Factors Participating in the Liver-Specific Expression of the Human Apolipoprotein A-II Gene and Their Significance for Transcription†

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ABSTRACT: We have shown previously that the hepatic and intestinal transcription of the human apolipoprotein A-II (apoA-II) gene in cell cultures is controlled by a complex set of regulatory elements A–N [Chambaz, et al. (1991) *J. Biol. Chem. 266*, 11676–11685; Cardot, et al. (1991) *J. Biol. Chem. 266*, 24460–24470]. In the present communication, we have assessed the functional importance of each of the regulatory elements. In addition, we have used DNA binding and competition assays and protein fractionation to identify the hepatic nuclear activities which are involved in the regulation of the human apoA-II gene. Such activities may be of general importance for the regulation of liver-specific genes. The DNA binding and competition analysis showed that the regulatory elements M, D, and F bind new activities which have not been identified in apolipoprotein or other liver-specific promoters. These activities have been designated AIIM1 and AIIM2 for element M, AIID1 and AIID2 for element D, and AIIF2 for element F. The activity AIIM2 is present in liver, but absent in CaCo-2 cells. A set of regulatory elements binds activities which resemble liver-enriched or ubiquitous factors previously shown to play important roles in the regulation of their target genes. Thus, element I binds to activities related to NF1, and elements L, C, D, G, AB, and F bind to C/EBPα as well as other heat-stable activities. The affinity of the bacterially expressed C/EBPα for the various apoA-II regulatory regions follows the order: AIIL $\sim$ AIIC > AIID > AIIF > AIIG > AIIAB. Protein fractionation showed that element J binds at least three hepatic nuclear activities and is also recognized by members of the nuclear receptor family, HNF4, EAR2, EAR3, and ARP1. Another liver-enriched factor, HNF1, was shown previously to bind to element H. Despite the importance of HNF1, HNF4, NF1, and C/EBPα in the regulation of numerous other target genes, deletion of the HNF1, NF1, and HNF4 and several C/EBP binding sites did not drastically affect the hepatic transcription of the apoA-II gene. Rather, the hepatic and intestinal transcription is affected severely by deletion of elements A, B, K, L, and N. In addition, the intestinal transcription is affected by deletion of elements C, J, and M. The *in vivo* physiological importance of these elements will require analysis of their function in transgenic animals. This analysis establishes the organization of several nuclear activities on the human apoA-II promoter. Our findings suggest that the expression of a liver-specific gene such as apoA-II is controlled by a combination of factors and that the relative importance of individual factors is determined by the promoter context. Maximum expression of the apoA-II gene requires the synergism of factors bound to the proximal and distal regulatory elements.

Human apolipoprotein A-II (apoA-II) is a major protein component of HDL (plasma concentration of 0.3–0.5 mg/mL) and is synthesized by the liver and, to a much lesser extent, by the intestine (Schonfeld et al., 1982; Hussain & Zannis, 1990). apoA-II has known protein (Brewer et al., 1972) and gene sequences (Knott et al., 1984; Lackner et al., 1984; Tsao et al., 1985; Sharpe et al., 1984) and exists in plasma as a dimer of two 77 amino acid long subunits linked by an intra-disulfide bond at residue 6 (Brewer et al., 1972). Following synthesis, apoA-II is subject to intra- and extracellular modifications, which include a new type of modification, O-glycosylation, propeptide cleavage, and cyclization of the N-terminal glutamine (Hussain & Zannis, 1990). Following secretion, apoA-II is mostly incorporated into lipoprotein particles containing apoA-I and apoA-II (LpAI: AII) (Cheung & Albers, 1984; Ohta et al., 1988; Fruchart & Bard, 1991). The functional significance of these particles has not been clarified. It has been reported that inbred strains of mice with increased apoA-II levels are associated with increased HDL size, suggesting that the plasma concentration of apoA-II may affect HDL structure and function(s) (Lusis, 1988; Doolittle et al., 1990). Overexpression of the human apoA-II gene in transgenic mice affected the size and the distribution of HDL particles, but did not affect the plasma HDL concentration (Schultz et al., 1991). Thus, the overall physiological significance of apoA-II remains unknown.

Recent studies have shown that the transcription of human apoA-II gene is controlled by a complex array of distal and proximal regulatory elements, designated A–N (Shelley & Baralle, 1987; Lucero et al., 1989; Chambaz et al., 1991; Cardot et al., 1991). The distal elements N–I, between nucleotides –903 and –680, are essential for cell type specific expression of the apoA-II gene, and they display enhancer type activity in hepatic cells when placed in front of heterologous promoters (Shelley & Baralle, 1987; Lucero et al., 1989; Chambaz et al., 1991). Deletion of element N drastically reduces hepatic and intestinal transcription. However, element N alone was not sufficient to enhance tran-

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Table I

deletion mutant	primers for amplification of the region upstream of the deletion	primers for amplification of the region downstream of the deletion
1. apoA-II (−911/+29) Δ(−32 to −42) (deletion A domain)	1a (nucleotide +34 to +9 upstream of *Hin*dIII site of the pUC19 polylinker (sense))	1b (apoA-II (+4 to +29) with *Xho*I and *Bam*HI restriction sites (CCATGGATCTGCTCGAG) (antisense))
	2a (apoA-II (−65 to −42) (antisense))	2b (apoA-II (−32 to −8) (sense))
2. apoA-II (−911/+29) Δ(−42 to −65) (deletion B domain)	1a	1b
	3a (apoA-II (−88 to −66) (antisense))	3b (apoA-II (−41 to −13) (sense))
3. apoA-II (−911/+29) Δ(−32 to −65)	1a	1b
	3a	2b
4. apoA-II (−911/+29) Δ(−110 to −126) (deletion C domain)	1a	1b
	4a (apoA-II (−154 to −127) (antisense))	4b (apoA-II (−154 to −83) Δ(−126 to −110) (sense))
5. apoA-II (−911/+29) Δ(−255 to −276) (deletion D domain)	1a	1b
	5a (apoA-II (−306 to −277) (antisense))	5b (apoA-II (−254 to −226) (sense))
6. apoA-II (−911/+29) Δ(−364 to −377) (deletion E domain)	1a	1b
	6a (apoA-II (−378 to −407) (antisense))	6b (apoA-II (−363 to −334) (sense))
7. apoA-II (−911/+29) Δ(−404 to −384) (deletion F domain)	1a	1b
	7a (apoA-II (−405 to −428) (antisense))	7b (apoA-II (−383 to −356) (sense))
8. A-II (−911/+29) Δ(−455 to −468) (deletion G domain)	1c	1b
	8a (apoA-II (−469 to −493) (antisense))	8b (apoA-II (−455 to −428) (sense))
9. apoA-II (−911/+29) Δ(−706 to −675) (deletion I domain)	1a	1b
	9a (apoA-II (−761 to −707) (antisense))	9b (apoA-II (−675 to −614) (sense))
10. apoA-II (−911/+29) Δ(−734 to −716) (deletion J domain)	1a	1b
	10a (apoA-II (−679 to −764) Δ(−734 to −716) (antisense))	10b (apoA-II (−711 to −676) (sense))
11. apoA-II (−911/+29) Δ(−853 to −829) (deletion M domain)	1a	1b
	11a (apoA-II (−802 to −881) Δ(−853 to −829) (antisense))	11b (apoA-II (−828 to −802) (sense))

scription (Shelley & Baralle, 1987; Chambaz et al., 1991). The middle region contains elements H–D, between nucleotides −573 and −255, and its deletion slightly reduced the transcription of the apoA-II gene in cells of hepatic and intestinal origin (Chambaz et al., 1991). The proximal region contains elements C–A between nucleotides −126 and −33, and its importance has not been assessed. Previous analysis showed that elements AB, K, and L bind to the heat-stable factor CIIIB1, which has been purified from hepatic nuclear extracts and is a transcription activator of the human apoC-III gene (Cardot et al., 1991; Ogami et al., 1991). Elements AB and K also bind to another heat-labile activity, designated AIIAB1, the importance of which is not known, and element L binds to proteins that recognize the CCAAT motif (Cardot et al., 1991). Element N predominantly binds an activity designated AIIN3, and element H binds an activity related to HNF1 (Chambaz et al., 1991). The importance of CIIIB1 and of CCAAT box activities for the transcription of the apoA-II gene was assessed by *in vitro* mutagenesis. Point mutations in domains L, K, and AB, which prevented the binding of CIIIB1, reduced transcription in hepatic and intestinal cells to 7% of control. Mutations that prevented the binding of CCAAT box binding proteins in domain L reduced hepatic and intestinal transcription to 30% of control (Cardot et al., 1991).

In the present communication, we have completed the characterization of the activities which bind to the regulatory regions C, D, E, F, G, I, J, and M of apoA-II and assessed their importance for hepatic and intestinal transcription. The arrangement and properties of the apoA-II promoter suggest that the liver-specific expression of the apoA-II gene is controlled by the synergistic interactions of factors which bind to the proximal and the distal regulatory elements. The multiplicity of the regulatory elements and the nuclear factors which participate in the regulation of a single gene is very interesting. It is possible that the interaction of different sets of factors within the apoA-II promoter may lead to different levels of transcription of the apoA-II gene.

EXPERIMENTAL PROCEDURES

Materials. All materials were purchased from sources described previously (Ogami et al., 1990).

Synthetic Oligonucleotides and DNA Amplifications. Oligonucleotides were synthesized by the solid-phase phosphite triester method using an automated oligonucleotide synthesizer (Applied Biosystems, Model 380-B). The oligonucleotides were purified by electrophoresis on 20% polyacrylamide/7 M urea gels and labeled with ^{32}P as described (Ogami et al., 1990). DNA fragments were generated by DNA amplification with the polymerase chain reaction (PCR). The primers used and the fragments generated are shown in Table I. PCR reactions were performed using a Perkin-Elmer automated thermocycler according to the manufacturer's specifications. The sequence of the fragments and their orientation in the final constructs were determined by DNA sequencing.

Plasmid Construction. The −911/+29 CAT construct containing the deletion of elements A–M was generated with PCR amplification of the pUC-AII plasmid (Chambaz et al., 1991). For the deletion of regions A, B, AB, D, E, F, G, and I, the sequences upstream and downstream of the intended deletion were amplified separately. For instance, for the deletion of element A, the region upstream of nucleotide −42 was amplified using oligonucleotides 1a and 2a as 5′ and 3′ primers, and the region downstream of nucleotide −32 was amplified using oligonucleotides 2b and 1b as 5′ and 3′ primers, respectively (Table I). The two amplified fragments for each deletion were digested with *Hin*dIII and *Xho*I, respectively, purified by electroelution from the agarose gel, and cloned into the corresponding sites of the pUCSH-CAT vector.

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

		ref
AIC	$^{-178}$AGCTTGCTGTTTGCCCACTCTATTTGCCCAGCCCCAG^{-142}	Papazafiri et al., 1991
AID	$^{-218}$GCCCCCACTGAACCCTTGACCCCTGCCCTGC^{-188}	Papazafiri et al., 1991
CIIIB	$^{-92}$GGTCAGCAGGTGACCTTTGCCCAGCG^{-67}	Ogami et al., 1990
CIIIC	$^{-138}$CCGCTTGCTGCATCTGGACA^{-119}	Ogami et al., 1990
CIIID	$^{-165}$GTCCTAGGGATTTCCCAATCTCCCGCCC^{-137}	Ogami et al., 1990
BA1	$^{-88}$CCCGGGAGGCGCCCTTTGGAGCTTTTG^{-62}	Kardassis et al., 1990a
BA2	$^{-61}$CAATCCTGGCGCTCTTGCAGCCTGGG^{-36}	Kardassis et al., 1990a
BA3	$^{-78}$GCCCTTTGGACCTTTTGCAATCCTGGCGCT^{-48}	Kardassis et al., 1990a
BC1	$^{-116}$GCCAGTGTAGAAAAGCAAA^{-99}	Kardassis et al., 1990a
α1AT	$^{-130}$ATCCCAGCCAGTGGACTTAGCCCCTGTTTGCTCCTCC^{-94}	DeSimone et al. 1987
HNF1/LFB1	$^{-70}$AGTATGGTTAATGATCTACAG^{-50}	Maire et al., 1989
NFY	$^{-92}$GGAACCAATGAAATGCGAGG^{-73}	Maire et al., 1989
NF1	$^{-133}$ACAATTTTTTGGCAAGAATATTAT^{-110}	Maire et al., 1989
A1bD	$^{-115}$TGGTATGATTTTGTAATGGGGTAGGA^{-90}	Maire et al., 1989
TK/CEBP	$^{-96}$GCGTCTTGTCATTGGCGAATTCG^{-74}	Dorn et al., 1987
AP1	$^{-110}$AGCCGCAAGTGACTCAGCGCGGGGCGTGTGCA^{-77}	Bos et al., 1988
AP2/3	$^{-255}$GTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAG^{-224}	Mitchell et al., 1987
OTF	$^{-74}$TTCCCAATGATTTGCATGCTCTCACT^{-49}	Scheidereit et al. 1987
AIIAB	$^{-67}$AGTCCTGTCACCTGACAGGGGGTGGGTAAACAGACA^{-32}	Figure 1
AIIC	$^{-128}$CTCCCCCATTTCTCCAACTTG^{-108}	Figure 1
AIID	$^{-278}$TGCTTCCTGTTGCATTCAAGTCCAAG^{-253}	Figure 1
AIIE	$^{-379}$ATAATGGAATAAAGACAC^{-362}	Figure 1
AIIF	$^{-406}$GATAAGGTTGAGAGATGAGATCTAC^{-382}	Figure 1
AIIG	$^{-471}$GATTTCAATTCCTTTCTC^{-454}	Figure 1
AIIH	$^{-572}$GTCTCATTACACATTAACTC^{-553}	Figure 1
AIII	$^{-706}$ATTCACCTCTTTTCCTGCCAGAGCCC^{-681}	Figure 1
AIIJ	$^{-739}$TGCCTTCAACCTTTACCCTGGTAG^{-716}	Figure 1
AIIK	$^{-760}$TAAGGTGATCAAATGACC^{-743}	Figure 1
AIIM	$^{-855}$ACCTCTCCCCCTCCCCCACCCCCAACAGGA^{-826}	Figure 1

Deletions B, AB, D, E, G, and F were generated in a similar manner using the primers listed in Table I. For deletion of domain C (−126 to −110), the region upstream of nucleotide −126 was amplified using oligonucleotides 1a and 4a as 5′ and 3′ primers, respectively. The region downstream of nucleotide −154 was amplified using 5′ and 3′ amplification primers 1b and 4b. The 4b primer extends from nucleotide −154 to −83 and contains the −126 to −110 deletion. An aliquot containing 4% of the two amplified regions was used for further amplification using the 5′ and 3′ amplification primers 1a and 1b. The amplified DNA was digested with *Hin*dIII and *Xho*I, purified by electroelution from the agarose gel, and cloned into the *Hin*dIII and *Xho*I sites of the pUCSH-CAT. The deletions in domains M and J were constructed in a similar manner using the amplification primers shown in Table I. The oligonucleotides carrying the deletion of domains J and M were 10a and 11a, respectively. apoA-II promoter plasmids were cotransfected with a β-galactosidase-containing plasmid in HepG2 cells by the calcium phosphate–DNA coprecipitation method (Graham & van der Eb, 1973). CAT and β-galactosidase assays were performed as described previously (Edlund et al., 1985; Gorman et al., 1982).

Preparation of Nuclear Extracts and Fractionation of Activities Bound to the Regulatory Elements J and D of the apoA-II Promoters. All buffers contained 1 mM DTT, 0.1 mM benzamidine, 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin added just prior to use. Nuclear dialysis buffer (NDB) contained 25 mM Hepes (pH 7.6), 5 mM $MgCl_2$, 0.1 mM EDTA, and 10% glycerol. All purification steps were carried out at 4 °C. Nuclear extracts were prepared from the livers of 10 rats (approximately 120 g of liver) as described (Gorski et al., 1986). The extracts (10 mL, 90 mg of protein) were dialyzed against two changes of NDB buffer containing 40 mM KCl and used for purification.

Q-Sepharose Chromatography. Five milliliters of dialyzed nuclear extracts were applied to a column (1.6 cm diameter, 20 cm length) of Q-Sepharose (10 mL bead volume) equilibrated in NDB buffer containing 40 mM KCl at a flow rate of 30 mL/h. The column was eluted stepwise with NDB containing 0.2–1 M KCl. In this and subsequent fractionations, fractions of 3 mL were collected and analyzed for DNA binding activity by a DNA binding gel electrophoretic assay.

Biorex-70 Chromatography. Another 5 mL of dialyzed nuclear extracts was applied to a column of Biorex-70 similar to that described above and equilibrated in NDB buffer containing 40 mM KCl. The column was eluted stepwise with NDB buffer containing 0.2–1 M KCl.

DNA Binding Gel Electrophoresis and Competition Assays. For this analysis, 6–10 μg of nuclear extracts was preincubated for 15 min at 4 °C in a 20-μL reaction containing 25 mM Hepes (pH 7.6), 8% Ficoll, 40 mM KCl, 1 mM DTT, 3 μg of double-stranded poly(dI–dC), and 5 mM $MgCl_2$ in the presence or absence of a 25–500-fold excess of competitor oligonucleotide sequences (Cardot et al., 1991). The sequences of synthetic oligonucleotides used as competitors are presented in Table II. Labeled, double-stranded apoA-II oligonucleotide (30 000 cpm) was then added, and the incubation was continued for 30 min at 4 °C. Free DNA and DNA–protein complexes were resolved on a 4% polyacrylamide gel in 1× TAE (1× TAE = 6.7 mM Tris, 3.3 mM sodium acetate, and 1 mM EDTA, pH 7.9). The gel was dried and exposed to X-ray film. C/EBPα cDNA (Landschulz et al., 1988) was the generous gift of Dr. McKnight of Carnegie Institution of Washington and was expressed in *Escherichia coli* and purified as described (Papazafiri et al., 1991). Extracts from COS-1 cells transiently transfected with HNF4, ARP1, EAR2, and EAR3 expression plasmids were prepared as described (Ladias et al., 1992).

RESULTS

Nomenclature of the Binding Activities. The activities which bind to the regulatory elements A–N of apoA-II are identified by (a) the name of the apoA-II gene (AII), (b) the regulatory element to which they bind (A, B, C, etc.), and (c) the mobility of the DNA protein complexes (1, 2, 3, etc.) going from the slowest to the fastest migrating complexes.

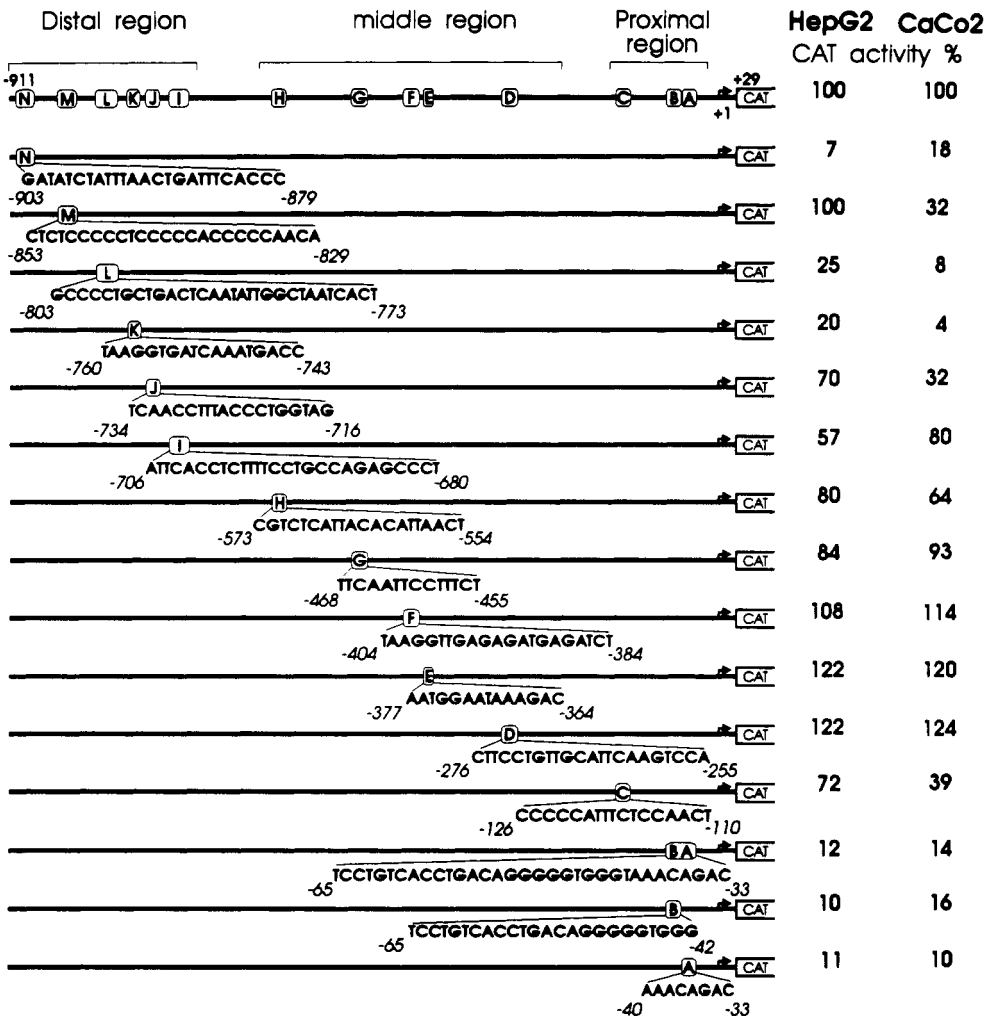

FIGURE 1: Effect of deletions of the apoA-II promoter elements A–N on the transcription of the reporter CAT gene in HepG2 and CaCo-2 cells.

Importance of the Regulatory Elements A–N for Transcription in Cells of Hepatic and Intestinal Origin. The deletions of the regulatory elements A–N affected differently the transcription of the reporter CAT gene in hepatic HepG2 and intestinal CaCo-2 cells (Figure 1). This analysis generally showed that deletion of elements of the middle regulatory region (elements J–C) did not dramatically affect hepatic transcription. The intestinal transcription was also not affected in these deletion mutants, with the exception of the deletion of elements J and C where the intestinal transcription was reduced to 32% and 39% of control, respectively. Deletion of the elements A, B, or both reduced hepatic and intestinal transcription to 10–16% of control, indicating that the factors which bind to this proximal region are essential for the transcription of the human apoA-II gene. With the exception of element M, deletion of the distal regulatory elements K–N reduced hepatic transcription to levels ranging from 7 to 25% of control and the intestinal transcription to levels ranging from 4 to 18% of control, indicating that this region is also essential for transcription. The deletion of element M reduced intestinal transcription to 32% of control and had no effect on hepatic transcription (Figure 1). The effects of deletions of elements A–N on transcription in HepG2 and CaCo-2 cells imply that hepatic and intestinal transcription may be controlled by different combinations of regulatory factors.

Identification and Characterization of the Activities Which Bind to Regulatory Element M in Liver and CaCo-2 Cells. The activities which bind to the proximal and distal apoA-II promoter elements were identified by DNA binding gel electrophoretic and competition assays using synthetic oligonucleotides as binding probes or competitors. This analysis showed that the regulatory element M (nucleotides −853 to −829) is recognized predominantly by two major hepatic activities, designated AIIM1 and AIIM2 (Figure 2A). It is interesting that the activity AIIM2 is present in rat liver nuclear extracts but is absent from CaCo-2 cell extracts (Figure 2B) and may represent a new liver-enriched factor. This factor has no effect on apoA-II transcription, but may be important for the transcription of other liver-specific genes. The observation that the deletion of element M reduced intestinal transcription to 32% of control implies that factor AIIM1 is important for intestinal transcription but has no effect on hepatic transcription. Competition analysis showed that both the AIIM1 and the AIIM2 activities are not competed out by oligonucleotides containing the binding sites of the previously described transcription factors C/EBP, NF1, HNF1/LFB1, and NFY (Maire et al., 1989) or by regulatory elements of apoB (BA2, BA3, and BC1), apoA-I (AID), apoC-III (CIIID), albumin (AlbD), and α1-antitrypsin (α1AT) (Ogami et al., 1990; Papazafiri et al., 1991; Maire et al., 1989; Kardassis et al., 1990a; DeSimone et al., 1987). At least two other fast migrating DNA protein complexes appear on the DNA binding gel which compete with oligonucleotides NFY, BA2, and AID (Figure 2A). The designations of the competitor oligonucleotides used are shown on the top of Figure 2, and their sequences are described in Table II. The competitor oligonucleotides used in this and subsequent figures define four groups of transcription factors: a liver-enriched factor

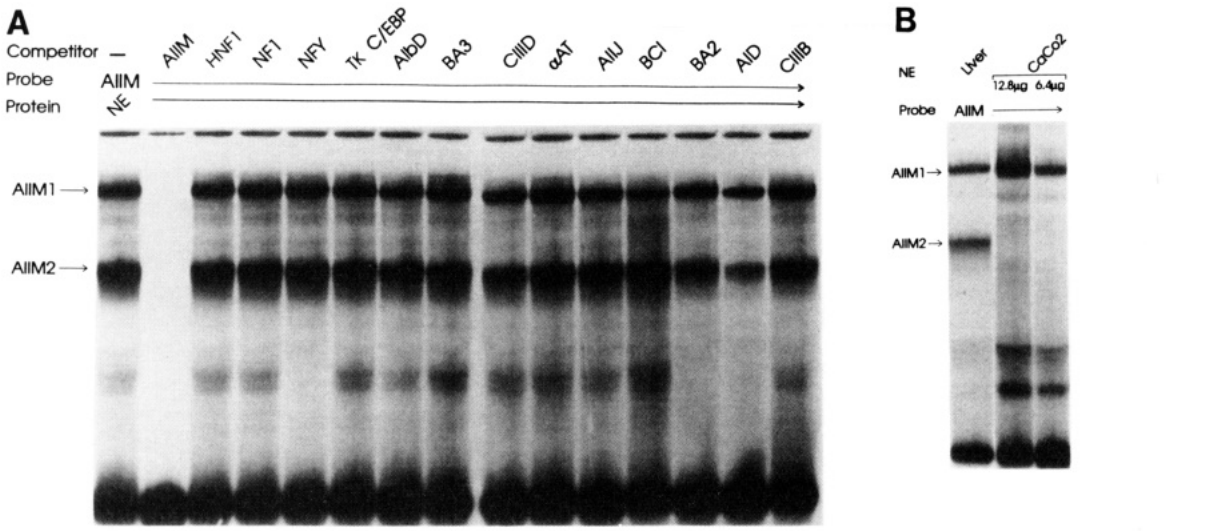

FIGURE 2: (A) DNA binding gel electrophoretic and competition assays with apoA-II promoter region M (–853 to –829) as probe. The double-stranded oligonucleotide corresponding to footprinting region M was labeled with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dGTP and [α-^{32}P]dCTP and used as a binding probe. In this panel and in subsequent figures (Figures 3–8), DNA binding and competition assays were performed with rat liver nuclear extracts as described in the Experimental Procedures. Competitor oligonucleotides were added in all except the first lane at 500-fold molar excess relative to the ^{32}P-labeled oligonucleotides. The oligonucleotides used are indicated by abbreviations at the top of the figure and are described in Table II. (B) DNA binding gel electrophoretic assay using apoA-II promoter region M as probe and nuclear extracts from rat liver or CaCo-2 cells. NE represents analyses performed with rat liver nuclear extracts.

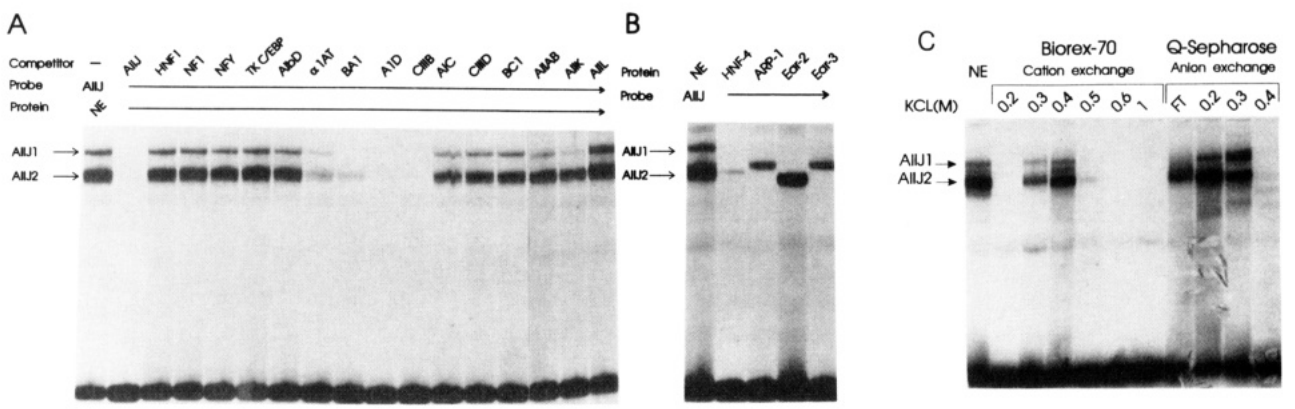

FIGURE 3: (A) DNA binding gel electrophoretic and competition assays with apoA-II promoter region J (–734 to –716) as probe. Labeling of the oligonucleotide probe and DNA binding and competition assays were performed with rat liver nuclear extracts as described in the Experimental Procedures. The competitor oligonucleotides were used at 500-fold molar excess relative to the ^{32}P-labeled probe. They are indicated by abbreviations at the top of the figure and are described in Table II. (B) DNA binding gel electrophoretic assay using apoA-II promoter region J (–734 to –716) as probe and rat liver nuclear extracts (NE) or factors HNF4, ARP1, EAR2, and EAR3 produced by expression of the corresponding cDNAs in COS-1 cells. (C) DNA binding gel electrophoretic assays of fractions eluted from the cation (Biorex-70) and anion (Q-Sepharose) exchange columns using the apoA-II promoter region J (–734 to –716) as probe. The KCl concentration at which the activities AIIJ1 and AIIJ2 elute is indicated at the top.

(HNF1/LFB1), nuclear receptors (AID, BAI, CIIIB, and α1-antitrypsin), CCAAT box binding proteins (AlbD, NFY, NF1, BA3, CIIID, and TKC/EBP), and miscellaneous factors (AP1, AP2/3, OTF, and CIIIB1) (Ogami et al., 1990; Papazafiri et al., 1991; Ladias et al., 1992; Maire et al., 1989; Kardassis et al., 1991a; DeSimone et al., 1987; Dorn et al., 1987; Bos et al., 1988; Mitchell et al., 1987; Scheidereit et al., 1987).

Identification and Characterization of the Hepatic Activities Which Bind to Regulatory Elements J (–734 to –716) and I (–706 to –680). DNA binding and competition assays showed that element J predominantly forms two DNA–protein complexes designated AIIJ1 and AIIJ2 (Figure 3A). Competition analysis showed that both complexes are competed out by oligonucleotides AID and CIIIB (Table II) (Ogami et al., 1990; Papazafiri et al., 1991). The AIIJ1 is competed out completely and the AIIJ2 is competed out partially by oligonucleotide BA1 (Table II) (Kardassis et al., 1990a). Both AIIJ1 and AIIJ2 complexes are competed out partially by the oligonucleotide α1AT, which corresponds to the regulatory region –130 to –94 of α1-antitrypsin (DeSimone et al., 1987). The competition experiments by the α1AT and BA1 oligonucleotides indicate that the regulatory element J is recognized by at least three distinct activities. Some of these activities also bind to the regulatory elements AID, BA1, and CIIIB of the human apoA-I, apoB, and apoC-III genes, respectively. Elements AID, BA1, CIIIB, and α1AT contain a direct, nonperfect repeat of the TGACCT motif, which is the site that is recognized by nuclear receptors (Martinez et al., 1991). Members of the orphan receptor family HNF4, EAR2, EAR3, and ARP1 (Sladek et al., 1990; Ladias & Karathanasis, 1991; Ritchie et al., 1990; Miyajima et al., 1988) bind to the nuclear receptor sites of the human apolipoprotein genes and regulate their transcription. HNF4 is a positive regulator and ARP1, EAR2, and EAR3 are negative regulators of these human apolipoprotein genes (Ladias et al., 1992). All of these elements are also recognized by factor NF-BA1, which was purified recently from rat liver nuclear extracts and was shown to be a positive regulator of the human apoB gene (Kardassis et al., 1990b).

Figure 3B shows the electrophoretic mobilities of the DNA–protein complexes of the AIIJ element with ARP1, EAR2, EAR3, and HNF4, expressed in COS-1 cells, along with the complexes formed with rat hepatic nuclear extracts. The electrophoretic mobility of AIIJ2 is similar to that of HNF4, but different from those of ARP1, EAR2, and EAR3. In addition, the fact that the AIIJ2 complex is not competed out by a large excess of oligonucleotide BA1 and α1AT implied that other binding activities with electrophoretic mobilities similar to that of HNF4 present in hepatic extract recognize the AIIJ element. The relationship of the activities responsible for the AIIJ2 and AIIJ1 complexes with members of the nuclear receptors requires further clarification by utilization of specific antibodies which recognize members of the hormone or orphan receptor families. Competition analysis also showed that the AIIJ1 and AIIJ2 bands are not competed out by oligonucleotides containing the binding sites of the previously described transcription factors C/EBP, NF1, HNF1, and NFY or by regulatory elements of apoB (BC1), apoA-I (AIC), apoA-II (AIIAB, AIIK, and AIIL), apoC-III (CIIID), and albumin (AlbD). The designations of the competitor oligonucleotides used are shown on the top of Figure 3, and their sequences are described in Table II. Overall, the DNA binding and competition analyses indicated that regulatory element J may be the binding site of factors similar or related to previously described hormone receptor family members.

Fractionation of the activities which bind to regulatory element J by cation (Biorex-70) exchange chromatography showed that both activities elute at 0.3–0.4 M KCl. Fractionation by anion exchange (Q-Sepharose) chromatography showed that a significant portion of the AIIJ2 activity elutes at 40 mM KCl (flow through, FT), whereas significant amounts of AIIJ2 and all of the AIIJ1 activity elute at 0.2–0.3 M KCl (Figure 3C).

DNA binding and competition analysis showed that regulatory region I forms three DNA–protein complexes, which are competed out by oligonucleotide NF1 (Table II) previously shown to bind transcription factor NF1 (Figure 4A) (Maire et al., 1989). Partial competition of binding is also observed with oligonucleotides AIC, BA3, and TKC/EBP, all containing a noncanonical CCAAT binding motif (Table II) (Dorn et al., 1987). The apoA-II –706 to –680 sequence in the antisense orientation has extensive homology with the –133 to –110 regulatory element of the albumin promoter, which contains the motif TGGCAA that is recognized by NF1 (Maire et al., 1989). The electrophoretic mobility profiles of the hepatic nuclear activities which bind to the NF1 (–133 to –110) oligonucleotide and the AII I (–706 to –680) oligonucleotide are similar (Figure 4B). A truncated NF1 form which contains the binding site of NF1 and was produced by the expression of a truncated rat NF1 cDNA using a vaccinia virus expression system (Gounari et al., 1990) binds to NF1 and AII I oligonucleotides (Figure 4C). The affinity of rat NF1 for its cognate sequence on the mouse albumin and human apoA-II promoters was determined by cross-competition experiments. Figure 4D shows the ability of the unlabeled NF1 and AII I oligonucleotides to compete for the binding of the truncated NF1 factor to the albumin promoter. The direct binding (Figure 4C) and competition experiments (Figure 4D) indicated that the NF1 affinity for the regulatory element of the human apoA-II gene is lower than that for its cognate sequence on the albumin promoter.

Low-Affinity Binding of Nuclear Activities to Footprint Regions G (–468 to –425), F (–404 to –384), and E (–377 to –364). The activities which bind to regions E, G, and F have been designated AIIE1, AIIG1, AIIF1, AIIF2, and AIIF3, respectively (Figure 5A–C). Competition experiments showed that the slower migrating AIIF1 activity, which appears as a broad band, is competed out by oligonucleotides containing the binding sites of the previously described transcription factors C/EBP, NF1, and NFY (Maire et al., 1989) and by regulatory elements of apoB (BA2, BA3), apoA-I (AIC), apoC-III (CIIID), and albumin (AlbD), which contain binding sites for CCAAT box binding proteins (Ogami et al., 1990; Maire et al., 1989; Kardassis et al., 1990a). However, the AIIF1 activity is not competed out by oligonucleotides α1AT and CIIIB, which contain binding sites for nuclear receptors (Ladias et al., 1992; Martinez et al., 1991), or by an oligonucleotide which contains the binding site of the liver-enriched factor HNF1/LFB1 (Maire et al., 1989). The faster migrating activity AIIF3 is competed out by oligonucleotide NF1, whereas activity AIIF2 is not competed out by any of the competitor oligonucleotides used. The binding of AIIE1 activity to footprint region E is very weak. The competition pattern of AIIG1 activity is identical to that observed with AIIF1 activity (data not shown). A faster migrating DNA–protein complex is observed in panels B and C which is not competed out with excess cold oligonucleotide and probably is the result of nonspecific binding. The designation of the competitor oligonucleotides used is shown on the top of Figure 5, and their sequences are described in Table II.

Identification and Characterization of the Hepatic Activities Which Bind to Regulatory Elements D and C of the Human apoA-II Gene. DNA binding and competition assays showed that regulatory region D of apoA-II forms a broad band that is the result of at least three superimposed DNA–protein complexes designated AIID1, AIID2, and AIID3 (Figure 6). AIID3 represents a broad band which may result from the binding to this region of one or more heat-stable activities. AIID1 and AIID2 represent heat-labile activities (data not shown). These activities become apparent when the heat-stable AIID3 activities are competed out. The activities which bind to regulatory element D were also fractionated by anion Q-Sepharose and cation (Biorex-70) exchange chromatography. Two additional activities elute at 0.3 and 1.0 M KCl from the Biorex-70 column and at 0.4 KCl from the Q-Sepharose column. These activities may exist in low abundance in the crude extracts and are concentrated in the indicated fractions during purification. Alternatively, they may represent degradation products of the AIID1, AIID2, and AIID3 activities. The AIID1 and AIID3 activities elute at different salt concentrations from the cation exchange column (Figure 6B) but cannot be separated by anion exchange chromatography.

DNA binding and competition experiments showed that the heat-stable activities are competed by oligonucleotides containing the binding sites of the previously described transcription factors C/EBP, NF1, HNF1, NFY, AP1, AP2/3, α1AT, and OTF (Maire et al., 1989; DeSimone et al., 1987; Bos et al., 1988; Mitchell et al., 1987; Scheidereit et al., 1987) and by regulatory elements of apoB (BA3), apoA-I (AIC), apoA-II (AIIAB, AIIC, AIIF, AIIG, and AIIL), and apoC-III (CIIID and CIIIC), albumin (AlbD). These apolipoprotein regulatory elements bind heat-stable factors possibly related to C/EBP family members. The heat-stable activities are not competed out by oligonucleotides BA1 and AID, which contain binding sites to nuclear receptors (Sladek et al., 1990; Ladias & Karathanasis, 1991; Ritchie et al., 1990; Miyajima et al., 1988; Kardassis et al., 1990a,b, 1992). The designations of the competitor oligonucleotides used are shown on the top of Figure 6, and their sequences are described in Table II. The AIID1 activity is competed out by oligonu-

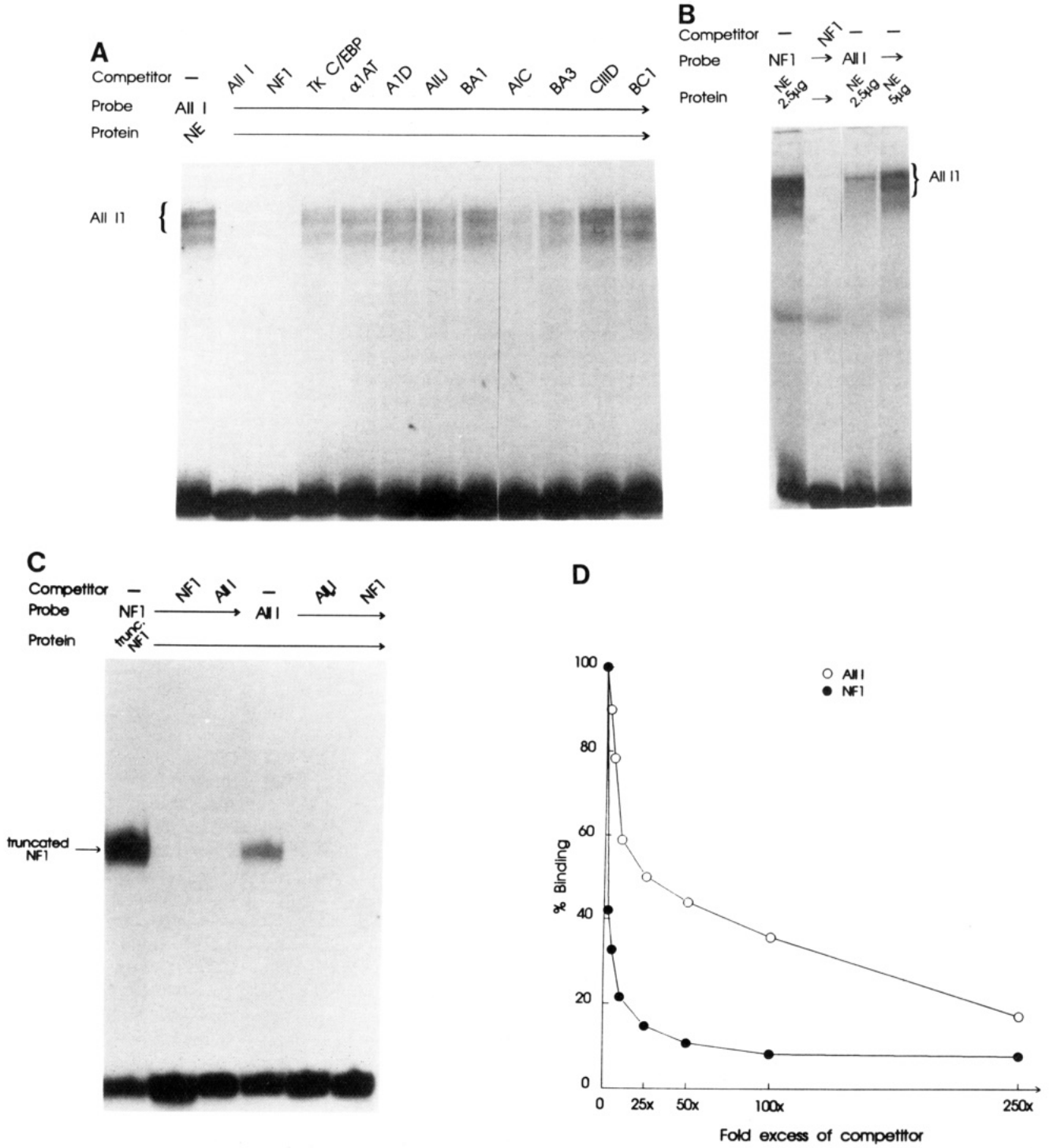

FIGURE 4: (A) DNA binding gel electrophoretic and competition assays with apoA-II promoter region I (–706 to –680) as probe. Labeling of the oligonucleotide probe and DNA binding and competition assays were performed with rat liver nuclear extracts as described in the Experimental Procedures. Competitor oligonucleotides were added in all except the first lane at 500-fold molar excess with respect to the ^{32}P-labeled oligonucleotides. They are indicated by abbreviations at the top of the figure and are described in Table II. (B) DNA binding gel electrophoretic and competition assays using the mouse albumin –133 to –110 promoter region (NF1 oligo) or apoA-II promoter region I (–706 to –680) as probes. (C) DNA binding gel electrophoretic and competition assays using the mouse albumin –133 to –110 promoter region (NF1 oligo) or apoA-II promoter region I (–706 to –680) as probes. DNA binding assays were performed with a truncated NF1 form, which contained the binding site of NF1 and was produced by expression of a truncated rat NF1 cDNA using a vaccinia virus expression system. (D) Competition of binding of NF1 activity to a probe containing the NF1 binding site on the mouse albumin promoter by oligonucleotides containing apoA-II promoter element I or the NF1 binding site on the albumin promoter. The complete sequences of the competitor oligonucleotides used are shown in Table II.

cleotides AIC and CIIIC as well as by the oligonucleotide TKC/EBP (Dorn et al., 1987). Finally, activity AIID2 is not competed out by any of the oligonucleotides of Table II.

DNA binding and competition experiments showed that regulatory region C of apoA-II forms two DNA–protein complexes designated AIIC1 and AIIC2. The complexes are retained after heat treatment of the extracts at 85 °C for 5 min (Figure 7). Complex AIIC1 appears as a broad band. Its competition pattern is very similar to that observed for activity AIID3 (compare Figures 6 and 7). Specifically, this activity is competed by oligonucleotides AlbD, AIC, BA3, CIIIC, TKC/EBP, etc., which bind heat-stable factors, but it is not competed by oligonucleotides BA1 and A1D, HNF1, and AII I, which bind nuclear receptors HNF1 and NF1,

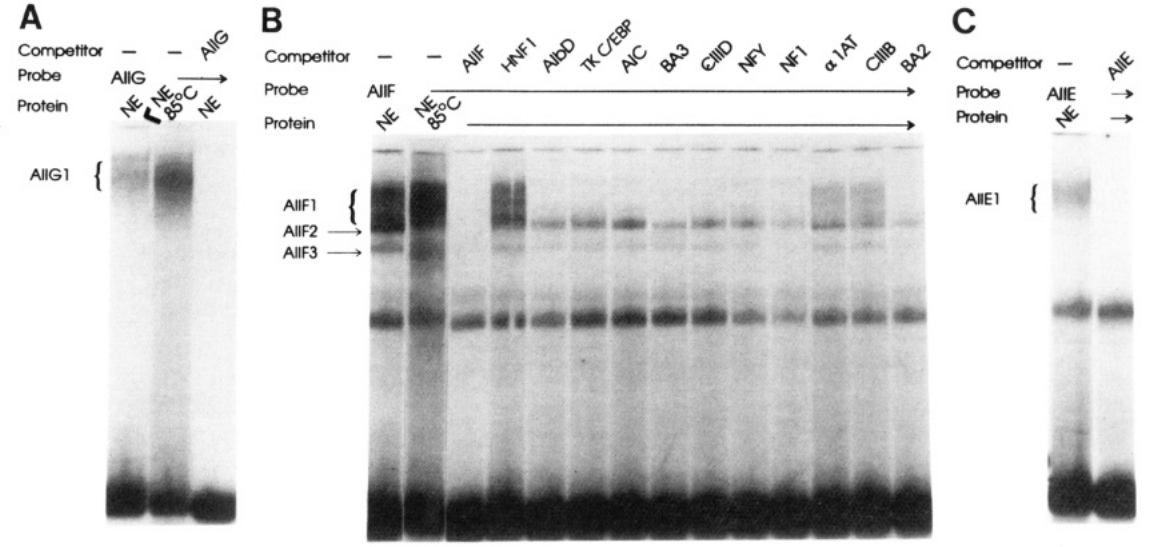

FIGURE 5: DNA binding gel electrophoretic and competition assays with apoA-II promoter regions E (–377 to –364) (A), F (–404 to –384) (B), and G (–468 to –455) (C). Labeling of the oligonucleotide probe and DNA binding and competition assays were performed with rat liver extracts as detailed in the Experimental Procedures. Reactions were performed in the presence of 15 μg of rat liver nuclear extracts in panels A and C and 10 μg in panel B. NE 85 °C indicates that nuclear extracts were heated at 85 °C for 5 min. The competitor oligonucleotides were used at 500-fold molar excess relative to the ^{32}P-labeled probe. They are indicated by abbreviations at the top of the figure and are described in Table II.

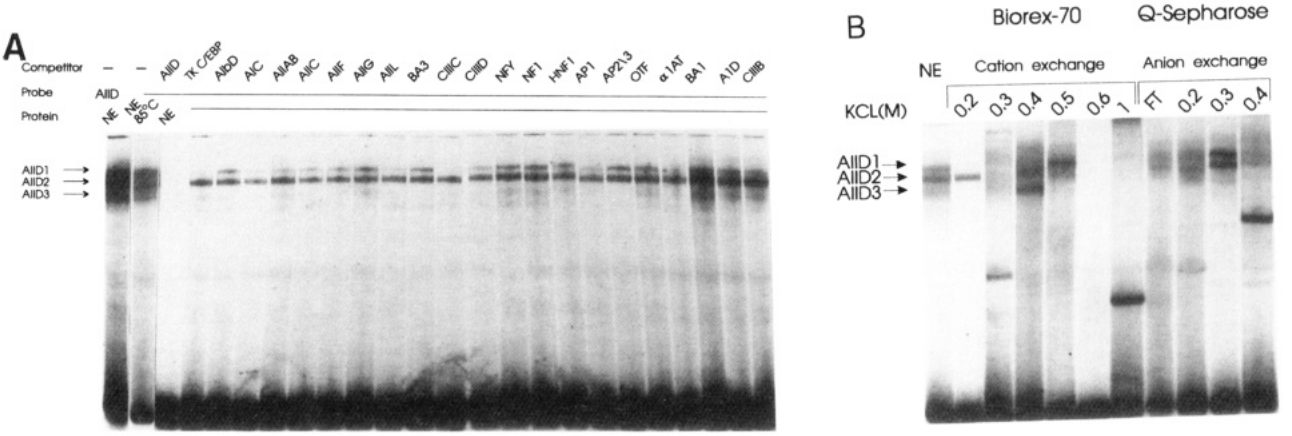

FIGURE 6: (A) DNA binding gel electrophoretic and competition assays with apoA-II promoter region D (–276 to –255). Labeling of oligonucleotide probe and DNA binding and competition assays were performed with rat liver nuclear extracts as detailed in the Experimental Procedures. NE 85 °C indicates that nuclear extracts were heated at 85 °C for 5 min. The competitor oligonucleotides were used at 500-fold molar excess relative to the ^{32}P-labeled probe. They are indicated by abbreviations at the top of the figure and are described in Table II. (B) DNA binding gel electrophoretic assays of fractions eluted from the cation (Biorex-70) and anion (Q-Sepharose) exchange columns using apoA-II promoter region D as a probe. The KCl concentration at which the activities AIID1, AIID2, and AIID3 elute is indicated at the top.

respectively. It is interesting that the HNF1 oligonucleotide which competes for the binding of AIID3 to regulatory element D does not compete for the binding of AIIC1 to regulatory region C. This suggests that AIIC1 and AIID3 activities may not be identical. In addition, AIIC1 activity is competed out by oligonucleotide NF1 but not by oligonucleotide AII I which, as shown in Figure 4A,B, binds to NF1. The heat-stable activity AIIC2 is competed out by the AlbD oligonucleotide, which contains the binding sites of C/EBPα and DBP (Maire et al., 1989). The designations of the competitor oligonucleotides used are shown on the top of Figure 7, and their sequences are described in Table II.

The activities which bind to regulatory elements N, K, L, and AB have been described previously (Chambaz et al., 1991; Cardot et al., 1991). The competition experiments of Figures 5–7 combined with previous findings suggest that regulatory elements C and L of apoA-II, C and D of apoC-III, C of apoA-I, and BA3 of apoB bind similar heat-stable activities (Chambaz et al., 1991; Cardot et al., 1991; Ogami et al., 1990, 1991; Papazafiri et al., 1991; Kardassis et al., 1990a, 1992). A common feature of these elements is that they contain sequences that are homologous to the T(orG)NNGC-(orT)AAT(orG) motif, which are the binding sites of C/EBP and other CCAAT box binding proteins (Table III) (Ryden & Beemon, 1989). The nature of these activities and their relationship to previously described factors require further clarification by utilization of specific antibodies recognizing the C/EBP family members. Their importance for the regulation of the different apolipoprotein genes requires *in vivo* expression studies using transgenic mice.

Affinity of C/EBP for Different Cognate Sites on the Human apoA-II Gene Promoter. DNA binding of C/EBPα produced by expression of C/EBP cDNA in bacteria (Landschulz et al., 1988; Papazafiri et al., 1991; Kardassis et al., 1992) showed that C/EBPα binds with different affinities to the regulatory regions AIIAB, AIIC, AIID, AIIF, AIIG, and AIIL (Figure 8A, Tables II and III). The affinity of C/EBPα for its cognate sequence on the human apoA-II promoter was determined by cross-competition experiments. Figure 8B shows the ability of unlabeled oligonucleotides to compete for

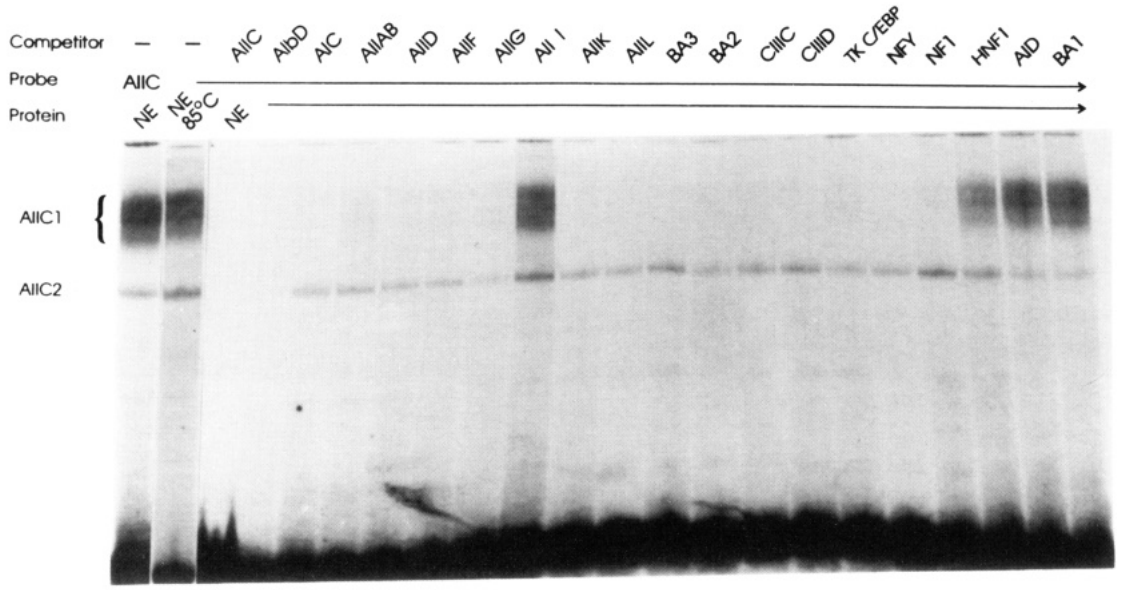

FIGURE 7: DNA binding gel electrophoretic and competition assays with apoA-II promoter region C (−126 to −110). Labeling of the oligonucleotide probe and DNA binding and competition assays were performed with rat liver nuclear extracts as detailed in the Experimental Procedures. Competitor oligonucleotides were added in all except the first two lanes at 500-fold molar excess with respect to the ^{32}P-labeled oligonucleotides. They are indicated by abbreviations at the top of the figure and are described in Table II.

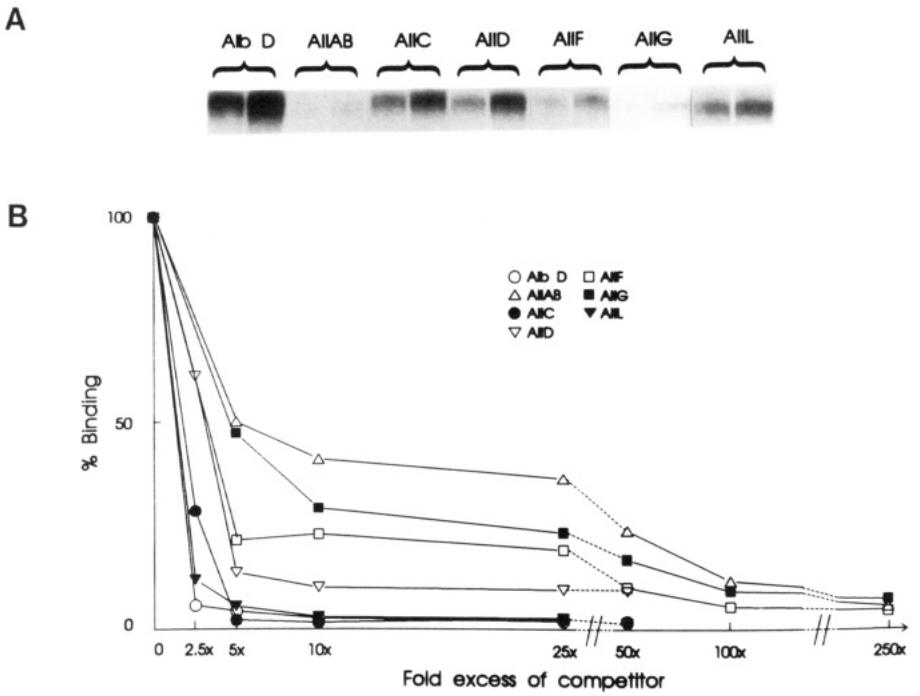

FIGURE 8: (A) DNA binding gel electrophoresis of C/EBPα produced by the expression of C/EBPα cDNA in bacteria to the regulatory region D of the mouse albumin promoter and regions AB, C, D, E, G, and L of the human apoA-II promoter. The abbreviations of the oligonucleotide probes used are indicated at the top of the figure and are described in Table II. (B) Competition of binding of C/EBPα to the AlbD regulatory region (Table II) by unlabeled oligonucleotides corresponding to the AlbD region and regions AB, C, D, E, G, and L of apoA-II. The complete sequences of the competitor oligonucleotides used are shown in Table II.

the binding of C/EBPα to AlbD oligonucleotide. On the basis of the concentration of cold competitor required to displace 50% of the labeled probe, the affinity of C/EBPα for its cognate sequence on the mouse albumin and apoA-II promoters follows the order AlbD > AIIL > AIIC > AIID > AIIF > AIIG > AIIAB. The importance of C/EBP proteins for the regulation of transcription of the apoA-II gene needs to be determined.

Organization of the Different Activities along the Regulatory Elements A–N of the apoA-II Promoter and Their Importance for Transcription. The organization of the different activities which participate in the regulation of transcription of the human apoA-II gene, their importance for transcription, and their putative paths of transcription activation are shown in Figure 9A,B. Such activities may be of general importance for our understanding of the combination of factors which control liver-specific gene expression. The effect of mutations in different elements (deletion or nucleotide substitutions), which affected the binding of specific factors, on hepatic and intestinal transcription is indicated at the bottom of Figure 9A. This assignment is based on the information presented in Figures 2–8, as well as in references (Chambaz et al., 1991; Cardot et al., 1991). Table III compares the consensus binding site of C/EBP with that found on the albumin promoter and several regulatory elements of the apoA-II, apoA-I, apoB, and apoC-III promoters. Finally, Figure

Table III

												ref
C/EBP consensus sequence			T	T G	N	N	G	C T	A	A	T G		Ryden et al., 1987
A1bD coding strand	5′-	-107	T	T	T	T	G	T	A	A	-99 T	-3′	Maire et al., 1988
AIIC noncoding strand	5′-	-113	T	G	G	A	G	- A	A	· A	-121 T	-3′	Table II
AIIL noncoding strand	5′-	-778	T	T	A	G	C	C	A	A	-786 T	-3′	Table II
AIID noncoding strand	5′-	-262	G	A	A	T	G	C	A	A	-270 C	-3′	Table II
AIIF coding strand	5′-	-399	T	T	G	A	G	A	G	A	-391 T	-3′	Table II
AIIG coding strand	5′-	-471	G	A	T	T	T	C	A	A	-462 T	-3′	Table II
AIIAB noncoding strand	5′-	-39	T	T	A	C	C	C	A	C	-47 C	-3′	Table II
AIC noncoding strand	5′-	-148	C	T	G	G	G	C	A	A	-156 A	-3′	Papazafiri et al., 1991
AIC noncoding strand	5′-	-161	T	G	G	G	G	C	A	A	-169 A	-3′	Papazafiri et al., 1991
BA2 noncoding strand	5′-	-40	G	G	C	T	G	C	A	A	-48 G	-3′	Kardassis et al., 1990
BA3 coding strand	5′-	-66	T	T	T	T	G	C	A	A	-58 T	-3′	Kardassis et al., 1990
CIIIC noncoding strand	5′-	-127	T	G	C	A	G	C	A	A	-135 G	-3′	Ogami et al., 1990
CIIID coding strand	5′-	-155	T	T	T	C	C	C	A	A	-147 C	-3′	Ogami et al., 1990

9B shows putative paths of transcriptional activation of the apoA-II gene. Figure 9A,B and Table III are designed to facilitate our discussion. The precise role of the different activities on the regulation of transcription of the apoA-II gene is the subject of ongoing studies.

DISCUSSION

Background. The transcription of eukaryotic genes is controlled by the binding of specific nuclear proteins (transcription factors) to specific regulatory gene sequences (usually referred to as promoter elements, enhancers, or silencers). These interactions control tissue-specific gene expression and gene expression during differentiation and development and that in response to intra- and extracellular stimuli such as hormones and metabolites.

Human apoA-II is a protein which is synthesized almost exclusively in the liver (Hussain & Zannis, 1990). Understanding the regulation of synthesis of this protein may be important because of its potential role in the reverse transport of cholesterol. Furthermore, the strict tissue-specific expression of this gene makes it a good model to study mechanisms which control liver-specific gene expression.

Recent studies have shown that the transcription of the apoA-II gene is controlled by a core of regulatory elements, which can act as a liver-specific enhancer (Lucero et al., 1989; Chambaz et al., 1991; Cardot et al., 1991). This core consists of regulatory elements N–I and is localized between nucleotides –903 and –680 of the apoA-II promoter (Chambaz et al., 1991; Cardot et al., 1991). Previous studies also showed that deletions or nucleotide substitutions within regulatory elements K and L, or both, reduced hepatic and intestinal transcription to low levels (Cardot et al., 1991). This indicated that factor CIIIB1 and other factors which bind within these regulatory elements are required for hepatic and intestinal transcription of the human apoA-II gene. A similar reduction in transcription was observed by deletion of the regulatory element N (Chambaz et al., 1991). The objectives of this study were to assess the importance of each of the 14 regulatory elements of the human apoA-II gene for intestinal and hepatic transcription *in vitro* and to identify the different hepatic activities which bind to them.

Hepatic Activities Involved in the Regulation of the Human apoA-II Gene. Identification of activities that recognize the apoA-II promoter has been facilitated by our recent characterization of activities that bind to the regulatory elements of other apolipoprotein promoters (Ogami et al., 1990, 1991; Papazafiri et al., 1991; Kardassis et al., 1990a, 1992), as well as general progress in the purification and characterization

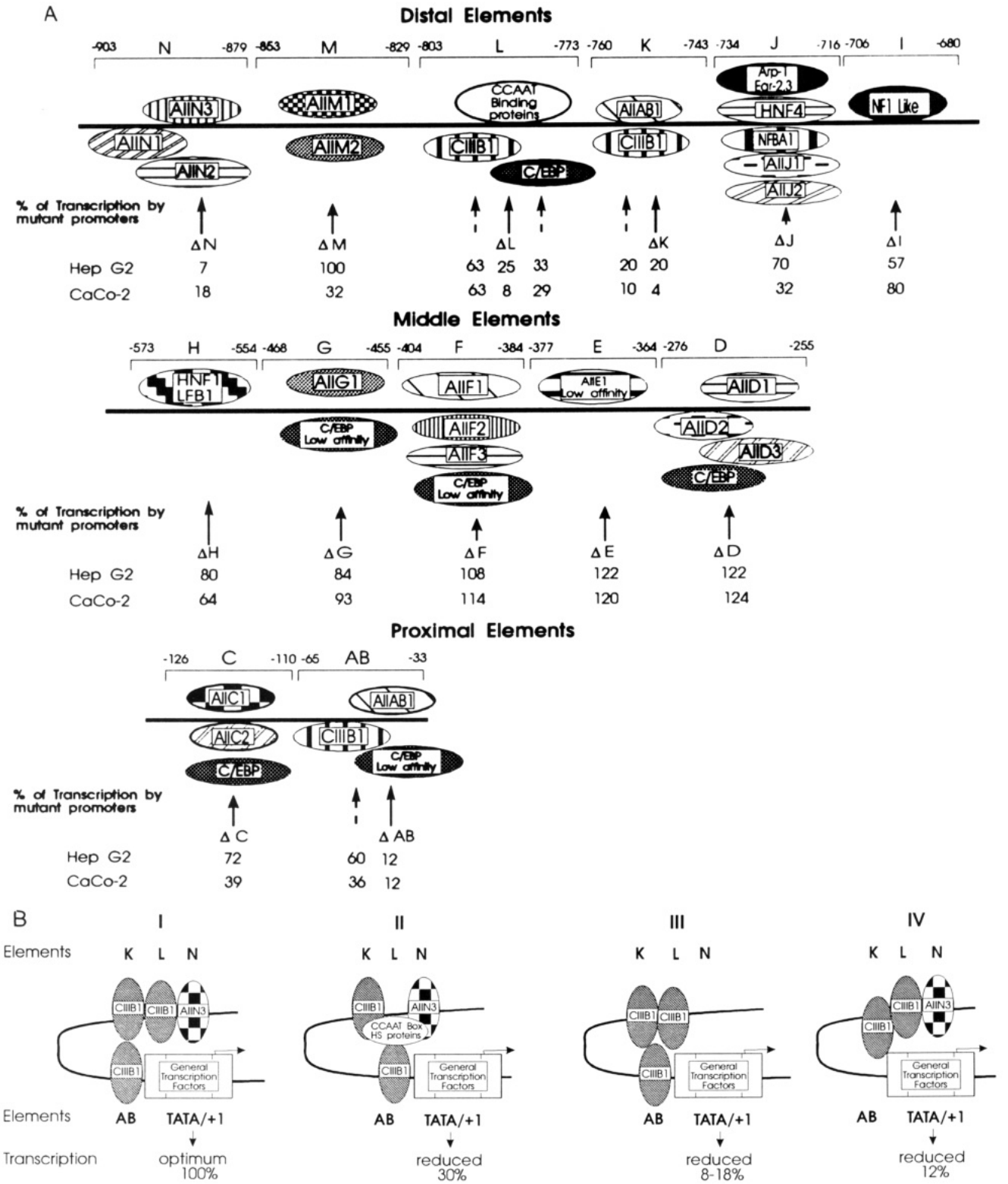

FIGURE 9: (A, top) Organization of the nuclear activities on the human apoA-II promoter elements. The boundaries of promoter elements A–N are indicated at the top of the figure. In this panel, the effect of deletions or nucleotide substitutions which affect the binding of the different factors on the promoter activity in HepG2 and CaCo-2 cells is indicated at the bottom of the figure. (B, bottom) Schematic presentation of putative regulatory paths which lead to different levels of transcriptional activation of the human apoA-II gene. Panel I shows optimal hypothetical interactions of CIIIB1 bound to elements L, K, and AB with the basal transcription factors which lead to optimal (100%) transcription. Panel II shows how hypothetical interactions of C/EBP (or other CCAAT binding proteins) bound to element L and CIIIB1 bound to elements K and AB with the basal transcription factors lead to reduced 30% transcription. Panels III and IV show how the elimination of factors AIIN3 and AB (by deletion of their cognate elements) disrupts the interaction of the remaining factors with each other and the basal transcription factors and thus further reduces the transcription to low levels (8–18%).

of several transcriptional activities (Hardon et al., 1988; Paonessa et al., 1988; Frain et al., 1989; Johnson & McKnight, 1989; Akira et al., 1990; Descombes et al., 1990; Gounari et al., 1990; Hooft van Huijsduijnen et al., 1990; Kardassis et al., 1990b, 1992; Mueller et al., 1990; Poli et al., 1990; Rangan & Das, 1990; Cladaras et al., 1991; Ogami et al., 1991; Ramji et al., 1991). On the basis of this information, a variety of oligonucleotides corresponding to the binding sites of known factors or to the regulatory sequences of apolipoproteins or other liver-specific genes were used in DNA binding

and competition assays. This analysis allowed the identification of (a) new activities, (b) activities such as CIIIB1, NF-BA1, and others which regulate other apolipoprotein genes, and (c) activities related to previously described factors C/EBP, HNF4, HNF1, and NF1.

Role of New Activities in the Regulation of Transcription of the Human apoA-II Gene. New activities bind to regulatory elements M (designated AIIM1 and AIIM2), D (designated AIID1 and AIID2), and F (designated AIIF2; Figure 9A). Some of these activities can be separated in different fractions by anion and cation exchange chromatography (Figures 3C and 6B). Deletion of element M reduced intestinal transcription to 32% of control without affecting hepatic transcription. This finding suggests that activity AIIM1 is important for optimum intestinal transcription of the apoA-II gene, whereas both AIIM1 and AIIM2 may not substantially affect the hepatic transcription of this gene. The factors which bind to element D have been designated AID1, AID2, and AIID3. The first two are new heat-labile factors and the latter is a heat-stable factor which may be related to the family of C/EBP proteins (Akira et al., 1990; Descombes et al., 1990; Poli et al., 1990; Cao et al., 1991; Williams et al., 1991).

Element D of the human apoA-II promoter contains a TGT-TGC motif which is similar to the cognate sites of EBP-40 and EBP-45. EBP-40 and EBP-45 interact with the human transferrin gene enhancer (Petropoulos et al., 1991). These proteins recognize the 5′-TGTTTGC-3′ motif present in element F of the mouse albumin promoter where C/EBP binds (Friedman et al., 1989). However, an antiserum directed against C/EBPα did not inhibit the formation of DNA–protein complexes of EBP-40 and EBP-45 with its cognate site of the human transferrin gene enhancer, indicating that these activities differ from that of C/EBP (Petropoulos et al., 1991).

A heat-stable activity designated AIIF1 also binds to regulatory element F. Deletion of element D or element F increased intestinal and hepatic transcription only slightly, suggesting that the factors which bind to these elements may not substantially affect hepatic and intestinal transcription *in vivo*. Deletion of element C reduced intestinal transcription to 39% of control, whereas it had a small effect on hepatic transcription. Element C is the binding site of two heat-stable activities. One (designated AIIC2) may be related to activities which bind to regulatory region D of the albumin promoter. The other, designated AIIC1, may be related to similar heat-stable activities which bind to regulatory element D of apoA-II. Some of these new activities described for the apoA-II promoter may contribute to optimum transcription but are not essential for hepatic transcription of the human apoA-II gene.

Role of Heat-Stable Activities and the C/EBP Family Member in the Regulation of Transcription of the Human apoA-II Gene. The heat-stable activities which bind to regulatory elements C, D, and F of apoA-II form broad bands following DNA binding gel electrophoretic assays (Figures 5–7). This may result from the binding of different homo- or heterodimeric forms. Such dimers may be formed among known or still unidentified C/EBP family members. Direct binding assays showed that purified C/EBPα produced by the expression of C/EBPα cDNA in bacteria binds with relatively high affinity to regulatory elements L, C, and D of apoA-II and with low affinity to regulatory elements F, G, and AB. DNA binding assays with crude hepatic nuclear extracts and competition experiments also suggest that besides C/EBPα other heat-stable activities bind with high affinity to regulatory elements L, C, and D and with low affinity to elements F, G, and AB.

C/EBPα can form homo- or heterodimers through its leucine zipper domain with other members of the C/EBP family, which include C/EBPβ, -γ, and -δ (Akira et al., 1990; Descombes et al., 1990; Poli et al., 1990; Cao et al., 1991; Williams et al., 1991). It has also been shown that C/EBPβ is involved in the regulation of hepatic genes during the acute phase response (Poli et al., 1990). Elements L and C contain the sequences TTAGCCAAT and TGGAGAAAT, respectively, which differ by one nucleotide (boldface type) from the consensus binding site of C/EBP [TT(orG)NNGC(orT)-AAT(orG)] (Ryden & Beemon, 1989). Elements D, F, G, and AB differ by two or more nucleotides from the consensus binding site of C/EBP (Table III). The relative higher binding affinity of C/EBPα for element D, as compared to element F, could be explained by the fact that the two mismatches in element F occur within the half-consensus site, GCAAT.

Cross-competition experiments showed that the heat-stable activities which bind to regulatory regions C and L of apoA-II may be similar to or identical with the activities which bind to regulatory regions C and D of apoC-III, C of apoA-I, and BA3 of apoB (see Table III). Two heat-stable nuclear activities, designated NF-BA2 and NF-BA3, have been fractionated from crude nuclear extracts and protected the same apoB regions as C/EBP (Kardassis et al., 1992). These activities bind to regulatory elements L of apoA-II and C and D of apoC-III (Cardot et al., 1991; Cladaras et al., 1991). However, it is possible that other heat-stable activities may, in addition, bind to regulatory regions D, F, G, and AB of the apoA-II promoter. The relationship of the heat-stable activities with the members of the C/EBP family will require further characterization by the utilization of specific antibodies which recognize the C/EBP family members.

The role of these proteins and different members of the C/EBP family in apoA-II gene transcription will require further *in vivo* and *in vitro* studies. Thus, cotransfection experiments of C/EBP expression plasmids with the wild-type and mutated promoters in elements C, D, and L will clarify the role of different C/EBP members for transcription in HepG2 and CaCo-2 cell cultures. Similarly, expression of wild-type and mutated constructs in elements C and L in transgenic mice will clarify the role of these elements in intestinal and hepatic transcription *in vivo*. Previous analysis showed that mutations which affected the binding of these proteins to the distal regulatory region (element L) of apoA-II promoter reduced both hepatic and intestinal transcription to 30% of the control (Cardot et al., 1991). Furthermore, the current study suggests that the binding of these proteins to the proximal region (regulatory element C) may also be important for optimum intestinal transcription of the human apoA-II gene. It will be interesting to assess whether mutations in these elements affect similarly the hepatic and intestinal transcriptions *in vivo*.

Role of CIIIB1 and HNF1 and NF1 in the Regulation of Transcription of the Human apoA-II Gene. The apoA-II promoter contains binding sites for previously described factors CIIIB1 and NF1 (Cardot et al., 1991). Previous studies showed that CIIIB1 binds to regulatory elements AB, K, and L of apoA-II and element B of apoC-III (Cardot et al., 1991; Ogami et al., 1991). Mutations which affected the binding of CIIIB1 to all of the apoA-II elements reduced hepatic and intestinal transcription to control levels, indicating that CIIIB1 is important for the regulation of the apoA-II gene (Cardot et al., 1991). However, mutations which prevented the binding of C/EBP family members and other CCAAT box proteins without affecting the binding of CIIIB1 reduced hepatic and intestinal transcription to 30% of the control. This indicates that either C/EBP family members or CIIIB1 bound to

element L may act synergistically with other factors bound to the proximal regulatory elements to promote tissue-specific transcription of the human apoA-II gene.

This hypothesis is further supported by the observation that deletion of element L, which precludes the binding of both CIIIB1 and C/EBP type activities to this region, reduced hepatic and intestinal transcriptions to 25% and 8% of control, respectively (Cardot et al., 1991). Previous studies also showed that deletion of the middle regulatory region (614–230) reduced hepatic transcription to 60% of the control, whereas it increased intestinal transcription 1.4-fold. Deletion of the individual elements within this region likewise causes small decreases (when elements H and G are deleted) or increases (when elements E, F, and D are deleted) in hepatic and intestinal transcription. It is very interesting that element H is a binding site for the liver-enriched factor HNF1/LFB1 and element I is a binding site for NF1 (Jones et al., 1987). HNF1 is a mammalian homeodomain transcription factor, which plays a very important role in the regulation of several liver-specific genes (Frain et al., 1989), whereas NF1 is a ubiquitous factor that has been implicated in gene transcription as well as in DNA replication (Paonessa et al., 1988; Jones et al., 1987). Deletion analysis of the apoA-II promoter showed that both HNF1 and NF1 contribute to the optimum transcription but are not essential for hepatic expression of the apoA-II gene.

Role of Nuclear Receptors in the Regulation of Transcription of the Human apoA-II Gene. Regulatory element J forms two DNA–protein complexes, designated AIIJ1 and AIIJ2. DNA binding and competition assays and protein fractionation showed that complex AIIJ2 consists of two activities, one of which is similar to the previously described factor NF-BA1 (Kardassis et al., 1990b). This factor binds to regulatory regions –88 to –62 of apoB, –87 to –72 of apoC-III, and –220 to –190 of apoA-I (Kardassis et al., 1990b) and acts as a positive regulator of these genes. Mutations in the apoB and apoC-III promoters, which reduced the binding of this factor to its cognate sequence, reduced apoB and apoC-III transcription to background levels (Ogami et al., 1991; Kardassis et al., 1990a, 1992). Regulatory element J is also a binding site for transcription factors HNF4, EAR2, EAR3, and ARP1 (Figure 3). These proteins belong to the orphan receptor subfamily, for which the activation ligand is not known, and can form homo- or heterodimers *in vitro* (Sladek et al., 1990; Ladias & Karathanasis, 1991; Ritchie et al., 1990; Miyajima et al., 1988). The first three proteins have 90% sequence homology in their DNA binding domains and approximately 50–60% homology with the DNA binding domain of HNF4 (Ladias et al., 1992). It has been shown that HNF4 is a positive regulator and that EAR2, EAR3, and ARP1 are negative regulators of the human apoC-III, apoB, and apoA-I genes (Ladias et al., 1992; Ladias & Karathanasis, 1991; Mietus-Snyder et al., 1992). Deletion of regulatory element J reduced hepatic and intestinal transcriptions by 64% and 32% of control, respectively, and abolished the activation of apoA-II by HNF4 (Cardot et al., 1991; Ladias et al., 1992).

Recently it was also shown that regulatory element J of apoA-II binds to heterodimers of thyroid hormone receptor β with *Drosophila chorion* transcription factor ultraspiracle (Khoury et al., 1992). It is interesting that neither ultraspiracle, which is a homologue of the mammalian transcription factor retinoic X receptor, nor thyroid hormone receptor β can bind to element J as homodimers. Thus, it is possible that different homo- or heterodimeric combinations of nuclear receptors may modulate the transcription of the apoA-II gene by binding to element J without being essential for the hepatic or the intestinal transcription of this gene.

Overall, systematic analysis of the apoA-II promoter did establish that, while elements A, B, K, and L are the most crucial for hepatic transcription, three additional elements (J, M, and C) play an important role in intestinal transcription. This indicates that the mode of intestinal and hepatic regulation must encompass similar as well as unique interactions among factors bound to different sites. Systematic analysis of the intestinal activities which bind to the 14 regulatory elements will be required to address this question. In addition to the observed differences between proximal hepatic and intestinal regulatory elements and factors, other differences may exist in the 5′ upstream regulatory region which can be only assessed by expression of the apoA-II gene in transgenic mice. Similarly, the transgenic mice approach will also be required to assess the role of elements H, I, and J, which are the binding sites of HNF1, NF1, and nuclear receptors for the hepatic and intestinal transcription of the human apoA-II gene *in vivo*.

Are There Multiple Paths of Transcriptional Activation for a Single Gene? The analysis of the apoA-II promoter shows the participation of several transcription factors in the regulation of a single gene (Figure 9A). Some of these factors may occupy identical or overlapping binding sites, indicating a redundancy of factors which can regulate this and possibly other genes. Our findings also suggest the cooperation of factors which bind to the proximal and distal regulatory elements. The contribution of a specific transcription factor, such as NF-BA1 or members of the orphan receptor subfamily, HNF1/LFB1, C/EBPα, etc., on the rate of transcription of a target gene depends on their relative positions on a specific promoter. For instance, previous studies have shown that HNF1/LFB1, HNF4, and members of the nuclear receptors play an important role in the regulation of other liver-specific genes, but are not essential for the hepatic and intestinal transcription of the human apoA-II (Ladias et al., 1992; Martinez et al., 1991; Frain et al., 1989). It is expected that the position of a factor on a specific promoter will determine its interactions with other factors as well as with the general transcription factors.

An important question is whether different combinations of proximal with distal regulatory factors can result in different levels of transcription of the apoA-II gene. A few such possibilities are suggested by our mutagenesis analysis and are shown in Figure 9B. In one hypothetical path (panel I), the interaction of CIIIB1 bound to elements I, K, and AB with the basal transcription factors leads to optimum apoA-II gene transcription. In another path (panel II), interaction of C/EBP or other CCAAT binding proteins bound to element L with CIIIB1 bound to elements K and AB and the basal transcription factors leads to 30% transcription. Finally, in two other cases (panels III and IV), deletion of elements N and AB, respectively, which are the binding sites of factors AIIN3 and CIIIB1, disrupts the synergism of the factors and/or their interaction with the basal transcription factors and diminishes hepatic and intestinal transcription. Several other combinations of factors may lead to different levels of intestinal and hepatic transcription. Elucidation of such mechanisms of gene activation will require isolation of the factors involved and precise probing on how the interaction(s) of a set of tissue-specific and ubiquitous factors can possibly enhance or suppress the transcription of a target gene.

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REFERENCES

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., & Kishimoto, T. (1990) *EMBO J. 9*, 1897–1906.

Bos, T. J., Bohman, D., Tsuchie, H., Tjian, R., & Vogt, P. K. (1988) *Cell 52*, 705–712.

Brewer, H. B., Jr., Lux, S. E., Ronan, R., & John, K. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1304–1308.

Cao, Z., Umek, R. M., & McKnight, S. L. (1991) *Genes Dev. 5*, 1583–1552.

Cardot, P., Chambaz, J., Cladaras, C., & Zannis, V. I. (1991) *J. Biol. Chem. 266*, 24460–24470.

Chambaz, J., Cardot, P., Pastier, D., Zannis, V. I., & Cladaras, C. (1991) *J. Biol. Chem. 266*, 11676–11685.

Cheung, M. C., & Albers, J. J. (1984) *J. Biol. Chem. 259*, 12201–12209.

Cladaras, C., Ogami, K., Kardassis, D., Hadzopoulou-Cladaras, M., & Zannis, V. I. (1991) *Circulation 84*, 109.

Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E., & Schibler, U. (1990) *Genes Dev. 4*, 1541–1551.

DeSimone, V., Ciliberto, G., Hardon, E., Paonessa, G., Palla, F., Lundberg, L., & Cortese, R. (1987) *EMBO J. 6*, 2759–2766.

Doolittle, M. H., LeBoeuf, R. C., Warden, C. H., Bee, L. M., & Lusis, A. J. (1990) *J. Biol. Chem. 265*, 16380–16388.

Dorn, A., Bollekens, J., Staub, A., Benoist, C., & Mathis, D. (1987) *Cell 50*, 863–872.

Edlund, T., Walker, M. D., Barr, P. J., & Rutter, W. J. (1985) *Science 230*, 912–916.

Frain, M., Swart, G., Monaci, P., Nicosia, A., Stämpfli, S., Frank, R., & Cortese, R. (1989) *Cell 59*, 145–157.

Friedman, A. D., Landschulz, W. H., & McKnight, S. L. (1989) *Genes Dev. 8*, 1314–1322.

Fruchart, J. C., & Bard, J. M. (1991) *Curr. Opin. Lipid 2*, 362–366.

Gorman, C. M., Moffat, L. F., & Howard, B. H. (1982) *Mol. Cell. Biol. 2*, 1044–1051.

Gorski, K., Carneiro, M., & Schibler, U. (1986) *Cell 47*, 767–776.

Gounari, F., De Franscesco, R., Schmitt, J., van der Vliet, P., Cortese, R., & Stunnenberg, H. (1990) *EMBO J. 9*, 559–566.

Graham, F. L., & van der Eb, A. J. (1973) *Virology 52*, 456–467.

Hardon, E. M., Frain, M., Paonessa, G., & Cortese, R. (1988) *EMBO J. 7*, 1711–1719.

Hooft van Huijsduijnen, R. H., Li, X. Y., Black, D., Matthes, H., Benoist, C., & Mathis, D. (1990) *EMBO J. 9*, 3119–3127.

Hussain, M. M., & Zannis, V. I. (1990) *Biochemistry 29*, 209–217.

Johnson, P. F., & McKnight, S. L. (1989) *Annu. Rev. Biochem. 58*, 799–839.

Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J., & Tjian, R. (1987) *Cell 48*, 79–89.

Kardassis, D., Hadzopoulou-Cladaras, M., Ramji, D. P., Cortese, R., Zannis, V. I., & Cladaras, C. (1990a) *Mol. Cell. Biol. 10*, 2653–2659.

Kardassis, D., Zannis, V. I., & Cladaras, C. (1990b) *J. Biol. Chem. 265*, 21733–21740.

Kardassis, D., Zannis, V. I., & Cladaras, C. (1992) *J. Biol. Chem. 267*, 2622–2632.

Khoury Christianson, A. M., King, D. L., Hatzivassiliou, E., Casas, J. E., Hallenbeck, P. L., Nikodem, V. M., Mitsialis, S. A., & Kafatos, F. C. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11503–11507.

Knott, T. J., Priestley, L. M., Urdea, M., & Scott, J. (1984) *Biochem. Biophys. Res. Commun. 120*, 734–740.

Lackner, K. J., Law, S. W., & Brewer, H. B., Jr. (1984) *FEBS Lett. 175*, 159–164.

Ladias, J. A., & Karathanasis, S. K. (1991) *Science 251*, 561–565.

Ladias, J. A. A., Hadzopoulou-Cladaras, M., Kardassis, D., Cardot, P., Cheung, J., Zannis, V., & Cladaras, C. (1992) *J. Biol. Chem. 267*, 15849–15860.

Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J., & McKnight, S. L. (1988) *Genes Dev. 2*, 786–800.

Lucero, M. A., Sanchez, D., Ochoa, A. R., Brunel, F., Cohen, G. N., Baralle, F. E., & Zakin, M. M. (1989) *Nucleic Acids Res. 17*, 2283–2300.

Lusis, A. J. (1988) *J. Lipid Res. 29*, 397–429.

Maire, P., Wuarin, J., & Schibler, U. (1989) *Science 244*, 343–346.

Martinez, E., Givel, F., & Wahli, W. (1991) *EMBO J. 10*, 263–268.

Mietus-Snyder, M., Sladeck, F. M., Ginsburg, G. S., Kuo, C. F., Ladias, J. A., Darnell, J. E., Jr., & Karathanasis, S. K. (1992) *Mol. Cell. Biol. 12*, 1708–1718.

Mitchell, P. J., Wang, C., & Tjian, R. (1987) *Cell 50*, 847–861.

Miyajima, N., Kadowaki, Y., Fukushige, S., Shimizzu, S., Semba, K., Yamanashi, Y., Matsubara, K., Toyoshima, K., & Yamamoto, T. (1988) *Nucleic Acids Res. 16*, 11057–11074.

Mueller, C. R., Maire, P., & Schibler, U. (1990) *Cell 61*, 279–291.

Ogami, K., Hadzopoulou-Cladaras, M., Cladaras, C., & Zannis, V. I. (1990) *J. Biol. Chem. 265*, 9808–9815.

Ogami, K., Kardassis, D., Cladaras, C., & Zannis, V. I. (1991) *J. Biol. Chem. 266*, 9640–9646.

Ohta, T., Hattori, S., Nishiyama, S., & Matsuda, I. (1988) *J. Lipid Res. 29*, 721–728.

Paonessa, G., Gounari, F., Frank, R., & Cortese, R. (1988) *EMBO J. 7*, 3115–3123.

Papazafiri, P., Ogami, K., Ramji, D. P., Nicosia, A., Monaci, P., Cladaras, C., & Zannis, V. I. (1991) *J. Biol. Chem. 266*, 5790–5797.

Petropoulos, I., Auge-Gouillou, C., & Zakin, M. (1991) *J. Biol. Chem. 266*, 24220–244225.

Poli, V., Mancini, F. P., & Cortese, R. (1990) *Cell 63*, 643–653.

Ramji, D. P., Tadros, M. H., Hardon, E. M., & Cortese, R. (1991) *Nucleic Acids Res. 19*, 1139–1146.

Rangan, V. S., & Das, G. C. (1990) *J. Biol. Chem. 265*, 8874–8879.

Ritchie, H. H., Wang, L. H., Tsai, S., O'Malley, B. W., & Tsai, M. J. (1990) *Nucleic Acids Res. 18*, 6857–6862.

Ryden, T. A., & Beemon, K. (1989) *Mol. Cell. Biol. 9*, 1155–1164.

Scheidereit, C., Heguy, A., & Roeder, R. G. (1987) *Cell 51*, 783–793.

Schonfeld, G., Patsch, W., Rudel, L. L., Nelson, C., Epstein, M., & Olson, R. E. (1982) *J. Clin. Invest. 69*, 1072–1080.

Schultz, J. R., Clift, S. M., & Rubin, E. M. (1991) *Circulation 84*, 395.

Sharpe, C. R., Sidoli, A., Shelley, C. S., Lucero, M. A., Shoulders, C. C., & Baralle, F. E. (1984) *Nucleic Acids Res. 12*, 3917–3932.

Shelley, C. S., & Baralle, F. E. (1987) *Nucleic Acids Res. 15*, 3801–3821.

Sladek, F. M., Zhong, W. M., Lai, E., & Darnell, J. E., Jr. (1990) *Genes Dev. 4*, 2353–2365.

Tsao, Y. K., Wei, C. F., Robberson, D. L., Gotto, A. M., Jr., & Chan, L. (1985) *J. Biol. Chem. 260*, 15222–15231.

Williams, S. C., Cantwell, C. A., & Johnson, P. F. (1991) *Genes Dev. 5*, 1553–1567.