Characterization of Multiple Enhancer Regions Upstream of the Apolipoprotein(a) Gene*

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Plasma concentrations of the atherogenic lipoprotein(a) (Lp(a)) are predominantly determined by inherited sequences within or closely linked to the apolipoprotein(a) gene locus. Much of the interindividual variability in Lp(a) levels is likely to originate at the level of apo(a) gene transcription. However, the liver-specific apo(a) basal promoter is extremely weak and does not exhibit common functional variations that affect plasma Lp(a) concentrations. In a search for additional apo(a) gene control elements, we have identified two fragments with enhancer activity within the 40-kilobase pair apo(a)-plasminogen intergenic region that coincide with DNase I-hypersensitive sites (DHII and DHIII) observed in liver chromatin of mice expressing a human apo(a) transgene. Neither enhancer exhibits tissue specificity. DHIII activity was mapped to a 600-base pair fragment containing nine DNase I-protected elements (footprints) that stimulates luciferase expression from the apo(a) promoter 10–15-fold in HepG2 cells. Binding of the ubiquitous transcription factor Sp1 plays a major role in the function of this enhancer, but no single site was indispensable for activity. DHIII comprises part of the regulatory region of an inactive long interspersed nucleotide element 1 retrotransposon, raising the possibility that retrotransposon insertion can influence the regulation of adjacent genes. DHII enhancer activity was localized to a 180-base pair fragment that stimulates transcription from the apo(a) promoter 4–8-fold in HepG2 cells. Mutations within an Sp1 site or either of two elements composed of direct repeats of the nuclear hormone receptor half-site AGGTCA in this sequence completely abolished enhancer function. Both nuclear hormone receptor elements were shown to bind peroxisome proliferator-activated receptors and other members of the nuclear receptor family, suggesting that this enhancer may mediate drug and hormone responsiveness.

Apolipoprotein(a) (apo(a))[1] is a large protein that is closely related in structure to plasminogen (1) and forms the characteristic protein constituent of the atherogenic lipoprotein(a) (Lp(a)). Many clinical studies have shown that high Lp(a) concentrations in plasma are associated with an increased incidence of arterial disease (2–4). The most recent prospective study reported that men whose Lp(a) concentration exceeded 35 nmol/liter had a nearly 3-fold greater risk of developing coronary artery disease (5). Transgenic mice that express human apo(a) show an increased propensity to develop fatty streak lesions when fed a high fat diet, which suggests that the protein may play a direct role in atherogenesis (6).

Apo(a) is synthesized in the liver (7, 8), and Lp(a) is probably formed by the covalent attachment of apo(a) to the apoB100 moiety of an LDL-like particle at the hepatocyte surface (9). In humans, interindividual plasma Lp(a) concentrations vary very widely, spanning a range from ~0.1 to more than 200 mg/dl (10, 11). Lp(a) concentrations are predominantly genetically determined (12, 13), and at least 90% of the variation in plasma concentration is attributable to inherited sequences within or closely linked to the apo(a) gene locus itself (14). However, the nature of the sequence variations responsible and the mechanisms by which they affect Lp(a) levels are not yet fully understood. The apo(a) gene exhibits extreme size polymorphism as a result of the presence of a hypervariable region that may contain between 12 and 51 tandem repeats of a sequence similar to kringle four of plasminogen (15). A general inverse relationship exists between the number of kringle four repeats and plasma Lp(a) levels (13), suggesting that apo(a) gene size partly determines the amount of circulating Lp(a). This relationship is likely to result from post-translational processes, since it has been shown that the residence time of immature apo(a) proteins in the endoplasmic reticulum and, consequently, their availability for incorporation into lipoproteins are inversely related to protein isoform size (16). However, identically sized apo(a) isoforms may be associated with up to 200-fold variations in apo(a) levels in plasma, suggesting that the apo(a) locus influences Lp(a) concentration through a mechanism that is independent of apo(a) gene size (11, 17). It is likely that much of this influence occurs at the level of transcription, since hepatic apo(a) mRNA abundance differs markedly between individuals in both primate and human populations (18–20), and apo(a) mRNA concentration has been shown to correlate with Lp(a) levels in cynomolgus monkeys (21). An understanding of the mechanisms that control transcription of

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[1] The abbreviations used are: apo, apolipoprotein; bp, base pair(s); DH, DNase I-hypersensitive; DR, direct repeat; GR, glucocorticoid receptor; kb, kilobase pair(s); LINE, long interspersed nucleotide element; Lp(a), lipoprotein(a); PPAR, peroxisome proliferator-activated receptor; UTR, untranslated region; YAC, yeast artificial chromosome; CMV, cytomegalovirus; LDL, low density lipoprotein; ORF, open reading frame; C/EBP, CCAAT enhancer-binding protein.

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the apo(a) gene is therefore important.

In a previous study, the minimal apo(a) promoter was mapped by deletion analysis, and basal transcription of the apo(a) gene was shown to be dependent on the binding of the liver-enriched transcription factor HNF-1α to a site in the untranslated region of the promoter (22). Since HNF-1α expression is limited to the liver and a few other tissues (23), this feature of the apo(a) promoter partly accounts for the liver specificity of transcription of the apo(a) gene. However the apo(a) promoter is extremely weak, directing only a very low level of basal transcription in transiently transfected HepG2 cells (22). Furthermore, a number of studies have failed to reveal functional polymorphisms in the apo(a) promoter of a magnitude that might account for the apo(a) allele size-independent variation in apo(a) concentrations (24–26). We hypothesized that optimal transcription of the apo(a) gene, like that of many genes might depend on the presence of one or more enhancer regions at some distance from the basal promoter. Therefore, to gain a greater understanding of the factors that control expression of the apo(a) gene and to identify regions that might harbor functional variations that affect apo(a) synthesis, we began to look for additional apo(a) gene regulatory elements. We focused our search on the 40-kb sequence between the apo(a) and plasminogen gene, since several hepatoma cell-specific DNase I-hypersensitive sites have been detected in this region (27), and such sites indicate an open chromatin conformation, which is often associated with active regulatory regions (28, 29). Here we report the identification of two enhancers upstream of the apo(a) gene and the characterization by site-directed mutagenesis, DNase I footprinting, and gel mobility shift assays of the *cis*-elements and *trans*-acting factors most critical for their function.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmid GL3F, consisting of bases −98 to +130 of the apo(a) 5′-flanking region fused upstream of the luciferase gene in pGL3-basic (Promega), was constructed by ligation of an *Xho*I-*Sac*I fragment of p(−98)GLA1 (22) into pGL3-basic digested with *Xho*I and *Sac*I. Fragments of the apo(a)-plasminogen intergenic region were subcloned into a *Kpn*I site immediately upstream of the apo(a) promoter insert in pGL3F. A region spanning DNase-hypersensitive site IV (Fig. 1) (27) consisting of ~3 kb of sequence immediately upstream of the previously cloned 1.4-kb apo(a) gene 5′-flanking region (30) was amplified from genomic DNA using the PromoterFinder DNA Walking Kit (CLONTECH). Briefly, libraries of purified genomic DNA fragments ligated to adaptor linkers were amplified with a primer complementary to the adaptors and a primer (5′-TGACAGAGCAAGAATGTCTCAG-GAAAG-3′) complementary to nucleotides −858 to −832 (relative to the start site of transcription) of the apo(a) 5′-flanking sequence (30). Products were subjected to a second amplification with a nested primer complementary to the adaptors and a primer complementary to nucleotides −1226 to −1197 of the apo(a) 5′-flanking sequence (5′-CGT-CAGTGCACTTCAACCGGGGTGAGAGTC-3′). After cloning into T-vector (Promega) the identity of the 3-kb product was confirmed by dideoxy sequencing. The product was excised from the T-vector as an *Nsi*I-*Sph*I fragment and ligated into *Kpn*I-digested pGL3F after the cohesive ends of both vector and insert were blunted with Klenow enzyme. Consecutive fragments comprising a further 28 kb of the intergenic region were excised from cosmid 2B (27) by digestion with *Kpn*I, isolated from agarose gels, and subcloned into the *Kpn*I site of pGL3F. Fragments representing the 5′ and 3′ termini of the cosmid clone (9.0 and 2.1 in Fig. 1) were ligated first into pBluescript KS+ (Stratagene) to provide an additional polylinker-derived *Kpn*I site for subcloning. Nested deletion mutants of the 1.8- and 2.0-kb *Kpn*I subclones in GL3F (pGL3F1.8K and pGL3F2K, Fig. 3) were synthesized by digestion with Exonuclease III and Mung Bean nuclease (Stratagene) according to the manufacturer's instructions. The enhancer core region of the 1.8-kb fragment (bases −1796 to −1215, 3′ terminus of each fragment designated as −1; see Fig. 3) was excised from deletion mutant p(−1215)GL3F1.8K by digestion with *Xba*I and *Kpn*I for ligation upstream of heterologous promoters. After blunting with Klenow enzyme, the fragment was subcloned into the *EcoICR*I site of pGL3F and pGL3 promoter (a luciferase reporter construct driven by the SV40 promoter (Promega)) and the *Sma*I site of pLDLRGL3 (a luciferase reporter construct driven by the LDL receptor gene promoter (31)). Corresponding constructs were made with a *Pvu*II-*EcoICR*I restriction fragment of the 2.0-kb enhancer (bases −1 to −618) that contained the enhancer core region (bases −1 to −186). The orientation of the inserts was verified by restriction digestion. Site-directed mutants of sequences within regions protected from DNase I digestion by the binding of nuclear proteins (Fig. 6) were synthesized using the Unique Site Elimination Mutagenesis kit (Pharmacia) as described (22). All mutated fragments were sequenced and subcloned into parent vectors that had not undergone the mutagenesis procedure. A deletion mutant of p(−1215)GL3F1.8K lacking the region containing footprint J (Fig. 6A) was constructed by removal of bases −1796 to −1697 from p(−1215)GL3F1.8K by digestion with *Kpn*I and *Ava*I and religation of the remaining vector after blunting with Klenow enzyme. To construct a deletion mutant lacking the region of footprint B (Fig. 6A), p(−1158)GL3F1.8K was digested with *Pvu*II, removing bases −1197 to −1156, and the parent vector was circularized with T4 DNA ligase.

Transient Plasmid DNA Transfection and Assays of Luciferase and β-Galactosidase Activity—Human hepatoma HepG2 cells and human cervical carcinoma HeLa cells were seeded in 24-mm wells at a density of 0.5×10^6 cells/well and transfected 24 h later with complexes containing 6 μl of Lipofectin (Life Technologies, Inc.), 0.4 μg of CMV-β-galactosidase vector (CLONTECH) as a control for transfection efficiency, and up to 2 μg of luciferase reporter plasmid/well as described previously (30). When the transcriptional activity of plasmids of different sizes was compared, equimolar amounts of plasmid were transfected, and where necessary the total mass of DNA added per well was adjusted to 2.4 μg by the addition of pBluescript. Lysates were prepared 48 h after transfection by the addition of 80 μl of reporter gene cell lysis buffer (Promega) per well and assayed for luciferase and β-galactosidase activity exactly as described (30).

DNase I Protection Analysis—Nuclear extracts were prepared from HepG2 cells as described (22). For DNase I footprint analysis, probe fragments were excised from nested deletion mutants of pGL3FK1.8 and pGL3F2K by restriction enzyme digestion and end-labeled by fill-in with Klenow enzyme incorporating the appropriate [α-^{32}P]dNTP. Probe A (−1158 to −1451) was an *Nar*I-*Xba*I fragment of p(−1158)GL3F1.8K; probe B (−1332 to −1505) was an *Nhe*I-*Not*I fragment of p(−1332)GL3F1.8K; probe C (−1423 to −1740) was a *Bssh*II-*Xba*I fragment of p(−1423)GL3F1.8K; probe D (−1517 to −1740) was a *Bssh*II-*Xba*I fragment of p(−1517)GL3F1.8K; and probe E (−1 to −618) was an *Xba*I-*Pvu*II fragment of pGL3F2K. DNase I protection analysis was performed as described (22).

Gel Mobility Shift Assay—The following oligonucleotide probes were synthesized for gel mobility shift assays: oligo EF, 5′-TAGGTAAACA-AAGCAGCCCGGAAGCTCGAACTGGGTGGAGCCCACC-3′, and mutant derivatives oligo mutE, 5′-TAGGTAAACAAAGCAGCCCGGAAGC-TCGAACTactagtAGCCCACC-3′, and oligo mutF, 5′-TAGGTAAAacta-gttGCCCGGAAGCTCGAACTGGGTGGAGCCCACC-3′; oligo I, 5′-TG-AAGGTTGAGCCAAAGGTTAGTCTCCTCCCCCTCCACTAGT-3′, and mutant derivatives oligo ImutA, 5′-TGAAGGTTGggatccAGGTTAGTC-TCCTCCCCCTCCACTAGT-3′, and oligo ImutB, 5′-TGAAGGTTGAGC-CAAAGGTTAGTCTCCTggatccCCACTAGT-3′; oligo II, 5′CTCCCATG-TTGACACAGGTCAAATCCTTGAACTCTGTTGCCCAAATAGCT-3′ and mutant derivative oligo IImutA, 5′-CTCCCATGTTGggatccGTCA-AATCCTTGAACTCTGTTGCCCAAATAGCT-3′ (mutated bases shown in lowercase throughout). Oligonucleotides complementary to each forward strand were designed to create 4-base overhangs after annealing, and the resulting double-stranded probes were labeled by fill-in with Klenow enzyme incorporating the appropriate [α-^{32}P]dNTPs. Gel mobility shift assays were performed as described by Wade *et al.* (22), except that the binding buffer contained 20 mM HEPES (pH 7.9), 60 mM KCl, 1 mM MgCl$_2$, 1 mM dithiothreitol, 66.6 μg/ml poly(dI-dC), and 10% (v/v) glycerol. When binding reactions were performed with recombinant Sp1 (Promega), poly(dI-dC) was omitted from the buffer. Antiserum to Sp1 was purchased from Santa Cruz Biotechnology, Inc., and antiserum to PPARα and PPARγ was from Affinity BioReagents, Inc.

DNase I Hypersensitivity Assays—Sites of hypersensitivity to DNase I digestion in mouse liver chromatin were detected as described by Magnaghi *et al.* (27).

RESULTS

Identification of Enhancer Elements in the Apo(a)-Plasminogen Intergenic Region—The presence of a number of HepG2-specific DNase I-hypersensitive sites in sequences between the

FIG. 1. **Identification of enhancer elements in the apo(a)-plasminogen intergenic region.** *Panel A*, schematic drawing of the apo(a)-plasminogen intergenic region. The genomic region examined for enhancer activity in this study is shown as a *horizontal box*, and other regions are shown as a *single line*. Individual fragments of the intergenic region that were subcloned into reporter constructs are shown as *hatched boxes* labeled with the fragment size in kb. *K*, *Kpn*I site; *E*, the *Eco*RI site marking the upstream end of the previously cloned 1.4-kb apo(a) 5′-flanking region (30); *X* and *B*, the *Xba*I and *Bam*HI sites used in DH site mapping (Fig. 2). Other *Eco*RI, *Bam*HI, and *Xba*I sites are not shown. The positions of the DH sites identified previously in HepG2 cells (27) and in this study in apo(a) YAC mouse liver (Fig. 2) are shown by *vertical arrows*. *Heavy lines* labeled *B* and *C* indicate the positions of probes used in DH site mapping (Fig. 2), and a *heavy line* labeled *L1* marks the position of the LINE-1 element. *Panel B*, schematic drawing of the apo(a)promoter-luciferase reporter gene constructs used to test the enhancer activity of fragments of the apo(a)-plasminogen intergenic region. *Panel C*, transcriptional activity of reporter constructs in HepG2 cells. HepG2 cells were transfected with equimolar amounts of the reporter constructs (up to 2 μg per 24-mm well) together with 0.4 μg of CMV-β-galactoside vector as a control for transfection efficiency. Luciferase and β-galactosidase activities were measured in cell lysates 48 h after transfection. Values are expressed as a percentage of the activity of GL3F, the luciferase reporter gene driven by the -98 to $+130$ apo(a) minimal promoter region alone, and represent the mean and S.E. of 4–6 independent transfections. The suffix *rev* indicates constructs containing fragments cloned in reverse orientation.
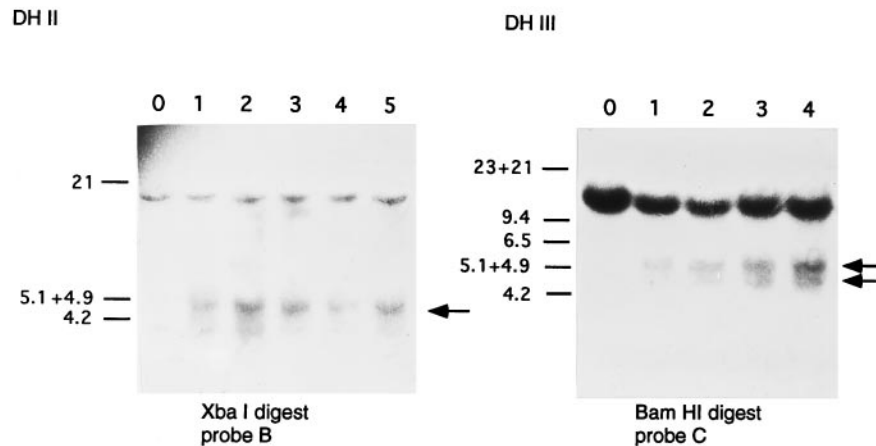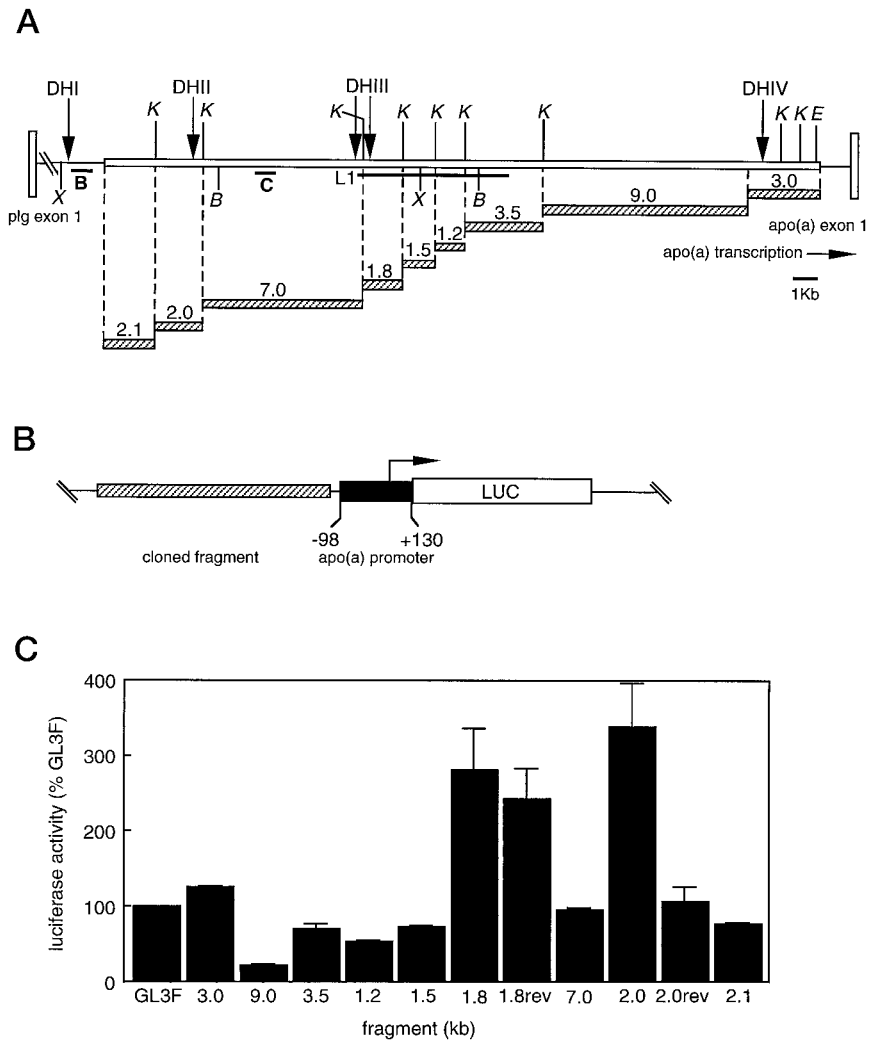
FIG. 2. **Mapping of DNase I-hypersensitive sites in the apo(a)-plasminogen intergenic region of apo(a) YAC transgenic mice.** Nuclei isolated from apo(a) YAC transgenic mouse liver cells were digested with DNase I for 5 min at 37 °C. Concentrations of DNase I used were as follows (in units/μl): (for DH II) *lane 0*, 0; *lane 1*, 0.28; *lane 2*, 0.7; *lane 3*, 1.12; *lane 4*, 1.4; (for DHIII) *lane 0*, 0; *lane 1*, 0.28; *lane 2*, 0.7; *lane 3*, 1.12; *lane 4*, 1.4; *lane 5*, 2.8. Genomic DNA was purified, digested with the indicated restriction enzymes, transferred to nylon filters after gel electrophoresis, and hybridized with probe B, a 600-bp *Bam*HI-*Hin*dIII fragment, to detect DHII or with probe C, a 700-bp *Eco*RI-*Sac*I fragment, to detect DHIII (27). *Numbered lines* indicate the positions of DNA size markers (kb), and DNase I-hypersensitive sites are shown by *arrows*. DHIII appears as a doublet.

apo(a) and plasminogen genes (27) suggested that this region may be of importance in the transcriptional regulation of the liver-specific apo(a) gene. We therefore investigated whether elements in the apo(a)-plasminogen intergenic region could

affect the expression of a luciferase reporter gene driven by the apo(a) minimal promoter region (positions -98 to $+130$ relative to the cap site) (22). Constructs containing consecutive fragments of the apo(a)-plasminogen intergenic region extend-

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FIG. 3. **Deletion analysis of the 1.8-kb and 2.0-kb enhancer fragments.** *Panel A*, transcriptional activity of deletion mutants of the 1.8-kb enhancer fragment. HepG2 cells were transfected with equimolar amounts of the indicated deletion mutants of construct pGL3F1.8K together with CMV-β-galactosidase as a control for transfection efficiency, and luciferase activity was measured in cell lysates after 48 h. Results are expressed as a percentage of the activity of GL3F and represent the mean and S.E. of three independent transfections. In the construct schematic drawings, the *hatched* portions represent the apo(a) minimal promoter, and the *black boxes* represent the extent of the enhancer sequences remaining in each construct. The nucleotide end point of each deletion is numbered from the most 3′ base pair of the enhancer fragment, which is designated −1. *Panel B*, schematic drawing of the positions of elements in the 1.8-kb enhancer fragment having positive or negative effects on transcription as revealed by the deletion analysis in *panel A*. The *bracket* indicates the restriction fragment used to synthesize 1.8-kb core enhancer constructs. *Panel C*, transcriptional activity of deletion mutants of the 2.0-kb enhancer fragment. Details are as in *panel A*. *Panel D*, schematic drawing of elements in the 2.0-kb enhancer fragment having a positive effect on transcription as revealed by the deletion analysis in *panel C*. The *bracket* indicates the restriction fragment used to synthesize 2.0-kb core enhancer constructs.
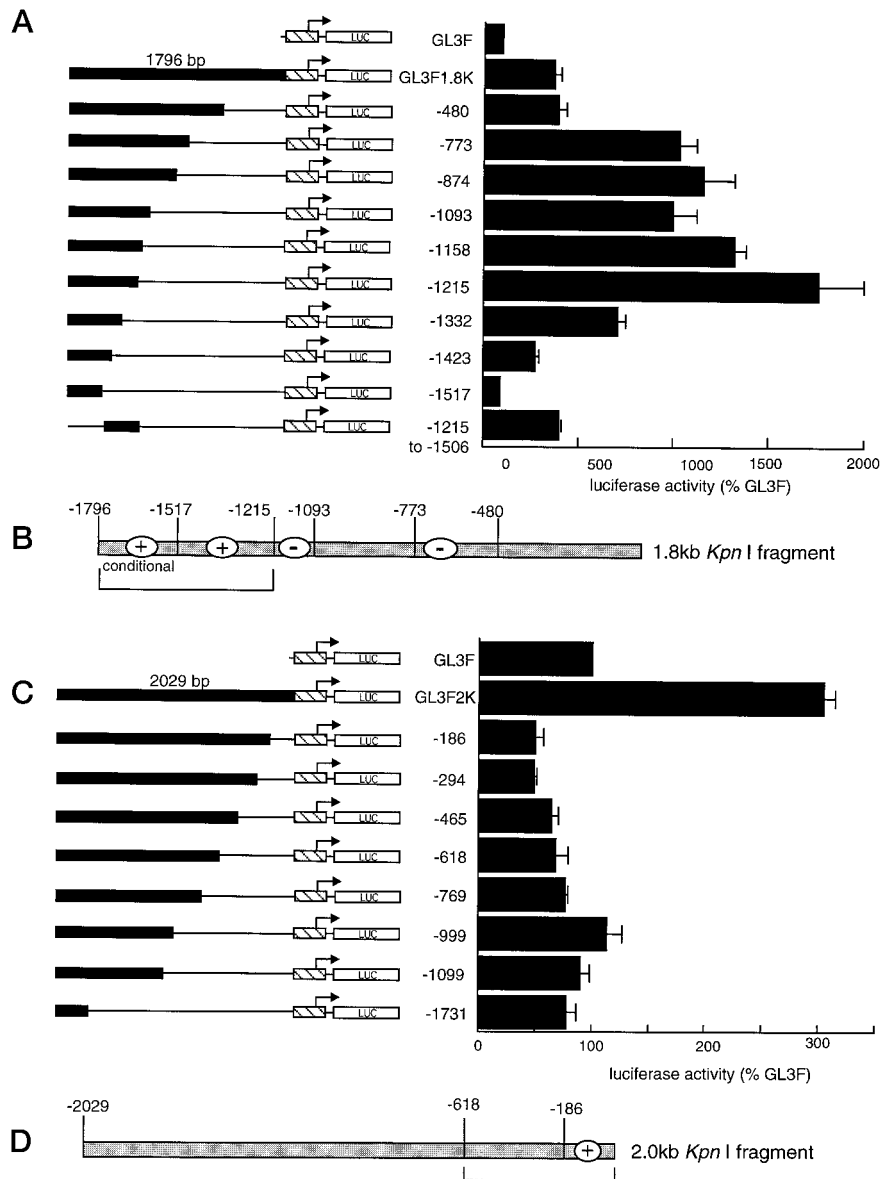
ing from the previously cloned 1.4-kb 5′-flanking region (30) to approximately 31 kb upstream of the apo(a) gene cap site were synthesized and assayed for luciferase expression in transiently transfected HepG2 cells (Fig. 1). One fragment (9.0-kb fragment, Fig. 1*C*) consistently repressed expression of the reporter gene to ∼20% of control values, a result not likely to be due to a methodological artifact related to its length, since no repression was observed with the comparably sized 7.0-kb fragment. This region may therefore contain negative regulatory elements. Two discrete *Kpn*I fragments enhanced luciferase expression from the apo(a) promoter ∼4-fold (1.8- and 2.0-kb fragments, Fig. 1*C*). When cloned in reverse orientation, the 1.8-kb fragment retained its ability to stimulate transcription, showing the orientation independence characteristic of enhancers, while the 2.0-kb fragment did not (Fig. 1*C*). However, the 2.0-kb fragment could not drive transcription of the luciferase reporter gene in the absence of the apo(a) minimal promoter (results not shown), suggesting that it is not itself a promoter, and the core region of this fragment was subsequently shown to exhibit enhancer-like orientation-independent activity (see below).

DNase I-hypersensitive Sites Are Present over Enhancer Elements in the Apo(a)-Plasminogen Intergenic Region of Trans-

genic Mice That Express the Apo(a) Gene—We examined chromatin isolated from the livers of transgenic mice containing stably integrated copies of a YAC clone spanning the apo(a) gene and including the complete apo(a)-plasminogen intergenic region.[2] These mice transcribe the apo(a) gene at high levels in a liver-specific manner,[2] suggesting that the regulatory regions necessary for appropriate apo(a) gene expression are present in the integrated sequences. Figs. 1 and 2 show that DNase I-hypersensitive sites DHII and DHIII are detectable in the apo(a)-plasminogen intergenic region in positions coincident with the sequences we have identified as having enhancer activity. These sites are not detectable in chromatin isolated from kidney or brain of the transgenic mice (results not shown).

Identification of the Core Enhancer Regions of the 1.8- and 2.0-kb Fragments by Deletion Mutagenesis—To map the positions of functional regulatory elements within the 1.8- and 2.0-kb *Kpn*I fragments, a series of deletion mutants of each fragment was constructed as shown in Fig. 3. Deletion of sequences from −1 to −1215 of the 1.8-kb fragment (the first base of the *Kpn*I site at the 3′-end of each fragment is designated

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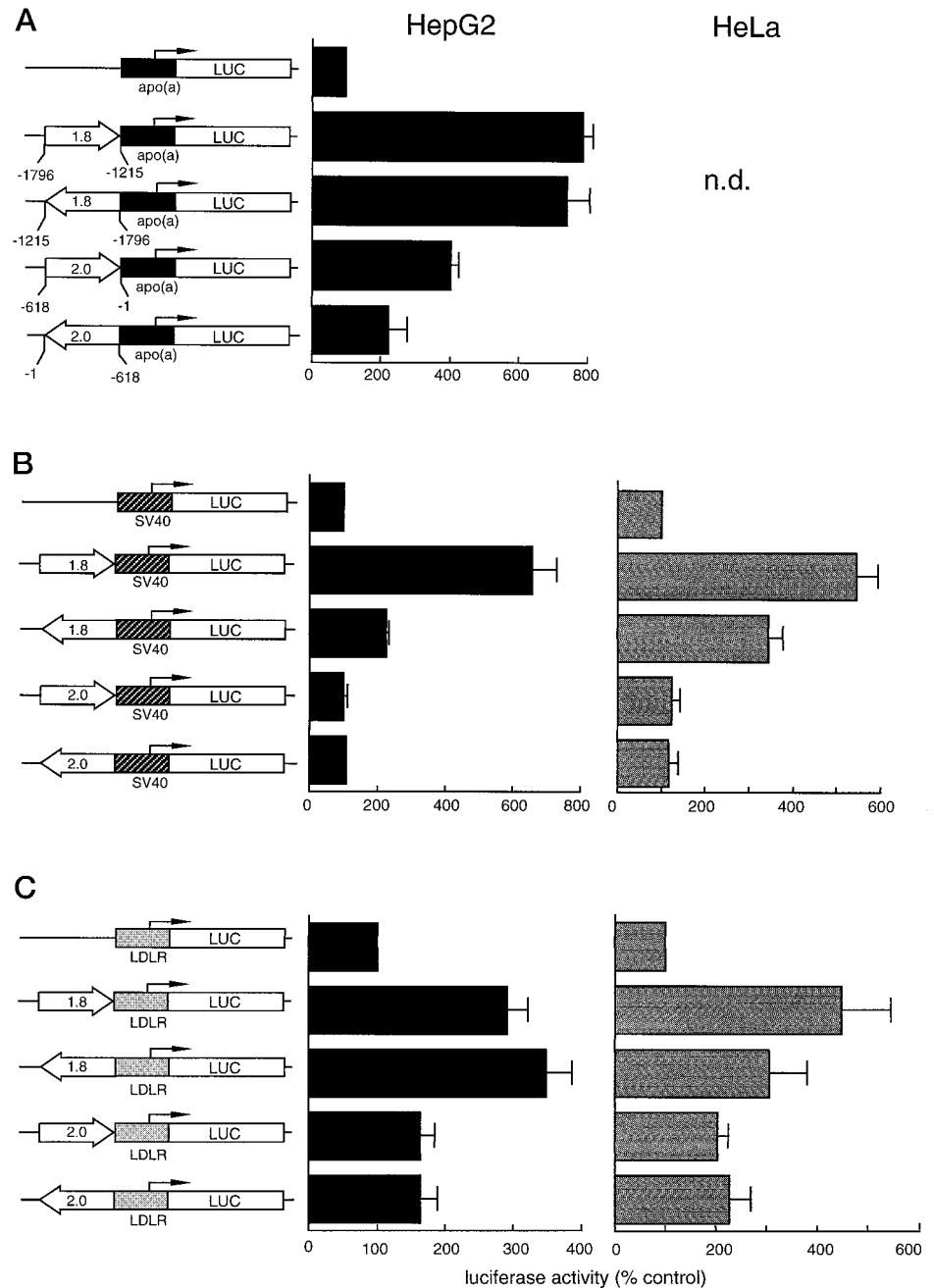[2] H-J. Muller and F. Acquati, manuscript in preparation.

FIG. 4. **Activity of enhancer core elements with heterologous promoters in HepG2 and HeLa cells.** Restriction fragments containing the the core elements of the 1.8- and 2.0-kb enhancers (Fig. 3) were excised and subcloned in both orientations upstream of the apo(a) minimal promoter (*panel A*), the SV40 promoter (*panel B*), and the LDL receptor promoter (*panel C*), as described under "Experimental Procedures." The constructs were transfected into HepG2 and HeLa cells, and luciferase and β-galactosidase activities were measured as described in the legend to Fig. 1. Results presented represent the mean and S.E. of three independent transfections. In the schematic drawings of the constructs, *unfilled arrows* show the orientation of the inserted restriction fragments.

−1) resulted in a ~5-fold increase in enhancer activity (Fig. 3*A*), indicating the presence of negative regulatory elements or interfering sequences in this region. Further deletions down to position −1517 reduced enhancer activity in a stepwise manner to zero, suggesting that all the elements necessary for maximal enhancer activity are present in the region between positions −1517 and −1215. However, a −1506 to −1215 *Nhe*I-*Xba*I fragment excised from deletion construct p(−1215)GL3FK1.8, which includes all of the nuclear protein binding sites that were subsequently detected between positions −1517 and −1215 (see below), exhibited enhancer activity that was only 20–30% of maximal (Fig. 3*A*). This indicates that the region from −1796 to −1512 contains elements that alone do not have enhancer activity but can interact with the downstream enhancer sequences to increase transcription (conditional positive region, Fig. 3*B*). The region from −1796 to −1215 of the 1.8-kb fragment therefore contains all of the elements necessary for maximal enhancer function. Compari-

son of the sequence of the 1.8-kb fragment and downstream regions with the GenBank™ data base revealed that it comprised part of a full-length 6-kb LINE-1 (32) retrotransposon element (Figs. 1*A* and 10; see "Discussion"). By a similar analysis of a series of deletion mutants in transient transfection assays, the core enhancer elements of the 2.0-kb fragment were shown to reside within sequences from −186 to −1 (Fig. 3, *C* and *D*).

Investigation of Cell Type and Promoter Specificity of the Core Enhancer Elements—To characterize the activity of the core enhancer elements of the 1.8- and 2.0-kb fragments with heterologous promoters and in a nonhepatic cell line, fragments containing the core elements of each enhancer were excised, blunted, and ligated in either orientation upstream of the apo(a) minimal promoter, the SV40 promoter, and the LDL receptor promoter. The resulting constructs were transfected into HepG2 cells and HeLa cells, a cell type that does not express the liver-specific apo(a) gene. Constructs containing
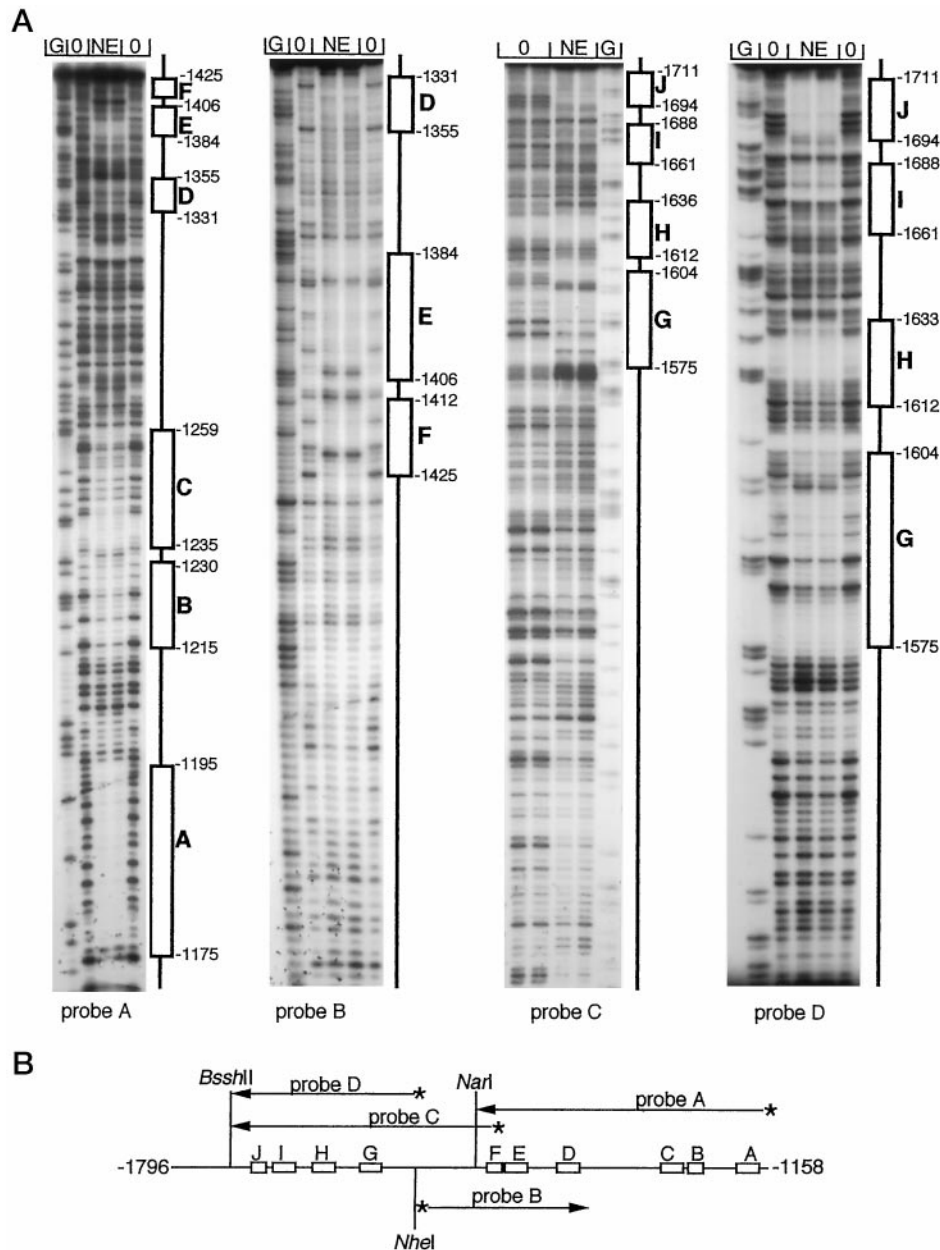
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FIG. 5. **DNase I protection of the core enhancer regions of the 1.8- and 2.0-kb fragments by HepG2 nuclear extract.** Restriction fragments were excised from the −1158 to −1796 region of the 1.8-kb enhancer (probes A–D, *panel B*) and the −1 to −618 region of the 2.0-kb enhancer (probe E, *panel D*) as detailed under "Experimental Procedures" and end-labeled. The *asterisks* on the probe schematic drawings in *panels B* and *D* mark the orientation of labeling. Fragments of the 1.8-kb enhancer (*panel A*) or the 2.0-kb enhancer (*panel C*) were digested with DNase I in the absence (*0*) or presence (*NE*) of 200 μg of HepG2 nuclear extract and fractionated on 6% polyacrylamide, 7 M urea gels beside Maxam-Gilbert G reaction sequencing ladders of the same fragments (G). Protected regions are *boxed*, and their nucleotide positions are indicated.

the apo(a) promoter alone did not express luciferase above the background value obtained with the promoterless reporter gene vector when transfected into HeLa cells; therefore, analysis of the effects of enhancer elements on apo(a) promoter activity was not undertaken in this cell line. The 1.8-kb core region enhanced luciferase expression from the apo(a) promoter in an orientation-independent manner in HepG2 cells but showed little cell type or promoter specificity, since it also enhanced luciferase expression driven by the SV40 or LDL receptor promoter in both cell lines (Fig. 4). For unknown reasons, the activity of the 1.8-kb core region appeared to be somewhat diminished in the reverse orientation with the SV40 promoter. The −1 to −618 restriction fragment containing the 2.0-kb enhancer core region previously identified by deletion analysis (bases −1 to −186) also enhanced luciferase expres-

sion from the apo(a) promoter in HepG2 cells in a largely orientation-independent manner. This region stimulated luciferase expression from the LDL receptor promoter only 2-fold or less in both HeLa and HepG2 cells and did not seem to be active with the SV40 promoter (Fig. 4). The 2.0-kb core element, like the 1.8-kb core element, did not markedly differ in its activity between the two cell lines.

DNase I Footprinting of the Core Enhancer Regions—To identify binding sites for transcription factors in the core elements of each enhancer, end-labeled probes spanning the core regions were incubated with nuclear extracts isolated from HepG2 cells and subsequently digested with DNase I. Ten footprints, designated A–J, were observed in the region from −1796 to −1158 of the 1.8-kb fragment (Fig. 5A). The strong footprint A may represent a negative regulatory element, since
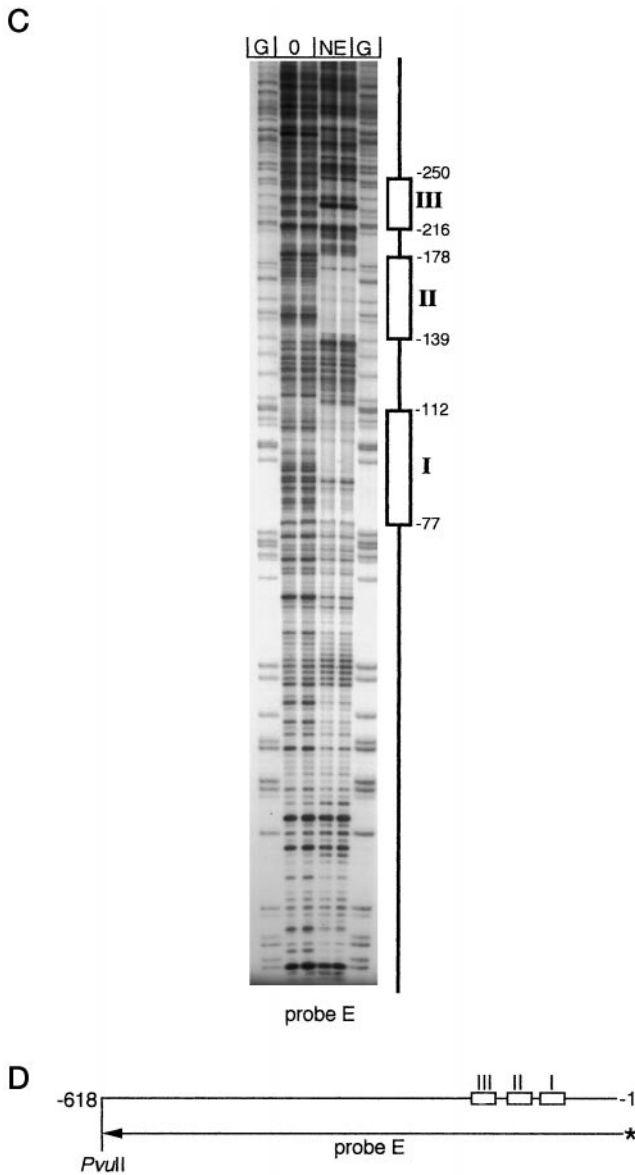
C

probe E

D

probe E

FIG. 5.—*continued*

its removal results in an increase in luciferase expression and it contains a DR-1 sequence that is homologous to the binding site for the transcriptional repressors COUP-TF and ARP-1 (33). Three footprints designated I, II, and III were detected within the −1 to −618 restriction fragment containing the −1 to −186 functional core region of the 2.0-kb enhancer (Fig. 5C). The sequence of footprint III is outside this functional core region, and its removal does not alter luciferase expression from reporter constructs (Fig. 3C); therefore, this element does not appear to mediate enhancer function.

Identification of Protein-binding Sites Mediating Major Effects on Enhancer Activity by Site-directed Mutagenesis—To investigate the functional significance of the protein binding sites that were detected by footprinting, a series of substitution or deletion mutants of these sites were synthesized. Deletion mutants of the 1.8-kb core enhancer lacking footprint B or J were constructed by the removal of the appropriate restriction fragments (*underlined* in Fig. 6A); other footprint sites in the 1.8-kb core region were mutated by the introduction of *Spe*I sites at the positions shown (Fig. 6A). Footprints I and II in the −1 to −186 functional core enhancer region of the 2.0-kb *Kpn*I fragment were mutated similarly by the substitution of *Bam*HI

sites (Fig. 6C). The effects of these mutations on enhancer activity are shown in Fig. 6, *B* and *D*. Removal of binding sites B and J (ΔmutB and ΔmutJ, Fig. 6B) from the 1.8-kb core region and substitution mutation of sites C and G had little effect on enhancer function. Mutation of sites D, H, and I reduced activity by approximately 50%. Sites E and F appear to be of the greatest significance for enhancer function, since mutation of either reduced activity by approximately 75%. Thus, individual protein binding sites in this element appear to contribute to enhancer activity with no single site being indispensable. In contrast, three of the four mutations in the protein binding sites of the −1 to −186 enhancer core region of the 2.0-kb fragment (mutIA, mutIB, and mutIIA) resulted in a complete loss of function (Fig. 6D), suggesting that the correct assembly and mutual interaction of several proteins on different sites within this element is essential for enhancement of transcription to occur.

Transcription Factor Sp1 Binds to the Site of Footprint E— Since site-directed mutagenesis showed that disruption of sites E and F in the 1.8-kb core region and sites IA, IB, and IIA in the 2.0-kb core region resulted in the greatest impairment of enhancer function, it was of interest to identify the nuclear proteins that bind to these sites and therefore play a critical role in enhancer activity. A radiolabeled oligonucleotide, oligo EF, containing the sequence of footprint sites E and F was incubated with nuclear extracts from HepG2 cells, and nuclear protein binding was analyzed by gel mobility shift assays (Fig. 7). Two major complexes were formed with this oligonucleotide (I and II, Fig. 7B, *lane 1*), which were sequence-specific, since they were abolished by an excess of the same oligonucleotide (Fig. 7B, *lane 2*) but not by unrelated oligonucleotides (Fig. 7C). An oligonucleotide, oligo mutE, containing the mutation in footprint E, which reduced transcriptional activity of the enhancer by 75% in transient transfection assays (Fig. 6B), failed to form complexes I and II, producing a complex of intermediate mobility (Fig. 7B, *lane 5*), and did not prevent formation of complexes I and II when added in competitive excess (Fig. 7B, *lane 3*), suggesting that this mutation prevents the binding of proteins important for enhancer function. However, an oligonucleotide, oligo mutF, containing the mutation of site F, which also resulted in a 75% reduction in enhancer activity in transient transfection assays, formed complexes indistinguishable from those observed with the wild type oligonucleotide EF (Fig. 7B, *lane 9*) and competed for complex formation as well as the wild type oligonucleotide (Fig. 7B, *lane 4*). Mutation of site F does not therefore appear to alter nuclear protein binding to this oligonucleotide, although it affects enhancer function significantly when present in a reporter construct (Fig. 6B). It is possible that additional proteins may bind to this site that are not detectable with the gel mobility shift protocol used here or that additional sequences flanking site F may need to be included in the oligonucleotide probe to permit protein binding to site F. Computer analysis of the sequence of oligo EF revealed homologies to known binding sites for transcription factors HrpF, c-Ets, E2F, GR, Sp1, CP2, Oct-1, AP-2, NF-κB, and C/EBP. Accordingly, to identify the proteins binding to oligo EF, double-stranded oligonucleotides representing consensus sequences for the binding of these transcription factors were synthesized (Table I) and tested for the ability to compete for complex formation (Fig. 7C). Only the oligonucleotide representing an Sp1 binding site was an efficient competitor of complexes I and II (Fig. 7C, *lane 6*), suggesting that both complexes are formed by the binding of Sp1 family proteins. When an antibody to Sp1 was included in the binding reaction, a supershift of band I was observed, confirming the presence of Sp1 in this complex (Fig. 7D, *lane 2*). Furthermore, a complex
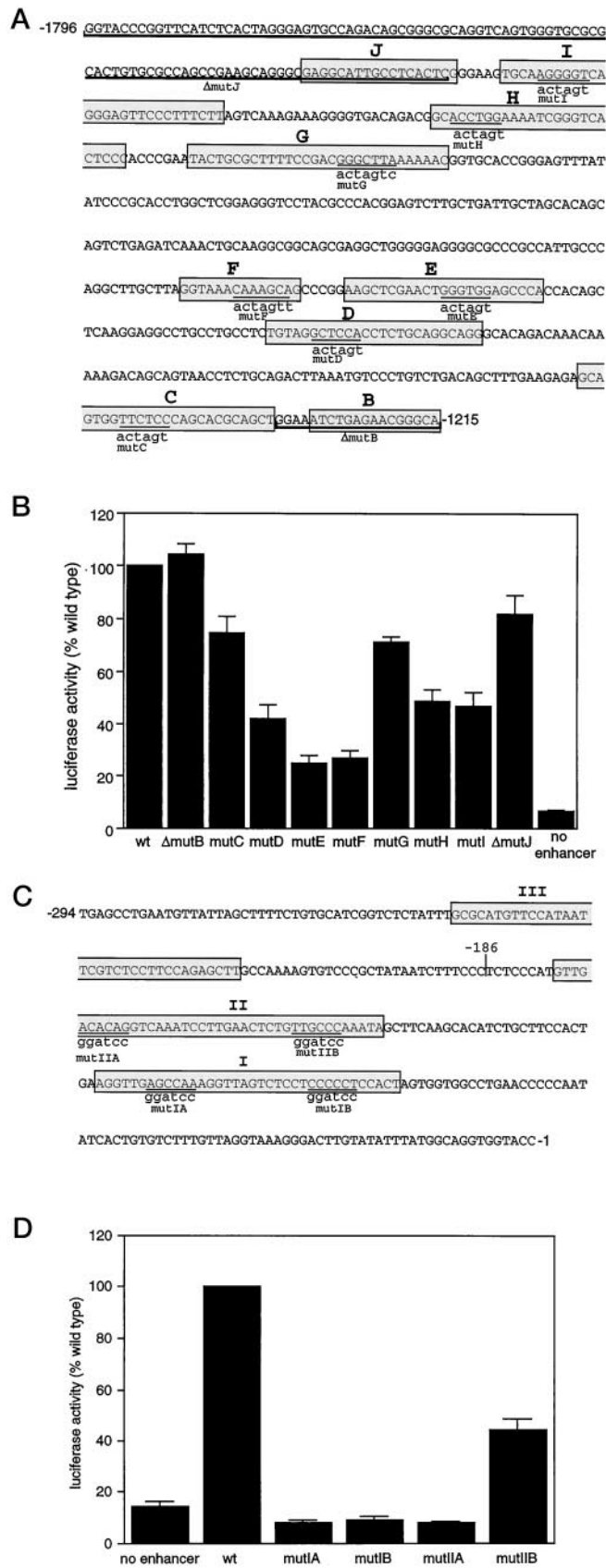
Apolipoprotein(a) Gene Enhancers

A -1796 <u>GGTACCCGGTTCATCTCACTAGGGAGTGCCAGACAGCGGGCGCAGGTCAGTGGGTGCGCG</u>

J　　　　　　　　　　　　　　　　　　**I**

<u>CACTGTGCGCCAGCCGAAGCAGGG</u>GAGGCATTGCCTCACTCGGGAAGTGCAAGGGGTCA
　　　　　　　　　ΔmutJ　　　　　　　　　　　　　　　actagt
　　　　　　　　　　　　　　　　　　　　　　　　　mutI

　　　　　　　　　　　　　　　　　　　H
GGGAGTTCCCTTTCTTAGTCAAAGAAAGGGGTGACAGACGGCACCTGGAAAATCGGGTCA
　　　　　　　　　　　　　　　　　　　　　　　　actagt
　　　　　　　　　　　　　　　　　　　　　　　　mutH

　　　　　　　　　　　　　G
CTCCGACCCGAATACTGCGCTTTTCCGACGGGCTTAAAAAACGGTGCACCGGGAGTTTAT
　　　　　　　　　　　　　　　　actagtc
　　　　　　　　　　　　　　　　mutG

ATCCCGCACCTGGCTCGGAGGGTCCTACGCCCACGGAGTCTTGCTGATTGCTAGCACAGC

AGTCTGAGATCAAACTGCAAGGCGGCAGCGAGGCTGGGGGAGGGGCGCCCGCCATTGCCC

　　　　F　　　　　　　　　　　　　　**E**
AGGCTTGCTTAGGTAAACAAAGCAGCCCGGAAGCTCGAACTGGGTGGAGCCCACCACAGC
　　　　　　　actagtt　　　　　　　　　　　　actagt
　　　　　　　mutF　　　　　　　　　**D**　　mutE
TCAAGGAGGCCTGCCTGCCTCTGTAGGCTCCACCTCTGCAGGCAGCGCACAGACAAACAA
　　　　　　　　　　　　　actagt
　　　　　　　　　　　　　mutD

AAAGACAGCAGTAACCTCTGCAGACTTAAATGTCCCTGTCTGACAGCTTTGAAGAGAGCA

　　　　C　　　　　　　　　　　　**B**
<u>GTGGTTCTCCCAGCACGCAGCT</u>GGAAATCTGAGAACGGGCA-1215
　　actagt　　　　　　　　ΔmutB
　　mutC

B

C -294 TGAGCCTGAATGTTATTAGCTTTTCTGTGCATCGGTCTCTATTTGCGCATGTTCCATAAT
　　　　　　　　　　　　　　　　　　　　　　　III

TCGTCTCCTTCCAGAGCTTGCCAAAAGTGTCCCGCTATAATCTTTCCCTCTCCCATGTTG
　　　　　　　　　　　　　　-186

　　II
ACACAGGTCAAATCCTTGAACTCTGTTGCCCAAATAGCTTCAAGCACATCTGCTTCCACT
ggatcc　　　　　　　　　　ggatcc
mutIIA　　　　　　　　　　mutIIB

　　　　　　　　　　　I
GAAGGTTGAGCCAAAGGTTAGTCTCCTCCCCCTCCACTAGTGGTGGCCTGAACCCCCAAT
　　ggatcc　　　　　　　　ggatcc
　　mutIA　　　　　　　　　mutIB

ATCACTGTGTCTTTGTTAGGTAAAGGGACTTGTATATTTATGGCAGGTGGTACC-1

D

FIG. 6. **Functional analysis of footprint regions in the 1.8- and 2.0-kb enhancers by site-directed mutagenesis.** *Panels A* and *C*, nucleotide sequences protected from DNase I digestion by HepG2 nuclear extracts. The sequences covered by footprints in the 1.8-kb enhancer core region (*panel A*) and the 2.0-kb enhancer core region (*panel C*) are *boxed*. Site-directed mutants of these fragments were constructed as detailed under "Experimental Procedures"; the position of

with similar mobility to complex I was observed when purified recombinant Sp1 was incubated with oligo EF, directly demonstrating the ability of Sp1 to recognize this sequence in the absence of accessory factors (Fig. 7*D*, *lane 5*).

Transcription Factors Sp1 and PPAR Bind to Footprint Site I—Gel mobility shift assays were also performed with an oligonucleotide, oligo I, spanning the sequence of footprint I from the 2.0-kb enhancer core region, and oligonucleotides ImutA and ImutB, which contain the previously described mutations of this sequence that abolish enhancer activity (Fig. 6*D*). Oligo I formed two major complexes, a and b, and one minor complex, c, with HepG2 nuclear extract (Fig. 8*B*, *lane 1*); these complexes result from the binding of sequence-specific proteins, since they were abolished by the addition of an excess of cold oligo I (Fig. 8*B*, *lane 2*) but not by oligonucleotides of an unrelated sequence (Fig. 8*C*). Complexes a and b result from the binding of proteins to a site disrupted by mutation IB, since radiolabeled oligo ImutB formed only complex c (Fig. 8*B*, *lane 5*) and two diffuse complexes that differ in mobility and intensity from a and b, possibly representing the fortuitous binding of other proteins to the mutated sequence. Furthermore, a molar excess of unlabeled oligo ImutB did not impair the formation of complexes a and b with oligo I (Fig. 8*B*, *lane 10*), indicating that the proteins forming complexes a and b cannot bind to the mutant oligonucleotide. Radiolabeled oligo ImutA formed complexes a and b but not c (Fig. 8*B*, *lane 3*), and cold excess oligo ImutA competed only for the binding of complexes a and b (Fig. 8*B*, *lane 9*), demonstrating that the mutation ImutA disrupts the binding site for proteins in complex c. Computer analysis of the sequence of footprint I revealed that sequences with close homology to the binding sites for transcription factors CP2, PPAR, AP-1, GATA-1, C/EBP, and E2F were clustered around site A and that sequences with close homology to binding sites for Sp1, H4TF, and C/EBP were clustered around site B. Competition studies were performed with oligonucleotides representing consensus sequences for the binding of these factors (Table I). Clearly, the Sp1 consensus binding sequence abolished complexes a and b (Fig. 8*C*, *lane 3*), while the PPAR consensus binding sequence abolished complex c (Fig. 8*C*, *lane 7*). The binding of complex c was also impaired by consensus sequences for the binding of E2F and, to a lesser extent, GATA-1. These results suggest that site B in footprint I is an Sp1 binding site and that site A is a PPAR binding site. These conclusions were confirmed by gel mobility shift assays using antiserum to Sp1 and PPAR and recombinant Sp1. A strong supershift is produced when antiserum against Sp1 is included with oligo I or oligo ImutA in the bandshift reactions but not with oligo ImutB (Fig. 8*D*, *lanes 2*, *5*, and *7*). In addition, purified recombinant Sp1 can bind to oligo I and ImutA but not oligo ImutB (Fig. 8*D*, *lanes 9*, *11*, and *12*). Thus, site B is a functional Sp1 binding site. To determine whether site A

each mutation is *underlined*, and the bases changed are in *lowercase type*. *Heavy underlines* in *panel A* indicate sequences removed from the core region of the 1.8-kb fragment by restriction digestion to construct deletion mutants ΔmutB and ΔmutJ. *Panel B*, transcriptional activity of site-directed mutants of the the 1.8-kb enhancer core region in HepG2 cells. HepG2 cells were transfected as detailed in the legend to Fig. 1 with luciferase reporter constructs containing the wild-type −1796 to −1215 region of the 1.8-kb enhancer fragment or its mutant derivatives fused upstream of the apo(a) minimal promoter. Luciferase activities are expressed as a percentage of the activity of the wild-type enhancer, and the results presented are the mean and S.E. of three independent transfections. *Panel D*, transcriptional activity of site-directed mutants of the 2.0-kb enhancer core region in HepG2 cells. HepG2 cells were transfected with luciferase reporter constructs containing the wild-type −618 to −1 region of the 2.0-kb enhancer fragment or its mutant derivatives detailed in *panel C*, fused upstream of the apo(a) minimal promoter. Experimental details are as in *panel B*.
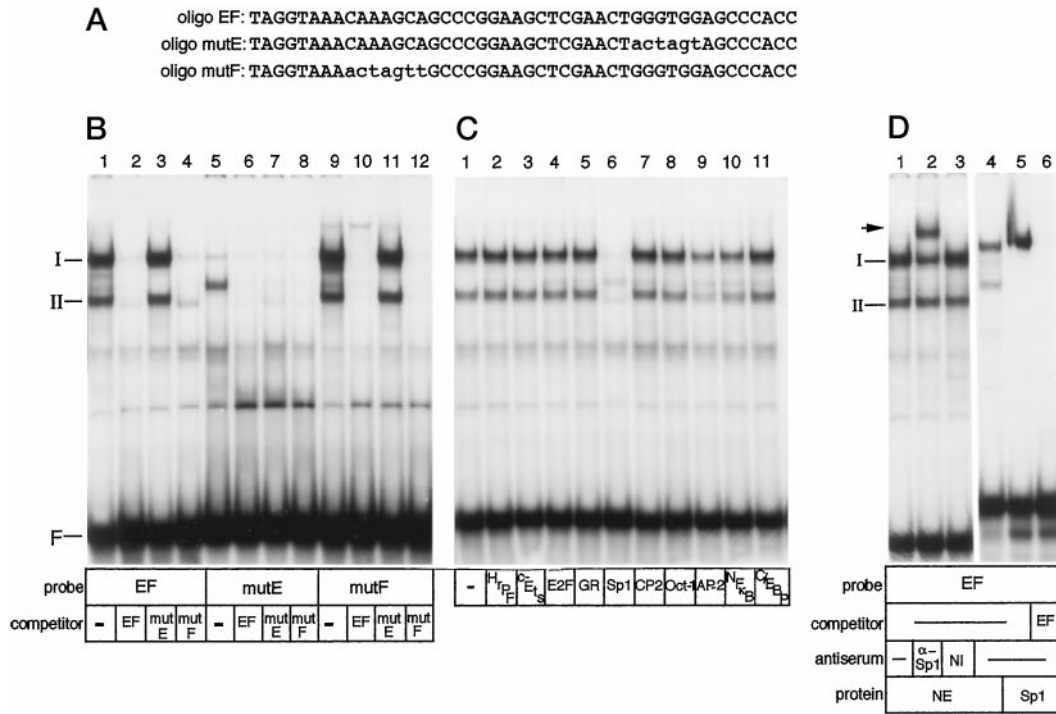
FIG. 7. **Gel mobility shift assay of footprints E and F in the core region of the 1.8-kb enhancer.** *Panel A* shows the sequence of a synthetic double-stranded oligonucleotide spanning footprints E and F in the −1796 to −1215 region of the 1.8-kb enhancer fragment, oligo EF, and oligonucleotides oligo mutF, and oligo mutE, containing previously described mutations of this sequence (Fig. 6*A*). Bases in the mutant oligonucleotides that differ from wild type are shown in *lowercase type*. *Panel B*, gel mobility shift assay of wild type and mutant footprints E and F. End-labeled oligonucleotides EF, mutF, or mutE were incubated with 4 μg of HepG2 nuclear extract in the presence or absence of a 100-fold molar excess of unlabeled competitor oligonucleotides as indicated. *I* and *II* indicate specific complexes, and *F* indicates free probe. *Panel C*, competition studies with oligonucleotides containing consensus binding sites for transcription factors. Oligo EF was incubated with 4 μg of HepG2 nuclear extract in the presence or absence of a 100-fold molar excess of unlabeled oligonucleotides containing binding sites for the transcription factors indicated *below each lane*. *Panel D*, identification of Sp1 binding to oligo EF. Oligo EF was incubated with 4 μg of HepG2 nuclear extract (*NE*) or purified recombinant Sp1 (*Sp1*) in the presence or absence of antiserum to Sp1 (*α-Sp1*), nonimmune serum (*NI*), or a 100-fold excess of unlabeled competitor (*EF*) as indicated. The supershifted band is shown by an *arrow*.

TABLE I
Sequences of transcription factor binding site competitor oligonucleotides

The core binding sequences for each factor are underlined.

Transcription factor	Sequence	Ref.
AP-1	CGCTTGATGAGTCAGCCGGAA	34
AP-2	GATCGAACTGACCGCCCGCGGCCCGT	35
C/EBP	GCTGCAGATTGCGCAATCTGCA	36
CP2	GAGCAAGCACAAACCAGCAATGAG	37
CREB[a]	AGAGATTGCCTGACGTCAGAGAGCTAG	38
E2F	TAAGTTTCGCGCCCTTTC	39
c-Ets	TCGAGCAGGAAGTGACGTCG	40
GATA-1	GTTGAAACAAGATAAGATCAAATTGA	41
GR	GATCTGTACAGGATGTTCTAGAT	42
HrpF	GAGGACACGGAAGTGACG	43
H4TF	GATCGGGGGAGGGGATCG	44
NF-κB	AGTTGAGGGGACTTTCCCAGGC	45
OCT-1	TGTCGAATGCAAATCACTAGAA	46
PPAR	CTAGCGATATCATGACCTTTGTCCTAGGCCTC	47
ROR-α[a]	TGGTTGACCTACTTATATATG	48
Sp1	ATTCGATCGGGGCGGGGCGAGC	49

[a] cAMP response element-binding protein.
[b] Retinoid-related orphan receptor-α.

binds PPAR, an antiserum that recognizes both PPARα and PPARγ was included in the bandshift reaction with oligo I. A clear supershift was observed confirming the presence of PPAR in the complex (Fig. 8*D*, *lane 14*). Since this shows that PPAR is certainly present in complex *c*, it is probable that the competitive impairment of the formation of this complex observed with binding sites for GATA-1 and E2F results from cryptic similarities to the PPAR binding site within these sequences.

However, these experiments do not formally exclude the possibility that complex *c* also contains GATA-1 and E2F proteins.

Transcription Factor PPAR Binds to Footprint II—Similar studies were performed to investigate the nuclear proteins binding to footprint site II in the 2.0-kb enhancer core. Oligo II forms three specific complexes with HepG2 nuclear extract (*x*, *y*, and *z*, Fig. 9*B*, *lanes 1* and *2*). Complexes *x* and *y* contain doublet or triplet bands, more clearly seen in the shorter exposure of Fig. 9*C*, and probably represent a number of different proteins bound to the probe. Oligo IImutA, which includes the mutated sequence previously shown to eliminate enhancer function in transient transfection assays (Fig. 6*D*), formed few of the complexes constituting *x* and *y* (Fig. 9*B*, *lane 3*), suggesting that the binding of these proteins is largely disrupted by this mutation. Computer analysis of the sequence of footprint II revealed high homologies to binding sites for AP-1, NF-κB, cAMP response element-binding protein, GATA-1, C/EBP, and the nuclear hormone receptors GR, retinoid-related orphan receptor-α, and PPAR. Gel shift competition studies were performed with unlabeled oligonucleotides representing consensus sequences for the binding of these transcription factors (Table I). The PPAR consensus oligonucleotide abolished complex *x* and much of complex *y* (Fig. 9*C*), while the retinoid-related orphan receptor-α binding sequence, which is highly related to the PPAR binding sequence (48) had a similar effect on the formation of complex *x* but had less effect on the formation of complex *y* (*lanes 5* and *6*). These results suggest that PPAR is a component of the shifted complexes observed with oligo II. The presence of PPAR in these complexes was indeed confirmed by a gel supershift assay with antiserum to PPAR (Fig.
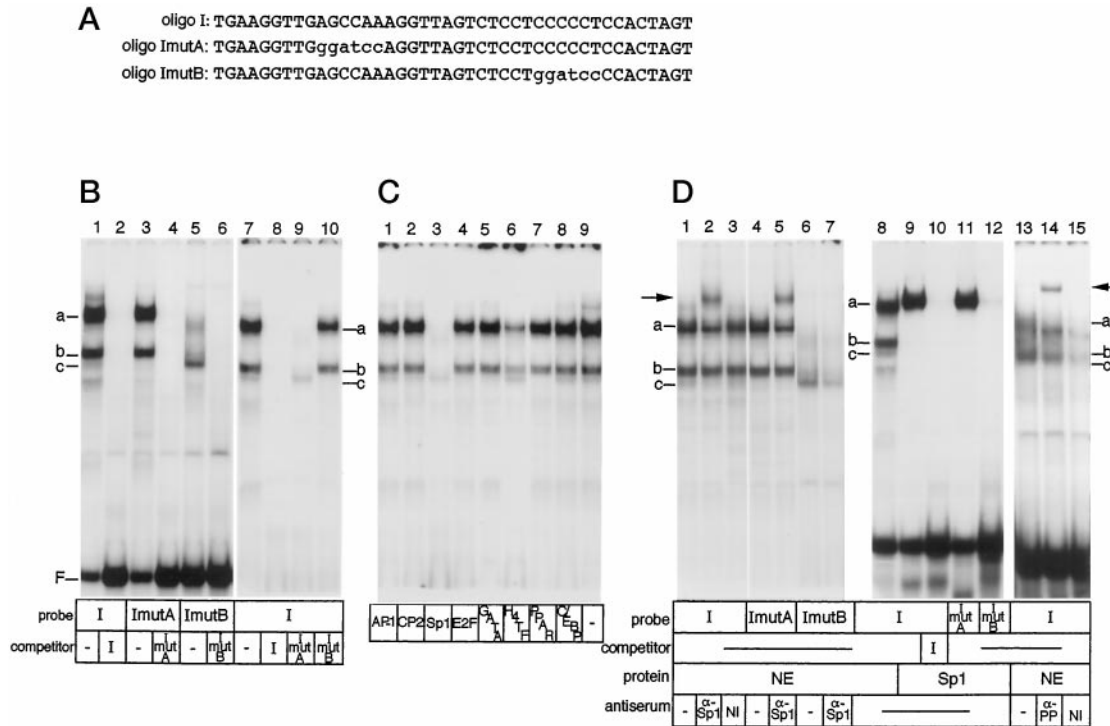
A
oligo I: TGAAGGTTGAGCCAAAGGTTAGTCTCCTCCCCCTCCACTAGT
oligo ImutA: TGAAGGTTGggatccAGGTTAGTCTCCTCCCCCTCCACTAGT
oligo ImutB: TGAAGGTTGAGCCAAAGGTTAGTCTCCTggatccCCACTAGT

FIG. 8. **Gel mobility shift assays of footprint I in the core region of the 2.0-kb enhancer.** *Panel A* shows the sequence of a synthetic double-stranded oligonucleotide, oligo I, spanning the sequence of footprint I in the −618 to −1 region of the 2.0-kb enhancer fragment, and oligonucleotides mutIA and mutIB containing the previously described mutations of this sequence (Fig. 6*C*). Bases that differ from wild-type sequence are shown in *lowercase type*. *Panel B*, gel mobility shift assays of wild-type and mutant footprint I. End-labeled oligonucleotides I, mutIA, or mutIB were incubated with 4 µg of HepG2 nuclear extract in the presence or absence of 100-fold molar excess of unlabeled competitor oligonucleotides as indicated. *a*, *b*, and *c* indicate specific complexes, and *F* indicates free probe. *Panel C*, competition studies with oligonucleotides containing consensus binding sites for transcription factors. Oligo I was incubated with 4 µg of HepG2 nuclear extract in the presence or absence of a 100-fold molar excess of unlabeled oligonucleotides containing binding sites for the transcription factors indicated *below* each *lane*. *Panel D*, identification of Sp1 and PPAR binding to oligo I. Oligos I, ImutA, and ImutB were incubated with 4 µg of HepG2 nuclear extract (*NE*) or purified recombinant Sp1 (*Sp1*) in the presence or absence of antiserum to Sp1 (*α-Sp1*), antiserum to PPAR (*α-PP*), nonimmune serum (*NI*), or a 100-fold excess of unlabeled competitor (*I*) as indicated. Supershifted bands are shown by *arrows*.

9*D*, *lane 2*). However, the multiple complexes observed suggest that several other proteins that recognize the PPAR consensus sequence also bind to the sequence of footprint II.

DISCUSSION

The transcription of many genes is driven only at a low level by proximal promoter elements, optimal expression often being dependent on the presence of one or more regulatory regions, frequently at some distance from the proximal promoter, which may also confer tissue specificity of expression (50–52). Such elements, termed enhancers, stimulate transcription from the proximal promoter in a distance- and orientation-independent manner and may be associated with the presence of DNase I-hypersensitive sites, indicating a chromatin structure that permits interaction of the elements with nuclear transcription factors (28, 29). In this study, we report the identification of two distinct regions ~20 and ~28 kb upstream of the transcription start site of the apo(a) gene that stimulate transcription from the apo(a) promoter in an orientation-independent manner and so exhibit the properties of enhancers. The positions of these enhancer elements coincide with DNase I-hypersensitive sites DHIII and DHII that are present in hepatoma cell chromatin but absent in DNA from nonhepatic cell lines, reflecting the liver-specific expression of the apo(a) gene (27). We have shown here (Fig. 2) that these sites are also observed in the liver chromatin of apo(a) YAC transgenic mice that contain the apo(a)-plasminogen intergenic region and express apo(a) at high levels in an appropriate liver-specific manner.[2] The demonstration that the functional enhancer activity we have detected with the transient transfection system colocalizes with

DNase I-hypersensitive sites present in liver *in vivo* is a strong indication that these regions are important in transcriptional regulation of the apo(a) gene. However, since neither enhancer appears to be cell type-specific (Fig. 4), they are not likely to contribute to the restriction of apo(a) gene expression to the liver through the binding of liver-specific transcription factors. It is possible that the chromatin structure at these sites is only permissive for interaction with transcription factors in differentiated hepatic tissue.

The DHIII enhancer region was contained within a 1.8-kb genomic fragment situated ~20 kb upstream of the transcription start site of the apo(a) gene. Enhancer activity mapped to a core region of ~600 bp that was active in either orientation with heterologous promoters (Fig. 4). Enhancer activity is dependent on the binding of nuclear factors to multiple elements within this sequence, with no single element being essential for activity (Fig. 6*B*). A mutation that prevents the binding of the transcription factor Sp1 to the site of footprint E within this core sequence reduces enhancer activity 4-fold, implicating Sp1 as a major contributor to enhancer function. A second factor also appears to bind to the site of footprint E, since we observed two major complexes (complexes I and II) with HepG2 nuclear extracts, whereas purified Sp1 protein produced only complex I (Fig. 7*B*). Competition studies showed that complex II is formed by a protein that recognizes the Sp1 consensus sequence and may therefore be one of the recently identified Sp1 family members, Sp2, Sp3, or Sp4 (53, 54). Sp1 is a ubiquitously expressed transcription factor that functions as a constitutive activator of housekeeping genes through binding to

FIG. 9. **Gel mobility shift assays of footprint II in the core region of the 2.0-kb enhancer.** *Panel A* shows the sequence of a synthetic double-stranded oligonucleotide, oligo II, spanning the sequence of footprint II in the −618 to −1 region of the 2.0-kb enhancer fragment, and oligonucleotide mutIIA containing the previously described mutation of this sequence (Fig. 6*C*). Bases that differ from wild-type sequence are shown in *lowercase type*. *Panel B*, gel mobility shift assays of wild-type and mutant footprint II. End-labeled oligonucleotides II or mutIIA were incubated with 4 μg of HepG2 nuclear extract in the presence or absence of a 100-fold molar excess of unlabeled competitor oligonucleotides as indicated. *x*, *y*, and *z* indicate specific complexes. Free probe has migrated off the gel in this panel. *Panel C*, competition studies with oligonucleotides containing consensus binding sites for transcription factors. Oligo II was incubated with 4 μg of HepG2 nuclear extract in the presence or absence of 100-fold molar excess of unlabeled oligonucleotides containing binding sites for the transcription factors indicated *below* each *lane*. *Panel C*, identification of PPAR binding to oligo II. Oligo II was incubated with 4 μg of HepG2 nuclear extract (*NE*) in the presence or absence of antiserum to PPAR (*α-PP*) or nonimmune serum (*NI*) as indicated. The supershifted band is shown by an *arrow*.
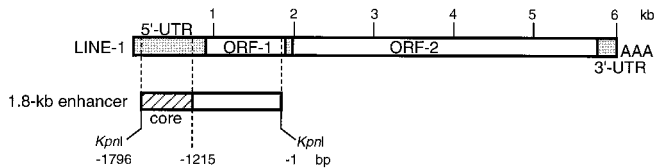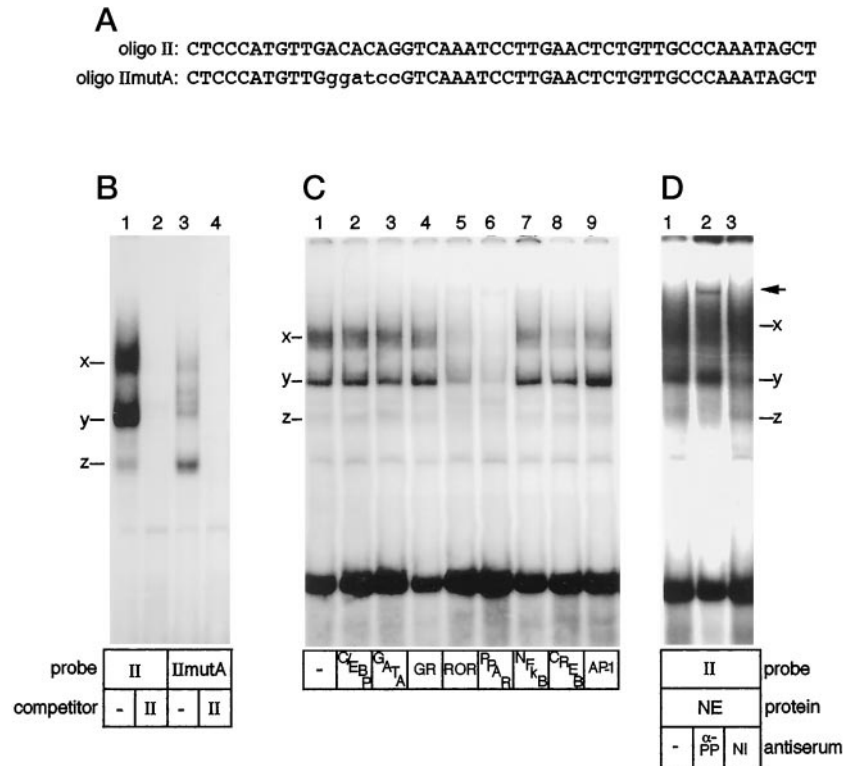

FIG. 10. **Schematic drawing showing the alignment of the 1.8-kb enhancer fragment with a consensus LINE-1 element.** *Shaded areas* in the LINE-1 element represent untranslated regions, and the *hatched area* in the 1.8-kb enhancer represents the enhancer core element, numbered as described under "Experimental Procedures" (Fig. 3). *AAA* indicates a poly(A) tail.

GC boxes in their proximal promoter regions (55, 56), but it has recently also been found to play an important role in the activity of several enhancers, including the apoE upstream regulatory element (57), the immunoglobulin-κ 3′-enhancer (58), and the intestinal enhancer region of the apoA1 gene (59).

Sequence comparison of the 1.8-kb *Kpn*I fragment containing the core enhancer region with the GenBank™ data base revealed to it to be part of a LINE-1 (32) retrotransposon repetitive element (Figs. 1 and 10). The full-length LINE-1 consensus sequence is approximately 6 kb, consisting of a 900-bp 5′-UTR, two open reading frames separated by a short noncoding region, and a 3′-UTR of 200 bp terminated by a poly(A) tail (32) (Fig. 10). In functional (transposable) LINE-1 elements, ORF1 codes for an RNA-binding protein and ORF2 codes for a reverse transcriptase with endonuclease activity (60, 61). However, only ~4000 of the 10^5 LINE-1 elements in the human genome are full-length, and of these most contain base changes that introduce premature stop codons into ORF1 or 2, rendering them nonfunctional (32, 62). The 1.8-kb *Kpn*I enhancer fragment (Fig. 10) comprises bases +97 to +907 of the 5′-UTR and the first 986 bases of ORF1 of a LINE-1 element that is complete but nonfunctional, since ORF1 is terminated by a stop codon corresponding to the codon for amino acid residue 75 (61). The 5′-UTR of a functional LINE-1 element was shown previously to contain an internal promoter that directs the

initiation of transcription from base +1 of the LINE-1 sequence (62). In this study (62), deletion of internal portions of the LINE-1 element 5′-UTR that are homologous to the 1.8-kb core enhancer fragment characterized here (Fig. 10) resulted in marked reduction of transcription from the LINE-1 promoter, suggesting that important regulatory elements are present within this sequence. However, no systematic footprinting analysis or site-directed mutagenesis of these regions had been performed to date. The findings reported here suggest that Sp1 may be important in the transcriptional regulation of the 40–60 currently active LINE-1 retrotransposons (63) in the human genome and in addition represent the first demonstration that a LINE-1 5′-UTR contains a potentially mobile enhancer element that is active with heterologous promoters. One possible effect of LINE-1 transpositions may therefore be to alter the regulation of nearby genes by the introduction of powerful enhancer elements at new sites in the genome. Transposed retroviral sequences have been shown to be the source of an androgen-responsive enhancer 2 kb upstream of the mouse complement-related *slp* gene (64) and a novel parotid-specific promoter/enhancer element in a human salivary amylase gene (65). Active LINE-1 retrotransposons may therefore prove to be an analogous source of mobile regulatory elements in human evolution.

The second enhancer element we have identified was finely mapped to a 186-bp region, which exhibited orientation-independent activity with the apo(a) promoter. Enhancer activity was completely lost when nuclear protein binding to site IA, IB, or IIA of footprints I and II within the core enhancer region was disrupted by site-directed mutagenesis (Fig. 6, *C* and *D*), suggesting that the simultaneous interaction of a number of proteins bound to separate sites on the enhancer is obligatory for transcriptional activation. We were able to identify some of the the proteins binding to these three functionally indispensable sites by gel mobility shift assays. Site B of footprint I (CTC-CTCCC) is an Sp1 binding site (Fig. 8) that is very similar to a recently identified Sp1 site in the Wilms tumor-1 gene (CTC-

CTCCT), which was reported to bind Sp1 more strongly than the established consensus (66).

We showed by a gel supershift assay that site A in footprint I and site A in footprint II bind PPAR (Fig. 8*C*). Although the antiserum used in the supershift assay reacts with both PPARα and PPARγ, we conclude that the protein binding to both sites is likely to be PPARα, since PPARα is expressed highly in liver and PPARγ is not (67). The consensus PPAR response element (47) is a direct repeat of the nuclear hormone receptor half-site AGGTCA separated by a 1-bp spacer (DR-1), and both footprint I site A (GAGCCAaAGGTTA) and footprint II site A (TTGA-CAcAGGTCA) contain variants of this sequence. PPARα is a ligand-activated member of the zinc finger nuclear hormone receptor superfamily that binds to its recognition sites as an obligate heterodimer with the retinoic acid-activated nuclear hormone receptor, retinoid X receptor (68, 69). PPARα is activated by long chain fatty acids, the fibrate-derivative hypolipidemic drugs, and leukotrienes (69–71) and can stimulate or repress transcription through DR-1 response elements in target genes, depending on the promoter context (47, 72). DR-1 sites may also bind additional members of the nuclear hormone receptor family, including HNF4, COUP-TF, and the two retinoic acid-activated receptors retinoid X receptor and retinoic acid receptor (33, 72, 73). Footprint II also contains an inverted repeat of the AGGTCA half-site separated by 6 bases (AGGT-CAaatcctTGAACT), which partially overlaps the DR-1 site. Inverted repeat elements with various spacings are recognized by other members of the nuclear hormone receptor family that bind as dimers, including the estrogen receptor, which may regulate transcription through elements consisting of inverted repeats separated by three base pairs, or multiple half-sites (74). Since several protein complexes are observed in bandshift assays with an oligonucleotide containing the sequence of footprint II and since most of these are abolished by competition with an excess of a DR-1 oligonucleotide (Fig. 9*C*), it is likely that a number of nuclear hormone receptors can interact with this site. It is beyond the scope of this preliminary characterization to identify all of these factors.

The juxtaposition of Sp1 and nuclear hormone receptor elements in the 2.0-kb core enhancer suggests that it may be capable of responding to drugs or hormones, since combinations of these elements have been shown to constitute hormone-responsive enhancers in a number of other genes. For example in the estrogen-induced cathepsin D gene, the c-*myc* gene, and the estrogen-induced creatine kinase gene, Sp1 stabilizes binding of the estrogen receptor to a single half-site by binding to an immediately adjacent sequence, the two elements comprising an estrogen-responsive enhancer (75–77). It is of interest that transcription of the apo(a) gene has been reported to be repressed in cynomolgus monkeys by gemfibrozil (78), a known activator of PPARα, in apo(a) YAC mice by estrogens and testosterone (79) and in primate hepatocytes by retinoic acids (80). It would be of interest to investigate whether the 2.0-kb core enhancer region is involved in mediating any of these responses.

In summary, we have identified two enhancers in the apo(a)-plasminogen intergenic region that could be important for the optimal expression of the apo(a) gene *in vivo* and have begun to elucidate the *trans*-acting factors that mediate their function. The studies reported here define regions that would be of interest to examine for the presence of functional polymorphisms affecting apo(a) gene expression. It would also be of interest to ask whether these enhancer regions can influence expression of the closely linked plasminogen gene. The ideal way to address these questions would be to create transgenic mouse lines containing intact and mutated versions of the

apo(a)-plasminogen intergenic region. Such studies are currently under way.

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Characterization of Multiple Enhancer Regions Upstream of the Apolipoprotein(a) Gene

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Page 30387: The footnote indicating the GenBank™ accession number omitted one of the numbers. The footnote should read: "*The nucleotide sequences reported in this paper have been submitted to the GenBank™/EBI Data Bank with accession numbers AF027597 and AF027598.*"

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