation domain which is recognized by the transcriptional coactivator CREB-


PROMOTER ELEMENTS AND FACTORS INVOLVED IN THE REGULATION OF THE HUMAN APOLIPOPROTEIN GENES
A
APO A-II
Distal Elements & Factors
Element N
Element M
H.R.E.
Element L
Element K
Element J
H.R.E.
Element I
-903
-879
-853
-829
-803
-773
-760
-743
-734
-716
-706
-680
AIIN3
AIIM1
AIIM2
C/EBP
USF
AIIAB1
SREBP1,2
USF
HNF4
Other Nucl. Rec.
NF 1
Liver specific enhancer
Middle Elements & Factors
Proximal Elements & Factors
Element HI
Element H
Element F,G
Element DE
Element D
Element CD
Element C
Element B
Element A
-594
-574
-573
-554
464
404
-352
-322
-276
--255
-178
--154
-126
-110
-64
-48
-40
-33
SREBP1
HNF1
C/EBP
SREBP1,2
AIIM2
SREBP1,2
C/EBP
SREBP1,2
AIIAB1
+33
+206
GABP/ Ets-1 related
AIID4
C/EBP
USF
RXR/ T3Rβ
Intron 2 silencer
B
APO B
5' Flanking APO B Gene Region
3' Intron Enhancer
ARP-1
C/EBP
HNF-1
-3067
-2734
-2470
-118
+52
+806
+940
Silencer
Proximal Elements and Factors
Intron 2 enhancer
Intron 3 enhancer
C
Element CB
Element A
Element E
-112
Site II
-94
-72
Site IV
-54
+33
Site VI
+52
-118
Site I
-98
-86
Site III
-62
-53
Site V
-33
HNF3
HNF4
C/EBP
C/EBP
C/EBP
Other Nucl. Rec.
C/EBP

D


APO B INTRON 2 ENHANCER
Element A to D
660
683
839
Elements & Factors
Element E
935
Element F
960
991
V
850
869
879
903
908
921
935
Y
HNF4
P-1
C/EBP
F
P-II
a,b,c,d
APO B INTRON 3 ENHANCER
1630
1648
1868
1892
1913
1931
X
W
Z
APO B SILENCER
H.R.E.
2801
2778
-3067
Arp-1
-2470

E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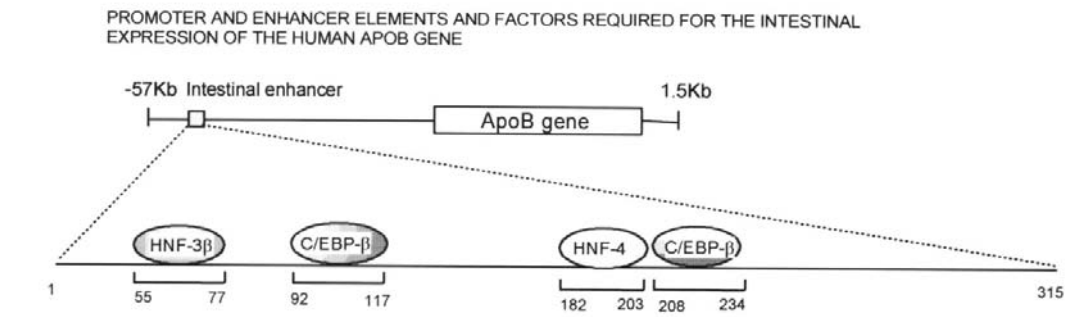
Third intron enhancer
-5262
-2762
+621
+2805
β-gal
-898
+121
3' MAR
5' distal MAR
5' proximal MAR
Second intron enhancer
Proximal promoter
-5Kb
1.5Kb
ApoB gene

F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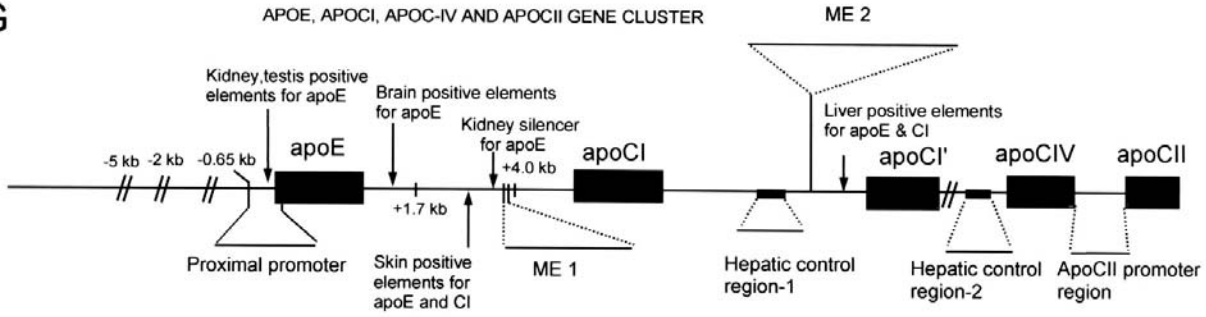
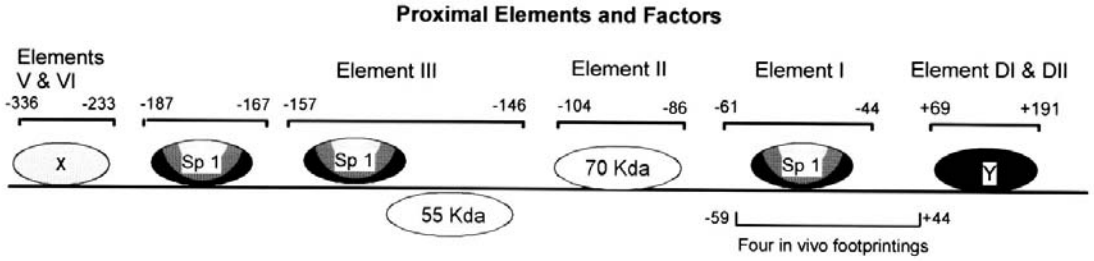
-57Kb Intestinal enhancer
1.5Kb
ApoB gene
HNF-3β
C/EBP-β
HNF-4
C/EBP-β
1
55
77
92
117
182
203
208
234
315

G


APOE, APOCI, APOC-IV AND APOCII GENE CLUSTER
ME 2
Kidney,testis positive elements for apoE
Brain positive elements for apoE
Kidney silencer for apoE
Liver positive elements for apoE & CI
-5 kb
-2 kb
-0.65 kb
apoE
+1.7 kb
+4.0 kb
apoCI
apoCI'
apoCIV
apoCII
Proximal promoter
Skin positive elements for apoE and CI
ME 1
Hepatic control region-1
Hepatic control region-2
ApoCII promoter region

APO E

H


Proximal Elements and Factors
Elements V & VI
Element III
Element II
Element I
Element DI & DII
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-187
-167
-157
-146
-104
-86
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-44
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Sp 1
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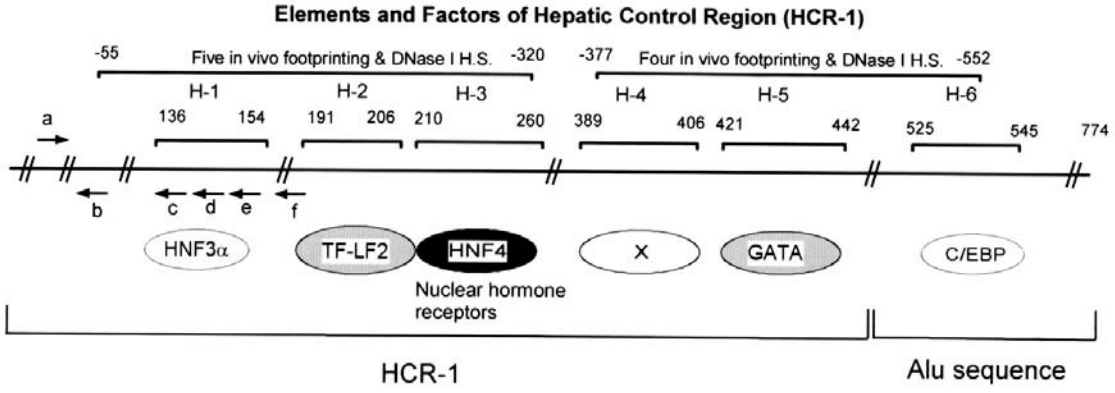
Sp 1
Y
55 Kda
-59
+44
Four in vivo footprintings

I


Elements and Factors of Hepatic Control Region (HCR-1)
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Five in vivo footprinting & DNase I H.S.
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-377
Four in vivo footprinting & DNase I H.S.
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H-1
H-2
H-3
H-4
H-5
H-6
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136
154
191
206
210
260
389
406
421
442
525
545
774
b
c
d
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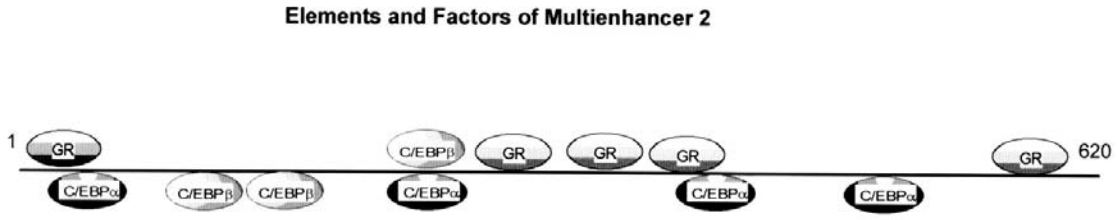
TF-LF2
HNF4
X
GATA
C/EBP
Nuclear hormone receptors
HCR-1
Alu sequence

J


Elements and Factors of Multienhancer 2
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C/EBPβ
GR
GR
GR
GR
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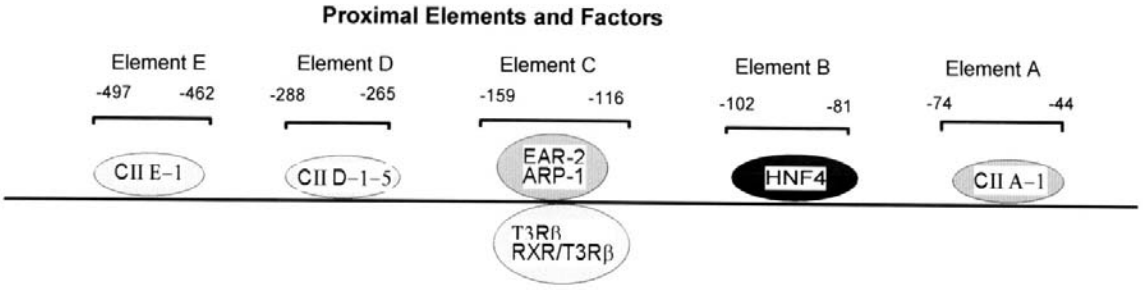
C/EBPα
C/EBPα

APO CII

K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-265
Element C
-159
-116
Element B
-102
-81
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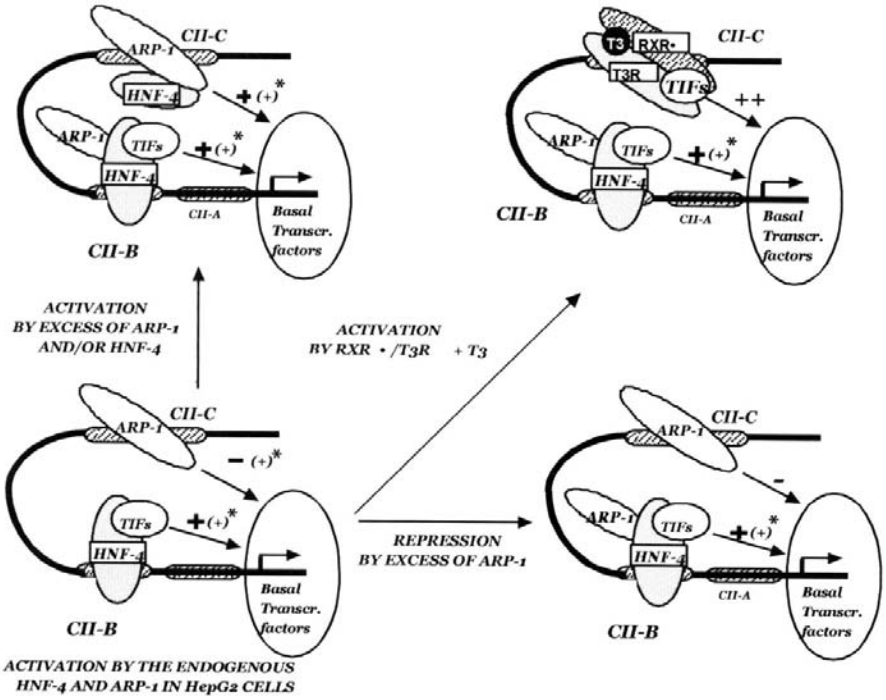
HNF4
CII A–1
T3Rβ
RXR/T3Rβ

L


CII-C
ARP-1
HNF-4
TIFs
CII-A
Basal Transcr. factors
CII-B
ACTIVATION BY EXCESS OF ARP-1 AND/OR HNF-4
ACTIVATION BY RXR • /T3R + T3
REPRESSION BY EXCESS OF ARP-1
ACTIVATION BY THE ENDOGENOUS HNF-4 AND ARP-1 IN HepG2 CELLS

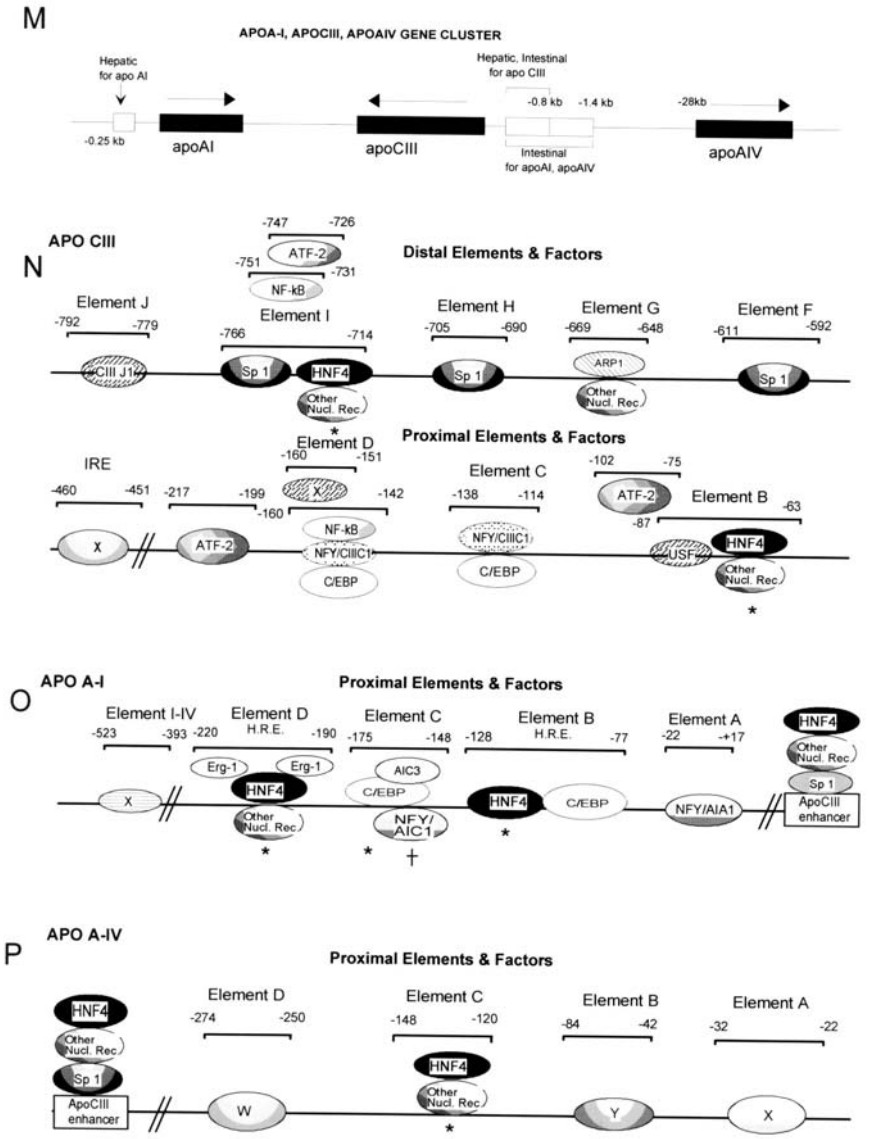


Figure 3A-P. Schematic representation of regulatory elements and transcription factors that participate in regulation of human apolipoprotein genes and putative transcriptional regulatory mechanisms. **Panels B, G and M**: Long distance regulatory elements of the apoB gene, the apoE/CI/CIV/CII gene cluster, and the apoA-I/CIII/A-IV gene cluster, respectively. In most cases, regulatory elements are designated by letters (A, B, etc.) starting from the transcription initiation site. Their boundaries are designated by brackets, with numbers specifying their location relative to the point of transcription initiation. The factors are designated by ellipsoids carrying their names. Asterisks indicate mutations that reduce transcription 1% to 14% (*) and 15% to 30% (**) of control. The symbol + indicates a mutation that increased transcription. The letters V, W, X, Y, and Z (in different panels) indicate nuclear activities of unknown identity. Erg-1 indicates early growth response-1; HS, heat-stable; IRE, insulin response element; SREBP-1, sterol response element binding protein-1; GABP, GA binding protein; Ets-1, E-twenty-six specific; MAR, matrix association region; and beta-gal, beta-galactosidase. The diagrams of A through O are based on references provided in the text. **Panels A, C, D, F, H, I, J, K, M, N, P**: Regulatory elements and factors controlling the transcription of human apoA-II (**Panel A**), apoB (**Panel C**), apoB introns 2 and 3 enhancer and apoB silencer (**Panel D**), apoB elements and factors required for hepatic expression (**Panel E**) apoB intestinal enhancer (**Panel F**), apoE (**Panel H**). Elements and factors of the HCR-1 and the multienhancer-2 (ME-2) of the apoE/CI/CIV/CII cluster (**Panels I and J** respectively), apoCII (**Panel K**), apoCIII (**Panel N**), apoA-I (**Panel O**), apoA-IV (**Panel P**). **Panel L:** Schematic representation showing the role of orphan and ligand-dependent nuclear receptors on the transactivation of the human apoCII promoter. The diagram assumes participation of transcriptional mediators/intermediary factors that exert positive or negative effects on the transcription. + and – indicate transactivation and repression of the promoter activity respectively. (+)* indicates that additional activation of the promoter results from DNA-bound HNF-4/ARP-1 interactions, and/or DNA-bound ARP-1/HNF-4 interactions. The diagram is based on Reference 40.

binding protein (CBP) (108). Upon cholesterol depletion, SREBP-2 family members are cleaved by two proteases at the lumen and the cytoplasmic site of the ER, respectively, to generate the N-terminal fragment (109-113). The SREBP-2 cleavage activating protein (SCAP) cleaves SREBP-2 between Leu522 and Ser523 (109). The processed N-terminal fragment of SREBP-1 and SREBP-2 containing the bHLHZip motif and the activation domain translocates to the nucleus and induces transcription of several genes involved in cholesterol biosynthesis and transport (102,114-116). The importance of SREBP-2 in cholesterol homeostasis has been further demonstrated in Chinese hamster ovary (CHO) cells which contain a 460 amino acid-long truncated SREBP-2 form that activates constitutively the LDL receptor and HMG-CoA synthase genes and confers resistance to 25-hydroxy cholesterol (117). SREBP-1 and SREBP-2 bind to several sites and transactivate the human apoA-II promoter *in vitro* (118,119) (figure 3A).

3.1.8. Upstream stimulatory factor (USF)

The upstream stimulatory factor (USF) appears to be the predominant bHLH/ZIP factor in liver nuclear extracts (120,121). Human USF, first designated as MLTF (major late transcriptional factor), was shown to stimulate transcription of the adenovirus major late promoter (120). Three USF isoforms have been described, USF1, USF2a and USF2b, with apparent molecular weights of 43, 44 and 38 kDa, respectively (122-124). USF1 and USF2a,2b are encoded by two different genes, USF2a and USF2b are generated by differential splicing. While ubiquitously expressed, USF has been involved in transcription of genes with restricted tissue specificity (124-125). USF binds to several sites on the apoA-II and apoCIII promoters and appears to play an important role in their regulation (figure 3A,N).

3.2. The basal transcription machinery: coactivators, corepressors

The class II genes are all transcribed by the RNA polymerase II (polII). This multicomponent enzyme and associated proteins make a complex of more than 70 polypeptides, comparable in size to a ribosome, that is necessary for gene transcription (126). Recent advances permitted the purification and cloning of many of the general transcription factors named TFIIA, B, D, E, F and H which have diverse functions and that participate in the initiation or elongation of transcription *in vitro* (127,128) (figure 2B). Studies in yeast identified a multicomponent complex named the mediator which is associated with the carboxy terminal domain (CTD) of the RNApolII (129-131) and contains nine polypeptides, the SRBs which are suppressors of RNApolB. Another multicomponent complex in yeast, called the switch/sucrose non-fermenting (SWI/SNF) complex, was implicated in the initiation of transcription. Proteins in this complex have the ability to remodel the chromatin and make it more accessible to the transcriptional machinery (132). The ubiquitous presence of the general transcription factors makes them potential targets for protein-protein interaction with transcriptional activators (133). The TFIID general factor is a complex of TATA box binding protein (TBP) and at least nine tightly associated polypeptides called TATA box binding protein-associated factors (TAFs) (134) which are targets for transcriptional activators. Multiple contacts between different transcription factors and their respective TAFs result in stabilization of the preinitiation complex and synergistic activation of transcription (135,136). Several coactivator proteins (137,138) or corepressors (139,140) have been shown to interact with the activation domains of numerous transcription factors (141-147).

Advances in the field have also identified histone acetylase activities in several coactivators and associated factors (148) which remodel the chromatin structure and facilitate the recruitment of all the proteins necessary for gene transcription. Existing hypotheses suggest that the transcriptional activation domains of transcription factors appear to facilitate the recruitment of the basal transcriptional apparatus to transcription initiation site (149-151).

4. METHODOLOGIES USED TO STUDY THE STRUCTURE AND FUNCTION OF TRANSCRIPTION FACTORS, AND THEIR ROLE IN THE TRANSCRIPTIONAL REGULATION OF GENES

Several experimental advances have facilitated the study of eukaryotic promoters and have led to the identification and characterization of several eukaryotic transcription factors. These include: (a) Definition of the long range regulatory elements which confer tissue specificity or developmentally regulated expression. This analysis utilizes transgenic mouse technologies described later in this review. (b) Definition of the promoter region a few kb upstream of the transcription initiation site which is necessary for gene transcription. This analysis monitors the expression of a reporter gene under the control of normal and mutated promoters following transfection of cell cultures. (c) Identification of the different factors which bind to a specific promoter region and definition of their binding sites on the DNA. For this purpose a variety of techniques are used including DNaseI footprinting, *in vivo* footprinting (152-154), gel electrophoretic mobility shift assays (155), supershift assays, DNA binding interference assays which involve modification of G residues by dimethyl sulfate (156) or T residues by oxidation with potassium permanganate (157). The relationship of a factor which binds to a specific regulatory element to previously described factors can be assessed by competition assays, by the direct comparison with the purified factor and by use of anti-factor antibodies in DNA binding assays. Finally, *in vitro* mutagenesis of the promoter region can be used to assess the importance of specific elements for transcription in cell cultures employing usually chloramphenicol acetyl transferase (CAT) or luciferase assays and *in vitro* transcription assays. This information can then be correlated with the ability of a mutated sequence to bind to the factor. The above methodologies also allow the purification of transcription factors and the cloning of cDNAs encoding them. A key step in the protein purification is a DNA sequence-specific affinity chromatography using concatamers of the DNA binding site of the factor as ligand (158). Two main approaches are employed for the isolation of cDNAs encoding mammalian transcription factors. The first involves screening of cDNA libraries with oligonucleotide probes corresponding to a partial protein sequence of the factor. The second approach involves screening of expression cDNA libraries with ^{32}P-labeled synthetic double-stranded oligonucleotides

corresponding to the DNA binding site of the corresponding factor or with appropriate antibodies (159). All known transcription factors are modular in nature and contain a DNA binding domain and a transcriptional activation domain (160). In addition, several factors contain a dimerization or multimerization domain which permits them to form homo- and heterodimers or multiprotein complexes. Finally, a variety of receptors for steroids, thyroids, retinoids, etc. contain a ligand-binding site (161). Isolation, expression and functional analysis of the cloned factors by *in vitro* mutagenesis provides the biological material required to study specific mechanisms which are responsible for transcriptional activation of eukaryotic genes.

In this review, we will present a summary of our current knowledge on the regulation of transcription of the apolipoprotein genes. Emphasis will be given to recent advances on the regulation of transcription of the human apoA-I, apoCIII, apoA-IV gene cluster and the human apoE, apoCI, apoCIV, apoCII gene cluster *in vivo* and on signaling mechanisms which affect apoCIII gene transcription.

5. *IN VITRO* STUDIES OF APOLIPOPROTEIN GENE REGULATION

5.1. Definition of the regulatory elements and the factors controlling apolipoprotein gene expression

Utilization of the techniques outlined above resulted in the mapping of the proximal regulatory elements of most of the apolipoprotein promoters and the factors which bind to them. The information obtained for the apoA-I, apoCIII, apoA-IV, apoB, apoA-II, apoCII and apoE is shown in figure 3A-N (162). The established names of known transcription factors are used. The names of unidentified factors is based on a) the name of the target gene; b) the element to which the factors bind; and c) the mobility of the DNA/protein complexes. Thus, the activities which bind to the regulatory element A-IC of the apoA-I promoter are designated AIC1, AIC2, AIC3, etc. (163). The slow-migrating activities which bind to the regulatory elements CIIIC and CIIID of apoCIII are designated CIIIC1 and CIIID1, etc. (164). The systematic analysis of 6 apolipoprotein promoters by us resulted in the identification of 37 regulatory elements (162,165). Four elements were also identified in the proximal apoE promoter, six elements in the hepatic control region of the apoE/apoCI/apoCIV/apoCII gene locus, six elements in the second intron enhancer of apoB, three elements in the third intron enhancer of apoB, and one element in the 5′ silencer of the apoB gene (162). A careful examination of the activities that have been identified indicates that several previously described factors participate in the transcriptional regulation of the apolipoprotein genes (figure 3A-P). This includes the liver-enriched factors C/EBP, HNF-1, HNF-3, HNF-4 (26,27,70,166) as well as ubiquitous nuclear factors (NF) such as NF1, NFY, SP1, GA-binding protein/E twenty-six-specific (GABP/Ets-1), SREBP and USF (table 2) (91,99,100,103,122,167,169).

Although figure 3A-P and table 2 indicate that several previously described transcription factors may recognize different apolipoprotein promoters, the arrangement of the factors within each promoter is unique. This unique arrangement of the regulatory elements and the factors bound to them (referred to as promoter context) may allow the formation of a unique and stereospecific DNA protein complex which contributes to the activation of the target gene.

5.2. Important elements and factors involved in the transcriptional regulation of the human apoA-II gene

ApoA-II synthesis is restricted to the liver and, to a much lesser extent, to the intestine (170). The apoA-II promoter contains a set of seven proximal and middle (AIIA to AIIH) and seven distal (AIII to AIIN) direct predominantly regulatory elements between nucleotides-903 and –33 of the apoA-II promoter (171-173) (figure 3A). This region is sufficient to restrict the apoA-II gene expression in the liver, and to a much lesser extent (5%) in the intestine of transgenic mice (174). The distal regulatory elements act as an enhancer which potentiates the strength of homologous apoA-II as well as the strength of heterologous liver-specific promoters (162,171). It appears that there is synergy between factors bound to the proximal promoter and the apoA-II enhancer in the transactivation of the apoA-II promoter/enhancer cluster (175).

Transfection experiments in HepG2 cells also showed that the first intron of the human apoA-II gene between nucleotides +38/+206 acts as silencer and reduces the strength of the apoA-II promoter (-911 to +38) to 15-18% of its original value in HepG2 and CaCo-2 cells. This region also reduces the strength of the heterologous thymidine kinase (TK) promoter (176).

5.2.1. Role of USF on the apoA-II promoter activity

An important role in apoA-II gene transcription is exerted by a heat-stable transcription factor, USF, which binds to the regulatory elements AIIAB, AIIK, and AIIL (figure 3A). This factor was previously designated CIIIB1, based on its ability to recognize the regulatory region CIIIB of the apoCIII promoter (177).

Simultaneous nucleotide substitutions which prevented the binding of CIIIB1 activity in elements AIIAB, AIIK and AIIL reduced the strength of the apoA-II promoter in HepG2 and CaCo-2 cells to 6-7% of control (175). Elements AIIAB and AIIK bind, in addition to USF, a heat-labile activity designated AIIAB1 (175). Element AIIAB also binds RXR alpha/T3R beta heterodimers. Deletion of elements AIIK, AIIL, or AIIAB1 reduce the promoter activity by 76 to 90% in HepG2 cells, and by 85 to 96% in CaCo-2 cells (178).

Cotransfection experiments in HepG2 cells established the functional significance of USF in apoA-II transcription. It was found that the minimal promoter containing the element AIIAB was transactivated by USF2a (179). In addition, all three E-boxes present in the proximal AIIAB and distal AIIK and AIIL elements were necessary for transactivation by USF2a. A dominant negative form of USF2a inhibited the transactivation of apoA-II promoter constructs containing the regulatory elements AIIAB, AIIK and AIIL of apoA-II. The USF1/2a heterodimer which is naturally expressed in the liver is as efficient as the USF2a homodimer in the transactivation of apoA-II promoter/enhancer constructs. Mutation within the proximal E-box of element AIIAB abolished the USF2a mediated transactivation (179).

Table 2. Binding sites for C/EBP, nuclear receptors, CIII-B1, HNF-1, and HNF-3 in apolipoprotein promoters

Factor	Gene	Element	Type of HRE (dr)
C/EBP	Human ApoA-I	AIC (-148 to -156) nc AIC (-161 to -169) nc	
	Human ApoA-II	AIIAB (-39 to -47) nc AIIC (-113 to -121) nc AIID (-262 to -270) nc AIIF (-399 to -391) c AIIG (-471 to -462) c AIIL (-778 to -786) nc	
	Human ApoB	BA2 (-40 to -48) nc BA3 (-66 to -58) nc	
	Human ApoCIII	CIIIC (-127 to -135) nc CIIID (-155 to -147) c	
Nuclear Receptor	Human ApoA-I	AIB (-132 to -117) nc AID (-210 to -190) nc	DR-1 DR-2
	Human ApoA-II	AIIJ (-733 to -721) nc	DR-1
	Human ApoB	BAI (-79 to -66) nc Reducer element (-2797 to -2786) nc	DR-3
	Human ApoCII	CIIB (-102 to -81) nc CIIC (-159 to -116) nc	DR-0 DR-4
	Human ApoCIII	CIIIB (-82 to -69) nc CIII-I_4 (-736 to--720) c CIIIG (-689 to -648) c	DR-1 DR-1 DR-0 and DR-5
	Human ApoA-IV	A-IVC (-142 to-127) c	DR-4
USF	Human ApoA-I I	AIIAB (-54 to -61) nc AIIK (-751 to -744) c AIIL (-799 to -792) c	
	Human ApoCIII	CIIIB (-86 to -79) c	
HNF-1	Human ApoA-II	AIIN (-896 to -882) c AIIH (-554 to -572) nc	
HNF-3	Human ApoB	BC (-95 to -106) nc	
SREBP-1 & 2	Human ApoA-II	AII (AIIAB, AIICD, AIIDE, AIIHI, AIIK)	
NF kappa B	Human ApoCIII	CIIID, CIII-I	
ATF-2	Human ApoCIII	CIII BC, CIII-I	
SP1	Human ApoCIII	CIIIF, CIIIH, CIII-I	

Cotransfection experiments in COS-1 cells also showed that HNF-4 synergizes with USF2a in the transactivation of the apoA-II promoter. In addition, it was shown that HNF-4 and USF2a bind to the enhancer cooperatively (179). This cooperativity may account for the transcriptional synergism observed between USF and HNF-4 in the transactivation of the apoA-II promoter.

5.2.2. Role of SREBP on the apoA-II promoter activity

DNA binding and footprinting assays showed that the cholesterol-regulated transcription factors SREBP-1 and SREBP-2 bind to the regulatory regions AIIAB, AIICD, AIIDE and AIIK. SREBP-1 also binds to the regulatory region AIIH (118,119) (figure 3A). These regions contain either palindromic (CANNTG) or direct repeat (CACCCCAC) motifs. DNA binding and interference assays using the AIIAB region as probe showed that the nucleotides of a decameric palindromic repeat RTCAMVTGMY and two 5′ T residues participate in DNA-protein interactions with SREBP-2. This binding motif overlaps with the binding site of USF (177). USF (CIIIB1) and SREBP cannot form heterodimers *in vitro* (119). Transient cotransfection experiments in HepG2 cells showed that WT SREBP-1 and SREBP-2 transactivated apoA-II promoter segments. SREBP-1 or SREBP-2 mutants lacking the amino terminal activation domain bound normally to their cognate site and repressed the apoA-II promoter activity (118,119). Repression was also caused by single or multiple amino acid substitutions which affected DNA binding. Repression by the DNA binding deficient mutants was abolished by deletion of the amino-terminal activation domain (1-90) of SREBP-1 or (1-93) of SREBP-2 indicating that repression may result from squelching of positive activator(s) which appear to recognize the activation domains of SREBP-1 and SREBP-2 (118,119). The *in vivo* role of SREBP-1 and SREBP-2 in apoA-II gene transcription has not been established.

5.2.3. Role of HNF-4 and other nuclear receptors on the apoA-II promoter activity

Important role in apoA-II gene regulation is also exerted by the HRE present on the regulatory element AIIJ of apoA-II (figure 3A). Element AIIJ contains an HRE with DR1 spacing which binds HNF-4 and other orphan and ligand-dependent nuclear receptors (27,38,179) (table 2). Cotransfection experiments in HepG2 cells showed that HNF-4 transactivated by 2.2-fold and ARP-1, EAR-2 and EAR-3 repressed by 60-65% the –911/+29 apoA-II promoter activity (38,178). Other studies showed that RXR alpha/T3R beta or Ultraspiracle (Usp)/T3R beta bind and transactivate the human apoA-II promoter by 7- to 8-fold in the presence of T3 (180). Transactivation requires complex interactions with HNF-1 and other factors which bind to upstream elements and utilizes the activation domain of T3R beta. Usp is the Drosophila homologue of RXR alpha. The RXR alpha/T3R beta and Usp/T3R beta bind to the regulatory elements AIIAB and AIIJ of the apoA-II promoter (180) (figure 3A; table 2). The apoA-II promoter is also transactivated by RXR alpha and RAR alpha homodimers indicating that hormone nuclear receptors play an important role in the regulation of the apoA-II gene. *In vivo* studies showed that fibrates increased the expression of the apoA-II in mice by activation of PPAR alpha which binds to element AIIJ and transactivates the mouse apoA-II promoter (181). The contribution of GAPBP and other factors which bind to the apoA-II promoter (179,182) in apoA-II gene transcription has been reviewed previously (162).

5.3. Important elements and factors involved in the transcriptional regulation of the human apoB gene.

5.3.1. Role of hormone nuclear receptors and C/EBP on the apoB promoter activity

The human apoB gene is localized in a 47.5 Kb region flanked by matrix association regions (MARs) (183). The proximal apoB promoter region between nucleotides –150 to +124 can direct the expression of a reporter gene in hepatic and intestinal cells but not in HeLa cells (184,185) (figure 3B,C), but was not sufficient to direct apoB gene transcription *in vivo* (186). *In vitro* mutagenesis of the proximal apoB promoter established that important for transcription were the regulatory elements BCB which bind HNF-3 between nucleotides –112/-95 and the regulatory element BA (185). This element contains an HRE with DR3 spacing and binds orphan and ligand-dependent nuclear receptors in the –86 to –62 region and C/EBP in the –72 to –54 region (table 2) (figure 3C). Mutations which prevented the binding of these factors to their cognate site reduced the promoter activity to 2-13% of its initial strength (figure 3C) (185). Other less important C/EBP binding sites are also present in the proximal promoter (figure 3C).

5.3.2. Role of distal regulatory elements on the hepatic expression of the apoB gene

Tissue culture experiments have shown that the second intron of the apoB gene between nucleotides +621 and +1064 enhances by 3- and 5-fold the strength of the apoB promoter in HepG2 and CaCo-2 cells, respectively but not in HeLa cells (187) (figure 3B). Similarly, the apoB sequence between nucleotides 1065 to 2977 enhances the strength of the apoB promoter approximately two-fold in HepG2 and CaCo-2 cells respectively (188). Finally, the region between nucleotides -3067 to -2734 represses the strength of the apoB promoter in CaCo-2 but not in HepG2 cells (189). These regions bind HNF-4, C/EBP and ARP-1 (figure 3D). Transgenic mouse experiments indicated that the second intron enhancer region is sufficient to direct expression of apoB promoter constructs in the liver but not in the intestine (187) (figure 3E). Incorporation of both the second and the third intron enhancers and sequences containing the 5′ and 3′ MARs in the apoB constructs increased their hepatic expression, but did not eliminate the integration-related position effects on the levels of the expression of the transgene. The inclusion of 5′ upstream negative regulatory region in this construct did not affect its hepatic expression *in vivo* (figure 3E) (186). Subsequent work showed that sequences located 5 Kb upstream and 1.5 Kb downstream of the apoB gene were sufficient for the hepatic expression of the apoB gene in transgenic mice (190,191).

5.3.3 Role of distal regulatory elements and factors required for the intestinal expression of the apoB gene

The intestinal expression of apoB required sequences located between 62 and 54 Kb upstream of the apoB gene. Recently, an intestinal enhancer was localized within 315 nucleotides approximately 56 Kb upstream of the apoB gene.

This region contains DNaseI hypersensitive sites specific for CaCo-2 cells. DNA binding assays identified binding sites of the liver-specific transcription factors HNF-4, HNF-3 beta, C/EBP beta. The enhancer could transactivate by approximately 2- to 4-fold a minimal promoter in the presence of either HNF-4, HNF-3 beta and C/EBP beta. The intestinal enhancer could also direct expression of an 80 Kb human apoB gene construct in the intestine when both segments were co-integrated in the DNA of transgenic mice (191) (figure 3F).

5.4. Important elements and factors involved in the regulation of the genes of the apoE/apoCI/apoCIV/apoCII cluster

The human apoE, apoCI, apoCIV, and apoCII genes are closely linked. The cluster of the four genes maps on the long arm of chromosome 19 and spans a 45 Kb region (192). The apoCI gene is located 5.5 Kb downstream of the apoE gene, and the apoCI′ pseudogene is located 7.5 Kb 3′ to the apoCI gene. The human apoCII gene is found 20 Kb downstream of the apoCI′ pseudogene and the human apoCIV gene is 0.55 Kb upstream of the apoCII gene (192,193) (figure 3G).

5.4.1. Proximal apoE regulatory elements and factors

Promoter deletions and footprinting analysis of the apoE promoter identified six proximal regulatory elements (I to VI) in the -336 to -44 region and two elements (DI and DII) within the +69 to +191 region (figure 3H) (194). DNA binding and competition assays using HeLa nuclear extracts showed that elements I, III, and IV are recognized by SP1. Element II is also recognized by a 70 kDa protein (figure 3H). Element III is recognized by a 55 kDa protein (194,195). Deletion analysis of the -246 to -81 region placed in front of the Herpes thymidine kinase promoter indicated that element III is important for transcription of the reporter CAT gene in CHO cells. Elements II and III are also essential for transcription of the apoE gene in hepatic cells (194). Similar information was obtained by *in vitro* transcription using the -383 to +73 region as a template and HeLa nuclear extracts (figure 3H). The *in vitro* transcription analysis also showed that element I contributes to the optimum transcription in HeLa cells (195).

5.4.2. Distal regulatory elements and factors

The distal regulatory elements which control the transcription of the apoE gene in different tissues have been determined using transgenic mice. Footprinting analysis combined with DNA binding and competition assays identified four regulatory elements within or 3′ of the HCR-1, that are designated H-1 to H-4 (figure 3I) (196). Element H-1 and the surrounding region contain four tandemly repeated motifs TGTTGC, in the antisense strand, designated c, d, e and f (figure 3I). In addition, two related sequences designated a and b are found 5′ upstream of footprint H-1. The TGTTTGC motif is found in the promoters of several genes expressed in the liver and binds a transcription factor which recognizes single-stranded DNA (197). The TTTG core of this motif is found in the core of the DNA-binding site of high mobility group proteins (198) which upon binding introduce pronounced bending to the DNA (199). Thus it is possible that binding of factors to these sites help in the formation of stereo specific DNA complexes involving the proximal and distal activators as well as the proteins of the basal transcription system. Element H-2 binds a factor designated TF-LF2 which recognizes the -480 region of the rat transferrin promoter (200). Element H-3 contains two direct repeat sequences AGGTCA(G)AGA-CCT which can be classified as an HRE with one spacer nucleotide shown in parenthesis. This sequence binds HNF-4 weakly, nevertheless it is possible that it binds with higher affinity other members of the orphan or ligand-dependent nuclear receptors (201). The factor which binds to H-4 element is unknown and has been designated X. Element H-5 contains a GATA motif found in the hemoglobin locus control region, which binds GATA-binding factors (202). Element H-6 is within an Alu family sequence which contains several DNaseI hypersensitive sites (196,203). This sequence binds activities related to the C/EBP family members. The sequence of the HCR-1 between nucleotides 1 to 461 which contain elements H-1 to H-5 is a functional HCR. However, a smaller region between nucleotides 6 to 325 which contains footprints H-1 to H-3 as well as a longer region between nucleotides 6 to 587 which contains all the elements H-1 to H-6 can direct liver specific expression of apoE in a relatively copy-independent manner (196). Subsequently, a second hepatic control region designated HCR-2 was identified 27 Kb 3′ of the apoE gene in the middle of the intergenic sequence between the apoCI′ pseudogene and the apoCIV gene (204) (figure 3G). *In vivo* footprinting identified seven footprints in liver nuclei extending from –55 nucleotides upstream to 302 nucleotides of the HCR-1 and five liver-specific DNaseI hypersensitive sites. Four *in vivo* footprints and five DNaseI hypersensitive sites were found in the 377 to 552 segment of the HCR-1 (203 and figure 3I). Four *in vivo* footprints in liver nuclei were identified in the proximal +44/-59 region of the promoter, one of them overlapping with the SP1 site of element I of the proximal promoter, and the next one covering the transcription initiation site (figure 3I) (203). Microsomal nuclease digestion of nuclei and ligation-mediated polymerase chain reaction (LMPCR) analysis gave 3 prominent bands at positions 3, 268 and 243, and 347 of the HCR-1 in liver, brain and kidney, suggesting an ordered positioning of the nucleosomes within this region (203).

Two other highly homologous regulatory regions which control apoE expression in macrophages and adipose tissue were identified recently. These regions were designated multi-enhancer 1 and 2 respectively (ME-1, ME-2). ME-1 and ME2 are located 3.3′ Kb and 15.9 Kb downstream of the apoE gene and contain 620 and 619 nucleotides respectively (205). Binding motifs for glucocorticoid receptor (GR) and C/EBP-alpha and C/EBP-beta were identified on ME-1 and ME-2 using the Transfac database (206) and are shown in figure 3J.

5.5 Important elements and factors involved in the regulation of the human apoCII gene

5.5.1. The role of nuclear receptors and the hepatic control region-1 on the apoCII promoter activity

The human apoCII promoter contains five regulatory elements designated CIIA (-74/-44), CIIB (-102/-81), CIIC (-159/-116), CIID (-288/-265) and CIIE (-497/-462) (165). Element CIIB contains an HRE with DR0 spacing that is recognized by HNF-4 but not by ARP-1 or EAR-3 whereas element CIIC contains an HRE with DR4 spacing which is recognized by ARP-1 and EAR-3 but not by HNF-4 (figure 3K, table 2). HNF-4 transactivated the apoCII promoter or the

apoCII promoter linked to the hepatic control region –1 (HCR-1) which is present in the apoE/CI/CIV/CII gene cluster (figure 3G). A double mutation in elements CIIB and CIIC that eliminated binding of HNF-4 or ARP-1 and EAR-3, respectively, to these sites abolished the enhancer activity of HCR-1 (165). The combined data suggest that the apoCII promoter/HCR-1 cluster can direct expression in cells of hepatic origin and that optimal enhancer activity requires synergistic interactions between factors bound to the distal HCR-1 and nuclear receptors bound to the two proximal hormone response elements. Transient cotransfection experiments showed that in the presence of T3, RXR alpha/T3R beta heterodimers transactivated the –205/+18 apoCII promoter 1.6- and 11-fold in HepG2 and COS-1 cells respectively. Transactivation requires the regulatory element CIIC which contains a thyroid hormone response element (40). This element also binds ARP-1 which, in normal concentrations, may repress the apoCII promoter activity. No transactivation was observed in the presence of 9-cis-RA. Mutations in the HNF-4 binding site on element CIIB and inhibition of HNF-4 synthesis in HepG2 cells by antisense HNF-4 ribozyme constructs decreased the apoCII promoter activity to 25-40% of the control, indicating that HNF-4 is a positive regulator of the apoCII gene. Although ARP-1 represses the apoCII promoter activity in HepG2 cells by binding to element CIIC, combination of ARP-1 and HNF-4 transactivated different apoCII promoter segments that contain element CIIB, which binds only HNF-4. Transactivation may result from HNF-4/ARP-1 interactions that elicit the transactivation functions of HNF-4. The findings indicate that depending on the specificity of the HRE, ARP-1 has the ability to act either as a repressor when it binds to DNA, or as an activator when it interacts via protein-protein interactions with HNF-4 that is bound to DNA. Such protein interactions could allow utilization of the activation domain of HNF-4 that is associated with ARP-1 to drive transcription (40). When RXR alpha/T3R beta heterodimers in the presence of T3 bind to the element CIIC, which is also recognized by ARP-1, the apoCII promoter activity is increased. This indicates that combination of different types of nuclear receptors may either stimulate or repress apoCII gene transcription. This hypothetical modulation of the apoCII promoter activity by orphan- and ligand-dependent nuclear receptors is shown in figure 3L.

5.6. Important regulatory elements and factors involved in the regulation of the human apoCIII gene

5.6.1. The role of hormone nuclear receptors and USF

The apoCIII gene is closely linked to the human apoA-I and apoA-IV genes (207). The apoCIII gene is localized 2.5 kb down-stream of the apoA-I gene and 5 kb upstream of the apoA-IV gene. The direction of transcription of the apoCIII gene is opposite to that of apoA-I and apoA-IV genes (figure 3M). The human apoCIII promoter contains a set of 4 proximal (CIIIA-CIIID) and six distal (CIIIE-CIIIJ) regulatory elements between nucleotides -792 to -25 (164) (figure 3N). Element CIIIB binds USF, previously designated CIIIB1 and hormone nuclear receptors in overlapping binding motifs (177,179). USF binds to an octameric CAGGTGAC sequence between nucleotides -86 to -79 of the coding strand. Hormone nuclear receptors bind to the HRE between nucleotides -82 to –69. This HRE has a DR1 spacing and binds strongly HNF-4, ARP-1, EAR-2 and EAR-3, heterodimers of RXR alpha with RAR alpha, and less efficiently, homodimers of RAR alpha and heterodimers of RXR alpha with T3R beta or PPAR alpha (38,164, 208-210).

5.6.2. The role of the distal regulatory elements, hormone nuclear receptors, and SP1 on the apoCIII promoter activity

The distal apoCIII promoter contains three binding sites for the ubiquitous transcription factor SP1, which recognizes the regulatory elements CIIIF, CIIIH, and CIII-I. The regulatory elements CIII-I4 (-736/-714) and CIIIG (-669/-648) of the apoCIII enhancer contain HREs which are recognized by various combinations of orphan and ligand-dependent nuclear receptors (38,164,208-210). Element CIIIG, which contains HREs with both DR0 and DR5 spacing, binds strongly on the DR0 HRE ARP-1 and EAR-3 (210). Element CIIIG also binds strongly, on the DR5 HRE, heterodimers of RXR alpha with either RAR alpha. or T3R beta and does not bind HNF-4 (208,210). Finally, element CIII-I4, which contains a DR1 HRE, binds strongly HNF-4, ARP-1, EAR-3, and RXR alpha/RAR alpha heterodimers and less efficiently, RXR alpha/T3R beta heterodimers (208,210). Cotransfection experiments have shown that HNF-4 and RXR alpha heterodimers in the presence of 9-cis RA act as positive regulators of the apoCIII promoter (38,210). A single activity designated CIIIJ1 binds to the regulatory element CIIIJ. This factor also binds to the regulatory elements CIII-I and CIIIF. Other factors which bind to the various apoCIII regulatory elements have been reviewed previously (162). A series of *in vitro* studies have pointed out that the distal apoCIII regulatory elements may act as homologous enhancers for apoCIII (164,208) as well as for the other two genes of the cluster (209,211,212). The *in vitro* experiments showed that deletion of the distal apoCIII promoter region reduced the strength of the proximal promoter by 75 to 95%, implying that this region may represent a transcriptional enhancer (164) (figure 4A). Further studies indicated that constructs which contain the upstream apoCIII regulatory elements CIIIF to CIIIJ could increase the strength of the other two promoters of the cluster, the apoA-I (209) (figure 4B) and apoA-IV (211) (figure 4C), as well as the strength of the heterologous apoB and AdML promoters (162,209) (figure 4D).

5.6.3. The role of other factors on the apoCIII promoter activity

The regulatory elements CIIIC and CIIID bind heat-stable activities as well as members of the C/EBP family. The CIIICD region also contains two binding sites for a new activity designated CIIIC1 (213) which has not been characterized further. Elements CIIID and CIII-I also bind NF-kappa-B, suggesting a potential role of this region in acute phase response (213,214). Element CIII-I and two other regions, designated BC and DE, bind the transcription factor activating transcription factor-2 (ATF-2), which can be activated by extracellular signals (215). The sequence -460 to -451 of apoCIII contains a motif TCCAAACATC which has high homology to an insulin response element (IRE) found in the phosphoenol pyruvate carboxy kinase (PEPCK) promoter (216). ApoCIII steady-state mRNA levels and transcription rates were elevated in diabetic rats and could be decreased significantly by treatment with insulin. Insulin also repressed the apoCIII promoter activity and it was proposed that the IRE of the apoCIII promoter is responsible for this transcriptional repression (217).

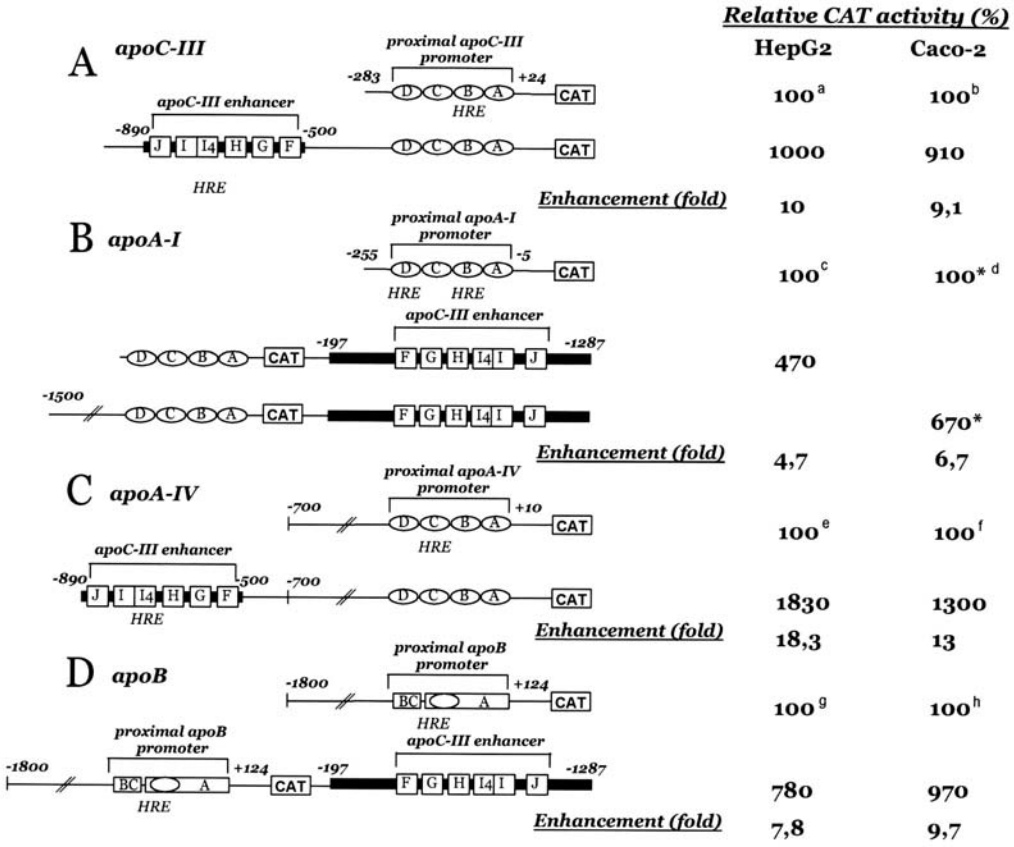


Figure 4A-D. Schematic representations showing the effect of the apoCIII enhancer on transcriptional activity of the apoCIII (**A**), apoA-I (**B**), apoA-IV (**C**), and apoB (**D**) promoters. The Figure establishes that the distal apoCIII regulatory elements CIIIF to CIIIJ enhance the strength of homologous and heterologous promoters. The letters a through h indicate reference values (100%) for each set of experiments. * indicates transactivation values obtained by cotransfection of the indicated promoter and vectors expressing HNF-4.

5.6.4 Effect of the apoCIII enhancer on the activity of the proximal apoCIII promoter in HepG2 and CaCo-2 cells

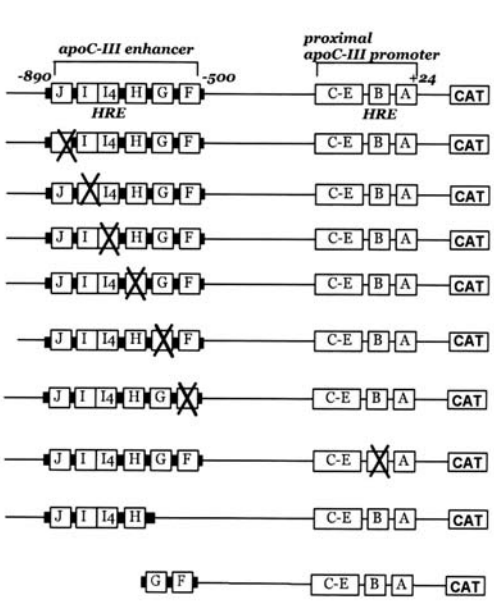
The contribution of the distal apoCIII regulatory elements and factors to intestinal and hepatic transcription of the apoCIII gene was assessed by mutations which eliminate the binding of these factors to their cognate site or by deletions of certain elements (figure 5A). This analysis yielded the following interesting generalizations: a) The apoCIII promoter strength in HepG2 cells is affected mostly by mutations in elements CIIIH, CIIIG and CIII-I4 (208,209). As shown in figure 3N, elements CIIIB, CIIIG and CIII-I4 bind nuclear hormone receptors and element CIIIH binds SP1. b) The intestinal transcription was not affected by mutations or deletions of element CIIIG (figure 5A).

Cotransfection experiments of HepG2 and CaCo-2 cells showed that HNF-4 transactivated the apoCIII promoter in HepG2 and CaCo-2 cells by approximately 6- to 8.4-fold (208). Transactivation was affected by mutations in the HREs of element B or deletion of upstream regions which contain element CIII-I4. It is interesting that although mutations or deletion of the element G did not affect the apoCIII promoter activity in CaCo-2 cells, it diminished the HNF-4-mediated transactivation of the promoter (figure 5A). These findings suggest that the communication of nuclear receptors and SP1 molecules bound to the proximal and distal sites are important for the transcriptional activation of the apoCIII enhancer/proximal promoter cluster (208).

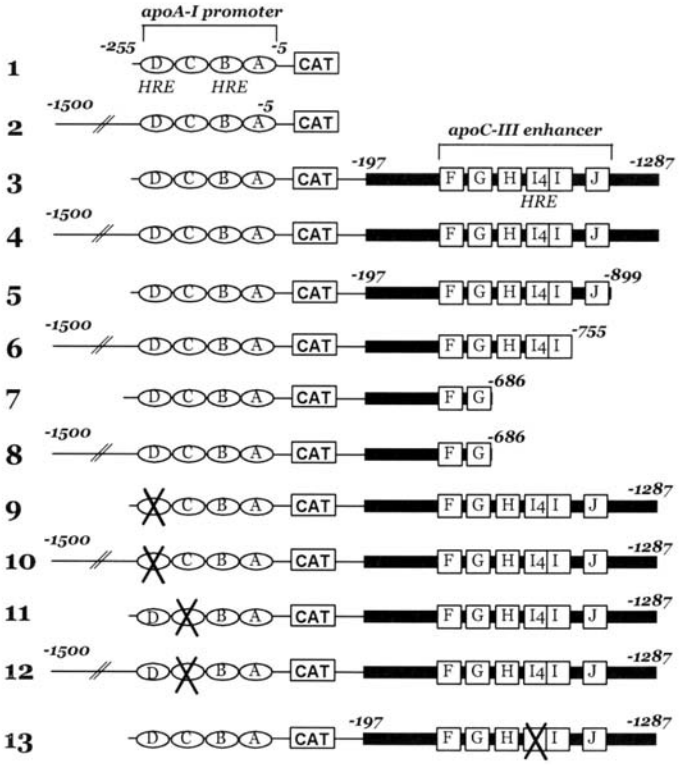
5.7. Important elements and factors involved in the regulation of the human apoA-I gene

5.7.1. The role of nuclear receptors

The human apoA-I promoter contains three proximal regulatory elements AIB (-128 to -17), AIC (-175 to -148), and AID (-220 to -190), which are required for transcription of the apoA-I gene in HepG2 cells (163). The regulatory elements AID and AIB contain HREs with DRR1 and DR2 spacing respectively, and bind to members of the steroid/thyroid receptor superfamily (200) (figure 3O) (table 2). Both elements AIB and AID contain DR1 HREs and bind HNF-4, other nuclear orphan receptors as well as the ligand-dependent nuclear receptors RXR-alpha, RXR-alpha/RAR-alpha and RXR-alpha/T3R-beta (218-221). Cotransfection experiments in HepG2 cells showed that RXR-alpha homodimers transactivated the wild-type promoter to 150% of control in the presence of 9-cis-RA, whereas RXR-alpha/T3R-beta heterodimers repressed transcription to 60% of control, in the presence of T3. RXR-alpha/RAR-alpha and HNF-4 did not affect the transcription driven by the proximal apoA-I promoter (220). Drastic mutagenesis which altered either part

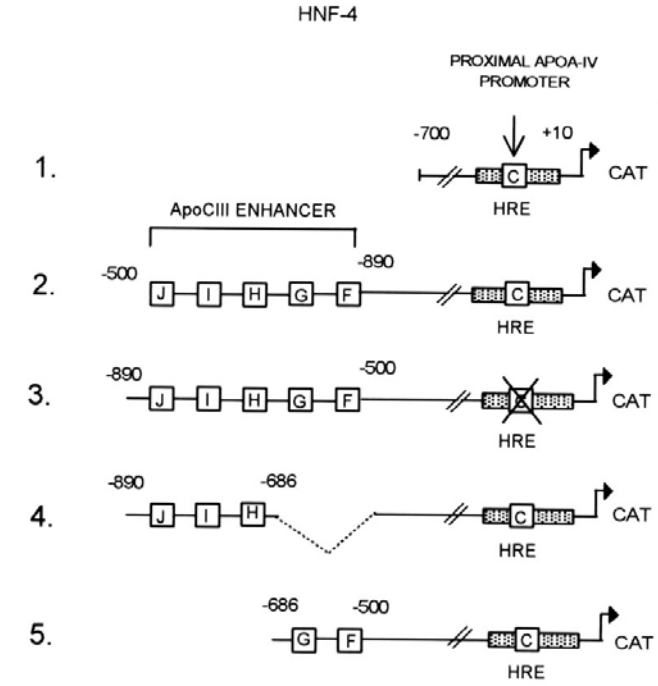


Relative CAT activity (%)				Enhancement (fold)		Transactivation (fold)	
HepG2		CaCo-2					
-HNF4	+HNF4	-HNF4	+HNF4	hepatic	intestinal	hepatic	intestinal
100	840	100	604	-	-	8,4	6
67	750	43	246	0,67	0,43	11	5
42	590	41	130	0,42	0,41	14	3
7				0,07			
14	130	25.1	91	0,27		9,3	3,6
27	250	105	173	0,27	1,05	9,3	1,7
57	710	54	221	0,57	0,54	12,5	4,1
5	21	9	24	0,05	0,09	4,2	2,7
25	80	160	210	0,25	1,6	3,2	1,3
9	110	9	24	0.09	0,09	12,2	2,7

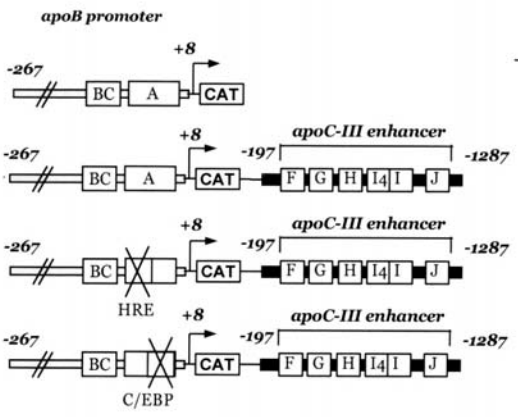


	Relative CAT activity (%)		Enhancement (fold)
	HepG2 - HNF-4	Caco-2 + HNF-4	
1	100		100
2		100	100
3	470 ± 100		4.7 *
4		670 ± 140	6.7
5	670 ± 153		6.7
6		270 ± 30	2.7
7	130 ± 45		1.3
8		170 ± 18	1.7
9	30 ± 5		0.3
10		50 ± 3	0.5
11	305 ± 70		3.1
12		700 ± 19	7.1
13	84 ± 19		0.8

THE DISTAL APOCIII REGULATORY ELEMENTS F TO J OF THE APO CIII PROMOTER ACT AS AN ENHANCER IN APOA-IV TRANSCRIPTION



	HepG2		Caco2		Transactivation (fold)	
	– HNF-4	+ HNF-4	– HNF-4	+ HNF-4	Hepatic	Intestine
1.	100	120	100	110	1.2	1.1
2.	1830	15660	1300	5010	8.5	3.9
3.	390	450	380	390	1.1	1
4.	300	2050	420	1020	6.8	2.4
5.	100	220	110	160	2.2	1.5



	Relative CAT activity (%)				
	HepG2	CaCo-2		Enhancement (fold)	Transactivation (fold)
	-HNF4	-HNF4	+HNF4	hepatic	intestinal	intestinal
	100	100	13000			13
	420	720		4.2	7.2	
	8	20	65	0.08	0.2	3.3
	210	240	680	2.1	2.4	2.8

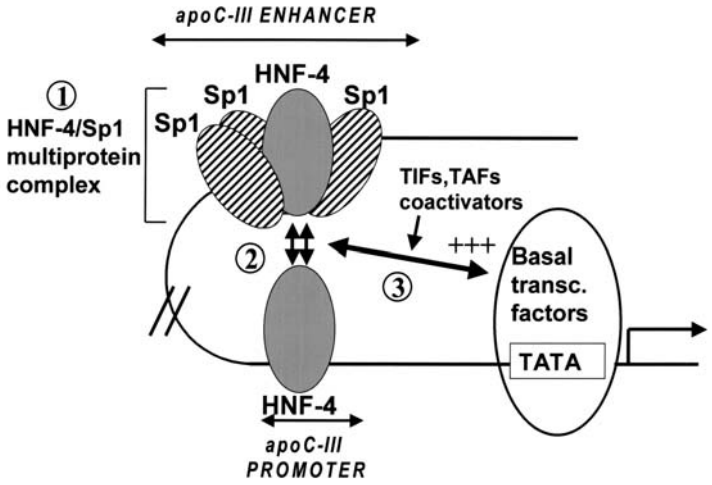


Figure 5A-E. Panel A: Effect of mutations in the proximal and distal apoCIII promoter on its strength and its transactivation by HNF-4. The Figure shows the effect of mutations which affected binding of the corresponding factors to their cognate sites, on the promoter strength and the HNF-4-mediated transactivation of the apoCIII promoter in HepG2 and CaCo-2 cells. Note that elements CIIIB and CIII-I_4 are essential for the transactivation in HepG2 and CaoCo-2 cells. Mutations in element CIIIG affect differently the hepatic and intestinal transactivation. **Panel B:** Effect of mutations on the strength of the -255 to -5 apoA-I promoter/apoCIII enhancer cluster in HepG2 cells and the transactivation of the -1500 to -5 apoA-I promoter/apoCIII enhancer cluster by HNF-4 in CaCo-2 cells. **Panel C:** effect of mutations on the strength of the apoA-IV promoter/apoCIII enhancer cluster in HepG2 and CaCo-2 cells and its transactivation by HNF-4. **Panel D:** Effect of mutations on the strength of the –267 to +8 apoB promoter/apoCIII enhancer cluster in HepG2 and CaCo-2 cells and its transactivation by HNF-4. The relevant regulatory elements of the apoA-I/apoCIII/apoA-IV promoter and the apoCIII enhancer and the factors bound to them have been described in Figure 3N-P. Mutated sites are indicated by X. Note in Panels B and D that the apoCIII enhancer can bypass mutations in the C/EBP binding site which inactivate the proximal apoA-I and apoB promoter. * in Panel B indicates that mutation in the SP1 sites reduced the promoter/enhancer activity 40 to 90% in HepG2 and CaCo-2 cells as compared to the wild-type promoter/enhancer. **Panel E:** Schematic representation showing the independent as well as synergistic contributions of protein complexes assembled on the proximal apoA-I promoter and the apoCIII enhancer on the transcription of the apoA-I gene. The diagram is based on the *in vivo* transcription data shown in Panels A-D as well as on the establishment of physical interactions between HNF-4 and SP1 on the apoCIII enhancer (228). The mechanism involves protein-protein interactions of the promoter and enhancer complexes via TAFs, TIFs and coactivators with the proteins of the basal transcription complex.

of both repeats in the HRE of element AIB or repeat 2 and the adjacent spacer region in the HRE of element AID, eliminated the binding of hepatic activities present in rat liver nuclei and reduced the promoter strength to approximately 5-7% of control. These findings suggest that both HREs are essential for optimal hepatic expression of the apoA-I gene and that the factors which occupy them may act alone or in synergy with other factors to increase transcription.

5.7.2. Other elements and factors

The regulatory region AIC is recognized by an activity designated AIC1 that may be related to NFY (Zannis, unpublished) as well as by C/EBP and an activity designated AIC3. The apoA-I promoter activity is also affected by the distal apoCIII regulatory region –800/-590, as will be discussed below, as well as by the –523/-393 apoA-I promoter region (212). Other factors shown to bind to the apoA-I regulatory regions have been reviewed previously (162).

5.7.3. Effect of apoCIII enhancer on the activity of the proximal apoA-I promoter in HepG2 and CaCo-2 cells

As shown in figure 4B, the apoCIII enhancer increases the strength of the proximal –255/-5 apoA-I promoter in HepG2 cells as well as the strength of a longer –1500/-5 promoter in CaCo-2 cells in the presence of HNF-4. The contribution of the apoCIII regulatory elements to the strength of the proximal apoA-I promoter in HepG2 cells was evaluated by deletions and 5′ mutations of the enhancer (209).

This analysis showed that deletion of the 5′ apoCIII promoter region extending to nucleotides -890 increased by 30% the activity of the apoA-I promoter/apoCIII enhancer cluster (figure 5B). The enhancer activity was nearly abolished by deletion of the regulatory elements CIIIJ, CIII-I and CIIIH (figure 5B). The promoter/enhancer activity was reduced to 40 to 45% of its value by mutations in elements CIIIH and CIIIG and to 55 to 70% of its value by mutations in elements CIII-I, CIIIJ or CIIIF (209). The ability of the apoCIII enhancer to activate transcription driven by the proximal apoA-I promoter is affected greatly by mutations in the regulatory element AID of apoA-I which contains an HRE (figure 5B). This mutation reduced the strength of the promoter/enhancer complex to 6% of its original value (163). In contrast, mutations in the regulatory element AIC of apoA-I which abolished the binding to this region of heat-stable activities related to C/EBP (163) reduced the strength of the promoter/enhancer complex only to 65% of its original value (figure 5B). The findings indicate that the activity of the apoA-I promoter/apoCIII enhancer is not

affected greatly by C/EBP and other activities which bind to the regulatory element AIC.

5.7.4. Effect of apoCIII enhancer and HNF-4 on the activity of the proximal apoA-I promoter in CaCo-2 cells

The proximal –255/-5 and the –1500/-5 nucleotides of the apoA-I promoter had low levels of activity in CaCo-2 cells both in the presence and the absence of the enhancer. This activity could be increased to levels comparable to that of HepG2 cells in the presence of HNF-4 (209). For this reason the -1500 apoA-I promoter/apoCIII enhancer CAT constructs were utilized in cotransfection experiments with plasmids expressing HNF-4 in order to assess the effects of mutations in the proximal HREs, as well as the distal apoCIII promoter elements on the activity of the apoA-I promoter/apoCIII enhancer in CaCo-2 cells. In general, the mutations affected the HNF-4 mediated transactivation of the -1500 apoA-I promoter/apoCIII enhancer cluster in CaCo-2 cells to the same extent as they affected the strength of the -255 to -5 apoA-I promoter/apoCIII enhancer cluster in HepG2 cells. The wild-type -1500 apoA-I promoter/apoCIII enhancer cluster was transactivated 6- to 7-fold by HNF-4. The transactivation was reduced to 40 and 45% of its original value by deletion of the regulatory element CIIIJ and by point mutations in element CIIIH, respectively, and to 25% of its original value by deletions of elements CIIIJ, CIII-I and CIIIH. Point mutations in elements CIIIJ or CIIIF and CIII-I reduced the transactivation of the promoter/enhancer cluster to 65-90% of its original value. Mutations in the regulatory element AID (HRE) of the proximal apoA-I promoter reduced the transactivation of the promoter/enhancer cluster to 7% of its original value whereas mutations in the regulatory element AIC of the proximal apoA-I promoter did not affect the HNF-4 mediated transactivation of the promoter/enhancer cluster in CaCo-2 cells (figure 5B). The combined findings suggest that the HNF-4-mediated transactivation of the apoA-I promoter/apoCIII enhancer cluster in HepG2 and CaCo-2 cells may be promoted by interactions between HNF-4 which binds to the HREs of the proximal apoA-I promoter and enhancer as well as SP1 which binds to three sites on the apoCIII enhancer. C/EBP does not appear to have a major effect on the activity of apoA-I promoter/enhancer constructs and their transactivation by HNF-4.

5.8. Important elements and factors involved in the transcriptional regulation of the human apoA-IV gene.

5.8.1. The role of hormone nuclear receptors

The apoA-IV gene displays a tissue-specific expression in primates with the intestine being the major and the liver the minor site of apoA-IV mRNA synthesis (222). The proximal –700/+10 apoA-IV promoter has very low activity in HepG2 and CaCo-2 cells (211). Ten-fold enhancement of the promoter activity was achieved in these cells by larger apoA-IV promoter constructs extending to nucleotides -3500 (223,224). The proximal apoA-IV promoter contains four regulatory elements: AIVA (-32 to -22), AIVB (-84 to -42), AIVC (-148 to -120), and AIVD (-274 to -250) (211) identified by footprinting using hepatic nuclear extracts. DNA binding, and competition assays showed that element AIVC contains an HRE with DR4 spacing which binds the orphan receptors HNF-4, ARP-1, and EAR-3 with similar affinity (Kd=4-7 nM) (figure 3P). Antibodies raised against HNF-4 which recognize only HNF-4, and COUP-TF which recognizes ARP-1 and EAR-3 but not HNF-4, supershifted part of the complex formed on the AIVC site (211).

5.8.2. Effect of the apoCIII enhancer on the activity of the apoA-IV promoter: The role of hormone nuclear receptors and SP1.

As shown in figure 4C, the strength of the proximal apoA-IV promoter increased by 18- and 13-fold in HepG2 and CaCo-2 cells respectively when the apoCIII enhancer (-500 to -890) which contains the regulatory elements CIIIF to CIIIJ (164,211) was linked to the proximal –700/+10 apoA-IV promoter (figure 4C). Mutagenesis of the construct showed that the ability of the apoCIII enhancer to increase the activity of the proximal promoter was practically abolished by a terminal 5′ deletion which removed elements CIIIJ, CIII-I and CIIIH (figure 5C; compare line 2 with lines 4 and 5). Furthermore, the activity of the promoter/enhancer construct was reduced to 12% and 18% of its original value in HepG2 and CaCo-2, respectively, by 3′ deletions of elements F and G (figure 5C; compare line 2 with line 4). This finding indicates that one or more proteins of the proximal apoA-IV promoter and the entire protein complex which assembles on the apoCIII enhancer is required for optimal enhancement of transcription. Mutagenesis of the HRE of element apoA-IV of the proximal promoter which abolished the binding of hormone nuclear receptors to this site reduced the activity of the promoter/enhancer construct to 21% and 29% of its original value in HepG2 and CaCo-2 cells, respectively (figure 5C; compare line 2 with line 3).

The role of HNF-4 in the transactivation of the apoA-IV promoter/enhancer construct was assessed by cotransfection experiments using HNF-4-expressing plasmids. It was shown that HNF-4 transactivated the apoCIII enhancer/apoA-IV promoter cluster by approximately 8.5- and 4-fold in HepG2 and CaCo-2 cells, respectively (figure 5C, line 2). The HNF-4-dependent transactivation of the apoCIII enhancer/apoA-IV construct was nearly abolished by mutations which eliminated the binding of HNF-4 to its cognate site on element AIVC and by deletion of the regulatory elements CIIIJ, CIII-I and CIIIH of the enhancer (figure 5C, compare line 2 with lines 3 and 5). In general, there was a correlation between the effect of the mutation on the apoCIII enhancer on the strength of the proximal apoA-IV promoter and the extent of the HNF-4 dependent transactivation of the promoter. Consistent with the findings in figures 5A and B, the data with the apoA-IV promoter of figure 5C suggest that the enhancement of transcription is mediated by complex interactions between Sp1 and HNF-4 which bind to the apoCIII enhancer, and hormone nuclear receptors bind to the HRE of the proximal apoAIVC promoter.

A preliminary report has shown that the full-length intergenic region between the apoCIII and apoA-IV genes allowed intestinal expression of the apoA-IV gene in transgenic mice, specifically the villus enterocytes, with a cephalo-caudal gradient (225). A subsequent work has suggested that the –700/+10 apoA-IV promoter sequence, in combination with the apoCIII enhancer, directed the expression of a reporter gene in the liver in enterocytes in a

pattern similar to the expression pattern of the endogenous apoA-IV gene (222,226). These data indicate the apoCIII enhancer is required for the intestinal expression of the apoA-IV gene and also contributes to the hepatic expression.

5.8.3. Effect of the apoCIII enhancer on the activity of the human apoB promoter

As discussed above, a common feature of several apolipoprotein promoters is that they contain one or two hormone response element HRE which bind orphan- and ligand-dependent nuclear receptors (38,162,210,220). The concept that the apoCIII enhancer operates through interactions between upstream activators bound to the enhancer and hormone nuclear receptors bound to proximal sites was further reinforced by cotransfection experiments involving heterologous promoters. As shown in figure 4D, the apoCIII enhancer increases the strength of the –1800/+124 apoB promoter in HepG2 and CaCo-2 cells approximately 8- and 10-fold respectively. Similarly, the strength of the proximal –267/+8 apoB promoter which was shown previously to contain both an HRE as well as a C/EBP binding site (185,227) increased by 4-fold in HepG2 cells and by 7-fold in CaCo-2 when it was linked to the apoCIII enhancer (figure 5D). Mutation in the HRE of the proximal apoB promoter abolished the activity of the promoter/enhancer cluster whereas mutation in the adjacent C/EBP binding site reduced the activity of the promoter/enhancer cluster to approximately 50% of its original value in HepG2 and 33% of its original value in CaCo-2 cells (209). The activity of the mutated promoter/enhancer construct increased to 52% of its original strength in CaCo-2 cells in the presence of HNF-4 (figure 5D).

Analysis of the effect of mutations on the proximal apoB promoter as well as on the apoA-I promoter discussed earlier (figure 5B) indicates that the apoCIII enhancer has the ability to bypass partially or totally the effect of mutations in proximal C/EBP binding sites. These mutations inactivate totally the proximal apoA-I and apoB promoters (163,185). However, the enhancer functions are greatly diminished by mutations in the proximal HREs of the apoA-I, apoCIII, apoA-I and apoB promoters as well as minimal promoters containing one or more HREs (figure 5A-D) (162,163,185).

5.8.4. Generalizations drawn from the mutagenesis of the apoCIII enhancer proximal apolipoprotein promoter constructs

The overall picture which emerges from the various studies indicates that the distal apoCIII regulatory elements act as a general modular enhancer which can potentiate the strength of proximal promoters or synthetic minimal promoters that contain one or more HREs (164,208,209,211). We have found recently that this potentiation may involve physical and functional interactions between hormone nuclear receptors and the factors which bind to the apoCIII enhancer (228). Similar to other systems, it is assumed that the hormone nuclear receptors which bind to the proximal HREs and the SP1 and the other factors which bind to the apoCIII enhancer form a stereo specific DNA-protein complex (13). These complexes may interact directly or indirectly via TAFs with the basal transcription complex, thus leading to the transcriptional activation of the target gene (13,15). Mutations in the enhancer or the proximal promoter which prevent the binding of one or more of the participating factors may affect the configuration of this complex and thus explain the reduction in the strength of the promoter/enhancer complex.

It is possible that SP1 which binds to elements CIIIF, CIIIH and CIII-I may act as an architectural component and serves to facilitate interactions among molecules of nuclear hormone receptors which bind to the proximal and distal sites. These interactions may be favorable or unfavorable, thus resulting in transcriptional synergism or transcriptional repression. Figure 5E shows putative interactions of SP1 and HNF-4 on the apoCIII enhancer which facilitate further synergism with the HNF-4 bound to the proximal promoter. This diagram is based on the *in vitro* studies shown in figures 4A-D and 5A-D as well as *in vivo* studies that are presented later in this review. Combinatorial interactions among factors have been described in other enhancers including the T-cell receptor alpha gene enhancer (198,228-230) and the virus induced human interferon beta enhancer (231,232). In the case of the T-cell receptor alpha enhancer binding of lymphoid enhancer factor-1 promotes interactions among the other factors which bind to the enhancer (198,228-230). In the case of the interferon beta enhancer binding of high mobility group 1Y (HMG1(Y)) protein increases the binding affinity as well as the interactions among the factors NF-kappa-beta, ATF-2 and interferon regulatory factor-1 (IRF-1) which also bind to the enhancer (231,232).

6. *IN VIVO* STUDIES OF APOLIPOPROTEIN GENE REGULATION

Gene regulation represents a complex biological event which involves a large number of transcription factors and an extensive set of proteins which constitute the basal transcription machinery. These proteins have to function with the constraints of the chromatin structure which has to be relaxed by deacetylation of histones. The tissue culture experiments represent a convenient approximation to assess the contribution of the transcription factors in gene transcription. Once a plausible transcriptional mechanism has been deduced from the *in vitro* studies, it will be essential that the putative mechanism is confirmed by *in vivo* studies using animal models. In other instances, it was shown that the distal regulatory elements of a specific gene such as beta-hemoglobin, apoB or apoE were not sufficient to drive faithfully tissue-specific expression *in vivo*. This limitation also necessitated the use of animal models to clarify how a specific gene is regulated *in vivo* (190,191,233,234). In this section, we will focus on *in vivo* regulatory mechanisms involving the apoE/apoCI/apoCIV/apoCII gene cluster and the apoA-I/apoCIII/apoA-IV gene cluster.

6.1. Contribution of tissue-specific enhancers in the transcriptional regulation of the genes of the apoE/apoCI/apoCIV/apoCII gene cluster *in vivo*

6.1.1. Identification of two regulatory regions which control hepatic expression of the apoE/apoCI/apoCIV/apoCII genes of the cluster

Initial studies showed that the proximal regulatory elements of apoE were not sufficient to direct liver-specific expression of the apoE gene *in vivo* (235). The distal

regulatory elements which control the transcription of the apoE gene in liver and different tissues have been determined using transgenic mice. High levels of expression of apoE in the kidney can be accomplished with a construct containing -650 nucleotides of the 5′ and 72 nucleotides of the 3′ region of the apoE gene (236) (figure 6A). Various regions in the intergenic sequence between apoE and apoCI are required for the expression of the apoE and apoCI gene in a variety of tissues tested including liver, testis, spleen, skin, submaxillary gland, kidney, brain, small intestine, heart, stomach, and pancreas (233) (figure 6A). This region also contains a silencer which inhibits the expression of apoE gene in the kidney in a construct containing 650 bp 5′ and 4 kb 3′ apoE sequences. Several elements contribute to the expression of apoE in kidney. A positive element is localized in the 5′ region between -2 to -.65 Kb, and a silencer is localized in the second intron of the apoE gene. Deletion of the second intron of the apoE gene allows expression in kidney in constructs containing .65 Kb 5′, and 1.7 Kb 5′ regulatory sequences. Expression of apoE in kidney is also abolished in constructs containing the -2 Kb upstream to +1.7 Kb downstream region by deletion of the regulatory element III which binds SP1 and the 55 KDa regulatory protein (195,233) (figures 3H and 6A). Finally, the region in the intergenic sequence between apoCI gene and apoCI′ pseudogene was originally shown to contain a 2 Kb element originating 4.4 Kb 3′ of the apoCI gene which was required for the hepatic expression of the human apoE and apoCI genes and was designated hepatic control region 1 (HCR-1) (233,236,237) (figure 6A). HCR-1 can also promote the hepatic expression of the apoA-IV gene in a construct which contains heterologous 2.4 Kb 5′ and 1.7 Kb 3′ sequences (233). The HCR-1-mediated expression of the apoE gene containing 5 Kb 5′ and 1.7 Kb 3′ region is significantly reduced by deletion of the regulatory element III (figures 3H and 6A). Deletion of elements I and II did not affect hepatic expression but permitted expression in other tissues (233). Thus the deletion of element I permitted high levels of expression in kidney and low levels of expression in lung, whereas deletion of element II permitted moderate levels of expression in kidney and lung and low levels of expression in spleen, heart, stomach, testis and brain (233). The minimal region of HCR-1 required for the hepatic expression of the apoE gene was mapped within 319 bp and is located approximately 15 Kb downstream of the apoE gene. HCR-1 contains several DNaseI hypersensitive sites, and has limited binding affinity for nuclear scaffold (196). Recently, a second hepatic control region designated HCR-2 was identified approximately 27 Kb 3′ of the apoE gene in the middle of the intergenic sequence between the apoCI′ pseudogene and the apoCIV gene (figure 6A). The HCR-2 has 85% sequence identity to the HCR-1 and is believed to have arisen from the duplication of HCR-1 (204). A 632 bp sequence containing HCR-2 can by itself direct hepatic transcription of a DNA segment containing the apoE gene including 5 Kb 5′ and 1.7 Kb 3′ sequences that lack the HCR-1 region (figure 6A). When both HCR-1 and HCR-2 were deleted from a transgenic construct extending approximately 5 Kb upstream of apoE and 20 Kb downstream of apoCII, the hepatic expression of apoCIV was abolished and that of the apoE, apoCI and apoCII was barely detectable. Deletion of either HCR-1 or HCR-2 did not affect the hepatic expression (238). It was suggested that HCR-1 has a dominant effect on the expression of apoE and apoCI and HCR-2 has a dominant effect for the expression of apoCIV and apoCII (238).

A variety of regulatory elements extending from 5 Kb 5′ of the apoE gene to 1 Kb downstream of the apoCI′ pseudogene also control positively or negatively the expression of the apoCI gene in different tissues. The hepatic expression requires sequences extending 3.1 Kb 5′ of apoCI and the intergenic sequences between the apoCI gene and the apoCI′ pseudogene which contains the HCR-1 (236,237). Deletions or additions in the 5′ and 3′ sequences cause different patterns of expression in various tissues studied in figure 6B.

The contribution of the factors which bind to the HCR-1 of apoE, to the hepatic expression of apoE and apoCI has not been assessed. Similar to the apoA-I/CIII/A-IV enhancer of figure 5E, it is possible to envision stereo specific DNA-protein interactions between SP1 and the other proteins that bind to the proximal promoter with the factors bound to HCR-1. Proteins which introduce DNA bending may orient properly the proximal and upstream activators and promote their interaction via TAFs with the proteins of the basal transcription complex.

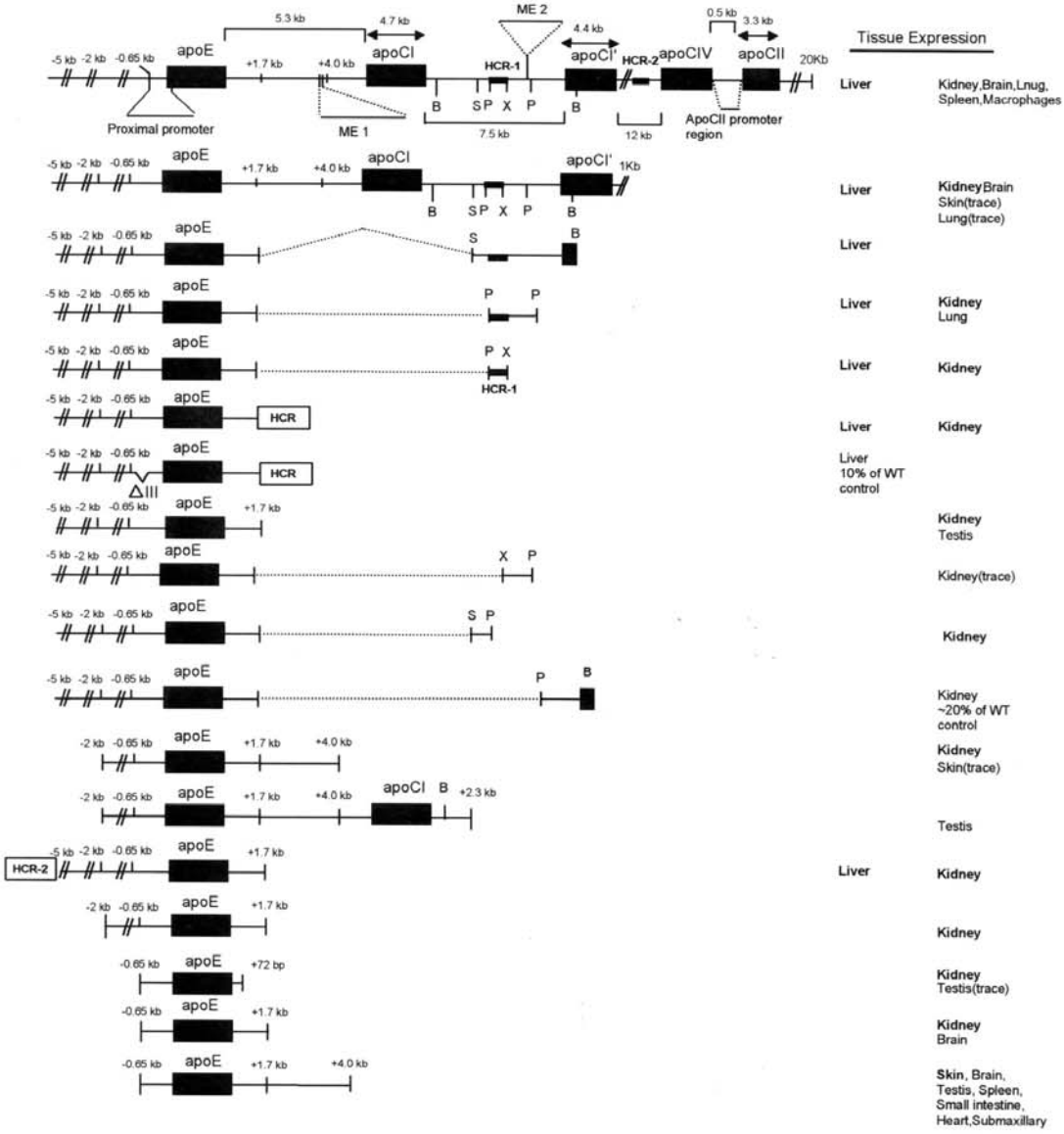
6.1.2. Identification of two regulatory regions which control the expression in macrophages and adipocytes of the genes of the apoE/apoCI/apoCIV gene cluster

Recently, two homologous enhancers designated multi-enhancer-1 and –2 (ME1 and ME2), were identified 3.3 and 15.9 Kb 3′ of the apoE gene respectively (figure 6C). Analysis of a series of transgenic mice expressing different apoE regulatory regions established that ME-1 and ME-2, which contain 620 and 619 nucleotides respectively, have equivalent ability to promote expression of the apoE gene in macrophages and adipocytes (205).

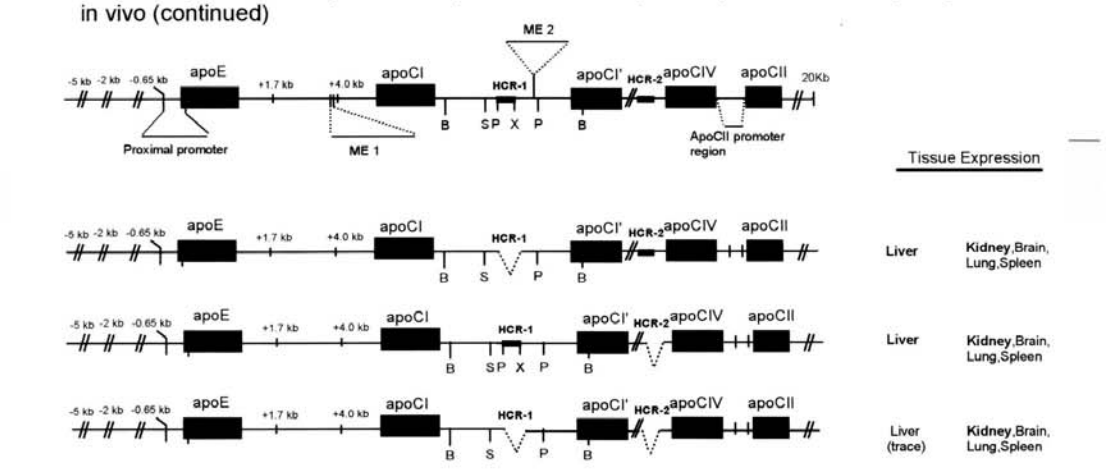
6.2. Role of hormone nuclear receptors SP1 and of a common enhancer on the regulation of apoA-I, apoCIII gene cluster

The *in vitro* studies outlined above have established that there is a linkage (207) and a common regulatory mechanism of the apoA-I/apoCIII/apoA-IV gene cluster (208-211). Each of the three genes contains hormone response elements (HREs) in its proximal promoter which bind orphan and ligand-dependent nuclear receptors with different specificities (38,209,210). As demonstrated in figures 4A-D and 5A-E the distal regulatory region of the apoCIII promoter –500/-890, which contains the regulatory elements CIIIF to CIIIJ, acts as a common enhancer for the three genes of the cluster (208,209,211). The enhancer also contains two HREs and three SP1 binding sites (208-210). Mutagenesis analysis showed that alterations, which prevent the binding of nuclear receptors to HREs, diminished the activity of the proximal promoters in cell cultures (163,208,211,220). Mutations in the HREs and some of the SP1 sites of the enhancer also significantly affected the enhancer activity in cell cultures (208,209,211). Other transcription factors (figure 3N-P) bind to proximal promoters of the apoA-I/apoCIII genes and may affect their overall activity and tissue specificity (163,164,208,211,212).

A. Regulatory elements required for hepatic and extrahepatic expression of the apoE gene in vivo



A. Regulatory elements required for hepatic and extrahepatic expression of the apoE gene in vivo (continued)



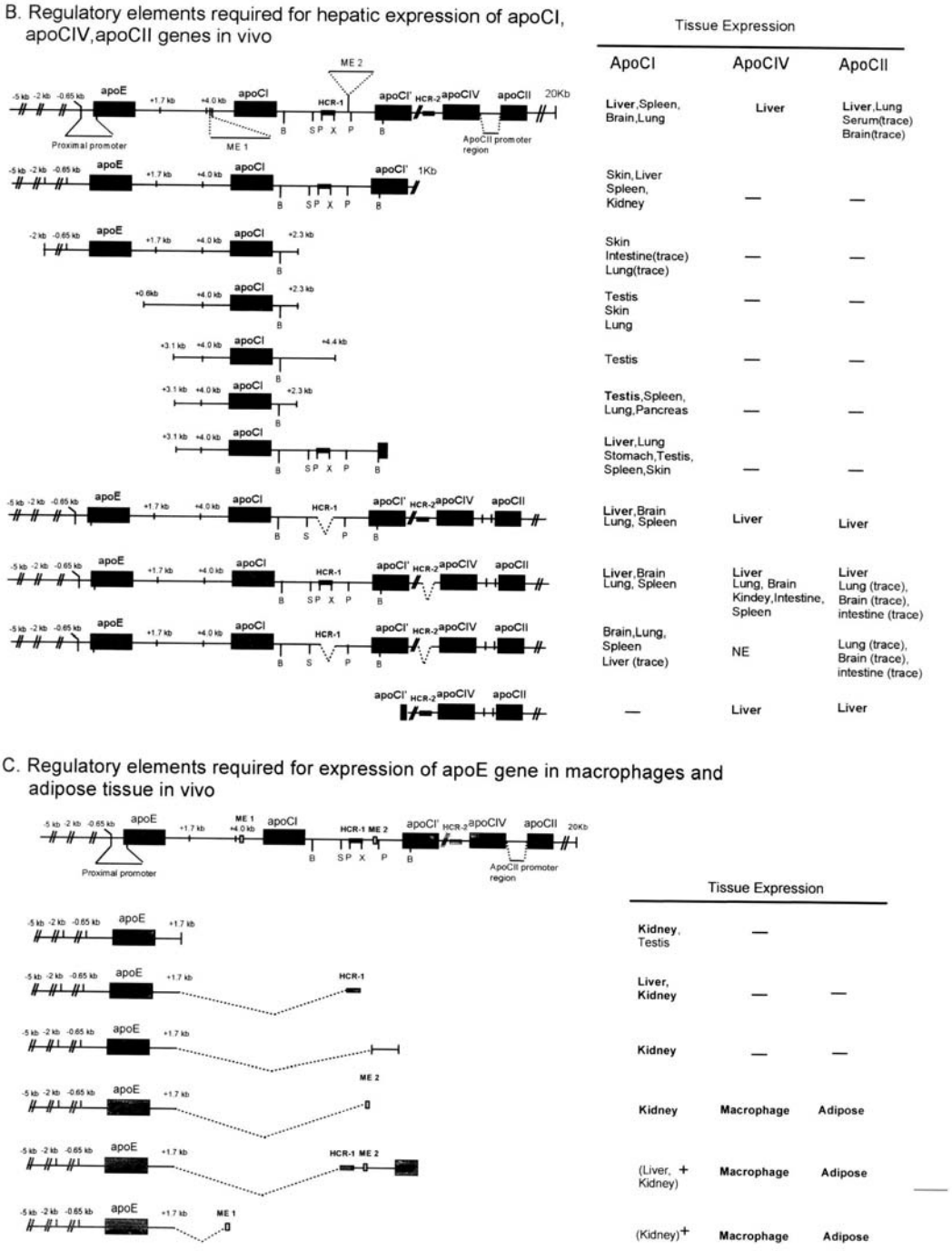


Figure 6A-C. Panel A: Schematic representation of the constructs containing the regulatory elements of the apoE/apoCI/apoCIV/apoCII cluster that are required for hepatic and extrahepatic expression of the human apoE gene. **Panel B:** Schematic representation of the constructs containing the regulatory elements of the apoE/apoCI/apoCIV/apoCII cluster that are required for the hepatic and extrahepatic expression of the human apoCI, apoCIV and apoCII genes. **Panel C:** Schematic representation of the constructs containing the regulatory elements of apoE/apoCI/apoCIV/apoCII cluster that are required for the expression of the apoE gene in macrophages and adipocytes. NE in Panel B indicates no expression. + in Panel C indicates that expression is inferred from the analysis of the initial construct extending 5 Kb upstream and 1.7 Kb downstream of apoE. In all panels, tissues with high levels of expression are indicated in bold type. – indicate analysis for the specific mRNA was not performed. The Figure is based on References 193, 196, 203-205, 232, 235-237.

6.2.1.Analysis of transgenic mice harboring wt and mutant promoter regions of the apoA-I/apoCIII gene cluster

As indicated earlier, the in vitro observations regarding gene regulation are meaningful only if they can mimic in vivo events. To obtain insight on the transcriptional regulation of the apoA-I/apoCIII/apoA-IV gene cluster, we have generated transgenic mice containing the WT and mutant regulatory regions of this cluster. Mutations were created to destroy the HREs of the apoA-I promoter or the apoCIII promoter and enhancer region. The transgenic mouse lines were designed to address the following questions: Which regulatory elements control the tissue-specific expression of the human apoA-I and apoCIII gene *in vivo*? How do mutations in the proximal HREs affect the promoter strength *in vivo*? How do mutations in the HREs of the enhancer affect the tissue-specific expression of the two genes *in vivo*?

Four sets of mouse lines expressing the WT and mutant apoA-I/apoCIII promoter enhancer transgenes (figure 7A-D) were generated and studied in detail. The first construct, designated AI WT CIII WT, contains the 2.1Kb of the 5′ regulatory sequence along with 1.81 Kb containing the entire coding sequence of apoA-I gene, a 1.6Kb segment of the intragenic sequence and the CAT gene in front of the –890/+24 regulatory sequence of the apoCIII gene. The second construct, designated AI WT CIII I4 Mut, contains a mutation in element I4 of the distal apoCIII enhancer (figure 7B). The third construct, designated A-I WT CIII (B+I4) Mut, contains mutations in the two HREs of elements B and I4 respectively, of the apoCIII promoter and enhancer (figure 7C). The fourth construct, designated A-I (B+D)Mut CIII WT, contains mutations in elements B and D of the proximal apoA-I promoter (figure 7D). In these constructs, the apoCIII gene was replaced by the CAT cDNA sequence (figure 7A-D).

The activity of the apoA-I promoter was determined by measuring the steady-state apoA-I mRNA levels in different tissues. The activity of the apoCIII promoter was determined by measuring the enzymatic activity of CAT in different tissues of transgenic mice (239).

6.2.2. Expression of the CAT gene used as a reporter of the apoCIII gene under the control of the WT and mutated apoCIII promoter and enhancer: The apoCIII promoter and enhancer confer correct hepatic and intestinal expression and low levels of renal expression of the apoCIII gene ***in vivo*****.**

Four different mouse lines carrying the WT construct displayed high levels of CAT activity in the liver and low levels of expression in the intestine and kidney and no expression in the lung, spleen, heart, brain, stomach, and in skeletal muscle (figure 8A). The levels of CAT activity in the intestine and kidney in mice expressing the WT construct was approximately 5% and 4%, respectively, of that observed in the liver (figure 8A). CAT expression in three mouse lines carrying the construct mutated in the two proximal HREs of the apoA-I promoter was similar to that of the lines carrying the WT constructs (compare figure 8D with figure 8A). The findings indicate that the elements of the proximal apoA-I promoter do not interfere with the activity of the apoCIII promoter and enhancer.

The analysis of the transgenic mice showed that the regulatory elements representing the –890/+24 apoCIII promoter/enhancer directed expression of the apoCIII gene in a pattern similar to that observed in fetal human tissues and rat and rabbit tissues (240-242). The only difference was that in mice expressing the CAT gene under the control of the WT apoCIII promoter and enhancer, there was a low level of ectopic expression in the kidney. It is possible that the construct utilized lacks a silencer which may be involved in the repression of the expression of the apoCIII gene in the kidney. Analysis of the relative expression of the reporter gene under the control of the apoCIII promoter and enhancer in proximal middle and distal segments of the intestine indicated that compared to the proximal segment, the expression was decreased slightly (to 77%) in the middle segment and significantly (to 25%) in the distal segment, indicating a gradient along the cephalo-caudal axis of expression.

6.2.3. The HREs of the proximal apoCIII promoter and enhancer are essential for its ***in vivo*** **activity in all tissues: The HRE of the enhancer alone control the intestinal expression of the apoCIII gene.**

The *in vitro* mutagenesis studies shown in figure 5A have established that mutations in the HREs of the regulatory element B of the proximal apoCIII promoter and element I4 of the apoCIII enhancer which bind HNF-4 diminished the activity of the apoCIII promoter/enhancer cluster in cell cultures (208,209) (figure 5A).

Analysis of the transgenic mice showed that mutations in element I4 of the apoCIII enhancer alone abolished the intestinal expression and reduced dramatically the hepatic expression by approximately 98% as compared to mice carrying the WT apoA-I promoter/WT apoCIII enhancer construct. The expression in kidney was barely detectable (figure 8B). The findings indicate that the HRE present on element I4 of the apoCIII enhancer which, as shown previously, binds HNF-4 (208,209) is essential for the intestinal and renal expression of the apoCIII gene. When the HRE of the enhancer is mutated, the proximal apoCIII promoter allows low levels of hepatic expression of the apoCIII gene. This observation is consistent with a previous finding which showed that a construct containing 200 bp of the apoCIII promoter could drive the hepatic transcription (243). Mutations in the HREs of elements B and I4 of the apoCIII promoter and enhancer abolished the expression of the CAT gene in all of the tissues tested (figure 8C), indicating that the HREs of element B of the proximal apoCIII promoter and I4 of the apoCIII enhancer are essential for the expression of the apoCIII gene in all tissues. Consistent with these findings, a recent report showed that the expression of the apoCIII gene is abolished in the fetal liver of mice in which the HNF-4 gene was inactivated by homologous recombination (244). Similar downregulation of the endogenous apoA-I mRNA was observed in hepatic and intestinal cell lines infected with a dominant negative HNF-4 form (245).

6.2.4. Analysis of transgenic mice expressing the apoA-I gene under the control of the WT apoA-I promoter/apoCIII enhancer: The proximal apoA-I promoter, in combination with the apoCIII enhancer, confers correct tissue-specific expression of the apoA-I gene ***in vivo*****.**

Previous studies had established that apoA-I is expressed abundantly in both the liver and intestine and at low levels in other tissues (240). The expression of the apoA-I gene in the four different mouse lines was determined by Northern blotting and S1 nuclease mapping. Analysis of four different

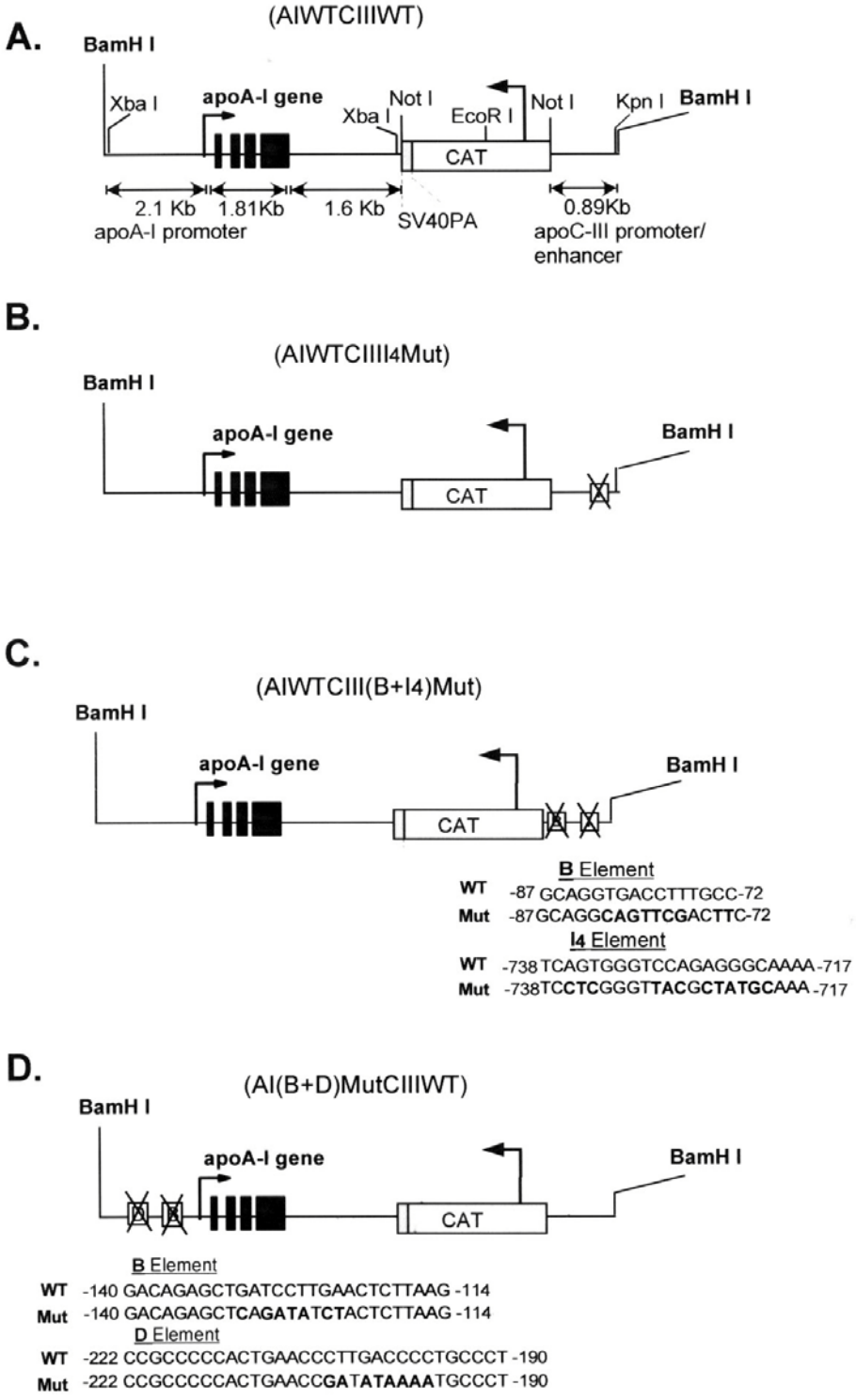


Figure 7A-D. Transgenic constructs containing wild-type (WT) (**Panel A**) and mutated apoCIII (**Panels B&C**) or apoA-I (**Panel D**) promoter segments. The names of the resulting transgenic lines harboring these transgenes are indicated in parentheses. The WT and mutant sequences of the HREs of the apoCIII promoter and enhancer and apoA-I promoter are shown in Panels C and D. Nucleotide substitutions in the mutated sequences are depicted in bold characters.

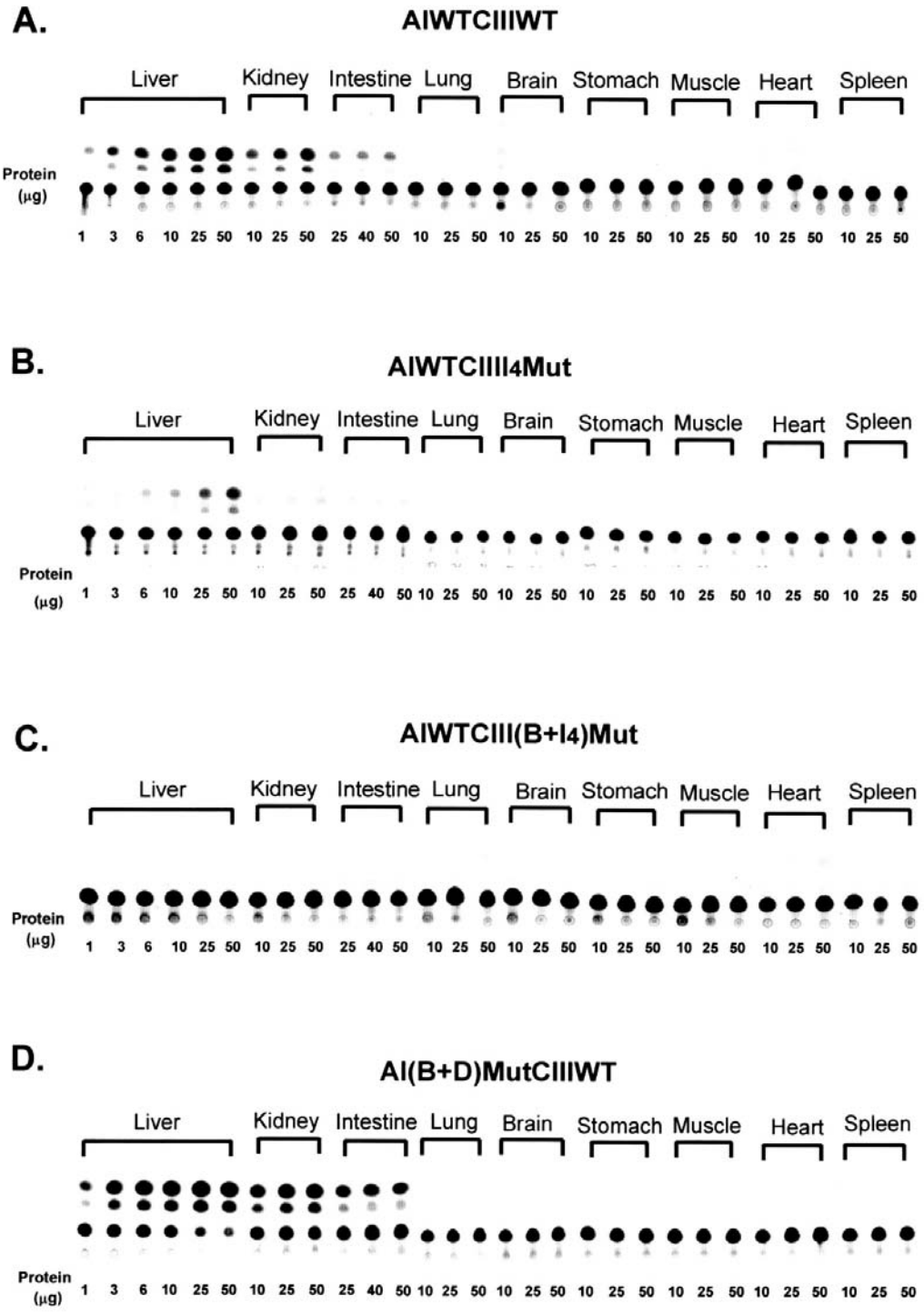
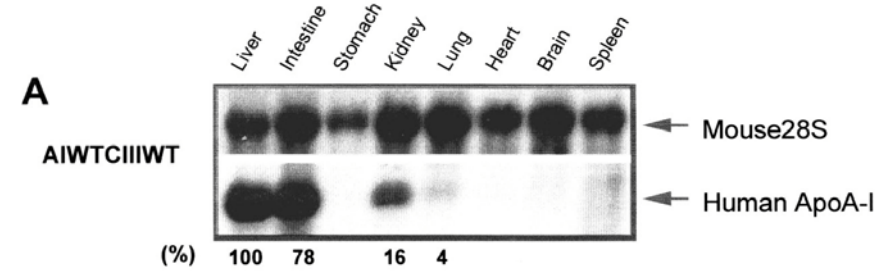


Figure 8A-D. CAT assays of transgenic mouse tissues expressing constructs containing the WT and mutated apoA-I and apoCIII promoter segments shown in Figure 7A-D. **Panel A:** Mice expressing CAT gene under the control of the WT apoA-I promoter/WT apoCIII promoter and enhancer. **Panel B:** Mice expressing the CAT gene under the control of WT apoA-I promoter/mutant apoCIII enhancer. **Panel C:** Mice expressing the CAT genes under the control of the WT apoA-I promoter/mutant apoCIII promoter and enhancer. **Panel D:** Mice expressing the CAT gene under the control of the mutant apoA-I promoter/WT apoCIII promoter and enhancer.



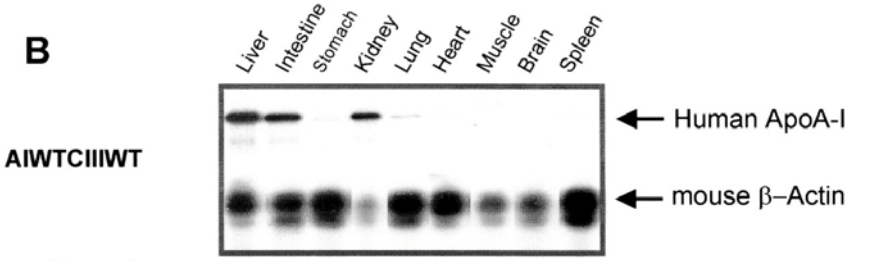


Figure 5.

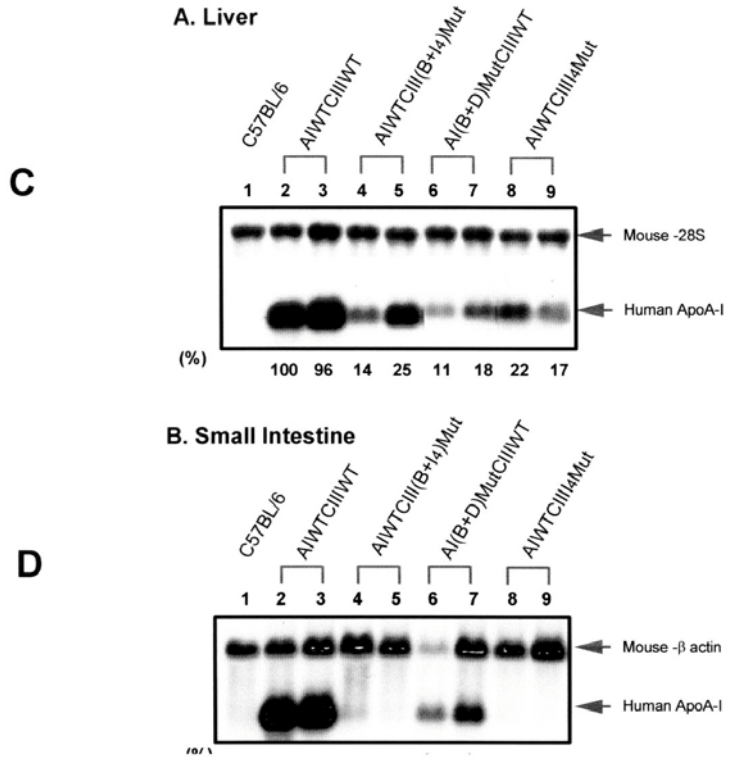


Figure 9A-D. Northern (**Panel A**) and S1 nuclease protection analysis (**Panel B**) of apoA-I mRNA obtained from tissues of transgenic mice expressing constructs containing the WT and mutated apoA-I and apoCIII promoter and enhancer constructs shown in Figure 7A-D. RNA was obtained from mice expressing the apoA-I gene under the control of the WT apoA-I promoter/WT apoCIII promoter and enhancer. **Panels C&D:** Northern blotting analysis of 10μg of total RNA obtained from liver (**Panel C**) and intestine (**Panel D**) of transgenic mice expressing cluster containing the WT and mutated apoA-I and apoCIII promoter and enhancer constructs shown in Figure 7A-D. The nylon membrane was hybridized simultaneously with the apoA-I mouse 28S RNA. (Panels A&C) or mouse beta-actin probe (Panel B-D). The ratio of apoA-I to 28S signal (Panels A-D) or apoA-I to beta-actin signal (Panels B-D) of samples obtained from mice carrying the WT apoA-I WT apoCIII construct was arbitrarily set to 100%. The ratios of apoA-I/28S or apoA-I/beta-actin mRNA for the other samples provide a measure of their abundance relative to those of the WT control.

mouse lines showed that in general the WT apoA-I promoter/WT apoCIII enhancer directs high levels of expression in the liver and intestine, moderate levels of expression in the kidney, and low levels of expression in the lung (figure 9A). S1 nuclease mapping also detected very low levels of expression in the stomach, heart, spleen and muscle (figure 9B). Very low levels of expression in the brain were observed in one out of four transgenic mouse lines. A previous study of transgenic mice has shown that the apoCIII enhancer was sufficient to direct the intestinal expression of the apoA-I gene, but did not restrict gene expression to the villus cells (246). The analysis of the transgenic mice carrying WT and mutated apoA-I promoter/apoCIII enhancer sequences, shown in figure 7A-C, has established that regulatory elements representing the –890/-500 apoCIII promoter and the +5/-2100 apoA-I promoter-directed expression of the apoA-I gene in a pattern similar to that observed in fetal human tissues (240), indicating that these regulatory regions contained the elements required for tissue-specific expression of the apoA-I gene.

6.2.5. The apoCIII enhancer confers intestinal expression and contributes to the hepatic expression of the apoA-I gene

Mutations in element I4 which precludes the binding of HNF-4 and other nuclear receptors to this site (209,210) abolished the intestinal and renal expression of the apoA-I gene and reduced the hepatic expression by approximately 80% as compared to mouse lines carrying the WT apoA-I WT apoCIII enhancer construct (figure 9C,D). Similarly, mutations in the HRE in the element I4 of the apoCIII enhancer suppressed the intestinal and renal expression of the apoA-I gene and reduced the levels of the hepatic expression by 80% as compared to mice carrying the WT apoA-I promoter WT apoCIII enhancer construct (figure 9C,D). This finding is consistent with an earlier report showing that a 256 bp proximal apoA-I promoter was sufficient for the hepatic expression of apoA-I in transgenic mice (1,247). The observation that the expression of the apoA-I gene in the liver is quantitatively similar in mice expressing the single mutation in element I4 or in the double mutation in elements B and I4 indicates that the element B of the proximal apoCIII promoter is not involved in the regulation of the neighboring apoA-I gene. The expression pattern of the apoA-I gene carrying mutations in the elements B and I4 or I4 alone in other tissues which display very low levels of expression, as determined by S1 nuclease mapping, remained qualitatively similar to those observed in tissues of mice expressing the WT apoA-I promoter WT apoCIII enhancer construct. The only exception was the stomach, where the expression was lost by the I4 or B and I4 mutations.

6.2.6. The apoCIII enhancer alone may independently contribute to the expression of the apoA-I gene in the liver, intestine and kidney

The *in vitro* mutagenesis studies shown in figure 5B have established that mutations in the HREs of the regulatory element D of the apoA-I promoter or element I4 of the apoCIII enhancer diminish the activity of the apoA-I promoter-/apoCIII enhancer cluster in HepG2 and CaCo-2 cells (209,210). A single mutation in the HREs of elements D and/or B, or both, also diminishes the activity of the proximal (-255/+5) apoA-I promoter in HepG2 cells (220).

The analysis of mutations in the two HREs on elements B and D of the proximal apoA-I promoter reduced the steady-state apoA-I mRNA levels in the liver and the intestine by 84% as compared to the expression in mice carrying the WT apoA-I construct (figure 9C,D). The pattern of expression of the apoA-I gene in the lung and heart in mice carrying the B+D mutation in the proximal apoA-I promoter was similar to that of mice carrying the WT construct apoA-I promoter WT apoCIII enhancer. No expression was detected in muscle and brain. A single mouse line carrying a triple mutation that altered the two HREs of elements B and D of the apoA-I promoter and the HRE on element I4 of the apoCIII enhancer abolished the expression of the apoA-I gene in all tissues tested (data not shown). The findings indicate that the apoA-I promoter cannot function in the absence of the intact HREs in the proximal promoters and the apoCIII enhancer regions. Consistent with these findings, a recent report has shown that expression of the apoA-I gene is abolished in the fetal liver of mice in which the HNF-4 gene was inactivated by homologous recombination (244). Similarly, downregulation of the endogenous apoCIII mRNA was observed in HepG2 cells infected with a dominant negative HNF-4 form (245). Finally, the expression of the apoCIII gene was abolished in all tissues by mutations in the three SP1 sites on elements F, H and I of the apoCIII enhancer (248).

6.2.7. Potential mechanisms of transcriptional enhancement *in vivo*

The *in vivo* studies showed that mutations in the two proximal HREs of the apoA-I promoter permitted expression of the apoA-I gene in the liver, intestine and kidney, and some of the minor tissues (figure 9C,D). The levels of the intestinal and hepatic expression of the apoA-I gene in the mouse lines carrying the mutations in the two HREs of the apoA-I promoter was approximately 15% of those observed in mice carrying the WT apoA-I promoter WT apoCIII enhancer. The findings of figure 9C,D indicate that hepatic and intestinal expression is still possible, albeit at lower levels, when the proximal apoA-I promoter is inactivated by mutations which prevent the binding of hormone nuclear receptors to the two proximal HREs. The most probable interpretation of these findings is that *in vivo* the apoCIII enhancer alone (figure 10B) can drive independently the hepatic and intestinal transcription of the apoA-I gene. Similarly, figure 9C also shows that the hepatic apoA-I expression in mouse lines carrying mutations in the HRE of the apoCIII enhancer which binds HNF-4 is approximately 20% of those observed in mice carrying the WT apoA-I promoter/apoCIII enhancer. The findings indicate that the proximal apoA-I promoter alone can drive independently the hepatic transcription of the apoA-I gene (figure 10A). When both the promoter and the enhancer are functional, the activity of the apoA-I promoter/apoCIII enhancer cluster is 100%, indicating *in vivo* synergistic interactions which lead to transcriptional enhancement (figure 10C).

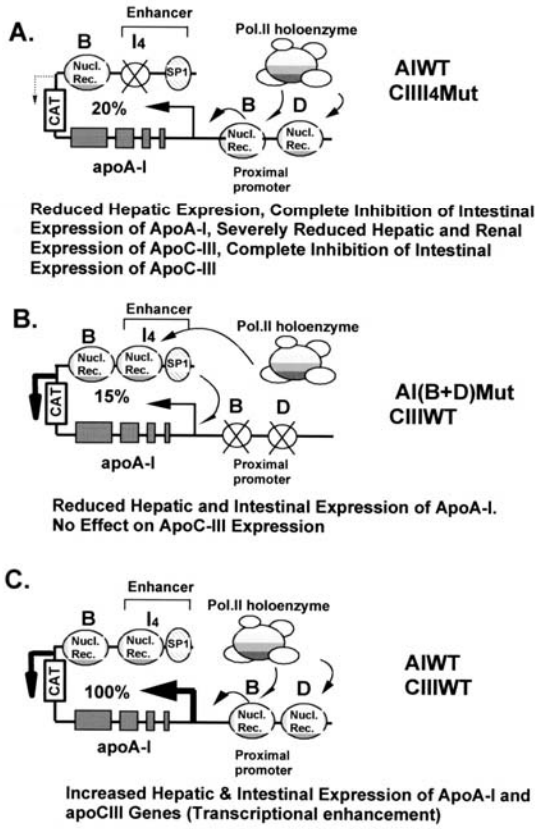


Figure 10A-C. Putative mechanisms by which the apoA-I promoter and enhancer cluster is activated in transgenic mice. **Panel A:** Gene expression when the HRE on element I_4 of the apoCIII enhancer is mutated. **Panel B:** Gene expression when the HREs on elements B and D of the apoA-I promoter are mutated. **Panel C:** Transcriptional enhancement when all the HREs of both the apoA-I promoter and the apoCIII enhancer are intact (WT).

7. SIGNALING PATHWAYS AND MECHANISM OF TRANSCRIPTIONAL ENHANCEMENT VIA PROTEIN-PROTEIN INTERACTIONS

7.1. Modulation of the apoCIII promoter activity by extracellular stimuli (cytokines and chemokines) and growth factors. Involvement of ATF-2, Jun and C/EBP delta

The apoCIII promoter contains three binding sites for the transcription factor ATF-2 (215). One site (-747/-726) present in the apoCIII enhancer is within the previously identified element CIII-I and has overlapping boundaries with the binding sites of SP1 (-766/-742) and HNF-4 (-734/-714). The other two sites have been designated CIIIDE (-217/-199) and CIIIBC (-102/-75). The CIIIBC site overlaps with the previously identified HNF-4 binding site (-87/-63) (figure 3N). Cotransfection experiments in HepG2 cells showed that ATF-2 transactivated the (–890/+24) apoCIII promoter 1.6-fold. In addition, mutations in the proximal CIIIDE, and distal CIII-I ATF-2 binding sites reduced the apoCIII promoter strength to 33 and 9% of control respectively, indicating that ATF-2 acts as a positive regulator (215). In contrast, to ATF-2, other members of the Jun family (c-Jun, JunB and JunD) caused a dose dependent inhibition of the (-890/+24) apoCIII promoter activity. ApoCIII promoter segments lacking the enhancer region were transactivated by Jun, suggesting that homodimers of Jun or heterodimers of Jun with other AP-1 partners or interactions of Jun with other factors bound to the apoCIII promoter/enhancer could be responsible for the observed repression of the apoCIII promoter/enhancer activity. Other studies have shown that Interleukin-1 (IL-1) repressed the apoCIII gene expression in HepG2 cells. These studies suggested that the IL-1 phosphorylated C/EBP delta which then binds to element CIIID and represses the apoCIII promoter activity (247). It was proposed that the binding of phosphorylated C/EBP delta to element CIIID interfered with the functions of the apoCIII enhancer (CaCo-2). Consistent with these findings, other studies also showed that binding of heterodimers of C/EBP delta with NF kappa B P50 to element CIIID repressed the apoCIII promoter activity (249,250).

These observations suggest that signaling mechanisms involving pro-inflammatory cytokines and other extracellular stimuli involving the Jun N-terminal kinase (JNK) pathway or the NF-kappa B pathway (figure 11A) may affect positively or negatively the apoCIII promoter activity. The stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK) pathway leads to the phosphorylation of the ATF-2 and the proto-oncogene c-Jun, which are members of the AP-1 family of transcription factors (251). Upon activation, homo- or heterodimers of ATF-2, as well as heterodimers of c-Jun, bind to "AP-1-like" target sites and affect transcription of the target genes (251-255). Similarly, key effectors in the TNF/IL-1 signal transduction pathway such as NF-kappa B-inducing kinase (NIK), I-kappa-kappa-beta (IkkB), I-kappa-beta (IkB) and NF-kappa-B (p65/Rel) (255-257) repressed apoCIII promoter activity to 10-20% relative to the control whereas dominant negative mutants of the above proteins increased apoCIII promoter activity to 200% (Kardassis & Zannis, unpublished). Furthermore, effectors of the NF-kappa-B pathway repressed the transcriptional activity of a GAL4-HNF-4 fusion protein in a mammalian GAL4-based transactivation system suggesting that these proteins somehow interfere with HNF-4 function. Interestingly, the repression of HNF-4-mediated transactivation by NF-kappa B was totally counteracted by overexpression of SMAD3 and SMAD4 proteins suggesting a negative crosstalk between TGF-beta and TNF-alpha/IL-1 signaling in the apoCIII promoter (Kardassis & Zannis, unpublished).

Further analysis showed that a dominant negative SMA/MAD4 (SMAD4) mutant which blocks the TGF beta signal transduction pathway repressed the apoCIII promoter activity by 50% (figure 11C), indicating the TGF-beta signaling may stimulate the apoCIII promoter activity. Finally, activation of the mitogen-activated protein kinase (MAPK) Erk1/2 (258) caused a 70 to 80% reduction in apoCIII transcription and the HNF-4 binding site in the apoCIII enhancer region (element I4) was found to be responsible for this regulation (259). These findings suggest that apoCIII can be regulated by signals acting through the mitogen-activated protein kinase (MAPK) pathway and that this regulation is mediated, at least in part, by changes in HNF-4 activity in hepatic cells.

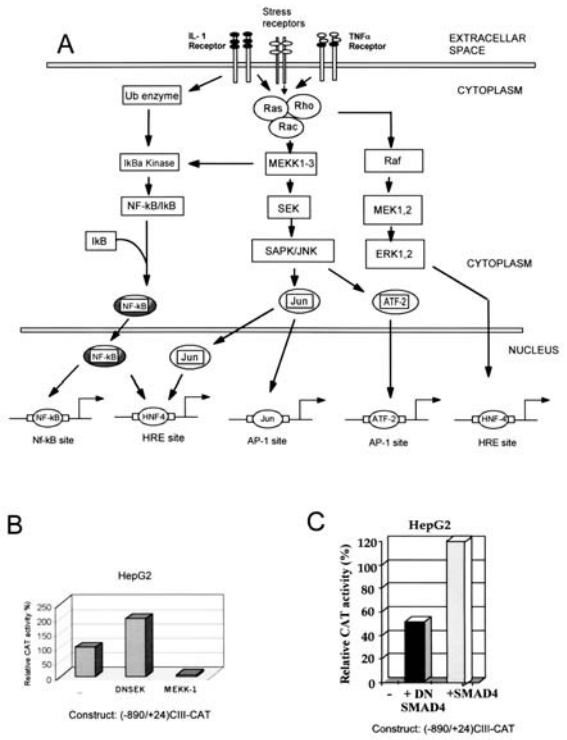


Figure 11A-C. Panel A shows signaling pathways leading to the activation of the Jun and NF kappa B. **Panel B:** cotransfection experiments showing transactivation of the human apoCIII promoter in HepG2 cells by a dominant negative SEK form (DNSEK) and repression by MEKK-1. **Panel C** shows repression of apoCIII promoter by a dominant negative form of SMAD. HepG2 cells were co-transfected with the human –890/+24 apoCIII promoter-CAT construct (2 μg) in the absence (-) or presence (4 μg) of expression vectors for wild-type (SMAD4) or a dominant negative SMAD4 form (SMAD4 1-514) in Panel C and a dominant negative form of SEK and MEKK-1 in Panel B, and the CMV-beta galactosidase plasmid (1 μg). In both Panels B and C, CAT activity was determined. The normalized, relative CAT activity (mean ± SEM) of at least two independent experiments performed in duplicate are shown in the form of a bar graph**.**

The involvement of the JNK pathway in the modulation of the apoCIII promoter activity has been supported by cotransfection of HepG2 cells with the reporter (-890/+24) apoCIII promoter plasmid and plasmids expressing MAPK/ErK-1/2 kinase-kinase-1 (MEKK-1) or a dominant negative form of SAPK/ErK kinase-1 (SEK) (260). This analysis showed that the MEKK-1 severely suppressed –890/+24 the apoCIII promoter and the dominant negative SEK transactivated two-fold the –890/+24 apoCIII promoter activity (figure 11B). The findings indicate that the activation of factors via the SAPK/JNK pathway may be responsible for the observed changes.

7.1.1. SMAD3 and SMAD3/4 transactivate strongly the human –890/+24 apoCIII promoter

To investigate the ability of different SMAD proteins to transactivate the apoCIII promoter, we performed transient transfection experiments in HepG2 cells using the –890/+24 apoCIII promoter CAT construct along with expression vectors for human SMAD 2, 3 and 4 proteins (figure 12A) independently or in combinations in the presence or absence of TGF beta (400 pM). As shown in Figure 12B, among the various SMAD proteins tested, SMAD3 showed the strongest transactivation potential (14-fold when transfected alone and 27-fold when co-transfected along with SMAD4). SMADs 2, 4 and 2/4 had a minor effect. A dominant negative SMAD3 mutant lacking the transactivation domain MH2 and part of the linker region (hSMAD3 1-220 figure 12A) was unable to transactivate the apoCIII promoter even when co-transfected with SMAD4 (figure 12B) (261).

7.1.2. The proximal and distal HREs present on the apoCIII promoter/enhancer may contribute independently to the SMAD mediated transactivation

The observation that SMAD3/SMAD4 could act as transcriptional activators of the –890/+24 apoCIII promoter prompted us to identify SMAD responsive elements in this promoter (figure 12C). Cotransfection experiments showed that a deletion of the apoCIII promoter extending to nucleotide –163 resulted in a reduction in both the constitutive and SMAD3/SMAD4-inducible apoCIII promoter activity. The fold transactivation of the apoCIII promoter by SMADs, however, was similar to that of the wild-type –890/+24 apoCIII promoter (20-fold versus 26-fold) (figure 12D). This finding indicates that the proximal –163/+24 apoCIII promoter contains SMAD responsive element(s) (figure 12C). A stronger transactivation by SMAD3/SMAD4 (50-fold) was achieved when the apoCIII promoter was further deleted to nucleotide –99 (figure 13A). In contrast, an apoCIII promoter fragment extending to nucleotide –55 was transactivated by SMAD3/SMAD4 proteins only 5-fold, suggesting that a putative SMAD3/SMAD4 responsive element is localized within the –99/-55 apoCIII promoter region. This region contains a previously identified and characterized HRE (element CIIIB between nucleotides –92 to -67) that serves as the binding site for various orphan and ligand-dependent nuclear receptors such as HNF-4, ARP-1, EAR-2, homodimers of RXR-alpha, and heterodimers of RXR-alpha with RAR-alpha, T3R-beta and PPAR-alpha among others (38,208-210) (figure 3N).

To test the role of the apoCIII enhancer (-790/-590) in SMAD3/SMAD4-mediated transactivation of the apoCIII promoter, a synthetic reporter construct containing the WT or mutated apoCIII enhancer fused with the heterologous AdML minimal promoter (-44/+1) (figure 12C) were used in cotransfection experiments. This analysis showed that SMAD3/SMAD4 transactivated the apoCIII enhancer/AdML CAT construct 5-fold, whereas they caused a small 1.8-fold transactivation on the AdML-CAT construct. Point mutations at the HRE (element I4) of the apoCIII enhancer did not affect the activity of the apoCIII enhancer/ minimal AdML promoter construct, but it diminished its transactivation by SMAD3/SMAD4 (1.7-fold for the mutant versus 5-fold for the WT enhancer minimal AdML promoter construct). This HRE binds strongly HNF-4, ARP-1, EAR-3 and RXR alpha/RAR alpha heterodimers (figure 3N) (210).

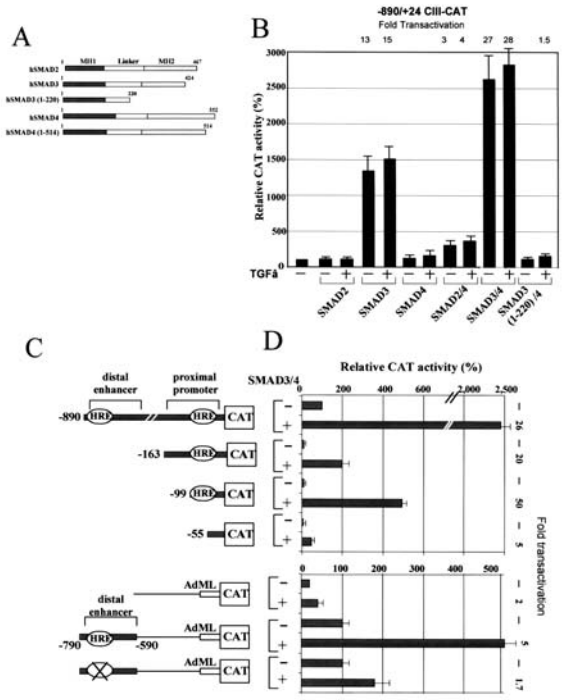


Figure 12A-D. Transactivation of the human apoCIII promoter segments in HepG2 cells by different forms of SMAD proteins. **Panel A** is a schematic representation of the human SMAD proteins utilized in the transactivation experiments presented in **Panel B**. MH1 and MH2 indicate the two SMAD homology domains shared by all SMAD family members. **Panel B** shows transactivation of the human –890/+24 apoCIII promoter by different SMAD proteins. HepG2 cells were co-transfected with the human –890/+24 apoCIII promoter-CAT construct (2 μg) in the absence (-) or presence (4 μg) of expression vectors for the indicated SMAD proteins and the CMV-beta galactosidase plasmid (1 μg). Experiments were performed in the presence and absence of 400 pM TGF beta. CAT activity was determined. The normalized, relative CAT activity (mean ± SEM) of at least two independent experiments performed in duplicate are shown in the form of a bar graph**. Panel C** is a schematic representation of apoCIII promoter-CAT reporter plasmids used in transactivation experiments. The positions of the HREs present on the distal enhancer and proximal apoCIII promoter are indicated. **Panel D** shows transactivation of different apoCIII promoter fragments by SMAD3 and SMAD4 proteins. In Panels B and D, the normalized, relative CAT activity (mean ± SEM) of at least two independent experiments performed in duplicate are shown in the form of a bar graph**.** The experiments were performed as described in Figure 11B.

7.1.3. SMAD proteins synergize with HNF-4 in the transactivation of the apoCIII promoter: Synergism requires the HNF-4 binding sites of the apoCIII promoter and enhancer

The possibility that SMAD3/SMAD4 proteins transactivate the apoCIII promoter by synergizing with HNF-4 that bind strongly to the proximal and distal HRE sites was tested with cotransfection experiments in HepG2 cells. This analysis showed a very strong synergistic transactivation of the proximal –99/+24 apoCIII promoter by HNF-4 and SMAD3/SMAD4 proteins. HNF-4 and SMAD3/SMAD4 alone transactivated the –99/+24 apoCIII promoter 2.5- and 7.5-fold respectively, for a total of 10-fold additive transactivation. However, the combination of HNF-4 and SMAD3/SMAD4 transactivated the promoter 69-fold (figure 13A). The observed 69-fold transactivation is not additive, but synergistic. Deletion of the apoCIII HRE in the -55/+24 apoCIII CAT construct abolished the synergistic transactivation of this promoter by the combination of HNF-4 and SMAD3/SMAD4 proteins. A single copy of the proximal apoCIII HRE (CIIIB, -92/-63), fused to a heterologous AdML promoter was used in cotransfection experiments. A comparable (28-fold) synergistic transactivation of this heterologous promoter was observed by the combination of SMADs and HNF-4 (figure 13A). A two-fold synergistic transactivation was also observed when HFN-4 and SMAD3/SMAD4 were co-transfected into HepG2 cells along with a reporter construct containing the –790/-590 apoCIII enhancer in front of the minimal AdML promoter (figure 13B). This synergistic transactivation was abolished by a mutation which prevents the binding of HNF-4 to its cognate HRE on the element I4 of the apoCIII enhancer (figure 13B). The findings indicate that functional interactions can occur between HNF-4 bound to the HREs of the proximal apoCIII promoter and the distal enhancer and SMAD3/4 proteins that cannot bind to these sites.

Cotransfections experiments were also performed with vectors expressing wild-type and two HNF-4 mutants. HNF-4 (227-455), lacks domains A (transactivation), domain B/C (DNA binding), domain D (linker), and part of domain E (ligand binding). HNF-4 (Delta 271-354), contains an internal deletion in the putative ligand binding domain of HNF-4 (domain E) (262). This analysis showed that both HNF-4 mutants were unable to transactivate the apoCIII promoter either alone or synergistically with SMAD3/SMAD4 proteins (261). These findings indicate that nuclear localization and DNA binding, as well as the presence of the 272-353 region of HNF-4, are essential for the functional cooperation of HNF-4 with SMADs. The overall findings of figure 13A,B support the hypothesis that SMAD3/SMAD4 proteins transactivate the apoCIII promoter via synergistic interactions with HNF-4 and possibly other orphan or ligand-dependent nuclear receptors bound to the proximal as well as the distal HREs.

It appeared that under the experimental conditions used, the steady-state concentration of the SMAD3 and/or SMAD3/SMAD4 proteins was limiting since overexpression of SMAD3/SMAD4 proteins alone could transactivate the apoCIII promoter by 26-fold. Increases in the concentrations of both HNF-4 and SMAD3/SMAD4 could transactivate different apoCIII promoter segments by 28- to 69-fold (figure 13A).

7.1.4. Antisense inhibition experiments indicate that HNF-4 levels may modulate the SMAD-mediated transactivation of the apoCIII promoter in HepG2 cells

The ability of SMAD3/SMAD4 proteins to transactivate the –890/+24 apoCIII promoter was tested under

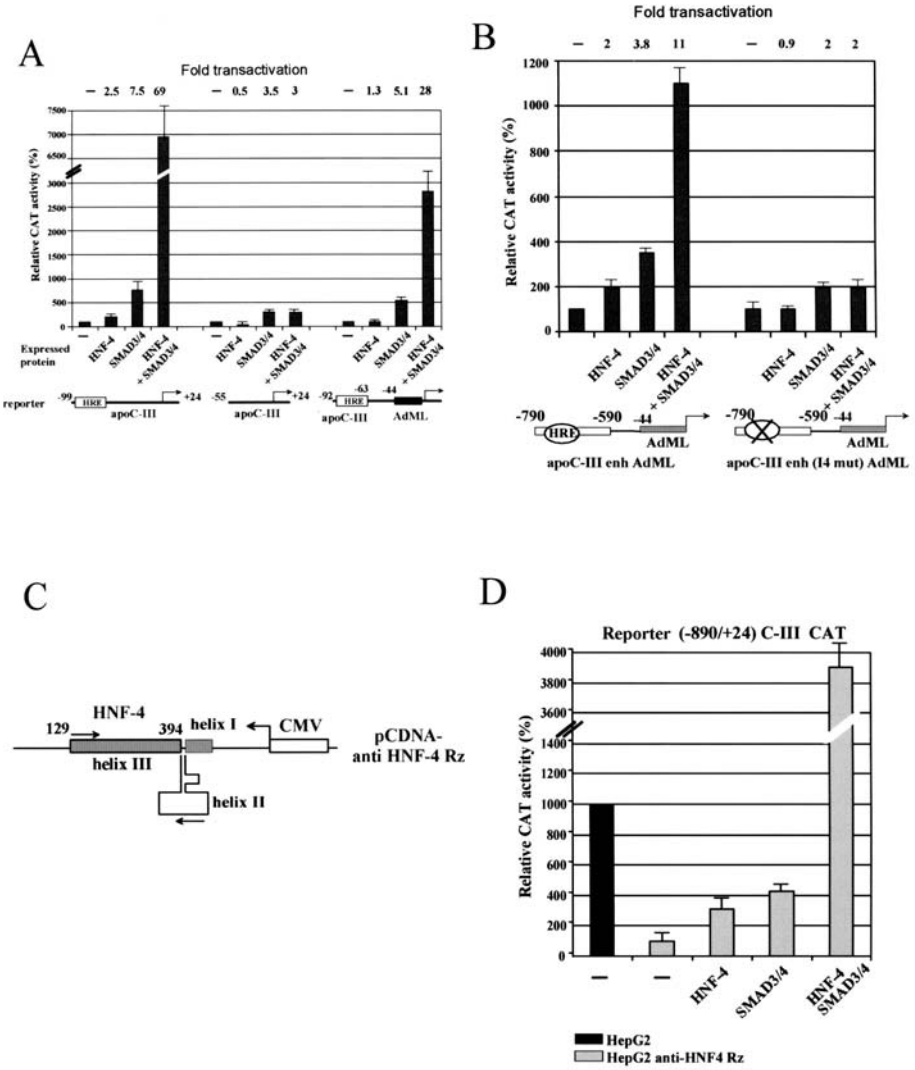


Figure 13A-D. Transactivation of apoCIII promoter segments by SMAD3/4 and HNF-4 in HepG2 cells and a HepG2 cell line expressing an HNF-4 ribozyme construct. **Panel A:** The apoCIII promoters used are: –99/+24 apoCIII, –55/+24 apoCIII and –92/-9 apoCIII AdML. **Panel B:** The apoCIII enhancer constructs used are: -790/-590 AdML, -790/-590 I_4Mut (AdML). In both Panels A and B, HepG2 cells were co-transfected with the apoCIII promoter construct (2 μg) either alone or in combination with expression vectors for human HNF-4, SMAD3 and SMAD4 proteins (1 μg) and the CMV-beta galactosidase plasmid (1μg). The normalized, relative CAT activity (mean ± SEM) of at least two independent experiments performed in duplicate are shown in the form of a bar graph**. Panel C** shows schematic representation of the pCDNA-anti-HNF-4 Rz expression vector used to generate the permanent HepG2 cell line expressing this construct. Numbers refer to the amino acid residues of the HNF-4 protein. Helices I to III correspond to double stranded regions in the anti-HNF-4 ribozyme. **Panel D:** Cotransfection and control experiments in a HepG2 cell line expressing an antisense HNF-4 ribozyme construct. Transfection conditions of the anti-HNF-4 Rz cell line are those described in Panels A and B. The normalized, relative CAT activity (mean ± SEM) of at least two independent experiments performed in duplicate are shown in the form of a bar graph.

conditions of reduction of the endogenous HNF-4 in HepG2 cells. In these experiments, a permanent HepG2 cell line was generated that expresses antisense HNF-4 sequences corresponding to amino acids 129 to 394 of the HNF-4 protein fused with the catalytic domain of hammerhead ribozymes (figure 13C). Quantitative reverse transcription (RT)-PCR showed expression of the antisense HNF-4 RNA. Western blotting showed that in the cell line expressing the antisense ribozyme, the expression of HNF-4 was greatly reduced. Co-transfection experiments showed that in the HepG2 cell line expressing the anti-HNF-4 ribozyme construct, the –890/+24 apoCIII promoter activity was reduced to ~10% of the control as compared to the parent HepG2 cell line. The apoCIII promoter was transactivated by either HNF-4 or SMAD3/SMAD4 to a minor extent (3- to 4-fold) compared to the transactivation of this promoter by the same factors in normal HepG2 cells (8-fold by HNF-4 (208), and 26-fold by SMAD3/SMAD4). However, co-expression of both HNF-4

and SMAD3/SMAD4 in the anti-HNF-4-Rz HepG2 cells resulted in a 39-fold transactivation (figure 13D). The findings suggest that in the HepG2 cells expressing the anti-HNF-4-Rz, the HNF-4 is rate-limiting for the activity of the apoCIII promoter. The promoter activity can be restored by increasing the concentration of HNF-4 and SMAD3/SMAD4 in the cell nucleus. Overall, the findings of figures 12A,B and 13A, B, and D indicate that the activity of the apoCIII promoter in HepG2 cells can be modulated by the relative abundance of HNF-4 and SMAD3/SMAD4.

7.1.5. SMAD proteins do not bind to the apoCIII promoter. Synergy is mediated by physical interactions between SMADs and HNF-4

The observation that HREs play an essential role for the synergistic transactivation of the apoCIII promoter by SMAD3/SMAD4 and HNF-4 suggested that SMAD proteins could either bind directly to the apoCIII HREs or cooperate functionally with HNF-4 receptors bound to these sites. To address whether the SMAD proteins can bind directly to the proximal apoCIII HRE, a gel electrophoretic mobility shift assay was performed using either HNF-4 expressed in COS-7 cells or a GST-SMAD3 fusion protein expressed in bacteria and purified by affinity chromatography. Probes for this analysis were either a synthetic double-stranded oligonucleotide containing the proximal apoCIII promoter element CIIIJ (–92 to –67) or a synthetic oligonucleotide containing a dimerized SMAD binding element (2 X CAGA) shown previously to bind SMAD3 proteins (263) (figure 14A). This analysis showed that HNF-4 binds efficiently to CIIIB probe in accordance with our previous findings (38) whereas GST-SMAD3 does not bind to this probe (figure 14B, lanes 2&4). Similarly, as expected, GST-SMAD3 binds efficiently to the dimerized CAGA probe whereas HNF-4 did not bind to this probe (figure 14B, lanes 1&3). These findings establish that SMAD3 cannot bind to the proximal apoCIII HRE and thus, DNA binding cannot explain the ability of SMAD3 protein to transactivate the –99/+24 apoCIII promoter which contains the proximal apoCIII HRE. The functional cooperation between HNF-4 and SMAD3/SMAD4 proteins on the human apoCIII HRE and the fact that SMADs do not bind to this DNA site suggested potential physical interactions among these factors. Physical interactions between SMADs and HNF-4 were examined using co-immunoprecipitation assays. For this analysis, COS-7 cells were co-transfected with expression vectors for myc-tagged SMAD3 and SMAD4 and FLAG-tagged HNF-4 protein. In each transfection, an expression vector for a constitutively active TGF beta type I receptor (CA-ALK5) was included (264) to allow activation of SMADs in COS-7 cells which do not respond to TGF beta. Expression of different proteins was monitored by Western Blotting of total COS-7 cell extracts using anti-myc or anti-FLAG monoclonal antibodies. To monitor the efficiency of the interaction assay, a co-transfection experiment was performed using myc-tagged SMAD3 and FLAG-tagged SMAD4 proteins. As expected, efficient interaction between SMAD3 and SMAD4 proteins was observed (lane 1). To test potential interactions between HNF-4 and SMAD proteins, total cell extracts from COS-7 cells co-transfected with myc-tagged SMAD3 (lane 2) or myc-tagged SMAD3 and SMAD4 proteins (lane 3) along with FLAG-tagged HNF-4 were subjected to immunoprecipitation with an anti-FLAG monoclonal antibody followed by Western blotting analysis of the immunoprecipitated proteins with an anti-myc monoclonal antibody that detects SMAD proteins. As shown in figure 14C (lanes 2 and 3), both SMAD3 and SMAD4 were co-immunoprecipitated with HNF-4 in this assay whereas no interaction between these proteins could be observed using an unrelated antiserum. The efficiency of the immunoprecipitation reaction was monitored by Western blotting analysis of the same blot shown in lanes 1-3 using the anti-FLAG antibody that detects HNF-4 and SMAD4 (figure 14C, lanes 4-6).

Physical interactions between HNF-4 and SMAD3/SMAD4 could also be demonstrated *in vitro* by using the GST-pull down assay. Specifically, fusion proteins consisting of human SMAD3 or SMAD4 and Glutathione S Transferase (GST-SMAD3, GST-SMAD4) or the GST portion alone were expressed in bacteria and coupled to Glutathione-Sepharose affinity beads (figure 14D, bottom). GST and GST-SMAD coupled beads were incubated with total cell extracts of COS-7 cells transfected with an expression vector for a myc-tagged human HNF-4. Following extensive washing of the beads, proteins bound to GST or GST-SMAD3 and GST-SMAD4 beads were eluted and analyzed by SDS-PAGE and Western blotting using an anti-myc antibody. As shown in figure 14D (top), HNF-4 could not bind to the GST-beads whereas it bound with high efficiency to the GST-SMAD3 beads and with low efficiency to GST-SMAD4 beads thus confirming specific physical interactions between these factors.

TGF-beta is expressed constitutively in HepG2 cells in an autocrine fashion and affects hepatic gene expression by binding to type I and type II TGF-beta receptors (265). Following TGF-beta stimulation, the type II receptor phosphorylates the type I receptor and the latter binds to and phosphorylates the *pathway-restricted* SMADs 2 and 3. The phosphorylated SMAD2 or 3 proteins subsequently bind to the common partner SMAD4, an event that triggers their translocation to the nucleus. There, they affect the transcription of target genes that are involved in important biological processes such as cell growth, differentiation, development, apoptosis and anti-inflammation and other cellular processes (266).

Our findings suggest that specific domains of HNF-4 bound to the HREs of the apoCIII promoter interact with SMAD3/SMAD4 complexes in the nucleus. These specific protein-protein interactions may in turn potentiate the interaction of HNF-4 with the proteins of the basal transcription machinery, thus leading to the activation of the apoCIII promoter. These putative protein-protein interactions that originate from TGF-beta signaling are shown in figure 15. Physical and functional interactions in TGF-beta/SMAD signaling pathway have also been described for other members of the nuclear receptor superfamily. It was recently shown, for instance, that SMAD3 acts as a coactivator of the Vitamin D receptor (VDR) in COS-1 cells (267). SMAD3 was also shown to form a complex with a member of the steroid receptor coactivator-1 protein family in the nucleus (268). Thus, SMAD3 may mediate cross-talk between vitamin D and TGF beta signaling pathways. In contrast, physical and functional interactions between SMADs and the glucocorticoid

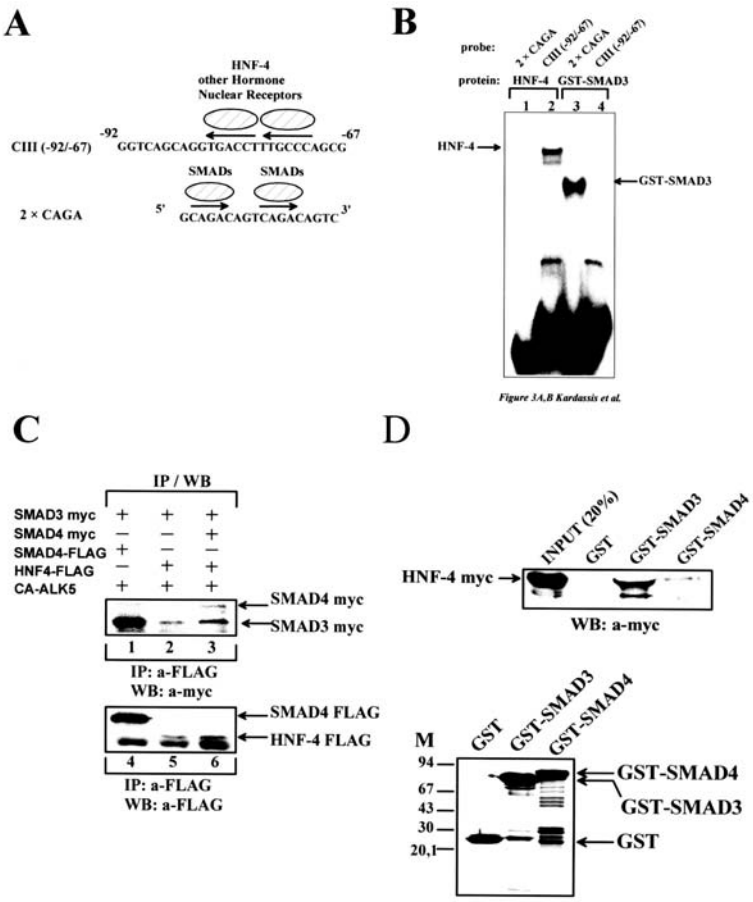


Figure 14A-D. Panel A shows the sequence of the oligonucleotides used as probes in the gel electrophoretic mobility shift assay shown in panel B. Arrows on top of the apoCIII –92/-67 promoter sequence indicate the location and orientation of the two half repeats of the HRE present in the element CIIIB of apoCIII. Arrows on the top of the 2 X CAGA sequence indicate the location and orientation of the two CAGA elements present in the synthetic oligonucleotide used as probe. **Panel B:** Gel electrophoretic mobility shift assays showing that SMAD3 cannot bind to the proximal apoCIII HRE but binds to the 2xGAGA probe shown in Panel A. The position of protein-DNA complexes is indicated. **Panel C** shows co-immunoprecipitation between HNF-4 and SMAD3 and SMAD4 which establish *in vivo* physical interactions among them. COS-7 cells were transfected with the indicated combinations of expression vectors. Forty-eight hours following transfection, cells were lysed and protein-protein interactions were analyzed by co-immunoprecipitation and Western blotting. IP indicates immunoprecipitation, WB indicates Western Blotting. **Panel D** shows GST pull-down experiments which confirm the physical interactions between HNF-4 and SMAD proteins. **Top:** Cell extracts from COS-7 cells transfected with a vector expressing myc-tagged HNF-4 were incubated with GST or the indicated GST-SMAD-coupled Sepharose beads. Bound proteins were analyzed by SDS-PAGE and Western blotting using an anti-myc antibody. Input represents 20% of the initial cell extract used in the binding experiments. The position of HNF-4 is indicated. **Bottom:** Bacterial expression of GST or GST-SMAD fusion proteins. Following coupling of GST fusion proteins to the Glutathione Sepharose beads, an aliquot of the affinity beads was analyzed for coupling efficiency by SDS-PAGE and Coomassie Brilliant Blue staining. M indicates molecular mass standards. Arrows indicate the position of the GST fusion proteins.

receptor resulted in an inhibition in the TGF beta responsiveness of the type-1 plasminogen activator inhibitor gene promoter (269).

8. CONCLUSIONS AND FUTURE DIRECTIONS

Transcription factors participate in the final step(s) of signal transduction pathways leading to transcriptional activation or repression of specific genes. The transcription of eukaryotic genes is a complex biological process; understanding this process will provide insights on how genes can be switched on and off in normal and pathologic states of an organism. As evidenced in this review, both *in vitro* as well as *in vivo* experiments are essential to identify the regulatory elements and the factors which are physiologically relevant. Both the *in vitro* and *in vivo* approaches have advantages and limitations and it is the combination of both approaches that can provide the optimal information. The identification of promoter and enhancer elements that are required for correct tissue-

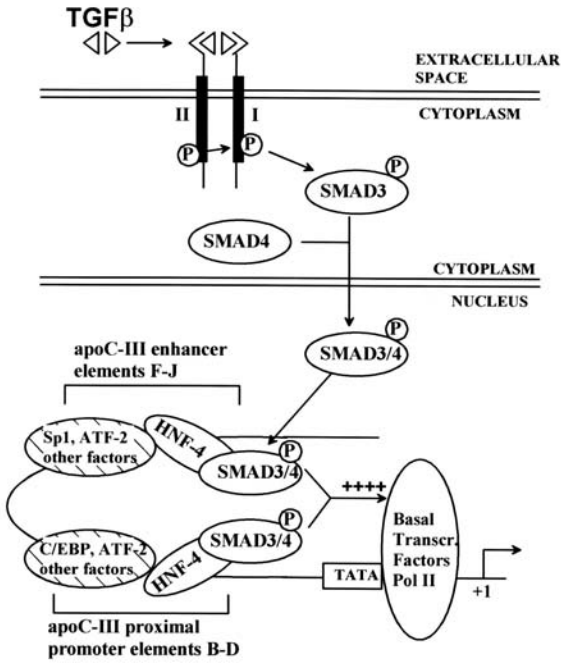


Figure 15. Schematic representation showing the pathway of TGF beta signaling and how interactions of specific SMAD proteins with HNF-4 transactivate the human apoCIII promoter. The binding of HNF-4, SP-1, ATF-2 and C/EBP on the apoCIII promoter and enhancer region has been established and are shown in Figure 3M.

specific expression *in vivo* is an essential early step that can be verified both by transgenic experiments as well as by *in vivo* footprinting techniques. The completion of the genome project will make the identification of the regulatory elements of important promoters, and the factors which occupy them, less tedious in the coming years. The importance of specific factors and the effects of their potential ligands can be assessed by promoter transactivation assays and inhibition of the synthesis of these factors in cell cultures by antisense and gene transfer methodologies using viral vectors. It will be interesting to see if specific patterns of promoter architecture involving specific multiprotein complexes which control tissue specificity can be deduced from this analysis.

Rapid scientific advances in structural biology have provided new insights into the domains of the transcription factors that are required for transcriptional activation, for DNA binding, for dimerization, and for interaction with ligands. In most cases, gene inactivation experiments have provided information on the importance of specific transcription factors. Rapid progress has also been made in the purification and structural analysis of the proteins of the basal transcription machinery and how the transcription factors communicate with the basal transcription factors through complex protein-protein and DNA-protein interactions. Although figures 3A-P indicate that the apolipoprotein promoters are recognized by a multitude of factors, the *in vivo* and *in vitro* approaches described above, make it possible to determine which of these factors are physiologically relevant.

The examples presented indicate that perhaps few factors may account for the functions and the specificity of a particular promoter. This may then allow determination of the precise protein/protein interactions and DNA/protein interactions within a specific promoter/enhancer cluster using structural biology and functional analyses, as has been done for the 30S and 50S ribosomes (270,271). From these studies the interaction of transcription factors with the basal transcriptional machinery may be deduced. The extracellular stimuli and the signaling mechanism which activate a specific gene are also important. As shown in this review, gene transcription may be altered by inducible factors which may or may not bind to the DNA.

Understanding the mechanism of transcriptional regulation may allow us in the long run to selectively switch on and off the apolipoprotein genes. As indicated, upregulation of apoA-I and possibly apoA-IV genes and/or downregulation of apoB, apoA-II and apoCIII genes may have beneficial effects in protecting humans from hyperlipidemia and atherosclerosis.

9. ACKNOWLEDGEMENTS

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