A Far-downstream Hepatocyte-specific Control Region Directs Expression of the Linked Human Apolipoprotein E and C-I Genes in Transgenic Mice*

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The human apolipoprotein (apo) E and apoC-I genes are located 5 kilobases apart in the same transcriptional orientation on chromosome 19, and they are expressed at high levels in the liver with lower levels of expression in selected other tissues. Analysis of a series of overlapping human apoE and apoC-I genomic fragments in transgenic mice revealed that the expression of these transgenes in the liver requires a common cis-acting regulatory domain. This hepatic control region (HCR) was localized to a 764-base pair region that is located about 18 kilobases downstream of the apoE promoter and about 9 kilobases downstream of the apoC-I promoter. All the transgenic animals that had been prepared with a construct that contained this region had relatively high levels of transgene expression in the liver, whereas constructs that lacked this region showed no expression in the liver. In situ hybridization studies showed that the HCR directed apoE and apoC-I transgene expression in hepatocytes. When the HCR from the apoE/C-I gene locus was ligated proximal to a human apoA-IV gene fragment, which is not normally expressed in the liver, the resulting apoA-IV/HCR fusion construct was expressed at high levels in the liver, indicating that the HCR could direct high level liver expression of a heterologous promoter/gene construct. Expression of the apoE transgene in the liver and kidney, and perhaps other tissues, required the presence of a nonspecific proximal enhancer element in the apolipoprotein E gene promoter, located between 161 and 141 bp relative to the transcription initiation site. However, the proximal apoE gene promoter, including this enhancer element, contained no sequences capable of directing hepatocyte expression in the absence of the HCR. Thus, the far-downstream HCR appears to contain all of the sequences necessary for determining high level liver-specific gene expression.

Apolipoprotein (apo) E is a structural component of several classes of mammalian lipoproteins, including chylomicron remnants, very low density lipoproteins (VLDL), and high density lipoproteins (1, 2). Human apoE is a single-chain polypeptide of 299 amino acids (Mr = 34,200) (3). Sialylation at threonine-194 accounts for a size polymorphism characteristic of approximately 20% of circulating apoE in human plasma (4, 5).

Apolipoprotein E has a major role in the redistribution of cholesterol and other lipids between peripheral tissues and the liver. It mediates the cellular uptake of specific lipoproteins through its interaction with the low density lipoprotein receptor and the low density lipoprotein receptor-related protein (2, 6). Through its interaction with these receptors, apoE mediates the clearance of chylomicron and VLDL remnant particles from the plasma. Certain natural variants of apoE that are defective in receptor binding are associated with type III hyperlipoproteinemia, a clinical disorder characterized by the accumulation of abnormal cholesteryl ester-rich remnant particles (known as β-VLDL) in plasma (2).

Apolipoprotein C-I is a 6.6-kDa protein, and it is a structural component of triglyceride-rich chylomicron and VLDL remnants as well as high density lipoprotein particles (1). The role that apoC-I plays in lipoprotein metabolism has been more obscure, although it can activate lecithin:cholesterol acyltransferase. Recent in vitro evidence suggests that apoC-I inhibits the apoE-mediated binding of β-VLDL to the low density lipoprotein receptor-related protein (7, 8), presumably by displacing apoE from the β-VLDL, although the mechanism of inhibition in vivo is unclear. Thus, it appears that apoE and apoC-I exert opposing effects on the metabolism of triglyceride-rich lipoproteins.

The human apoE and apoC-I genes are linked closely on chromosome 19, where they are separated by 5.3 kb of intergenic DNA (9). An apoC-I' pseudogene is located 7.5 kb downstream of the apoC-I gene (9). All three genes are in the same transcriptional orientation. The highest levels of apoE and apoC-I gene expression occur in the liver (10–14), which accounts for as much as 90% of their circulating levels in the plasma (15). The apoE gene is expressed at lower levels in many tissues (16, 17), with moderate expression in the skin and brain, and lower expression in several other tissues, including the kidney. In comparison, the apoC-I gene is expressed at moderate levels in the skin, with trace levels or no expression in the brain and other tissues.

Previously, we compared several lines of transgenic mice that were prepared with a series of overlapping gene fragments spanning over 50 kb of the human apoE/C-I gene locus (18). These studies revealed that the expression of the apoE and apoC-I genes is controlled by an array of tissue-specific cis-acting regulatory elements that are distributed over a 20-kb interval, including a liver-specific control region that is present in the apoE promoter and is required for determining high level liver-specific gene expression.
region spanning both of these apolipoprotein genes (17, 18).

We now show that a common far-downstream regulatory region is required for expression of both the human apoE and apoC-I genes in the liver. This liver control region, which directs transgene expression to hepatocytes, is capable of directing liver expression of a heterologous promoter construct, and its presence is essential for high level expression of the two upstream apolipoprotein genes in the liver. In addition, our results indicate that a proximal enhancer element of the apoE promoter, that does not confer cell-type specificity, is required for tissue-specific elements to direct expression of the apoE gene in the liver, kidney, and perhaps in some other tissues.

EXPERIMENTAL PROCEDURES

Preparation and Analysis of Transgenic Mice—DNA fragments for microinjection were purified by restriction endonuclease digestion, agarose gel electrophoresis, phenol extraction, and ethanol precipitation, and then diluted to 1 μg/ml in 5 mM Tris, pH 7.4, 0.2 mM EDTA (19). Single-cell embryos from ICR × ICR-bred mice were microinjected essentially as described (20), except that injection needles were beveled and siliconized before use. The embryos surviving microinjection were transferred to the oviducts of pseudopregnant ICR female mice. Offspring were weaned at 3 weeks of age, and DNA was prepared from a 1-cm portion of the tails (20). Transgenic founder (F0) animals, averaging 30% of the littermates derived from implanted embryos, were identified by Southern blot analysis (21) and mated with nontransgenic ICR mice to generate transgenic F1 progeny. Southern blot analysis indicated that the number of integrated copies of the transgene varied between 1 and 90. Additional Southern blot mapping confirmed the absence of any gross rearrangements in any of the gene constructs transferred to the transgenic mice. The animals used for all RNA and protein analyses were F1 transgenic heterozygous males between 6 and 10 weeks of age.

Preparation and Analysis of Total RNA—Total cellular RNA was isolated as described (22). Antisense RNA probes for human apoE, apoC-I, apoA-IV, or tubulin mRNAs were transcribed using T3 RNA polymerase in the presence of [32P]UTP (23). Of the various fragments from the apoC-I/C-I' intergenic region, which were microinjected in the same HEGl transgenic mouse line (described under "Results") was included in each gel as a standard for comparisons between autoradiograms. The expression of endogenous mouse apoE, apoC-I, or tubulin RNA was monitored for each tissue sample by RNase protection analysis. For all constructs, from two to four independent lines of transgenic mice were analyzed, and expression patterns were consistent within each line for each construct. Immunoblot analysis (18) of plasma from all transgenic mice served with apoE gene constructs that had 5 kb or 30 kb of the 5'-flanking sequence and the same length of the 3'-flanking sequence (18). These results suggest that kidney silencer activity may be present in the larger CI.361 construct. Subsequent experiments (described below) support this possibility, with potential silencing activity located near the hepatic control region as well as elsewhere in the apoE/C-I gene locus.

The hepatic control region, as defined by the constructs in Fig. 1, is contained within a 4-kb Sphl-BamHI genomic fragment, consisting of 3.2 kb of the 5'-flanking sequence upstream of the apoC-I gene. To localize further the regulatory sequences that control liver-specific expression of the apoE gene, seven different fragments from the hepatic control region were ligated downstream of the apoE gene, and these constructs were analyzed in transgenic mice (Fig. 2). This 10-kb apoE genomic fragment (HEGl) had been shown previously to be expressed at high levels in the kidney and low levels in the testis, with no detectable expression in the liver (18). Of the various fragments from the apoC-1/C-1' intergenic region that were ligated to the 3' end of the HEGl construct, three of the resultant constructs (LE1, LE2, and LE6) were expressed in the transgenic mouse liver (Fig. 2). These transgene constructs demonstrated that the sequences controlling liver expression of the apoE gene were contained within a 764-bp PstI-XbaI fragment (LE6) located approximately 2 kb upstream of the apoC-1' pseudogene. Neither of the neighboring DNA fragments (LE5 and LE7) directed liver expression of the apoE gene in transgenic mice.

The levels of expression of the seven fusion constructs in the kidney varied markedly, depending on which DNA fragment from the hepatic control region was used (Fig. 2). Expression with the LE7 fragment closely resembled that of
A hepatic control region of the ApoE/C-I gene locus

**Fig. 1.** A distal, common regulatory element controls liver expression of the human apoE and apoC-I genes in transgenic mice. Upper panel, apolipoprotein E and C-I gene constructs used to produce transgenic mice. The relative locations and intergenic distances (in base pairs) between the apoE gene, apoC-I gene, and apoC-I' pseudogene are illustrated in the CI.361 construct. The exons of each gene are indicated by the solid boxes, and the introns are indicated by Roman numerals. Negative numbers indicate the approximate length of the 5'-flanking sequence adjacent to the first exon. Positive numbers indicate the length of the 3'-flanking sequence adjacent to the fourth exon. The CI.361 construct is derived from the human genomic insert of a cloned cosmid (9). The HESS1 construct is an SphI fragment from CI.361 DNA. To make HECI1, a SphI-SalI fragment containing the apoE gene was ligated to a SalI-BamHI fragment with the apoC-I gene. The CLSSp fragment was prepared by complete digestion of CI.361 with SspI followed by partial digestion with SphI. The CLSE construct is a SalI-EcoRI fragment of CI.361 DNA. Lower panel, representative RNase protection analysis of human apoE and apoC-I mRNA in various tissues of transgenic mice are shown for each construct. As indicated under “Experimental Procedures,” the same amount of total tissue RNA was examined in every case, and similar autoradiogram exposures are shown here. Northern blot assays (19) performed as additional controls for RNA integrity showed endogenous mouse apoE mRNA having the predicted size in the livers of HESS1 and HECI1 transgenic mice and apoC-I mRNA of predicted size in the livers of CLSSp transgenic mice (data not shown). Transgenic human apoE mRNA and apoC-I mRNA in livers of CI.361 transgenic mice and transgenic human apoE mRNA in the kidneys of HESS1 transgenic mice were shown to have the predicted size by Northern blot analysis (data not shown). The results for constructs CI.361 and CI.SE have been published previously (10), but they are included here for convenience in identifying the location of the hepatic control region.

The parent HEG1 construct (18). A 3.8-kb SphI-BamHI fragment (LE1) that directed high level liver expression resulted in complete silencing of kidney expression. In contrast, a 1.7-kb PstI-PstI subfragment (LE2) of the larger 3.8-kb fragment directed high liver expression as well as kidney expression. These results suggest that the large LE1 fragment contains sequences that reduce or silence expression in the kidney. Apparent kidney silencer activity also was found upstream of the hepatic control region within the 2.1-kb BamHI-SphI fragment (LE3), and reduced kidney expression was observed with the LE5 fragment.

To identify the cell types within the liver and kidney that expressed the apoE transgenes, cross-sections of tissues from selected transgenic mice were analyzed by in situ hybridization. Analysis of liver cross-sections from an F1 transgenic mouse harboring the CI.361 cosmid construct revealed that the human apoE transgene was expressed in hepatocytes (Fig. 3, top left). In contrast to the endogenous mouse apoE gene, which exhibited a more uniform level of expression throughout all hepatocytes (Fig. 3, bottom left), the human apoE transgene was expressed at widely variable levels in different hepatocytes, resulting in a "patchy" appearance. The human apoC-I transgene showed a similar pattern of expression, and comparable results were obtained when F1 transgenic mice containing different liver-expressing constructs (Fig. 2) were analyzed (data not shown). Because the downstream liver control region directs expression to hepatocytes, we have designated it the hepatocyte control region, or HCR.

Analysis of kidney cross-sections from an F1 HEG1 transgenic mouse by in situ hybridization revealed that the human apoE transgene was expressed in epithelial cells of the proximal convoluted tubules and Bowman's capsule (Fig. 3, top left).
The endogenous mouse apoE gene was not detected in the kidney by in situ hybridization (Fig. 3, bottom right) probably because of its relatively low expression levels (17). In this regard, a previous report indicated that baboon apoE was expressed in low amounts in kidney proximal tubule epithelia (12).

The Hepatocyte Control Region Directs Liver Expression from a Heterologous Promoter—The ability of the HCR to direct liver expression of a human apoA-IV promoter/gene construct was investigated. The human apoA-IV gene was chosen as a reporter construct because it is not normally expressed in the liver (24). The only tissue in which the human apoA-IV gene is expressed in man is the small intestine (24), and normal expression of this gene in the small intestine of transgenic mice requires an intestinal control element located about 5 kb upstream of the gene (25).

A human apoA-IV genomic fragment with 2.4 kb of the 5′-flanking sequence and 1.7 kb of the 3′-flanking sequence was employed as a reporter gene because it was not expressed in the liver of transgenic mice (Fig. 4) (25). When the 1.7-kb PstI-PstI fragment (LE2) from the apoE/C-I HCR was ligated downstream of this apoA-IV genomic fragment, the resulting apoA-IV-HCR fusion transgene was expressed at high levels in the liver of transgenic mice (Fig. 4). Thus, the HCR can direct hepatic expression via a heterologous promoter, confirming that the HCR contains all necessary sequence information to specify gene expression to the liver.

**A Proximal Enhancer Element of the Apolipoprotein E Promoter Is Required for High Level Expression in the Liver and Other Tissues**—The distal HCR may direct transcription of the apoE and apoC-I genes by interacting directly with regulatory elements in the promoters of each gene. Therefore, the potential activities of three known transcriptional control elements in the proximal promoter of the apoE gene (26, 27) were examined for their ability to modulate the action of the HCR. These proximal promoter elements had been demonstrated previously to be required for optimum transcriptional activity of the apoE promoter by means of a cell-free transcription assay (26). An element (known as PET) located at −161 to −141 bp relative to the transcription start site acted as a nonspecific enhancer in vitro, and it was the most potent of the three elements in stimulating transcription rates. The
other two elements, located at -101 to -89 bp and -59 to
-45 bp, had no enhancer activity, and they were less effective
in stimulating transcription rates.

Fig. 5 shows the consequence of deleting proximal promoter
regulatory elements on the expression of the apoE HEG1LE1
construct in transgenic mice, which typically was expressed
at high levels exclusively in the liver (Fig. 2). Deletion of the
regions from −59 to −45 bp (HEGD4LE1) or −101 to −89 bp
(HEGD5LE1) had no significant effect on the high amount
of apoE expression in the liver, but permitted moderate to
high expression in the kidney and low to moderate expression
in some other tissues (Fig. 5). Deletion of the PET element
located between −161 and −141 bp (HEGD1LE1) resulted in
a near extinction of apoE training expression in the liver and
essentially no expression in other tissues. The low levels of
expression observed for HEGD1LE1 that are shown in Fig. 5
may be a consequence of the relatively high copy number of
the transgene (see legend to Fig. 5).

The effect of the deletion of promoter regulatory sequences
on the expression of control HEG1 constructs that lacked the
HCR was examined (Fig. 6). Expression of HEG1 occurred
normally at high levels in the kidney and low levels in the
testes (17, 18). When transgene constructs with deletions of
the regions from −59 to −45 bp (HEG1D4) or −101 to −89

Fig. 3. In situ hybridization of transgenic and control mouse tissues. Cross-sections 7 μm thick from the livers of F1 C1.361
transgenic mice, the kidneys of F1 HEG1 transgenic mice, and from both tissues of control mice were examined by in situ hybridization with
antisense RNA probes to human or mouse apoE mRNA. The dark areas show the location of human and mouse apoE mRNA. The three left
panels show liver cross-sections, and the three right panels show kidney cross-sections (both at 125X magnification). The top panels show
transgenic tissue cross-sections probed for human apoE mRNA; middle panels, nontransgenic control mouse tissues probed for human apoE
mRNA; lower panels, nontransgenic control mouse tissues probed for mouse apoE mRNA.
Fig. 4. The hepatocyte control region of the apoE/C-I gene locus directs liver-specific expression from a heterologous promoter. A human apoA-IV genomic fragment was ligated to the 1.7-kb PstI-PstI HCR-containing fragment of the human apoE/C-I gene locus. The control apoA-IV gene fragment and the fusion construct were microinjected into mouse embryos in separate experiments. The resulting transgenic mice were analyzed by RNase protection analysis of RNA prepared from various tissues. Representative results are shown for each construct. RNase protection analysis using a radioactive mouse tubulin antisense probe showed that each tissue examined contained equivalent amounts of RNA for each of the above transgenic lines (data not shown).

Fig. 5. Effect of promoter deletions on expression of the human apoE gene in various tissues of transgenic mice. Specific regions of the apoE promoter, indicated by open rectangles, were deleted from the HEG1LE1 construct. The nucleotides that were deleted are indicated by triangles and negative numbers that denote their positions relative to the start site of transcription. Representative RNase protection analysis results are shown for each construct. The transgenic lines harboring the HEGD4LE1 and HEGD5LE1 constructs contained about 5 and 10 copies, respectively, of the integrated transgene. Of three transgenic lines analyzed for the HEGD1LE1 construct, only the one shown here was expressed. This line contained about 50 copies of the transgene. Two additional transgenic lines harboring 5-10 copies of HEGD1LE1 also were analyzed, and they had no detectable expression in the liver, kidney, and other tissues (data not shown).
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FIG. 6. Effect of promoter deletions on expression of the HEG1 construct. The indicated regions of the apoE promoter were deleted (as described in the legend to Fig. 5) from the HEG1 construct. Representative RNase protection analysis results are shown for each construct following expression in transgenic mice.

CONSTRUCTION

HEG1D4 -5000 \( \Delta -59 / -45 \) I II III +1500

HEG1D5 -101 / -89

HEG1D1 -161 / -141

Liver Testis Skin Submaxillary Kidney Brain Small Intestine Heart Stomach Pancreas

HEG1D4

HEG1D5

HEG1D1

FIG. 7. Expression of human apoE gene constructs with various lengths of the 5'-flanking sequence. Five apoE gene constructs with decreasing lengths of the 5'-flanking sequence were analyzed in transgenic mice. The four constructs with the shortest portions of the 5'-flanking sequence resulted in identical expression patterns, which are shown by the HE43H construct data that are presented here. Representative RNase protection patterns are shown for the HESH1 and HE43H constructs.

DISCUSSION

The distal downstream hepatocyte control region (HCR) of the apoE and apoC-I gene is unusual among known liver regulatory elements in that it is required for detectable levels of expression. The results of the transgenic mouse study (Fig. 7) showed that 650 bp or less of the 5'-flanking sequence was not sufficient to direct expression of the intact apoE gene to the kidney despite the presence of the PET enhancer. Thus, expression of the intact apoE gene in the kidney appears to require a positive regulatory element located between 2000 and 650 bp.

However, constructs containing 650 bp of the 5'-flanking sequence and 1.7 kb of the 3'-flanking sequence, but with only the first intron (HE54H) or the third intron (HE563H) of the apoE gene, were expressed at a high level in the kidney (Fig. 8). This finding suggests that the second intron of the apoE gene may contain an element that silences expression of the apoE gene in the kidney. However, further studies beyond the scope of this manuscript would be required to demonstrate unequivocally that the second intron does contain a specific silencer element. The expression of the HE54H and HE563H constructs indicates that the apparent positive element located between 2000 and 650 bp that was necessary for expression of the intact apoE gene in the kidney is not an absolute requirement for kidney expression. This upstream element may function only to counteract the apparent silencer activity in the second intron.
of expression of both upstream human apolipoprotein genes in
the liver of transgenic mice, and it appears to be the sole
determinant of the expression of both genes in the liver.
There appear to be no proximal promoter elements for the
apoE or apoC-I genes that can direct liver-specific expression
without the presence of the HCR.

Studies of several other mammalian genes in transgenic
mice have revealed that regulatory elements in the proximal
promoter are sufficient to confer liver-specific expression,
although maximal expression requires a distal upstream
enhancer. For example, this arrangement is found for the gene
that encodes transthyretin, where 300 bp of the 5'-flanking
sequence alone results in relatively low levels of expression in
the liver, and high levels of expression require the presence
of an enhancer found about 2 kb upstream (28). The genes
encoding human apoA-I and apoC-III are expressed in the
liver when only 256 and 200 nucleotides of the proximal 5'-
flanking region next to each gene, respectively, is included in
the constructs used to produce transgenic mice (29, 30).

Both promoters of the closely linked albumin and α-feto-
protein genes contain proximal elements that direct low levels
of expression in the liver, but high level expression in the
liver is controlled by downstream enhancer elements that are
located farther upstream of each gene (31, 32). The
liver-specific enhancer for the albumin gene is located
10 kb upstream of its promoter. For the α-fetoprotein gene,
three enhancer elements located between 1 kb and 7 kb
upstream of the transcription start site are required for proper
levels of tissue-specific expression in transgenic mice. In
contrast, neither the apoE promoter nor the apoC-I promoter
appears to contain sequences that are capable of directing
expression in the liver, and the apoE and apoC-I genes share
a common downstream liver control region. Furthermore,
the HCR of the apoE/C-I gene locus directs strong liver-specific
tissue expression from a heterologous promoter, whereas
the albumin enhancer element has been reported to function
efficiently only with the albumin promoter (32).

Examination of independent lines of transgenic mice pre-
pared with 23 different constructs (Figs. 1, 2, 7, and 8 (17)),
that contained the apoE or the apoC-I gene but without the
far-downstream HCR, revealed no detectable expression of the
transgene in the liver. In contrast, transgenic mice made
with 6 different constructs (Figs. 1, 2, and 4) having the HCR
showed relatively high levels of transgene expression in the
liver. Immunoblot analysis of the plasma of 11 independent
lines of liver-expressing transgenic mice that were hetero-
gous for the apoE or apoC-I transgene showed circulating
human apoE or apoC-I at equivalent amounts or up to 5-fold
greater amounts than normal human plasma levels (data not
shown). Together, these findings suggest that the HCR can
direct relatively high levels of gene expression in the liver.

This property is similar to that observed for the β-globin
locus control region (LCR), which directs high level erythroid-
specific expression of the ε, γ, and β-globin genes within the
globin gene cluster on chromosome 11 (33). In addition, the
β-globin LCR functions efficiently outside of its normal gene
 locus, as demonstrated by its ability to direct high level
erythroid-specific expression of the human α-globin gene in
transgenic mice (34). The LCR of the β-globin gene cluster
appears to function by making the chromatin throughout the
β-globin locus more accessible in erythroid cells (35, 33).
Perhaps the HCR of the apoE/C-I gene locus plays a similar
role in hepatocytes, producing an active chromatin configu-
ration in which the absolute level of transcription is deter-

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lished results.
ments that modulate apoE gene (and apoC-I gene) expression may be missing from the constructs used to make the transgenic mice, resulting in a somewhat more patchy appearance than the endogenous apoE gene expression pattern.

In contrast to the defined location of the liver control region, expression of the apoE transgene in the kidney was influenced by a number of different regions of the apoE/C-I gene locus. Several segments of the locus were associated with a silencing of expression of the apoE gene in the kidney. These segments included a region within the second intron of the mouse apoE gene, which suggests that there may be differences in the regulatory control of the human and mouse apolipoprotein genes. In contrast, all constructs that contained the HCR were expressed at high levels in the liver, consistent with the normal expression of the apoE and apoC-I genes in both mouse and man.

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REFERENCES