Identification of a Cis-Acting Negative DNA Element Which Modulates Human Hepatic Triglyceride Lipase Gene Expression

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ABSTRACT: The promoter fragment −1550/+129 of the human hepatic triglyceride lipase (HTGL) gene drives the expression of the CAT gene in HepG2 cells, albeit at very low levels. Transient transfections in HepG2 and HeLa cells of 5′ deletion constructs indicated that the regulatory elements that control this expression are located in the proximal region of the gene. DNase I footprint analysis with DNA fragments spanning the region −483 to +129 and rat liver nuclear extracts identified eight protected regions, four upstream of the transcription initiation site (A, −28 to −75; B, −96 to −106; C, −118 to −158; D, −185 to −255) and four in the first exon of the gene (E1, −5 to +20; E2, +36 to +55; E3, +58 to +83; E4, +86 to +107). DNA binding and footprinting analysis demonstrated that the region −75 to −43 within footprint A binds to the liver-specific transcription factor HNF1. The region +28 to +129 contains a functional negative regulatory element (NRE) since deletion of this region results in a 17-fold increase in CAT activity. The NRE can act independent of orientation and position and repress transcription driven by heterologous promoters. DNA binding assays using native and fractionated liver nuclear extracts identified two transcription factors that bind to element E2 and also to element E3. A dinucleotide mutation in element E2 which causes derepression of the HTGL gene by 10-fold also abolishes the binding of these two activities. Transfection experiments showed that deletion of the NRE allows expression of reporter constructs in HeLa cells, indicating that the NRE may play a determinant role for the expression of HTGL gene in hepatic cells.

Triglyceride lipases are members of a dispersed gene family of lipolytic enzymes that are biologically active in absorption, transport, and metabolism of dietary lipids (Ameis et al., 1990; Cai et al., 1989). The endothelial-bound lipases, lipoprotein lipase and hepatic triglyceride lipase (HTGL), in addition to their function in hydrolyzing triglyceride rich lipoproteins, regulate the production and metabolism of several lipoproteins including remnant particles, intermediate density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Defects in their structure or biosynthesis may contribute to alterations of the plasma lipid transport system and therefore to the etiology of atherosclerosis.

HTGL is synthesized by the liver parenchymal cells and secreted into the sinusoids, where it is bound to glucosaminoglycans on the endothelium cell surface (Jackson, 1983). The function of hepatic lipase is the hydrolysis of triglycerides in IDL to produce LDL, and that of phospholipids and triglycerides in HDL_2 to produce HDL_3 (Kinnumen, 1984; Rao et al., 1982). The primary sequence of rat and human hepatic lipase has been derived from the corresponding sequence of cDNA clones (Stahnke et al., 1987; Komaromy & Schotz, 1987; Martin et al., 1988; Datta et al., 1988). The human hepatic lipase protein consists of 499 amino acids preceded by a signal peptide of 22 (Martin et al., 1988) or 23 (Stahnke et al., 1987; Datta et al., 1988) amino acids. The mature protein consists of 476 amino acids with a predicted molecular mass of 53 kDa (Stahnke et al., 1987; Martin et al., 1988; Datta et al., 1988). The hepatic lipase gene contains nine exons separated by eight introns. The gene spans over 60 kb, with the exons accounting for only 1.6 kb of its total length, and it maps at the q^{21} region of chromosome 15 (Datta et al., 1988; Sparkes et al., 1987). Hepatic lipase activity has been detected in several extrahepatic tissues, including adrenal gland and ovary (Doolittle et al., 1987). Northern blot analyses indicated the presence of hepatic lipase mRNA exclusively in the liver but not in other tissues, suggesting that hepatic lipase originates in liver and is transported to the extrahepatic tissues (Komaromy & Schotz, 1987; Semenkovich et al., 1989).

The synthesis of HTGL has been shown to be regulated during development, exhibiting a biphasic pattern (Semenkovich et al., 1989). Hepatic lipase mRNA was detected in liver six days before birth at a level 15–31% that in adult liver (Semenkovich et al., 1989; Chajek et al., 1977) and increased 7.9-fold at the time of birth and 7.5-fold between days 13 and 24 (weaning period) (Semenkovich et al., 1989). Hepatic lipase mRNA was shown to be moderately decreased (20% of control) in hypothyroid rats (Staels et al., 1990). Increases in the levels of hepatic lipase mRNA have been reported in HepG2 cells exposed to heparin (Busch et al., 1989) and also under conditions which perturb cellular cholesterol homeostasis (Busch et al., 1990).

Hormonal factors including T3 (Hulsmann et al., 1977; Jubelin et al., 1978; Murase & Uchimura, 1980), glucocorticoids (Hulsmann & Dubelaar, 1986), insulin (Knauer et al., 1982), estrogens (Applebaum et al., 1977), and androgens (Applebaum-Bowden et al., 1987; Ehnholm et al., 1975) have also been reported to affect hepatic lipase activity, but no studies on hepatic lipase mRNA levels or transcription have been reported. Notably, estrogens decrease HTGL activity in postheparin plasma, whereas androgens increase HTGL activity, and this is followed by a decrease in HDL_2 cholesterol. The observation that high HDL_2 levels protect against the development of atherosclerosis suggests a potential role of hepatic lipase in HDL regulation and the pathogenesis of

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atherosclerosis. Indeed, a strong negative correlation was demonstrated between levels of HDL_2 cholesterol and HTGL activity (Kuusi et al., 1989; Patsch et al., 1987).

Very little is known about the regulatory expression of the hepatic lipase gene. The sequences of 5′ upstream promoter regions for both the human (Ameis et al., 1990; Cai et al., 1989) and rat hepatic lipase (Sensel et al., 1990) genes have been characterized, and possible regulatory elements were identified by searching for known consensus sequences (Ameis et al., 1990; Cai et al., 1989; Sensel et al., 1990). In this study, we examined the role of 5′ and 3′ flanking sequences in determining the levels and tissue specificity of HTGL gene expression. Using transient transfections, we showed that the promoter region −483 to +28 supports the hepatic specific transcription of the HTGL gene. A negative regulatory element (NRE) identified downstream of the transcription start site appears to have properties of a general silencer. Two chromatographically separable nuclear activities E2-TF1 and E2-TF2 were identified by DNA binding assays that are associated with the NRE. A dinucleotide mutation which abolished binding of these two activities increased transcription of the reporter gene by 10-fold, indicating that E2-TF1 and E2-TF2 may play a significant role for the expression of the HTGL gene.

MATERIALS AND METHODS

Construction of Plasmids Containing Unilateral Deletions. The HTGL promoter fragment −1550 to +129 was generated by the polymerase chain reaction (PCR) methodology using the oligonucleotide primers PCRHL−1550 (5′-TAAGCTTCTAGAGGTACC GGCTCACTTGCAAACCAGAGTGATATTTTAAAACT-3′) and PCRHL+129R (5′-ATGATATCTCGAG GGTTTCAGGCTTTGTCCAAGGGCACTTGATTGG-3′) and 1 ng of phage DNA or 1 μg of human lung DNA as template. Lambda phages containing the 5′ upstream region of the human HTGL gene were isolated by screening a human genomic library in λ Dash (Stratagene). After 35 cycles of amplification, the amplified fragment was digested with *Xba*I and *Xho*I, gel purified, and cloned into the *Xba*I and *Xho*I sites of pUCSH-CAT vector (Ogami et al., 1990). Similarly, the HTGL promoter plasmids containing the 5′ progressive deletions extending from nucleotide −880 to −281 (Figure 1) were constructed using the PCR methodology. Fragments −880 to +129, −785 to +129, −682 to +129, −554 to +129, −483 to +129, −384 to +129, and −281 to +129 were generated using each of the 5′ primers PCRHL−880 (5′-TAAGCTTCTAGAGGTACC TCTCTTCTTTATTATGTCTACTGTCCGTTATCCAAGTCC-3′), PCRHL−785 (5′-TAAGCTTCTAGAGGTACC CAGTGCTTGGCACCAGTAGGCATACCAAAA-3′), PCRHL−682 (5′-TAAGCTTCTAGAGGTACC TGGTCGCCTTTTCCCTACCTGATTTTGCTGA-3′), PCRHL−584 (5′-TAAGCTTCTAGAGGTACC TACTCTAGGATCACCTCTCAATGGGTCACTT-3′), PCRHL−483 (5′-TAAGCTTCTAGAGGTACC GACCACAAGCATCACCAATTTCACTGAACCC-3′), PCRHL−384 (5′-TAAGCTTCTAGAGGTACC CTGCAATTTGAAACACCACTTTCCTGAGCCAGC-3′), and PCRHL−281 (5′-TAAGCTTCTAGAGGTACC GGCAGAATTTCCAAACACAACACAGTAGCTTTAA)-3′ with the reverse primer PCRHL+129R in a PCR reaction using 1 ng of plasmid −1550/+129 as template DNA. The oligonucleotides PCRHL−281 to PCRHL−1550 have at their 5′ end sequences that correspond to the restriction endonucleases *Hind*III and *Xba*I and Asp718. The reverse primer PCRHL+129R also has a 5′ flanking sequence that corresponds to the restriction endonuclease *Xho*I. The underlined nucleotides are sequences corresponding to the 5′ upstream region of the HTGL gene (Ameis et al., 1990) and Figure 3G. After amplification, the fragments were digested with the restriction enzymes *Xba*I and *Xho*I, gel purified, and cloned into the *Xba*I and *Xho*I sites of the pUCSH-CAT vector. The mutant −483/+28 was obtained by digesting plasmid −483/+129 with *Xba*I and *Sma*I. The fragment −483 to +28 was gel purified and cloned into the pUCSH-CAT vector at the *Xba*I and *Sma*I sites.

Construction of Plasmids Containing Heterologous Enhancers. The SV40 72 base pairs (bp) repeat spanning from position 91 to 289 was isolated by *Xba*I and Asp718 digestion from plasmid pT25 (Cladaras et al., 1987) and cloned into the *Xba*I and Asp718 sites of plasmid −281/+129, to yield plasmid −281/+129[SV40], and plasmid −483/+28, to yield plasmid −483/+28[SV40]. The human apolipoprotein AII

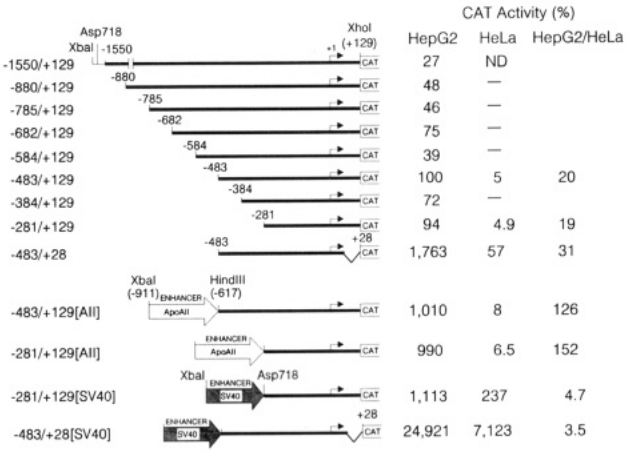

FIGURE 1: Effect of HTGL promoter mutants on the transcription of the promoterless CAT gene in HepG2 and HeLa cells. CAT activities are expressed relative to those achieved with the −483 to +129 construct. The apoAII and SV40 enhancers are represented by arrows. Numbers are with respect to the transcription start site (+1), and numbers in parentheses represent positions of nucleotides present in the apoAII promoter. CAT activity of HTGL promoter constructs in HepG2 and HeLa cells was calculated relative to the activity obtained by the pSV2CAT plasmid. ND, not detectable.

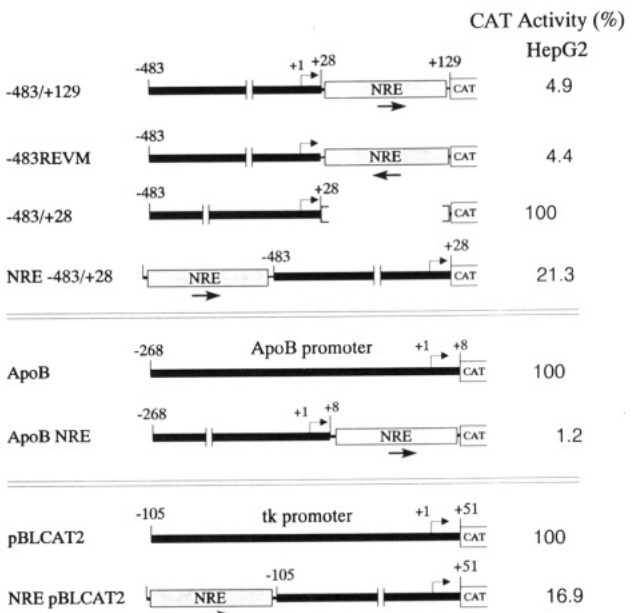

FIGURE 2: Effect of the NRE on the transcription of HTGL, apoB, and thymidine kinase (tk) promoter constructs. Arrows indicate orientation of the NRE.

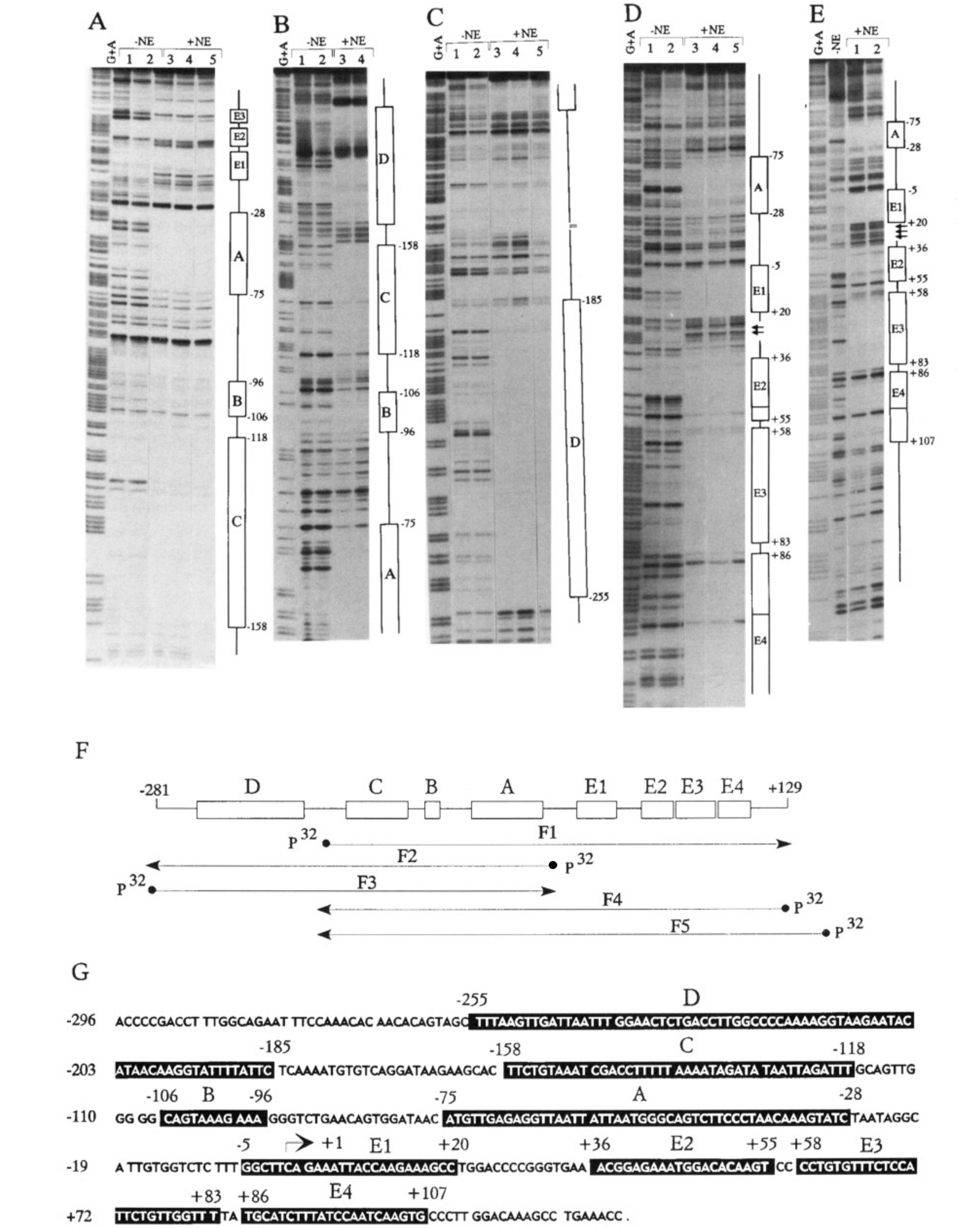
A
B
C
D
E
G+A
-NE
+NE
1 2 3 4 5
F
-281 D C B A E1 E2 E3 E4 +129
F1
F2
F3
F4
F5
P 32
G
-255 D
-296 ACCCCGACCT TTGGCAGAAT TTCCAAACAC AACACAGTAGC TTTAAGTTGATTAATTT GGAACTCTGACCTTGGCCCCAAAAGGTAAGAATAC
-185 -158 C -118
-203 ATAACAAGGTATTTTATTC TCAAAATGTGTCAGGATAAGAAGCAC TTCTGTAAAT CGACCTTTTT AAAATAGATA TAATTAGATTT GCAGTTG
-106 B -96 -75 A -28
-110 GG GG CAGTAAAG AAA GGGTCTGAACAGTGGATAAC ATGTTGAGAGGTTAATT ATTAATGGGCAGTCTTCCCTAACAAAGTATC TAATAGGC
-5 +1 E1 +20 +36 E2 +55 +58 E3
-19 A TTGTGGTCTC TTT GGCTTCA GAAATTACCAAGAAAGCC TGGACCCCGGGTGAA ACGGAGAAATGGACACAAGT CC CCTGTGTTTCTCCA
+83 +86 E4 +107
+72 TTCTGTTGGTT T TA TGCATCTTTATCCAATCAAGTG CCCTT GGACAAAGCC TGAAACC .

FIGURE 3: Panels A–E. DNase I footprinting analysis of the hepatic lipase promoter fragment −281 to +129 performed with rat liver nuclear extracts and the ^{32}P-labeled fragments F1–F5 (shown in panel F), respectively. −NE and +NE indicate reactions performed in the absence or in the presence of nuclear extracts, respectively. G+A are chemical cleavages of the same DNA fragments used in footprinting analysis. Open boxes indicate regions protected from DNase I digestion. In lanes 1 and 2 of panels A–D, the reactions were treated with 12.5 and 25 ng, respectively, of DNase I. In lanes 3, 4, and 5 of panesl A–D and lanes 1 and 2 of panel E, the amounts of nuclear extracts used were 40, 50, and 60 μg, respectively. Panel F. Schematic representation of ^{32}P-labeled promoter fragments F1–F5 used in footprinting reactions along with the HTGL promoter fragment −281 to +129 and DNase I-protected regions D–E4 shown as open boxes. The filled circles indicate ^{32}P-labeled sites. Panel G. Sequence of the HTGL promoter fragment −296 to +129 and of protected regions D–E4 shown enclosed in dark boxes. The arrow indicates the transcription start site. In all panels, numbers are with respect to the transcription start site.

(apoAII) tissue-specific enhancer region −911 to −617 (Chambaz et al., 1991) was isolated as an *Xba*I/*Hind*III fragment and cloned with the *Hind*III/*Xho*I −483 to +129 or −281 to +129 HTGL fragments into the *Xba*I and *Xho*I sites of pUCSH-CAT.

Construction of Plasmids Containing the NRE. The NRE fragment (+29 to +115) was obtained by PCR amplification using the primers HLREV (5′-ATATATCTAGACTCGAG GGGTGAAACGGAGAAATGGACAC-3′) and HLREVC (5′-ATATAGGATCCTCGAGGGTACCGATATC TCCAAGGGCACTTGATTGGATA-3′). The fragment was digested with (a) *Eco*RV and *Xho*I, (b) *Xba*I and Asp718, (c) *Xho*I, and (d) *Bam*HI and *Xba*I and cloned into plasmid (a) −483/+28 digested with *Sma*I and *Xho*I to yield plasmid −483REVM, (b) −483/+28 digested with *Xba*I and Asp718 to yield plasmid NRE −483/+28, (c) apoB −268/+8 (Kardassis et al., 1990) digested with *Xho*I to yield plasmid apoB NRE and (d) pBLCAT2 (Luckow & Schutz, 1987) digested with *Bam*HI and *Xba*I to yield plasmid NRE pBLCAT2. The underlined sequences in HLREV and HLREVC correspond to nucleotides +29 to +51 and +115 to +94, respectively.

Construction of Plasmids Containing Internal Deletions and Substitution Mutations. Plasmid ΔE2 containing deletion of the region E2 was generated as follows. The HTGL promoter fragment A was generated by PCR amplification using as 5′ primer the oligonucleotide PCRHL-483 and as 3′ primer the oligonucleotide PCRHL-DE2C (5′-GAATGGAGAAACACAGGGGTTCACCCGGGGTCCA-3′). The PCRHL-DE2C corresponds to the region +85 to +25 with the nucleotides from +36 to +55 deleted. Similarly, the HTGL promoter fragment B was generated using as 5′ primer the oligonucleotide PCRHL-DE2 (5′-TGGACCCCGGGTGAACCCCTGTGTTTCTCCATTC-3′) and as 3′ primer the oligonucleotide 3′-CAT-26 (5′-CTCCATTTTAGCTTCCTTAGCTTCTG-3′). The PCRHL-DE2 is the reverse complement of the PCRHL-DE2C and also contains the deletion of nucleotides from +36 to +55. The oligonucleotide 3′-CAT-26 initiates approximately 40 bp downstream of nucleotide +129. Aliquots containing 2% of each of the amplified regions (fragments A and B) were mixed and used for another round of PCR amplification in the presence of PCRHL-483 and 3′-CAT-26. The *in vivo* ligated fragment was digested with *Xba*I and *Xho*I, gel purified, and cloned into the pUCSH-CAT vector at the *Xba*I and *Xho*I sites to yield plasmid ΔE2. Similarly, this methodology was used to construct reporter plasmids: (a) -483E2M using the primers PCRHL-483, PCRHL-E2M (5′-GGGTGAAACGGAGAAACAGACACAAGTCC-3′), PCRHL-E2MC (5′-GGACTTGTGTCTGTTTCTCCGTTTCACCCG-3′), and 3′-CAT-26; (b) ΔE3 using the primers PCRHL-483, PCRHL-DE3 (5′-GAAATGGACACAAGTCCTATGCATCTTTATCCAATCAA-3′), PCRHL-DE3C (5′-TTGATTGGATAAAGATGCATAGGACTTGTGTCCATT-3′), and 3′-CAT-26; (c) ΔE4 using the primers PCRHL-483, PCRHL-DE4 (5′-GTGTTTCTCCATTCTGTTGGTTTTAGCCCTTGGAC-3′), PCRHL-DE4C (5′-GTCCAAGGGCTAAAACCAACAGAATGGAGAAACAC-3′), and 3′-CAT-26; and (d) E2REV using the primers PCRHL-483, PCRHL-INV (5′-GGTGAAACTTGTGTCCATTTCTCCGTCCCCTGTGTTTCTCCATTCTGTTGGT-3′), PCRHL-INVC (5′-GGACGGAGAAATGGACACAAGTTTCACCCGGGGTCCAGGCTTTCTTGGT-3′), and 3′-CAT-26. All constructs were characterized by DNA sequencing analysis.

Cell Transfections and CAT Assays. Plasmids were transfected into HepG2 (human hepatoma) and HeLa (cervical carcinoma) cells and assayed for their ability to promote transcription of the promoterless chloramphenicol acetyltransferase (CAT) gene. HepG2 and HeLa cells were maintained as stocks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. All transient transfections were performed in 60% confluent 60-mm dishes using the calcium phosphate DNA coprecipitation method (Graham & Van Der Eb, 1973). The transfection mixture contained 12 μg of promoter plasmid DNA and 5 μg of RSV β-gal plasmid. Cells were harvested 42 h later and lysed by freeze–thawing. The β-galactosidase activity of the cell lysates was determined as previously described (Edlund et al., 1985), and the values were used to normalize variabilities in the efficiency of transfection. CAT activities were determined using ^{14}C-chloramphenicol and acetyl–CoA as previously described (Gorman et al., 1982). The nonacetylated and acetylated chloramphenicol forms were separated on IB2 silica gel plates using chloroform/methanol 95:5 for development. The radioactive spots, detected by autoradiography, were cut from the thin-layer plates and counted. CAT enzyme levels that exhibited more than 60% conversion of acetylated product were diluted and reassayed for CAT activity in the linear range. The CAT values represent the average of at least three independent transfection experiments.

Labeling of DNA Fragments for Footprinting. The HTGL promoter fragments F1–F5 shown in Figure 3, Panel F, were end-labeled using the PCR procedure. The DNA fragments F1–F5 were generated by PCR amplification using the 5′ upstream [PCRHL-183 (5′-CAAAATGTGTCAGGATAAGAAGCACTTCTGTAAA-3′), PCRHL-281] and 3′ reverse primers [PCRHL+129R, PCRHL-24R (5′-ATTAGATACTTTGTTAGGGAAGACTGCCCATTAATAATTAACC-3′), and 3′-CAT-26]. Twenty-five picomoles of 5′ or 3′ primer was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase prior to use. PCR reactions were performed in 25 μL total volume containing 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 1.25 mM of each dNTPs, 1 ng of template DNA, 25 pmol of cold primer, 25 pmol of ^{32}P-labeled primer, and 2 units of Taq DNA polymerase. After 25 cycles of amplification, the labeled fragments were gel purified and used in DNase I footprint assays.

DNase I Footprint Assays. Rat liver nuclear extracts were prepared as previously described (Gorski et al., 1986). DNase I footprinting assays were performed in a 20-μL reaction containing 25 mM HEPES, pH 7.6, 40 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1 μg poly(dIdC) competitor DNA, and 40–60 μg of rat liver nuclear extracts. After 15 min on ice, 1–2 ng of end-labeled fragment was added, and the incubation continued for 90 min on ice. Two microliters of DNase I (Worthington) were added to a final concentration of 12.5–100 ng, and the digestion was allowed to proceed for 5 min on ice. The reaction was stopped by the addition of 4 μL of 125 mM Tris–HCl, pH 8.0, 125 mM EDTA, and 3% SDS. Forty micrograms of proteinase K and 10 μg of carrier tRNA were added, and the reaction mixture was incubated for 30 min at 65 °C. The DNA was precipitated with 1 volume of 5 M ammonium acetate and 2 volumes of ethanol, resuspended in 98% formamide dye, electrophoresed on a 6% polyacrylamide/urea gel, and analyzed by autoradiography. DNase I footprinting with recombinant HNF1, generously provided by Dr. R. Cortese,

was performed with 5–10 units of factor, 50–200 ng of poly(dIdC) competitor, and 20 μg of BSA as described above.

DNA Binding Gel Mobility Shift Assays. Gel mobility shift assays were performed in a 20 μL reaction volume containing 25 mM HEPES, pH 7.6, 40 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 3 μg of poly(dIdC), 6–10 μg of rat liver nuclear extracts or 1–3 μL of fractionated extracts, and 1 pmol of ^{32}P-labeled double-stranded probe. The sequences of oligonucleotides used in DNA binding gel mobility shift and competition assays are the following: HL-A1 (5′-CATGTTGAGAGGTTAATTATTAATGGGCAGTCTT-3′), HL-E2 (5′-TGAAACGGAGAAATGGACACAAGTCCCC-3′), HL-E3 (5′-CCCCCTGTGTTTCTCCATTCTGTTGGTTTTA-3′), and HL-E4 (5′-TATGGATCTTTATCCAATCAAGTG-3′). After being incubated at 4 °C for 30 min, the reaction mixture was loaded onto a 4% polyacrylamide gel in TAE buffer (6.7 mM Tris, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA) and electrophoresed at 10 V/cm of gel for 2–3 h at 4 °C (Strauss & Varshavsky, 1984). After electrophoresis, the gel was dried and analyzed by autoradiography.

Fractionation of Nuclear Extracts on Ion-Exchange Chromatography Columns. Rat liver nuclear extracts (60 mg) were loaded onto a 10 mL column (BioRex-70, Q-Sepharose, S-Sepharose) in NDB buffer (25 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM benzamidine, 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin) containing 40 mM KCl. The column was washed with the same buffer and eluted with a step gradient of KCl (0.04–0.6 M) in NDB buffer. DNA binding gel mobility shift assays were performed on peak fractions using oligonucleotides HL-E2 and HL-E3.

RESULTS

Promoter Elements Required for Hepatic Transcription of the Human Hepatic Triglyceride Lipase (HTGL) Gene. To define the DNA sequences that regulate hepatic lipase gene expression, we have initially assessed the effect of sequential promoter deletions on the transcription of the promoterless CAT gene in HepG2 and HeLa cells. For this analysis, the HTGL promoter region −1550 to +129 was cloned in front of the promoterless CAT gene using the pUCSH-CAT vector (Ogami et al., 1990) (Figure 1). Transient transfections in HepG2 and HeLa cells showed that the −1550 to +129 promoter region has weak promoter activity in HepG2 cells, as compared with the activity obtained by the pSV2CAT promoter plasmid (data not shown), and no measurable activity in HeLa cells. Previous studies have reported that the levels of hepatic lipase are extremely low, and the low levels of protein may reflect low levels of mRNA. In Northern blot analyses, specific HTGL mRNA transcripts have been detected only with 8–10 μg of rat liver poly(A) mRNA (Komaromy & Schotz, 1987; Semenkovich et al., 1989). In addition, solution hybridization analysis performed with mRNA from HepG2 cells detected 2.5 transcripts per cell of specific HTGL mRNA (Busch et al., 1990). Therefore, the low levels of mRNA indicate that the HTGL promoter is a weak promoter, and this may be reflected by the low levels of activity produced from the −1550/+129 HTGL promoter fragment in HepG2 cells. Albeit the low levels of transcription, this promoter fragment contains important elements for the regulation and tissue-specific expression of the gene.

Reporter plasmids with further 5′ progressive deletions extending from nucleotide −880 to nucleotide −281 were constructed and are shown in Figure 1. Transient transfections of the deletion mutants in HepG2 cells and analyses of the cell lysates for CAT activity showed that as the deletions progressed from −1550 to −483, the activity increased by approximately 3.7-fold (Figure 1). Further deletions to −281 did not significantly alter the activity obtained by the −483/+129 mutant (Figure 1). The CAT analysis of the deletion mutants indicated that the regulatory elements that control the hepatic expression of the HTGL gene are located in the proximal region between nucleotides −483 to +129. The gradual increase in promoter strength as the deletions progress from −1550 to −483 indicated the presence of elements that may have a small negative influence in promoter activity.

HTGL Gene Transcription Is Strongly Repressed by the Presence of a Negative Regulatory Element (NRE) Present in the First Exon of the Gene from Nucleotides +29 to +129. To test the importance of DNA sequences present downstream of the transcriptional start site, a 3′ deletion mutant was constructed that lacks nucleotides +29 to +129. This mutant designated, −483/+28, was further tested for its ability to drive the expression of the CAT gene in HepG2 cells. The CAT analysis showed that the transcriptional activity of the −483/+28 plasmid increased by approximately 17-fold as compared to the activity obtained by the −483/+129 plasmid. These results revealed that the region +29 to +129 contains elements (NRE) that can strongly repress transcription of the HTGL gene in HepG2 cells (Figure 1).

To test whether the NRE is also functional in cells of extrahepatic origin, transient transfections with reporter plasmids −483/+129, −281/+129, and −483/+28 were also performed in HeLa cells. Although no measurable activity was obtained with plasmid −1550/+129, a small but detectable CAT activity was observed in cell lysates of HeLa cells transfected with the −483/+129 and −281/+129 plasmids (Figure 1). In addition, a 10-fold increase in CAT activity was observed in cell lysates of HeLa cells transfected with the −483/+28 plasmid, lacking the NRE sequences, indicating that the NRE can repress transcription of the HTGL gene in both HepG2 and HeLa cells. The CAT activities of HTGL promoter constructs −483/+129, −281/+129, and −483/+28 obtained in HepG2 and HeLa cells were calculated relative to the activities obtained by the pSV2CAT plasmid, which is transcribed equally well in both cell types. As seen in Figure 1, removal of the NRE resulted in a 10-fold increase in CAT activity in HeLa cells; however, expression in HepG2 cells was 20–30-fold higher than the activity obtained in HeLa cells. These results revealed that two major regions are important for the hepatic expression of the HTGL gene. The one region, −483 to +28, contains elements that can drive transcription of the gene predominantly in hepatic cells, and the other region, +29 to +129, contains the NRE that strongly represses the transcription driven by the −483 to +28 promoter fragment in both HepG2 and HeLa cells. Therefore, the NRE plays a determinant role for the expression of the HTGL gene in hepatic cells.

NRE Can Repress Transcription of the HTGL Reporter Constructs Containing Heterologous Enhancers. To test the effect of heterologous enhancers on the transcription of the HTGL gene, we constructed plasmids −483/+129[AII], −281/+129[AII], and −281/+129[SV40] (Figure 1). Plasmids −483/+129[AII] and −281/+129[AII] contain the tissue-specific (liver and intestine) enhancer of the apolipoprotein AII (apoAII) gene (Chambaz et al., 1991; Lucero et al., 1989; Shelley & Baralle, 1987) cloned immediately upstream of the HTGL promoter fragments −483 to +129 and −281 to +129. Plasmids −281/+129[SV40] and −483/+28[SV40] contain the SV40 72 bp repeat cloned upstream of the HTGL

fragments –281 to +129 and –483 to +28 respectively. Transfections in HepG2 and HeLa cells showed that constructs carrying the apoAII tissue-specific enhancer are significantly expressed only in HepG2 cells, while constructs carrying the SV40 enhancer were expressed in both HepG2 and HeLa cells. Transfections in HepG2 cells and CAT analysis showed that both enhancers potentiated the HTGL promoter activity driven by the fragments –483 to +129 and –281 to +129 by approximately 10-fold. In contrast, the SV40 enhancer potentiated the HTGL promoter activity driven by the –483 to +28 fragment lacking the NRE by 240-fold, indicating that the NRE can strongly repress transcription driven by the SV40 enhancer.

NRE Can Act Independent of Position and Orientation and Repress Transcription Driven by Heterologous Promoters. To determine whether orientation of the NRE is important for repression, the NRE was reversed in plasmid -483REVM. As seen in Figure 2, plasmids –483/+129 and -483REVM with the NRE present in the sense or reverse orientation, respectively, gave the same CAT activity after transfection in HepG2 cells. The CAT activity obtained from both plasmids was 20-fold lower than that obtained by the –483/+28 reporter plasmid lacking the NRE. These results indicated that the NRE represses transcription of the HTGL reporter plasmids to the same extent when present in either orientation.

To test whether repression is maintained when NRE is moved upstream of the transcription start site, we constructed plasmid NRE-483/+28. In this plasmid, the NRE was removed from its position and cloned upstream of nucleotide –483 in the sense orientation. As seen in Figure 2, the promoter plasmids carrying the NRE either upstream or downstream of the promoter fragment –483 to +28 repressed the transcription by 5- and 20-fold, respectively, indicating that the NRE can act independent of its position. However, the transcriptional repression is more potent when the NRE is present downstream of the transcription start site.

To test whether the NRE can repress transcription driven by heterologous promoters, we placed the NRE in the apoB and thymidine kinase (tk) promoters. In the apoB NRE plasmid, the element is positioned downstream of the transcription start site at nucleotide +8, and in the NREpBLCAT2 plasmid, the element is positioned upstream of nucleotide –105 in the thymidine kinase promoter. These constructs were transfected into HepG2 cells, and the CAT activity was measured relative to the parental plasmids apoB –268/+8 and pBLCAT2. As seen in Figure 2, transcription of the apoB promoter is repressed by 80-fold and that of the tk promoter is repressed by 7-fold. These data indicated that NRE can repress transcription driven by the heterologous promoters, and the repression is more potent when the element is present downstream from the transcription start site, consistent with the repression observed in the HTGL promoter. These data demonstrated that the NRE can act independent of orientation and position and also repress transcription from heterologous promoters.

DNase I Footprinting Analysis Identified Eight Protected Regions Present in the Proximal Region. The regulatory elements controlling the hepatic transcription of the HTGL gene were further defined by DNase I footprinting analysis using rat liver nuclear extracts (Figure 3, Panels A–E). DNA fragments spanning the promoter region –483 to +129 were end-labeled and used in DNase I footprinting assays. This analysis identified eight protected regions between nucleotides –255 and +107 as the DNA binding sites for nuclear factors (Figure 3, Panel F). Four elements are located upstream of the transcription initiation site (A, –28 to –75; B, –96 to –106; C –118 to –158; D, –185 to –255), and four in the first exon of the gene (E1, –5 to +20; E2, +36 to +55; E3, +58 to +83; E4, +86 to +107) (Figure 3, Panels A–E). As shown in Figure 3G, all the DNA elements that interact with nuclear factors are localized in the proximal promoter region of the HTGL gene.

Identification of a Nuclear Activity Which Binds to the Regulatory Region A (–75 to –28) of the Human HTGL Gene. As was previously suggested, the region –483 to +28 contains elements that support transcription of the HTGL gene predominantly in hepatic cells. The region –65 to –53 within the footprint A contains the motif 5′-GTTAATTAT-TAAT-3′, which is an inverted repeat of the sequence GTTAAT. This sequence has homology with the consensus sequence 5′-GTTAATNATTAAC-3′ of the liver-specific factor HNF1 (Courtois et al., 1988). DNA binding gel mobility shift assays with rat liver nuclear extracts and a double-stranded oligonucleotide (HL-A1) corresponding to the region (–74 to –43) showed that this region binds a single activity (Figure 4, Panel A). The activity is preserved following heat treatment of the nuclear extracts at 85 °C for 5 min. Competition experiments showed that binding of this activity to the HL-A1 oligonucleotide is competed by the double-stranded oligonucleotide AlbB (Maire et al., 1989), containing the HNF1 binding site of the rat albumin promoter, and by the double-stranded oligonucleotide AIIH (Chambaz et al., 1991), containing the HNF1 binding site of the human apoAII promoter (Figure 4, Panel A). Furthermore, DNA binding assays performed with different concentrations of rat liver nuclear extracts showed that the HNF1 factor present in these extracts binds to the A region of hepatic lipase with stronger affinity than it binds to the albumin B region (Figure 4, Panel B). The affinity of binding for the HL-A1 oligonucleotide is approximately 3 times higher than that for the AlbB oligonucleotide. To directly address the question of whether HNF1 binds to oligonucleotide HL-A1, DNA binding gel mobility shift assays were performed with a truncated form of HNF1 produced by expression of the corresponding cDNA using a vaccinia virus expression system (Frain et al., 1989). This analysis showed that truncated HNF1 is capable of binding to the AlbB oligonucleotide as well as to the HL-A1 oligonucleotide (Figure 4, Panel C). In addition, DNase I footprinting using the truncated HNF1 protein demonstrated that the region –43 to –75 within the footprinting area A binds to the HNF1 protein (Figure 5). From the data presented in Figures 4 and 5, we suggest that the region designated A1 (–43 to –75) of the HTGL promoter is recognized by factor HNF1.

Analysis of the NRE. DNase I footprinting analysis revealed the presence of three elements (E2, E3, and E4) within the NRE (Figure 3, Panel G). To identify the importance of each element, we constructed reporter plasmids containing internal deletions of elements E2, E3, and E4 in the –483 to +129 promoter fragment (Figure 6). Transient transfections in HepG2 cells showed that deletion of element E2 in plasmid ΔE2 resulted in a 10-fold increase in CAT activity, while deletion of elements E3 and E4 in plasmids ΔE3 and ΔE4 resulted in low levels of CAT activity, comparable to that obtained with the –483/+129 reporter construct. However, deletion of element E2 alone did not increase the levels of CAT activity to the same extent as deletion of the NRE, indicating that elements E3 and E4 may be required for maximal repression.

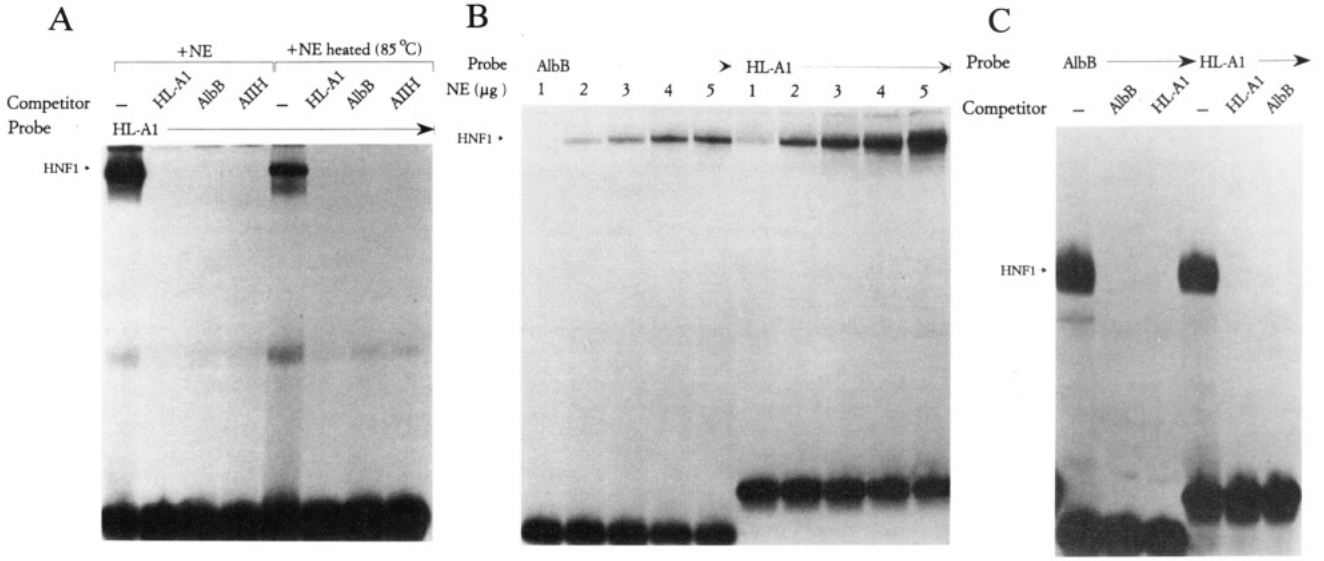

FIGURE 4: DNA binding gel mobility shift and competition assays of the HTGL promoter region A1 (–75 to –43). Double-stranded synthetic oligonucleotides corresponding to sequences –75 to –43 in the HTGL promoter (HL-A1) and to sequences –70 to –50 of the albumin promoter (Alb B) were labeled with the Klenow fragment of DNA polymerase in the presence of [^{32}P]dGTP and [^{32}P]dCTP. DNA binding reactions were performed with rat liver nuclear extracts (+NE, Panels A and B), extracts heated at 85 °C for 5 min (+NE heated at 85 °C, Panel A), and a purified truncated HNF1 protein, produced by expression of the corresponding cDNA using a vaccinia virus expression system (Panel C). Competitive oligonucleotides were used at 200-fold excess. In all panels, the top band represents the DNA–protein complex and the bottom band the free oligonucleotide.

To determine whether element E2 could operate in reverse orientation, plasmid E2Rev was constructed, in which the sequence from nucleotides +36 to +55 was inserted in its exact position at reverse orientation. Transfections into HepG2 cells showed that the CAT activity was not significantly altered, indicating that the repression effect obtained by element E2 is retained even in its opposite orientation (Figure 6). In addition, a substitution mutation of element E2 that alters nucleotides TG to CA at positions +45 and +46 resulted in a 10-fold activation similar to that obtained by deletion of element E2.

Characterization of the Activities Which Bind to the Regions E2 (+36 to +55), E3 (+58 to +83), and E4 (+86 to +107) of the NRE. Element E4 contains the canonical CCAAT motif that has been implicated for the binding of several transcription factors including the families of C/EBP (Williams et al., 1991; Cao et al., 1991), NF1 (Santoro et al., 1988; Rupp et al., 1990) and NFY (van Huijsduijnen et al., 1990; Li et al., 1992) proteins and the recently characterized CDP repressor protein (Neufeld et al., 1992). Elements E2 and E3 have extensive sequence homology with element E4 and, therefore, may bind common nuclear activities (Figure 7A). To assess whether C/EBP may bind to those regions, DNA binding gel mobility shift assays were performed with bacterially expressed C/EBP (Kardassis et al., 1992) and double-stranded oligonucleotides corresponding to elements E2, E3, and E4. As shown in Figure 7B, C/EBP can bind to element E3, but it has lower affinity for element E4 and much lower affinity for element E2.

To investigate the specific interaction of hepatic nuclear factors with elements E2, E3, and E4, we used DNA binding gel mobility shift assays. These assays, performed with double-stranded oligonucleotides E2, E2M, E3, E4, and rat liver nuclear extracts, are shown in Figure 8, Panels A–D. Element E2 binds predominantly two nuclear activities designated E2-TF1 and E2-TF2 (Figure 8, Panel A). Competition experiments showed that the binding of E2-TF1 and E2-TF2 is not competed by oligonucleotides NF1, TK C/EBP, and NFY, which are recognized by nuclear factors NF1, C/EBP, and NFY, indicating that the activities E2-TF1 and E2-TF2 are not related to the CCAAT binding proteins C/EBP, NF1, and NFY (Dorn et al., 1987; Maire et al., 1989). Competition experiments also showed that the binding of nuclear activities E2-TF1 and E2-TF2 is not competed by the mutant oligonucleotide E2M, indicating that the dinucleotide mutation has destroyed the ability of that region to bind these two factors. In addition, direct binding experiments performed with oligonucleotide E2M (Figure 8, Panel B) showed that this oligonucleotide does not bind nuclear activities E2-TF1 and E2-TF2. As shown previously (Figure 6), the E2M mutation, when introduced into the HTGL promoter construct –483/+129, resulted in a 10-fold increase in CAT activity. This 10-fold increase was also obtained by the ΔE2 deletion mutant, indicating that E2-TF1 and E2-TF2 may play a major role in repressing the HTGL promoter in HepG2 and HeLa cells. Competition experiments also showed that the complexes formed by factors E2-TF1 and E2-TF2 are competed by oligonucleotide E3, indicating that these factors may also interact with element E3 (Figure 8, Panel A).

DNA binding gel mobility shift assays performed with oligonucleotide E3 identified seven shifted complexes (Figure 8, Panel C), indicating that multiple factors interact with this element. Two of the complexes were identified as the E2-TF1 and E2-TF2 nuclear activities since they cross-compete with the E2 oligonucleotide. A rather diffused shifted complex, designated E3-TF7, with slower electrophoretic mobility was also observed, indicating interaction of more than one activities which give rise to multiple overlapping complexes. The E3-TF7 complexes are effectively competed by the oligonucleotides NF1, TK C/EBP, and NFY, indicating the presence of CCAAT-related proteins.

DNA binding gel mobility shift assays using oligonucleotide E4 identified mainly two major complexes, designated E4-TF1 and E4-TF2 (Figure 8, Panel D). Competition experiments showed that the binding of nuclear activities that form complexes E4-TF1 and E4-TF2 is not competed by oligonucleotides E2 and E3, indicating that the factors binding to element E4 do not recognize elements E2 and E3. Complexes E4-TF1 and E4-TF2 are competed either by oligonucleotides NFY and TK-C/EBP, while complex E4-TF1 is competed

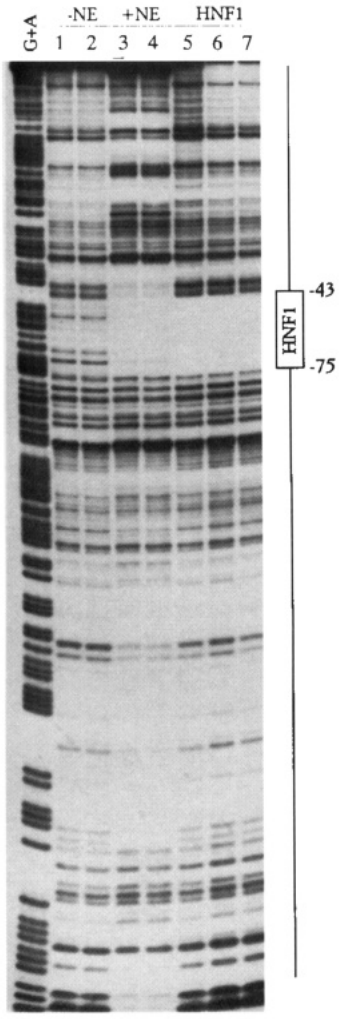

FIGURE 5: DNase I footprinting analysis of the hepatic lipase promoter fragment F1 (shown in Figure 2, Panel F) performed with rat liver nuclear extracts and recombinant HNF1 protein. In lanes 1 and 2 (–NE), reactions were performed in the absence of nuclear extracts and treated with 12.5 and 25 ng of DNase I, respectively. In lanes 3 and 4 (+NE), reactions were performed with 40 and 50 μg of nuclear extracts. In lanes 5, 6, and 7 (+HNF1), reactions were performed with 5, 7.5, and 10 units of recombinant HNF1. One unit of HNF1 is the activity that binds to 50 pg of radiolabeled probe.

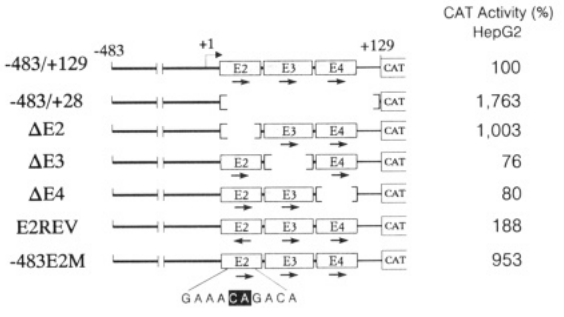

FIGURE 6: Effect of deletion and substitution mutations in elements E2, E3, and E4 on the transcription of the HTGL gene. CAT activities are expressed relative to those achieved with the –483 to +129 construct. The substitution mutation ATG to CAG is shown as enclosed in a dark box.

also by oligonucleotide AlbD, indicating that element E4 may bind more than one nuclear activities related to the CCAAT box-binding proteins.

DNA binding gel mobility shift assays using the E2 and E3 elements as probes were also performed with rat liver nuclear extracts fractionated on three ion-exchange chromatography columns: BioRex-70, Q-Sepharose, and S-Sepharose. The

A

Element	Position	Sequence
HLE4	+86 +107	5′ TGCATCTTTATCCAATCAAGTG 3′
HLE3	+58 +83	5′ CCTGTGTTTCTCCATTCTGTTGGT 3′
HLE2	+55 +36	5′ ACTTGTGTCCATTTCTCCGT 3′

B

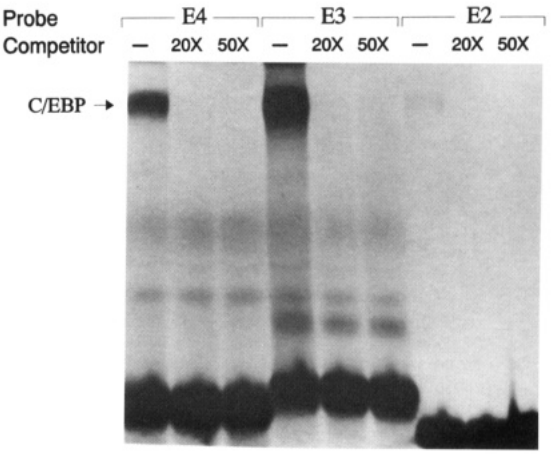

FIGURE 7: Panel A. Sequence homology of elements E2, E3 and E4. Homologous regions are boxed. Panel B. DNA binding gel mobility shift and competition assays of the HTGL promoter regions E4 (+86 to +107), E3 (+58 to +83), and E2 (+36 to +55) performed with partially purified C/EBP protein expressed in bacteria. Arrow shows the position of the DNA–protein complex.

results of these analyses are shown in Figure 9, Panels A and B. The activities E2-TF1 and E2-TF2 can be chromatographically separated using the Q-Sepharose column, where E2-TF1 elutes with 40 mM KCl and E2-TF2 with 400 mM KCl. These two activities were also monitored using the E3 element as a probe and were found to elute in the same fractions, confirming the result obtained from the binding and competition experiments (Figure 9, Panel B). The binding of factor E2-TF1 in element E3 was readily visible in the 0.3 M fraction of the S-Sepharose column. The low intensity of E2-TF1 complex on the fractions eluted from the BioRex-70 and Q-Sepharose columns may be due to the presence of other transcription factors that mask its binding to element E3. The observation that nuclear activities E2-TF1 and E2-TF2 can be chromatographically separated using the Q-Sepharose column indicated that they are distinct binding activities. In addition, the results from the fractionation experiments showed that the activities forming complexes E3-TF2,3 and E3-TF4,6 can be also chromatographically separated.

The results of the binding experiments are summarized in Figure 10. Elements E2 and E3 can both bind the nuclear activities E2-TF1 and E2-TF2, which have been implicated in the transcriptional repression of the HTGL gene. Elements E3 and E4 both bind C/EBP and factors possibly related to the family of CCAAT binding proteins. In addition, element E3 binds transcription factors E3-TF2,3 and E3-TF4,6.

DISCUSSION

In the present study, deletion analysis showed that transcription of the HTGL gene in hepatic cells is controlled by the interaction of factors that bind to two regulatory regions, –483/+28 and +29/+129, that have opposing transcriptional effects. DNase I footprinting analysis identified eight protected regions between nucleotides –255 and +107. Elements

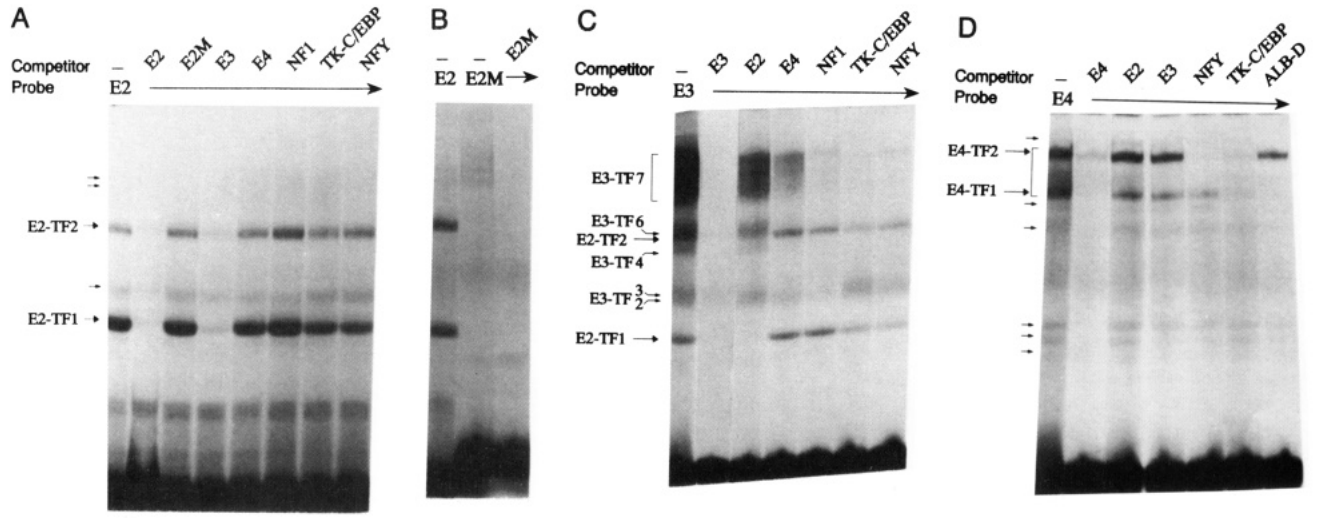

FIGURE 8: DNA binding gel mobility shift analysis of radiolabeled double-stranded synthetic oligonucleotides E2 (Panel A), E2M (Panel B), E3 (Panel C), and E4 (Panel D) using rat liver nuclear extracts in the presence and in the absence of competitor oligonucleotides as indicated. The concentration of competitor oligonucleotides used was 200 ng. DNA–protein complexes formed are indicated by arrows.

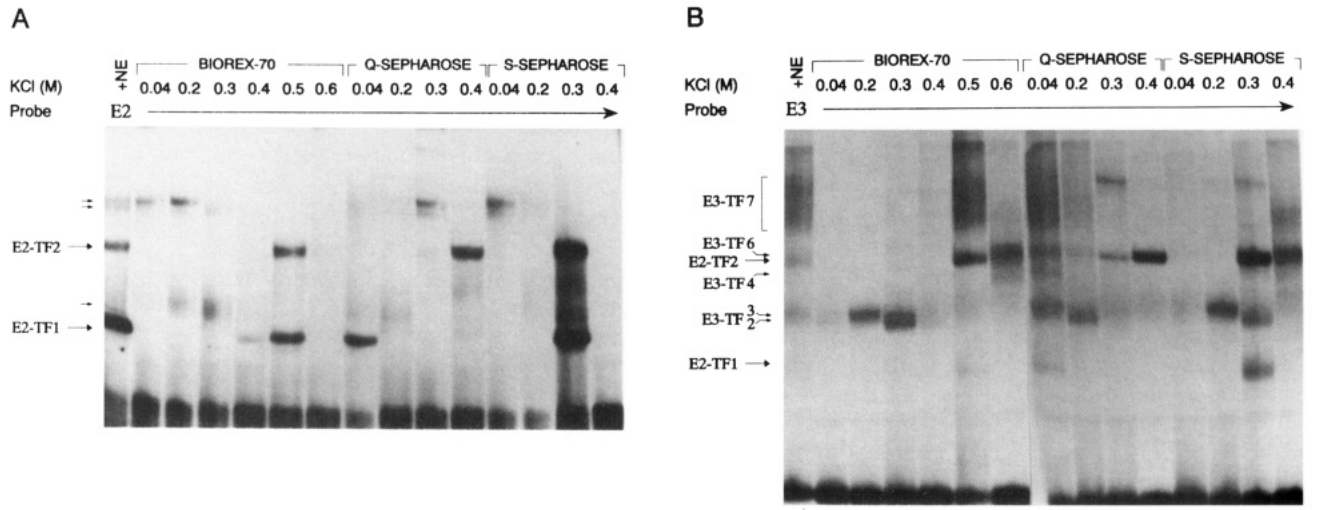

FIGURE 9: DNA binding gel mobility shift analysis of the fractions eluted from the BioRex-70, Q-Sepharose, and S-Sepharose columns. The double-stranded synthetic oligonucleotides E2 (Panel A) and E3 (panel B) were used for the DNA binding assays.

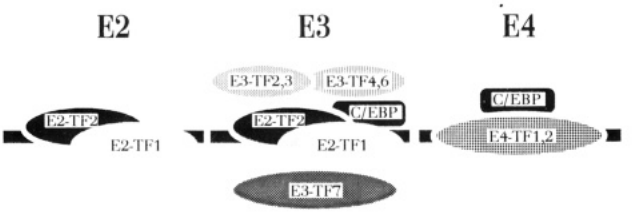

FIGURE 10: Schematic representation of nuclear activities interacting with regulatory elements present in footprinting regions E2, E3, and E4.

A, B, C, D, and E1 present in the region –483/+28 enhance expression of the gene predominantly in hepatic cells, while elements E2, E3, and E4 present in the region +29/+129 repress transcription of the gene in any cell type. Therefore, transcriptional regulation of the HTGL gene results by the coordinated action of factors that bind to these elements and fluctuations of the HTGL gene transcription can be the result of signals that are mediated by these elements.

Element A has perfect homology with the consensus of the liver-enriched transcription factor HNF1 (Courtois et al., 1988). The observation that HNF1 binds to element A with approximately 3 times stronger affinity than to element B present in the albumin promoter suggests participation of HNF1 in the regulation of the HTGL gene.

A reporter construct lacking nucleotides +29 to +129 resulted in a dramatic (15–20-fold) increase in promoter activity, indicating the presence of a strong negative regulatory element (NRE) in the first exon of the gene. Removal of the NRE allows expression in HeLa cells, indicating that the NRE is also functional in HeLa cells.

NREs have been implicated in the transcriptional regulation of a number of eukaryotic genes. Such negative cis-acting elements have been detected in genes like the retinol binding protein (RBP) (Colantuoni et al., 1987), mouse a-fetoprotein gene (Muglia & Rothman-Denes, 1986), chicken lysozyme (Steiner et al., 1987; Baniahmad et al., 1987), c-myc (Remmers et al., 1986), vimentin (Farrell et al., 1990), adipocyte-specific gene (Hunt et al., 1986), and intercellular adhesion molecule 1 (Degitz et a., 1991). In the retinol binding protein (RBP) gene, the negative element is also responsible for the tissue specificity. In c-myc and vimentin genes, the negative elements act as silencers since they repress transcription from a promoter irrespective of orientation or location within the same plasmid. In addition, the silencer present in type IV collagenase gene is enhancer dependent and cell type specific (Frisch & Morisaki, 1990).

Characterization of the NRE present in the HTGL gene revealed that the element functions in an orientation- and position-independent manner, suggesting that it is acting as a silencer. Moreover, the NRE represses transcription driven by heterologous promoters (apoB, thymidine kinase), implying that it functions as a general negative regulator of basal gene transcription.

DNase I footprinting analysis revealed the presence of elements E2, E3, and E4 within the NRE. Deletion of element E2 increased the promoter activity by 10-fold. Although element E3 binds the E2-TF1 and E2-TF2 activities, deletion of element E3 did not result in an increase in CAT activity as it was observed with the deletion of element E2. This was probably due to the fact that element E3 also binds the CCAAT-related proteins, and this competition of binding masks the negative activity contributed by the E3 element. These results indicated that element E2 plays a dominant negative role, while elements E3 and E4 may be required for maximum repression. In addition, a dinucleotide substitution mutation within element E2 increased the promoter activity to the same extent as the deletion of element E2. DNA binding and competition assays performed with rat liver nuclear extracts identified two sequence-specific DNA binding proteins, designated as E2-TF1 and E2-TF2, that interact with both elements E2 and E3. Binding of both activities is abolished by the dinucleotide substitution mutation (E2M) on element E2, indicating that both E2-TF1 and E2-TF2 play a crucial role in the repression of HTGL gene transcription.

Three different mechanisms have been proposed for negative control of gene transcription in eukaryotes (Levine & Manley, 1989; Renkawitz, 1990; Goodbourn, 1990). In the first mechanism, repression occurs by direct competition between repressors and activators for common or overlapping DNA binding sites. In the second mechanism, negative control of gene transcription occurs by the neutralization of activators. In the third mechanism, negative regulation is conferred by the presence of negative cis-acting elements that can either be promoter specific or act as silencers.

Investigation for sequence homology of element E2 with other elements acting as negative regulatory elements (NRE) or silencers revealed extensive homology with the silencing element present in the −294 to −251 region of human ε-globin gene (Cao et al., 1989; Gutman et al., 1992; Raich et al., 1992) and that present in the −435 to −410 region of the stearoyl–CoA desaturase 2 (SCD2) gene (Swick & Lane, 1992). The silencer element of the ε-globin gene is essential for its embryonic expression, since removal of the element from its promoter allows expression of the embryonic form in definitive erythroid cells. The silencing element of the SCD2 gene was capable of repressing transcription from both SCD2 and SV40 reporter constructs in preadipocytes and HeLa cells but not in differentiated adipocytes. Transcriptional repression was coincidental with the presence of a developmentally programmed protein detected by Southwestern blot analyses in crude nuclear extracts from preadipocytes and HeLa cells but not in nuclear extracts from differentiated adipocytes.

Element E2 also has sequence similarity with various core motifs of elements from the adeno-associated virus P5 promoter (Shi et al., 1991), the mouse ribosomal protein promoter (Hariharan et al., 1991), the molony murine leukemia virus LTR (Flanagan et al., 1992), and the immunoglobulin kE3′ enhancer (Park & Atchison, 1991). Recently, a transcriptional repressor, termed YY1 (Shi et al., 1991), NF-E1 (Park & Atchison, 1991), δ (Hariharan et al., 1991), and UCRBP (Flanagan et al., 1992), was cloned from human and mouse cells that specifically binds to these elements and down-regulates expression of their corresponding promoters. Although it is not known whether this repressor protein is recognized by the E2 element, our data show that two chromatographically distinct activities from hepatic extracts bind to this element. An intriguing possibility is that several NRE binding proteins may exist that recognize very similar types of motifs, like the family of CCAAT binding proteins. Cloning and characterization of factors E2-TF1 and E2-TF2 may provide important information for the transcriptional regulation of the HTGL gene and, in general, for the mechanism of transcriptional repression. Studying the transcriptional regulation of HTGL and the factors and signals that modulate its expression may contribute to our understanding on the influence of HTGL in HDL metabolism and cholesterol homeostasis.

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