Expression of the Human Apolipoprotein E Gene in Cultured Mammalian Cells*

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Catherine A. Reardon, Yun-Fai Lau†, Young-Ki Paik, Karl H. Weisgraber, Robert W. Mahley, and John M. Taylor‡

From the Gladstone Foundation Laboratories for Cardiovascular Disease, Cardiovascular Research Institute, and the Howard Hughes Medical Institute, Departments of Physiology, Medicine, and Pathology, University of California, San Francisco, California 94140-0808

The gene for human apolipoprotein (apo-) E was isolated from a human genomic library constructed in the cosmid shuttle vector pCV108. The transient expression of the apo-E gene was examined in cultured mammalian cells 48 h following calcium phosphate-mediated gene transfer. The expression of the cloned human apo-E gene, which contained between 0.7 and 29 kilobases of 5′-flanking DNA, was not restricted to human cells or to cultured cells derived from tissues that have been shown to synthesize apo-E. Several independent mouse L cell stable transfectants with the human apo-E gene integrated into their genome were selected on the basis of G418 resistance, which is conferred by the selectable gene marker in the cosmid vector. The levels of human apo-E mRNA found in the stable transfected mouse L cells ranged from undetectable to a level comparable to that found in the human liver. The size of the apo-E mRNA observed in the stable transfectants was identical to that found in the liver, indicating that the mouse L cells were capable of correctly processing the human apo-E gene transcripts. The integrated human apo-E genes had not undergone major rearrangements or deletions during transfer, and the level of apo-E mRNA found in the different stable transfected cells correlated directly with the number of integrated copies of the human apo-E gene. The stable transfected L cells secreted authentic human apo-E into the medium. The secreted protein interacted specifically with antibodies to human plasma apo-E. The secreted apo-E interacted with lipid (presumably phospholipids), floated at 1.00 g/ml, and bound with high affinity to the apo-B,E(LDL) receptor on fibroblasts.

Apolipoprotein (apo-) E is a component of several classes of plasma lipoproteins (for review, see Refs. 1–3). It mediates the catabolism of specific lipoproteins through high-affinity binding to hepatic and extrahepatic receptors (3–5). The receptor binding domain has been localized to an arginine-rich region centered in the vicinity of residues 140–146 of the protein (6–8). Human apo-E has a complex isoelectric focusing pattern that is due to the presence of multiple alleles for apo-E at a single gene locus and to post-translational modifications involving the addition of sialic acid residues (9, 10). Variants of apo-E in which neutral amino acids substitute for arginine and lysine residues within the receptor binding region exhibit decreased receptor binding capacity and are associated with type III hyperlipoproteinemia (11–14).

The liver is the major site of synthesis of apo-E (15). Mouse peritoneal macrophages (16) and human monocyte-derived macrophages (17) also have been shown to synthesize apo-E. Recently, apo-E mRNA has been detected in tissues of various peripheral organs, including the brain, adrenal, spleen, ovary, testis, and kidney (18–20). Immunocytocchemical analysis has localized apo-E to astrocytic glial cells of the central nervous system and to nonmyelinating glial cells of the peripheral nervous system (21). It has been estimated that 10 to 20% of apo-E in the circulating plasma may originate from peripheral organs (18). The apo-E synthesized by the tissues in peripheral organs may be involved in the redistribution of cholesterol and other lipids among cells and tissues.

Human apo-E is a M, ~34,000 protein composed of 299 amino acids of known sequence (22). It is initially synthesized with an 18-amino acid signal peptide that is removed cotranslationally (23, 24). The nucleotide sequences of the mRNA for human apo-E have been determined (25, 26) and rat (27) apo-E are known. Recently, the nucleotide sequence of the gene for human apo-E has been determined (28,29) and the gene has been mapped to chromosome 19 (29). The mRNA coding sequence is interrupted by three introns, which are located in the 5′-nontranslated region of the mRNA, in the signal peptide region within the codon for Gly, and within the codon for Arg140 in the mature plasma protein region. The structure of the human apo-E gene with respect to the number and location of the introns is similar to that found in genes for human apo-A-I and apo-C-III (30–32), as well as for apo-A-II (33).

Since the metabolism of cholesterol and other lipids may be affected by the expression of the apo-E gene, examination of the molecular basis of its regulation was begun. The human apo-E gene was isolated from a cosmide genomic DNA library, and the expression of the cloned gene was examined in cultured mammalian cells after both transient and stable transfections.

EXPERIMENTAL PROCEDURES

Materials—Radioisotopes and the plasmid pAT153 were purchased from Amersham Corp. ENHANCE was obtained from New England Nuclear. [3H] restriction endonucleases, T4 DNA ligase, and Klenow...
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Nitrocellulose paper for blots was obtained from Schleicher & Schuell, and HATF nitrocellulose filters were obtained from Millipore Corp. Cell culture media, trypsin EDTA solution, and G418, a neomycin analogue, were purchased from Gibco. Fetal bovine serum was obtained from Flow Laboratories, serum-free medium (HB101) was obtained from Collaborative Research, and thioglycolate (BBB E EH) was obtained from Avanti Polar Lipids (Birmingham, AL). Yeast RNA and phenylmethylsulfonyl fluoride were purchased from Sigma, and leupeptin was obtained from P-L Biochemicals.

Cells and Cell Cultures—Male Swiss-Webster mice were obtained from Charles River Breeding Laboratories (Wilmington, MA) and given free access to Purina rat chow. Cultured FQAH cells were maintained in modified Eagle’s medium. All media were supplemented with 10% fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml.

Isolation of Human Apolipoprotein E Cosmid Clones—Construction of the cosmid shuttle vector pCV108 and the preparation of the human genomic library have been described previously (34). The cosmid library was screened according to the high-density bacterial colony method of Hanahan and Meselson (35) using a 32P-labeled restriction endonuclease fragment derived from a full-length cloned human apo-E eDNA (33). Approximately 1 × 108 colonies were screened, and 16 positive clones were selected and isolated. A HindIII restriction endonuclease fragment of pCHEG1 containing the apo-E gene, described below, was cloned into the HindIII site of the plasmid pAT153 (37). The recombinant plasmid was introduced into Escherichia coli RII. Recombinant cosmids and plasmids were isolated by the alkaline-sodium dodecyl sulfate method (38), extracted with phenol/chloroform, and purified on CaCl2 gradients.

DNA Transfections—Cultured mammalian cells were transfected with the human apo-E-gene-containing cosmid DNAs by the calcium phosphate coprecipitation method of Graham and Van der Eb (39) as modified by Wigler et al. (40). To isolate stable transfecants, nontransfected L cells, grown at 1 × 104 cells/ml containing 200 pg of G418/ml for 20–24 h. Then, 5 pg of cosmid DNA in 1 ml of the DNA-calcium phosphate coprecipitate were added to the medium, and the cells were incubated for 4 h. The cells were shocked for 2 min with 15% glycerol in phosphate-buffered saline (140 mM NaCl, 8 mM NaH2PO4, 1.5 mM KH2PO4, and 3 mM KCl, at pH 1.5) (41), washed with phosphate-buffered saline, and allowed to grow for an additional 48 h. At this time the cells were subcultured and diluted 1:20 into medium supplemented with 400 μg of G418/ml (42). After 2 weeks of G418 selection, individual colonies were isolated and grown to mass culture. The cells were maintained in medium containing 400 pg of G418/ml. For transient expression, cells were seeded at 4 × 104 cells/75-cm2 flask, and the cells were transfected with 20 pg of DNA in 1 ml of DNA-calcium phosphate coprecipitate essentially as described above. Forty-eight hours after glycerol shock, total cellular RNA was prepared and analyzed as described below. Chloroquine was included at 100 μM.

Preparation and Analysis of RNA—Total cellular RNA was isolated from the cultured cells according to the guanidine thiocyanate procedure of Chirgwin et al. (44). Dot blot analysis was performed as described (18). Aliquots of total RNA (5.0, 2.0, 1.0, and 0.5 μg) were adjusted to 3 μg of RNA/sample with yeast RNA and denatured in 1.0 M formaldehyde, 0.9 M NaCl, and 0.09 M sodium citrate at 55°C for 15 min. The samples were diluted into 20 volumes of 3 M NaCl containing 0.3 M sodium citrate and applied to nitrocellulose filters using a vacuum manifold (Schleicher & Schuell). For Northern blot analysis (45), the RNA was denatured in 1% glyoxal, electrophoresed through a 1.1% agarose gel, transferred to nitrocellulose paper, and hybridized with cDNA probes.

Cosmid DNA Rescue and Apolipoprotein E Gene Copy Number—Total cellular DNA was extracted from the stable L cell transfecants and human leukocytes as described (46). The transfected cosmid DNA was excised from the genomic DNA and transfected into bacteria by in vitro packaging with extracts from lyogenic bacteriophage as described (44). The apo-E gene copy number was estimated by biomolecular hybridization experiments essentially as described (27, 47); human leukocyte DNA was used as the standard. Aliquots of genomic DNA (1–20 μg) were denatured in 0.1 N NaOH containing 2 M NaCl for 2 min at 100°C, neutralized with 3 volumes of 1.0 M Tris (pH 8.0) containing 2 M NaCl, diluted with 15 volumes of 3 M NaCl containing 0.3 M sodium citrate, applied to nitrocellulose filters using a template manifold, and hybridized as described above. Blots were quantitated by densitometric scanning of several different exposures of the autoradiograms of the hybridized filters with a Beckman DU-8 spectrophotometer.

Protein Labeling—The cultured cells were grown in 75-cm2 tissue culture flasks in HB101 serum-free medium until ~75% confluent. The cells were labeled in Dulbecco’s modified Eagle’s medium containing 40 μM unlabeled methionine and 100 μCi of [35S]methionine/ml in the absence of serum for various lengths of time at 37°C. The medium was adjusted to 100 μM phenylmethylsulfonyl fluoride and 1 μg of leupeptin/ml before immunoprecipitation with rabbit anti-human apo-E serum and was analyzed on 5–20% polyacrylamide-sodium dodecyl sulfate gels (25).

Isolation of Macrophages—Peritoneal macrophages were harvested from thioglycollate-stimulated mice (48) and cultured for 24 h in Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum. The macrophages were then incubated with 100 μg of acetylated LDL protein/ml (17) for 24 h before being labeled with [35S]methionine as described above.

Lipoprotein Isolation and Potassium Bromide Density Gradient—To isolate total lipoproteins, cell culture medium was adjusted to a density of 1.215 g/ml with KBr and centrifuged at 55,000 rpm for 48 h. The d < 1.215 g/ml fraction was dialyzed against 25 mM NH4HCO3. 1.215 to 1.21 g/ml range, and centrifuged at 27,000 rpm in a Beckman SW28 rotor at 4°C for 60 h. Fractions (1 ml) were collected from the bottom of the tube and aliquots were examined for radioactivity. The density of each fraction was determined from the refractive index.

Receptor Binding Assay—To prepare apo-E for analysis, the apo-E secreted from the L cell transfecant L 1.1 into HB101 serum-free medium was partially purified on a heparin-Sepharose column (49). The medium was collected, concentrated 70- to 100-fold with an Amicon Ultrafiltration apparatus (Amicon) in the presence of 100 μM phenylmethylsulfonyl fluoride and 1 μg of leupeptin/ml, dialyzed against 10 mM NH4HCO3 containing 0.1% 2-mercaptoethanol, and applied to a heparin-Sepharose column. The bound proteins were eluted with a linear gradient between 0.01 and 0.7 M NH4HCO3. Elution of apo-E was monitored by quantitative radiomunnoassay. The partially purified apo-E was complexed with DMPC, and the complexes were purified on KBr gradients (50). Competitive binding assays were performed on human fibroblasts with 125I-labeled LDL as described (6).

RESULTS

Isolation of Human Apolipoprotein E Genomic Cosmid Clones—Restriction endonuclease analysis and Southern blot hybridization revealed that the 16 positive apo-E-gene-containing cosmid clones selected and isolated were of two types. A partial restriction endonuclease map showing the location of the apo-E gene in the genomic DNA insert of both types of cosmid clones is illustrated in Fig. 1. Both clones contained the entire apo-E gene. In pCHEG1, the apo-E gene was located at the 3′ end of the DNA insert and contained approximately 29 kilobases (kb) of 5′-flanking genomic DNA. In pCHEG2, the apo-E gene was located at the 5′ end of the

pCHEG1

pCHEG2

5 10 15 20 25 30 35

Nucleotides x 10^3

Fig. 1. Partial restriction map of pCHEG1 and pCHEG2.

The restriction map was determined by restriction endonuclease digests and Southern blot analysis (42). The location of the human apo-E gene in the genomic DNA insert in each cosmid clone is shown by the solid box. The restriction endonuclease sites are as follows: B, BamHI; E, EcoRI; and H, HindIII. Not all sites for these enzymes in the apo-E gene region are indicated. The HindIII fragment of pCHEG1 containing the apo-E gene was subcloned into pAT153.
DNA insert and contained approximately 0.7 kb of 5′-flanking genomic DNA. The partial restriction endonuclease map of the apo-E gene isolated from the cosmids genomic library was the same as that reported previously (28) for a human apo-E gene isolated from a λ genomic library.

**Transient Expression of the Transfected Apolipoprotein E Gene in Cultured Mammalian Cells**—To determine whether the apo-E gene in both cosmids clones was functional, the expression of the gene upon transfection of the cosmids DNA into cultured mammalian cells was examined. Several human and heterologous cultured cells (Table I) were transfected with both types of cosmids DNAs containing the apo-E gene by the calcium phosphate coprecipitation technique. Transient expression of the exogenous gene was determined 48 h after transfection. None of the cell lines contained detectable amounts of endogenous apo-E mRNA.

The transfected human apo-E gene was expressed in all of the cultured human cell lines, as well as in two mouse macrophage lines (J774.2 and P388D1), mouse fibroblasts (L cells), and African green monkey kidney cells (Cos7). It was not expressed in a rat hepatoma line (PuAH) or Chinese hamster ovary cells. Although the level of expression of the human apo-E gene in the different cell lines varied, the apo-E mRNA level for a given cell line was approximately the same for both types of apo-E-gene-containing cosmids DNAs. The apo-E mRNA transcribed in several of the transfected cell lines was examined on Northern blots and was found to be identical in size to the apo-E mRNA observed in the human liver (data not shown).

To characterize further the transcription unit of the human apo-E gene, a 10.5-kb HindIII fragment of pCHEG1 was cloned into the plasmid pAT153. This subclone contained the entire apo-E gene, with 5.2 kb of 5′-flanking genome DNA and 1.6 kb of 3′-flanking genomic DNA. The same cultured cells were transfected with this subclone. The transcription pattern and level of expression observed using this clone were virtually identical to those observed for the larger pCHEG1 and pCHEG2 cosmids clones. The results of the transient expression assays indicate that the regulatory sequences necessary for the transcription of the apo-E gene are located within 0.7 kb of 5′-flanking sequence and 1.6 kb of 3′-flanking sequence.

**Stable Expression of the Transfected Human Apolipoprotein E Gene in Mouse L Cells**—The human apo-E gene was introduced into mouse L cells to examine the expression of the transfected apo-E gene in greater detail. Mouse L cells were chosen because their endogenous apo-E gene is not expressed and because these cells can be readily adapted to grow in a defined serum-free medium, which would facilitate the characterization of the apo-E gene products.

Mouse L cells were transfected with both types of apo-E-gene-containing cosmids. Since the pCV108 cosmid vector (34) contains the SV2-neomycin selectable marker gene (42), stable transfectants were selected using the neomycin analogue G418. Nine G418-resistant clones transfected with pCHEG1 and six G418-resistant clones transfected with pCHEG2 were selected for analysis. Expression of the human apo-E gene in these clones was determined by examining the cellular RNA for the presence of human apo-E mRNA (Fig. 2). Eight of nine pCHEG1 L cell transfectants expressed the human apo-E gene, with the level of apo-E mRNA observed in the transfectants varying from 1% (L 1.4) to 100% (L 1.1) of that found in the human liver, as determined from densitometric scans of the dot blots seen in Fig. 2. Cultured mouse cells transfected with pCHEG2 also yielded stable transfectants that expressed the human apo-E gene at levels comparable to those observed in pCHEG1 stable transfectants, as illustrated by L 2.3 of Fig. 2. Therefore, it appears unlikely that distal 5′-flanking sequences that are not present in the pCHEG2 human genomic insert are essential for apo-E gene expression.

Expression of the human apo-E gene in these clones was stable. The level of apo-E mRNA in the L cell transfectants remained constant even after the cells were maintained in selection medium for several months. In addition, maintaining the cells in a defined serum-free medium for several months had no significant effect on the level of apo-E mRNA in these cells. Transfection of the human apo-E gene and isolation of stable cell lines did not activate the endogenous mouse apo-E gene in any of the L cell transfectants, as determined by hybridization of RNA dot blots with a mouse apo-E cDNA probe (data not shown).

![Table I](image)

**Table I. Transient expression of the human apolipoprotein E gene in cultured mammalian cells**

Expression of the apo-E gene was determined by dot blot analysis of the total cellular RNA isolated from the cells 48 h after transfection with human apo-E-gene-containing cosmid DNA (see "Experimental Procedures").

| Cell line | Description          | Apo-E gene expression |
|-----------|----------------------|-----------------------|
| A-431     | Human epidermoid carcinoma | +                     |
| BaGl      | Human fibroblast      | +                     |
| HeLa      | Human epithelial carcinoma | +                   |
| U-937     | Human monocyte-like   | +                     |
| J774.2    | Mouse macrophage-like | +                     |
| P388D1    | Mouse macrophage-like | +                     |
| L         | Mouse fibroblast      | +                     |
| Cos7      | Monkey kidney         | +                     |
| PuAH      | Rat hepatoma          | –                     |
| CHO       | Chinese hamster ovary | –                     |

![Fig. 2](image)

**Fig. 2. Relative level of human apo-E mRNA and gene copy number in L cell transfectants.** Total cellular RNA from the L cell transfectants was examined by dot blot hybridization. The indicated concentrations of RNA were denatured, and the samples were applied to nitrocellulose filters using a template manifold. Total human liver RNA was used as the standard. Gene copy number was estimated by dot blot analysis of genomic DNA isolated from the L cell transfectants; DNA from human leukocytes was used as the standard. Both RNA and DNA blots were hybridized with a 32P-labeled full-length human apo-E cDNA probe, and the blots were quantitated by densitometric scanning of the autoradiograms. ND, not determined.
Expression of the Human Apolipoprotein E Gene

The human apo-E gene transcripts appeared to be processed correctly by the L cell transfectants. Total RNA from human liver, control L cells, and L cell transfectants containing either pCHEG1 or pCHEG2 were examined by Northern blot analysis (Fig. 3). The human apo-E mRNA in both L cell transfectants was identical in size to the apo-E mRNA found in the human liver. In addition, the apo-E mRNA from transfected cells was polyadenylated, since it could be isolated on a poly(U)-Sepharose column (data not shown).

Analysis of DNA in the L Cell Transfectants—The number of copies of the human apo-E gene in several L cell transfecteds was determined by genomic DNA dot blots (Fig. 2). Assuming that there is only a single copy of the human apo-E gene per haploid genome (28), the apo-E gene copy number was found to vary from 1 to 11 in the L cell transfectants that were examined. In addition, there was a direct correlation between the number of copies of the human apo-E gene and the level of human apo-E mRNA in the different L cell transfecants.

Because several laboratories have reported that exogenous DNA sequences can be modified substantially following transfection into mammalian cells (51–53), the possibility that the transfected apo-E genes in the stable L cells were modified was investigated by cosmid DNA rescue (34). In this technique, when three or more transfected genomic cosmid DNA units are integrated tandemly into the cellular genome, the cosmid DNA can be recovered from the genomic DNA by in vitro λ bacteriophage packaging. After subsequent infection of a suitable bacterial host, the rescued cosmid DNA is recovered as a plasmid, and its restriction endonuclease pattern can then be directly compared with that of the original transfecting cosmid DNA for alterations that may affect gene expression.

Restriction endonuclease digestion and Southern blots of pCHEG1 DNA and cosmid DNA rescued from pCHEG1 L cell transfectants indicated that no major rearrangements or deletions of the human apo-E cosmid DNA had occurred during gene transfer (Fig. 4). The restriction pattern of the cosmid DNA rescued from the stable transfecants was identical to that of the original transfecting cosmid DNA.

Characterization of Human Apolipoprotein E Secreted from Stable Transfected Mouse L Cells—The L 1.1 transfectant was examined to determine whether the human apo-E mRNA that it contained was capable of functioning as a template for the synthesis of a secrtable apo-E. As shown in Fig. 5, apo-E was immunoprecipitated from the medium of [35S]methionine-labeled L 1.1 cells, whereas no labeled proteins were immunoprecipitated from the medium of the control L cells. Human apo-E was a major secreted protein of the mouse L cell transfecant, representing ~1 to 2% of total secreted proteins. The secreted human apo-E was found in two immunoprecipitated bands of Mr, ~35,000 and 36,000 (Fig. 5). These forms often are found in highly sialylated apo-E. Immunobots showed that the electrophoretic mobility of this protein was slightly slower than that of plasma apo-E (data not shown). The two-dimensional electrophoretic pattern (data not shown) of the human apo-E secreted by the transfected cells was consistent with the presence of multiple sialic acid residues on the secreted apo-E; the presence of these residues is characteristic of nascent apo-E (17, 54, 55). Thus, the mouse L cells apparently are capable of post-translationally modifying the human apo-E that they synthesize.

The apo-E secreted by the transfected L cells floated at d < 1.21 g/ml, indicating that it was complexed with lipid. Mouse L 1.1 cells, grown for several generations in serum-free medium were incubated with [35S]methionine to label the secreted proteins. The medium obtained 20 h after addition of the [35S]methionine was adjusted to d = 1.21 g/ml with KBr and centrifuged as described under “Experimental Procedures.” Most of the immunoprecipitable apo-E floated at d < 1.21. The actual density of flotation of the apo-E secreted

![Fig. 3. Northern blot analysis of human apo-E mRNA from L cell transfectants. Total cellular RNA (20 µg) from human liver (lane 1), control L cells (lane 2), and the L cell transfectants L 1.1 (lane 3) and L 2.3 (lane 4) were denatured in 10% deionized glyoxal in the presence of 25 mM sodium phosphate buffer, pH 6.5, for 1 h at 50 °C and electrophoresed through a 1.1% agarose gel in 25 mM sodium phosphate buffer, pH 7.0. After transfer to a nitrocellulose filter, the blot was treated with 20 mM Tris, pH 8.0, at 100 °C for 5 min to remove completely the glyoxal adduct before hybridization with a 32P-labeled human apo-E cDNA clone. The nucleotide lengths of HindIII-digested fragments of SV40 DNA are indicated as size markers.

![Fig. 4. Southern blot hybridization of cosmid DNA rescued from total DNA of pCHEG1 stable L cell transfectants. The pCHEG1 (denoted by the c below lane 1) and the rescued cosmid DNA were digested with EcoRI and BamHI (A and B) or HindIII (C and D) and electrophoresed on 0.8% agarose gels. The gels were stained with ethidium bromide (A and C) and then denatured and transferred by blotting to nitrocellulose filters. The blots were hybridized with a 32P-labeled human apo-E cDNA clone (B and D).]
Expression of the Human Apolipoprotein E Gene

We have isolated two genomic clones containing the human apo-E gene from a cosmid genomic library and characterized the expression of the gene after transfection into cultured mammalian cells. Although the two clones contain different amounts of 5’- and 3’-flanking genomic DNA, the apo-E gene in both clones was expressed with approximately equal efficiency in the transient expression assays to produce human apo-E mRNA that was identical in size to that found in the authentic apo-E, the ability of the secreted apo-E to compete with LDL for binding to the receptor (50% displacement: L cell apo-E, 0.125 μg of protein/ml; plasma apo-E3, 0.062 μg of protein/ml).

DISCUSSION

To determine whether the apo-E secreted by the L cell transfectant possessed biological activity similar to that of authentic apo-E, the ability of the secreted apo-E to compete with 125I-LDL for binding to apo-B,E(LDL) receptors on human fibroblasts was examined. The human apo-E that was secreted from L.11 cells into serum-free medium was partially purified on a heparin-Sepharose column and complexed with DMPC. The apo-E secreted by the mouse L cells eluted from the column at a similar density, d = 1.05,(0.08) g/ml, and apo-E from both sources floated in parallel with the transfected L cell apo-E at a density of 1.21 (0.06). For comparison, [35S]methionine-labeled proteins secreted by mouse peritoneal macrophages and floated at d < 1.21 were examined on the KBr density gradient in parallel with the transfected L cell apo-E at a density of 1.21. As shown in Fig. 6, apo-E from both sources floated at a similar density, d ~ 1.09. Analysis of aliquots of the peak fractions of both gradients by sodium dodecyl sulfate-polyacrylamide gels confirmed that apo-E was present in these fractions. No radiolabeled proteins were detected in any of the density fractions from medium of nontransfected control L cells.

By the transfected cells was determined by subjecting the d < 1.21 fraction to further centrifugation on a KBr density gradient (d = 1.006-1.21). For comparison, [35S]methionine-labeled proteins secreted by mouse peritoneal macrophages and floated at d < 1.21 were examined on the KBr density gradient in parallel with the transfected L cell apo-E at a density of 1.21. As shown in Fig. 6, apo-E from both sources floated at a similar density, d ~ 1.09. Analysis of aliquots of the peak fractions of both gradients by sodium dodecyl sulfate-polyacrylamide gels confirmed that apo-E was present in these fractions. No radiolabeled proteins were detected in any of the density fractions from medium of nontransfected control L cells.

To determine whether the apo-E secreted by the L cell transfectant possessed biological activity similar to that of authentic apo-E, the ability of the secreted apo-E to compete with 125I-LDL for binding to apo-B,E(LDL) receptors on human fibroblasts was examined. The human apo-E that was secreted from L.11 cells into serum-free medium was partially purified on a heparin-Sepharose column and complexed with DMPC. The apo-E secreted by the mouse L cells eluted from the column at the same ionic strength as that of plasma apo-E. As shown in Fig. 7, the apo-E secreted from the L cell transfectant effectively competed with LDL for binding to the receptor (50% displacement: L cell apo-E, 0.125 μg of protein/ml; plasma apo-E3, 0.062 μg of protein/ml).

Amounts of 5’- and 3’-flanking genomic DNA, the apo-E gene in both clones was expressed with approximately equal efficiency in the transient expression assays to produce human apo-E mRNA that was identical in size to that found in the

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [35S]-labeled proteins secreted by control and transfected L cells. Control L cells (lanes 1 and 3) and transfected L 1.1 cells (lanes 2 and 4) in 75-cm2 flasks were labeled with 100 μCi of [35S]methionine/ml for 6 h at 37 °C in the absence of serum. Aliquots of the medium containing 2 X 104 cpm of trichloroacetic acid-precipitable material were immunoprecipitated with anti-human-apo-E antibodies (25). Total medium, 2 X 104 cpm/lane (lanes 1 and 2), and the immunoprecipitates (lanes 3 and 4) of the medium were electrophoresed on 5-20% polyacrylamide-sodium dodecyl sulfate gradient gels, and this fluorogram was prepared (25).

FIG. 6. Flotation density of secreted apo-E. Control L cells (○), transfected L 1.1 cells (■), and acetoacetylated LDL-loaded mouse peritoneal macrophages (△) were labeled in the absence of serum with 100 μCi of [35S]methionine/ml for 20 h. The medium was adjusted to d = 1.21 g/ml with KBr, and the [35S]-labeled lipoprotein fractions were isolated, dialyzed against 25 mM NH.HCO3, and layered on top of linear 34-ml KBr gradients of d = 1.006-1.21 g/ml. The gradients were centrifuged at 27,000 rpm in a Beckman SW28 rotor at 4 °C for 60 h. Fractions (1 ml) were collected from the bottom of the tube. Aliquots of each fraction were examined for radioactivity, and the density of each fraction was determined by refractive index measurements. (Density of the fractions is indicated by the solid line.)

FIG. 7. Comparison of the ability of apo-E3-DMPC and L 1.1 apo-E-DMPC to compete with human 125I-LDL for binding to normal fibroblasts. Purified plasma apo-E3 (○) and L 1.1 apo-E (■) partially purified on a heparin-Sepharose column were complexed with DMPC and then further purified on KBr gradients (50). The amount of apo-E in the isolated apo-E-DMPC complexes was determined by quantitative radioimmunoassay. Human fibroblasts were incubated in Dulbecco’s modified Eagle’s medium containing 10% human lipoprotein-deficient serum for 48 h before use. Each dish received 2 ml of the same medium with 2 μg of 125I-LDL protein/ml and the indicated concentrations of apo-E- phospholipid complexes. After a 2-h incubation at 4 °C, the cells were washed extensively and the 125I-LDL that was specifically bound to the cells was determined. The 100% control value was 116 ng of 125I-LDL bound/mg of cellular protein.
human liver. This observation indicates that the regulatory sequences necessary for the expression of the apo-E gene are located in the gene itself or within ~0.7 kb of 5′-flanking sequence or 1.6 kb of 3′-flanking sequence.

Expression of the human apo-E gene was not restricted to human cell lines or to cultured cells derived from tissues that synthesize apo-E. This finding suggests that tissue-specific transcription factors are involved in the regulation of apo-E gene expression, then these factors are present in more than one cell type. Different results might be expected for other apolipoproteins, which are synthesized in fewer tissues.

The inability of some cell lines to express the human apo-E gene does not result from any inability to introduce the exogenous DNA into these cells. For example, several independently selected stable transfectants of the F9AH cell line were prepared with the apo-E-gene-containing cosmid pCHEG1. These stable transfectants contained several copies of the human apo-E gene, as determined by genomic DNA dot blots, and they expressed the neomycin-resistant marker gene, but none of the clones expressed the apo-E gene. This finding suggests that even transient expression of the apo-E gene in the transfected DNA is suppressed by these cells or that these cells may lack an essential transcription factor required for apo-E gene expression.

The expression of the apo-E gene in several stable transfected mouse L cells that were selected on the basis of G418 resistance has also been characterized. The exogenous human gene was transcribed, and the mRNA was processed properly in the L cell transfectants to produce functional mRNA. The transfected cell apo-E mRNA comigrated with human liver apo-E mRNA during agarose gel electrophoresis, and it was translated into a protein that was secreted into the medium and interacted specifically with antibodies to human plasma apo-E. That this protein was authentic apo-E was demonstrated by its having an apparent molecular weight similar to that of plasma apo-E, by its ability to bind to a heparin-Sepharose affinity column and elute at the same ionic strength as plasma apo-E, and by its ability to compete with LDL for binding to the apo-B,E(LDL) receptor on human fibroblasts with an affinity similar to that of authentic human apo-E.

The stable L cell transfectants express the human apo-E gene at different levels. There are several possible explanations for the large variation in the level of human apo-E mRNA observed in these cells. In general, the expression of exogenous genes can be affected by rearrangements or deletions of the transfected DNA that can occur during gene transfer and stable selection, by the number of copies of the transfected DNA integrated into the genome of different transfected cells, and by the site of integration of the transfected DNA into the chromosomal DNA of the cell. It appears that no major rearrangements or deletions of the human apo-E gene occurred during gene transfer. The apo-E-gene-containing cosmId DNA that was rescued from the L cell transfectants had a restriction endonuclease pattern identical to that of the original transfected DNA. Furthermore, in the stable transfectants examined, apo-E gene expression did not appear to be influenced by the site of integration. Therefore, the differences in the level of human apo-E mRNA in the various L cell transfectants appear to be a direct function of the number of integrated copies of the human apo-E gene.

A cell line (mouse L cells) that does not normally secrete lipoproteins has been shown to be capable of secreting human apo-E associated with lipid upon transfection with the corresponding exogenous gene. Since the apo-E particles secreted from the transfected L cells have a flotation density identical to that of apo-E secreted from mouse peritoneal macrophages (17), it is likely that the apo-E secreted from the L cells is complexed with phospholipid as well. There are several possible sources of the lipid that is associated with the secreted apo-E. Nascent apo-E may become associated with lipid during intracellular transport and processing, or it may acquire lipid during or after secretion from either the cell membrane or from other lipid in the surrounding medium. The latter possibility seems unlikely, since iodinated delipidated human plasma apo-E incubated with L cells in serum-free medium did not become associated with lipid (data not shown). It will be of interest to determine whether other apolipoproteins secreted from L cells after gene transfer are also associated with lipid. In cells that secrete apolipoproteins, the association of the apolipoproteins with lipid may depend most on the properties of the protein moieties themselves and not on special assembly mechanisms of the cells.

The ability to express efficiently the human apo-E gene in heterologous cultured mammalian cells will facilitate investigation of the regulatory elements associated with the expression of the human apo-E gene. Sequences upstream from the transcription initiation site, as well as sequences within introns, can be analyzed for their role in the expression of the apo-E gene.

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