Structure of the Hepatic Control Region of the Human Apolipoprotein E/C-I Gene Locus*

Qi Dang‡§, David Walker‡, Stacy Taylor‡, Charles Allan‡§, Peter Chin‡, Jianglin Fan‡§, and John Taylor‡§¶

From the ‡Gladstone Institute of Cardiovascular Disease, §Cardiovascular Research Institute, and ¶Department of Physiology, University of California, San Francisco, California 94141-9100

The specificity of expression in the liver of the human apolipoprotein (apo) E/C-I gene locus is determined by a hepatic control region (HCR) that is located 15 kilobases downstream of the apoE gene. DNase I footprint studies of this sequence using nuclear extracts identified a region of the HCR that is enriched in nuclear protein-binding sites. Nuclease analysis of chromatin revealed liver-specific DNase I-hypersensitive sites that were associated with this region, and additional liver-specific nuclease-sensitive sites associated with the apoE gene were identified. The HCR domain has a limited binding affinity for the nuclear scaffold. The specific domain required for liver expression was tested by ligating subfragments of the HCR to the apoE gene and examining their activity in transgenic mice. A segment of 319 nucleotides that contained several potential regulatory sequences was required for full activity of liver-specific transcription with shorter segments yielding much lower levels of expression in the liver. All constructs that contained a fully active HCR were expressed in approximately a copy-dependent manner, suggesting that transgene expression was independent of integration position. Taken together, the properties of the HCR are consistent with its function as a locus control region for the liver-specific expression of the apoE gene.

Human apolipoprotein (apo) E, a 299-amino-acid glycoprotein of M, = 35,000 (1), is a major component of various plasma lipoprotein classes, including chylomicron remnants, very low density lipoproteins, and high density lipoproteins (2, 3). It facilitates the redistribution of cholesterol from peripheral tissues to the liver and is required for the receptor-mediated uptake of chylomicron remnants (2, 4). Although apoE is produced by many different tissues, more than 90% of the circulating apoE is located 7.5 kb still further downstream. The apoE-I gene (3.3 kb) is found about 16 kb downstream of the apoE-I pseudogene. Each of these genes contains four exons, with the introns located in similar intragenic positions, suggesting that this gene family evolved from a common ancestor (1, 9).

Expression of the apoE gene in the liver was shown initially by Simonet et al. (1, 11, 12) to require the presence of a distal downstream tissue-specific enhancer. Subsequent studies by Simonet et al. (12) demonstrated that this hepatic control region (HCR) was located 19 kb downstream of the transcription start site of apoE gene and 9 kb downstream of the beginning of apoC-I gene. The HCR contained the sequences necessary to direct expression of both the apoE and apoC-I genes to hepatocytes with no liver specificity contributed by the promoters of either gene (13). Any construct that lacked the HCR was not expressed, even at low levels, in the transgenic mouse liver. Full liver-specific activity of the HCR was provided by a 0.77-kb fragment (construct LE6 in Ref. 13). However, the presence of a previously characterized enhancer element, that lacks tissue specificity, in the promoter of the apoE gene (14, 15) was required for transcriptional activation. These results suggested that interactions of a unique hepatocyte-specific combination of distal elements in the HCR with an activator sequence in the promoter directed the expression of the apoE/C-I/C-II locus in the liver.

The current study extends our earlier findings (13) and characterizes more precisely (16) the features of the HCR that define its activity. We find that a genomic fragment of about 300 nucleotides is required for full levels of HCR activity, that at least three nuclear protein binding sequences are involved, and that the HCR is characterized by prominent liver-specific DNase I hypersensitivity. These results further define the unique qualities of the HCR that determine liver-specific activity.

MATERIALS AND METHODS

Preparation of Nuclear Extracts

Nuclei and nuclear extracts from mouse liver and other tissues were isolated by the method of Gorski et al. (17) with minor modifications as described (15). HeLa cell nuclear extract was prepared by the method of Dignam et al. (18).
In Vitro Translation of Transcription Factor

Transcription factors C/EBPβ, HNF-3α, HNF-1, and HNF-4 were synthesized with a riboprobe transcription/translation system (Promega, Milwaukee, WI) according to the manufacturer's suggested protocol. C/EBPβ cDNA (a gift of S. McKnight, Tularik Co., South San Francisco, CA), HNF-3α cDNA (a gift of R. Costa, University of Illinois Medical Center, Chicago, IL), HNF-1 cDNA (a gift of G. Crabtree, Stanford University, Palo Alto, CA), and HNF-4 cDNA (a gift of F. Sladek, University of California, Riverside, CA) were cloned into vectors pBSKK and pGEM-1, respectively, and then used as templates.

Preparation of DNA Fragments

DNA Fragments for Footprinting—To generate single end-labeled DNA probes, the 774-bp LE6 HCR fragment (13) was cloned in pBSKK (Stratagene, San Diego, CA). Subfragments of the HCR were excised from the vector by restriction endonucleases that generated 5'-protruding ends, and the subfragments were purified by polyacrylamide gel electrophoresis (PAGE). The fragments were end-labeled with [α-32P]dCTP by the Klenow fragment of DNA polymerase, then purified with NE Nsorb columns (DuPont NEN), and stored in TE buffer (10 mM Tris, pH 7.4, and 1 mM EDTA).

Oligonucleotides—Complementary oligonucleotide pairs were synthesized via a nucleic acid synthesis system (Millipore, MA), then annealed in a buffer containing 67 mM Tris-HCl, pH 7.6, 13 mM MgCl2, 6.7 mM dithiothreitol, 1.3 mM spermidine, and 1.3 mM EDTA. The annealed products were purified byPAGE, then labeled by T4 DNA kinase in the presence of [γ-32P]ATP (19). Oligonucleotide sequences were:

1. TCCCTAATAAGGGCCAACATTGAGACACCAAC; 2. ACCGAGCTCTGCCTGAGGACACCCAAC; 3. TGGGCTGAGGTCAATCCCAAC; 4. GACCTGCTTGGCTGACACCCAAC; 5. AGGCCTGTATTCCCTCAGAC; 6. AATGATATGACGACCCATCTGATTAGGGTTTTG; 7. CCAGGCTGGAGTCAGTGACACAATCTCAGCCTGTTTCCCCATCTGTACAATG; 8. AGCCTGTTTCCCCATCTGTACAATG.

Preparation of DNA Probes—For mapping DNase I-hypersensitive sites, DNA fragments were purified by agarose gel electrophoresis, then end-labeled with [α-32P]dCTP by random primers and the Klenow fragment of DNA polymerase as described (19). For mapping nuclear scaffold-associated regions, plasmids containing HCR DNA fragments were digested with restriction enzymes, end-labeled with [α-32P]dCTP and Klenow fragment, and purified by column chromatography (Schleicher & Schuell). HCR Subfragments for Cloning—Different fragments of the 774-bp HCR (13) were amplified by polymerase chain reaction (PCR), with HindIII sites generated on the 5'-end and SpeI sites generated on the 3'-ends. The amplification products were digested with HindIII and SpeI, purified by agarose gel electrophoresis, and cloned into pBSKK. The 10-kb HE1 human genomic fragment (11), which contains the complete apoE gene sequence together with 5' of 5'-flanking and 1.7 kb of 3'-flanking sequences, was inserted adjacent to the 5'-ends of the HCR constructs. The polyclonal sequence of the fragments, as well as the complete HCR, were confirmed by direct DNA sequence analysis using oligonucleotides (19).

Gel Shift Assays

The assays were carried out essentially as described (15). Nuclear extract was incubated with 4 μg of poly(dI:dC) at 20 °C for 5 min in 20 μl of nuclear dialysis buffer. Where appropriate, 50 ng of specific oligonucleotides were added to each reaction and incubated at 20 °C for 15 min. Then, 0.5 ng of end-labeled oligonucleotide was added to the extract and incubated at 30 °C for 30 min. Samples were resolved by electrophoresis in 5% polyacrylamide gels in 30/2× TBE buffer (90 mM Tris borate, 2 mM EDTA), then the gel was dried and examined by autoradiography.

Mapping of DNase I-hypersensitive Sites—Nuclei were isolated, as described (17), from different tissues of transgenic mice bearing the LE1 transgene construct (13). This construct contained the intact human apoE gene together with 5' of 5'-flanking sequence, 1.7 kb of 3'-flanking sequence, and the LE1 HCR-containing fragment (a 3.8-kb SplH/BamHI fragment located 14 kb downstream of the apoE gene that contained the complete HCR activity). The nuclear pellet was resuspended in buffer B (5 mM Tris-HCl, pH 7.4, 0.15 mM spermidine, 80 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) containing 5% glycerol.

The nuclei were diluted to 10 A260/ml with Buffer A (without EDTA) containing 5% glycerol and 5 mM MgCl2. Then, 0.5-ml aliquots were incubated on ice for 10 min with different amounts of DNase I. The reactions were quenched by addition of 25 μl of 0.5 M EDTA, 25 μl of 5 M NaCl, 5 μl of 10% SDS, 12.5 μl of 1 M Tris, and 10 μl of 10 mg/ml proteinase K and incubation at 50 °C for 1 h. Aliquots were extracted with phenol, and DNA was precipitated with ethanol and then resuspended in 0.1 × TBE buffer. DNA was digested with appropriate restriction enzymes, resolved by electrophoresis in a 0.8% agarose gel, and transferred to nylon membranes. The membranes were hybridized to radioactively labeled DNA probes at 42 °C overnight, washed to a final stringency of 0.1 × SSC (0.15 mM NaCl, 15 mM sodium citrate) and 0.1% SDS at 55 °C for 30 min, and then examined by autoradiography.

Generation and Analysis of Transgenic Mice

The apoE gene/HCR constructs were used to generate transgenic mice as described (27). Nuclei were injected into one-cell-stage embryos at 3 weeks of age. DNA was extracted from tail segments, and transgenic founders were identified by Southern blot analysis (28). The copy number of the incorporated transgene was determined by quantification of the Southern blots via a Fuji Bas1000 Bio-imaging analyzer (Fuji Photo Film Co., Ltd., Japan) using human genomic DNA as a standard, following hybridization using a human apoE hybridization probe (11).

Total cellular RNA was isolated from various tissues of transgenic mice and examined by RNase protection analysis as described (29) followed by quantitation via the Fuji Bas1000 Bio-imaging analyzer. The quantity of the total cellular RNA in each reaction was monitored by comparison to the amount of mouse β-actin measured by the same assay using a mouse β-actin antisense RNA probe (Ambion, TX). For each construct, at least two, and up to five, independent lines were analyzed.

RESULTS

Nucleotide Sequence of the HCR

Since it contained all of the regulatory information required for full levels of expression of the apoE gene in the liver, the sequence of the 774-nucleotide length of the LE6 HCR fragment was determined (Fig. 1) (13). The 3'-terminal portion of the LE6 fragment has an 83% identity in the 3' to 5' orientation in comparison to the corresponding regions of an Alu family consensus sequence (30, 31). The typical Alu family member is composed of two closely related segments separated by a short A-rich sequence of slightly variable length with a second variable length A-rich sequence at the 3' terminus. In the LE6 fragment, the complete HCR activity. The nuclear pellet was resuspended in Buffer B (5 mM Tris-HCl, pH 7.4, 0.15 mM spermidine, 80 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) containing 5% glycerol.
fragment of the HCR, nucleotides 462–666 consist of the 3’-terminal two-thirds of an Alu sequence family, and nucleotides 667–774 consists of the middle one-third of a separate Alu sequence family. The 5’ portion of the HCR fragment, consisting of 461 nucleotides, does not contain any portion of an Alu sequence family, nor any other repeat family, as evaluated by hybridization to genomic DNA (data not shown).

Binding of Nuclear Proteins to the HCR

DNase I Footprinting—To examine the binding of nuclear proteins to the HCR region, DNase I footprinting was performed along the entire length of the 774-bp HCR fragment using both mouse liver and cultured HeLa cell nuclear extracts. Six distinct footprints were revealed (Fig. 2), with three footprints clustered near the 5’ end of the HCR. Footprints 1, 4, 5, and 6 were protected by nuclear factors that appeared to be somewhat enriched in the liver. Footprints 2 and 3 were protected by factors that were abundant in both HeLa cell and mouse liver nuclear extracts. The protected region for footprint 1 was longer on the 3’ strand than on the 5’ strand.

DNase I hypersensitive sites were detected at footprint regions as a consequence of nuclear protein binding (Figs. 1 and 2). These sites were abundant in the region around footprint 1. It is noteworthy that this region has four tandem copies of the TGTTTGC motif and two upstream closely related sequences, indicated by the arrow bars in Fig. 1. In all but one of these sequences, a DNase I hypersensitive site was present in the TGTTTGTG motif between the two G nucleotides at a T adjacent to a G. The most prominent hypersensitive site detected in the 3’ strand (nucleotide 114) was located within one of these motifs although an unambiguous footprint was not detected here. The hypersensitive site at nucleotide 156 in footprint 1 was prominent in both DNA strands. Several DNase I hypersensitive sites were detected elsewhere in the HCR fragment where clear footprints were not observed, possibly reflecting weak or unstable protein binding. Some DNase I hypersensitive sites were detected in the Alu family sequence region. Both footprint 5, adjacent to the Alu sequence, and footprint 6, within the Alu sequence, had nearby hypersensitive sites. Since subsequent studies (described below) showed that this region was not required for HCR activity, the significance of DNase I sensitive sites in the Alu sequence domain remains unclear.

Gel-shift Assays—Protein binding to the footprint sequences was confirmed by gel-shift assays using mouse liver nuclear extracts and double-stranded oligonucleotides corresponding to these regions (Fig. 3). Since some of these sequences contained homologies to known transcription factor binding motifs, the gel-shift assay was used to investigate potential relationships. In addition, known transcription factors associated with enhanced liver expression were synthesized and used in this assay to investigate their potential role in HCR action. The radioactively labeled footprint 1 oligonucleotide had prominent gel-shift bands, and low levels of these bands were still detectable in the presence of a 100-fold excess of nonlabeled competitor. The footprint 1 sequence did not bind C/EBP, HNF1, or HNF4 (data not shown), but it did bind HNF3α, yielding a gel-shift band that corresponded to the major band produced by liver extracts (Fig. 3, panel 1). Footprint 2 contains an identity of eight nucleotides with a sequence in the TF-LF2 element located at nucleotide –480 in the promoter of the rat transferrin gene (21). In gel-shift as-

---

**Fig. 1. Sequence analysis of LCR region.** Shaded boxes represent footprint regions. Arrows with letters indicate the TGTTTGC motif. Triangles indicate DNase I-hypersensitive sites revealed by in vitro footprinting study. The line above nucleotides 462 to 774 indicates Alu family sequence homology.
says, an oligonucleotide corresponding to the transferrin regulatory sequence competed effectively for nuclear protein binding by the HCR footprint 2 probe (Fig. 3). This result suggests that the TF-LF2 factor (or factor family) recognizes the HCR footprint 2 sequence. The footprint 2 sequence did not bind C/EBP α, HNF1, HNF3α, or HNF4 (data not shown).

The 5' portion of footprint 3 has a limited homology to the consensus binding sequence for HNF4 (22); however, the HNF4 produced by an in vitro transcription/translation system bound very weakly to the footprint 3 oligonucleotide (data not shown). Nevertheless, the oligonucleotide of the footprint 3 sequence was an effective competitor for HNF4 binding to its own consensus high affinity binding sequence (22). These results suggest that a member of the HNF4 factor family may contribute to generating footprint 3. Although footprint 3 extended over a length of 41 nucleotides, only one major gel-shift band was observed. The footprint 3 sequence did not bind C/EBPα, HNF1, or HNF3α (data not shown).

Gel-shift assays confirmed the binding of a nuclear protein to the footprint 4 sequence (Fig. 3, panel 4). However, the factors HNF1, HNF3α, HNF4, and C/EBP did not bind to this element (data not shown).

Footprint 5 of the HCR contains a GATA motif. This motif is a core sequence in regulatory elements that bind a family of erythroid transcription factors most closely identified with globin gene promoters and the globin locus control region (32–35). However, GATA-binding factors also have been identified in nonerythroid tissue (24). An oligonucleotide corresponding to a high affinity GATA-1 binding site in the β-globin promoter (24) was used as a competitor in HCR gel-shift assays. The major gel-shift band produced by the footprint 5 oligonucleotide with liver extract was competed by the erythroid GATA-1 oligonucleotide (Fig. 3), indicating a potential role for a GATA binding factor in HCR function.

The sequence of footprint 6 has a limited homology to the consensus binding sequence of C/EBP (23). Protein produced by a C/EBPα expression vector bound to the footprint 6 oligonucleotide, yielding a band in the gel-shift assay that corresponded to one of the liver extract-generated bands (Fig. 3). Thus, it is likely that a member of this transcription family interacts with this sequence.

Mapping of DNase I-hypersensitive Sites in Chromatin—Regulatory sequences from actively transcribed genes are commonly hypersensitive to nuclease cleavage within the chromatin of the nucleus (36). Therefore, we investigated the presence of DNase I-hypersensitive sites (DHS) in the HCR as well as in the associated apoE gene construct using nuclei from transgenic mice that had been generated with the HEG.LE1 con-
Hepatic Control Region of the Apolipoprotein E Gene

Fig. 3. Gel shift assays. Mouse liver nuclear extract or in vitro translated nuclear factors were incubated with end-labeled dinucleotides that were synthesized according to footprint sequences and DNA binding consensus sequence of nuclear factors TF-LF2 (21) and HNF-4 (22), respectively. Unlabeled oligonucleotides were used as specific competitors. The samples were analyzed by PAGE, and autoradiograms of dried gels are shown. ML = mouse liver.

To determine if tissue-specific nuclease cleavage sites were associated with the HEG1LE1 transgene, isolated intact nuclei from the liver, brain, and kidney of transgenic mice were incubated briefly with DNase I. Then, the DNA was extracted and hybridized to probes from different regions of the construct. As shown in Fig. 4, panels A-D, and summarized in Fig. 5, 20 nuclease-sensitive sites were identified in chromatin that were distributed throughout the transgene. Most sites were detected in all tissues examined. However, five sites were liver-specific: one in the proximal promoter, two in the second intron, one in the HCR domain, and one just 3' of the LE6 HCR segment. The most prominent liver-specific DNase I-sensitive sites were DHS 11 in the second intron and DHS 17 in the 5'-terminal of the LE6 HCR segment. The most prominent liver-specific footprint 1 site was in the 5'-terminal 122 nucleotides (LES12) and footprint 2 (LES11) resulted in a 4-fold to more than 10-fold reduction in HCR activity. A construct having only the 5'-terminal 118 nucleotides (LES14) showed no expression in the liver (data not shown). The LES13 fragment that deleted 72 nucleotides of the 5' portion of the HCR but retained footprints 1, 2, and 3 sequences together with nearly all of the 5' DNase I-hypersensitive sites had low activity in the liver, equivalent to that of the LES12 fragment. The LES6 construct had no detectable liver expression, and a comparison of its activity to that of the LES3 construct, in which deletion of the 5'-terminal 122 nucleotides is the only difference, suggests that this segment is essential to the activity of the HCR. Taken together, these results indicated that the minimum region required for HCR activity includes the footprint 1 sequence and the adjacent DNase I-hypersensitive regions, as contained in the LES11 fragment.

Association of the HCR with the Nuclear Scaffold—The possibility that the HCR may associate with the nuclear scaffold was investigated by a rebinding assay (26). In this assay, the LE6 fragment or a subfragment of LE6 (Fig. 8), mixed together with a construct consisting of the HEG1 apoE gene and plasmid fragments (13), were radioactively labeled, then incubated with an isolated nuclear scaffold preparation. The DNA fragments that did not bind to the nuclear scaffold were separated subsequently by centrifugation, and the distribution of the bound radioactivity in the pellet (scaffold) and the nonbound radioactivity free in the supernatant fractions were resolved by agarose gel electrophoresis. As shown in Fig. 8, radioactivity near the top of the gel corresponded to the HEG1

Footprint Extract (10 μg) Competitor (60ng) Probe (0.5ng)

1 1 1 1 1 2 2 2 3 3 3
2 2 2 2 2 3 3 3 4 4 4
3 3 3 3 3 4 4 4 5 5 5
4 4 4 4 4 5 5 5 6 6 6
5 5 5 5 5 6 6 6
6 6 6 6 6

FIG. 3. Gel shift assay. Mouse liver nuclear extract or in vitro translated nuclear factors were incubated with end-labeled dinucleotides that were synthesized according to footprint sequences and DNA binding consensus sequence of nuclear factors TF-LF2 (21) and HNF-4 (22), respectively. Unlabeled oligonucleotides were used as specific competitors. The samples were analyzed by PAGE, and autoradiograms of dried gels are shown. ML = mouse liver.
apoE gene construct and plasmid DNA, and radioactivity near the bottom of the gel corresponded to the HCR DNA. The 774-nucleotide LE6 HCR fragment and the LES3 subfragment showed the greatest association with the nuclear scaffold in the pellet. In comparison, essentially no LES2 or LES8 (nucleotides 462–774 consisting of the complete Alu family segment) fragments were found in the pellet. Similarly, HEG1 apoE gene fragments showed relatively little binding to the nuclear scaffold. These preliminary findings suggest that the unique sequence portion of the HCR has a low, but specific, affinity to the nuclear scaffold whereas the apoE gene and its proximal flanking regions do not associate significantly with the nuclear scaffold.

**DISCUSSION**

Previous studies in our laboratory mapped the liver-specific HCR of the human apoE/C-I locus to a 774-bp fragment (LE6) located about 19 kb downstream of apoE promoter and about 9 kb downstream of apoC-I promoter (13). The expression of apoE and apoC-I genes in the liver of the transgenic mice were totally dependent on this distal regulatory region. In the absence of the HCR, there was no expression of the apoE gene in the liver. No sequences in the promoter, even extending 30 kb upstream, were found that conferred expression of the apoE gene (or the apoC-I gene) in the liver. The finding that all of the liver expression capability of the apoE gene is contained entirely within the downstream HCR makes this tissue specificity unique among genes that are expressed in the liver. The typical mechanism for the hepatic expression of other genes positive promoter elements that provide low levels of liver expression, with higher levels of transcription in the liver dependent upon distal enhancer sequences. The results presented here have described several distinct properties of the apoE gene HCR that contribute to its unique activity.

Nuclear protein DNase I footprint analysis and gel-shift assays of the LE6 HCR revealed several different protein families.
iliesthatbindtothisfragment. Most of these families consist of multiple members, often present together in the same cell type, binding to DNA sequences that are characteristic of a particular family. Thus, members of the HNF3 and HNF4 transcription factor families may influence HCR activity, whereas the potential roles of the GATA-binding and the C/EBP families are unclear. Further study beyond the scope of this preliminary investigation would be required to identify the particular member in each factor family that binds to the HCR domain. The HNF3, HNF4, and C/EBP transcription factor families contribute to the liver-specific expression of many genes (i.e. most of the genes listed in Table II). In data not shown here, we determined that the frequently used liver transcription factor HNF-1 (generated by in vitro translation of a cDNA expression vector) does not bind to any footprint sequence in the HCR.

Furthermore, none of the transcription factors investigated in this study bind to footprint sequences that were previously identified (1, 15) in the apoE gene promoter (data not shown), consistent with its inability to direct gene expression to the liver (13–15).

The unique sequence domain at the 5' end of the LE6 fragment (nucleotides 1–461) appears to constitute the functional HCR. The minimum length having full activity is a 319-nucleotide region located at the 5' terminus. This segment contains three major footprints, two of which are liver-specific, and prominent DNase I-hypersensitive character, which is also liver-specific. Analysis of subfragments of the HCR in apoE transgene constructs demonstrates that shorter fragments have a 4-fold or greater reduction in transcriptional activity. Thus, while the footprint 1 element together with adjacent nuclease-sensitive regions can direct liver-specific expression, full activity requires a sequence of about 300 nucleotides.

Three copies of the TGTTTGC motif are found in the footprint 1 sequence, and three more copies are located nearby. Five of these sequences are associated with DNase I-hypersensitive sites, and together they probably account for the prominent liver-specific nuclease hypersensitivity that was detected in whole nuclear chromatin at this location (Fig. 4, panel D, site 17). The highly conserved TGTTTGC motif is found in the promoters and/or enhancers of many genes that are expressed in the liver at moderate to high levels (Table II). In the human transferrin gene promoter, this motif binds a transcription factor that is specific for single-stranded DNA (37). It is of interest that the TTTG motif is conserved in the core DNA binding sequence of high mobility group (HMG) proteins (38). The HMG proteins act by introducing pronounced bending in DNA at the binding site (39), an action expected to result in enhanced DNase I hypersensitivity. Perhaps, the binding of nuclear proteins to the TGTTTGC motif in the footprint 1 sequence of the HCR may induce a substantial conformational change in the DNA duplex.

Nuclease-sensitive sites are a characteristic feature of transcriptionally active chromatin in eukaryotes (reviewed in Ref. 40), and they are associated with a wide variety of regulatory elements. We mapped 20 DNase I-hypersensitive sites in whole nuclei that were distributed throughout the HEG1.LE1 transgene, with five of them being specific to the liver. Since HEG1 alone, without an HCR domain, is not expressed in the liver (11), it was notable to find one liver-specific site in the proximal promoter and two liver-specific DNase I-hypersensitive sites in the second intron. These regions may participate in the hepatic
expression of the apoE gene, even though transgene expression data shows that the far downstream HCR is sufficient for directing liver tissue-specific transcription.

The shortest HCR construct analyzed that resulted in full liver-specific activity was the 319-nucleotide LES2 subfragment. A comparison to the 3.8-kb LE1 parent HCR fragment (13) indicates that transgenic mice generated with either construct yield similar apoE mRNA levels per gene copy number (Table 1). The range of 2–70 transgene copies in the six independent lines examined for these two constructs suggests that HCR activity is relatively independent of the position of construct integration into the genome. This property is similar to the action of the locus control region of the human β-globin gene (41, 42).

Position-independent expression of exogenous genes in stably transfected cultured cells or transgenic mice has been shown to be a consequence of the presence of nuclear scaffold binding regions (41, 42). The finding of limited nuclear scaffold binding character in the unique sequence portion of the HCR, but not in other regions of the HEG1 apoE gene fragments, is consistent with the observed HCR action. Perhaps sequences adjacent to this portion of the HCR are required for conferring

**TABLE I**

Expression levels of human apo E gene constructs in transgenic mice

| Constructs | LE1 | LES2 | LES3 | LES6 | LES7 | LES11 | LES12 | LES13 |
|------------|-----|------|------|------|------|-------|-------|-------|
| Transgenic lines | 1   | 1    | 2    | 3    | 4    | 5     | 1     | 2     |
| Transgene copy no. | 70  | 12   | 19   | 17   | 2    | 57    | 19    | 15    |
| Liver mRNA level/transgene copy no. | 0.36 | 0.13 | 0.14 | 0.35 | 0.26 | 0.35  | 0.23  | 0.01  |
| Average mRNA level/transgene copy no. | 0.36 | 0.25 | 0.39 | <0.01| 0.22  | 0.06  | 0.06  | 0.03  |

* Independent founder lines were used for analysis. The LE1 transgenic animals used for these studies were from the HEG.LE1 line described previously by Simonet et al. (13). It was one of three independent lines generated from the same construct, all of which had equivalent expression levels.

**TABLE II**

A common sequence motif in a footprint sequence of the apoE gene hepatic control region

| Gene | Location | Sequence | Refs |
|------|----------|----------|------|
| h-ApoE | 1,067 | GATGTGTTGCTCCTCC | 43 |
| h-Albumin | 1,081 | TCTGTTGCTCCTCC | 46 |
| h-Transferrin | 1,151 | GATGTGTTGCTCCTCC | 47 |
| m-Apolipoprotein B | 1,106 | ATCTGTTGCTCCTCC | 48 |
| m-Albumin | 1,125 | ATCTGTTGCTCCTCC | 49 |
| h-Hepatitis B virus | 1,126 | ATCTGTTGCTCCTCC | 50 |
| h-A1-Antitrypsin | 1,127 | ATCTGTTGCTCCTCC | 51 |
| h-Transferrin | 1,128 | ATCTGTTGCTCCTCC | 52 |
| m-Apolipoprotein B | 1,129 | ATCTGTTGCTCCTCC | 53 |
| m-Albumin | 1,130 | ATCTGTTGCTCCTCC | 54 |

**Fig. 8. Affinity of the HCR for the nuclear scaffold.** Plasmids carrying HEG.LE6 and subfragments of LE6 were digested with HindIII and XbaI, then end-labeled with [32P]dCTP. The nuclear scaffolds were isolated from ICR mouse liver nuclei, then digested with HindIII, BamHI, and XhoI. Probes and digested scaffolds were incubated at 37°C for 1 h, then pellets and supernatants were separated by centrifugation. DNA from each fraction was extracted, then resolved by electrophoresis in 1.0% agarose gels. The gels were dried and autoradiographed. Samples were labeled as T = total probe, S = 1/2 supernatant fraction, and P = pellet fraction.

The results of our current study, taken together with previously published data (11–13), argue that a domain of nucleosomal DNA standard.

The action of the locus control region of the human β-globin gene (41, 42).

**FIG. 8.** Affinity of the HCR for the nuclear scaffold. Plasmids carrying HEG.LE6 and subfragments of LE6 were digested with HindIII and XbaI, then end-labeled with [32P]dCTP. The nuclear scaffolds were isolated from ICR mouse liver nuclei, then digested with HindIII, BamHI, and XhoI. Probes and digested scaffolds were incubated at 37°C for 1 h, then pellets and supernatants were separated by centrifugation. DNA from each fraction was extracted, then resolved by electrophoresis in 1.0% agarose gels. The gels were dried and autoradiographed. Samples were labeled as T = total probe, S = 1/2 supernatant fraction, and P = pellet fraction.

more stringent position independence.

Our results differ from the recently reported findings of others (16). They employed nuclear extracts from cultured hepatoma cells to identify two DNase I footprints in a fragment that corresponded to our nucleotides 191–223, but they did not detect footprint 1. They also concluded that a 154-bp fragment (corresponding to our nucleotides 78–231) constituted the liver-specific enhancer. Their use of cultured tumor cells, potential differences in nuclear extract protocols, as well as a transgene test vector with a comparatively short 5'-flanking sequence, may have led to their different conclusions regarding the nature of the HCR.

The results of our current study, taken together with previously published data (11–13), argue that a domain of nucleotides 6–325 is required for full HCR activity. The footprint 1...
sequence region is essential for HCR function, and it is associated with prominent liver-specific DNase I hypersensitivity. It also seems likely that DNase I nuclease-sensitive sites adjacent to the HCR, within the second intron, and within the proximal promoter region may facilitate HCR activity. In addition, a potential nuclear scaffold attachment capability may contribute to HCR action by distilling position independent gene expression in the liver. While these additional elements may contribute to the unique character of the HCR, the required information that determines the liver-specific expression of the apoE gene is contained within the unique combination of elements that constitute the far downstream HCR.

Acknowledgments—We thank Susannah White for help with manuscript preparation, Gary Howard for editorial review, and John Carroll and Amy Corder for graphics.

REFERENCES
1. Paik, Y.-K., Chang, D. J., Reardon, C. A., Davis, G. E., Mahley, R. W., and Taylor, J. M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3445–3449
2. Mahley, R. W., Innerarity, T. L., Rall, S. C., Jr., and Weisgraber, K. H. (1984) J. Lipid Res. 25, 1277–1294
3. Mahley, R. W. (1988) Science 240, 622–630
4. Brown, M. S., and Goldstein, J. L. (1986) Science 232, 34–47
5. Strittmatter, W. J., Saunders, A. M., Schmechel, D., Pericak-Vance, M., Enghil, J., Salvesen, G., and Roses, A. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1977–1981
6. Saunders, A. M., Strittmatter, W. J., Schmechel, D., St. George-Hyslop, P. H., Pericak-Vance, M. A., Joo, S. H., Rosi, B. L., Gusella, J. F., Crapper-D. M., McPherson, J., Bruns, G. A. P., Karathanasis, S. K., and Breslow, J. L. (1990) J. Lipid Res. 31, 572–581
7. Macdonald, R. J., Swift, G. H., Przybyla, A. E., and Chirgwin, J. M. (1987) Methods Enzymol. 152, 219–227
8. Schmid, C. W., and jelinek, W. R. (1982) Science 216, 1065–1070
9. Zaret, K. S., Liu, J.-K., and DiPersio, C. M. (1990) Nucleic Acids Res. 19, 5237–5245
10. Lauer, S. J., Walker, D., Elshourbagy, N. A., Reardon, C. A., Levy-Wilson, B., and Taylor, J. M. (1988) J. Biol. Chem. 263, 1169–1178
11. Zhang, D.-E., Hoyt, P. R., and Papoian, U. S., Proc. Natl. Acad. Sci. U. S. A. 87, 5469–5473
12. Zaret, K. S., Liu, J.-K., and DiPersio, C. M. (1988) J. Mol. Cell. Biol. 8, 3362–3391
13. Godbout, R., Ingram, R. S., and Tilghman, S. M. (1988) Mol. Cell. Biol. 8, 1169–1178
14. Zaret, K. S., Liu, J.-K., and DiPersio, C. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 87, 5469–5473
15. Trujillo, M. A., Letovsky, J., and Siddiqui, A. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3797–3801
16. Grange, T. J., Ruza, J., Rigaud, G., and Pictet, R. (1993) Nucleic Acids Res. 11, 131–139
17. Hepatic Control Region of the Apolipoprotein E Gene