Purification and Characterization of Nuclear Factors Binding to the Negative Regulatory Element D of Human Apolipoprotein A-II Promoter: A Negative Regulatory Effect Is Reversed by GABP, an Ets-Related Protein†

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ABSTRACT: We have previously shown that transcription of the human apolipoprotein A-II (apoA-II) gene is controlled by a complex set of regulatory elements [Cardot et al. (1993) *Biochemistry 32*, 9080–9093]. We have also identified previously described, as well as new activities which bind to these elements and influence to varying degrees the transcription of the apoA-II gene. DNA binding and competition assays indicated that element D binds three new activities, designated AIID1, AIID2, and AIID4, as well as an activity related to C/EBP. Activities AIID1, AIID2, and AIID4 were purified and characterized further in order to determine their function on the transcriptional regulation of human apoA-II gene. SDS–PAGE analysis as well as photoaffinity cross-linking of the affinity-purified AIID2 showed that it consists of three proteins with molecular masses ranging between 54 and 63 kDa. The amino acid sequence of tryptic peptides obtained from AIID2 protein bands revealed that it is homologous to GABP, an Ets-related protein. Similar analysis showed that affinity-purified AIID4 has an apparent molecular mass of 130 kDa. AIID1 activity was purified partially; in addition to binding to the apoA-II promoter, AIID1 also binds to the regulatory element C of apoCIII and may play a role in the transcriptional regulation of both genes. Methylation interference of G residues and permanganate modification of T residues indicated that the binding sites of AIID2 and AIID4 were contiguous on element D. However, the binding site of AIID1 overlaps with the binding sites of both AIID2 and AIID4. This suggests that the binding of AIID1 and AIID2 or of AIID1 and AIID4 may be mutually exclusive, whereas AIID2 and AIID4 may bind simultaneously. Transcription from a minimal promoter containing elements AB, C, and D of apoA-II increased 1.5- to 1.6-fold when element D is deleted, as well as by promoter mutations which eliminated the binding of both AIID1 and/or AIID4 to element D, but permitted the binding of AIID2/GABP. The findings suggest that element D has a negative regulatory role on apoA-II gene transcription when it is occupied by protein AIID1 and/or AIID4. This negative effect is reversed when element D is occupied only by the regulatory factor AIID2/GABP.

Human apolipoprotein A-II (apoA-II) is a major protein component of HDL and is synthesized by the liver and to a much lesser extent by the intestine (Schonfeld et al., 1982; Brewer et al., 1972; Hussain & Zannis, 1990). Recent genetic studies in mouse and humans have pointed out that apoA-II is a risk factor for atherosclerosis (Lusis, 1988; Doolittle et al., 1990; Warden et al., 1993a). Overexpression of mouse apoA-II in transgenic mice results in elevated HDL cholesterol and the development of fatty streak lesions as compared to normal mice (Warden et al., 1993b). Transgenic mice overexpressing both human apoA-I and apoA-II are protected less against atherosclerosis in response to an atherogenic diet as compared to transgenic mice overexpressing apoA-I alone. However, both the apoA-I and apoA-I/A-II expressing mice were less susceptible to diet-induced atherosclerosis than the control C57BL/B mice (Schultz et al., 1993). These *in vivo* observations are further supported by *in vitro* experiments. ApoA-II-containing HDL lipoparticles do not stimulate lecithin–cholesterol acyltransferase (Vanloo et al., 1992; Bolin & Jonas, 1993) and behave as a poor cholesteryl ester substrate donor for the cholesteryl ester transfer protein (Lagrost et al., 1994; Mowri et al., 1993) as compared to apoA-I-containing HDL particles. Although the role of LpA-I as compared to LpA-I/A-II particles on efflux of cholesterol from cells remains unclear (Barbaras et al., 1987; Johnson et al., 1993; Oikawa et al., 1993), the existing data are consistent with the hypothesis that apoA-II-containing HDL may be a risk factor for coronary artery disease through a mechanism which is not fully understood at the present time.

We and others (Shelley & Barralle, 1987; Lucero et al., 1989; Chambaz et al., 1991; Cardot et al., 1991, 1993) have previously shown that the transcription of the human apoA-II gene is controlled by a complex array of distal and proximal regulatory elements, designated A to N, that have been divided in three functional regions. The distal region (–911/–614) consists of elements N to I and displays an enhancer-type activity in HepG2 cells when placed in front of heterologous promoters (Shelley & Baralle, 1987; Hadzopoulou-Cladaras & Cardot, 1993). The middle region (–573/–255) consists of elements F to D, and the proximal region (–126/–33) consists of elements C to A. Recently, we have also characterized several previously described, as well as new transcription factors which bind to these elements (Cardot et al., 1993). More specifically, an important regulatory role is exerted by factor CIIIB1 (Ogami et al., 1991) which binds to the regulatory elements AB, K, and L. An important role

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

AIID	$^{-278}$TGCTTCCTGTTGCATTCAAGTCCAAG^{-253}	Cardot et al., 1993
AIIDM1	$^{-278}$GTAGGCCTGTTGCATTCAAGTCCAA^{-253}	this study
AIIDM2	$^{-278}$TGCTTCCTGTTGTCTTACCGTCCAAG^{-253}	this study
AIC	$^{-178}$AGCTTCCTGTTTGCCCACTCTATTTGCCCAGCCCCAG^{-142}	Papazifiri et al., 1991
AID	$^{-218}$GCCCCCACTGAACCCTTGACCCCTGCCCTGC^{-188}	Papazifiri et al., 1991
CIIIB	$^{-92}$GGTCAGCAGGTGACCTTTGCCCAGCG^{-67}	Ogami et al., 1990
CIIIC	$^{-138}$CGCTTGCTGCATCTGGACA^{-119}	Ogami et al., 1990
TK/CEBP	$^{-96}$GCGTCTTGTCATTGGCGAATTCG^{-74}	Dorn et al., 1987
A1bD	$^{-115}$TGGTATGATTTTGTAATGGGGTAGGA-3′	Maire et al., 1989
NF1	$^{-133}$ACAATTTTTTGGCAAGAATATTAT^{-110}	Maire et al., 1989
HNF1	$^{-70}$AGTATGGTTAATGATCTACAG^{-50}	Maire et al., 1989
HNF3	$^{-111}$GTTGACTAAGTCAATAATCAGA^{-90}	Costa et al., 1989
PEA3	5′-GATCTCGAGCAGGAAGTTCGAT-3′	Bhat et al., 1992

is also exerted by a new factor designated AIIN3 which binds to the regulatory element N. Simultaneous nucleotide substitutions which prevented the binding of CIIIB1 activity in elements A, B, K, and L reduced hepatic and intestinal transcription to 6–7% of control (Chambaz et al., 1991; Cardot et al., 1991). Deletion of element N also reduced hepatic transcription to 8% of the control. We have also identified high-affinity binding sites for activities related to C/EBP family members on elements C and L as well as low-affinity binding sites for such activities on elements D, F, G, and AB (Cardot et al., 1993). Binding sites for the NF1 have been identified on element I. Finally, binding sites for the previously described liver-enriched factors HNF1 (Frain et al., 1989) and HNF4 (Sladek et al., 1990) have been localized on elements H and J, respectively (Cardot et al., 1993). Site J is also recognized by Arp-1, EAR-2, and EAR-3 (Cardot et al., 1993) as well as homo- and heterodimers of nuclear receptors. Systematic mutagenesis of the apoA-II promoter has shown that deletion of most of the regulatory elements of apoA-II decreases to different levels the transcription of a reported CAT gene in HepG2 and CaCo-2 cells. However, a small but reproducible increase in transcription is observed when elements D, C, and E are deleted (Cardot et al., 1993). In the present study, we have purified and characterized the activities AIID1, AIID2, and AIID4 which bind to element D of apoA-II and determined their role on the negative regulation of the human apoA-II gene. We have found that the binding of AIID1 and/or AIID4 to element D is associated with reduced transcription, whereas the binding of AIID2/GABP is associated with increased transcription.

EXPERIMENTAL PROCEDURES

Materials. The Klenow fragment of DNA polymerase I, T4 DNA ligase, and restriction enzymes were purchased from New England Biolabs. Polynucleotide kinase and double-stranded poly(dI-dC) were purchased from Pharmacia. [α-^{32}P]dCTP and [α-^{32}P]dGTP (3000 Ci/mmol) were obtained from Amersham, and [γ-^{32}P]ATP (>5000 Ci/mmol) and [^{14}C]chloramphenicol (60 mCi/mmol) were purchased from ICN. Protease inhibitors, nitrophenyl β-D-galactopyranoside, potassium permanganate, Q-Sepharose (anion exchanger), and cyanogen bromide-activated Sepharose 4B were purchased from Sigma. Bio-Rex-70 (cation exchanger), acrylamide, *N*,*N*′-methylenebis(acrylamide) (electrophoresis grade), and protein markers were obtained from Bio-Rad. Bromodeoxyuridine cyanoethyl phosphoramidite was purchased from Millipore. The sequencing kit was purchased from United States Biochemical.

Synthetic Oligonucleotides. Synthesis, purification, annealing, and labeling of synthetic oligonucleotides were performed as described previously (Ogami et al., 1990).

Plasmid Construction. The –298/+29 CAT plasmid was generated as described previously (Chambaz et al., 1991). An apoA-II fragment, –298/+29, was obtained by DNA amplification of the pUC-AII as a template (Chambaz et al., 1991). The 5′ primer was PCRAII-1 ($^{-298}$GGG AAA TAA CTT GGA ATC CTG CTT CCT GTT GCA TTC AAG TCC A^{-255}), and the reverse 3′ primer was PCRAII-2 (ATC CTG CTC GAG^{+29} CAG CGT CTC TGT CCT TGG TG^{+10}) containing an *Xho*I site. The amplified fragment was digested with *Xho*I, purified by electroelution, and cloned into the *Sma*I and *Xho*I sites of the pUCSH-CAT vector (Ogami et al., 1990). The sequence of the recombinant vector was validated by DNA sequencing. Nucleotide substitution mutants were also generated by PCR amplification using, as 5′ primer, the mutated oligonucleotides PCRAIIM2 (ATC TAG AAA GCT $^{-281}$TCC GTA GGC CCT GTT GCA TTC AAG TCC AA-3′) and PCRAIIM4 (CTA GAA AGC T^{-281}TC CTG CTT CCT GTT GTC TTA CCG TCC AAG GAC) and, as 3′ primer, the oligonucleotide PCRAII-2. The amplified DNA fragment was digested with *Hin*dIII and *Xho*I and cloned into the corresponding sites of the pUCSH-CAT.

Cell Transfection and CAT Assay. These different plasmids were cotransfected with the RSV β-Gal plasmid in HepG2 cells using the calcium phosphate–DNA coprecipitation method (Graham & van der Eb, 1973). CAT assays were performed as described previously (Gorman et al., 1982); β-galactosidase activity was determined to normalize for variabilities in the efficiency of transfection (Edlund et al., 1985).

DNA Binding Gel Electrophoresis and Competition Assays. Nuclear extracts or various chromatographic fractions were preincubated for 15 min at 4 °C in a 20-μL reaction solution 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 25- to 500-fold excess of competitor oligonucleotide (Fried et al., 1981). Labeled double-stranded oligonucleotide, corresponding to the element D of apoA-II promoter or to the mutated AIIDM2, AIIDM4 (60 000 cpm), was then added and the incubation carried on 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, 1 mM EDTA, pH 7.9). The gel was dried and exposed to X-ray film. The sequences of the synthetic oligonucleotides used as competitors are presented in Table 1.

Purification of Nuclear Factors. All buffers contained 1 mM dithiothreitol, 0.1 mM benzamidine, 2 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin which was 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. The purification scheme is shown in Figure 1. Nuclear extracts were prepared from livers of 190 rats as described (Gorski et al., 1986). The extracts were pooled (300 mL, 1.729 g of protein), dialyzed twice against NDB containing 40 mM KCl,

and submitted to successive purification steps. AIID binding activities of the different fractions were analyzed by DNA-binding gel electrophoresis. Dialyzed nuclear extracts were applied to a Q-Sepharose column (5 × 30 cm; 290 mL bead volume), equilibrated in NDB containing 40 mM buffer at a flow rate of 480 mL/h. (The AIID3 activity was recovered in the 0.2 M KCl fraction. DNA binding competition experiments showed that this activity is related to C/EBP, and thus it was not purified further.) AIID1, AIID2, and AIID4 activities were recovered in the fraction eluting at 0.3 M KCl from Q-Sepharose. The 0.3 M KCl fraction was dialyzed against NDB, applied to a Bio-Rex-70 column (5 × 30 cm; 200 mL) equilibrated in NDB containing 40 mM KCl, and eluted stepwise with a 0.2–1 M KCl gradient in NDB.

DNA sequence-specific affinity chromatography was then performed according to Kadanoga and Tjian (1986), using as a ligand the double-stranded synthetic oligonucleotide AIID (Table 1). Bio-Rex-70 fractions eluted at 0.2 and 0.3 M KCl which contain the AIID2 and AIID4 activities, respectively, were dialyzed against NDB containing 40 mM NaCl, adjusted with 100 μg/mL sonicated herring sperm DNA and 10 μg/mL dI-dC DNA, and were loaded onto the DNA sequence-specific affinity chromatography columns equilibrated in NDB containing 40 mM NaCl. The column was eluted with a step gradient of 0.5 and 1 M NaCl in NDB. The AIID2 and AIID4 activities were recovered in the 0.5 M NaCl fractions of the affinity column.

Partial Amino Acid Sequencing. Final purification for amino acid sequencing was achieved by applying the sample to an SDS–polyacrylamide gel and electroblotting to PVDF membrane (Immobilon, Millipore). The membrane was stained with Coomassie blue, and the protein bands were excised for *in situ* tryptic digestion. The eluted tryptic peptides were fractionated by reverse-phase HPLC chromatography. The material in selected peaks from the tryptic digest was analyzed further by peptide sequencing.

SDS–Polyacrylamide Gel Electrophoresis and Photoaffinity Cross-Linking. SDS–polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970). Nuclear activities were submitted to 12% PAGE in the presence of low molecular weight protein markers. Gels were stained with 0.25% Coomassie Brilliant blue R-250. Photoaffinity cross-linking of affinity-purified AIID2 and AIID4 proteins to their cognate DNA sequence was performed as described (Cereghini et al., 1988), using a labeled double-stranded oligonucleotide corresponding to the regulatory element D (–278 to –253) of the apoA-II promoter in which all the T residues of the coding strand were substituted with 5-bromodeoxyuridine residues (Ogami et al., 1990).

Methylation Interference Assay and Thymidine-Specific Modification by $KMnO_4$. Methylation interference assays were performed as described (Ogami et al., 1990). Thymidine DNA modifications were performed as described by Truss et al. (1990). Each strand of the synthetic oligonucleotide corresponding to the regulatory element D of the apoA-II promoter was labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase. DNA was dissolved in 30 mM Tris-HCl buffer (pH 8) containing 2 μg of salmon sperm DNA and 0.25 mM KCl, and the samples were incubated 10 min at room temperature. Reactions were stopped by mixing 50 μL of stop buffer (1.5 M sodium acetate, pH 7.0, 1.0 M 2-mercaptoethanol) and 180 μL of distilled water. After ethanol precipitation, each labeled strand was annealed with its unlabeled complementary strand. Methylated and modified-thymidine probes were incubated with purified factors and analyzed by preparative DNA binding gel electrophoresis. Complexed and free oligonucleotides were detected by autoradiography, excised from the gel, purified by electroelution, and treated with 1 M piperidine at 90 °C for 30 min (Maxam & Gilbert, 1977). Samples were subsequently analyzed by 20% polyacrylamide/urea gels using polyacrylamide electrophoresis followed by autoradiography.

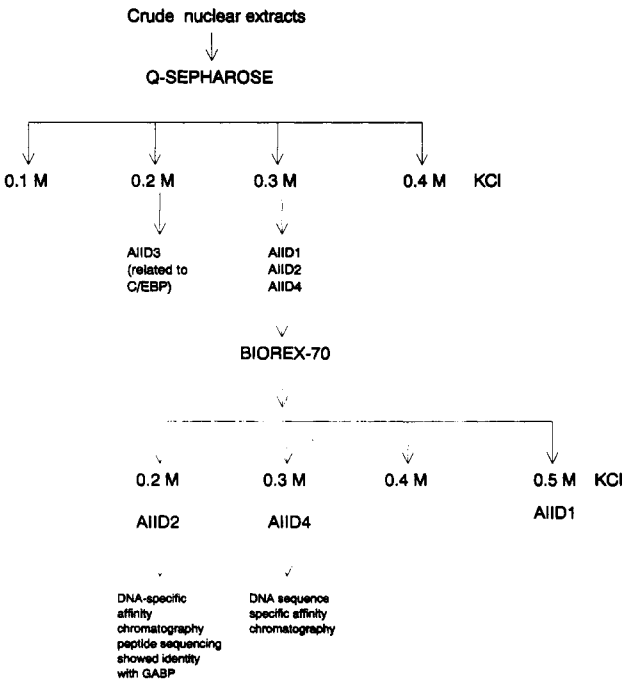

FIGURE 1: Schematic representation of the simultaneous purification of transcription factors AIID1, AIID2, AIID3, and AIID4.

RESULTS

Purification of the Hepatic Nuclear Activities Which Bind to Regulatory Element D of the Human ApoA-II Gene. We have previously reported that element D of apoA-II forms a broad band composed of at least three superimposed DNA complexes: two major heat-labile activities AIID1 and AIID2, and a heat-stable complex AIID3, which results from the binding of C/EBP-related proteins (Cardot et al., 1993). Figures 1 and 2A,B show the fractionation of these binding activities by two successive steps of ion-exchange chromatography. The anion-exchange Q-Sepharose column separates the AIID3 which elutes at 0.2 M KCl from the other activities (AIID1, AIID2, and AIID4) which elute at 0.3 M KCl (Figure 2A). Using cation-exchange chromatography on a Bio-Rex-70 column, AIID2 activity elutes at 0.2 M KCl, AIID4 at 0.3 M KCl, and AIID1 at the 0.5 M KCl fraction (Figure 2B). AIID4 represents an additional activity which was revealed by DNA binding gel electrophoresis assay of the 0.3 M KCl fraction of the Q-Sepharose Bio-Rex-70 column (Figure 2A,B). This activity apparently exists at a low abundance in the crude nuclear extracts and was further concentrated in this fraction. Alternatively, AIID4 could represent a degradation product of another activity. Such degradation may occur during the purification process. AIID1 activity purified by Bio-Rex-70 chromatography also binds strongly to the regulatory region C of apoCIII promoter (Figure 2B). Previous DNA binding competition experiments also showed that AIID1 activity is competed out by oligonucleotides corresponding to the C regulatory region (–160 to –142) of apoCIII (Ogami et al., 1990; Cardot et al., 1993). The purification of this activity was not pursued further in this study.

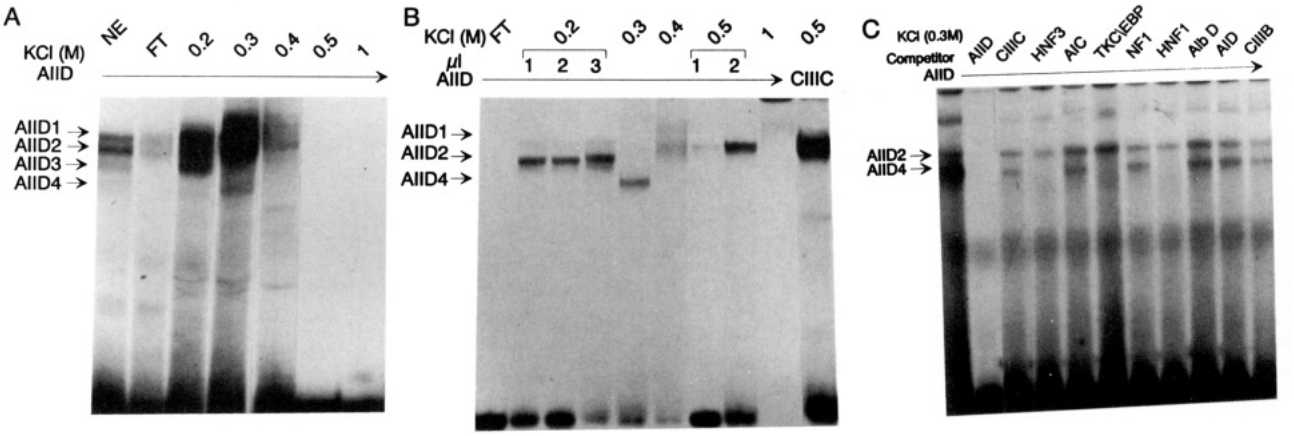

FIGURE 2: DNA binding and competition assays of the fractions eluted from the anion (Q-Sepharose) and cation (Bio-Rex-70) columns. The probe used was the double-stranded oligonucleotide corresponding to the AIID sequence (Table 1) and was labeled with ^{32}P as described under Experimental Procedures. The assays follow the order of the purification step used. Panel A: Fractionation of crude rat liver nuclear extracts on the Q-Sepharose column. Note that AIID3 eluted at 0.2 M KCl and that the remaining activities eluted at 0.3 M KCl. Panel B: Fractionation of the activities eluting at 0.3 M KCl from the Q-Sepharose column on Bio-Rex-70 column. Note that AIID2, AIID4, and AIID1 elute at 0.2, 0.3, and 0.5 M KCl, respectively. Also note that the AIID1 activity binds to the regulatory elements C of apoCIII. The KCl concentrations at which the activities AIID1, AIID2, AIID3, and AIID4 elute are indicated at the top. NE indicates rat liver crude nuclear extracts; FT indicates flow through. Panel C: DNA binding gel electrophoretic assays and competition analysis of partially purified AIID4 using AIID oligonucleotide as probe (Table 1). The competitor oligonucleotides were utilized at 100-fold excess. They are indicated by abbreviations at the top of the figure and are described in Table 1. The top DNA–protein complex represents the AIID2 activity which is present in low concentration in the 0.3 M KCl fractions of the Bio-Rex-70 column. Note that complete competition of AIID4 is achieved with oligonucleotides containing the binding sites of HNF3 and HNF1. The oligonucleotide TK/CEBP binds to activities related to C/EBP and an unidentified activity designated NFY* (Dorn et al., 1987) of the thymidine kinase promoter. None of the oligonucleotides used competed for the binding of AIID2.

Table 2: Purification of Nuclear Factor AIID2 from Rat Liver[a]

purification step	total protein (mg)	vol (mL)	total act. (units)	sp act. (units/mg)	purifn (*x*-fold)	yield (%)
nuclear extract	1729	300	24 000	13.9	–	100
Q-Sepharose	305	285	20 520	67.3	4.8	85
Bio-Rex-70	47	105	5 546	118	8.5	23
affinity 3×	0.03	20	3 208	109,933	7,693	13.4

[a] The activity of each fraction is expressed in units, where 1 unit is defined as the amount of protein bound to 1 ng of oligonucleotide probe.

DNA binding competition assays using the 0.3 M KCl fraction of the Bio-Rex-70 column showed that complexes AIID4 and AIID2 represent different binding activities (Figure 2C). AIID4 was competed out by oligonucleotides containing the binding sites of HNF3 and HNF1 and by oligonucleotide TKC/EBP which contains the binding site of C/EBP and another factor related to NFY* (Dorn et al., 1987) (Table 1). In contrast, AIID2 was not competed by a variety of oligonucleotides containing the binding sites of previously described activities which are encountered in hepatic extracts. The mobility of the AIID4 complex was different from that of the complex formed by purified HNF1 (Chambaz et al., 1991). In addition, regulatory element D displays only partial homology with the consensus HNF1 and HNF3 binding sites (Frain et al., 1989; Tronche et al., 1992; Raymondjean et al., 1991; Costa et al., 1991). Competition analysis also showed that AIID4 complex is not competed out by oligonucleotides containing the binding site of transcription factors C/EBP (Ryden & Beemon, 1989) and NF1 (Jones et al., 1987) or the regulatory elements C and D of apoA-I and B and C of apoCIII promoters which were characterized previously (Ogami et al., 1990; Kardassis et al., 1992, 1993). Recent studies have shown that the regulatory elements D of apoA-I and B of apoCIII represent binding sites for HNF4 as well as homo- and heterodimers of nuclear receptors (Zannis et al., 1992, 1993) (Table 1). To understand the role of AIID2 and AIID4 activities in apoA-II gene transcription, these factors were purified to near homogeneity using ion-exchange and affinity chromatography.

Affinity Purification, Photoaffinity Cross-Linking, and Partial Protein Sequence of AIID2. The purification procedure of factor AIID2, summarized in Table 2, was monitored by gel electrophoretic mobility shift assays (Figure 3A) and SDS–PAGE (Figure 3B). This procedure resulted in a purification of approximately 7693-fold and an overall yield of 13.4% (Table 2). SDS–PAGE analysis of affinity-purified AIID2 revealed three predominant polypeptide bands, designated α, β, and γ (Figure 3B, lane 6), with molecular masses ranging from 63 to 54 kDa. These three bands may correspond to the same protein with different post-transcriptional modifications or to three proteins that may associate to form the AIID2 binding activity.

In order to verify that the proteins with molecular mass 54–63 kDa represent the AIID2 binding activity, the affinity-purified proteins were cross-linked to bromodeoxyuridine-containing oligonucleotides corresponding to the D regulatory element of apoA-II promoter. As seen in Figure 3C, the photoaffinity-labeled AIID2 proteins have an apparent molecular mass ranging between 64 and 73 kDa. The difference in the apparent M_r of AIID2, determined in Figure 3, panels B and C, respectively, can be accounted for by the cross-linking of the –278 to –253 DNA sequence of apoA-II promoter ($M_r \sim 10\,000$) to the AIID2 factor.

Microsequencing of tryptic peptides of AIID2α subunit gave three peptide sequences which perfectly matched residues 151–165, 167–174, and 350–359 of the mouse GABPα, which is an Ets-related protein (Table 3) (Lamarco et al., 1991). GABP consists of three subunits: α, β_1, and β_2, with molecular masses of 56, 41, and 37 kDa, respectively (Thompson et al., 1991; Lamarco et al., 1991). The amino-terminal peptide sequence of the AIID2β subunit represented a perfect match to the mouse GABPα residues 26–47. We presume that the difference in the NH_2 terminus between GABP and this AIID2β subunit may represent some form of proteolytic processing. The AIID2γ subunit digest gave one peptide whose

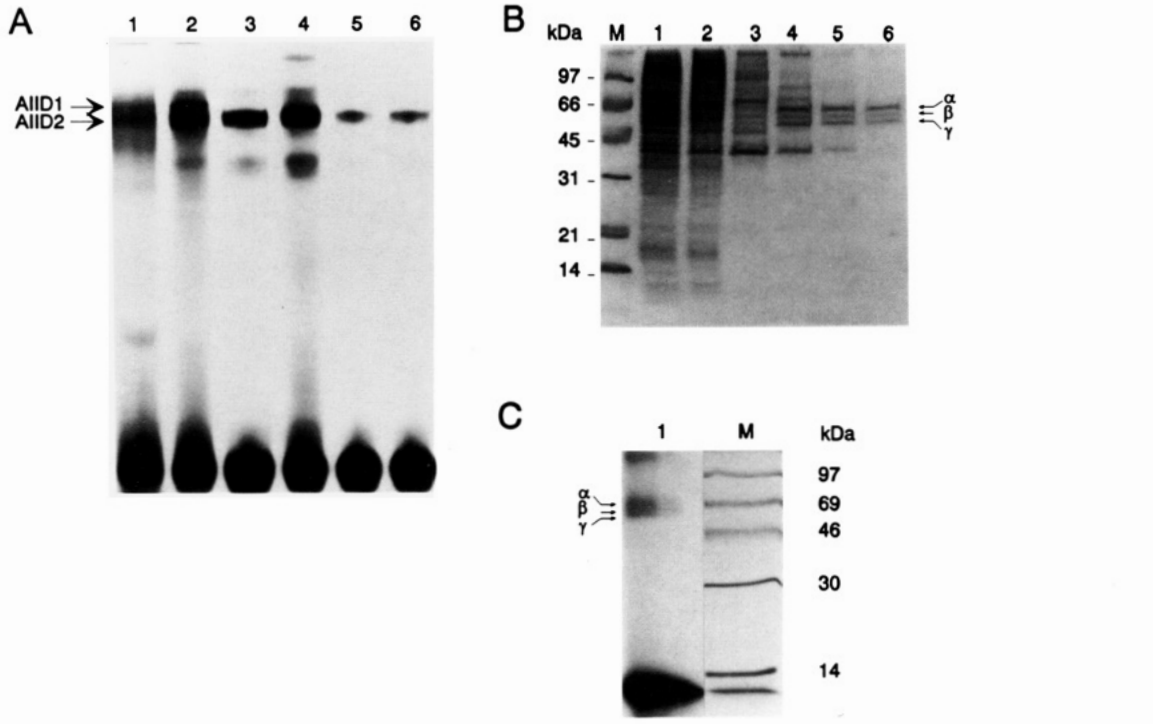

FIGURE 3: Affinity purification and photoaffinity cross-linking of AIID2 activity. Panel A: DNA binding assays of the AIID2 activity eluted from the ion-exchange and the affinity column using the AIID oligonucleotide as probe (Table 1). Lane 1, crude liver nuclear extracts; lane 2, fractions eluted in 0.3 M KCl from Q-Sepharose; lane 3, fractions eluted in 0.2 M KCl from Bio-Rex-70; lanes 4–6, fractions eluted in 0.5 M NaCl from the DNA-specific affinity chromatography columns: the 1st, 2nd, and 3rd affinity columns,respectively. Panel B: 12% SDS–polyacrylamide gel electrophoresis of AIID2 eluted from the ion-exchange and the affinity column. Lane M indicates protein markers as follows: phosphorylase *b* (97.4 kDa); serum albumin (66 kDa); ovalbumin (46 kDa); bovine carbonic anhydrase (30 kDa); and egg white lysozyme (14.3 kDa). Lanes 1–6 contain the same samples described in panel A. Panel C: 12% SDS–polyacrylamide gel electrophoresis of purified factor AIID2 cross-linked to the AIID oligonucleotide (–278 to –253) which contain substitutions of T residues with bromodeoxyuridine. Lane M indicates ^{14}C-labeled protein markers as follows: myosin (200 kDa); phosphorylase *b* (97.4 kDa); serum albumin (69 kDa); bovine carbonic anhydrase (30 kDa); and egg white lysozyme (14.3 kDa).

Table 3: Comparison of the Amino Acid Sequence of Tryptic Peptides Obtained from AIID2 Subunits with GABP Subunit Sequence (Lamarco et al., 1991)[a]

AIID2α peptide1 :		HITTISDETSEQVTR
GAPBα :	151	HITTISDETSEQVTR 165
AIID2α peptide2 :		LNQPELVAQK
GAPBα :	350	LNQPELVAQK 359
AIID2α peptide3 :		AAALEGYR
GAPBα :	167	AAALEGYR 174
AIID2β-N terminus:		SIVEQTYAPAEXVXQAIDINE
GABPα :	26	SIVEQTYTPAECVSQAIDINE 46
AIID2γ peptide1 :		PLLMAAXE
GABPβ$_1$/β$_2$:	74	PLHMAASE 81

[a] Residues in AIID2 that differ from GABP are underlined.

sequence differs in one amino acid from the mouse GABPβ subunit residues 74–81 (Table 3). However, it should be noted that the estimated molecular mass of AIID2γ (54 kDa) is considerably higher than that of the original reporter GABPβ proteins (Thompson et al., 1991). Other GABPβ isoforms, designated nuclear respiratory factor 2 (NRF2) subunits β_1 and β_2, have been reported with molecular masses of 48 and 46 kDa, respectively (Virbasius et al., 1993). Another reason for the observed differences in the reported molecular mass of GABP isoforms may be related to species differences or the existence of different isoforms.

Affinity Purification and Photoaffinity Cross-Linking of AIID4. AIID4 activity recovered in the 0.3 M KCl fraction of the Bio-Rex-70 column (Figure 4A) was further purified by DNA sequence-specific affinity chromatography. The overall yield of AIID4 could not be determined because of the low abundance of this activity in crude nuclear extracts. After two cycles of DNA affinity chromatography, SDS–PAGE analysis showed partial purification with three predominant polypeptides, designated a, b, and c, with an approximate molecular mass of 31, 66, and 130 kDa, respectively (Figure 4B). SDS–PAGE analysis of photoaffinity cross-linked purified AIID4 revealed a protein with an apparent molecular mass of 140 kDa (Figure 4C), which suggests that the 130-kDa protein may be responsible for the AIID4 binding activity. The purification and the photoaffinity cross-linking data established that AIID4 does not represent a degradation product of AIID2.

Base-Specific Interaction of the AIID1, AIID2, and AIID4 Proteins with the Regulatory Element D. Methylation interference and thymidine DNA modification analysis were performed with purified fractions AIID1, AIID2, and AIID4 in order to determine the relationships of these activities with the transcriptional factors the binding sites of which compete out their binding to regulatory element D. The interference of the modified G and T residues in the binding of AIID1, AIID2, and AIID4 is displayed in panels A, B, and C of Figure 5, respectively. The base-specific interactions of AIID1, AIID2, and AIID4 factors on regulatory element D are summarized in Figure 5D. The binding sites of AIID2 and AIID4 were located between –278 and –272, and between –271, and –259, respectively. The binding site of AIID1 has been localized between residues –276 and –262 and overlaps partially with the binding of both AID1 and AID2.

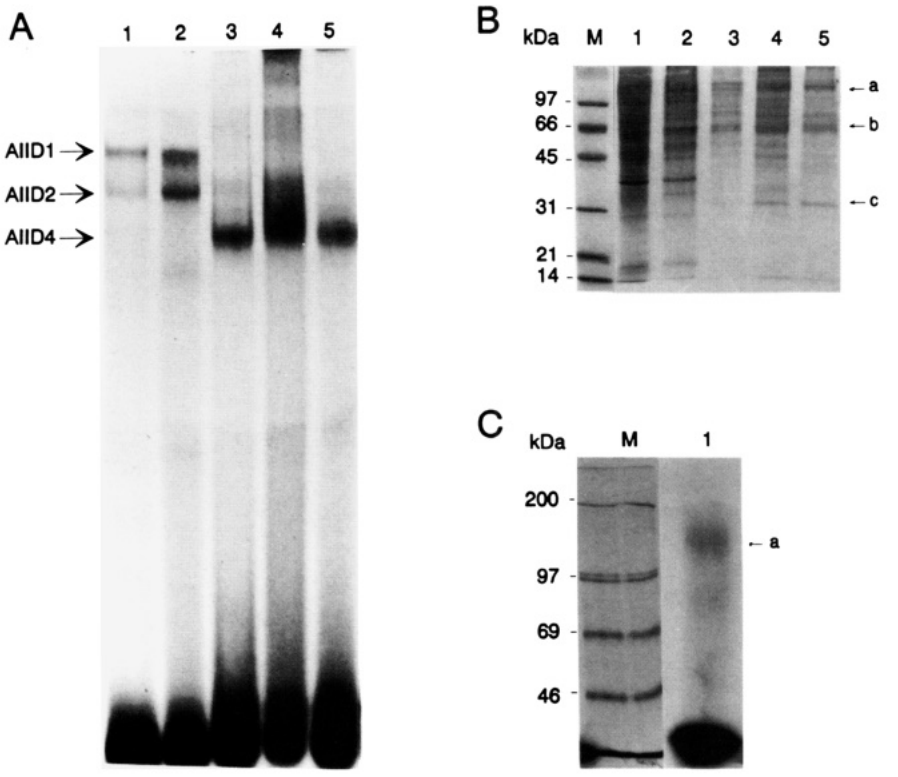

FIGURE 4: Affinity purification and photoaffinity cross-linking of AIID4 activity. Panel A: DNA binding assays of the AIID2 activity eluted from the ion exchange and the affinity column using the AIID oligonucleotide as probe (Table 1). Lane 1, crude liver nuclear extracts; lane 2, fractions eluted in 0.3 M KCl from Q-Sepharose; lane 3, fractions eluted in 0.3 M KCl from Bio-Rex-70; lanes 4 and 5, fractions eluted in 0.5 M NaCl from the DNA-specific affinity chromatography columns: 1st and 2nd affinity columns, respectively. Panel B: 12% SDS–polyacrylamide gel electrophoresis of AIID4 eluted from the ion-exchange and the affinity column. Lane M indicates protein markers indicated in the legend of Figure 3B. Lanes 1–6 contain the same samples described in panel A. Panel C: 7% SDS–polyacrylamide gel electrophoresis of affinity-purified factor AIID4 cross-linked to the AIID oligonucleotide (–278 to –253) substitution, which contain T residues with bromodeoxyuridine. Lane M indicates ^{14}C-labeled protein markers as indicated in the legend of Figure 3C.

Effects of Nucleotide Substitution Analysis within Regulatory Element D on the Binding of Nuclear Activities and on Gene Transcription. The methylation interference and the thymidine DNA modification analysis allowed us to design point mutations of the regulatory element D which alter the contact points of AIID1, AIID2, or AIID3 with the DNA. Such mutations are expected to prevent the binding of the corresponding factors to their cognate site. Analysis of the effects of these mutations on the transcriptional activity of the promoter provided information on the relative contribution of individual factors on the regulation of transcription of the apoA-II gene. Figure 6A–C shows DNA binding and competition experiments using the mutated oligonucleotides AIIDM2 and AIIDM4 of Table 1 as templates. As expected from methylation interference and thymidine modification experiments, mutation in the AIID2 binding site abolished the binding of AIID2 activity from crude nuclear extracts (Figure 6A) as well as from affinity-purified AIID2 protein (Figure 6B). Mutation in the AIID4 binding site abolished the binding of AIID1 activity from crude nuclear extracts (Figure 6A) as well as the activity of AIID4 from the 0.3 M KCl fraction of the Bio-Rex-70 column (Figure 6C). This analysis indicates that the binding of AIID1 is mutually exclusive with the binding of both AIID2 and AIID4 whereas the AIID2 and AIID4 may bind simultaneously. The nucleotide substitution contained in oligonucleotides AIIDM2 and AIIDM4 was also introduced into apoA-II promoter–CAT plasmids, and their effects on apoA-II promoter activity were tested by transient transfection in HepG2 cells. This analysis showed that the AIIDM2 mutation that abolished the binding of AIID2 but allowed the binding of AIID1 and AIID4 displayed the same activity as the wild-type construct. In contrast, the AIIDM4 mutation that affected the binding of AIID1 and AIID4 but allowed the binding of AIID2 increased transcription by 1.6-fold as compared to the wild-type constructs. An increase of 1.5-fold on transcription is also observed when element D is deleted (Figure 7). These results suggest that the inhibitory effect of regulatory element D depends on the binding of AIID1 or AIID4 on element D and that the binding of AIID2/GABP alone exerts a positive regulatory effect on transcription.

DISCUSSION

Background. Apolipoprotein genes are expressed in a tissue-specific pattern, which could be modulated in response to various stimuli. Controlling their expression requires a clear understanding of the combination of factors responsible for their transcription. The control of the apoA-II gene expression appears to be very important since several groups have presented new *in vivo* experiments which implicate apoA-II as a risk factor in the development of coronary heart disease (Warden et al., 1993a,b; Schultz et al., 1993). In addition, *in vitro* studies support a negative role of apoA-II on the activity of the proteins involved in the reverse transport of cholesterol (Vanloo et al., 1992; Bolin & Jonas, 1993; Mowri et al., 1993; Lagrost et al., 1994) through a mechanism which is not fully understood at the present time. We and others (Shelley & Baralle, 1987; Lucero et al., 1989; Chambaz et al., 1991; Cardot et al., 1991, 1993) have shown previously that the transcription of the human apoA-II gene is controlled by a complex array of distal and proximal regulatory elements, designated A to N, that have been divided into three functional regions. We have also characterized several groups of hepatic activities which bind to these regulatory elements and regulate

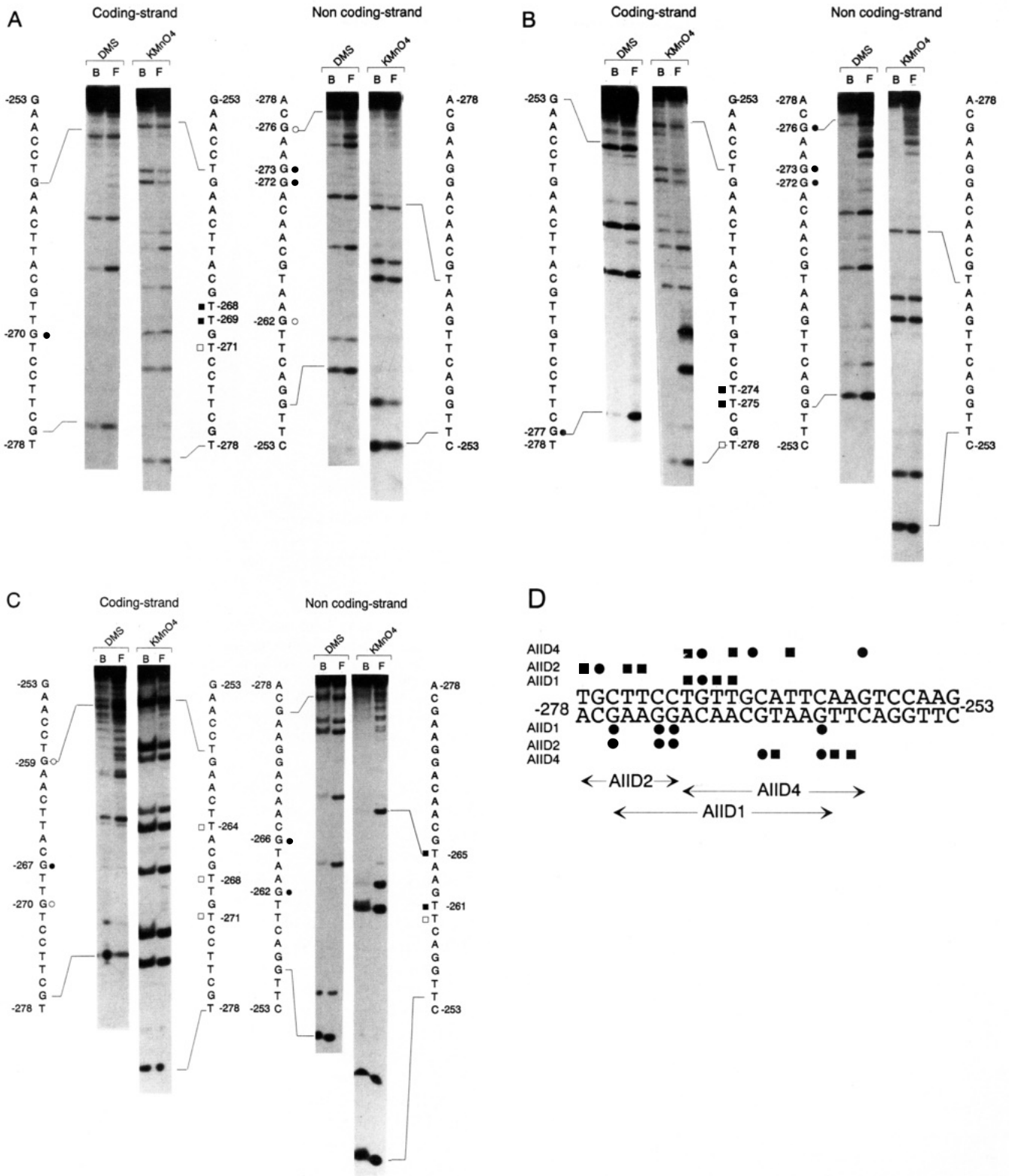

FIGURE 5: Methylation interference by $KMnO_4$ modification of residues on the element AIID (–278 to –253) of apoA-II using fractions containing AIID1 (panel A), AIID2 (panel B), and AIID4 (panel C). The analyses were performed following labeling of the coding strand and noncoding strand as indicated on the top of the figure. F, free probe; B, probe recovered from the DNA–protein complexes with AIID1, AIID2, and AIID4 following modification of the DNA with DMS or $KMnO_4$. The solid circles and open circles indicate the position of guanidine residues with strong and weak interference of binding, respectively. Residue –277 (panel A) appears to be affected slightly by methylation. However, mutation at this site did not affect the binding of AIID1. The solid squares and open squares indicate the position of thymidine residues with strong and weak interference of binding, respectively. The numbers indicate the positions of nucleotides with respect to the transcription initiation site. Panel D: Summary of the chemical modification interference patterns with AIID1, AIID2, and AIID4.

the apoA-II gene transcription. Analysis of the effects of mutagenesis on the apoA-II promoter, in transcription, suggests that deletion of elements J, H, I, G, and C reduced hepatic transcription to 60–80% of control, whereas deletion of element D increased slightly, but reproducibly, the hepatic transcription 1.2-fold, indicating that the factor(s) which recognize this element may exert a negative control on apoA-II gene transcription. The combined data from this and a previous study (Cardot et al., 1993) show that element D binds, in addition to C/EBP (AIID3), new activities which

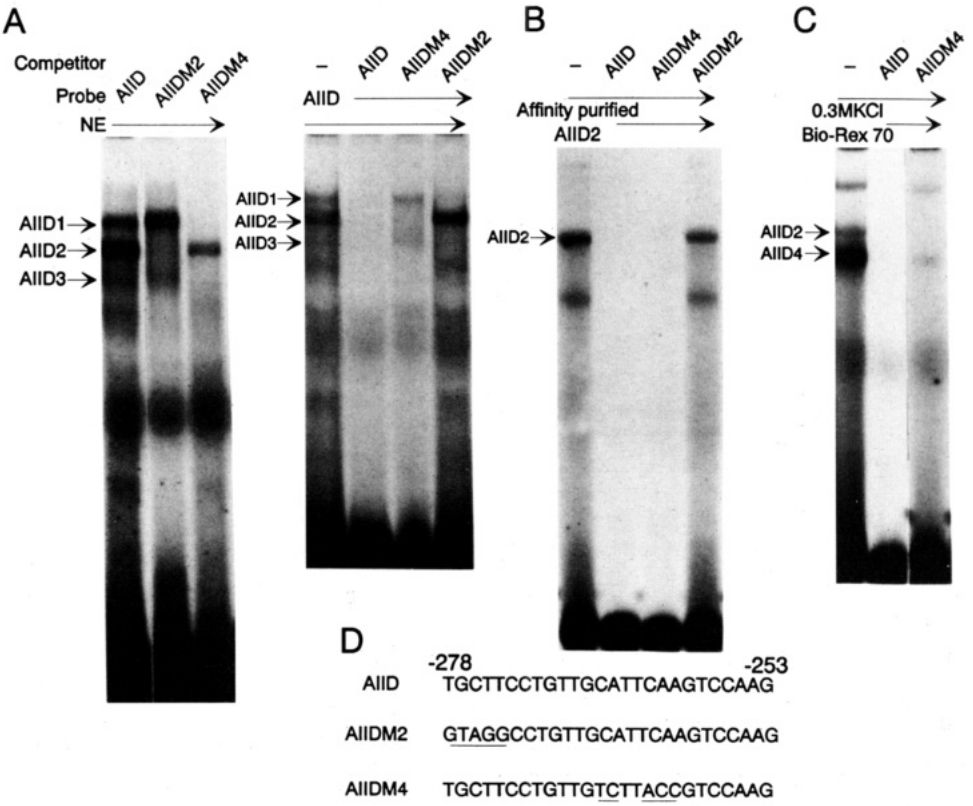

FIGURE 6: Effect of nucleotide substitution mutations of apoA-II promoter region D on binding of the nuclear activities AIID1, AIID2, and AIID4. DNA binding and competition assays were performed using the wild-type and mutated oligonucleotide sequences shown at the bottom of the figure. The following fractions were used for DNA binding and competition assays (panel A), crude nuclear extracts (the left side shows direct binding and the right shows competition assays) (panel B), affinity-purified AIID2 (panel C), and DNA fractions eluted at 0.3 M KCl from Bio-Rex-70 column (panel D). The competitor oligonucleotides shown in Table 1 were utilized at 100-fold excess. The alterations relative to the wild-type probe are underlined. Note in panels A–C that AIIDM2 mutation eliminated the binding of AIID2 and the AIIDM4 mutation eliminated the binding of AIID1 and AIID4. The fast migrating DNA protein observed in panel B most likely represents a proteolytic degradation product of AIID2.

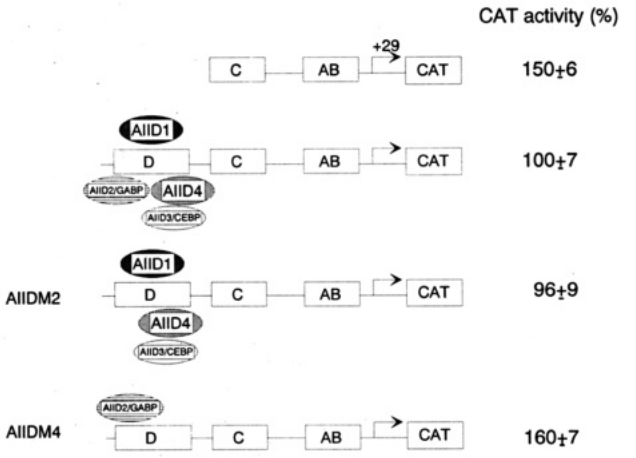

FIGURE 7: Effect of nucleotide substitutions in the AIID elements on transcription of reporter CAT gene in HepG2 cells. CAT analysis was performed as described under Experimental Procedures. CAT values represent the average of triplicate experiments. The binding sites of AIID1, AIID2/GABP, and AIID4 have been established on the basis of interference of binding by chemical modification of G or T residues and DNA binding and competition analyses.

could not be competed out by oligonucleotides containing the binding sites of known hepatic factors. To understand the specific role of these activities on the regulation of transcription of the human apoA-II gene, we have proceeded with their purification and characterization from rat liver nuclei.

Proteins Related to GABP/Ets Participate in the Hepatic Regulation of the Human ApoA-II Gene. After three steps of DNA-specific affinity chromatography, the purified protein AIID2 generated three bands on SDS–PAGE designated α, β, and γ with molecular masses ranging between 54 and 63 kDa. However, these three purified proteins formed a single DNA–protein complex with element D, as determined by DNA binding gel electrophoresis assays. Comparison of the partial amino acid sequences of AIID2α and -β peptides showed an identity of AIID2α,β to GABPα, whereas AIID2γ peptide differs in one amino acid from the mouse GABPβ. GABP is a transcription factor which consists of three subunits and has been originally characterized as an activator of herpes simplex virus immediate early gene (Lamarco & McKnight, 1989). More recently, GABP was shown to be an activator of rat or mouse cytochrome *c* oxidase subunit IV gene (Virbasius et al., 1993; Carter et al., 1992). Different observations strengthened the identity of AIID2 and GABP proteins. First, the AIID2 binding site contains the AGGAA motif which is characteristic of the recognition site of members of the family of Ets proteins to which GABP belongs. DNA binding and competition experiments showed that AIID2 activity was efficiently competed out by an excess of oligonucleotide PEA3 (Table 1), which contains the Ets recognition motif (data not shown) but could not be competed out by oligonucleotide (AIC) corresponding to element C of apoA-I (Cardot et al., 1993). AIC matches the binding site of AIID2 except for a single G to C substitution in the essential GGAA core of the Ets recognition sequence (Table 1). This paper shows for the first time that proteins related to the Ets gene superfamily may participate in the regulation of a liver-specific gene.

Protein Unrelated to GABP/Ets May Bind Simultaneously or Competitively to Regulatory Element D and Modulate

Table 4: Homology between Human Apolipoprotein A-II Regulatory Element D and Regulatory Elements of Human Apolipoprotein A-I, CIII, and B

$^{-278}$TGCTTCCTGTTGCATTCAAGTCCAAG^{-253}	AIID
**** *****	(Cardot et al., 1993)
$^{-178}$AGCTTGCTGTTTGCCCACTCTCTATTTGCCCAGCCCAG^{-142}	AIC
**** ***	(Papazafiri et al., 1991)
$^{-138}$CCGCTTGCTGCATCTGGACA^{-119}	CIIIC
* ** ** **	(Ogami et al., 1990)
$^{-93}$CACCTGTTTGCTTTTCTACACTGGCTTCTCTTT^{-124}	BCB
	(Kardassis et al., 1990)

ApoA-II Gene Transcription. The anion- and cation-exchange chromatography of the hepatic nuclear extracts identified a new activity which recognized the regulatory element D, which was designated AIID4. We initially hypothesized that this activity may represent a degradation product of one of the activities which bind to the regulatory element D. Denaturing SDS–PAGE analysis of the fraction(s) obtained by successive DNA sequence-specific affinity chromatography showed that the most purified fraction contained three predominant bands, a, b, and c, with approximate molecular masses of 130, 66, and 31 kDa, respectively. However, photoaffinity cross-linking generated a 140 kDa protein, thus suggesting that at least the 130 kDa subunit is a component of AIID4. The size of the purified protein also excludes the possibility that AIID4 is a degradation product of AIID2 or the other proteins which bind to the regulatory element D. The observed competition of AIID4 with oligonucleotides containing the binding site of HNF1 most likely results from the partial homology of the HNF1 binding site with the sequence $^{-265}$ATTCAAG^{-259} which is present within the AIID4 binding site (Courtois et al., 1988). Similarly, the competition with oligonucleotides containing the binding site of HNF3 can be explained by the partial homology of the binding site of AIID4 (TGTTG-CATTCAAG) with the consensus binding site of HNF3 (TATTGAC/$_T$ TTA/$_T$G) (Costa et al., 1991; Raymondjean et al., 1991).

Important insights into the mode of action of the proteins which bind to the regulatory element D were obtained by analysis of their contact points with the DNA by methylation interference and modification of the thymidine by $KMnO_4$. This analysis showed that the binding sites of AIID2/GABP and AIID4 were contiguous on element D. However, the binding sites of both AIID2/GABP and AIID4 overlap with the binding site of AIID1. It should also be noted that the regulatory element D contains a weak binding site for C/EBP (GAATGCAAC) on the noncoding strand, between nucleotides −262 and −270 (Cardot et al., 1993). This sequence differs from the consensus binding site of C/EBP (Ryden et al., 1987) at three positions, which are underlined (Cardot et al., 1993). It is interesting that the binding site of C/EBP overlaps with the binding site of AIID1 and AIID4 but not with that of AIID2/GABP. This suggests that the binding of AIID2/GABP and AIID1 may be mutually exclusive, whereas AIID2/GABP and AIID1 or AIID2/GABP and AIID3/C/EBP may bind simultaneously. *In vitro* mutagenesis of element D showed that mutations that affected the binding of AIID1, AIID4, and AIID3/C/EBP, but allowed that of AIID2, resulted in a 1.6-fold transcriptional activity of the mutated promoter as compared to the wild-type promoters. This suggests that one or more among the factors AIID1, AIID4, or AIID3/C/EBP may act as negative regulators of transcription in contrast to factor AIID2, which appears to act as a positive regulator.

This study and a previous study (Cardot et al., 1993) illustrate the complexities which underlie the regulation of apoA-II. This complexity is the result of both multiple regulatory elements and multiple factors which can occupy each of these elements. It appears, though, that each element, depending on the availability of factors which recognize it, can make its own unique contribution on gene transcription. Given the low abundance of AIID4 in crude nuclear extracts, as well as the weak affinity of the C/EBP family members for element D, it is reasonable to speculate that the negative regulatory control may be exerted by the binding of protein AIID1 to the regulatory element D. The fact that negative regulatory factors which bind to element D can influence the transcription of the apoA-II gene is supported further by the small, but reproducible, decrease in promoter activity when element D is deleted (Figure 7) (Cardot et al., 1993). The contribution of the AIID1 and AIID4 factors involved will require their positive identification either by peptide sequencing or by expression cloning of the cDNAs encoding for them.

The regulatory element D contains the motif $^{-278}$TGCTTCCTGTTGC^{-266} which has partial sequence homology with the regulatory elements CIIIC of apoCIII, BCB of apoB, and AIC of apoA-I (Table 4). Thus, it is possible that some of the activities described here may also play a role in the regulation of the apoA-I and apoCIII genes. On the other hand, activities such as HNF3, which has been reported to bind to element BCB of apoB (Kardassis et al., 1992; Paulweber et al., 1993), does not bind the regulatory element D of apoA-II.

Potential Mechanisms Controlling Gene Transcription and Tissue Specificity. Different mechanisms have been proposed for the negative control of gene transcription in eukaryotes (Renkawitz, 1990). One of these mechanisms results from a direct competition between repressors and activators for overlapping DNA binding sites. In this article characterization of the binding motifs of three activities which occupy the regulatory element D of apoA-II suggests that the most possible mechanism of the observed negative regulatory effect is the displacement of GABP/Ets-related proteins by a negative regulatory protein AIID1. The determination of the role of these factors and of their interplay in the negative regulation of apoA-II gene requires derivation of the complete cDNA sequence of the factors involved and analysis of their function by an *in vitro* transcription system.

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