Two Hepatic Enhancers, HCR.1 and HCR.2, Coordinate the Liver Expression of the Entire Human Apolipoprotein E/C-I/C-IV/C-II Gene Cluster*

(Received for publication, August 22, 1997)

Charles M. Allan‡§, Stacy Taylor‡, and John M. Taylor‡§¶

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

We show that the liver-specific expression of all four genes in the human apolipoprotein (apo) E/C-I/C-IV/C-II gene cluster in transgenic mice is determined by the coordinate action of two distinct hepatic control regions (HCR). These enhancers are positioned 15 kilobases (kb) (HCR.1) and 26 kb (HCR.2) downstream of the apoE gene. To investigate the action of each HCR, transgenic mice were generated with a 70-kb human genomic fragment that contained the complete apoE gene cluster or with this fragment modified by the specific deletion of HCR.1, HCR.2, or both HCR domains. Hepatic expression of all four apolipoprotein genes was observed in transgenic mice in which either HCR.1 or HCR.2 was deleted, but no transgene expression was found in the liver in the absence of both HCR domains. The overall patterns of transgene expression suggested that HCR.2 was the dominant element for apoC-IV and apoC-II expression and that HCR.1 was dominant for the apoE/C-I expression. No liver-specific transcriptional activity was identified for the proximal promoter of any gene in the cluster; all liver-specific activity was associated with HCR.1 and HCR.2. Thus, the HCRs of the apoE gene cluster constitute unique regulatory domains for determining the requirements for apolipoprotein gene expression in the liver.

The human apolipoprotein (apo) E, apoC-I, apoC-IV, and apoC-II genes are located in the same transcriptional orientation within a 44-kb cluster on chromosome 19 (1–3). These genes share many structural and sequence characteristics (3–5), suggesting that they evolved from a common ancestral gene; and they are evolutionarily related to the apoA-I, apoA-IV, and apoC-III genes located in an 18-kb cluster on chromosome 11 (5–7). The human apoE gene cluster also contains an apoC-I’ pseudogene (4), located between the apoC-I and apoC-IV genes, which appears to have arisen from a duplication event early in the primate lineage, approximately 39 million years ago (8, 9).

The apoE gene cluster encodes amphipathic lipid-binding proteins that have evolved distinct functions in lipid metabolism. ApoE mediates lipoprotein clearance from the plasma by acting as a ligand for the low density lipoprotein receptor (10, 11), the low density lipoprotein receptor-related protein (12), and cell surface proteoglycans (13). A role for apoE in neuron growth and homeostasis, as well as in the pathology of Alzheimer’s disease, has been suggested by recent studies (for review, see Ref. 14). ApoC-II, a specific cofactor for lipoprotein lipase, has an important role in the hydrolysis of lipoprotein triglycerides (15). Although its precise function is uncertain, apoC-I may inhibit the apoE-mediated cellular uptake of lipoproteins (16, 17), which would suggest a role in modulating lipoprotein catabolism. The function of apoC-IV, the most recently described member of this protein family, remains unknown (3). Although apoC-IV is not detected in normal human plasma (18), there is evidence that it may participate in lipid metabolism. The expression of human apoC-IV in transgenic mice resulted in hypertriglyceridemia (18), and normal rabbit plasma lipoproteins were recently found to contain a human apoC-IV homolog (19).

The primary site of synthesis of apoE, C-I, and C-II is the liver (4, 20, 21); nearly all apoE circulating in plasma is derived from the liver (22). Human apoC-IV mRNA is also expressed in the liver, although at considerably lower levels than the closely linked apoC-II gene (3). Because the hepatic expression of these apolipoprotein genes is fundamental to their proposed roles in lipid metabolism, we have investigated the regulatory elements required for their liver-specific expression. Transgenic mice expressing human genomic fragments of this cluster were used to identify and characterize a hepatic control region (HCR.1) that is required for liver-specific expression of the apoE and apoC-I genes (23, 24). HCR.1 is located 18.4 and 9.5 kb downstream of the 5’ ends of the apoE and apoC-I genes, respectively, between the apoC-I gene and apoC-I’ pseudogene (see Fig. 1). The full activity of HCR.1 appears to be provided by a 319-bp sequence (24), although subfragments have significant activity (24, 25). HCR.1 contains at least seven distinct regulatory elements that are cooperatively involved in directing high level, liver-specific expression of the human apoE transgene (24, 26). We recently identified in the apoE gene cluster a second HCR-like sequence (HCR.2) has an 85% nucleotide identity to the functional 319-bp HCR.1 sequence (27). The HCR.2 sequence is located 11 kb downstream of HCR.1 and 6 kb upstream of the closely linked apoC-IV and apoC-II genes. HCR.2 probably arose during the ancestral duplication event that formed the apoC-I’ pseudogene. We demonstrated that HCR.2 has retained the necessary sequences for directing high level, liver-specific expression of the human apoE gene in transgenic mice (27). These studies suggested that at least two hepatic enhancers are involved in the liver-specific expression of the human apoE gene.

Here, we report further studies of the roles of HCR.1 and
Two Hepatic Enhancers Control the ApoE Gene Cluster

HCR.2 in the liver-specific expression of each gene in the apoE gene cluster. First, to extend our previous investigation of the apoE and apoC-I gene loci (23), we determined if proximal flanking sequences in the apoC-IV and apoC-II gene loci could direct expression of these genes in the liver. We have identified all sequences within the apoE gene cluster which controlled liver-specific transcription of these genes. Second, we used RecA-assisted restriction endonuclease (RARE) cleavage to delete either or both of the HCR domains to determine their actions in the expression of each gene in the cluster.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Human Genomic DNA Clones—A cosmide clone (pCCV-111), shown previously to contain the complete human apoC-II gene, was a gift from Dr. Lawrence Chan (Baylor University, Houston, TX) (28). After digestion with HindIII, a 12-kg fragment (CII/H3) containing the entire apoC-II gene with 7 and 3 kb of 5′- and 3′-flanking sequences, respectively, was isolated during electrophoresis in 0.75% agarose using NaA45 membranes as recommended (Schleicher & Schuell). Purified CII/H3 DNA was ligation to a 774-bp genomic fragment containing HCR.1 (23). The HCR.1.CII/H3 construct was isolated by electrophoresis in 1.0% SeaPlaque GTG agarose (FMC Bio-products, Rockland, ME). A gel slice containing the construct was digested with PstI (FMC), and the DNA was purified using a DNA clean-up kit (Promega, Madison, WI).

A 12-kb plasmid clone (p1.198) containing the entire human apoC-I/C-IV/C-II gene cluster was identified by screening a human P1 genomic library (Genome Systems, St. Louis), and the 90-kb genomic insert was mapped as described (3). A 70-kb genomic fragment (198.KK) containing all four apolipoprotein genes was derived from purified after pulsed field gel electrophoresis for microinjection as described above. Purified p1.198 and 12E DNA was linearized by EcoRI digestion using the restriction endonuclease 5′-GACCTGGGAGACTACCTGGGAATTCCAGGGG-GGACATCTTGTCACCTACTCTTGTC (to protect the recombinable EcoRI site from methylation) and EcoRI digestion. The isolated 12-kb fragment was subcloned into a modified pBR322 vector that lacked a BamHI site in the polylinker. A 1.2-kb fragment containing the HCR.2 sequence was removed from 12E DNA by digestion with BamHI and BssHI. The modified 12E DNA was ligated, cleaved with EcoRI, and purified for reintroduction into linearized 198–12E DNA. Plasmids containing the insert were screened with BamHI, EcoRI, and HindIII digestion and separation of DNA fragments by electrophoresis on 0.7% agarose gel. A P1 clone containing the modified 12E insert with the deleted HCR.2 sequence was denoted p1.198–HCR.2. Plasmid DNA was digested with KpnI, and the genomic 198.KK fragment was isolated for microinjection after pulsed field gel electrophoresis.

This RARE cleavage procedure was repeated to delete the HCR.2 sequence from the P1 clone already lacking HCR.1 (p1.198–HCR.1) to obtain a P1 clone that lacked both the HCR.1 and HCR.2 sequences, referred to as p1.198–HCR.1&2. A genomic KpnI fragment, 198–HCR.1&2.KK, was prepared for microinjection after isolation by pulsed field gel electrophoresis.

Generation of Transgenic Mice—Transgenic animals were generated as described (3), using the inbred FVB/N strain. Constructs CII/H3 and HCR.1.CII/H3 (2–3 ng/μl) were microinjected in 10 nl Tris-HCl, pH 7.4, 0.1 mM EDTA containing 30 μM spermine and 70 μM spermidine. Human genomic fragments were used directly for microinjection.

RESULTS

RNA Preparation and RNase Protection Analysis—Total RNA from tissues of F1 transgenic mice and from cultured HepG2 cells was isolated using guanidine thiocyanate (32). Tissue RNA was obtained from at least two independent transgenic lines of each construct. Anti-sense RNA probes for human apoE and apoC-1 mRNA were transcribed as described (25), as were probes for apoC-II and apoC-IV mRNA using [α-32P]UTP (800 Ci/mmol, NEN Life Science Products) in the presence of T3 or T7 RNA polymerase (Stratagene, San Diego). RNase protection analysis was performed as described (33), using 5 μg of total cellular RNA/sample. Protected fragments were resolved by electrophoresis in 6% polyacrylamide gels containing 7 M urea and detected by autoradiography of the dried gels. A mouse actin mRNA probe (pTRI-actin, Ambion) was used in this assay to confirm that equivalent levels of RNA were present in each tissue sample. Relative levels of hepatic expression (per transgene copy number) of human apoC-II mRNA in different transgenic lines were quantitated with a Fujix Bas1000 Bio-imaging Analyzer (Fuji Photo Co., Ltd., Japan). Normal FVB/N mouse genomic DNA was supplemented with known amounts of a genomic fragment containing the human apoC-II gene and used as standard. A mouse actin probe (pTRI-actin, Ambion) was used to compare the relative amounts of mouse genomic DNA added to each well. To determine that no major rearrangements or deletions had occurred after the integration of human DNA into the mouse genome, tail DNA was digested with restriction enzymes and analyzed by Southern blot analysis with probes specific for human apoC-I, apoC-II, apoC-IV, or apoC as described (3).

Transgenic Mice Generated with Genomic Fragments Containing the Human ApoC-IV/C-II Gene Cluster—Recent studies showed that expression of the human apoE and apoC-I
Fig. 1. Expression of human apoC-IV and apoC-II mRNA in transgenic mice. Transgenic animals were generated with the human genomic constructs CII.H3, HCR.1.CII.H3, or 199.MM. Total RNA (5 μg) from each tissue was analyzed by RNase protection, with protected fragments resolved by electrophoresis in 6% polyacrylamide gels containing 7 M urea. Autoradiograms of the dried gels show that human apoC-II and apoC-IV mRNA were not found in mouse liver. Small amounts are seen in brain and lung. Analysis of mouse actin mRNA confirmed that equivalent levels of RNA were present in each tissue sample of each transgenic line. The human probes do not cross-react with mouse apoC-II or apoC-IV mRNA in this assay.

genes in the liver was not specified by their proximal promoter sequences; expression in the liver required a distal sequence, referred to as the hepatic control region or HCR.1 (23). To determine if the downstream human apoC-IV and apoC-II genes also required distal elements for hepatic expression as do apoE and apoC-I, transgenic mice were generated using CII.H3, a 13-kb genomic fragment that contained the entire human apoC-II (28) and apoC-IV genes (3). Independent lines of CII.H3 transgenic mice were examined for human apoC-II and apoC-IV mRNA expression in the liver and five other tissues. Human apoC-IV and apoC-II mRNA were not detected by RNase protection analysis in the liver, although trace levels of human apoC-II expression were present in the brain and spleen (Fig. 1).

To determine if the HCR.1 sequence could confer hepatic expression of the human apoC-IV/C-II genes in transgenic mice, a 774-bp fragment containing HCR.1 (23) was ligated to CII.H3. Transgenic mice generated with this construct expressed human apoC-II mRNA in the liver (Fig. 1) and human apoC-IV mRNA in the livers with lower levels of human apoC-IV mRNA in four other tissues examined (Fig. 1). Thus, the HCR.1 sequence was able to direct hepatic expression of both the apoC-IV and apoC-II genes at relative levels that appeared to reflect their expression in vivo (3). The extrahepatic expression of the apoC-IV/C-II genes suggests that the 774-bp HCR.1-containing fragment conferred low levels of transgene transcription in other tissues. Whether this extrahepatic expression involves specific regulation by elements within the functional 319-bp HCR domain or is the result of activation of other distinct elements within the construct has yet to be determined.

In the human apoE/C-I/C-IV/C-II gene cluster, the HCR.2 domain is located just –6 kb upstream of the apoC-IV gene (27). HCR.2 directed high level, liver-specific expression of a human apoE gene construct in transgenic mice that was equivalent to the activity of HCR-1 (27). However, the proximity of HCR.2 to the apoC-IV and apoC-II genes suggested that it might be more important for the hepatic expression of these genes. To investigate this possibility, a 43-kb genomic fragment generated by MluI digestion containing the human apoC-IV/ C-II gene cluster and HCR.2 sequence, but not the HCR.1 sequence, was isolated from the 5’ end of the P1 clone p1.199. Southern blot analysis of p1.199 DNA showed that no major gene rearrangements or deletions were present in the human genomic insert; human apoC-II and HCR.2 probes identified genomic fragments of p1.199 that were equivalent to genomic fragments of the recently characterized p1.198 clone (3), as shown in Fig. 2. Nucleotide sequence analysis revealed that the 5’ end of the p1.199 genomic insert terminated at a Sau3AI site located 5.26 kb upstream of HCR.2 and within the third intron of the apoC-I’ pseudogene (data not shown). The 43-kb MluI fragment of p1.199 (199.MM) contained the human apoC-IV/ C-II genes and extended ~20 kb downstream of the apoC-II gene. Transgenic mice generated with the 199.MM genomic fragment were examined for expression of human apoC-II and apoC-IV mRNA in six different tissues. RNase protection analysis of independent lines showed that human apoC-II and apoC-IV mRNA were expressed in the liver (Fig. 1), demonstrating that the HCR.2 sequence can direct hepatic expression of both apolipoprotein genes.

Recent work showed that apoC-IV mRNA was present in the livers of humans, mice, and rabbits but not in the intestine of humans (18), the intestine, kidney, spleen, brain, lung, heart, and testis of mice (34), or the intestine, heart, kidney, brain, or skeletal muscle of rabbits (19). In agreement with these studies, the apoC-IV construct 199.MM was expressed only in the liver. Other experiments showed that the human and rat apoC-II genes are expressed primarily in the liver and at low levels in enterocytes of the small intestine (35–37). We found that human apoC-II mRNA was detected at trace levels in the intestine of 199.MM transgenic mice but not in the other tissues examined. The lack of extrahepatic expression of human apoC-IV and apoC-II mRNAs in 199.MM animals also was consistent with the expression of both genes in transgenic mice prepared with the larger 198.KK fragment, as described below. By comparison, HCR.1.CII.H3 mice that contained only HCR.1 expressed the human apoC-IV transgene at higher than expected levels in nonhepatic tissues. Considered together, these observations suggest that the HCR.2 region may normally dominate the transcriptional regulation of the closely linked apoC-IV/C-II genes.

The above findings raised the possibility that ligation of distal regulatory domains to the relatively small genomic fragments may result in artifactual expression of transgenes (i.e., the HCR.1.CII.H3 construct in Fig. 1). Therefore, a 70-kb genomic KpnI fragment of the p1.198 clone (198.KK) that included the entire apoE gene cluster (Fig. 2) was used to generate transgenic mice. Analysis of mouse genomic DNA by Southern blotting and PCR screening indicated that all four transgenes were present in 198.KK animals (Fig. 2), and no rearrangements or deletions of the human apoE gene cluster were detected by detailed restriction endonuclease mapping (data not shown).

RNase protection analysis detected human apoE, apoC-I, apoC-IV, and apoC-II mRNAs in the livers of 198.KK mice (Fig. 3). The expression patterns of the four apolipoprotein transgenes in the different tissues examined were consistent among independent lines of 198.KK mice. In addition, the relative expressions of human apoC-IV and apoC-II mRNAs in transgenic 198.KK mouse livers were similar to that previously
observed in human liver (3) with apoC-IV being expressed at relatively low levels. Thus, the 198.KK genomic fragment contained all of the necessary sequences for the hepatic expression of the complete human apoE/C-II gene cluster.

Transgenic Mice Generated with p1.198 DNA Modified by RARE Cleavage—To investigate the possibility that HCR.1 and HCR.2 may contribute individually to the hepatic expression of the entire gene cluster, we prepared additional DNA constructs by using RARE cleavage to delete each HCR sequence of the p1.198 genomic insert. Deletion of HCR.1 and HCR.2 was confirmed by analysis of restriction endonuclease fragments by agarose gel electrophoresis and Southern blotting (Fig. 2) as well as by PCR (data not shown). Human genomic KpnI fragments lacking HCR.1 (198–HCR.1.KK), HCR.2 (198–HCR.2.KK), or both HCRs (198–HCR.1&2.KK) were used to generate transgenic mice.

Independent transgenic 198–HCR.1.KK lines that contained HCR.2 and lacked HCR.1 expressed human apoE, apoC-I, apoC-IV, and apoC-II mRNAs in the liver (Fig. 3). Thus, the deletion of HCR.1 did not abolish the liver-specific expression of any of the four apolipoprotein genes. These findings indicate that the HCR.2 sequence is capable of directing the hepatic transcription of all four genes at this cluster and suggest that the HCR.1 sequence is not an absolute requirement for the liver-specific expression of any of the genes. The relative levels of human apoC-II mRNA in the livers of 198.KK or 198–HCR.2.KK transgenic mice were determined by RNase protection assay and scanning densitometry (Table I). Compared with the apoC-II expression levels of 198.KK mice, the 198–HCR.1.KK mice had consistently lower levels of hepatic apoC-II mRNA. Based on the number of hepatic enhancers per transgene, a lower hepatic apoC-II mRNA level would be expected for 198–HCR.1.KK animals; the 198–HCR.1&2.KK mice have just one HCR (HCR.2) domain to direct the liver expression of the four genes, whereas the 198.KK animals have two HCR domains to direct liver expression of the four genes.

Transgenic mice generated with 198–HCR.2.KK that contained HCR.1 but lacked HCR.2 expressed human apoE, apoC-I, apoC-IV, and apoC-II mRNAs in the liver (Fig. 3). Thus, the deletion of HCR.2 did not selectively eliminate the hepatic expression of any apolipoprotein gene in this cluster. Therefore, either HCR.1 or HCR.2 can direct the liver-specific expression of the entire human apoE gene cluster. It is noteworthy that,
Two Hepatic Enhancers Control the ApoE Gene Cluster

TABLE I
Comparison of the relative expression levels of human apoC-II mRNA (per transgene copy no.) in the livers of independent lines of transgenic mice

| Construct and line | Copy no. | Human apoC-II mRNA/copy no. |
|-------------------|----------|-----------------------------|
| 199.MM            | 2        | 155.3                       |
| 2708              |          |                             |
| 2750              | 3        | 135.0                       |
| 198.KK            | 4        |                              |
| 4091              | 10       | 96.4                        |
| 4092              | 1        | 202.5                       |
| 4470              | 10       | 100.0                       |
| 198–HCR.1.KK      | 11       | 40.0                        |
| 2908              |          |                             |
| 2952              | 11       | 79.3                        |
| 2990              | 4        | 38.0                        |
| 198–HCR.2.KK      | 2        | 31.1                        |
| 3280              |          |                             |
| 3283              | 53       | 54.9                        |
| 3290              | 21       | 76.3                        |

Expression levels of human apoC-II mRNA were determined by RNase protection analysis as described under “Experimental Procedures.” ApoC-II mRNA expression values are indicated relative to the hepatic apoC-II mRNA levels of 198.KK animals (line 447), arbitrarily set to 100 for comparison. Transgenic copy numbers were determined by slot-blot analysis of mouse genomic tail DNA, as described under “Experimental Procedures.”

We have shown that two cis-acting liver enhancers, HCR.1 and HCR.2, direct the hepatic expression of the entire human apoE/C-I/C-IV/C-II gene cluster. Our results demonstrate that each HCR can individually coordinate the hepatic expression of all four apolipoprotein genes, demonstrating that the liver expression of this gene cluster is more complicated than believed previously (40). The ability of both enhancers to coordinate the simultaneous expression of all four apolipoprotein genes provides a unique model to investigate gene regulation at a multigene cluster.

Transgenic animals were prepared specifically to determine the liver expression requirements of the recently described human apoE/C-I/C-IV/C-II gene cluster. Our results demonstrate that each HCR can individually coordinate the hepatic expression of all four apolipoprotein genes, demonstrating the ability of both enhancers to coordinate the simultaneous expression of all four apolipoprotein genes in vivo.

In contrast, in vitro studies using artificial promoter-reporter gene constructs showed that the apoC-II gene promoter directed transcription in cultured hepatoma cell lines (40, 41). This in vitro apoC-II promoter-driven expression may reflect the use of highly sensitive reporter gene constructs or an abnormality associated with transformed cell lines. Our previous studies showed that the expression of transfected human apoE and apoC-I genes in cultured hepatoma cells was highly variable between cell lines (42). In addition, genomic constructs that directed apoE gene expression in cultured hepatoma cells were not expressed in vivo in the livers of transgenic mice (42, 43).

The human apoE gene cluster contains two evolutionarily related hepatic enhancers, HCR.1 (23) and HCR.2 (27), both of which direct the liver-specific expression of the human apoE gene in transgenic mice. We now show that the HCR.1 sequence is also capable of directing expression of the downstream human apoC-IV and apoC-II genes in the livers of transgenic mice. The ability of HCR.1 to confer liver transcription of the human apoC-II gene is consistent with recent in vitro studies using artificial promoter-reporter gene constructs (40, 41), which indicated that HCR.1 can enhance transcription of the isolated apoC-II gene promoter. Our current work further demonstrates that human apoC-IV/C-II genomic frag-

2 C. M. Allan, and I. M. Taylor, manuscript in preparation.
ments containing HCR.2, in the absence of HCR.1, are expressed in the livers of transgenic mice. Therefore, either HCR.1 or HCR.2 appears to be able to interact with the proximal promoters of the human apoC-IV and apoC-II genes to coordinate hepatic transcription.

To extend the above observations, we prepared a 70-kb genomic fragment (198.KK) that contained all the necessary sequences to direct liver expression of the complete human apoE gene cluster. In addition to directing liver expression, the nonhepatic expression of all four transgenes in 198.KK mice was consistent with the previously reported endogenous expression of each gene. Human apoE mRNA was detected in the kidney, spleen, brain, and lung (Fig. 3), as well as in the adipose tissue, skin, and testis of transgenic mice (data not shown). The relative level of apoE mRNA in the kidney was higher than expected (20, 44); however, our previous work showed that kidney expression of human apoE transgenes can be influenced by multiple silencing elements located both upstream and downstream of the apoE gene (43, 45, 46). It is possible that the 198.KK fragment lacked a far distal kidney silencing element, resulting in increased levels of kidney apoE expression. Human apoC-I mRNA detected in the spleen, brain, and lung of transgenic mice may reflect the presence of resident macrophages in these tissues since macrophages synthesize both human apoE and apoC-I (4, 38). There was little expression of the apoC-IV and apoC-II genes in nonhepatic tissues of the 198.KK transgenic mice, consistent with earlier studies that showed that both genes are expressed primarily in the liver (19, 36, 37).

The RARE cleavage technique was used to remove selectively the HCR.1 or HCR.2 sequence of the 198.KK fragment. Analysis of transgene expression in fragments lacking HCR.1 or HCR.2 revealed that either HCR sequence individually could coordinate the hepatic expression of the entire apoE gene cluster. Furthermore, the deletion of both HCR.1 and HCR.2 virtually abolished the hepatic expression of all four apolipoprotein genes. Thus, this study provides strong evidence that the two HCR domains are the only liver enhancers in the 198.KK fragment and further demonstrates that the presence of at least one HCR is essential for the liver-specific expression of these genes. Previous studies reported that no liver enhancers were located within 30 kb upstream of the apoE gene (23), and the present work indicates that no liver enhancer lies within 18 kb downstream of the apoC-II gene. Thus, the two HCR domains located in a 92-kb genomic segment containing all known genes of the human apoE gene cluster appear to confer liver-specific control of the entire cluster.

Although either HCR could individually direct the hepatic expression of the complete gene cluster, the nonhepatic expression patterns of the apoC-IV and apoC-II genes in different transgenic lines suggested that the HCR.2 domain was more likely to regulate the transcription of these two genes in particular. In the presence of HCR.2, human apoC-IV and apoC-II mRNA were detected, as expected, mainly in the liver with little or no hepatic expression. However, under the control of HCR.1 alone, the apoC-IV and apoC-II genes were expressed at significant levels in nonhepatic tissues. A 774-bp, HCR.1-containing fragment used to prepare the HCR.1.CII.H3 construct appeared to contain undefined elements that directed hepatic expression of both apoC-IV and apoC-II genes. It remains to be determined if these extrahepatic elements are found in the functional 319-bp HCR.1 domain (24) or represent distinct, closely linked elements. The HCR.2 region does not appear to have these extrahepatic elements, since HCR.2 alone does not direct apoC-IV and apoC-II transgene expression in nonhepatic tissues. In the presence of both HCR.1 and HCR.2, apoC-IV and apoC-II transgene expression in the six tissues examined was found to resemble the correct expression patterns observed when the transgenes were directed by HCR.2 alone. Therefore, transcriptional regulation of the apoC-IV and apoC-II genes appeared to be dominated by the HCR.2 domain. The ability of HCR.2 to control this downstream portion of the cluster may simply reflect its proximity to the apoC-IV and apoC-II genes. The presence of HCR.2 may also insulate their promoters from upstream distal elements that direct extrahepatic expression. Thus, the deletion of HCR.2 may have enabled these distal elements, located in or near the HCR.1 domain, to direct apoC-IV and apoC-II transgene expression in nonhepatic tissues, as was observed in 198–HCR.2.KK mice (Fig. 3). Similarly, the proximity of the HCR.1 domain to the upstream apoE and apoC-I genes may allow it to dominate the transcription of these genes: they are normally expressed at higher levels in nonhepatic tissues relative to the apoC-IV and apoC-II genes.

The demonstration of two functional and evolutionarily related hepatic enhancers in this human cluster that account for the complete liver-specific activity of the cluster provides a unique model to investigate further the regulation of gene expression at a complex multigene cluster. Both HCR domains coordinate the simultaneous expression of all four apolipoprotein genes in the liver, which is unlike the temporal expression pattern of other well characterized gene clusters, such as the developmental stage-specific liver expression of the albumin gene cluster (47, 48) or the β-globin genes in erythrocytes (49, 50). In addition, the human apoE/C-I/C-IV/C-II gene cluster has an absolute requirement for these distal enhancers for any detectable liver-specific expression. The proximal promoter elements of all four apolipoprotein genes do not direct hepatocyte expression without the presence of an HCR domain. In contrast, the promoters of the liver-specific transthyretin gene (51) and of the albumin and α-fetoprotein genes (52, 53) can direct low level expression even in the absence of known enhancer sequences. Another noteworthy feature of the human apoE gene cluster is the proposed duplication event that gave rise to two copies of the HCR sequence (27) and the apoC-I gene (4). The duplication of a ~10-kb fragment containing the ancestral HCR and apoC-I gene is estimated to have occurred 39 million years ago, early in the primate lineage (8, 9). Consistent with this model, the corresponding apoE gene clusters of the mouse (54) and rat (55) lack the equivalent of the human apoC-I pseudogene. Thus, the presence of two functional HCR domains is likely to be a unique characteristic of the primate apoE gene cluster, and the corresponding apoE gene cluster of nonprimate species is likely to be controlled by a single HCR domain.

In summary, our present work demonstrates that either HCR.1 or HCR.2 can direct liver expression of the entire human apoE gene cluster and that the presence of at least one HCR is essential for any significant liver expression. The apoE/C-I/C-IV/C-II gene cluster constitutes a unique model for understanding how distal enhancers mediate gene expression in the liver.

Acknowledgments—We thank Dr. Stephen Young for help with purification of large genomic fragments from P1 clones and Dr. Jan Borén for help with the RARE technology. Thanks also to John Carroll and Amy Corder for assistance with graphics, Gary Howard and Stephen Ordway for editorial assistance, and Angela Chen for manuscript preparation.

REFERENCES
1. Sorci-Thomas, M., Wilson, M. D., Johnson, F. L., Williams, D. L., and Rudel, L. L. (1989) J. Biol. Chem. 264, 9039–9045
2. Myklebost, O., and Rogne, S. (1988) Hum. Genet. 78, 244–247
3. Allan, C. M., Walker, D., Segrest, J. P., and Taylor, J. M. (1995) Genomics 28, 291–300
4. Lauer, S. J., Walker, D., Elsheibagy, N. A., Reardon, C. A., Levy-Wilson, B.,
Two Hepatic Enhancers Control the ApoE Gene Cluster

29119

5. Li, W.-H., Tanimura, M., Luo, C.-C., Datta, S., and Chan, L. (1988) J. Biol. Chem. 263, 7277–7286

10. Mahley, R. W. (1988) Science 240, 255–257

16. Sehayek, E., and Eisenberg, S. (1991) J. Biol. Chem. 266, 12127–12134

15. Breckenridge, W. C., Little, J. A., Steiner, G., Chow, A., and Poapst, M. (1978) J. Biol. Chem. 253, 2565–2571

28. Wei, C.-F., Tsao, Y.-K., Robberson, D. L., Gotto, A. M., Jr., Brown, K., and Wang, K. (1995) J. Clin. Invest. 96, 2245–2250

32. MacDonald, R. J., Swift, G. H., Przybyla, A. E., and Chirgwin, J. M. (1987) Methods Enzymol. 152, 219–227

33. Gilman, M. (1995) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 4.7.1–4.7.8, John Wiley & Sons, New York

54. Hoffer, M. J. V., Hofker, M. H., van Eck, M. M., Havekes, L. M., and Frants, R. R. (1995) Genomics 23, 110–115

55. Shen, P., and Howlett, G. J. (1992) Arch. Biochem. Biophys. 297, 345–353