Expression of the Human Apolipoprotein E Gene Is Regulated by Multiple Positive and Negative Elements

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Apolipoprotein E (apoE), unlike the other major lipoproteins, is synthesized in a variety of tissues. We examined which regions of the human apoE gene contributed to its tissue-specific expression using HepG2 and HeLa cells as examples of expressing and nonexpressing tissues, respectively. Regions between -360 bp and -80 bp and within the first intron were shown to be necessary for full expression activity in HepG2 cells by a nuclease protection assay which demonstrated correct transcriptional initiation of the transfected constructions. To fine map the regulatory regions, we constructed a series of deletions fused to the reporter gene chloramphenicol acetyltransferase. We discovered eight regions which had a positive effect on expression and three regions that had a negative effect on expression, in both HepG2 and HeLa cells. In addition we found three regions which had a tissue-specific negative effect on expression in HeLa cells and one region with a tissue-specific positive effect in HepG2 cells. A DNase I protection assay revealed eight footprints within the proximal 5'-flanking sequence and the first intron. Seven of these footprints fell within closely defined regions with positive expression activity. Sequence analysis of these footprint elements revealed the presence of previously identified elements and two novel elements related to each other, identified here as B1 and B2. We also defined another repeated sequence, the A element; all three of the tissue-nonspecific negative regions contained this element or sequences with homology to it. In the context of a heterologous promoter, a synthetic oligonucleotide containing the B1 and B2 elements behaved like a classical enhancer, having a positive effect on expression, even when placed at a distance. This effect was neutralized by a different synthetic oligonucleotide containing an A element repeat.

Apolipoprotein E (apoE) is a major constituent of very low density lipoprotein and can be found associated with all of the major classes of lipoprotein particles (1). ApoE, which can associate with lipoproteins in vitro, has a higher affinity for the low density lipoprotein (LDL) receptor than apolipoprotein B (2), and in addition there may be a specific hepatic apoE receptor (3). Via these receptors apoE may play a crucial role in cholesterol transport. In humans there are three common isoforms of apoE due to genetic polymorphism at this locus (4, 5). One of these isoforms, apoE2, has an amino acid substitution in the domain which binds to the LDL receptor and leads to a decreased binding affinity (6). Homozygosity of the allele coding for this isoform is associated with familial type III hyperlipoproteinemia, characterized by elevated plasma lipids, xanthomas, and atherosclerosis (7).

apoE is synthesized in a variety of tissues and in this way it differs from the other major apolipoproteins that are synthesized almost exclusively in the liver and small intestine. In nonhuman primates apoE mRNA has been shown to be present in the following tissues, in order of their relative abundance; liver, adrenal, spleen, brain, mesenteric lymph node, and kidney; and the extrahepatic message might account for up to 40% of the total body apoE mRNA (8). Synthesis and secretion of apoE has been found in Rhesus monkey aortic smooth muscle cells, as well as in rodent testis and ovary (9). The synthesis of apoE has been shown to be regulated during development (10) and by nutritional or hormonal factors. Rats fed high fat diets have an increased plasma apoE concentration, increased apoE synthesis in liver slices, and increased hepatic apoE mRNA (11). However, in mice fed high fat diets, the resulting increase in plasma apoE is not accompanied by an increase in hepatic apoE mRNA (12). The mouse F9 teratocarcinoma cell line increases or decreases its synthesis of apoE when induced to differentiate in vitro, with the properties of visceral or parietal endoderm, respectively (13). ApoE is synthesized and secreted by mouse peritoneal macrophages (14), and apoE synthesis is induced during the differentiation of human blood-derived monocyte-macrophages in tissue culture (15). ApoE synthesis and secretion are increased in macrophages by cholesterol loading (14, 16), due to an intracellular increase in free cholesterol (17). The secreted apoE can then associate with high density lipoprotein as it acquires more cholesterol (18) and presumably tag it for hepatic clearance in a process called reverse cholesterol transport.

ApoE mRNA is synthesized in the human hepatoma cell line HepG2, but it is not detectable in either the epithelial HeLa cell line or the monocyte U937 cell line (16, 19). Our laboratory has previously characterized the human apoE gene, and sequenced over 1000 base pairs (bp) of the 5'-flanking sequence (20). In the present study we attempted to define DNA elements within the apoE gene and its flanking sequence that promote its transient expression in transfected HepG2 cells (as a model system of an expressing tissue), and restrict
its expression in HeLa cells. We show here that the apoE promoter region is very complex, hosting an array of positive and negative elements within the 5′-flanking region and also within the first intron. Three regions were identified that inhibited expression in HeLa cells but not in HepG2 cells. By a DNase I protection assay we have identified specific sequences that control expression from nuclear extracts of HepG2 cells. Several of these protein binding DNA elements lie within those regions having activity in the transient expression system.

MATERIALS AND METHODS

Plasmid Constructions—Constructions were made according to standard recombinant DNA techniques (21). A series of constructions were made starting with the previously isolated human apoE λ clone (20). A HindIII linker was inserted into the first exon at a unique AatII site, 24 bp 3′ to the start of transcription. This series was cloned into pUC18 and contained various amounts of 5′ sequence and the entire apoE gene through to an EcoRI site 628 bp 3′ to the polyadenylation site. Constructions containing the chloramphenicol acetyltransferase (CAT) gene were derived from the plasmid pKT (22). Some constructions contained a portion of the adenovirus major late promoter and sequences from −416 to −199 bp 3′ to the start of transcription were derived from pSA (23). Oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method on an Applied Biosystems model 381A synthesizer. Complementary oligonucleotides were annealed and ligated into plasmid vectors. Bat11 (IBI, fast form) deletions were performed, and the resulting DNA ends were flushed with T4 polynucleotide kinase and HindIII linkers. Oligonucleotides were annealed and ligated into plasmid vectors. Bat11 (IBI, fast form) deletions were performed, and the resulting DNA ends were flushed with T4 polynucleotide kinase and HindIII linkers. Oligonucleotides were annealed and ligated into plasmid vectors. Bat11 (IBI, fast form) deletions were performed, and the resulting DNA ends were flushed with T4 polynucleotide kinase and HindIII linkers. Oligonucleotides were annealed and ligated into plasmid vectors.

Cell Culture and Transfections—HepG2 and HeLa cells were grown in minimal essential medium containing 5% and 10% fetal calf serum, respectively. The day before transfection, confluent flasks were subcultured by trypsinization and 1.3–10^6 cells were plated per 60-mm tissue culture dish, or 0.3–10^6 cells/100-mm dish. Calcium phosphate-DNA coprecipitates were prepared by adding dropwise a 250 mM calcium chloride solution to an equal volume of vortexing Hepes-buffered saline solution (280 mM sodium chloride, 50 mM Hepes, 1.5 mM sodium phosphate, pH 7.10) containing plasmid plus carrier and sonicated salmon sperm DNA. The precipitates were added, 0.35 ml, containing 10 μg each of test and reference plasmid constructions and 5 μg carrier DNA, per 60-mm dish or 1 ml, containing 30 μg of test construction plasmid and 15 μg each of reference plasmid and carrier DNA, per 100-mm dish, and allowed to incubate with the cells for 16–24 h. HepG2 cells were shocked with a 15% glycerol solution in phosphate-buffered saline for 3 min at room temperature; this treatment increased transfection efficiency by 4–6-fold. Both cell lines were washed twice with serum-free minimal essential medium and replenished with growth medium. Cells were harvested on day 2 or 3 following transfection.

5′ Nucleotide Protection Assay—Cytoplasmic RNA was prepared from cells harvested by scraping from an 100-mm tissue culture dish. The cells were lysed by vortexing in 0.5 ml of a solution containing 10 mM Tris, 150 mM sodium chloride, 1.5 mM magnesium chloride, and 0.65% Nonidet P-40, pH 7.5. The nuclei were pelleted, and the supernatant was removed to a fresh tube containing 20 μl of 50 mM EDTA and 20 μl of a 20% sodium dodecyl sulfate solution. This was followed by three extractions with 0.5 ml of a 1:1 mixture of phenol and chloroform. One ml of ethanol was added to the aqueous layer, and the RNA concentration was determined from an aliquot by absorbance at 260 nm following precipitation and dissolution in water. A DNA probe was designed that was complementary to the 5′ end of apoE from −5000 to −741 bp. Hybridization buffer contained 50% deionized formamide, 0.4 M sodium chloride, 40 mM Pipes, 1 mM EDTA, pH 6.4. After heating at 65°C for 15 min to denature the probe, the annealing took place at 50°C overnight. The following day 180 μl of ice-cold 50 mM sodium acetate, 300 mM sodium chloride, 6 mM zinc acetate, pH 4.5 containing 300 units of 5′ nuclease were added, and the tubes were incubated for 90 min at 37°C. The protected probe fragments were ethanol precipitated, washed, dried, and resuspended in a solution containing the hybridization buffer (10% glycerol, 50% formamide, 1× TBE, 50 mM sodium hydroxide, 1 mM EDTA, 0.1% each of bromophenol blue and xylene cyanol). After boiling for 5 min, the samples were loaded onto 8% polyacrylamide gels containing 6 M urea and 1× TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0). Autoradiographs from the gel were scanned as the dried gels were analyzed with a LKB scanning laser densitometer. Control experiments determined that the calculated areas under a peak was correlated linearly with the radioactivity applied. For each sample the ratio of the expression of the test gene to that of the reference gene was calculated in order to normalize for transfection efficiency.

CAT and β-Galactosidase Assays—Cells scraped from 60-mm dishes were disrupted by 4 cycles of freezing and thawing in 120 μl of freeze-thaw buffer (250 mM sucrose, 10 mM Tris, 10 mM EDTA, pH 7.4). After centrifugation for 5 min at 12,000 × g at 4°C, the supernatant was transferred to fresh tubes, and the protein concentration was determined using the Bio-Rad protein assay. For each amount of protein were assayed for β-galactosidase and CAT activities. β-Galactosidase activity was determined spectrophotometrically at 420 nm using onithosponynyl-galactoside as the substrate as described by Miller (25). CAT activity was determined as described by Gorman et al. (26). The percent of the chloramphenicol substrate acetylated was determined by liquid scintillation counting. The CAT activity of each sample was corrected for transfection efficiency by dividing by its corresponding background subtracted β-galactosidase activity.

DNase I Protection Assay—Various DNA probes were end labeled with [γ-32P]ATP and T4 polynucleotide kinase. Each probe was digested with a restriction enzyme to yield the final probe fragment labeled at only one end. After gel purification the probe (10,000 cpm) was incubated with either albumin, as a control, or various amounts of HepG2 nuclear extract, prepared according to the method of Dignam et al. (27). The 50 μl of reaction mixture contained, in addition to the probe and extract, 20 mM Hepes, pH 7.5, 60 mM potassium chloride, 4 mM magnesium chloride, 0.5 mM dithiothreitol, 10% glycerol, 0.04 mM ATP, and 1 μg of poly(d)IIC). The mixture was incubated for 15 min on ice followed by 3 min at room temperature and treated with DNase I for 1 min by the addition of 50 μl of a solution containing 5 mM calcium chloride, 1 mM EDTA, and 0.12 mg/ml DNase I. DNase I digestion was halted by the addition of 100 μl of a solution containing 0.6 M sodium acetate, 1% sodium dodecyl sulfate, 20 mM EDTA, and 100 μg/ml glycerol. The DNA was phenol extracted and ethanol precipitated before running on a 6% polyacrylamide, 6 M urea sequencing gel. In order to define the position of the protected regions, a G + A sequence ladder was prepared from each probe (28), and run alongside the reaction products in the gel.

RESULTS

ApoE Expression Mediated by the Proximal 5′-Flanking Region and the First Intron—An apoE transient expression system was developed which allowed us to map, in the context of the entire gene, which sequences were necessary to yield maximal expression in the HepG2 human hepatoma cell line. We designed a series of apoE gene constructions with a HindIII linker inserted into the first exon at +24 nucleotides relative to the start of transcription. Upstream of this linker we placed either the remainder of the first exon with various amounts of 5′ sequence or internal deletions (test constructions) or the SV40 early promoter (reference construction). Using a DNA probe made from apoE sequences containing the inserted linker, we were able to distinguish in a nuclease protection assay the RNA synthesized by the endogenous apoE gene from the RNA produced by the transient expression of both the test promoter construction and the reference SV40 expression construct (Fig. 1). This assay also demonstrated that the test gene constructions initiated transcription at the previously identified mRNA cap site (20). This initial study revealed that the 5′ sequences from −5000 to −360 bp (relative to the transcriptional start site) could be deleted with very
expression which initiated upstream of the apoE cap site, presumably in the adjoining plasmid sequence (Fig. 1B). We also identified a deletion in the first intron which decreased expression to 36%.

Multiple Positive and Negative Regulatory Regions within 5' Proximal Flanking Region and the First Intron Mediate Expression in HepG2 Cells—For our fine mapping of the sequences which govern the expression of the apoE gene, we switched to gene fusion constructions in which the 5' end of the apoE gene at position +806, which is in the second exon but precedes the translation initiation codon, was fused to the coding sequences of the CAT gene in the pKT1 vector. The parent construction pEC1, whose expression the other constructions were compared to, contained the apoE sequences up to -506 bp (Fig. 3). A series of constructions with various internal deletion end points were transfected into HepG2 cells as well as into HeLa cells. Along with the test constructions, each plate was cotransfected with the β-galactosidase-expressing plasmid pH110 (24) which served as a reference for transfection efficiency. The results of this analysis (Fig. 4) revealed a very complex array of regulatory regions within 360 nucleotides upstream of transcription initiation and within the first intron.

Concentrating first on the series with their 5' deletion end point at -360 bp, positive and negative regions of activity were defined by successive deletions. The first positive region was defined by the deletion extending from -360 to -314 bp (62% of the expression of the parent construction in HepG2 cells). The next positive region was defined between -277 and -254 bp by comparing the deletion between -360 and -277 bp (64% expression) with deletion extending from -360 to -254 bp (47% expression). However, deleting even further to -175 bp resulted in a greater amount of relative expression (73%), thereby defining a negative region (a region whose deletion causes increased expression) between -254 and -175 bp. The next two deletions in this series, from -360 to -161 bp and from -360 to -125 bp, define two more positive regions (61 and 42% expression, respectively) from -174 to -161 bp and from -161 to -125 bp. The deletion extending from -362 to -113 bp expressed at 110%, thereby defining a negative regulatory region between -125 and -113 bp. The deletion covering -362 to -80 bp caused a decrease in expression to 26%, defining another positive region between -113 and -80 bp. The region between -80 and -40, which contains two copies of the consensus binding site for transcription factor SP1, also has a positive effect on expression (compare deletions -362 to -80 at 26% expression with -362 to -40 at 9% expression).

Many of these positive and negative regulatory regions were confirmed and better defined by more specific deletions. For instance very strong positive regions (those which when deleted decreased expression to below 50%) were mapped by deletions spanning -177 to -161 bp (36% expression), -163 to -125 bp (14% expression), and -102 to -80 bp (10% expression). The positive element within the first intron, first identified by the S1 nuclease protection assay (Fig. 2), was deletion mapped by the CAT assay to within the 12 nucleotides between +75 and +87 bp (45% expression).

The region between -254 and -175 had weakly negative activity as described above. As this region was further characterized, it proved to be extremely complex. We interpret the results to indicate the presence of two positive and two negative elements within this region. The two positive elements were within the regions defined by the deletions spanning -243 to -227 bp (52% expression) and -186 to -175 bp (80% expression). One of the negative elements was placed...
the deletion of both positive and negative regulatory regions. As discussed below.

The net expression of other constructions was reflective of the deletion of both positive and negative regulatory regions. For example, when the region from -163 to -113 bp was deleted, there was 56% expression compared to the 14% expression of the deletion from -163 to -125, revealing again the effect of the negative region between -125 and -113 bp. However, the deletion from -163 to -113 bp expressed as well as the deletion from -243 to -227 bp (111% expression). In this case the presence of the upstream negative elements (−254 to −243 bp and −207 to −186 bp) could account for this difference. The use of these gene fusion constructions as a measure of transcriptional activity was verified by the comparison of the −360 to −80 bp deletion which expressed at 26 ± 6%, with the −80-bp construction of the S1 nuclease experiment which expressed at 37 ± 16% (Figs. 2 and 4).

Tissue-specific Regulatory Elements within the 5'-Flanking Region and First Intron—The results stated in the previous section were all determined in HepG2 cells; and, quite similar results were obtained in HeLa cells for most of the deletions with end points spanning −360 to +87 bp. However, there were three differences observed within this region between HepG2 and HeLa cells. 1) There appeared to be a tissue-specific negative element between −227 and −207 bp, which when deleted caused a 75% increase in expression in HeLa cells only (Fig. 4, compare activity of deletions from −243 to

FIG. 2. Transient expression of various apoE gene test gene constructions in HepG2 cells as measured by the S1 nuclease protection assay. The relative expression of the various constructions shown was determined in comparison to the −1000 bp construction. For each determination, the level of test gene expression was normalized to the transcription efficiency of the SV40-driven reference gene. The results show the mean relative expression ± S.E. for N number of determinations.

FIG. 3. Plasmid constructions containing the CAT gene. A, pKTI is a promoterless CAT expression vector. It was constructed from pKT (22) by repairing the BamHI site at the 3' end of the CAT gene and by deleting the poly linker region from the SaeI site to the HindIII site and replacing with a HindIII linker. pEC1 contains a 1.3-kilobase apoE gene promoter from −506 to +834 bp (Bul fragment) inserted into pKTI at the unique HindIII site just upstream of the CAT gene. This insert contained the entire first exon (hatched box) and first intron (IVS-1), as well as a portion of the second exon preceding the translation initiation codon. B, pCT is a CAT expression vector containing an enhancerless version of the adenovirus major late promoter. It was constructed from pKTI by cutting with PscI, chewing back the overhanging ends with T4 polymerase, cutting with HindIII, and ligating in the 143-bp SmaI to HindIII fragment of pSA (23). The dotted box represents the remaining pUC18 poly linker region from EcoRI to Sall. E, B, and H represent sites for EcoRI, BamHI, and HindIII, respectively.

upstream of −243 bp, therefore between −243 and −254 bp, since the deletion from −272 to −227 bp restored expression activity when compared to the deletion from −243 to −227 bp (93 versus 52% expression). The second negative element was placed between −207 and −186 bp. This placement was based upon the following results. 1) The construction deleting from −243 to −207 bp expressed at roughly the same level as the deletion from −243 to −227 bp; therefore, there was no element between −227 and −207 bp. 2) The construction deleting from −243 to −175 bp also expressed as well as the deletion from −243 to −227 bp; yet, this construction spans both of the positive regions defined above. The expression of this construction was expected to decrease to account for the deletion of the additional positive element between −186 and −175 bp. Since the expected decrease in expression was not observed, we inferred that the region between −207 and −186 contained a negative element. The placement of a negative element between −207 and −186 was confirmed in HeLa cells as discussed below.

The net expression of other constructions was reflective of the deletion of both positive and negative regulatory regions. For example, when the region from −163 to −113 bp was deleted, there was 56% expression compared to the 14% expression of the deletion from −163 to −125, revealing again...
The presence of the general negative element placed between only marginal positive activity in HeLa cells helped to confirm that in HepG2 cells while expression decreased only marginally in HeLa cells to 88%. That this particular region had only marginal positive activity in HeLa cells helped to confirm the presence of the general negative element placed between −207 and −186 bp in the previous section. The construction deleting from −243 to −207 bp expressed at 96% in HeLa cells while the deletion spanning from −243 to −175 bp increased expression to 147%. The region between −163 and −125, although a strong positive element in both cell lines, appeared to be a stronger element in HeLa cells (Fig. 4, compare deletions from −360 to −125 bp and from −163 to −125 bp in both cell lines).

Two elements outside of the proximal 5′-flanking region which had negative effects on expression in HeLa cells but not on HepG2 cells were also detected 1) in the first intron between +88 and +349 bp, where a deletion with these end points increased expression almost 2.5-fold in HeLa cells with little effect in HepG2 cells, and 2) in sequences between −506 and −1000 bp, where adding these sequences decreased expression almost 5-fold in HeLa cells only.

It was not possible to directly compare apoE-driven CAT expression in the HepG2 and HeLa cells by using the CAT activity normalized to the reference β-galactosidase activity since the ratio of the half-lives of their respective mRNAs and/or proteins may vary greatly. A direct comparison was accomplished using the S1 nuclease protection assay described above. Since mRNA products of both the test and reference constructions were identical except for their extreme 5′ ends, their half-lives were expected to be similar within each cell line, and the ratio of their respective mRNA levels determined by the S1 nuclease assay would provide a more accurate determination of their relative transcriptional efficiencies. The −5000-bp construction was expressed in HeLa cells at 28% of the level compared to the expression in HepG2 cells. In contrast, the −506-bp construction expressed in HeLa cells at 64% of the level observed in HepG2 cells. This result verifies the presence of the tissue-specific distal 5′ negative region which was found to be between −1000 and −506 bp in the apoE-CAT fusion constructions. Therefore, the lack of expression of the endogenous apoE gene in HeLa cells may be partially, but not entirely explained by the sequence of the apoE gene and 5000 bp of its 5′-flanking sequence.

HepG2 Nuclear Extract Protects Elements within Positive Regulatory Regions from DNase I Digestion—We added HepG2 nuclear extracts to labeled DNA probes which extended through the proximal 5′-flanking region and a portion of the first intron. Specific elements were protected by factors within the nuclear extract from digestion by DNase I. Fig. 5 shows the resulting footprints and their positions relative to the start of transcription. Six footprints were detected in the 5′-flanking region, labeled with Roman numerals starting with the most proximal (I) to the most distal (VI). There were also two footprints in the first intron, labeled DI and DII, for the downstream proximal and distal footprint, respectively. The schematic drawing in Fig. 6 shows that the specific elements protected by footprints I–VI and DI all lie within positive regulatory regions which were defined by expression activity in the experiments described in Fig. 4.

The Footprint Elements within Regions of Positive Activity Are Related to Each Other or Previously Defined Elements—Table I shows the elements within the positive regions which were protected by a HepG2 nuclear extract from DNase I digestion. The element protected by footprint VI shows 85% homology with a previously identified serum-responsive element found in the human c-fos gene (29). A 67-kilodalton protein has been identified which binds to this element in the c-fos gene and related elements in cytoskeletal actin genes (30). The region protected by footprint I contains one copy of the GC box consensus sequence for binding to the transcription factor SP1 (31, 32). Footprints II, III, V, DI, and the upstream half of footprint IV, all within regions of positive expression activity, define two related elements, B1 and B2. Both of these elements, shown in Table I, contain the core CCCA and end with the dinucleotide TC. The B1 element is protected by footprints II and III; both of these footprints are over regions with very strong positive expression activity. A B2 element with a one-base mismatch from the consensus occurs adjacent, but in an inverted orientation, to the B1
TABLE I

| Sequence elements in the ApoE gene | Positive elements within the ApoE gene | Footprint |
|------------------------------------|---------------------------------------|-----------|
| **Sequence in apoE gene**           |                                      |           |
| C-fos serum responsive element (29) | -338 CCTCTCCAGATTACATTCATC             | VI        |
|                                    | -318 GATGTCATTATAGGACATC               |           |
|                                    | -173 GGTCAAAAAGACC                    | IV        |
|                                    | -162 GGTCANNNTGACC                     |           |
| **Sequence in apoE gene**           |                                      |           |
| Vitellogenin estrogen responsive element (33) | -173 GGTCANNNTGACC | I |
| **SP1 consensus sequence (31)**     | GGCCGG                                  |           |
| **ApoE gene B1 element**            | GCCCCACCTC                             | II, III   |
| **ApoE gene B2 element**            | GGCCCCAGNNTC                           | II, IV, V, DI |
| **5' proximal negative element in the apoE gene** | **CTCT** | |
| **ApoE gene A element**             | **CCCCTCG**                            |           |
| **Sterol-responsive element repeat 3 (34)** | **AAAATCTCTCCCCTC** | |

The Three Negative Regulatory Regions, Common to Both Cell Lines, Contain Elements Homologous to Each Other and to the Sterol-responsive Element of the LDL Receptor—The most 5' proximal region which had strong negative expression activity in both HepG2 and HeLa cells was deletion mapped between -125 and -113 bp. A sequence motif partially contained within this region, between -120 and -109 bp (antisense strand), is repeated a total of three times between -143 and -37 bp. The consensus of this sequence, defined as the A element, is shown in Table I. The second copy of this sequence occurs between -143 and -132 bp, adjacent to and in the opposite orientation of the first copy. The third copy is located between the TATA box and the 5' proximal GC box at position -49 to -37 bp (antisense strand) (Fig. 7). The two upstream negative regions that were also common to both cell lines spanned from -254 to -243 bp and from -207 to -186 bp. Both of these regions at least partially contain a sequence related to the A element. The sequences spanning from -261 to -249 bp and from -263 to -192 bp are identical to the A element consensus sequence at 8 of 12 positions, and we have therefore named these sequences A' elements. The first 10 bases of the 12-base A element consensus sequence is identical to the last 10 bases of the third repeat of the sterol responsive element found within the LDL receptor promoter (Table I) (34).

Conservation of Positive and Negative Elements between the Rat and Human Apolipoprotein E Genes—We found similar sequence motifs in the published rat apoE gene sequence (35) to all of the human apoE elements listed in Table I. An 82% homology between the human and rat apoE gene sequence has been reported for the 140 bp 5' to the transcriptional start site (35). Alignment of these genes also shows a 63% homology in the region from +1 to +106 bp, which is comprised of the untranslated first exon and 55 bp of the first intron. Similar sequence elements to the human apoE elements protected by footprints I–VI and footprint DI are found at similar positions in the rat gene.

A Synthetic A Element Neutralizes the Effect of a Synthetic B1-B2 Element on the Expression of a Heterologous Promoter—The strong negative element disrupted by the deletion between -125 and -113 bp contains one copy of the A element and is adjacent to the strong positive element mapped to -102 to -80 bp which contains one B1 element and one B2 element in opposite orientations. We prepared two double-stranded synthetic oligonucleotides; oligonucleotide A encompassed the inverted repeat of the A element (-144 to -108 bp), and oligonucleotide B contained the inverted B1 and B2 elements protected by footprint II (-106 to -82) (Fig. 8). These oligonucleotides were cloned in various combinations into the polylinker region of pCT, an expression vector in which the CAT gene is driven by the adenovirus major late promoter (Fig. 3). The transient expression of various constructions was analyzed in HepG2 cells (Fig. 8). The SV40 enhancer cloned into the polylinker of pCT increased expression about 90-fold. One copy of the A oligonucleotide had little effect on expression. However, two copies of the A oligonucleotide decreased expression to 36%. A single copy of the B oligonucleotide inhibited expression, while two copies of the B oligonucleotide decreased expression to 1%.
FIG. 7. Annotated sequence of the human apoE gene promoter region. The location of the footprints are shown above the sequence within brackets, and the hypersensitive site is denoted by HS over three arrows. The boxed elements correspond to the following: SeRE, the element with homology to the c-fos gene serum responsive element; SP1, the GC box element which can bind to the transcription factor SP1; ERE, the element with homology to the estrogen-responsive element; TATA, the TATA box element; B1 and B2, two related elements with positive expression activity; A, the consensus of an element repeated three times between -143 and -37 bp, at least one of which is associated with negative expression activity; A', two elements within regions of negative activity which are identical to the A consensus element at 8 of 12 positions. A left pointing arrow over an A, B1, or B2 element indicates that the consensus sequence is found on the antisense strand. The sequence identified by an arrow with a U over it denotes a 10-bp direct repeat.

cleotide, corresponding to the positive element protected by footprint II, increased CAT expression by over 7-fold. The constructions with one copy of both the A and B oligonucleotides (in either orientation) expressed only as well as the parent construction pCT. Therefore, one copy of the A oligonucleotide was able to block the positive effect that the B oligonucleotide had on CAT expression in the context of this heterologous promotor. The B oligonucleotide gave a dosagedependent increase of CAT expression. Two copies of the B oligonucleotide increased CAT expression more than twice as much as the single copy did. Two copies of the B oligonucleotide were able to act as a classical enhancer, increasing CAT expression when a 550-bp spacer of phage λ DNA was inserted between the oligonucleotides and the heterologous promotor.

DISCUSSION

In this study we have identified multiple regions with positive and negative activities upon the expression of apoE-CAT gene constructions. We defined seven regions within 360 bp of the apoE gene transcriptional start site, and one region within the first intron, that had a positive effect on expression in both the apoE-expressing HepG2 cell line and in the nonexpressing HeLa cell line. All but one of these non-tissue-specific positive regions were associated with DNA-binding proteins which resulted in footprints in the DNase I protection assay. We also identified three regions in the proximal 5'-flanking sequence that had negative effects on expression in both cell lines. These three regions each contained a sequence motif similar to the A element, which was defined as the consensus of a sequence repeated three times between -143 and -37 bp. In addition there were three tissue-specific negative regions which decreased expression specifically in HeLa cells, and one region which had a tissue-specific positive effect on expression in HepG2 cells. All of these add up to 15 separate regions which influence the expression of the apoE gene. The question arises as to why should any gene be endowed with such a complex array of positive and negative elements. We speculate that the multitude of elements in the apoE promotor provides for the complex regulation of the expression of this gene by three separate conditions, tissue type, intracellular cholesterol level, and other nutritional or hormonal factors.

ApoE is expressed in a variety of tissues at different levels.
For instance in fetal human tissues apoE mRNA was measured at these levels relative to the level in the fetal liver: adrenal and macrophages, 74-100%; gonads and kidney, 12-15%; spleen, brain, thymus, ovaries, intestine, and pancreas, 3-9%; heart, 1.5%; and stomach, striated muscle, and lung, <1% (15). The specific amounts and combinations of various transacting factors which interact with the multiple positive and negative elements of the apoE gene may, in part, determine the level of apoE expression in these tissues. The three tissue-specific negative regions which we defined in the apoE gene join a growing list of similar structures found in viral enhancers (36, 37), the rat insulin 1 gene (38), the murine immunoglobulin H gene (39), and the rat α-fetoprotein gene (40). Chromatin structure may play an additional role in the tissues which do not express the apoE gene. This is exemplified by the observation that although HeLa cells did not express their endogenous apoE gene, they expressed the transfected apoE gene constructions, albeit at 28% of the level found in HepG2 cells. 

ApoE may have a dual role in cholesterol transport, either to transport cholesterol into cells via their LDL receptors, as is the case in steroidogenic tissues (41), or to aid in the hepatic clearance of secreted cholesterol, as occurs in cholesterol loaded macrophages (42). Therefore, different cell types may regulate the transcription of the apoE gene by different mechanisms in response to their cholesterol status. In primary rat ovarian granulosa cells, the steroidogenic hormone follicle-stimulating hormone, and agents which increase its intracellular messenger cyclic AMP, increase steroid hormone synthesis from cholesterol and also apoE secretion (41). The postulated purpose of this increased apoE secretion is to locally provide apoE to high density lipoprotein, thereby generating a cholesterol-rich ligand which can then be recognized and internalized via the granulosa cell’s LDL receptors (41). In contrast cholesterol-loaded macrophages, in a primary culture system, increase their apoE synthesis in response to elevated levels of intracellular free cholesterol (17). An in vitro system that may be similar to the cholesterol-loaded macrophage has been studied. Optic nerve sheath apoE secretion increases after nerve damage in rats (43, 44). The increase in apoE synthesis has been localized to phagocytic cells, and is also thought to be due to an increase in cellular cholesterol, and plays a role in mobilizing cholesterol from damaged nerve sheaths (45). The apoE promoter region with its multiple positive and negative elements seems well suited for the contrasting regulatory needs of cholesterol importing and exporting tissues. The A element, with its homology to the sterol-responsive element of the LDL receptor, is repeated three times in the apoE promoter between -143 and -37 bp and is a likely candidate for the site of regulation by cholesterol. Two other regions with similar sequences, the A' elements, occur further upstream in the promoter within regions of negative activity and may also play a role in the regulation of apoE expression by cholesterol. The A element, partially contained within the region of negative activity between -125 and -113 bp, is adjacent to the strongly positive B1-B2 element inverted repeat (-104 to -84 bp). This positive-negative element pair may be analogous to the situation in the human β-interferon gene where a positive element is adjacent to negative regulatory sequences that prevent expression prior to induction (46).

Various nutritional and hormonal factors have been reported to influence apoE synthesis in HepG2 cells. We have observed that the removal of serum from cultures of HepG2 cells resulted in a dramatic specific decrease in apoE mRNA levels (data not shown). The addition of either linoleic acid or a complex hormone mixture including fatty acids to HepG2 cells in the presence of serum-free medium results in an increase in apoE secretion (47). In contrast, insulin added to serum-free medium causes a decrease in apoE secretion (48), although this may be due to a nonspecific decrease of protein secretion by insulin in HepG2 cells (49). It is possible to test which serum factors promote apoE expression and whether these factors regulate the expression of the apoE gene via one of the regulatory elements identified in this report. For example, the apoE element with homology to the c-fos serum-responsive element may be involved in the regulation of apoE expression by serum factors.

Estrogen, at pharmacological doses, has been reported to increase apoE secretion, synthesis, and apoE mRNA levels (50, 51), as well as LDL receptor activity (52) in HepG2 cells. Whether or not the estrogen effect on apoE expression is of physiological significance in the liver has yet to be determined. One might expect to find regulation of apoE expression, at physiological doses of estrogen, in tissues with high levels of estrogen receptors. We speculate that any estrogen response would be mediated by the DNA element at -174 to -163 bp which is similar to the estrogen responsive consensus element (39).

In light of the complex regulation of apoE expression by tissue type, lipids, and hormones, it is not surprising that the apoE gene contains multiple cis-acting loci which may serve as binding sites for the proteins that regulate apoE transcription. Other eukaryotic genes have been described with multiple regulatory elements, including immunoglobulin genes (53), the α-1 antitrypsin gene (54, 55), and the metallothionein genes (56-58). At this time we are not aware of any other gene described which has as many regulatory regions as the 15 identified here. As these genes and many others are analyzed at a detailed level, the presence of a complex array of regulatory elements may prove to be a common feature of eukaryotic promoters.

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