Identification and Characterization of Transcriptional Regulatory Regions Associated with Expression of the Human Apolipoprotein E Gene*

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Multiple cis-acting regulatory elements have been mapped within a 1-kilobase fragment spanning nucleotides -651 through +356 of the human apolipoprotein E gene using a transient expression system based on the chloramphenicol acetyltransferase gene as well as DNase I footprinting techniques. A 651-base pair 5'-flanking region of the human apolipoprotein E gene was capable of directing chloramphenicol acetyltransferase gene expression over a 48-fold range among the various cultured cell lines tested. Deletion analysis of this 651-base pair upstream region linked to either the chloramphenolic acetyltransferase gene or the intact apolipoprotein E structural sequences revealed at least three regulatory domains within the proximal 383 nucleotides. One of these domains contained a GC box footprinting assay showed that specific sequences within two of these elements (-193 to -124 and +44 to +262) bind proteins in nuclear extracts from HepG2 and Chinese hamster ovary cells. A protein footprint also was identified for a GC box element at nucleotides -59 to -45. Thus, control of apolipoprotein E gene expression is the result of a complex interaction of several different regulatory elements.

Human apolipoprotein (apo)E is a major component of various lipoprotein classes in mammals (1–3). It is a single-chain polypeptide of 299 amino acids (M, 34,000) and it is synthesized mainly in the liver (5). Studies of its tissue distribution have shown that various extrahepatic tissues, including brain, adrenal, spleen, ovary, testis, and kidney, contain abundant levels of apoE mRNA (6–8). Mouse peritoneal macrophages (9) and human monocyte-derived macrophages (10) also have been shown to contain apoE mRNA. Related studies have demonstrated that transfection of the intact human apoE gene into various cultured mammalian cells of both hepatic and non-hepatic origin leads to production of a biologically active apoE protein (11).

Apolipoprotein E mediates the cellular uptake of cholesterol, and it serves as a ligand for the apoE receptor (LDL). There are three major isoforms of apoE (E2, E3, and E4), which represent different alleles at a single genetic locus (14, 15). These isoforms are the result of specific cysteine-arginine interchanges in the primary structure (1, 3).

The nucleotide sequences of the mRNAs for human (16, 17), rat (18), and mouse apoE (19) are known. The human apoE gene has been mapped to chromosome 19 (20), and the nucleotide sequences of the gene for human apoE (20, 21) and rat apoE (22) have been determined. Each sequence has four exons and three introns, and the human gene is 3597 nucleotides in length. Recently, it was found that the apoE gene is linked closely to the apoC-II gene (23).

The 5'-flanking region of the human apoE gene has several striking structural features (21); however, the function or specific roles of these sequence elements in apoE gene expression have not been determined. To understand the potential role of these elements in the transcriptional regulation of the apoE gene, we have examined the 5'-flanking region as well as the first intron of the apoE gene using transient expression systems involving chimeric apoE gene/chloramphenicol acetyltransferase (CAT) gene recombinants and DNase I footprinting assays. The identification and partial characterization of specific sequences that modulate apoE gene expression are reported here.

**EXPERIMENTAL PROCEDURES**

**Construction of Recombinant Plasmids**—For the analysis of the promoter activity of the 5'-flanking region of the human apoE gene,

1. The abbreviations used are: apo, apolipoprotein; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; RSV, Rous sarcoma virus; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; kb, kilobase pairs; bp, base pair(s); LDL, low density lipoprotein; CHO, Chinese hamster ovary; SRE-42, sterol regulatory element. EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; URE, upstream regulatory element, IRE, intron regulatory element.
which is located 617 bp upstream of the TK promoter, producing two plasmids, pHAEN-10 (correct orientation) and pHAEN-11 (reverse clone pUCE4 (21) and ligated into plasmid pLSl to produce pHAE-CAT1 (Fig. 1A). Nucleotide positions of apoE gene fragments refer to the previously determined sequence of the apoE gene (21).

Plasmids containing progressively shorter sequences at the 5' end were generated by Bal31 exonuclease treatments. Briefly, pHAE-CAT1 was digested with BglIII and treated with Bal31 for different lengths of time. The Bal31 reactions were stopped by adding 20 mM EGTA, heating the solution to 66 °C for 15 min, and then precipitating with ethanol. The resulting fragments were ligated with Bal31 linkers (8-mer, New England Biolabs) under conditions recommended by the supplier. The ligation products, which had various lengths of the 5'-flanking sequence, were excised by BglII-Sac1 digestion and purified on a 5% acrylamide gel and then were ligated into the pLSl vector. The precise end points of these deletion mutants were determined by DNA sequencing using the method of Maxam and Gilbert (25) or deoxyribonucleotide chain termination (26).

For the analysis of the enhancer activity of regions of the human apoE gene, three CAT vectors were used: pTK1 (Fig. 1B), pTK10 (same as pTK1 but lacking the 600-bp pBR322 sequence between polylinker sites and the TK promoter), and pA,CAT2 (see Fig. 4). In both the pTK1 and pTK10 plasmids, the expression of the CAT gene is under the control of the TK promoter of the herpes simplex virus. In pTK1 (same as pTE1 in Ref. 24), fragments of the apoE gene were inserted into the BglIII site located 600 bp upstream of the TK promoter, whereas in pTK10 the inserts were placed immediately 5' to the TK promoter (BglII site). A starting plasmid, pHAE-10, was constructed by insertion of a 1-kilobase (kb) fragment of the apoE gene (−651 to +356) into pTK1 in the normal transcriptional orientation relative to the TK promoter. Plasmid pHAEN-11 was constructed similarly, but the same fragment was inserted in the reverse orientation. Subfragments within the 1-kb insert of pHAE-10 were generated by restriction endonuclease digestion and Bal31 treatment, followed by addition of the BglIII linkers. These subfragments were then inserted into pTK1 and pTK10 vectors in both orientations.

To compare the activity of the enhancer elements when positioned 5' or 3' of the CAT gene, the plasmid pA,CAT, was used. The CAT gene in this plasmid was under the control of the enhancerless SV40 promoter (27). The pA,CAT, vector was digested with BglIII for the 5'-end-positioned inserts and with BamHI for the 3'-end-positioned inserts, respectively, and then was ligated to apoE gene fragments that contained BglIII linkers at each end (see Fig. 4). The resulting plasmids, pSVTK-CAT and pSVE-CAT, were constructed by ligation of the SV40 enhancer segment (a 400-bp insert isolated from pSV2-CAT (28) by HindIII-PuII digestion) into the polylinker region (HindIII-BglII sites) of pTK1 and pHAE-CAT1, respectively. The SV40 enhancer segment in pSVTK-CAT and pSVE-CAT is situated in the antisense orientation against the CAT coding sequence. To construct deletions of portions of the 5'-flanking region of the human apoE gene, a 10.5-kb HindIII fragment of plasmid pCHEG-1(11) was digested with BamHI-HindIII. A 5.2-kb fragment containing 14 bp of the 5'-flanking sequence, 3.6 kb of the apoE gene, and 1.6 kb of the 5'-flanking sequence was inserted into the BamHI-HindIII sites of pAT153 to produce pHEG-10 (Fig. 1C). The 5'-flanking fragments of the apoE gene to be examined were excised from the apoE/CAT recombinants that are listed in Fig. 2A by BglII-Sac1 digestion and ligated into the BamHI-Sac1 sites of pHEG-10 to generate plasmids pHEG-13, pHEG-14, and pHEG-15 (Fig. 2B), respectively. Plasmid pHEG-12 was constructed by ligating the SpI-Sac1 fragment (~3000 to +73) that was isolated from a genomic subclone, pUCE4 (21), into SpI-Sac1 sites of pHEG-10.

Gene Transfer of Recombinant Plasmids—Twenty-four h before

Fig. 1. Construction of apoE/CAT expression recombinants. Panel A, a 724-bp fragment (−651 to +73) of the human apoE gene sequence was excised by BglII-Sac1 digestion of the genomic clone pUCE4 (21) and ligated into plasmid pLS1 to produce pHAEN-CAT1. This plasmid (pLS1) was derived from pTK1 (same as pTE1 in Ref. 24) by removal of the TK promoter (24) by BglII-Sac1 digestion. Panel B, a 1001-bp fragment of the human apoE gene was excised by BglII and ligated into the BglII site of the vector pTK1, which is located 617 bp upstream of the TK promoter, producing two plasmids, pHAE-10 (correct orientation) and pHAE-11 (reverse orientation). Panel C, a 5.2-kb apoE gene-containing fragment was excised by BamHI-HindIII digestion of a 10.5-kb cloned fragment (11) and inserted into BamHI-HindIII sites of pAT153 to produce pHEG-10. This plasmid was used for construction of the 5' deletions of the apoE gene as described under "Experimental Procedures." The resulting recombinants are shown in Fig. 2B (B, BamHI; P, EcoRI; H, HindIII; S, SacI; Sp, SphI; X, XbaI).
Human Apolipoprotein E Gene Regulation

Table 1

| Cell line | Relative activity (n = 3) % |
|-----------|-----------------------------|
| CHO       | 48.5 ± 3.8                  |
| HepG2     | 28.1 ± 4.7                  |
| L         | 27.3 ± 2.9                  |
| CaCo-2    | 22.2 ± 5.3                  |
| J774.2    | 4.5 ± 1.5                   |
| HeLa      | 5.2 ± 1.6                   |
| BaGL      | 5.6 ± 2.1                   |
| U-937     | 3.4 ± 0.3                   |
| Fv4AH     | 1.0 ± 0.4                   |
| Fv4AH     | 1.0 ± 0.2                   |

Preparation and Analysis of RNA—Total cellular RNA was isolated from cultured cells according to the procedure of Chirgwin et al. (33), and RNA dot blot analysis was performed using 50 µg of protein, 0.6 µg of a-nitrophenyl-ß-D-galactopyranoside in 600 µl of total reaction volume as described (24).

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FIG. 2. Promoter activity of deletion fragments of the 5′-flanking region of the apoE gene. Panel A, apolipoprotein E promoter-CAT gene recombinants. The numbers to each side of the solid bars indicate the positions in base pairs relative to the transcription initiation site. The hatched box represents the first exon. The vertical bars above and numbers beneath the uppermost bar indicate the position of each pair of repeated sequences and GC boxes as described in Ref. 21. The values in the columns on the right represent the means of results that were obtained from two separate experiments. The relative CAT activities of the different deletion constructs are expressed as a percentage of that of pHAE-CAT1 (−651 to +73). To obtain relative CAT activities, the specific activity of each construct was normalized to β-galactosidase activity before comparison (i.e., normalized CAT activity = CAT specific activity/β-galactosidase activity). Panel B, deletion of portions of the 5′-flanking region of the intact apoE gene. The gene is not drawn to scale. Chinese hamster ovary cells were transfected with 15 μg of each plasmid and processed for preparation of RNA and dot blot analysis as described under “Experimental Procedures.” The RNA blot was hybridized with a 32P-labeled full length human apoE cDNA probe (HinfI fragment, 1.1 kb, Ref. 17), and the blots were quantitated by densitometric scanning of the autoradiograms.

uptake within a cell line, the cultured cells were cotransfected with a vector containing the RSV-directed β-galactosidase gene (pRSV-β-galactosidase) (24). The β-galactosidase activity in each cell extract was measured, and CAT activity (determined by apoE gene promoter effectiveness) was normalized to this value. To compensate for differences in transfection efficiency among cell lines, each cell line was transfected separately with another control vector containing the RSV promoter ligated to the CAT gene and cotransfected with pRSV-β-galactosidase. These genes were assayed as described above. The promoter activity of the apoE gene fragment then was determined relative to RSV promoter activity. The RSV promoter was chosen for both control vectors because it displays little cell specificity (28).

The results of this experiment are summarized in Table I, which shows the relative apoE promoter-driven CAT activities of the different cell lines. Overall, CAT expression over a 48-fold range was observed, with promoter activity being greatest in CHO cells. These results indicate that a CAT gene recombinant containing 651 bp of the 5′-flanking sequences of the human apoE gene allows relatively efficient expression of the gene in CHO, HepG2, L, and CaCo-2 cells. In a separate control experiment, the apoE gene fragment inserted in the antisense orientation in a second test vector, pHAE-CAT10, showed no expression in any of the cell lines examined (data not shown). The CHO and HepG2 cells were used subsequently for more detailed analyses of the regulatory elements within the 5′-flanking region of the human apoE gene. The CHO cells were employed because of their high apoE promoter activity, and the hepatoma-derived HepG2 cells were used because of the normally high expression of the apoE gene in the liver.

Identification of the Sequences That Are Required for Gene Expression—To identify specific sequences in the 5′-flanking region that are significant for human apoE gene expression, portions of the 5′ ends of this region were progressively deleted by Bal31 nuclease treatment. The structures resulting from the deletions are outlined in Fig. 2A, as are their activities. In both CHO and HepG2 cells, deletion of the 268 nucleotides between −651 and −383, which contains part of an AluI sequence, had little effect on apoE promoter-directed CAT activity. Deletion of the region between nucleotides −383 and −212 resulted in almost a 2-fold reduction in CAT activity. This region contains two types of directly repeated elements: 5′-TCCAGAT-3′ (−355 to −349, −335 to −329, and −268 to −262) and 5′-CAGGAAAGGA-3′ (−312 to −303 and
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Expression of the Human Apolipoprotein E Genes Containing Different Lengths of the 5'-flanking Sequence—To corroborate the results with the CAT gene recombinant plasmids, CHO cells were transfected with human apoE gene constructs containing different lengths of the 5'-flanking sequence (Fig. 2B). The endogenous apoE gene is not expressed in this cell line (11). Total cellular RNA isolated from the transfected cells was examined for the presence of human apoE mRNA by dot blot analysis. Cells transfected with the human apoE gene containing only the proximal 51 bp of the 5'-flanking sequences showed an mRNA level that was about 8-fold less than those of the gene that contained 383 bp of the upstream sequence (Fig. 2B). This finding was consistent with the results obtained from the promoter deletion analysis (Fig. 2A).

Deletion of the nucleotides between -3000 and -383 resulted in a slight increase in the expression of the apoE gene, which suggested that an upstream negative transcription element might have been removed. However, when fragments from this region were tested subsequently with a test vector that was employed for enhancer assays (described below), a negative effect on gene expression could not be demonstrated (data not shown).

Identification of Apolipoprotein E Gene Enhancer Elements—To determine whether regulatory elements in the 5'-flanking region of the apoE gene, as well as sequences in the intragenic regions of the apoE gene, can function as transcription enhancers, a series of recombinants in the vectors pTK1 and pTK10 were constructed (see “Experimental Procedures”). In these vectors, the expression of the CAT gene is under the control of the TK promoter of the herpes simplex virus, which is active in most cultured cells (24). The use of these two vector systems enabled us to determine the effects of putative enhancer sequences on the TK promoter located either adjacent to (pTK10) or 600 bp distant from (pTK1) the putative enhancer. In addition, several fragments were inserted in either orientation relative to the direction of transcription to examine the effect of orientation on the potential enhancer fragments.

First, we examined the CAT activity of plasmids pHAEN-10 and pHAEN-11, which have a 1-kb insert spanning nucleotides -651 and +356 (including the first exon and 312 nucleotides of the first intron) ligated into vector pTK1. In both CHO and HepG2 cells this fragment stimulated the activity of the endogenous apoE gene.
One element, termed upstream regulatory element 2 (URE2), was found within the first intron and is located between residues -366 and -246. Another element, termed upstream regulatory element 1 (URE1), was found within a 219-bp segment between residues -193 and -246 of this fragment. The third element, termed intron regulatory element 1 (IRE1), was found within the first intron and is located in a 219-bp segment between residues +44 and +262. These three elements had promoter-enhancing activity in both orientations. Other 5'-flanking and intragenic regions, including distal upstream sequences (-3000 to -651), portions of the first and second introns (+561 to +1439), and the third intron (+1852 to +2907), were tested, but they failed to show any enhancer activity in these assays (data not shown).

Although enhancers can often act over very large distances, there are many examples of enhancers that exhibit substantially reduced activity when separated from the test promoter (24, 38-42). To examine the effect of distance on the activities of the apoE enhancer elements, the appropriate fragments were ligated into plasmid pTK10 in a location immediately proximal to the TK promoter (i.e. there was no 600-nucleotide spacer). The observed enhancement was essentially identical to that observed when the spacer was present (data not shown), suggesting that the action of these enhancer-like elements is relatively independent of distance under these conditions.

To determine whether these enhancer-like elements can act when positioned at the 3' end of the CAT gene and with a different promoter, another test vector (pAEP,CAT), which contained an enhancerless SV40 promoter (27), was employed. The three putative enhancer elements, URE1, URE2, and IRE1, were inserted either immediately 5' to the enhancerless SV40 promoter or at the 3' end of the CAT gene and transfected into CHO cells. The results shown in Fig. 4 indicate that URE1 was able to enhance SV40 promoter activity for the SV40 promoter, although the effect from the 3' position (about a 2-fold enhancement) was about half that from the 5' position. URE2 possessed little activity at either position, suggesting that it may be influenced by the associated promoter, perhaps through specific interactions of trans-acting factors. Thus, it was concluded that URE2 has conditional enhancer-like activity.

Because the cloning of many of these inserts into the CAT vectors involved adding BglII linkers on either end, we examined whether the synthetic linkers themselves had an effect on CAT gene activity. Fragments from the second intron of the apoE gene as well as 300- and 600-bp fragments from bacteriophage DNA (i.e. φX174 RF DNA) were prepared and ligated with the BglII linkers and then inserted into the same CAT gene vectors. None of these control fragments exhibited enhancer activity or any other effect on the transcription of the CAT gene (data not shown).

### Table II

| Plasmid     | Fragment                  | Relative activitya |
|-------------|---------------------------|--------------------|
|             | CHO | HepG2 | HeLa | L |
| pHAEN-1     | -366 to -246 (URE2)       | 4.2 ± 1.5         | 4.9 ± 2.6 | 2.5 ± 0.5 | 2.2 ± 0.1 |
| pHAEN-2     | -246 to -81 (URE1)        | 9.4 ± 2.6         | 6.9 ± 2.1 | 5.0 ± 1.8 | 7.3 ± 2.9 |
| pHAEN-3     | -81 to -11                | 1.5 ± 0.4         | 1.8 ± 0.1 | 1.0 ± 0.2 | 1.4 ± 0.5 |
| pHAEN-4     | +44 to +262 (IRE1)        | 4.8 ± 1.4         | 3.3 ± 0.6 | 0.7 ± 0.3 | 1.7 ± 0.2 |
| pSVTK-CAT   | SV40 enhancer             | 34.6 ± 3.7        | 25.1 ± 2.8 | 16.9 ± 1.2 | 19.3 ± 2.6 |
| pTK1 (control) | 1.0 | 1.0 | 1.0 | 1.0 |

a Relative activity is expressed as -fold stimulation over the control, pTK1. All enhancer constructs used for this experiment have the 5' to 3' orientation with respect to the test TK promoter.
Promoter fragment, containing 81 nucleotides of the apoE gene. Recombinants—To verify that the products of the CAT gene sequence) (lanes 1 and 2 in Fig. 5), facilitating detection without influencing the initiation site. The enhancer test vector, pHAEN-2, which contains URE1 600 bp upstream of the TK promoter, generated a 134-bp transcript (71 nucleotides of the CAT gene sequence and 63 nucleotides of the TK gene sequence) that also indicated correct transcription initiation (lane 6 in Fig. 5) (24). Furthermore, the relative intensity of the transcript band produced by DNA-containing URE1 is about 6-fold stronger than that produced by the TK promoter itself (compare lanes 5 and 6 in Fig. 5). This finding is consistent with the enhancer test results obtained from the CAT enzyme activity assay (Fig. 3).

Cell-type Specificity of the Apolipoprotein E Enhancers—An important property of some viral and cellular enhancers is that they often exhibit host, or cell-type, specificity (24, 35–42). To test whether the apoE enhancer sequences also exhibit cell-type specificity, the TK promoter/CAT constructs containing regulatory elements URE1, URE2, and IRE1, as well as the DNA segment spanning −81 to −15 as a negative control, were transfected individually into four different cultured cell lines (Table II). A control plasmid, pSVTK-CAT, which contained the relatively strong SV40 enhancer located 600 bp upstream from the TK promoter, also was tested. The URE1 and URE2 segments were active in all cell lines tested. The IRE1 element showed equivalent activity in CHO and HepG2 cells, no activity in HeLa cells, and little activity in L cells. Thus, the three apoE gene enhancer-like elements showed variations in their activity among different cultured cells. However, the biological roles of these elements remain to be determined. It is noteworthy that the ratio between the magnitude of stimulation by the apoE URE1 domain enhancer and the SV40 enhancer in each cell line remained relatively constant among all four cell lines (3-4-fold). In this regard, the activities of the other apoE enhancers showed much greater variation in the different cell lines. In addition, the URE1-containing fragment was able to stimulate TK promoter activity more than 4-fold overall in several other cell lines tested (data not shown).

DNase I Footprinting of the Apolipoprotein E Enhancer Regions—To test the possibility that nuclear protein factors may interact with the apoE gene regulatory elements that were identified by the above studies, nuclear extracts prepared from HepG2 cells were assayed for the presence of sequence-specific binding activities by using the DNase I footprinting analysis of apoE gene fragments by HepG2 (A) and CHO (B) nuclear proteins. Panel A, apolipoprotein E DNA probes were 3' end-labeled at nucleotide −15 for the 5' strand, and at nucleotide −366 for the 3' strand. Panel B, the same fragment was 5' end-labeled at nucleotide −366 for the 5' strand (by kinase reaction) and 3' end-labeled at nucleotide −366 for the 3' strand (by Klenow reaction). Lanes 1 and 2, G and G + A reactions of Maxam and Gilbert sequencing reactions; lane 3, no nuclear extract; lane 4, 36 µg of nuclear extract; lane 5, 72 µg of nuclear extract. Sequences protected from DNase I digestion are indicated by the brackets, with the nucleotide positions at the boundaries indicated on the right.

Primer Extension Analysis of Chloramphenicol Acetyltransferase Gene Recombinants—To verify that the products of the CAT gene reflected correctly initiated mRNA transcripts and that CAT activity measurements in this system do indeed reflect events at the RNA level, total cellular RNA from cells that were transfected with the CAT gene constructs were examined by primer extension analysis. Total RNA from CHO cells transfected with the promoter test vectors pHAECAT1 (containing 651 nucleotides of proximal 5'-flanking sequence of the apoE gene) or pSVE-CAT (SV40 enhancer in pHAECAT1 located 600 bp upstream from the promoter) produced a prominent band 150 nucleotides in length (79 nucleotides of the apoE gene sequence and 71 nucleotides of the CAT gene sequence) (lanes 1 and 2 in Fig. 5), corresponding to the length predicted for the correct RNA transcription initiation site. The same results were obtained when a shorter promoter fragment, containing 81 nucleotides of the apoE gene 5'-flanking sequence, was examined (data not shown). The SV40 enhancer stimulated the RNA transcription signal by greater than nine times (compare lanes 1 and 2 in Fig. 5), facilitating detection without influencing the initiation site.

FIG. 7. Mapping of the 5'-flanking sequences of the human apoE gene. The hatched boxes indicate the apoE gene fragments that exhibited enhancer-like activity, as indicated in Fig. 3. The protein binding sites determined by the DNase I footprinting assay presented in Fig. 6 are represented by the ovals enclosed by a dark line. The oval without the surrounding line indicates weaker protein binding. The nucleotide sequences in the protected region are shown, with the protein site underlined by the shaded bar. The numbers indicate the nucleotide sequence in base pairs relative to the apoE transcription initiation site. The arrows indicate regions of reverse symmetry, and the asterisks indicate mismatched positions in these regions.
permit apoE gene transcription in several cell lines. It was those tested for the activity of the 651-nucleotide promoter fragment of the human apoE gene. However, the promoter fragment may have been active because of the elimination of either upstream or downstream negative control elements. In this regard, examination of various 5'- and 3'-proximal and distal apoE gene fragments with the pTK1 vector did not demonstrate negative elements in this test system. Further studies with alternative test vectors may be required to determine additional control elements.

In promoter deletion studies, there is a good correlation between the results obtained from the CAT enzyme assay using the expression of the chimeric apoE promoter-CAT gene and the measurement of cellular RNA levels obtained from transfection of the intact apoE genes containing various lengths of the 5'-flanking region. These results (Fig. 2, A and B) suggest that the region between -393 and -15 contains at least three domains, possibly with multiple elements, that affect apoE gene expression. An additional regulatory domain was found subsequently in the first intron. From the analysis of various fragments in these regions, a 168-bp fragment between -246 and -81 was found to have relatively strong enhancer activity. The level of basal expression of control vectors was enhanced by this DNA sequence up to nearly 10-fold in HepG2 and CHO cells. Much of this enhancer activity was found within a 69-bp fragment (-193 to -124, URE1). Furthermore, this upstream regulatory element (URE1) acted in a distance-, orientation-, and position-independent manner, characteristics seen in many viral enhancers (24, 46-50). The URE1-containing DNA sequence was active in all cells tested. This lack of cell-type specificity of URE1 may correlate with the wide tissue distribution of apoE mRNA in various tissues of mammals (6). In contrast to URE1, URE2 and IRE1 exhibited partial cell-type specificity (IRE1) and position or promoter dependence (URE2).

Although the general mechanism of enhancer action is not clear, it appears to be coupled with the binding of specific protein factors in the nuclei of cells in which they are active (24, 39, 51, 52). The results presented here show that nuclear proteins bind specifically to a region within URE1. The sequence of the URE1 protein binding region (Fig. 7) contains inverted repeated sequences (-164 to -159, -152 to -147, 5'-AACCTCTATGGCCGCCACCTCTTC-3'). It is not clear whether this feature plays a role in these sequences being recognized by the protein factor(s).

The protein binding region within URE1 appears to be associated directly with the enhancer activity of this domain. A synthetic oligonucleotide of 30 nucleotides spanning region -169 to -149 and containing the URE1 footprint sequence acted as an enhancer when examined with the pTK1 test vector (data not shown). However, the 30-mer was only 60% as active as the URE1-containing fragment (-246 to -81). This finding suggests that the enhancer activity of this domain may be the result of complex interactions between adjacent sequences, not just a simple protein-DNA binding interaction.

Both HepG2 and CHO cells showed a prominent protein footprint in the same portion of URE1. Thus, this region of
the apoE promoter may contribute significantly to its activity in these two diverse cell types. However, the lack of expression by the endogenous apoE gene in CHO cells suggests that additional protein factors and sequences are involved in gene expression.

A prominent protein footprint (−59 to −45) was found at the proximal GC box, indicating that the GC box is likely to be active in stimulating apoE gene transcription. In contrast, no protein binding was observed for the two distal GC boxes (−77 to −68 and −280 to −271), suggesting that they may not play a role in apoE expression. This probability correlates with the observation that the nucleotides surrounding the hexamer GC core would influence these GC boxes to be weak binding sites for Sp1 (53).

There is a striking homology between parts of the protein binding domains of URE1 and IRE1 and the Sp1 binding sequence (Table III). Nine nucleotides in 12-nucleotide sequences from URE1 (−157 to −146) and IRE1 (−169 to +180) are identical, and 8 of 10 nucleotide positions in URE1 and 7 of 10 positions in IRE1 are identical to the corresponding positions of the Sp1-binding GC box (10 nucleotides) consensus sequence (37). Nevertheless, it is unlikely that Sp1 binding by itself is responsible for the enhancer activities of URE1 and IRE1, for two reasons. First, there is no evidence that GC box sequences alone have enhancer activity in any viral or cellular genes. In the case of the apoE gene, the fragment containing the proximal GC box (−59 to −45), whose footprint as seen in Fig. 6 suggests a high affinity for Sp1 binding, did not stimulate TK promoter-directed CAT activity when placed in enhancer test vectors (Fig. 9). Second, the protein binding regions of URE1 and IRE1 are much longer than observed for the proximal GC box, indicating that other proteins in addition to Sp1-like proteins may be bound to these two regions. However, there may be an interaction between Sp1, which might be bound to these GC box-like sequences, and adjacent URE1-specific and IRE1-specific binding proteins that are directly responsible for enhancer activity.

The identity between 9 of 12 nucleotide positions in portions of URE1 and IRE1 and repeat 3 of the sterol regulatory region (SRE-42) of the LDL receptor gene (53, 54) is also interesting. The SRE-42 element in the 5′-flanking region of the LDL receptor gene is the cholesterol-responsive sequence associated with the down regulation of the LDL receptor gene. It is surprising to find a homologous component of part of the SRE-42 sequence in the apoE gene, since increased intracellular cholesterol levels have been associated with increased levels of apoE mRNA (55, 56). Perhaps the activities of these sequences are modulated by closely related proteins that bring about diverse effects through interactions with additional unrelated proteins and by different affinities of DNA binding. It is also possible that the repeat 3 element in the SRE-42 domain is not involved directly in the cholesterol responsive-ness of the LDL receptor gene. It may be that repeat 3 of SRE-42 and sequences of URE1 and IRE1 bind a protein in common that modulates the overall activity of these regulatory domains. In this regard, the Sp1 binding sequence (Table III) suggests that an Sp1-like protein might be involved in the activities of these regulatory domains.

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