Complex Interactions between SP1 Bound to Multiple Distal Regulatory Sites and HNF-4 Bound to the Proximal Promoter Lead to Transcriptional Activation of Liver-Specific Human APOCIII Gene†

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ABSTRACT: Footprinting analysis of the human apoCIII promoter identified a set of four proximal (A−D) and six distal (E−J) regulatory elements between nucleotides −792 and −25 [Ogami, K., et al. (1990) *J. Biol. Chem. 265*, 9808−9815]. The distal regulatory elements of the apoCIII gene increase by 10-fold the strength of the homologous as well as of heterologous proximal promoters. Required for such transcriptional enhancement is the presence of an intact hormone response element (HRE) on the proximal promoter which binds a variety of nuclear hormone receptors. To understand the mechanism of this transcriptional activation, we identified the nature and the importance of the factors which bind to the upstream regulatory elements of the apoCIII promoter by DNA binding, competition, supershift, and transient transfection assays. These analyses showed that the upstream apoCIII promoter contains multiple binding sites for the ubiquitous transcription factor SP1, which recognizes the regulatory elements F, H, and I. The regulatory element G represents a specialized HRE which is recognized by the orphan receptors ARP-1 and EAR-3 but not by HNF-4. A single activity designated CIII J1 binds to the regulatory element J. The same or a similar activity binds as a minor component to the regulatory elements F and I where SP1 is the predominant binding activity. Finally, a minor activity designated CIII I5 binds to the regulatory element I. *In vitro* mutagenesis of the distal promoter showed that mutations in elements H and G which affect the binding of SP1 and ARP-1 and EAR-3, respectively, had the most severe effect and decreased the promoter strength in HepG2 cells to 14% and 26% of control, respectively. The promoter strength was similarly decreased in CaCo-2 cells to 25% of control, by mutations in element H, but either was slightly affected or increased 1.6-fold by mutations in element G. HNF-4 transactivated 8- and 6-fold hepatic and intestinal transcription, respectively, driven by the full-length apoCIII promoter. The transactivation patterns in HepG2, CaCo-2, and HeLa cells were different; however, in all cases the transactivation pattern was significantly affected by mutations in elements B, G, and H. The findings suggest the involvement of a complex mechanism in the regulation of transcription of the human apoCIII gene. A major role is exerted by HNF-4 or other nuclear hormone receptors that bind to the regulatory element B as well as by SP1 or by related factors that bind to upstream regulatory sites. It appears that the binding of multiple SP1 molecules to these sites directly or indirectly increases the binding or the activation potential of HNF-4, thus promoting the transcriptional activation of the apoCIII gene.

Human apoCIII is a 79 amino acid protein of known primary structure (Shulman et al., 1974; Brewer et al., 1974) and is a major component of very low density lipoprotein and a minor component of high-density lipoprotein (Herbert et al., 1982). The gene and cDNAs encoding human apoCIII have been isolated and sequenced (Protter et al., 1984; Sharpe et al., 1984; Karathanasis et al., 1985). The apoCIII, apoA-I, and apoA-IV genes are closely linked (Karathanasis, 1985) and have been mapped on the long arm of chromosome 11 (Cheung et al., 1984). ApoCIII has been implicated by *in vitro* experiments in the modulation of the catabolism of triglyceride-rich lipoproteins (Krauss et al., 1973; Brown & Bajinksy, 1972; Windler et al., 1980; Shelburne et al., 1980; Quarfordt et al., 1982). It has been shown recently that overexpression of the human apoCIII gene in transgenic mice results in severe hypertriglyceridemia (Ito et al., 1990; de Silva et al., 1994) with fasting triglyceride levels exceeding 950 mg/dL (Ito et al., 1990) and accumulation in plasma of apoB-48 containing lipoprotein remnants (de Silva et al., 1994), suggesting a potential role of apoCIII in the catabolism of triglyceride-rich lipoproteins *in vivo*.

ApoCIII gene expression is tissue specific (Zannis et al., 1985; Lenich et al., 1986; Haddad et al., 1986) and developmentally regulated (Haddad et al., 1986).

Footprinting analysis of the apoCIII promoter identified a set of four proximal (A−D) and six distal (E−J) regulatory elements between nucleotides −792 and −25 (Reue et al., 1988; Ogami et al., 1990). DNA binding and competition assays established the different activities which recognize the proximal regulatory region. Element B contains an HRE[1] and recognizes more than one activity, and these activities bind in overlapping binding motifs. One of the activities is

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Table 1: Sequences of Oligonucleotides Used in DNA Binding and Competition Experiments

Oligo	Sequence[a]
CIII B	-96-TCGACACTGGTCAGCAGGTGACCTTTGCCCAGCGCCCTGG-61
CIII BM1	-96-TCGACACTGGTCA**TATATT**GACCTTTGCCCAGCGCCCTGG-61
CIII BM5	-96-TCGACACTGGTCAGCAGGTGAC**GACAGA**CCAGCGCCCTGG-61
CIII F	-612-TCGACAGGGTGGGGGCGGGTGGGG-592
CIII FM1	-616-GCACACAG**TTAT**GGGGCGG**TATC**GGGCTHC-586
CIII FM2	-611-CAGGGTGGG**TAAT**GGTGGGG-592
CIII FM3	-620-TGGGGCACA**GGGATCCGTAGGCAT**GGGGGCTGCTGGGT-581
CIII G	-669-TCGACCTTGGCTTCTCCACCAACCCC-648
CIII GM1	-672-CGGCCTT**ACAG**TCTCCACCAACCCC-648
CIII GM3	-673-GCGGCCT**TGGCTGAGA**CACCAACCCCT-647
CIII GM4	-779-CTCTGAGCGGCCTT**ACAGGTCTCGAG**CAACCCCTGCCCT-642
CIII H	-705-TCGAGTGGAGGGAGGGGCAA-690
CIII HM1	-714-GGGAGCCTGGTGT**CTGTCTT**GGCAAAGGC-685
CIII I	-766-TCGAGAGACCAGCTCCTCCCCCAGGGATGTTATCAGTGGGTCCAG-726
CIII IM1	-769-CATGAGACCAGCT**AAGATCT**CAGGGATGTTATCAGTGGGTCCAG-726
CIII IM2	-769-CATGAGACCAGCTCCTCCCCA**CTCTAGA**TTATCAGTGGGTCCAG-726
CIII J	-792-TCGAGTGGGGGCTGGGGA-779
CIII JM1	-798-TCAGTCCTGGTGT**TCTAGTT**GGAGGGCCC-773
HNF1 TRH	-47-TCGAGGCTGAAGTCCAAAGTTCAGTCCCTTCGC-69
SP1	TCGATTCGATCGGGGCGGGGCGAGC

[a] The wild-type sequences were obtained from Ogami et al. (1990). Alterations of sequences in the mutated oligonucleotides are in boldface type and underlined.

the HNF-4, and the other is a heat-stable factor of $M_r = 41$ kDa which has been purified to homogeneity and has been designated CIIIB1 (Ogami et al., 1991). Mutagenesis of this element indicated that factors HNF-4 and CIIIB1 are both positive regulators; however, the former has greater activation potential than the latter. In addition to HNF-4, the HRE of apoCIII is recognized by other members of the orphan receptor family: ARP-1, EAR-2, and EAR-3 (Miyajima et al., 1988; Ladias et al., 1992; Myetus-Snyder et al., 1992). Cotransfection experiments showed that HNF-4 transactivates apoCIII transcription approximately 7−8-fold, whereas ARP-1, EAR-2, and EAR-3 inhibited transcription to less than 10% of control (Ladias et al., 1992). In this paper we have characterized the activities which bind to the distal regulatory elements of apoCIII and have assessed their individual contribution to transcription. We demonstrate that the hepatic and intestinal transcription of apoCIII and the transactivation of the apoCIII promoter depend on complex interactions between nuclear hormone receptors bound to the HRE(s) and SP1 and other minor activities bound on multiple sites to the distal apoCIII promoter.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. Plasmid apoCIII-CAT containing the −890 to +24 nucleotide region of the human apoCIII gene upstream of the chloramphenicol acetyltransferase cDNA has been previously described (Ogami et al., 1990). Mutant promoter constructs were generated by a PCR-based mutagenesis method using the wild-type promoter as template and mutated sense or antisense oligonucleotides (Table 1) as internal primers. PCR reactions were performed by using either the M-13 reverse primer (Pharmacia) as external primer and the mutated antisense or the antisense CIIIB and mutated sense oligonucleotides. Both amplified products were gel-purified, mixed in a 1:1 molar ratio, and subjected to a second amplification using the M-13 reverse and antisense CIIIB primers. These secondary products were sequentially digested with *Xba*I and *Bsu*361 and inserted into the same sites of the apoCIII-CAT plasmid. To obtain the ΔFG mutant, the wild-type plasmid was digested by *Stu*I, and the vector-containing fragment was religated. The ΔHIJ mutant was constructed by partial *Stu*I digestion of apoCIII-CAT, followed by complete digestion with *Xba*I. After the ends were filled in by the Klenow fragment of DNA polymerase I, the vector-containing fragment was religated. pCBHNF-4 was created by subcloning the *Eco*RI/*Hin*dIII fragment of pMT-HNF-4, containing the entire open reading frame of the rat HNF-4 cDNA, into the Asp-718/*Hin*dIII sites of pCB-6 expression vector (Ktistaki et al., 1994; Anderson et al., 1989). All clones were verified by DNA sequencing using T7 polymerase (Sequenase).

Electrophoretic Mobility Shift Assays. Nuclear extracts from rat liver, spleen, and kidney were prepared (Lichsteiner et al., 1989). Similarly, nuclear extracts from HeLa, Cos-1, HepG2, and CaCo-2 cells were prepared by previously described procedures (Dignam et al., 1983). Double-stranded oligonucleotides were labeled by filling in the overhanging ends with T7 polymerase (Sequenase) in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP. Radiolabeled probes (0.1 ng) were used in DNA binding reaction mixtures containing 20 mM HEPES, pH 7.9, 50 mM KCl, 2 mM $MgCl_2$, 4 mM spermidine, 0.02 mM Zn-acetate, 10% glycerol, 0.5 mM dithiothreitol, 2 μg of poly(dIdC), and 5−10 μg of protein-containing nuclear extract. When indicated, 100-fold molar excess cold competitor oligonucleotide was also included. The reaction mixture was incubated on ice for 15 min without probe, followed by a 30-min incubation in the presence of radiolabeled probe. When indicated, 1 μL of polyclonal SP1 antibody (Santa Cruz Biotechnology) (diluted to 0.1 mg/mL protein concentration in PBS containing 1 mg/mL BSA) was preincubated with the nuclear extract at room temperature for 10 min before the binding reaction was started. Protein-bound and free probes were separated by electrophoresis in 4% native polyacrylamide gels and visualized by autoradiography.

Cell Culture and Transfections. Monolayer cultures of HepG2, HeLa, and CaCo-2 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10%

[1] Abbreviations: HNF-1, hepatic nuclear factor 1; HNF-4, hepatic nuclear factor 4; HRE, hormone response element; AdML, adenovirus major late promoter; TAFs, TATA box binding protein associated factors.

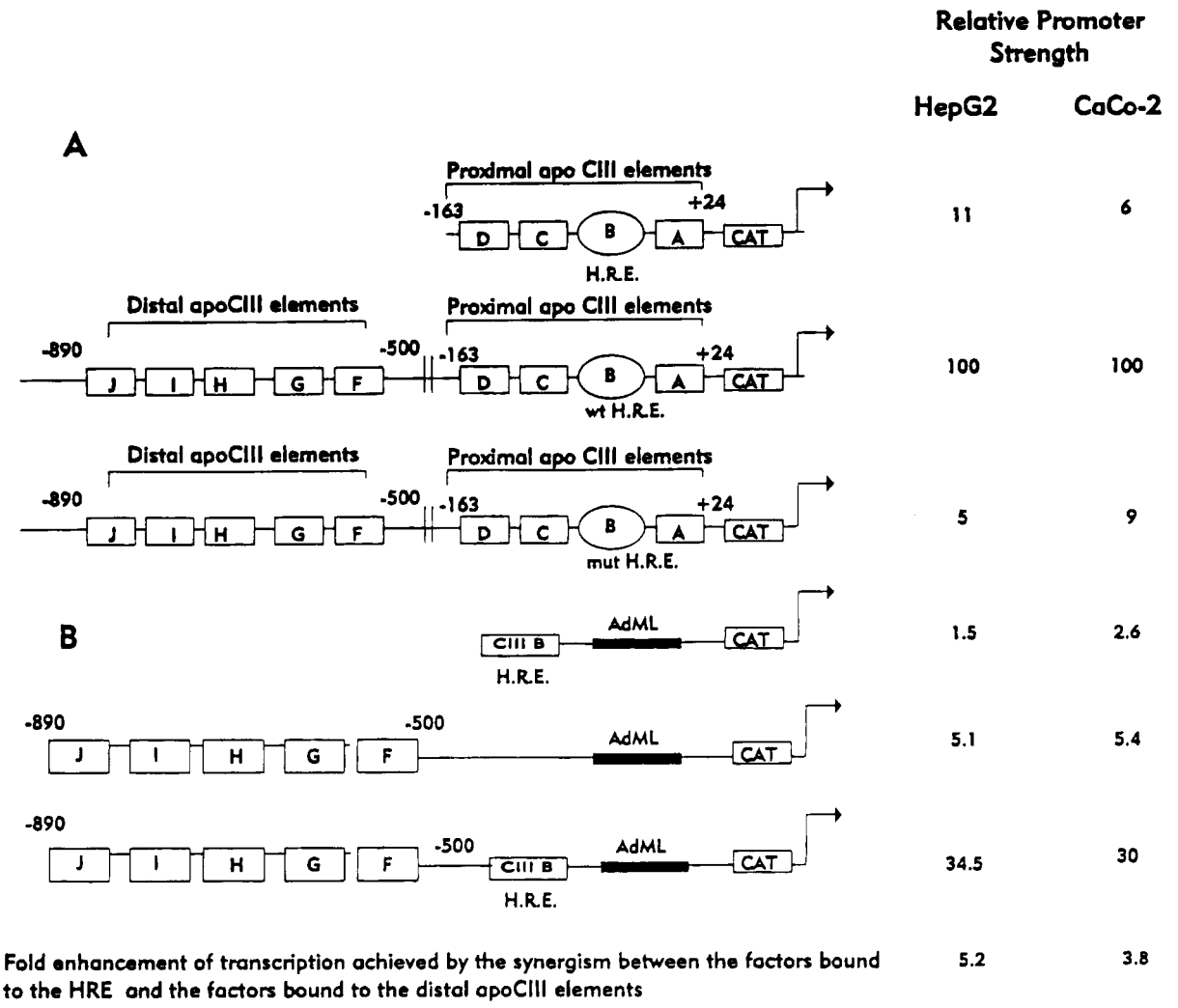


FIGURE 1: Activation of the homologous and heterologous promoters by the distal regulatory elements of apoCIII. Panel A shows the effects of the distal apoCIII regulatory elements on the activation of the core apoCIII promoter containing a wild-type or a mutated HRE. Panel B shows the effect of distal regulatory elements F–J and proximal element B of apoCIII (HRE) on the activation of the heterologous AdML promoter in HepG2 and CaCo-2 cells. Note that the HRE and the distal regulatory elements of the human apoCIII gene act synergistically to increase the transcription of the heterologous promoter 4- and 5-fold, respectively, in CaCo-2 and HepG2 cells.

and 20% heat-inactivated fetal calf serum, respectively. Twenty-four hours before transfection, the cells were seeded at 50–60% confluency into 35 mm diameter dishes. Two micrograms of reporter constructs, 2 μg of either pCB-HNF-4 or empty pCB-6 vector, and 3 μg of pRSV-βGal plasmid (Edlund et al., 1985) were introduced into the cells by the calcium phosphate coprecipitation method (Graham & Van der Eb, 1973). Forty-eight hours later the cells were harvested and subjected to three consecutive freeze–thaw cycles. Chloramphenicol acetyltransferase activities were assayed by using constant amounts of protein as described previously (Gorman et al., 1982). The β-galactosidase activity in the cellular extracts were measured as described (Edlund et al., 1985), and the values were used to normalize the variations in transfection efficiencies. Incubation times were carefully selected by titration and kinetic experiments to ensure that the enzyme reactions were performed in the linear range. The values reported represent the average of four to six determinations obtained in two or three independent experiments.

RESULTS

The Distal ApoCIII Regulatory Elements Enhance the Strength of the Homologous and Heterologous Promoters which Contain a Proximal HRE. Previous analysis of the apoCIII promoter showed that deletion of the distal regulatory elements decreased the strength of the core promoter, which consists of elements A–D, to approximately 11% of control in HepG2 cells and 6% in CaCo-2 cells, indicating that these elements may act as homologous enhancers in apoCIII transcription (Ogami et al., 1990). This enhancement is abolished when the proximal HRE is mutated. These changes in apoCIII promoter strength are illustrated in Figure 1A. Further analysis showed that activation of transcription can be achieved in the minimal adenovirus major late promoter (AdML) by combination of the proximal HRE and the distal regulatory elements F–J (Ktistaki et al., 1994). Thus, as shown in Figure 1B, cloning of the HRE upstream of the minimal AdML promoter does not produce substantial activation of this promoter in HepG2 or CaCo-2 cells. The upstream apoCIII region provides some activation; however, the combination of the two provided approximately 5-fold greater activation in hepatic cells than the sum of the activation achieved by either the HRE or the distal apoCIII regulatory element alone. This synergistic activation applies for both the homologous and heterologous promoters in either HepG2 or CaCo-2 cells (Figure 1). These findings indicate that, in both the homologous and heterologous promoters,

enhancement of transcription is mediated by synergistic interactions between the factors which bind to the HRE and the factors which bind to the distal apoCIII regulatory elements.

To understand the mechanism of this transcriptional synergism, we must have a clear understanding of the factors which bind to these elements and how binding (or lack thereof) of these factors affects transcription. As indicated, recent studies have established that the proximal apoCIII regulatory element, which is an HRE, binds the orphan nuclear receptors HNF-4, ARP-1, EAR-2, and EAR-3 as well as homo- and heterodimers of RXR, which has as a ligand 9-*cis*-retinoic acid (E. Tzameli and V. I. Zannis, unpublished). The factors which bind to the distal regulatory elements were identified in the present study by DNA binding and supershift assays. Their importance was assessed by analysis of the promoter strength (using CAT assays) following mutagenesis which altered the DNA binding site of each specific factor as indicated below.

Activities Related to the Ubiquitous Factor SP1 Bind to Three Neighboring Elements, F, H, and I, on the Distal ApoCIII Promoter, and a New Activity Binds to Regulatory Element J. DNA binding assays showed that the regulatory element H forms three DNA−protein complexes with rat liver nuclear extracts. The nature of the factors which bind to these elements was initially assessed by competition experiments. This analysis showed that all three complexes formed with element CIII H were competed completely by oligonucleotides corresponding to elements CIII H and CIII I (Table 1) as well as by an SP1 binding oligonucleotide. An oligonucleotide corresponding to element CIII F competed out fully the formation of complex 3 and partially that of complexes 1 and 2, whereas an oligonucleotide corresponding to element CIII J did not compete out any of the complexes (Figure 2A). This finding suggested that the factors which bind to regulatory elements H, I, and F of the apoCIII promoter may be related to SP1. Competition experiments were also performed using mutated oligonucleotides HM1 and IM1 which do not bind SP1. This analysis indicated that oligonucleotides containing the mutations HM1 and IM1 (Table 1) did not compete any of the three complexes, indicating that these mutations affected the binding of all three complexes to their cognate sites on elements H and I. Mutations HM1 and IM1 altered the G(A)GGAGGGG sequence present in both oligonucleotides which conforms with the consensus binding site of SP1 (Kadonaga et al., 1987). Additional evidence in favor of the binding of SP1 to element H and other distal apoCIII regulatory elements was obtained by DNA binding and competition assays using the oligonucleotide SP1 as probe which contains the binding site of the transcription factor SP1 (Courey & Tjian, 1992). This analysis produced the same DNA−protein complexes and competition patterns as those observed when the oligonucleotide corresponding to regulatory element CIII H was used as a probe (Figure 2B). The binding of SP1-related activities to regulatory element I was confirmed directly by DNA binding and competition experiments similar to those performed with regulatory element H. This analysis showed that element I forms seven DNA−protein complexes. Complexes 1, 2, and 4 are competed out fully by oligonucleotides CIII H and SP1 and partially by oligonucleotide CIII F (Table 1), indicating that they may result from the binding of SP1 or related activities. Complex 3 is competed out completely by oligonucleotides CIII F and CIII J, indicating that this activity also recognizes these two regulatory elements (Figure 2C). Complex 5 is not competed by several competitor oligonucleotides used and may represent a new activity, which was designated CIII I5. Two other minor complexes, 6 and 7, are formed. Complexes 6 and 7 have competition patterns similar to those of CIII I3 and CIII I4, respectively, and may originate from their degradation products.

DNA binding and competition assays also established binding of SP1 to regulatory element F. This element forms three DNA−protein complexes. The two major complexes, 1 and 2, are competed out by oligonucleotides CIII H, CIII I, and SP1, indicating that the factor responsible for these complexes is related to SP1. The third minor complex is competed out only by oligonucleotides CIII I and CIII J (Figure 2D). Since oligonucleotides CIII F and CIII J were shown in Figure 2C to compete the CIII I3 activity, this finding indicates that the minor CIII F3 and CIII I3 activities, as well as the CIII J1 activity which binds to the regulatory element J, may be related. Finally, other evidence in support of this interpretation is provided by competition of the activities which bind to regulatory element J by oligonucleotides CIII I and CIII F, which are discussed further in Figure 4. Henceforth all of these activities are referred to as CIII J1. The competition experiments with mutant oligonucleotides FM1, FM2, and FM3 (Table 1) indicate that the FM1 mutation affected the binding of minor activity 3, whereas the FM2 and FM3 mutations affected the binding of major activities 1 and 2, which are related to SP1, but not that of activity 3, which is different from SP1. The data to this point suggest that SP1 binds on three distinct sites located on regulatory elements F, H, and I, respectively.

The recognition of regulatory elements H, I, and F by activities related to SP1 was demonstrated by direct binding to them of purified SP1. Utilization of probes with the same activity suggests that elements H and I represent strong SP1 binding sites, whereas element F represents a weaker SP1 binding site (Figure 2E).

DNA binding experiments with nuclear extracts prepared from different rat tissues as well as HeLa and CaCo-2 cells showed that the activities which are related to SP1 are ubiquitous, while the activity which binds to regulatory element J is absent from spleen (Figure 2F). This finding is also consistent with the ubiquitous nature of SP1. Finally, the binding of SP1 or related activities to regulatory elements CIII F, CIII H, CIII I, and SP1 was demonstrated by DNA binding gel electrophoretic assays in the presence of SP1 antibodies (Santa Cruz Biotechnologies). This analysis resulted in the supershift of the slowest migrating activity which binds to these regulatory elements (Figure 3). The finding can be explained by the fact that the antibodies used were raised against a peptide corresponding to the 520−538 amino acid sequence of SP1 and that the fastest migrating complexes which bind the SP1, CIII F, CIII H, and CIII I oligonucleotides represent modified forms or degradation products of SP1. Alternatively the faster migrating complexes observed in Figure 3 may represent proteins related to SP1 (Imataka et al., 1992; Kingsley & Winoto, 1992; Hagen et al., 1992). The combined data of Figures 2 and 3 indicated that activities related to SP1 bind to three neighboring elements, F, H, and I, on the distal apoCIII promoter.

As explained above, the DNA binding and competition

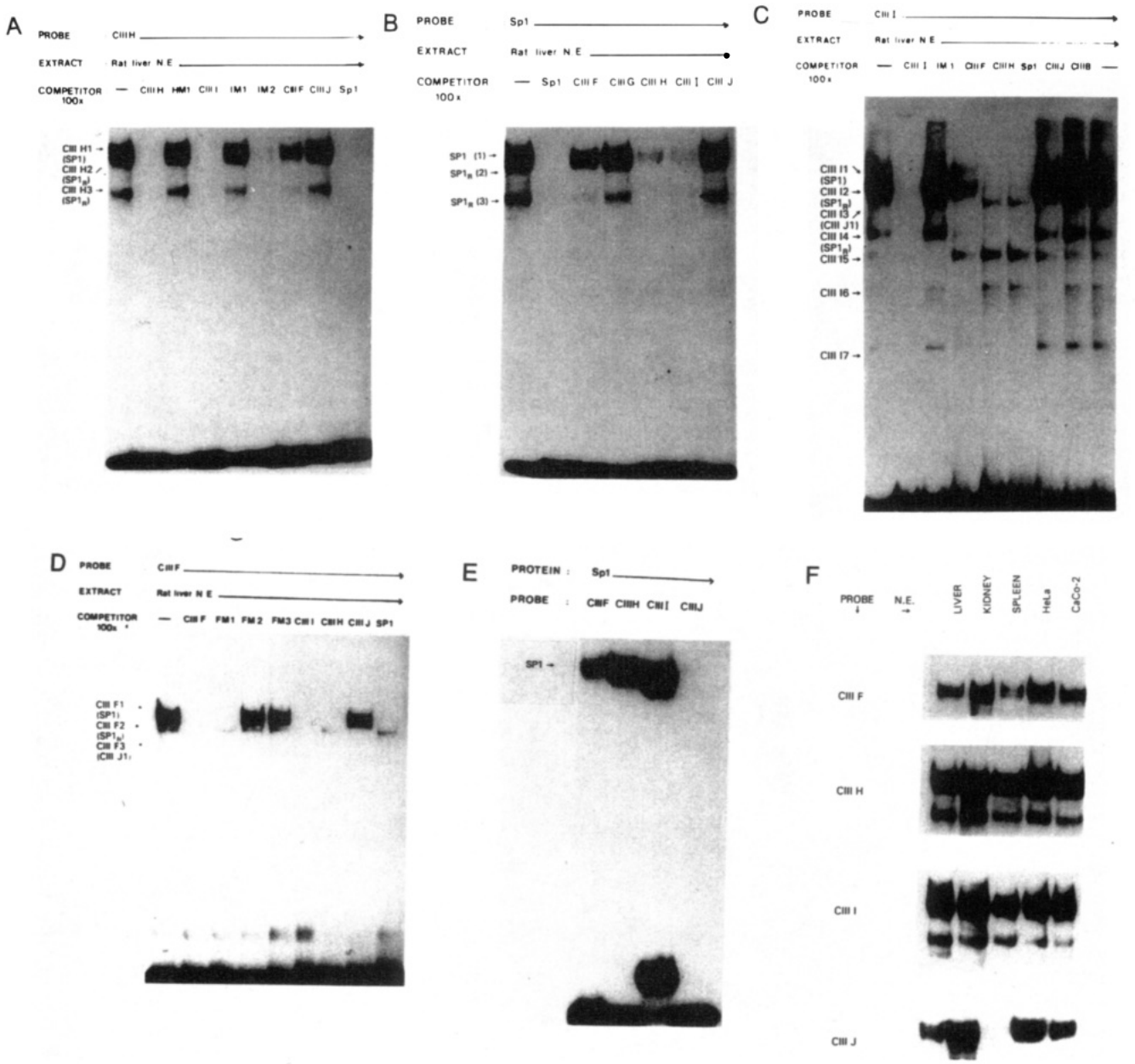


FIGURE 2: Panel A: DNA binding gel electrophoresis and competition assays with apoCIII element H (−705 to −690) as probe. The double-stranded oligonucleotide corresponding to footprinting region H of the apoCIII promoter (Ogami et al., 1990) was labeled with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP and used as binding probe. In this panel and in subsequent figures DNA binding and competition assays were performed with rat liver nuclear extracts, cell extracts, or purified fractions as described in Experimental Procedures. Competitor oligonucleotides were added in all lanes except the first at 100-fold molar excess relative to the ^{32}P-labeled oligonucleotides. The oligonucleotides used are indicated by abbreviations at the top of the gel and are described in Table 1. Note that the DNA-protein complexes formed with element H are competed out by an SP1-binding oligonucleotide as well as by oligonucleotides corresponding to regulatory element CIII I and partially by element CIII F. Panel B: Binding assays using an oligonucleotide containing the SP1 binding site as probe and rat liver nuclear extracts. Note that the DNA-protein complexes formed with the CIII H or SP1 probe are similar and are competed by oligonucleotides corresponding to the apoCIII regulatory elements CIII I and CIII F. Panel C: Binding and competition assays using the apoCIII promoter element I (−766 to −726) as probe. The competitor oligonucleotides used are indicated by abbreviations at the top of the gel and are described in Table 1. Note that certain DNA-protein complexes are competed by the SP1, CIII I, and CIII F oligonucleotides (Table 1). Panel D: Binding and competition assays using the apoCIII promoter region F (−611 to −592) as probe. The competitor oligonucleotides used are indicated by abbreviations at the top of the gel and are described in Table 1. Note that certain DNA-protein complexes formed with CIII F are competed out by the SP1, CIII I, and CIII H oligonucleotides. The slowly migrating complex may be equivalent to complex 1 or 2 of Figure 1A or may represent a mixture of the two. Panel E: Binding assays using purified SP1 and regulatory elements F, H, I, and J as probes. Note that purified SP1 binds to regulatory elements F, H, and I but does not bind to regulatory element J. Panel F: Binding assays using regulatory elements F, H, and J as probes and nuclear extracts prepared from rat liver, kidney, and spleen, HeLa cells, and CaCo-2 cells. Note that the activities which are related to SP1 are ubiquitous, while the activity which binds to element J is absent from spleen.

experiments of Figure 2E showed that element J does not bind SP1. Further analysis established that this element binds a single activity which is present in liver, kidney, HeLa, and CaCo-2 cells but absent from spleen (Figure 2F and Figure 4). Consistent with the experiments of Figure 2C,D, the activity bound to regulatory element J is competed by oligonucleotides CIII F and CIII I but is not competed by oligonucleotides CIII H and SP1 which bind activities related to SP1, (Figure 4). This novel activity has been designated CIII J1. The competition with oligonucleotides CIII J and

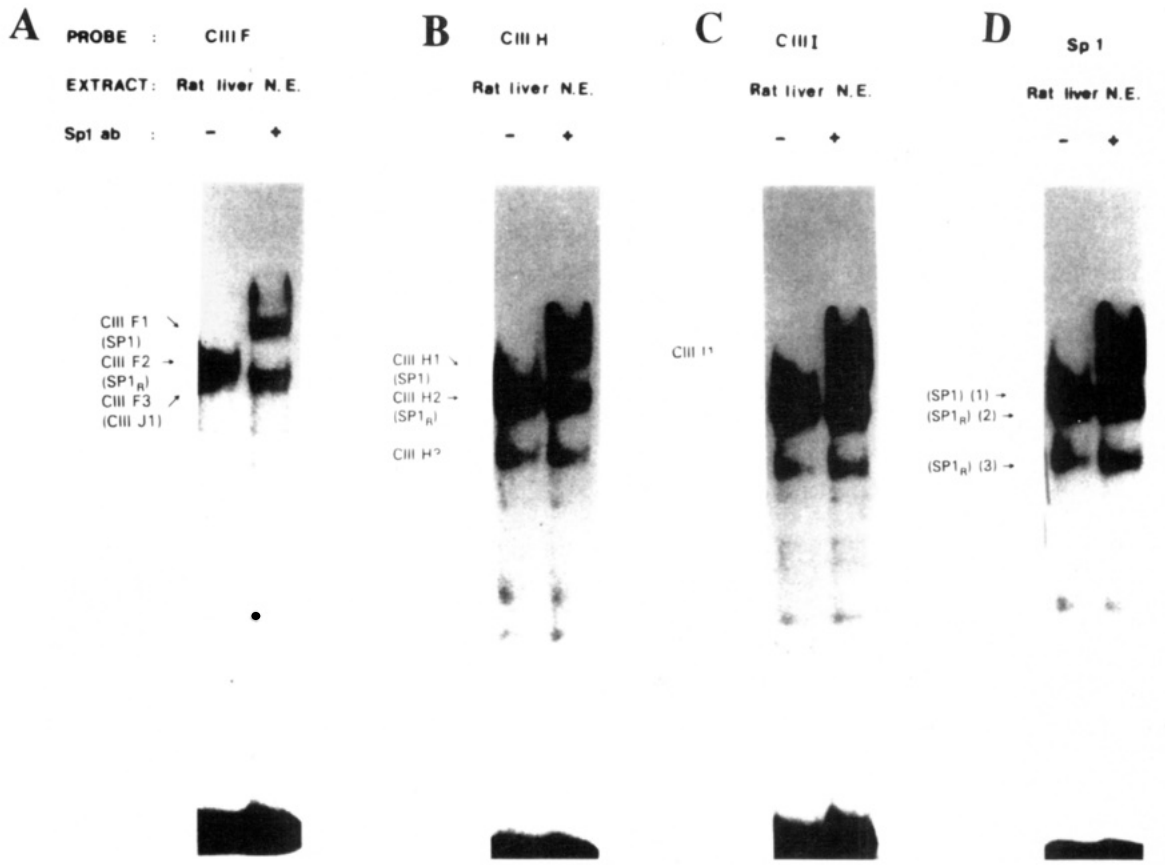


FIGURE 3: DNA binding gel electrophoresis assays using the CIII F, CIII H, CIII I, and SP1 oligonucleotides of Table 1 as probes in the presence or absence of antiserum raised against the synthesis peptide corresponding to amino acids 520−538 of SP1. Note the supershift of the slowest migrating DNA-protein complexes by these antisera.

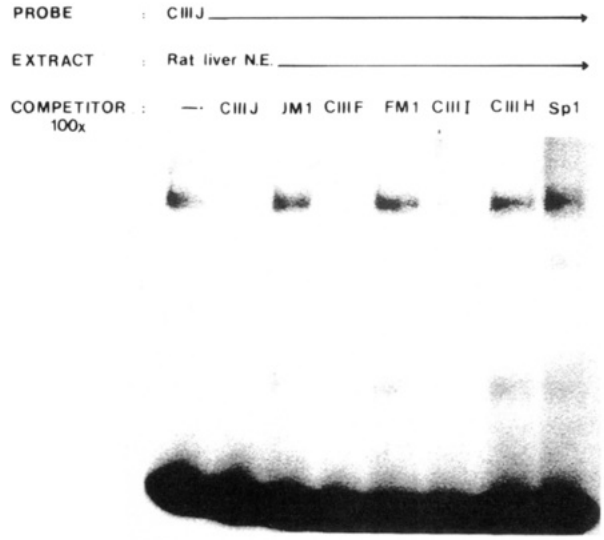


FIGURE 4: DNA binding gel electrophoresis and competition assays with apoCIII promoter element J (−792 to −779) as probe. The competitor oligonucleotides used are indicated by abbreviations at the top of each gel and are described in Table 1. Note that this probe forms a single DNA-protein complex which is not competed by any of the competitor oligonucleotides used.

CIII F is stronger than that with oligonucleotide CIII I. This indicates that factor CIII J1 may have a lower affinity for element I as compared to that of element J or F.

Regulatory Element G of ApoCIII Represents a Novel HRE which Binds the Orphan Receptors ARP-1 and EAR-3 but not HNF-4. DNA binding and competition experiments showed that regulatory element G forms a single DNA−protein complex with rat liver nuclear extracts designated CIII G1. This complex is competed by oligonucleotide sequences (CIII B, CIII BM1, and TRH of Table 1) which contain binding sites for nuclear hormone receptors (Figure 5A). Thus oligonucleotides CIIIB and TRH correspond to the HREs found in the proximal promoters of apoCIII and HNF-1, respectively, and contain a direct nonperfect repeat (Ogami et al., 1990; Kritis et al., 1993). This repeat conforms with the consensus binding site (TAGCCCT)X2 of orphan hormone receptors (Ladias et al., 1992; Martinez et al., 1991). Competition analysis of the CIII G1 complex with the mutant oligonucleotides indicated that mutations GM4 and GM1 and to a lesser extent mutation GM3 (Table 1) affected the binding of this activity to its cognate site on element G. Direct binding assays using extracts of Cos-1 cells expressing either ARP-1, EAR-3, or HNF-4 showed that ARP-1 and EAR-3 bind to this element, whereas HNF-4 does not bind (Figure 5B). The fact that orphan receptors such as ARP-1 may recognize the regulatory element G in hepatic cells has been further supported by the isolation of a full-length ARP-1 cDNA clone from a λgt11 cDNA expression library using a concatenated ^{32}P-labeled element G as a probe (J.-M. Lacorte, V. I. Zannis, and I. Talianidis, unpublished). The findings indicate that element G represents a specialized HRE which recognizes the orphan receptors ARP-1 and EAR-3 or related factors but does not recognize the liver-enriched factor HNF-4 (Ladias et al., 1991; Miyajima et al., 1988; Sladek et al., 1990).

Organization of the Different Activities on the Distal Regulatory Elements of the Human ApoCIII Promoter. The organization of the different activities which bind to the distal elements which participate in the regulation of the hepatic transcription of the human apoCIII gene is shown in Figure 6A. This arrangement of transcription factors is based on

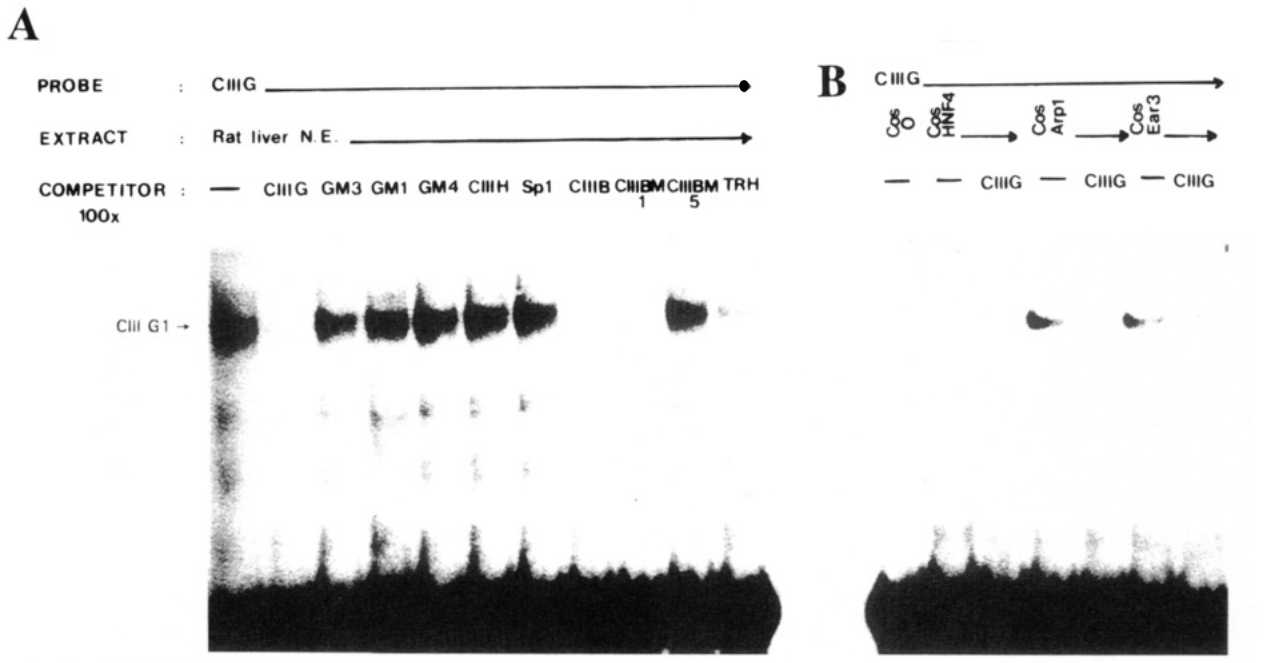


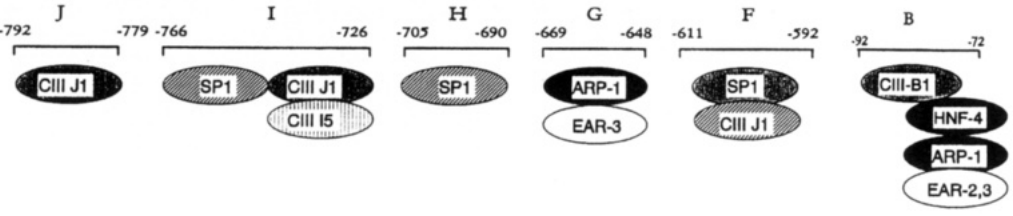
FIGURE 5: Panel A: DNA binding gel electrophoresis and competition assays using the apoCIII promoter element G (−669 to −648) as probe and crude nuclear extracts. The competitor oligonucleotides used are indicated by abbreviations at the top of the figure and are described in Table 1. Panel B: DNA binding gel electrophoresis assay using the apoCIII promoter region (−664 to −648) as probe and factors HNF-4, ARP-1, and EAR-3 produced by expression of the corresponding cDNAs in COS-1 cells. Note that regulatory element G binds ARP-1 and EAR-3 but does not bind HNF-4.

the findings of Figures 2−5. A unique feature of the distal apoCIII promoter is that SP1 or related factors constitute the major activity which occupies the distal regulatory elements and binds to three neighboring sites on elements F, H, and J, respectively. Element J binds a single activity, designated CIII J1. This or a related activity also binds as a minor component to regulatory elements I and F. Another minor activity, designated CIII I5, binds to regulatory element I. Finally, the orphan receptors ARP-1 and EAR-3 or related activities bind to regulatory element G. The factors occupying some of these elements in intestinal as well as in other nonhepatic cells may be different.

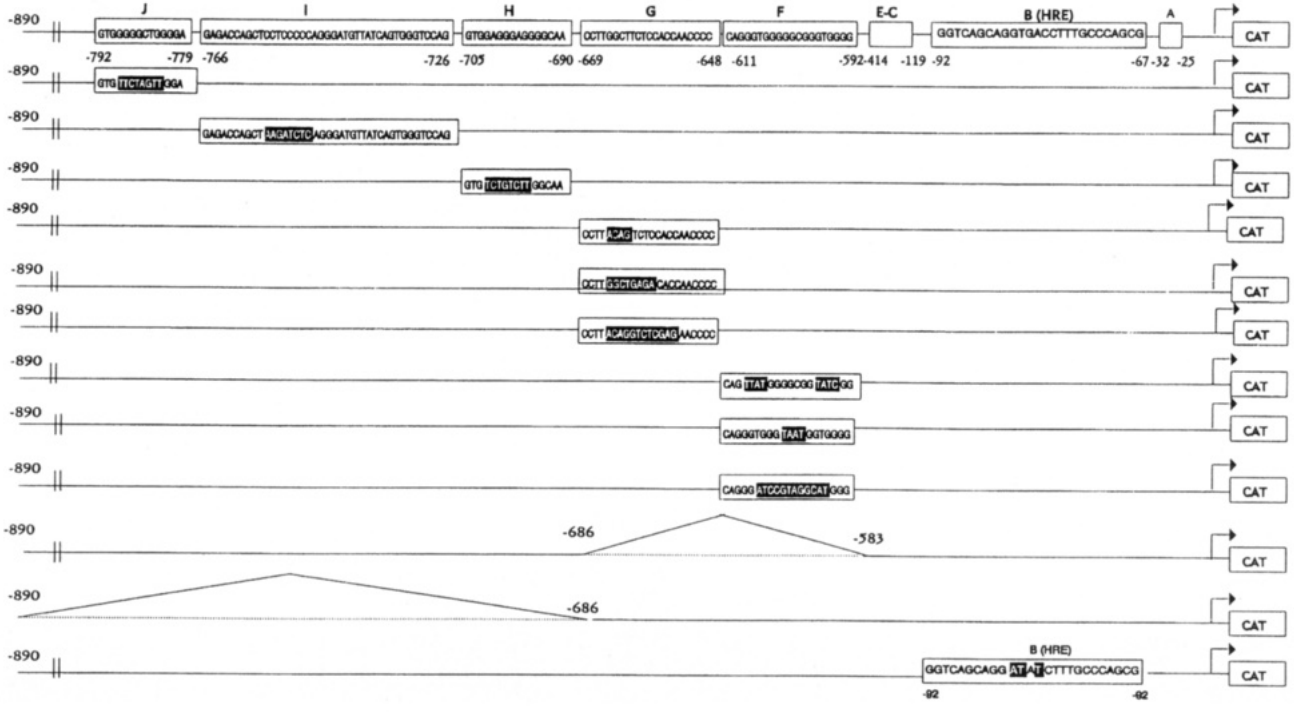
Contribution of the ApoCIII Regulatory Factors to the Strength of the ApoCIII Promoter. To assess the contribution of the factors which occupy distal apoCIII regulatory elements to intestinal and hepatic transcription, we either introduced mutations which eliminate the binding of these factors to their cognate site or deleted certain elements of the distal promoter and have generated the mutant apoCIII promoter−CAT constructs shown in Figure 6B. Transfection of these constructs in HepG2 and CaCo-2 cells provided information on the effect of specific mutations on the promoter strength. This analysis yielded the following interesting data: (a) The hepatic transcription of apoCIII is affected mostly by mutations in elements B, H, and G. As shown in Figure 6A, elements B and G bind nuclear receptors, and element H binds activities related to SP1. (b) Hepatic transcription and intestinal transcription are affected differently by mutations in element G. Thus point mutations in element G (enclosed in a box) or deletion of elements G and F reduced hepatic transcription to 26% of the control. In contrast, intestinal transcription was either unaffected by point mutation to element G or increased 1.6-fold by deletion of elements G and F. The findings indicate that different combinations of factors which occupy the distal regulatory elements of the apoCIII promoter are required for optimal transcription in hepatic and intestinal cells. Similar to the hepatic transcription, mutations in elements B and H affected severely the intestinal transcription. (c) Both hepatic and intestinal transcription were also reduced significantly by point mutations in regulatory elements F, I, and J or by deletion of the 5′ elements H, I, and J. Elements I, H, and F bind mainly activities related to SP1. Elements B and G bind nuclear hormone receptors, and element J binds a new activity designated CIII J1. The findings indicate that optimal activation of the apoCIII promoter in hepatic and intestinal cells requires positive regulatory factors which bind to the HRE as well as all positive regulatory factors which bind to the distal regulatory elements F−J. As indicated in Figure 6A, the proximal HRE of the apoCIII promoter can bind several different combinations of nuclear hormone receptors. Some of them, like HNF-4, act as positive regulators; others, like ARP-1, EAR-2, and EAR-3, act as repressors (Ladias et al., 1992).

Contribution of the Factors Bound to Distal ApoCIII Regulatory Elements to HNF-4-Mediated Transactivation of the ApoCIII Promoter. We have used cotransfection experiments of cells with the reporter apoCIII promoter plasmids of Figure 6B and an HNF-4-expressing plasmid to assess the role of this specific factor in the activation of the apoCIII promoter in different cell types. This analysis showed that HNF-4 transactivated the apoCIII promoter in HepG2, CaCo-2, and HeLa cells 8-, 6-, and 17-fold, respectively (Figure 6B, right panel). Deletion of either the 5′ elements J, I, and H or the 3′ elements G and F of the distal promoter resulted in much reduced transactivation of 0.8- and 1.1-fold, respectively, in HepG2 cells (Figure 6B, right panel). The 5′ region contains two SP1 binding sites on elements I and H, and the 3′ region contains one weak SP1 binding site on element F and an orphan receptor binding site on element G. Reduction in transactivation by deletion of 5′ or 3′ elements is also observed in CaCo-2 and HeLa cells, although the deletion of elements F and G had less severe effects on the HNF-4-mediated transactivation of CaCo-2 cells as

A



B



	RELATIVE CAT ACTIVITY %				
	Promoter Strength		Transactivation by HNF-4		
	HepG2	CaCo-2	HepG2 +HNF-4	CaCo-2 +HNF-4	HeLa +HNF-4
WT	100	100	840±140	604±89	1680±170
JM1	67±8	43±9	750±140	246±48	1090±140
IM1	42±17	41±6	590±70	130±19	2210±270
HM1	14±5	25±8	130±20	91±12	370±50
GM1	26 ±5	87±6	140±30	111±16	210±70
GM3	86±7	89±7	490±30	168±49	970±190
GM4	27±6	105±12	250±30	173±38	230±60
FM1	57±6	54±5	710±4 0	221±103	1640±230
FM2	78±9	75±10	450±40	253±45	720±120
FM3	73±6	59±8	460±50	355±98	–
ΔFG	25±3	160±25	80±20	210±20	269±40
ΔHI J	9±4	9±3	110±50	24±5	180±8
BM2	5±3	9±4	21±10	24±12	–

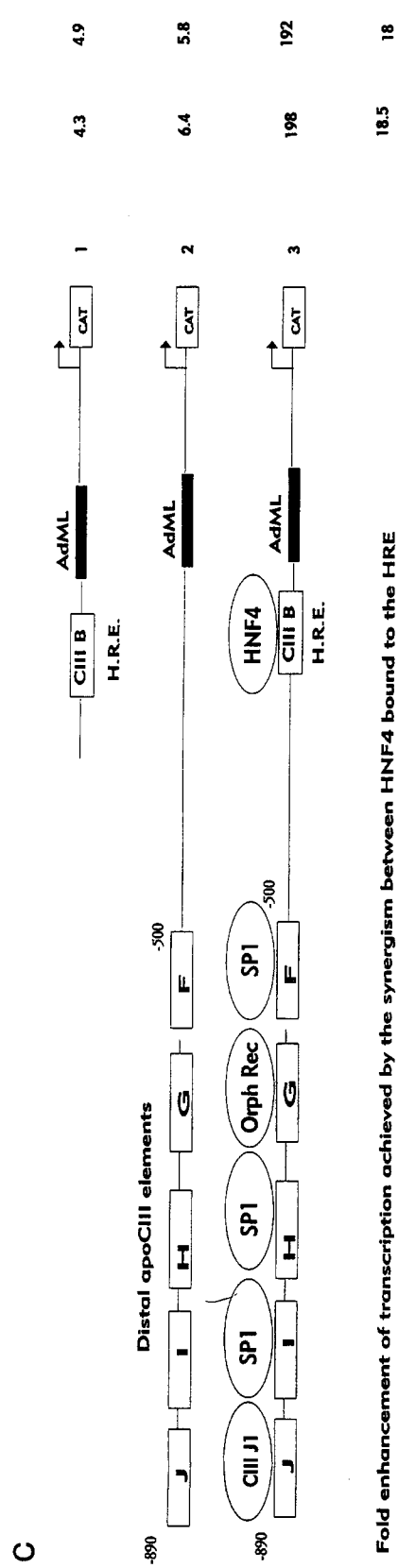


FIGURE 6: Panel A shows the organization of the nuclear factors of the apoCIII promoter on the distal regulatory elements of the apoCIII promoter and the proximal HRE. The boundaries of promoter elements F–J are indicated at the top of the panel. The factors are designated by ellipsoids carrying their names. Panel B shows the effect of mutations described in the left side of the panel which affected the binding of the corresponding factors to their cognate sites on the promoter strength and the HNF-4-mediated transactivation of the apoCIII promoter in different cell types. Note that elements B and H are essential for the transactivation in hepatic, intestinal, and HeLa cells. Some mutations in other elements affect differently the hepatic and intestinal transactivation. Panel C shows the effect of distal regulatory elements F–J and proximal element B of apoCIII (HRE) on the activation of the heterologous AdML promoter in HepG2 and CaCo-2 cells in the presence of HNF-4. Note that the HRE and the distal regulatory elements of the human apoCIII gene act synergistically to increase the transcription of the heterologous promoter. The enhancement of transcription is 18-fold in the presence of HNF-4 in HepG2 and CaCo-2 cells. HNF-4 alone increases the strength of the heterologous promoter, which contains an HRE, only 3-fold in hepatic and intestinal cells, respectively.

compared to either HepG2 or HeLa cells. Reduction in HNF-4-mediated transactivation is also observed by several point mutations which eliminate the binding of the corresponding factor to its cognate site as shown in Figure 6A. The overall mutagenesis analysis of the distal promoter suggests that neither the 3′ half of the distal promoter, which contains regulatory elements F and G, nor the 5′ half of the enhancer, which contains elements H, I, and J, is sufficient by itself to provide optimal HNF-4-mediated transactivation. Rather, the optimal HNF-4-mediated transactivation requires complex synergistic interactions between HNF-4 and several factors which bind to distal regulatory elements F–J.

These synergistic interactions, which may take place in the presence of HNF-4 in the cell nucleus, can be better visualized using heterologous constructs consisting of apoCIII regulatory elements linked to the minimal AdML promoter. This analysis indicated that the minimal AdML promoter linked either with apoCIII element B, which contains an HRE, or with distal apoCIII elements F–J is transactivated moderately by HNF-4 (Ktistaki et al., 1994). Comparison of Figures 1B and 6C shows that the promoter activity of the HRE-containing AdML construct increased approximately 3-fold, whereas the activity of the AdML constructs containing distal regulatory elements F–J increased only 1.25-fold in the presence of HNF-4. However, linkage of both elements to the 5′ region of the minimal AdML promoter provided 18-fold greater transactivation in hepatic and intestinal cells than the sum of the transactivations achieved by the HRE and distal apoCIII regulatory elements alone (Figure 6C). The findings indicate that the small enhancement in transcription achieved by the binding of HNF-4 to its cognate HRE site does not completely account for its transactivation effects (from 1.5 to 4.3) in the presence of HNF-4 (Figures 1B and 6C). Rather the observed transactivation by HNF-4 can be accounted for by synergistic interactions between HNF-4 bound to the HRE and SP1 and other activities bound to the distal apoCIII regulatory elements.

HNF-4-Mediated Transactivation of the ApoCIII Gene Is Different in Different Cell Types. The proximal element B of the apoCIII promoter (HRE) can be recognized by a variety of nuclear receptors present in different cell types. Cotransfection with HNF-4 generates an excess of this factor in the cell nucleus and may lead to the occupation of the proximal HRE mostly by HNF-4. Under these conditions the analysis of the effects of mutations of the distal regulatory elements on the HNF-4-mediated transactivation may provide information on the potential cross communication of the factors bound to the distal promoter with the HNF-4 bound to the HRE in different cell types. As shown in the right panel of Figure 6B, mutations in element B, G, and H affected significantly the HNF-4-mediated transactivation in HepG2, CaCo-2, and HeLa cells. However, mutations in element I reduced severely the transactivation in CaCo-2 cells, and mutations in element J reduced transactivation in CaCo-2 and HeLa cells without affecting transactivation in HepG2 cells. Interestingly, a mutation in element I increased transactivation 1.3-fold in HeLa cells as compared to the wild-type construct. The mutations which affected significantly the HNF-4-mediated transactivation in different cell types are enclosed in boxes in Figure 6B. The results of the point mutations suggest that interaction of a single SP1 molecule bound to element H, I, or J and HNF-4 bound to element B is not sufficient for optimal transacti-

vation of the apoCIII promoter in different cell types. Optimal transactivation requires complex interactions among SP1 and other activities which bind the upstream regulatory elements. It is speculated that, similar to other systems, the proteins bound to the promoter enhancer region form a stereospecific DNA−protein complex which provides the activating functions to the basal transcription complex (Tjian & Maniatis, 1994). The observation that the same mutations (i.e., mutations in element G or J) may affect differently the promoter strength and the HNF-4-mediated transactivation suggests that the interactions among the factors forming the promoter/enhancer complex are different in different cell types.

The putative interactions of factors bound to regulatory elements F−G which may lead to transcriptional activation of the apoCIII gene in hepatic and intestinal cells are shown schematically in Figure 7A,B. The arrows illustrate that the indicated factors, either directly via protein−protein interactions or indirectly via the involvement of TAFs (TATA box binding protein associated factors), increase the transactivation potential of HNF-4 and thus increase apoCIII gene transcription. Strong and weak interactions are indicated by one, two, or three plus signs. Figure 7C shows putative mechanisms of transcriptional repression. Repression is indicated by the minus signs. Such repression may, for instance, occur by binding of ARP-1, EAR-2, or EAR-3 to proximal regulatory element B (HRE). Similar repression may occur by binding of negative regulators to any of the upstream regulatory sites.

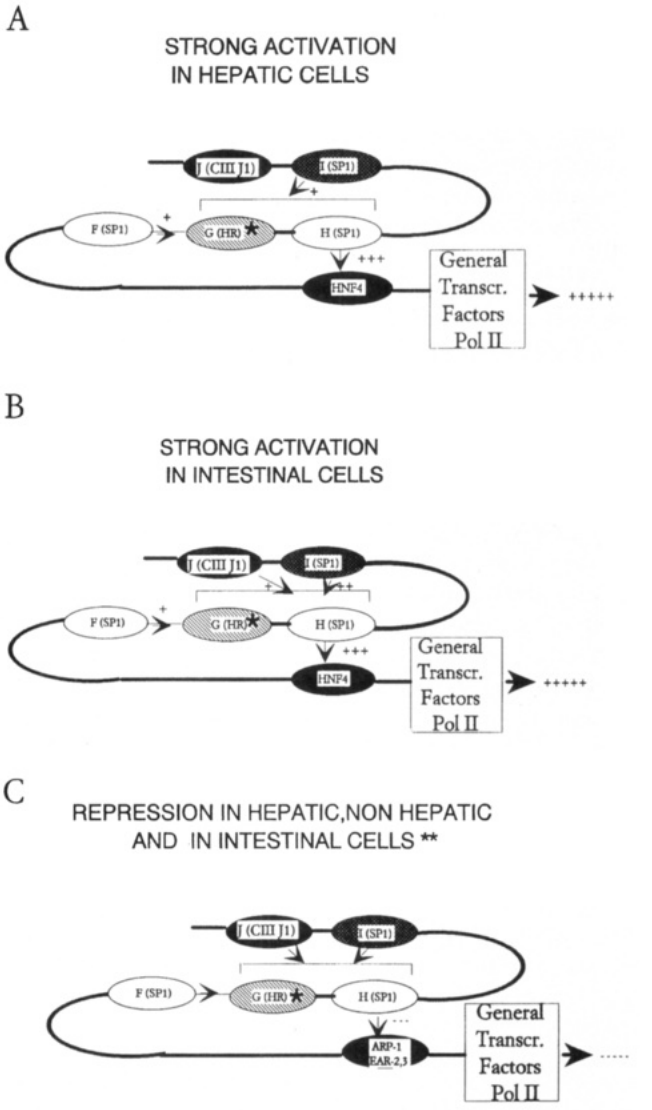


FIGURE 7: Schematic representation of mechanisms which lead to either transcriptional activation of the apoCIII promoter in HepG2 and CaCo-2 cells (panels A and B, respectively) or its repression in HeLa cells (panel C). One, two, or three + symbols indicate moderate, strong, and very strong putative interactions among the indicated factors. Arrows indicate direct or indirect contributions of the indicated factors to the HNF-4-mediated transactivation. Brackets indicate involvement of one or more factors in these interactions. The diagrams are based on the data of Figure 5. The presence of HNF-4 in the liver and intestine has been reported (Sladek et al., 1990). The presence of CIII J1 or related activities in intestine, liver, and HeLa cells is based on Figure 1F. An asterisk (*) indicates that different types of nuclear hormone receptors may occupy this site. Two asterisks (**) indicate that repression may also occur by the binding of negative regulators to any of the regulatory elements H, F, G, I, and J.

DISCUSSION

Factors Recognizing the ApoCIII Promoter Region and Their Significance. Early *in vitro* experiments combined with recent *in vivo* studies with transgenic mice suggest that apoCIII plays an important role in the catabolism of triglyceride-rich lipoproteins (Krauss et al., 1973; Brown & Bajinsky, 1972; Windler et al., 1980; Shelburne et al., 1980; Quarfordt et al., 1982; Ito et al., 1990; de Silva et al., 1994).

The present study established that SP1 or related activities are the predominant factors which bind to elements F, H, and I. This assignment is based on several lines of evidence, including direct binding of SP1 to these sites, competition of binding with SP1 binding oligonucleotides, supershift of the DNA−protein complex with antibodies, and the ubiquitous tissue distribution of this binding activity. It also established that the orphan receptors ARP-1 and EAR-3 bind to element G and that a new and so far uncharacterized activity, designated CIII J1, binds to element J. These data combined with previous findings provide a clear understanding of the activities which occupy the proximal and distal regulatory elements of the human apoCIII promoter.

The contribution of individual factors which bind to the distal apoCIII promoter to hepatic and intestinal transcription was assessed by promoter mutations which eliminate the binding of the factors to their cognate sites and affect the promoter strength. This analysis showed that the promoter strength was affected severely in both HepG2 and CaCo-2 cells by mutations in elements B, which binds nuclear hormone receptors, and H, which binds SP1. However, mutations in the two other SP1 binding sites, on elements F and I, respectively, as well as mutations in the binding site of factor CIII J1 on element J also affected the promoter strength to a lesser extent in both HepG2 and CaCo-2 cells.

Cotransfection experiments using homologous or heterologous promoter constructs suggest complex interactions between SP1 and other factors bound to the distal sites, nuclear receptors such as HNF-4 bound to the proximal HRE. This complex cross-communication of factors results in synergistic transactivation of the apoCIII promoter.

Several of the conclusions of this study are based on cotransfection experiments in cells of hepatic (HepG2) and intestinal (CaCo-2) origin. It is possible, however, that these tumor-derived cells may not have the full complement of factors found in hepatocytes and enterocytes and thus may not mimic precisely the *in vivo* properties of the hepatic and intestinal cells.

Potential Contribution of SP1 in ApoCIII Gene Transcription. The present study points out the importance of the distal SP1 and orphan receptor binding sites of the distal promoter for the transcriptional activation of the human apoCIII gene. Previous studies have shown that SP1 is a ubiquitous transcription factor which activates several cellular and viral promoters that contain the consensus binding site sequence 5′-G(T)G(A)GGC(A)GG(T)G(A)G(A)C(T)-3′ (Briggs et al., 1986; Kadonaga et al., 1987; Courey & Tjian, 1992). Subsequently three other SP1-related genes, SP2, SP3, and SP4, have been reported with similar DNA binding specificities and activation properties (Imataka et al., 1992; Kingsley & Winoto, 1992; Hagen et al., 1992). Different activation domains of the SP1 molecule are responsible for the distinct functions and the formation of protein−protein complexes between SP1 bound to proximal and distal sites (Courey et al., 1989). Similar protein−protein interactions between SP1 and other factors may promote synergistic activation of a given promoter. Evidence for the latter type of direct interaction between SP1 and the BPV enhancer E2 protein has been reported (Li et al., 1991).

The nature of the synergistic interactions between SP1 and HNF-4 or other nuclear hormone receptors is not known. Similar to other systems, it is assumed that the factors which bind to the apoCIII promoter/enhancer region form a stereospecific DNA−protein complex (Gill et al., 1994; Tjian & Maniatis, 1994). This complex may directly or indirectly interact with the basal transcription complex via TAFs thus leading to the transcriptional activation of the target apoCIII gene. The current study indicates that the enhancement of the transactivation functions of HNF-4 is mostly affected by the SP1 molecule which binds to the regulatory element H. However, optimal enhancement requires also the binding of two other SP1 molecules at the neighboring elements I and F. It is possible that SP1 molecules facilitate the communication of HNF-4 with the components of the basal transcription system and vice versa. It is also possible that both HNF-4 and one or more SP1 molecules communicate simultaneously with the components of the basal transcription, thus leading to synergistic activation of transcription. Recent studies indicate that HNF-4 can synergize with a wide variety of transcription factors including C/EBP (Metzger et al., 1993), CREB (Nitsch et al., 1993), and HNF-1 (Guerra et al., 1993). This is reminiscent of the derivatives of the yeast activator GAL4, which can stimulate transcription in synergy with various mammalian transcription factors such as the glucocorticoid receptor, ATF, and USF (Carey et al., 1990; Lin et al., 1988, 1990).

Potential Contribution of Orphan Receptors Bound to Regulatory Element G in ApoCIII Transcription. The functioning of the upstream apoCIII promoter elements to induce HNF-4-mediated transactivation may also require interactions of HNF-4 with *trans*-acting factors other than SP1 or its family members. In hepatic cells mutation in element G, which is a binding site for the orphan receptors ARP-1 and EAR-3, but not for HNF-4, also diminished promoter activity and HNF-4-mediated transactivation. This finding is interesting in light of previous observations showing that ARP-1 and EAR-3, when bound to proximal element B, repress the apoCIII promoter (Ladias et al., 1992; Myetus-Snyder et al., 1992). In addition, studies on several other promoters, including apoB, apoA-I, apoA-II, apoA-IV (Ladias et al., 1992; Ktistaki et al., 1994), L-type pyruvate kinase (Guerra et al., 1993), ornithine transcarbamylase (Kimura et al., 1993), and the cellular retinol binding protein (Nakshatri et al., 1994), showed that these factors modulate transcription in a negative direction. Although repressor activity of a transcription factor can be achieved by several different mechanisms, it is well-documented that in all cases when ARP-1 and EAR-3 act as repressors the negative effect is being exerted by competition with HNF-4 or other nuclear hormone receptors for the same binding site (Ladias et al., 1992). Since element G binds ARP-1/EAR-3 but not HNF-4, it is possible that ARP-1 and EAR-3 may have a dual regulatory role on apoCIII transcription. When they are overexpressed, they inhibit promoter activity by replacing the essential activator (HNF-4) in proximal site B; on the other hand, their binding to distal element G may be necessary for HNF-4-dependent activation of the apoCIII promoter.

Tissue Specificity Is Determined by Different Interactions among Factors Bound to the Proximal and Distal ApoCIII Promoter Elements. Taken together, our results suggest the involvement of a rather complex mechanism in the regulation of the apoCIII gene in hepatic cells. HNF-4 or other nuclear receptors bind to proximal element B, which is an HRE. Its binding or activation potential is potentiated by SP1 and orphan receptors which bind to distal regulatory elements H and G, respectively. Additional minor activities bound to elements F and I are also necessary for maximal promoter activity and HNF-4-mediated transactivation, albeit to a lesser degree. The possible interactions between the factors involved in the optimal transcriptionally active complex on the apoCIII promoter in hepatic cells are summarized in a model shown in Figure 7A.

A somewhat different model may apply for the transcriptional regulation of apoCIII gene in intestinal cells, the major difference being that the mutations in element G did not influence or increased slightly transcription. HNF-4-mediated transactivation, however, was severely affected not only by the mutations in elements H and G but also by mutations in elements F, I, and J. These observations may imply that in CaCo-2 cells factors binding to element G may not be as crucial for the promoter activity as in HepG2 cells. On the other hand, they are necessary for HNF-4-mediated transactivation, suggesting that in this cell line a different type of communication may exist between the factors binding to the distal and proximal apoCIII regulatory elements. A putative model of interaction of the factors involved in intestinal cells is shown in Figure 6B. The HNF-4-mediated transactivations in HeLa and HepG2 cells were similar with two exceptions: (a) mutations in element J decreased significantly transactivation only in HeLa cells, and (b) a mutation in element I decreased slightly transactivation in HepG2 cells, while it increased 1.3-fold the transactivation in HeLa cells as compared to the wild-type promoter.

Downregulation or repression of apoCIII transcription may also occur in different cell types. Figure 7C shows that such repression may occur when hormone response element B is occupied by ARP-1, EAR-2, or EAR-3. In principle, downregulation or repression of transcription could also occur if element H or the other SP1 binding sites are occupied by negative regulators. SP3, for instance, which is a member of the SP family, was shown recently to act as a transcriptional repressor (Hagen et al., 1994). Finally, repression may occur due to the lack of putative coactivators which bridge SP1 with HNF-4 or the basal transcription factors through protein−protein interactions.

The exact mode of interaction of SP1 and orphan receptors with HNF-4 which results in the transcriptional activation of the apoCIII promoter will be the topic of future studies.

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