Multiple Tissue-specific Elements Control the Apolipoprotein E/C-I Gene Locus in Transgenic Mice*

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To investigate the mechanisms controlling tissue-specific expression of the human apolipoprotein (apo) E/C-I gene locus, human apoE and apoC-I gene constructs containing various lengths of the 5'-flanking or 3'-flanking region were used to create transgenic mice. Several essential tissue-specific regulatory elements were identified in the region between the apoE and the apoC-I genes, as well as in a distal domain found downstream of the apoC-I gene. Most notably, transcription of both the apoE and apoC-I genes in the liver, their major site of expression, required downstream regulatory elements, possibly located within a common regulatory domain more than 2 kilobases 3' of the apoC-I gene (about 14 kilobases downstream of the apoE gene promoter). In the region between the apoE and apoC-I genes, a single strong positive element directed apoE and apoC-I gene expression in the skin. The intergenic region also contained elements that stimulated apoE gene expression in the brain and silenced apoE gene expression only in the kidney. These results demonstrate that multiple independent regulatory elements control expression of the human apoE/C-I gene locus in various tissues. Transgenic mice expressing human apoC-I in the liver exhibited plasma triglyceride levels that were 2-3-fold higher than those in control mice, an effect not found when transgenic human apoE was produced. This result suggests that apoC-I may modulate the metabolism of triglyceride-rich lipoproteins.

Apolipoprotein (apo) E is a major structural component of several mammalian lipoproteins, including chylomicron remnants, very low density lipoproteins, and high density lipoproteins (1). Apolipoprotein E plays an important role in cholesterol transport and in chylomicron remnant clearance by the liver. Human apoE is a single-chain protein of 299 amino acids (Mr = 34,200) (2), which is encoded by a gene of 3,597 nucleotides that has 4 exons and 3 introns and is located on chromosome 19 (3, 4). The apoC-I gene (4,653 nucleotides) is located 5.3 kilobases (kb) downstream of the apoE gene, in the same transcriptional orientation (5). An apoC-I' pseudogene (4,387 nucleotides) is located 7.5 kb downstream of the apoC-I gene (5), also in the same transcriptional orientation.

While the expression of most apolipoproteins is restricted primarily to the liver and intestine (6, 7), apoE is expressed in a wide distribution of tissues. The major source of circulating apoE is the hepatocyte (6-8). Apolipoprotein E is also produced by specific cell types in several peripheral tissues (9-15), perhaps reflecting local metabolic requirements. It is a major product of astrocytic glial cells in the central and peripheral nervous systems (9), as well as of terminally differentiated macrophages (10, 11). Apolipoprotein E is also produced by ovarian granulosa cells (12), growth-arrested smooth muscle cells (13), cultured keratinocytes (14), and specific epithelial cells in many tissues (8).

To further our understanding of the mechanisms controlling expression of the human apoE gene in different tissues, the tissue-specific regulatory elements that control expression of the human apoE/C-1/C-I' gene locus were investigated in transgenic mice. In this paper, we report that several independent elements that control expression in various tissues are located downstream of the apoE gene. Tissue-specific elements that determine expression in the liver and the skin appear to control both the apoE and apoC-I genes.

MATERIALS AND METHODS

Preparation and Analysis of Transgenic Mice—DNA fragments intended for microinjection were purified by CsCl gradient centrifugation, 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 (16). Single-cell embryos from ICRxICR-bred mice were microinjected essentially as described (17). The embryos surviving microinjection were transferred to the oviducts of pseudopregnant ICR female mice. Offspring were weaned at 3-4 weeks of age, and DNA was prepared from a 1-cm portion of their tails (17). Transgenic founder (F0) animals, averaging 27% of the offspring derived from implanted embryos, were identified by Southern blot analysis (18) and mated with control mice to generate transgenic F1 progeny. Serial dilutions of transgenic mouse DNA followed by Southern blot analysis indicated that the number of integrated copies of the transgene varied between 1 and 70. Additional Southern blot mapping confirmed the absence of any gross rearrangements in any of the gene constructs transferred to the transgenic mice. Approximately 25% of the founders were mosaic. Therefore, the animals used for all RNA and protein analyses were transgenic F1 males at 6 and 10 weeks of age.

Preparation and Analysis of Total RNA—Total cellular RNA was isolated as described (19). Antisense RNA probes for either human or mouse apoE mRNA or human or mouse apoC-I mRNA were transcribed using T3 RNA polymerase in the presence of [32P]UTP (16) from cDNA fragments that had been cloned in Bluescribe vectors (Stratagene, San Diego, CA). Ribonuclease protection analysis was performed as described (20). Protected fragments were analyzed by electrophoresis in 6% polyacrylamide gels containing 7 M urea, followed by autoradiography of dried gels. Transgenic and control mouse RNAs were analyzed with human and mouse probes in separate assays.

RESULTS AND DISCUSSION

The apoE and apoC-I gene constructs depicted in Fig. 1 were used to prepare transgenic mice as described (17). Total cellular RNA was isolated from 12 tissues of F1 transgenic...
progeny, and apoE mRNA or apoC-I mRNA distribution patterns were examined by RNase protection analysis (Fig. 2). The markedly different apoE gene expression patterns observed for the HEG1 and HESH1 constructs (high kidney expression, low liver, and brain expression) compared with the CI.361 construct (high liver expression, moderate kidney, skin, and brain expression) suggested that important regulatory elements controlling apoE gene expression were located more than 1.7 kb downstream of the apoE gene.

To identify these elements, apoE gene constructs containing 650 base pairs of 5' flanking sequence, the first intron, and different lengths of 3' flanking region were expressed in transgenic mice. A construct containing only 72 base pairs of 3' flanking region (HE54) was expressed at high levels in the kidney and low levels in the testis, but in no other tissues. Extending the 3' flanking region to 1.7 kb downstream (HE54H) resulted in low levels of apoE gene expression in the brain, suggesting that a cis-acting element required for brain expression is located in the proximal 1.7 kb of the 3' flanking sequence. This construct resulted in an expression pattern similar to that of the HEG1 construct, suggesting that introns 2 and 3 may not contain tissue-specific control elements.

Extending the 3' flanking sequence from 1.7 to 4.0 kb downstream (HE54.4) had a marked effect on the pattern of apoE gene expression in different tissues: apoE expression in the kidney was silenced, and the skin had a high amount of human apoE mRNA. This construct showed moderate levels of apoE mRNA expression in the brain and yielded low levels in other tissues. No expression was detected in the liver. With the exception of a lack of liver synthesis, the expression pattern of this construct closely resembled that of the endogenous mouse apoE gene (Fig. 2), which was expressed at high levels in the liver, at moderate levels in the skin and brain, and at lower levels in other tissues. These results indicate that the region between the apoE and apoC-I genes contains several independent regulatory elements that control expression in different tissues.

The absence of apoE gene expression in the liver of transgenic mice harboring apoE gene constructs that lacked the apoC-I gene suggested that expression of apoE in the liver required elements proximal to, within, or downstream of the apoC-I gene. To localize the liver regulatory element of the apoC-I gene, a series of human apoC-I gene constructs (Fig. 1) that contained various lengths of 5' and 3' flanking sequence were tested in transgenic mice. The apoC-I gene constructs CI.SS, CI.SB, and CILE were expressed in several peripheral tissues at low levels, but no apoC-I mRNA was detected in the liver.

Transgenic mice harboring either of the two apoC-I constructs with the longest 5' flanking sequence (CI.361 and CILE) expressed apoC-I mRNA at noticeably higher levels in the skin. These results were in agreement with the previous findings with HE54.4 apoE transgenic mice, suggesting that a common skin-specific enhancer located between the apoE and apoC-I genes influences the expression of both genes. This element appears to be located between 1.7 and 2.2 kb downstream of the apoE gene.

CI.SE and CI.SC, the two apoC-I gene constructs that contained the entire region between the apoC-I gene and the apoC-I' pseudogene, were expressed at high levels in the liver of transgenic mice and at lower levels in several peripheral tissues, including the lung, testis, and stomach. Thus, regulatory elements controlling expression of the apoC-I gene in the liver, as well as the stomach, are located between 2.3 and 8.0 kb downstream of the apoC-I gene, most likely between the apoC-I gene and the apoC-I' pseudogene. While the possibility remains that an element required for apoE gene expression in the liver is located proximal to or within the apoC-I gene, this element would have to direct high levels of hepatic apoE gene expression without affecting apoC-I gene expression in the liver. Because this possibility seems quite unlikely, we propose that the downstream region controlling hepatic apoC-I gene expression contains an element that acts over a distance of at least 15 kb to stimulate high level expression of the apoE gene in the liver.

Nine independent lines that harbor one of the three constructs having the apoC-I/C-I' intergenic region (CI.361, CI.SE, CI.SC) expressed high levels of the human transgene(s) in the liver. In contrast, in more than 25 independent lines of transgenic mice containing human apoE or apoC-I gene constructs that lack the apoC-I/C-I' intergenic region (the other eight constructs in Fig. 1), no transgene expression was detectable in the liver. This finding differs from observations for other transgenes expressed in the liver, including the genes for transthyretin (23), albumin (24), α-fetoprotein (25), apoA-I (26), and apoC-III (27), which show expression in the liver when fewer than 500 nucleotides of the proximal 5' flanking region are included in the injected constructs. Thus, it appears that the far-downstream regulatory domain of the apoE/C-I gene locus is essential for specifying transcription of these genes in the liver.
The expression pattern of the CI.361 transgene construct differed somewhat from the expression pattern of the endogenous mouse apoE and apoC-I genes (Fig. 2). Whereas mouse apoE mRNA was present at variable levels in all tissues analyzed, human apoE mRNA from the CI.361 transgene was present at high levels in the liver and low levels in the kidney and brain, with longer exposures revealing trace levels in the skin and testis. Similarly, apoC-I mRNA from the transgene was detected at high levels in the liver and at low levels in the skin, spleen, and kidney, but it was not detected in several tissues that contained limited amounts of mouse apoC-I mRNA (i.e., submaxillary, lung, and testis). These findings raise the possibility that important regulatory elements controlling expression of the apoE/C-I gene locus may reside outside the sequences contained in the CI.361 construct. Some differences between the expression patterns of the transgenes and endogenous genes also may reflect variations in control of the mouse and human genes. Furthermore, each transgene construct has an inherently different content of potential positive and negative control sequences, whose combined

| Pedigree | Plasma cholesterol | Plasma triglycerides |
|----------|-------------------|---------------------|
| CLSC 650 (3) | 164 ± 24 | 460 ± 80 |
| CLSC 647 (6) | 144 ± 10 | 284 ± 48 |
| Control (3) | 89 ± 18 | 155 ± 66 |

* Numbers in parentheses represent the number of F1 transgenic heterozygotes and nontransgenic control animals analyzed.
interactions may affect the level of gene expression in individual tissues.

The different expression patterns for the gene constructs used in this study indicate that expression of the apoE/C-I gene locus is controlled by a complex interaction of several independent tissue-specific transcriptional enhancers and silencers. It seems likely that certain regulatory elements will dominate control of gene expression, whereas the influence of other regulatory sequences, perhaps cryptic elements, is observed only upon removal of a more physiologically important element. All of the tissues for which we have identified specific regulatory elements in this study have been shown previously to contain apoE mRNA or immunoreactive apoE (6–11). The finding of a downstream silencer that appears to specifically reduce the effect of a strong positive element in the promoter suggests that apoE production in this tissue may be highly regulated. Although we have obtained transgene expression in most tissues that express the endogenous apoE and C-I genes, a thorough understanding of the mechanisms controlling expression of this gene locus will be facilitated through study of tissue-specific transcriptional enhancers and silencers. It seems likely that certain regulatory elements will serve only upon removal of a more physiologically important element.

An initial analysis of transgenic lines harboring constructs having only the apoC-I gene, but with sufficient downstream sequences to specify liver expression, revealed that these animals had elevated plasma cholesterol and triglyceride levels compared with control animals (Table 1). Groups of three and six F1 heterozygotes from two separate transgenic lines (fed mouse chow ad libitum) carrying the CI.Sc construct had plasma cholesterol levels that were 50–80% higher than that of a control group of three and plasma triglyceride levels that were 2–3-fold higher than in the control group. In contrast, transgenic animals with both the human apoE and apoC-I genes (CI.361) had normal plasma levels of triglycerides and cholesterol (data not shown). These results are consistent with the recent finding in vitro (28) that apoC-I can displace apoE from lipoprotein particles, which would be expected to interfere with lipoprotein uptake by cells and lead to hyperlipidemia.

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