Contribution of the Hormone-Response Elements of the Proximal ApoA-I Promoter, ApoCIII Enhancer, and C/EBP Binding Site of the Proximal ApoA-I Promoter to the Hepatic and Intestinal Expression of the ApoA-I and ApoCIII Genes in Transgenic Mice†

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Received September 30, 2003; Revised Manuscript Received January 20, 2004

ABSTRACT: We have generated and studied the pattern of expression of transgenic mouse lines carrying the human apoA-I and apoCIII gene cluster mutated at different sites. In two lines, we have either mutated the hormone-response element (HRE) of element G of the apoCIII enhancer or the C/EBP binding site of the proximal apoA-I promoter. In a third line, we have mutated the two HREs of the apoA-I promoter and the HRE of the apoCIII enhancer. Mutations in the HRE of element G reduced the hepatic and intestinal expressions of the reporter chloramphenicol acetyltransferase (CAT) gene (which substituted the apoCIII gene) to 4 and 13% of the wild-type (WT) control, whereas the hepatic and intestinal expressions of the apoA-I gene were reduced to 92 and 25% of the WT control, respectively. A mutation in the C/EBP site increased the hepatic and intestinal expressions of the apoA-I gene approximately 1.25- and 1.6-fold, respectively, and did not affect the expression of the CAT gene. The mutation in the three HNF-4 binding sites of the apoA-I promoter/apoCIII enhancer nearly abolished the expression of apoA-I and the reporter CAT gene in all tissues. These findings establish the importance of the HREs for the hepatic and intestinal expressions of the apoA-I and apoCIII genes and suggest that C/EBP does not play a central role in the expression of the apoA-I gene.

Earlier studies established that there is linkage and a common regulatory mechanism of the apoA-I, apoCIII, and apoA-IV gene cluster (*1*). There are two common features in this cluster. The first feature is that the distal regulatory region of the apoCIII promoter is an enhancer that increases the strength of the neighboring genes in vitro (*2*−*5*). The second feature is that all three genes of the cluster have one or more HREs[11] in their proximal promoters (*6*−*9*).

There are two such elements in the apoA-I promoter, one in the apoCIII, and one in the apoA-IV promoter (*6*−*9*). The apoCIII enhancer also has two HREs, which differ in their receptor specificity, and has three SP1 binding sites (*4*).

In terms of receptor specificity, the HRE of the proximal CIII promoter binds HNF-4, which is an orphan nuclear receptor. It also binds other orphan nuclear receptors and a variety of ligand-dependent nuclear receptors with different affinities (*7*). One of the two HREs of the enhancer on element I4 binds HNF-4, other orphan nuclear receptors, and ligand-dependent nuclear receptors with different affinities. The other, on element G, does not bind HNF-4 but binds other orphan receptors and different combinations of ligand-dependent nuclear receptors with different affinities (*7*). Both of the HREs of the proximal apoA-I bind HNF-4, other orphan receptors, and a variety of ligand-dependent nuclear receptors with different affinities (*8, 9*).

Previous in vitro studies have shown that the apoCIII enhancer increases the strength of the proximal apoA-I promoter 13-fold and the strength of apoA-IV promoter 10-fold in HepG2 cells (*3, 5*). The apoCIII enhancer also increases, approximately 4−10-fold, the strength of different proximal apoCIII promoter segments (*2*).

Systematic in vitro mutagenesis of different sites of the promoter/enhancer cluster and determination of the promoter activity by CAT assays showed that mutations in the HNF-4 binding sites, which prevent binding of nuclear receptors to these sites, affect the activity of apoA-I, apoCIII, and apoA-IV promoter/enhancer cluster in HepG2 cells (*2*−*9*). We have smaller but significant reduction in the activity of the promoter/enhancer cluster by mutations in individual SP1 sites of the enhancer (*4, 5*). One additional regulatory region of the apoCIII enhancer on element G was shown to affect the activity of apoCIII and the apoA promoters in vitro (*4, 5*). Finally, the activity of the proximal apoA-I promoter in

† This work was supported by the National Institutes of Health Grant HL33952 and by Kos Pharmaceuticals (Miami, FL). The costs of publication of this paper were defrayed in part by the payment of page charges. This paper must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] Abbreviations: apo, apolipoprotein; ARP-1, apolipoprotein AI regulatory protein; CAT, chloramphenicol acetyltransferase; EAR-2, vErb-A related protein 2; HDL, high-density lipoprotein; HNF, hepatocyte nuclear factor; HRE, hormone-response element, RARα, all-trans retinoic acid receptor α; T3Rβ, thyroid hormone receptor β; WT, wild type; DR, direct repeat; mut, mutated; Fo, founder(s).

10.1021/bi035764g CCC: $27.50
Published on Web 04/09/2004

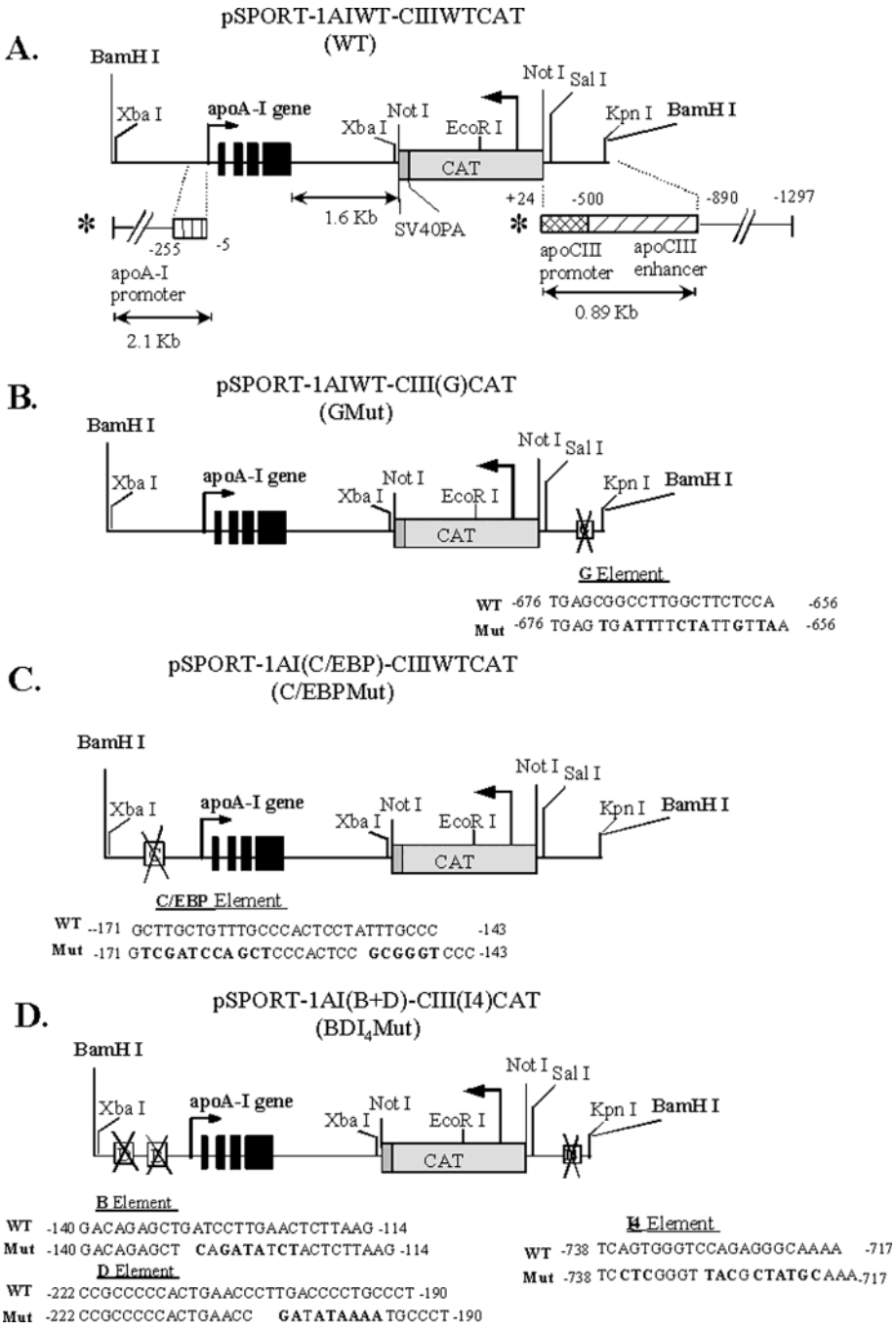


FIGURE 1: pSPORT-1 plasmid derivatives containing WT (A) and mutated (B−D) constructs of the apoA-I and apoCIII gene cluster. The following mutations were generated: mutations in the HRE on element G of the apoCIII enhancer (B); mutations in the C/EBP binding site of the proximal apoA-I promoter (C); mutations in the two HREs of the apoA-I promoter and the HRE on element I4 of the apoCIII enhancer (D). The names of the resulting transgenic lines harboring these transgenes are indicated in parentheses. Nucleotide substitutions in the mutated sequences are depicted by bold characters underneath each construct. The regions of the apoCIII promoter and enhancer or the apoA-I promoter and apoCIII enhancer utilized for in vitro experiments (*4*, *5*) are indicated by asterisks underneath the WT construct.

vivo was affected by mutations in element C, which binds C/EBP (*5*, *6*).

The most recent in vivo studies have shown that the HNF-4 binding site of the apoCIII enhancer is required for the intestinal expression of apoA-I and apoCIII genes and enhances synergistically the hepatic transcription of the apoA-I and apoCIII genes in vivo (*10*, *11*). 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 hepatic transcription (*11*, *12*).

The objective of this paper was two-fold. First, to investigate the effects of the HRE on element G of the apoCIII enhancer and the effects of the C/EBP binding site of the proximal apoA-I promoter on the transcription of the apoCIII and apoA-I genes, using transgenic mouse models. Second, to determine by the same approach whether other regulatory elements besides HREs could sustain the expression of the apoA-I gene.

MATERIALS AND METHODS

Plasmid Constructions. The WT construct pSPORT-1 AI WT-CIII WT CAT plasmid shown in Figure 1A, which contains the apoA-I and apoCIII regulatory sequences and has the apoCIII gene replaced by the reporter CAT gene,

Table 1: Oligonucleotide Sequence of Primers Used in PCR Amplifications

name	sequence	location of sequence
CIII 890K	ATAGGTACCGTTCCTCCCAGTTGCTCC	CIII −910/−893 antisense containing a *Kpn*I site
CIII 24E	AAAGAATTCCAGCTGCCTCTAGGGATG	CIII +27/+10 antisense containing an *Eco*RI site
PN2	AAAGAATTCCTGCCTCTAGGGATGAACTGAGCA	CIII +24/+1 sense containing an *Eco*RI site
PBN1	ATAGGTACCGGATCCGTTCTTCCCAGTTGCTCCCACAGC	CIII −890/−868 sense containing a *Kpn*I and *Bam*HI site
CIII IP-1	TTTGCATAGCGTAACCCGAGGATAACATCCCTGGGGGAGGAGCT	CIII −737/−760 antisense mutagenic
CIII IP-2	CTCGGGTTACGCTATGCAAATAGGGAGCCTGGTGGAGG	CIII −720/−687 sense mutagenic
CIII GP-5	TGATTTTCTATTGTTAACCAACCCCTGCCCTACACTCAGG	CIII −672/−633 sense mutagenic
CIII GP-3	GTTGGTTAACAATAGAAAATCACTCAGAGCCCGAGGCCTTTGCCCC	CIII −651/−696 antisense mutagenic
AICM6	AGCTGGATCGACTGCGGGGGCGTGCAGGGCAGCCC	A-I −167/−201 antisense mutagenic
AICM7	CCCTGCACGCCCCCGCAGTCGATCCAGCTCCCACTCGCGGGTCCCAG	A-I −195/−149 sense mutagenic
AI2100-3	TCGCAGTCTCTAAGCAGCCAGCTCTTG	A-I +10/−15 antisense mutagenic
AI XMAI-2	AACCCCGACCCCACCCGGGAGACCTG	A-I −270/−249 sense mutagenic containing an *Xma*I site

has been described (*10*). To generate the constructs containing the apoA-I and apoCIII gene cluster and introduce mutations in the HRE on element G of the enhancer, initially the human −890/+24 apoCIII promoter was amplified using the apoCIII promoter plasmid pSPORT-CIII WT CAT (*10*) as a template and pairs of external and mutagenic primers of Table 1. The external primers PBN1 and PN2 introduced *Kpn*I and *Eco*RI sites at the 5′ and 3′ ends of the −890/+24 apoCIII promoter/enhancer region. The mutagenic primers CIII GP5 and CIII GP3 introduced the mutation in element G. After digestion with *Kpn*I and *Eco*RI, the amplified and mutagenized fragment was cloned into the corresponding sites of the pSPORT-1 plasmid (Invitrogen). The *Eco*RI site of this plasmid was previously blunted by *Eco*RI digestion and filling of the 5′ protruding end using the Klenow fragment of the DNA polymerase I to generate the modified pSPORT-1 CIII WT plasmid. The pSPORT-1 CIIIG mut plasmid thus generated was digested with *Bam*HI and *Sal*I to excise a 0.89 kb fragment containing the mutated apoCIII promoter. The pSPORT-1 AI WT-CIII WT CAT plasmid (*10*) was digested with *Bam*HI and *Sal*I to release the 4 kb plasmid vector, a 7.31 kb fragment containing the CAT and the apoA-I gene, and a 0.89 kb *Sal*−*Bam*HI fragment corresponding to the WT apoCIII promoter. The final pSPORT A-I WT-CIII G CAT plasmid was generated by a triple ligation of the 4 kb pSPORT vector, the 7.31 kb fragment containing the apoA-I and the CAT gene, and the 0.89 kb promoter containing the G mutation (*10*).

To generate the construct containing the C/EBP mutation, a pBluescript apoA-I plasmid, consisting of a 5.5 kb genomic sequence (*1*, *13*) containing the entire apoA-I gene and a 2.1 kb promoter segment, was digested with *Alf*II and *Eco*RI to generate a 3.6 kb fragment that included the apoA-I gene. The same plasmid was digested with *Xma*I and *Eco*RI to release a 4.7 kb fragment containing the original pBluescript plasmid along with 1.8 kb of the apoA-I promoter. To mutagenize the C/EBP binding site of the apoA-I promoter, we used as a template a previously described WT apoA-I promoter pUC-SN-A-I CAT plasmid that contains the M4 mutation in the C/EBP site (*6*). This plasmid was mutagenized using the oligonucleotides AIX MAI-2 and AI 2100.3 as 5′ and 3′ external primers and the AICM6 and A-I CM7 as the 5′ and 3′ mutagenic primers. This mutagenesis eliminated the C/EBP binding site of element C of the proximal apoA-I promoter. The PCR product that contains the mutant promoter was digested with *Xma*I and *Alf*II, and a 137 bp fragment containing the mutated C/EBP site was isolated.

This 4.7 kb *Xma*I *Eco*RI fragment, the 3.6 kb *Alf*II *Eco*RI fragment, and the 137 bp *Xma*I *Alf*II fragment containing a C/EBP mutation were used in a triple ligation to generate the pBluescript AI C/EBP mut plasmid. This plasmid was digested with *Xba*I to release a 5.5 kb fragment containing the apoA-I gene and 2.1 kb of the mutated apoA-I promoter. This fragment was used to replace the corresponding 5.5 kb sequence of the pSPORT-1 AI WT-CIII WT CAT construct (*10*). To generate the transgenic construct containing mutations in the HREs of elements B and D of the apoA-I promoter and element I4 of the apoCIII enhancer, we digested the plasmid pSPORT-1 AI WT-CIII(I4) CAT with *Bam*HI and *Eco*RI. This digestion released a 1.16 kb fragment containing the I4 mutation. Similarly we digested the plasmid pSPORT-1 AI (B+D)-CIII WT CAT with *Bam*HI and *Eco*RI to release a 6.9 kb fragment containing the mutant apoA-I promoter, the apoA-I gene, and the 3′ portion of the CAT gene. The 1.16 kb fragment obtained from the first digestion, the 6.9 kb fragment obtained from the second digestion, and the 4.1 kb pSPORT-1 vector following digestion with *Bam*HI were joined in a triple ligation to generate the pSPORT-1 AI (B+D)-CIII(I4) CAT plasmid.

Heterozygous transgenic mice carrying the WT and the three mutant apoA-I and apoCIII promoter constructs were generated using standard transgenic mice methodologies (*10*, *14*). To obtain fertilized oocytes, C57BL/6 female embryo donor mice at 6−8 weeks of age were injected with pregnant mare's serum followed by injection of human chorionic gonadotropins 48 h later. The female embryo donors were mated with C57BL/6 males, and on the following day, the fertilized oocytes were recovered in a M2 medium (*14*). DNA of the constructs containing the WT and the three mutant apoA-I and apoCIII promoter constructs was excised from the corresponding pSPORT-1 constructs, separated by agarose gel electrophoresis, electroeluted, and purified. The DNA of the transgene was dissolved in 10 mM Tris-HCl at pH 7.4 and 0.1 mM EDTA at a concentration of 2 ng/μL and was microinjected into the pronuclei of fertilized oocytes. Embryos which survived microinjection, were maintained for 30 min in a M2 medium (*14*) and then implanted into the oviduct of pseudopregnant female mice (Swiss Webster) mated previously with vasectomized male mice (Swiss Webster). A total of 30 embryos were implanted into each

female pseudopregnant mouse. Recipient mice progressed through gestation, and approximately 70% of them gave birth to transgenic mice. Typically, about 20−30% of the littermates were identified as transgenic founders (Fo) by analysis of DNA isolated from tail biopsies of 4-week old mice. Identification was based on Southern blotting of mouse genomic DNA following *Eco*RI digestion and hybridization of the blot with the 978 bp apoA-I probe. This probe contained 540 bp of exon 4 and 438 bp of the intergenic sequence between the apoA-I and apoCIII genes. This analysis detects an 8.2 kb band in the transgenic mice that corresponds to the microinjected transgene. F1 progeny of transgenic mice were obtained through breeding of the Fo founders. Three to four transgenic mouse lines were generated per construct to overcome positional effects and ensure that the pattern of expression is characteristic of a specific construct. The abbreviated names of the transgenic lines are shown in parentheses over each of the constructs of panels A−D of Figure 1.

The number of transgene copies incorporated into the genome of each transgenic founder was determined by Southern blotting and a comparison of the intensity of the bands formed when increasing amounts of the transgene were diluted in nontransgenic DNA.

Lipid, Lipoprotein, and ApoA-I Profile of Transgenic Mice. Levels of total serum cholesterol and serum TG were determined using commercially available enzymatic kits (Sigma) (*10*). The mean ± standard deviation for an individual mouse line and all of the lines expressing the same construct is provided. For fast protein liquid chromatography (FPLC) analysis of serum samples, 12 μL of serum were diluted 1:5 with phosphate-buffered saline (PBS). The sample was then loaded onto a Sepharose 6 column in a SMART micro FPLC system (Amersham Pharmacia Biotech) and eluted with PBS. A total of 25 fractions of 50 μL each were then collected and analyzed for cholesterol levels as described (*15*).

RNA Isolation, Blotting, and CAT Assays. Total cellular RNA was isolated from liver and other tissues using the guanidine isothiocyanate method and the Qiagen RNA/DNA kit and purified using a medium-size column. RNA was eluted with high salt buffer, precipitated by 2-propanol, and dissolved in RNase-free water. Equal quantities of RNA (10 μg) were separated by electrophoresis in 1.0% agarose−formaldehyde gels. After the RNA was transferred to a HyBond-N+ nylon membrane (Amersham Pharmacia Biotech), the RNA was cross-linked to the filter by UV irradiation (Stratalinker, Stratagene) at 0.12 J/cm^2 for 1 min. The apoA-I probe for hybridization is 438 bp in length. This probe contains 290 bp of exon 4 of the human apoA-I gene and 148 bp of the intergenic sequence between the apoA-I and apoCIII genes and does not cross-hybridize with the mouse apoA-I mRNA. The mouse 28S rRNA probe was obtained from Ambion (Austin, TX). This oligonucleotide probe contains 43 nucleotides of highly conserved sequences among higher eukaryotes. The 28S rRNA probe was labeled by 5′-end labeling using the T4 polynucleotide kinase method. The apoA-I mRNA signal of each sample was normalized by dividing it with the 28S rRNA signal. Hybridization reactions contained (1−2) × 10^6 cpm (^{32}P)-labeled DNA per mL of buffer. The unhybridized probe was removed by washing at 65 °C with 2× SSC and 0.1% SDS, followed by 15−30 min washes with 1× SSC and then with 0.5× SSC, as needed. Quantitation of X-ray film was performed by a phosphoimager (Molecular Dynamics) using the ImageQuant program. The relative levels (%) of apoA-I mRNA represents the ratio of apoA-I to the 28S ribosomal RNA signal.

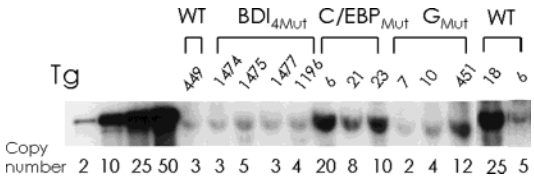


FIGURE 2: Gene copy number of mouse lines expressing WT and mutated apoA-I and apoCIII promoter/enhancer constructs. The abbreviated names of the transgenic mouse lines analyzed are shown on the top of the figure and are described in detail in Figure 1. Numbers on the top indicate the mouse lines analyzed. The copy number was determined as described in the Materials and Methods.

Table 2: Lipid Profiles in Control and Transgenic Mice[a]

constructs	mouse line	total cholesterol (mg/dL)	triglyceride (mg/dL)
C57BL/6		61	42
WT	449	171	81
	18	137	56
	6	167	98
G mut	7	99	84
	10	117	103
	451	125	76
C/EBP mut	6	194	184
	21	146	95
	23	201	82
I4 BD mut	1474	59	63
	1475	92	45
	1477	86	69
	1196	66	78

[a] Mouse plasma was collected by pooling the blood from two to three mice of each line in plastic tubes containing EDTA to achieve a final concentration of 5 mM EDTA.

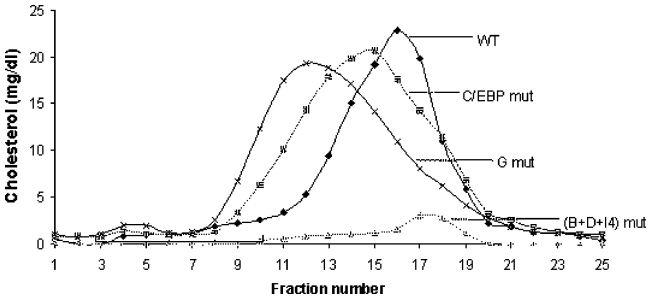


FIGURE 3: (A−D) FPLC profile of plasma of mice carrying the WT and mutated apoA-I and apoCIII promoter/enhancer constructs as indicated in the figure. The sites of the mutations of the G, C/EBP, and (B+D+I4) mutants are shown in Figure 1.

CAT Assays. For the CAT assay, F1 mice were sacrificed at 2 months of age. Tissues were collected and used immediately or were frozen and stored at −80°C. CAT assays were performed as described (*10*, *16*).

RESULTS

Generation and Characterization of Transgenic Mice Harboring Mutations in the Proximal ApoA-I Promoter and ApoCIII Enhancer. Recent studies in transgenic mice established the importance of the HNF-4 and SP1 binding sites of the apoCIII enhancer as well as the importance of the

Table 3: CAT Activity in Tissues of Transgenic Mice Expressing the Reporter Gene under the Control of WT or Mutated ApoCIII and ApoA-I Promoter Segments

construct	mouse line	copy number	*N*	liver (mean ± SD)	intestine (mean ± SD)	kidney (mean ± SD)
WT	449	3	3	5045 ± 960	159 ± 35	59 ± 11
	6	25	4	7985 ± 1000	486 ± 110	171 ± 36
	6	5	5	9855 ± 185	141 ± 4	123 ± 18
average				8029 ± −2074	261 ± −174	123 ± −48
B+D+I4 mut	1474	3	3			
	1475	5	3	271 ± 19		
	1477	3	4	150 ± 16		
	1196	4	3			
average				216 ± −94		
G mut	7	2	1	211 ± 42	33 ± 6	45 ± 6
	10	4	3	234 ± 31	45 ± 7	81 ± 5
	451	12	4	103 ± 15	29 ± 3	34 ± 3
average				164 ± −65	36 ± −8	51 ± −21
C/EBP mut	6	20	1	8960 ± 795	168 ± 20	350 ± 41
	21	8	3	3340 ± 430	59 ± 14	206 ± 46
	23	10	3	12210 ± 1080	92 ± 32	797 ± 53
average				8170 ± −3950	116 ± −78	451 ± −270

proximal HREs of the apoA-I and apoCIII promoters for the intestinal and hepatic expression of the apoA-I and apoCIII genes. To understand further how the apoA-I and apoCIII genes are regulated, we have generated three additional lines of transgenic mice and compared them with the mouse lines that carry the WT construct (parts A−D of Figure 1). The first line carries a mutation in the HRE on element G of the apoCIII enhancer (Figure 1B). This element binds some orphan and ligand-dependent nuclear receptors but does not bind HNF-4. The second line carries mutations in the C/EBP binding site on element C of the apoA-I promoter (Figure 1C). The third line carries mutations in the two HREs of the proximal apoA-I promoter and the HRE on element I4 of the apoCIII enhancer (Figure 1D). All three HREs bind orphan and ligand-dependent nuclear receptors.

The three transgenic lines (G mut) have 2, 4, and 12 copies of the transgene, respectively. The three transgenic lines (C/EBP mut) have 20, 8, and 10 copies of the transgene, respectively. The four transgenic lines (B D I4) mut have 3, 5, 3, and 4 copies of the transgene, respectively. The three transgenic mouse lines expressing the WT construct have 3, 25, and 5 copies of the transgene respectively (Figure 2).

Lipid and ApoA-I Levels of Mice Expressing the ApoA-I Transgene Under the Control of the WT and Mutant ApoA-I and ApoCIII Promoter and Enhancer Regions. The plasma lipids of mice expressing the human apoA-I gene were determined as explained in the Materials and Methods. The triglyceride levels, with one exception, were within the normal range (45−103 mg/dL) (Table 2). In one of the C/EBP cell lines, the triglycerides were 184 mg/dL. Cholesterol levels in mice expressing the WT and the C/EBP mut were in the range of 137−200 mg/dL. Low cholesterol levels were detected in mice expressing the G mut (99−125 mg/dL) and even lower levels in mice expressing the BDI mut (59−92 mg/dL) as compared to the WT control (Table 2).

FPLC fractionation of the mouse plasma showed that practically all of the HDL was in the HDL region (Figure 3). As expected, the FPLC profile of the (B+D+I4) mutant that does not express human apoA-I was similar to that of the apoA-I-deficient mice.

Effect of Mutations in the Regulatory Elements of the ApoA-I and ApoCIII Gene Cluster on the Activity of the Reporter CAT Gene. The activity of the apoCIII promoter and enhancer in the WT and mutated constructs was measured by the activity of the reporter CAT gene, which replaced the apoCIII gene in the transgenic construct, as described in the Materials and Methods. This analysis showed that a mutation in the HRE of element G of the apoCIII enhancer (which does not bind HNF-4) reduced the hepatic expression of the CAT gene to 4% of the WT control and reduced the intestinal and renal expressions to approximately 13 and 30% of the WT control, respectively. The findings indicate the importance of this element for the intestinal and hepatic expressions of the apoCIII promoter/enhancer cluster (Table 3). As expected, a mutation in the C/EBP binding site of the apoA-I promoter did not affect the hepatic or intestinal expressions of the CAT gene, but in two of the mouse lines, it caused a significant increase in the renal expression as compared to the WT control lines (Table 3).

A mutation in the two HREs of the proximal apoA-I promoter and the HRE of the apoCIII enhancer that binds HNF-4, abolished the expression of the CAT gene in all tissues and reduced the hepatic expression to approximately 1% of the WT control (Table 3). The findings confirm that the HNF-4 binding site of the apoCIII enhancer is required for the intestinal expression and greatly enhances the hepatic expression of the apoCIII gene.

Effect of Mutations in the Regulatory Elements of the Proximal ApoA-I Promoter and ApoCIII Enhancer on the Expression of the Human ApoA-I Gene. The activity of the apoA-I promoter and apoCIII enhancer in mice expressing the WT and the mutated constructs was assessed by the steady-state apoA-I mRNA levels as described in the Materials and Methods. This analysis showed that the mutation in the HRE on element G of the apoCIII enhancer (the site that does not bind HNF-4) had only a minor effect on the hepatic expression of apoA-I, but it greatly reduced the intestinal and testicular expressions and increased the renal expression of the apoA-I gene (Figure 4B). Comparative analysis of the relative levels of expression indicated that, in mice mutated in the HRE of element G, the hepatic, intestinal, and renal expressions were 92, 25, and 350% of the WT control, respectively (Figure 5A). The findings indicate that the apoCIII enhancer has a modulatory effect on the tissue-specific expression of the apoA-I gene. A

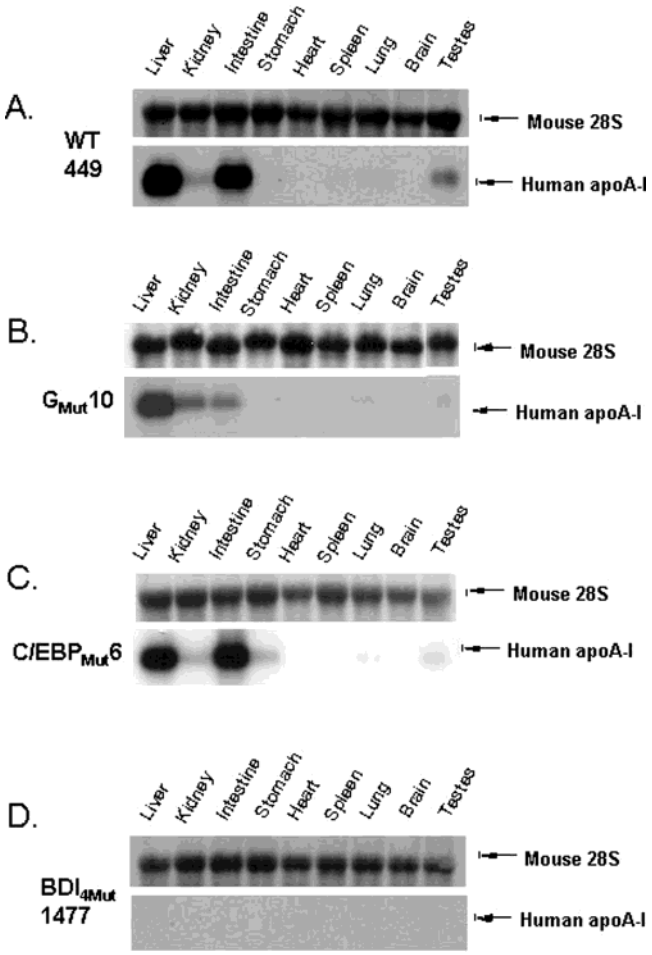


FIGURE 4: Representative Northern blots of apoA-I mRNA obtained from tissues of transgenic mice expressing constructs containing the WT and mutated apoA-I and apoCIII promoter and enhancer shown in Figure 1. (A) mice expressing the apoA-I gene under the control of the WT apoA-I promoter/WT apoCIII promoter and enhancer. (B) mice expressing the apoA-I gene under the control of the WT apoA-I promoter and apoCIII enhancer mutated in the HRE of element G. (C) mice expressing the apoA-I gene under the control of the apoA-I promoter mutated in the C/EBP binding site and the WT apoCIII promoter/enhancer. (D) mice expressing the apoA-I gene under the control of the mutant apoA-I promoter and mutant apoCIII enhancer. The mutations were introduced in the two HREs of the proximal apoA-I promoter on elements B and D and the HRE on element I4 of the apoCIII enhancer. The abbreviated names of the transgenic lines described in Figure 1 are used. Northern blotting was performed as described in the Materials and Methods. These panels show the relative apoA-I mRNA levels in different tissues of representative mouse lines. Mouse 28S RNA was used as a control. Comparative levels of apoA-I mRNA in the liver, intestine, and kidney of different mouse lines are shown in Figure 5.

mutation in the C/EBP binding site on element C on the proximal apoA-I promoter increased to some extent the intestinal, hepatic, and renal expressions of the apoA-I gene. The expression in the other tissues had minor changes compared to the WT controls (Figure 4C). Comparative analysis showed that, in mice mutated in the C/EBP binding site, the hepatic, intestinal, and renal expressions increased 1.26-, 1.59-, and 2.12-fold, respectively (Figure 5B). In the WT mice, the expression in the kidney, stomach, lung, and testes was estimated to be 11, 2, 4, and 12% of the hepatic expression, respectively (Figure 4A). Comparison in the three mouse lines carrying the C/EBP mutation showed that the expressions in the stomach were 0, 4, and 10%, lung, 2, 3, and 2%, and testes 5, 8, and 10% as compared to the hepatic expression.

A mutation in the HRE of the proximal apoA-I promoter and the HRE of the apoCIII enhancer (the sites that bind HNF-4) nearly abolished the expression of the apoA-I gene in all tissues (Figure 4D). In only two of the four mouse lines, there was trace expression in the liver (Figure 5C) and, in one of those lines, traces of expression in the testes. The findings suggest very strongly that the transcription of the apoA-I gene in all tissues depends on different types of nuclear receptors, which occupy the two HREs of the proximal promoter and apoCIII enhancer.

Figure 6 compares the in vitro and in vivo data of apoA-I and apoCIII gene regulations following mutagenesis in the proximal promoter and apoCIII enhancer region. Parts A−D of Figure 7 provide putative mechanisms of transcriptional activation of the apoA-I promoter/apoCIII enhancer cluster in vivo based on all of the available data in transgenic mice compiled in Figure 7E.

DISCUSSION

In this paper, we used transgenic mice carrying the WT and mutated apoA-I and apoCIII promoter/enhancer constructs to investigate the in vivo effects of the HRE on element G of the apoCIII enhancer and the effects of the C/EBP binding site of the proximal apoA-I promoter on the transcription of the apoCIII and apoA-I genes. We also used transgenic mice carrying in vivo constructs mutated in the HREs of the proximal apoA-I promoter and apoCIII enhancer to determine whether other regulatory elements besides HREs could sustain the expression of the apoA-I and apoCIII genes in vivo. Previous studies have shown that the nuclear receptors are highly conserved among species and bind with equal efficiency to HREs in vitro (*17*−*19*). Thus, we do not expect that expression of the human apoA-I and apoCIII genes in mice will affect their expression. Furthermore, the CAT cDNA used does not contain any regulatory elements and is not expected to affect gene expression.

Effect of Mutations on the in Vivo and in Vitro Regulation of the ApoCIII Gene. The current paper shows that both the hepatic and intestinal expressions of the CAT gene (which is a reporter for the apoCIII gene) were greatly affected by mutations in the HRE of element G of the apoCIII enhancer. This indicates that nuclear receptors other than HNF-4 that bind to this element are crucial for the intestinal and hepatic expressions of the apoCIII gene.

It also showed that the activity of the apoCIII promoter mutated in both the HRE of element I4 of apoCIII and elements B and D of apoA-I was approximately 1% of the WT control. Similar levels of expression were observed previously by a single mutation in element I4 of the apoCIII enhancer (*10*). These findings indicate that the proximal apoCIII promoter alone can promote low levels of gene transcription and that the hepatic transcription of apoCIII is driven mainly by the enhancer. The in vivo pattern of intestinal expression of the apoCIII gene following mutagenesis in element G of the apoCIII enhancer correlates with that observed in vitro. However, the pattern of hepatic expression differs drastically. Previous in vitro studies showed that point mutations in element G of the apoCIII

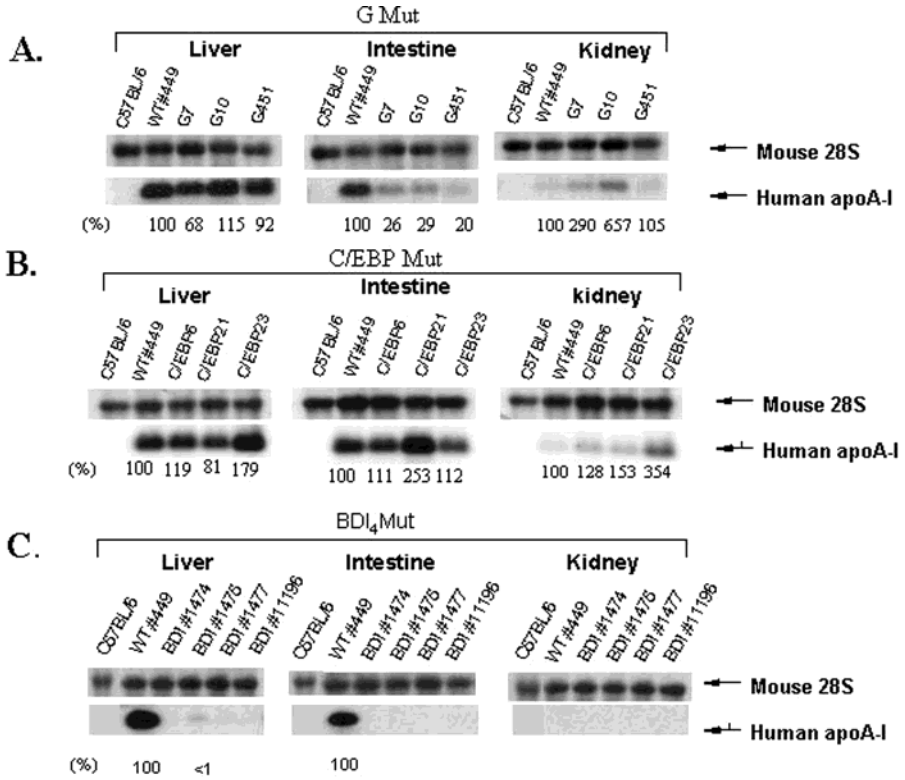


FIGURE 5: Northern blotting analysis of 10 μg of the total RNA obtained from the liver (A), intestine (B), and kidney (C) of transgenic mice expressing constructs containing the WT and mutated apoA-I and apoCIII promoter and enhancer shown in Figure 1. The nylon membrane was hybridized simultaneously with the apoA-I mouse 28S RNA as described in the Materials and Methods. All panels contain samples obtained from C57BL/6 mice, one line of mice carrying the WT promoters and enhancer, and three of the four lines of mice containing the mutated promoter/enhancer cluster as follows: (A) mice expressing the apoA-I gene under the control of the WT apoA-I promoter and apoCIII enhancer mutated in the HRE of element G. (B) mice expressing the apoA-I gene under the control of the apoA-I promoter mutated in the C/EBP binding site and the WT apoCIII promoter/enhancer. (C) mice expressing the apoA-I gene under the control of the mutant apoA-I promoter and mutant apoCIII enhancer. The mutations were introduced in the two HREs of the proximal apoA-I promoter on elements B and D and the HRE on element I4 of the apoCIII enhancer. The abbreviated names of the transgenic lines are shown on top of each lane. The ratio of apoA-I/28S signal of samples obtained from mice carrying the WT apoA-I WT apoCIII construct was arbitrarily set to 100%. The ratios of apoA-I/28S mRNA for the other samples provide a measure of their abundance relative to those of the WT control.

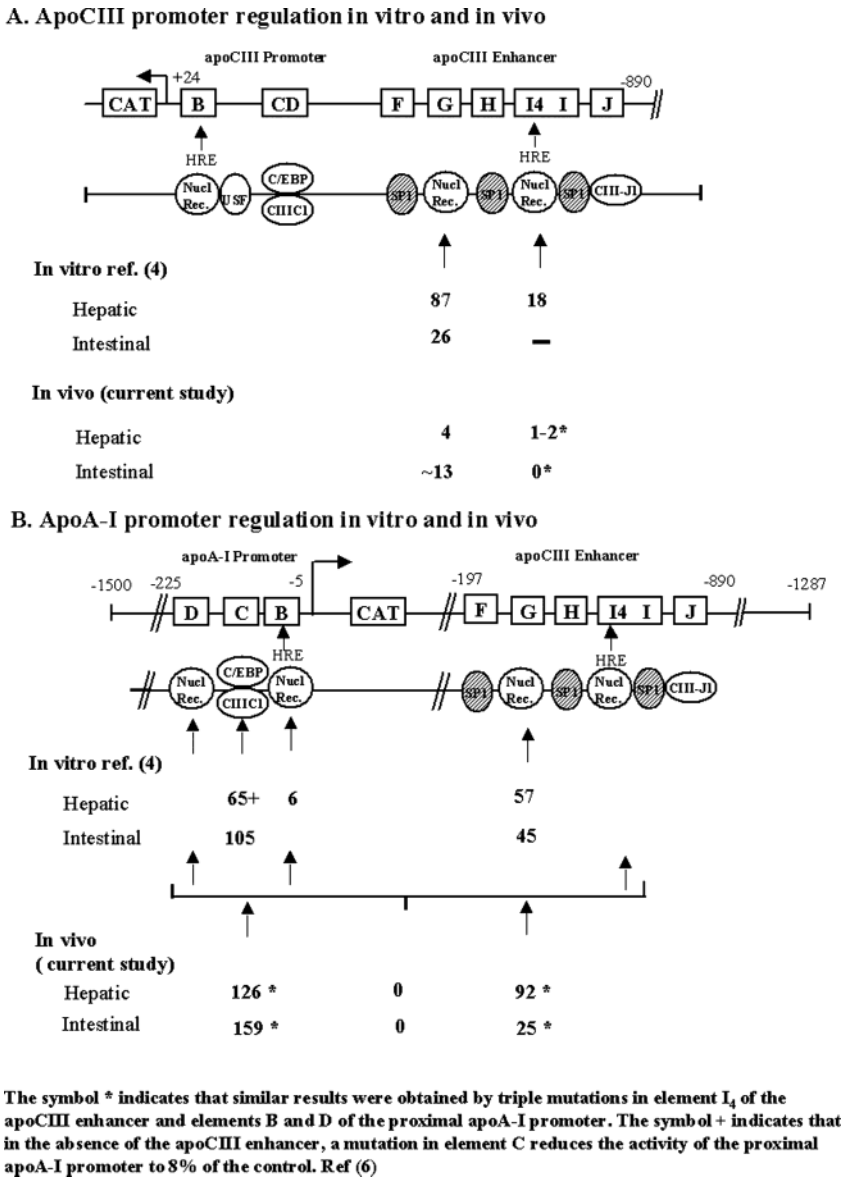
enhancer reduced the activity of the apoCIII promoter/enhancer to 26 and 87% of the control in HepG2 and CaCo-2 cells, respectively (*4*). The in vitro studies showed only a small (13%) reduction in the promoter activity in cells of hepatic origin, whereas the in vivo studies show a 96% reduction in the hepatic expression of the reporter CAT gene, which substitutes for the apoCIII gene (Figure 6A). As expected, the mutation in the C/EBP site of the apoA-I promoter did not significantly affect the expression of the reporter CAT gene.

Effect of Mutations of the C/EBP Binding Site of the Proximal ApoA-I Promoter and the HREs of the Promoter and Enhancer on the Expression of ApoA-I Gene in Vitro and in Vivo. The current paper shows that a mutation in element G of the apoCIII enhancer selectively reduced the intestinal expression but did not significantly affect the hepatic expression. These findings suggest that nuclear hormone receptors, which bind to this site of the apoCIII enhancer, affect intestinal transcription but have a limited effect on the hepatic transcription. The in vitro data had suggested that these mutations in element G reduced the activity of the promoter/enhancer cluster in HepG2 and CaCo-2 cells to 57 and 45%, respectively (Figure 6B). Thus, in the case of the apoA-I and apoCIII expressions, the in vitro data do not accurately reflect the observed in vivo pattern of expression of the apoA-I-mutated apoA-I (parts A and B of Figure 6).

The analysis of the mice carrying mutations in the C/EBP binding site of the proximal apoA-I promoter indicated that in some mouse lines the mutations either did not affect or increased the intestinal, hepatic, and renal expressions of the apoA-I gene. This finding strongly suggests that C/EBP may not play a central role in the in vivo regulation of the apoA-I gene.

In vitro studies had previously shown that mutagenesis of the C/EBP binding site dramatically decreased the in vitro activity of the proximal apoA-I promoter to 8% of the WT control (*6*). However, the effect of this mutation was offset when the mutated promoter was linked to the apoCIII enhancer (*5*). Thus, the activity of the mutant promoter/enhancer construct was 65 and 105% in HepG2 and CaCo-2 cells, respectively, as compared to the WT construct (*5*).

The observation that this triple mutation in the HREs of the proximal apoA-I promoter and apoCIII enhancer eliminated the expression of the apoA-I gene in all tissues highlights the critical role of the nuclear receptors for the overall expression of the apoA-I gene and suggests that other factors such as C/EBP are not sufficient to sustain the activity of the proximal apoA-I promoter.



The symbol * indicates that similar results were obtained by triple mutations in element I_4 of the apoCIII enhancer and elements B and D of the proximal apoA-I promoter. The symbol + indicates that in the absence of the apoCIII enhancer, a mutation in element C reduces the activity of the proximal apoA-I promoter to 8% of the control. Ref (6)

FIGURE 6: Comparison of the in vivo and in vitro regulation of the apoA-I and apoCIII promoter/enhancer constructs. (A) Schematic representation of the WT and mutated −890/+24 apoCIII promoter/enhancer construct. (B) Schematic representation of the WT and mutated −1500/−5 apoA-I promoter/−500/−800 apoCIII enhancer regions. Both panels show the binding specificity of the different regulatory sites in vitro. Factors are symbolized by ovals. Numbers in parentheses indicate the percent activity of the promoter/enhancer cluster in vitro when it is mutated so that the indicated factors do not bind to the mutated site (*4*, *5*).

Overall Mechanism of Transcription of the Human ApoA-I and ApoCIII Genes. Previous studies in transgenic mice showed that the level of hepatic and intestinal expressions of the apoA-I gene in the mouse lines carrying the mutations in the two HREs of the apoA-I promoter was approximately 15% as compared to the WT control. These findings indicate that hepatic and intestinal expressions are still possible, at lower levels, when the proximal apoA-I promoter is inactivated by mutations that prevent the binding of nuclear hormone receptors to the two proximal HREs. The most probable interpretation of these findings is that in vivo the proximal apoCIII enhancer alone (Figure 7A) can independently drive the hepatic and intestinal transcriptions of the apoA-I gene. Similarly, the hepatic expression of apoA-I in mouse lines carrying mutations in the HRE of the apoCIII enhancer, which binds HNF-4 or the three SP1 sites of the enhancer, is approximately 15−20% of those observed in mice carrying the WT apoA-I promoter/apoCIII enhancer (Figure 7B). The findings indicate that the proximal apoA-I promoter alone can independently drive the hepatic transcription of the apoA-I gene. These mutations abolish the intestinal expression of the apoA-I gene. As shown in this paper, when the proximal apoA-I promoter and apoCIII enhancer are inactivated by mutations in the HREs that bind HNF-4, the expression of the apoA-I gene is abolished in all tissues (Figure 7C). On the other hand, when both the promoter and enhancer are functional, the activity of the apoA-I promoter/apoCIII enhancer cluster is not additive (15 + 20 = 35%) but rather synergistic (100%) (Figure 7D). This synergism has been documented by in vivo as well as in vitro studies (*4*, *5*, *10*, *12*). On the basis of this paper, the C/EBP binding site of the proximal apoA-I promoter and the HRE on element G of the apoCIII enhancer do not appear to substantially influence the hepatic expression of the apoA-I gene (Figure 7E). Glutathione-*S*-transferase (GST) pull down and coimmunoprecipitation experiments established protein−

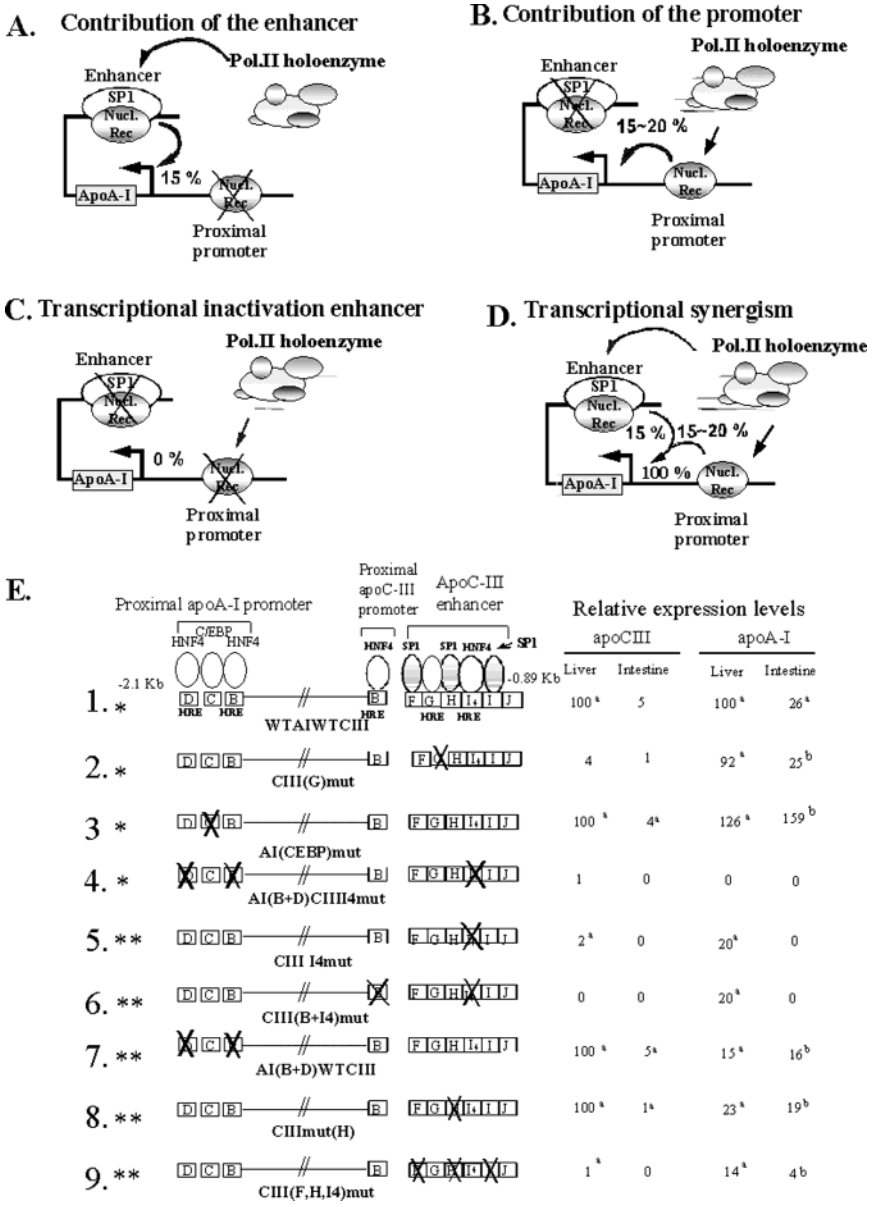


FIGURE 7: Putative mechanism of transcriptional activation of the apoA-I promoter/apoCIII enhancer in vivo. (A) shows the independent contribution of the nuclear receptors and the SP1, which bind to the apoCIII enhancer. (B) shows the independent contribution of the nuclear nuclear hormonereceptors, which bind to the proximal apoA-I promoter. (C) shows the complete inactivation of the promoter/enhancer cluster by mutations in the HREs of the proximal apoA-I promoter and in the HRE on element I4 of the enhancer. (D) shows enhancement of the activity of the apoA-I promoter/enhancer cluster as a result of the synergistic interaction of nuclear receptors and SP1 molecules, which bind to the apoCIII enhancer and the proximal apoA-I promoter. (E) represents a summary of the expression of WT and mutant constructs of the apoA-I and apoCIII gene cluster in transgenic mice, which provides the experimental data in support of the models proposed in A−D. The single asterisk indicates that lines 1−4 summarize the data of the current paper. The double asterisk indicates that lines 5−9 summarize the data of previous studies (*10*, *12*). (a) indicates the percent expression relative to the expression of the WT construct in the liver, and (b) indicates the percent expression relative to the expression of the WT construct in the intestine.

protein interactions between SP1 and HNF-4 (*20*). These protein−protein interactions and the allosteric effects that they elicit may increase the activation properties of the individual factors (*21*, *22*) and facilitate the recruitment of the proteins required for the remodeling of the chromatin and initiation of transcription (*23*−*25*).

The intestinal expression of the apoA-I gene seems to depend entirely on the activity of the apoCIII enhancer. Thus, when the enhancer is inactivated by mutations in the HNF-4 binding site of the apoCIII enhancer, the intestinal expression of the apoA-I gene is abolished. In addition, the intestinal expression of the apoA-I gene is affected severely by mutations in the element G and the SP1 binding site(s) of the apoCIII enhancer (*10*, *12*) but not by mutations in the C/EBP binding site of the proximal promoter (Figure 7E).

Similar regulatory mechanisms that apply for the apoA-I gene also apply for the apoCIII gene.

Implication of This Paper for the Selective Regulation of the ApoA-I and ApoCIII Genes. The findings of the current paper strongly suggest that, like the apoA-I gene, transcription of the apoCIII gene depends on combinations of nuclear receptors that bind to proximal and distal HREs as well as on members of the SP1 family of transcription factors.

Previous studies showed that the expression of the apoA-I, apoCIII, and apoA-IV genes is abolished in the fetal liver

of mice in which the HNF-4 is inactivated by homologous recombination (*26*). On the other hand, liver-specific inactivation of HNF-4 diminished the hepatic expression of apoCIII but did not affect the expression of the apoA-I gene (*27*). These data indicate that different combinations of nuclear hormone receptors recognize the proximal apoA-I promoter and apoCIII enhancer and control the transcription of the apoA-I and apoCIII genes. This concept is further supported by other studies that show differences in the regulation of the apoA-I and apoCIII genes when various nuclear hormone receptors are inactivated. For instance, inactivation of PPARα in mice is associated with decreased levels of hepatic apoA-I mRNA, plasma apoA-I, and HDLc levels, whereas the expression of the apoCIII gene is not affected (*28*). On the other hand, liver-specific inactivation of the RXRα gene in mice is associated with increased expression of the apoA-I and apoCIII genes (*29*). The observed differences in receptor specificity for the apoA-I and apoCIII genes might provide potential treatments to upregulate apoA-I and downregulate apoCIII gene expression. Treatments that might selectively increase plasma apoA-I and decrease plasma apoCIII levels are expected protect against atherosclerosis.

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BI035764G