Abstract Among inbred mouse strains there is a striking genetic variation in the levels of apolipoprotein A-IV (apoA-IV) mRNA in the liver, although intestinal mRNA levels vary only twofold in these strains. In the present study we have characterized the apoA-IV expression phenotypes in strains C57BL/6J and 129/J, and investigated the molecular basis for the genetic variation. We report that the two strains differ eight- to tenfold both in the levels of apoA-IV mRNA and in the rate of apoA-IV protein synthesis in liver. Presumably due to the increased synthetic rate, strain 129 exhibits a threefold higher concentration of apoA-IV protein in the circulation. mRNA synthesis and turnover studies indicate that both transcriptional and post-transcriptional events contribute to the genetic variation in steady state apoA-IV mRNA levels. An analysis of the levels of apoA-IV mRNA derived from 129 and C57BL/6 alleles in F1 mice indicates that the genetic control of apoA-IV mRNA levels involves both cis-acting elements linked to the apoA-IV gene, and genetically distinct trans-acting factors. Genetic variation in mouse apolipoprotein A-IV expression is determined pre- and post-transcriptionally.

Supplementary key words apolipoprotein expression • transcriptional regulation • post-transcriptional regulation

Cholesterol, triglycerides, and other lipids are transported in the plasma in the form of lipoprotein particles. These particles also contain lipid-binding polypeptides, known as apolipoproteins, which serve as structural components and have specific functions in lipid metabolism. The levels of lipoproteins and apolipoproteins in the circulation are influenced by a combination of hereditary and environmental factors (reviewed in ref. 1). Defects in apolipoprotein biosynthesis may contribute to disorders in lipid transport and development of coronary artery disease. To better understand the factors that regulate apolipoprotein expression, we have begun to characterize naturally occurring genetic variations in apolipoprotein A-IV (apoA-IV) expression in mouse strains.

ApoA-IV is synthesized in liver and intestine, and is a protein constituent of triglyceride-rich and high density lipoproteins (HDL). Although the physiological role of apoA-IV is not well understood, several activities for this protein have been demonstrated in vitro. For example, it is a potent activator of the enzyme lecithin:cholesterol acyltransferase, which functions in the esterification of HDL cholesterol (2). ApoA-IV can also serve as a co-factor for the interconversion of HDL subclasses (3), and enhance the activation of lipoprotein lipase in conjunction with apoC-II (4). It has been proposed that apoA-IV may promote removal of excess cholesterol from peripheral tissues, since apoA-IV-containing particles increase the rate of cholesterol efflux from cultured cells (5, 6), and apoA-IV is a ligand for HDL binding to hepatocytes (6-8). Recent studies indicate that apoA-IV present in mesenteric lymph after a lipid meal may act as a physiological signal for satiation (9).

We have previously described genetic variations in apoA-IV expression levels among inbred and wild-derived mouse strains. In a survey of 13 inbred strains, basal levels of apoA-IV mRNA in liver varied dramatically, with the largest difference occurring between strains C57BL/6J and 129/J (10). In contrast to these differences in liver, apoA-IV mRNA levels in intestine vary less than twofold between these two strains. The response of liver apoA-IV mRNA levels to diet also appears to be genetically determined, with some strains exhibiting an increase and others a decrease in apoA-IV mRNA after a diet rich in fats (10). Genetic analysis of this variation using recombinant inbred lines should provide insights into the genetic control of apoA-IV expression.

Abbreviations: HDL, high density lipoproteins; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; DRB, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole.

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In addition to genetic variation in apoA-IV expression levels, mouse strains exhibit variation in the structure of apoA-IV mRNA and protein. In a survey of several Mus musculus inbred strains and related Mus species, three apoA-IV isoforms were identified (11). Determination of the underlying nucleotide sequence revealed that a common site for genetic variation in apoA-IV structure is a series of tetra-amino acid repeats near the carboxyl-terminus of the protein. The variant forms of apoA-IV differ in the number of these repeats, presumably due to deletion or insertion mutations. Of eight inbred strains examined, apoA-IV from C57BL/6 and six other strains each had four repeat units, while strain 129 had only three. Genetic polymorphism in apoA-IV protein structure has been detected in other species as well, including humans, baboons, horses, and dogs (12-14). A relatively common variant of human apoA-IV, apoA-IV-2, differs from the predominant isoform by a single amino acid substitution within one of the carboxyl repeat units (15). Bio-physical studies indicate that the apoA-IV-2 isoform exhibits distinctive physical and functional characteristics in vitro, including greater hydrophobicity, greater stability in solution, and greater efficiency of LCAT activation (8). In human population studies, apoA-IV-2 has been correlated with significantly higher HDL cholesterol and lower triglyceride levels (reviewed in ref. 12).

In the present study, we have further characterized the apoA-IV expression phenotypes in strains C57BL/6 and 129, and sought to determine the molecular basis for genetic variation in apoA-IV mRNA levels. In this regard, we have examined apoA-IV mRNA levels, rates of protein synthesis, and circulating protein levels. In addition, the underlying mechanism responsible for the quantitative variation affecting apoA-IV mRNA levels has been investigated by determining rates of mRNA synthesis and turnover. Finally, we have investigated the genetic basis for the variation in apoA-IV expression and find that both the apoA-IV structural gene and distinct trans-acting gene(s) influence apoA-IV mRNA levels.

METHODS

Animals

Inbred mouse strains C57BL/6J and 129/J were obtained from the Jackson Laboratory (Bar Harbor, ME). (C57BL/6 x 129)F1 mice were bred in-house. Mice were maintained on Purina Mouse Chow and housed with a 12-h light/dark cycle. Studies were performed with female mice 3–8 months of age. Mice were fasted 2 h prior to tissue or blood collection, and killed between 9:00 and 11:00 AM.

Plasmid DNAs

For quantitation of mRNAs, cDNAs for mouse apoA-IV (16), mouse β-actin (17), mouse albumin and 28S RNA (C. H. Warden and A. J. Lusis, unpublished), and rat apoA-I (provided by Dr. S. K. Karathanasis) were used. Plasmid p(wt)A-IV3', used to produce control apoA-IV mRNA transcripts for the RNA solution hybridization assay, was constructed as follows: a full length apoA-IV cDNA derived from C57BL/6 mRNA (II) was digested with Hinc II and Rsa I, releasing a fragment corresponding to nucleotides 1146-1358 (numbering according to reference 11). This fragment was cloned into the Sma I site of pGEM2 (Promega).

mRNA preparation and Northern blot analysis

Total RNA was prepared from whole liver and isolated hepatocytes by extraction in guanidine hydrochloride (18). RNA (10 µg) was electrophoresed through 1% agarose containing formaldehyde, transferred to nylon membranes, and hybridized to 32P-labeled cDNA probes as described previously (10). RNA levels were quantitated by densitometry. ApoA-IV and apoA-I mRNA levels were normalized to signals for β-actin and/or 28S ribosomal RNA.

RNA solution hybridization assay

RNA quantitation by solution hybridization was performed as described by Durnham and Palmiter (19), with modifications. Briefly, aliquots of total mouse liver RNA (10 µg, 20 µg, and 40 µg) were suspended in 10 µl 0.2 × SET (0.2% SDS/2 mM Tris·HCl, pH 7.5/1 mM EDTA). Twenty µl of hybridization solution (1 M NaCl/33 mM Tris·HCl, pH 7.5/6.6 mM EDTA/0.2% SDS) containing 10,000 cpm of 32P-labeled oligonucleotide probe for apoA-IV or 28S ribosomal RNA (see below) was added. Samples were heated at 65°C for 10 min and incubated at 45°C for 16 h to allow formation of RNA-DNA hybrids. Unhybridized probe was digested with 16 U S1 nuclease (Gibco-BRL) in 1 ml S1 nuclease buffer (0.3 M NaCl/30 mM sodium acetate, pH 4.6/3 mM zinc acetate) for 1 h at 37°C. S1 nuclease-resistant hybrids were precipitated with 10 µl 100% trichloroacetic acid on ice for 1 h, and collected by filtration through GF/C glass fiber filters (Whatman). Filters were washed thoroughly with 5% trichloroacetic acid, dried, and placed in scintillation vials. Precipitates were released from filters by treatment with 1 ml Solvable (DuPont) at 55°C for 30 min, and radioactivity was determined in 9 ml Formula 989 cocktail (DuPont).

Sequences of antisense oligonucleotide probes used are as follows: mouse 28 S ribosomal RNA, 5'-GGCCAAGGCGGATCTCCCTGGC-3'; mouse apoA-IV, 5'-GGTTTGGGCCTGACCTGCTCC-3' (this sequence is invariant for strains C57BL/6 and 129). Oligo-
nucleotide probes were end-labeled with T4 polynucleotide kinase and [32P]ATP by standard methods. In each assay, yeast tRNA was included as a control for non-specific binding of oligo probes; background for tRNA samples was typically < 0.05% of input counts. To allow conversion of cpm of S1-resistant hybrids to absolute amounts of apoA-IV mRNA, known quantities (1.3 fmol, 3.3 fmol, 6.6 fmol, and 13.2 fmol) of a synthetic apoA-IV mRNA transcript were included in each assay. Unlabeled nuclear transcription run-off assay

Freshly isolated hepatocytes were prepared and pulse-labeled as described previously (21). Briefly, livers were perfused with chelator (1 mM EGTA) followed by collagenase (0.05% type V collagenase, Sigma). Hepatocytes were washed 3 times by low-speed centrifugation (50 g 2 min), and then incubated in a Dubnoff metabolic shaker at a concentration of about 10^7 cells/ml KRB-HEPES (Kreb's Ringer Bicarbonate supplemented with 10 mM HEPES) for 5 min before initiating the pulse (200 mCi/ml [35S]methionine). After 5, 10, 15, and 20 min, equal aliquots of 10^6 cells were quickly removed, pelleted, and lysed with 0.5 ml of ice-cold lysis buffer (3% Triton X-100, 0.1% N-lauryl sarcosine, 1 mM PMSF, 0.15 M NaCl, 0.1 M Tris, pH 7.5). Cell lysates were stored at ~80°C.

Rates of synthesis measurements

Rates of synthesis were also examined by administering 1 mCi [35S]methionine (in 0.15 M NaCl) intravenously in the tail vein of mice. Ten minutes after injection, mice were killed and the liver was quickly perfused with 0.15 M NaCl, removed, and homogenized in ice-cold lysis buffer containing 10 µg/ml cycloheximide. Total time from injection of label to liver homogenization was about 7 min.

Total protein synthesis was estimated by determining the incorporation of [35S]methionine into trichloroacetic acid-precipitable material. Apolipoproteins A-I, A-II, and A-IV were immunoprecipitated from hepatocyte and liver lysates with rabbit antiserum directed against purified mouse apoA-I and A-II (21), or bacterially produced mouse apoA-IV fusion protein (11). Immunoprecipitations were performed as described (22), and analyzed by electrophoresis in 12% (apoA-IV) or 14% (apoA-I and A-II) SDS-polyacrylamide gels. Relative apolipoprotein synthetic rates were determined by excising appropriate regions from dried gels, rehydrating in 1 ml 80% Protosol (New England Nuclear), and counting in 10 ml scintillant (Econoﬂuor (NEN)-Protosol-glacial acetic acid 20:1:0.04).

Immunoblotting

Individual mouse plasma samples (10 µl of 1:200 dilutions) were separated by electrophoresis through a 15% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to Amersham ECL membrane in transfer buffer (0.02 M Tris-HCl, pH 7.4, 0.15 M glycerol, 20% methanol) using a Bio-Rad Semi-Dry Blotter at 15 volts for 30 min. Blots were blocked overnight in TBST (0.01 M Tris-HCl, pH 7.4, 0.9% NaCl, 0.5% Tween-20) containing 5% non-fat dry milk, and then treated with 15% hydrogen peroxide for 15 min. Apolipoproteins were detected by incubating blots with antiserum against mouse apoA-IV fusion protein (11) (diluted 1:2500 in TBST) or apoA-I (21) (diluted 1:7500) for 2 h. Blots were washed 3 times for 10 min in TBS + 0.3% Tween-20, and then incubated for 1 h with horseradish peroxidase conjugated to goat anti-rabbit IgG (Amersham) diluted 1:7500 in TBS + 0.1% Tween-20. Final washes were in TBS + 0.3% Tween-20 (3 times for 5 min), followed by TBS + 0.1% Tween-20 (3 times for 5 min). Apolipoprotein bands were detected by chemiluminescence after incubation for 60 sec with Amersham ECL Detection Reagents, followed by exposure to Amersham Hyperﬁlm-ECL.

Nuclear transcription run-off assay

Nuclei were prepared from mouse liver according to Gorski, Carneiro, and Schibler (23). Nuclei from each liver were resuspended in 50 µl of nuclei storage buffer (20 mM Tris-HCl, pH 9.0, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% (v/v) glycerol), and stored at ~80°C. On the day of the experiment, nuclei were thawed on ice, and elongation reactions were performed at 26°C for 10 min (17). Radiolabeled RNA was isolated as described by Celano, Berchtold, and Casero (24). An equal number of counts from each run-off reaction (3 x 10^6 cpm/ml) was hybridized to plasmids that had been immobilized on a nylon filter. Plasmids used were pBS (Stratagene) containing no insert, or containing cDNA for mouse apoA-IV and for mouse actin. Hybridization signals were quantitated by densitometry.

mRNA turnover

Hepatocytes were isolated from C57BL/6 and 129 mice, suspended in culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin) at 3 x 10^6 cells/ml, and plated onto collagen-coated flasks. Cells were incubated at 37°C in 5% CO2 for 12 h, at which time cells were 80% confluent and had spread out to form junctional complexes. Culture medium was replaced with or without the addition of actinomycin D (10 µg/ml), and cells were incubated for timed intervals (0, 6, 12, 24, and 48 h). At the designated intervals, cells were rinsed with DMEM, lysed with guanidine thio-
 Allele-specific apoA-IV mRNA assay

A PCR amplification/endonuclease digestion assay was used to distinguish apoA-IV mRNA transcripts derived from C57BL/6 and 129 alleles in F1 mice based on a 12-nucleotide deletion in the 129 mRNA (11). Total mouse liver RNA was prepared by extraction in guanidine (18). RNA samples (10 µg) were treated with RQ1 DNase (Promega) at 37°C for 15 min to degrade any contaminating DNA. The RNase inhibitor RNasin (20 units, from C57BL/6 and 129 alleles in cyanate, and RNA was extracted. Relative mRNA concentrations were determined by Northern blotting followed by densitometry. Experiments were also performed with additional concentrations of actinomycin (5 and 15 µg/ml), and with the drug DRB (5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole, 100 µM, Calbiochem), with the same results as shown for actinomycin D at 10 µg/ml.

For the isolation of newly synthesized RNA, hepatocytes were cultured for 12 h as described above, and incubated with 4-thiouridine (100 µM; Sigma) and 5,6-[3H]uridine (20 µCi/ml). At the end of 1 h, total RNA was extracted. Newly synthesized RNA, which had incorporated the 3H and thiol labels, was purified from a portion of each total RNA sample by mercurated agarose affinity chromatography (25). The amount of radioactivity in each sample was determined by scintillation counting, and an equal number of counts of total RNA and newly synthesized (column purified) RNA from each hepatocyte sample were analyzed on Northern blots. Relative amounts of apoA-IV and apoA-I mRNA were determined by densitometry.

Allele-specific apoA-IV mRNA assay

One-tenth of the resulting cDNA was used for PCR. The primers used for PCR are as follows: 5'-CCTCTTTCAAGGACAAACTGCCTGCTCCTGA-3' (nucleotides 272-291, forward) and 5'-GGTTTGGGCTGCACCTGTGG (nucleotides 1254-1267, reverse). The reverse primer, which hybridizes 37 nucleotides downstream of the 12-nucleotide deletion in apoA-IV mRNA from strain 129, was end-labeled with T4 polynucleotide kinase and [32P]ATP. During the exponential phase of PCR amplification, the total amount of radioactivity incorporated from end-labeled primer is proportional to product copy number, and is independent of product size (26). We determined that amplification was within the exponential phase when PCR was terminated after 15 cycles (94°C for 0.5 min, 55°C 0.5 min, 72°C 1 min, extended to 10 min for the final cycle). To allow easy distinction between PCR products derived from C57BL/6 and 129 mRNA, PCR products were digested with endonuclease Hae III in buffer containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT. The restriction digest generated a labeled fragment of 49 nucleotides from C57BL/6 mRNA and 37 nucleotides from 129 mRNA. Samples were electrophoresed in a gel containing 7 M urea and 7% polyacrylamide (acrylamide-bis-acrylamide, 19:1) in 1 x TBE buffer (8.8 mM Tris borate, 8.8 mM boric acid, 2.0 mM EDTA). The gel was dried and exposed to Kodak XAR film at ~80°C. To quantitate counts incorporated into allele specific products, appropriate regions of the gel were excised, rehydrated, and counted in scintillant as described above.

RESULTS

Genetic variation in hepatic apoA-IV protein synthesis and plasma apoA-IV levels

In a previous study, inbred mouse strains were found to exhibit genetic variation in the levels of apoA-IV mRNA in liver, with the most extreme difference in mRNA levels occurring between strains C57BL/6 and 129 (10). To determine whether the difference in mRNA levels is reflected in apoA-IV protein synthetic rate, the relative levels of apoA-IV mRNA and protein synthesis were measured in the two strains. ApoA-IV mRNA levels were determined both by Northern blots and a solution hybridization assay, and normalized to β-actin mRNA and 28S ribosomal RNA, respectively. Densitometric quantitation of bands on Northern blots indicated that apoA-IV mRNA levels in liver were approximately 10-fold higher in 129 than in C57BL/6; apoA-I mRNA levels were approximately 1.5-fold higher in strain 129 (Fig. 1).

As the result from Northern blots was considerably different than the previous report of 90-fold higher levels of apoA-IV mRNA in 129 than C57BL/6 (10), we also measured apoA-IV mRNA levels using a solution hybridization assay. With this method, specific mRNA species hybridize to a radiolabeled oligonucleotide probe in solution; after removal of excess probe by S1 nuclease digestion, RNA-DNA hybrids are collected by precipitation and directly quantitated by scintillation counting.
This technique also allows determination of absolute mRNA levels by inclusion of a known amount of synthetic apoA-IV mRNA transcript produced from cloned cDNA sequences using a bacterial RNA polymerase (see Methods). The assay was performed with known amounts of the synthetic apoA-IV mRNA, and with aliquots of total liver RNA (10 µg, 20 µg, and 40 µg) from C57BL/6 and 129 mice. To ensure that the assay was specific, yeast tRNA samples were included and typically produced a background of less than 0.05% of input probe. To normalize samples from individual mice, 28S ribosomal RNA was also quantitated by solution hybridization. The levels of apoA-IV mRNA present in C57BL/6 and 129 liver are shown in Fig. 2. In agreement with the Northern blot analysis, these results show that 129 livers contained an average of 8.3-fold higher levels of apoA-IV mRNA than C57BL/6. This was true for mRNA prepared from numerous animals over the course of several months, and for RNA from both whole liver and isolated hepatocytes (not shown). The basis for the discrepancy from the previous study is unknown, but may include differences in methodology and non-genetic factors (see Discussion).

The relative rates of apolipoprotein synthesis were measured in hepatocytes from C57BL/6 and 129. Freshly isolated hepatocytes were pulse-labeled with [35S]methionine for timed intervals, and apolipoproteins A-I, A-11, and A-IV were immunoprecipitated and analyzed on SDS-polyacrylamide gels (Fig. 3). The relative rates of protein synthesis were determined by quantitating label in the immunoprecipitated protein bands and expressing counts as a fraction of total acid-precipitable material. As shown in Fig. 3 and Table 1, the two strains exhibited similar rates of synthesis for apolipoproteins A-I and A-11. However, the rate of [35S]methionine incorporation into apoA-IV was approximately 8.5-fold higher in hepatocytes isolated from 129 compared to C57BL/6 mice. This difference in liver apoA-IV synthetic rate was confirmed by in vivo labeling experiments, in which mice were injected intravenously with [35S]methionine for 15 min. Immunoprecipitation of apoA-IV and apoA-I from the liver of these animals confirmed that apoA-IV was specifically increased in the livers of strain 129 compared to C57BL/6 (not shown). These results demonstrate that the genetic variation between strains C57BL/6 and 129 occurs at both

Fig. 3. Relative rates of apolipoprotein synthesis in C57BL/6 and 129 liver. Freshly isolated hepatocytes from C57BL/6 and 129 were pulse-labeled with [35S]methionine for 5, 10, 15, and 20 min. Total protein synthesis was estimated by determining [35S]methionine incorporation into trichloroacetic acid-precipitable material and was linear with respect to pulse time. Apolipoproteins A-IV, A-I, and A-II were immunoprecipitated from equivalent amounts of total protein and analyzed by electrophoresis on SDS-polyacrylamide gels. The apoA-IV protein bands from C57BL/6 were visible on longer exposures of the autoradiogram (not shown).

### Table 1. Rates of apolipoprotein synthesis in C57BL/6 and 129 hepatocytes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Strain</th>
<th>Relative Rate of Synthesis</th>
<th>Ratio 129/C57BL/6</th>
<th>% of total synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td>C57BL/6</td>
<td>0.109 ± 0.014</td>
<td>1.2</td>
<td>28.0</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>129</td>
<td>0.130 ± 0.017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-II</td>
<td>C57BL/6</td>
<td>0.273 ± 0.039</td>
<td>0.8</td>
<td>35.9</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>129</td>
<td>0.215 ± 0.030</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-IV</td>
<td>C57BL/6</td>
<td>0.002 ± 0.001</td>
<td>8.5</td>
<td>0.3</td>
</tr>
<tr>
<td>ApoA-IV</td>
<td>129</td>
<td>0.017 ± 0.003</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isolated hepatocytes from C57BL/6 and 129 mice were labeled and apolipoproteins were quantitated as described in Methods. Relative rates of synthesis for apolipoproteins A-I, A-II, and A-IV are expressed as a percentage of the [35S]methionine incorporated into total protein. Incorporation of label was linear over 20 min and each determination represents the average ± standard deviation for four time points.
Apo A-IV in the blood is derived from both hepatic and intestinal synthesis (27). As demonstrated previously, however, intestinal apoA-IV mRNA levels do not vary significantly between strains C57BL/6 and 129 (10). We were interested to determine whether the genetic variation in hepatic apoA-IV synthesis alone could have an effect on the level of circulating apoA-IV. The relative levels of apoA-IV in the plasma of C57BL/6 and 129 mice were determined by immunoblot analysis and quantitated by densitometry. Fig. 4 shows that the apoA-IV concentration in plasma from 129 mice is approximately 1.8-fold higher than C57BL/6. Thus, the genetic variation in liver apoA-IV expression appears to influence the steady state concentration of apoA-IV in the blood, although it is possible that genetic differences in apoA-IV catabolism could also contribute to higher plasma levels in strain 129.

Genetic variation in apoA-IV expression occurs at both transcriptional and post-transcriptional levels

Steady state mRNA levels represent a balance between rates of transcription, transport, and turnover. To investigate whether a genetic variation in efficiency of apoA-IV gene transcription contributes to the difference in C57BL/6 and 129 mRNA levels, nuclear run-off assays were performed. Liver nuclei were isolated and nascent transcripts were allowed to elongate in the presence of radiolabeled-UTP. The elongated transcripts were then hybridized to filters containing cDNA clones for apoA-IV and actin, as well as a plasmid without insert as a control. As shown in Fig. 5, the labeled RNA transcripts did not hybridize to the control plasmid DNA, but did hybridize specifically to the apoA-IV and actin cDNAs. The relative amounts of apoA-IV and actin mRNA run-off transcripts were determined by densitometry. When normalized to actin, the rate of apoA-IV transcription in liver nuclei from strain 129 was 1.8-fold higher than in C57BL/6 nuclei (average of duplicate assays). This difference does not reflect the 8- to 10-fold difference between C57BL/6 and 129 steady state mRNA levels, and indicates that post-transcriptional events, such as mRNA turnover, may also contribute to the genetic variation.

Post-transcriptional events that may influence mRNA concentration include nuclear mRNA processing, transport from the nucleus, and degradation in the cytoplasm. Northern blot analysis of RNA prepared from isolated hepatocyte nuclei showed no evidence for an accumulation of unprocessed apoA-IV mRNA, and the genetic variation in apoA-IV mRNA levels was apparent in RNA prepared from hepatocyte cytoplasm (data not shown). These results suggest that neither mRNA processing nor transport events could account for the quantitative variation in apoA-IV mRNA levels, and that perhaps a difference in apoA-IV mRNA turnover rate may occur in the two strains.

A standard approach to measure mRNA half-life is to treat cells with drugs that inhibit transcription, and determine the amount of the specific mRNA remaining at timed intervals. We incubated freshly isolated hepatocytes from C57BL/6 and 129 mice with actinomycin D to inhibit mRNA transcription, and determined the levels of apoA-IV, apoA-I, and albumin mRNA remaining at timed intervals. Although apoA-I mRNA levels diminished approximately 50% over 24 h, apoA-IV and albumin mRNA levels remained relatively constant, indicating that apoA-IV mRNA turnover is slower than the other two mRNAs. This difference may be due to differences in apoA-IV mRNA stability, which could be influenced by post-transcriptional events such as mRNA degradation.

Post-transcriptional events that may influence mRNA concentration include nuclear mRNA processing, transport from the nucleus, and degradation in the cytoplasm. Northern blot analysis of RNA prepared from isolated hepatocyte nuclei showed no evidence for an accumulation of unprocessed apoA-IV mRNA, and the genetic variation in apoA-IV mRNA levels was apparent in RNA prepared from hepatocyte cytoplasm (data not shown). These results suggest that neither mRNA processing nor transport events could account for the quantitative variation in apoA-IV mRNA levels, and that perhaps a difference in apoA-IV mRNA turnover rate may occur in the two strains.
mRNA levels did not decrease significantly, even after 48 h (data not shown). Similar results were obtained when the transcription inhibitor DRB was used in place of actinomycin D (data not shown). These observations suggest that apoA-IV mRNA is long-lived, and that normal turnover of apoA-IV mRNA requires continued synthesis of proteins that become depleted in transcription-inhibited cells.

The results described above indicated that the traditional methodology for mRNA half-life determination is not adequate for studies of apoA-IV mRNA turnover. We therefore used an alternative method that allows an estimation of mRNA half-life based on the simultaneous measurement of mRNA synthesis and steady state concentration within the same sample. With this approach, newly synthesized mRNA is tagged by incorporation of thiolated nucleotide triphosphates and separated from preexisting RNA by affinity chromatography (25, 28). The concentrations of mRNAs of interest are determined in both the newly synthesized fraction and in an analogous amount of total RNA. Since steady state mRNA concentration is determined by the rates of both mRNA synthesis and mRNA turnover, information about two of these parameters within the same sample (steady state and synthesis) provides information about the third parameter (turnover).

We used this technique to compare the relative apoA-IV mRNA half-life in hepatocytes from C57BL/6 and 129 mice. The relative concentrations of steady state and newly synthesized apoA-IV mRNA were determined as described under Methods. As shown in Fig. 6, the steady state concentration of apoA-IV mRNA is approximately 11-fold higher in strain 129 compared to C57BL/6. In contrast, the concentration of apoA-IV mRNA synthesized during a 1-h period and isolated from an equivalent amount of total RNA is only 1.5-fold higher in strain 129, in good agreement with the nuclear run-off data. As a control, apoA-I mRNA levels were also determined and were found not to differ significantly between the strains (not shown). These results indicate that, in addition to a difference in apoA-IV mRNA transcription rate, a genetic variation in apoA-IV mRNA half-life contributes to the difference in hepatic apoA-IV mRNA levels in strains C57BL6/6 and 129.

A combination of cis- and trans-acting genetic elements control apoA-IV mRNA levels

To determine whether the variation in apoA-IV mRNA levels is controlled by the apoA-IV gene locus, we examined the expression of each allele in (C57BL/6 x 129) F1 animals. If mRNA levels are controlled entirely by cis-acting elements linked to the apoA-IV gene, the 129 allele should show 8- to 10-fold higher expression than the C57BL/6 allele. However, if apoA-IV mRNA levels are influenced by trans-acting factors that act equally on both alleles, equal amounts of 129 and C57BL/6 mRNA should be produced. Alternatively, a trans-acting factor that exerts an allele-specific effect on the parental apoA-IV genes might give rise to a novel expression pattern in the F1 mice.

ApoA-IV mRNA derived from C57BL/6 and 129 alleles can be distinguished by virtue of the 12 nucleotide deletion in the mRNA from strain 129 (11). This polymorphism allowed us to resolve apoA-IV mRNA transcribed from 129 and C57BL/6 alleles using a PCR/restriction enzyme assay (see Methods). Liver RNA from both parental strains and F1 mice were used as templates for cDNA synthesis, followed by PCR amplification. PCR products were labeled at one end by incorporation of a radiolabeled primer, and PCR was terminated during the exponential phase of amplification. Under these conditions, the amount of radioactivity incorporated from the labeled primer is directly proportional to product copy number, and could be used to compare the concentrations of mRNA derived from the two apoA-IV alleles (26). The PCR products were digested with a restriction enzyme that cuts near the deletion polymorphism to produce different sized fragments for the two apoA-IV alleles.

As shown in Fig. 7, the assay products derived from C57BL/6 (49 nucleotides) and 129 mRNA (37 nucleotides) were easily distinguished on denaturing polyacrylamide gels. To verify that products of the assay were derived exclusively from RNA and not from contaminating genomic DNA, control reactions were performed using
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Reduced, indicating that DNA does not contribute to the assay products produced from RNA samples. In contrast, levels is complex. As a higher concentration of apoA-IV transcripts is derived from the 129 allele than from the C57BW6 alleles was 3:1 (Fig. 7, lanes e-g), as determined from BL/6 and 129 apoA-IV mRNA in (C57BL/6 x 129)F1 mice. Liver RNA from C57BL/6, 129, and (C57BL/6 x 129)F1 mice were used as templates for a PCR/restriction enzyme assay to distinguish between C57BL/6 and 129 apoA-IV mRNA (see Methods). Products of the assay were resolved by electrophoresis on a denaturing polyacrylamide gel. Sizes of products from C57BL/6 (49 nucleotides) and 129 mRNA (37 nucleotides) are indicated by arrows. Templates used for the PCR/restriction enzyme reactions are as follows: (a) 129 liver RNA, omission of reverse transcriptase in first step of the assay; (b) apoA-IV cDNA plasmid containing C57BL/6 apoA-IV sequence; (c) 129 liver RNA; (d) C57BL/6 liver RNA; (e-g) (C57BL/6 x 129)F1 liver RNA. The signal corresponding to C57BL/6 mRNA in lane (d) was visible on longer exposures of the autoradiogram (not shown).

129 RNA with the omission of reverse transcriptase (Fig. 7, lane a). Under these conditions, no product was produced, indicating that DNA does not contribute to the assays. Under these conditions, no product was produced, indicating that DNA does not contribute to the assaying products produced from RNA samples. In contrast, when cDNA containing C57BL/6 apoA-IV sequence is included, the expected 49-nucleotide product is produced (Fig. 7, lane b). With liver RNA from 129 and C57BL/6, products of the expected sizes and of intensities that reflect the 10-fold difference in mRNA concentration between strains were produced (Fig. 7, lanes c and d). In F1 mice, the ratio of apoA-IV mRNA derived from 129 and C57BL/6 alleles was 3:1 (Fig. 7, lanes e-g), as determined by scintillation counting of bands isolated from the gel. This indicates that the genetic control of apoA-IV mRNA levels is complex. As a higher concentration of apoA-IV transcripts is derived from the 129 allele than from the C57BL/6 allele, cis-acting elements linked to the apoA-IV gene play a role in determining mRNA levels. However, because the ratio of apoA-IV mRNA derived from the two alleles was less than the 8- to 10-fold difference in mRNA levels observed in the parental strains, genetically distinct trans-acting factors are also involved.

DISCUSSION

Eucaryotic gene expression is controlled at many levels, including mRNA transcription, turnover, and translation. These processes may be modulated by a variety of genetic and environmental factors. The variation in apoA-IV mRNA levels among inbred mouse strains provides an interesting model for the identification of genetic determinants of apolipoprotein expression. In this study, we have investigated the genetic and molecular bases for a variation in hepatic apoA-IV mRNA levels between strains C57BL/6 and 129. The difference in mRNA levels occurs in RNA prepared from both total liver and from isolated hepatocytes, indicating that the variation is not due to aberrant expression in another cell type found in liver, such as Kupffer, endothelial, or fat-storing (Ito) cells.

The reason for a discrepancy between our estimate of an 8- to 10-fold variation in mRNA levels and the previous report of a 90-fold variation (10) is not clear; however, there are several distinctions between the two studies that may contribute, including number of mice examined, differences in methodology, and the influence of environmental and non-genetic factors. In the previous study, several mouse strains were surveyed for apoA-IV mRNA levels using only a small number of mice (2-7) from each strain (10). In contrast, we have assayed apoA-IV mRNA levels in at least 30 mice from each of the two strains over the course of several months with two independent quantitation methods. During this time, we have also observed that non-genetic variation may affect apoA-IV mRNA levels, with individual animals occasionally showing a specific aberration in apoA-IV mRNA concentration although other mRNA levels appear normal. Numerous studies have previously demonstrated that apoA-IV expression is acutely responsive to dietary fat content and to hormonal factors (9, 27, 29-31), which, in turn may be influenced by age, gender, cage effects, and season of the year. Mice used in both the current and previous studies were maintained on a commercial mouse chow diet for which the fat composition is not specified by the manufacturer, and which is known to vary from batch to batch (32). Additional variables mentioned above are not specified in the previous study, making it difficult to rule them out as contributing factors. Most important for the current study is the fact that all of these variables were controlled over the course of our study, and that all apoA-IV mRNA and protein measurements were internally consistent.

The higher concentration of apoA-IV mRNA in strain 129 liver is directly reflected in the rate of apoA-IV protein synthesis, as determined by pulse-label experiments. Strain 129 also has 3-fold higher levels of apoA-IV in the circulation. It is not known whether this is a direct consequence of the increased hepatic synthesis, or is influenced by additional factors, such as apoA-IV turnover rates in
plasma. Much of the apoA-IV in blood is associated with HDL, and it has been implicated in the interconversion of HDL subfractions and reverse cholesterol transport (3, 6, 33). In human population studies, a particular apoA-IV isoform (A-IV-2) has been associated with higher HDL and lower triglyceride levels (34). As described previously, apoA-IV from strain 129 differs in structure from that of C57BL/6 and several other inbred strains (11). Both 129 apoA-IV and human apoA-IV-2 differ from the more prevalent isoforms for each species in having variations within a repeated amino acid motif near the carboxyl-terminus of the protein. Thus, strain 129 may represent a useful model for studying the effects of elevated expression of a variant apoA-IV protein on various aspects of lipid transport.

The molecular basis for the genetic variation in apoA-IV mRNA levels involves both transcriptional and post-transcriptional processes. In transcription run-off experiments with isolated liver nuclei, the relative rate of apoA-IV transcription was 1.8-fold higher in 129 than in C57BL/6. As this difference does not account for the 8-to 10-fold higher steady state mRNA concentration in 129 liver, we investigated the relative half-life of apoA-IV mRNA in hepatocytes from the two strains. Using the standard approach of treating hepatocytes with the transcription inhibitors actinomycin D and DRB, levels of apoA-IV mRNA did not diminish, although levels of apoA-I mRNA decreased by 50%. This phenomenon, in which specific mRNA species do not decay in the presence of transcription inhibitors, has been described for a number of mRNAs, including albumin, tyrosine amino transferase, transferrin receptor, myosin heavy chain, and insulin growth factor-1 (28, 35). This effect is most common with long-lived mRNA species, and presumably occurs because transcription of all genes is inhibited, thus preventing synthesis of proteins required for mRNA turnover. The fact that apoA-IV mRNA levels do not decrease after 24 h in culture suggests that, in comparison to apoA-I and albumin mRNA, apoA-IV mRNA is long-lived, or that factors required for its turnover differ from those required for several other abundant liver mRNAs.

With the use of a thiol-nucleoside labeling technique, it was possible to compare the apoA-I mRNA half-life in hepatocytes from C57BL/6 and 129. Newly synthesized mRNA was labeled with thiolated UTP and isolated by affinity chromatography. ApoA-IV mRNA concentrations were then determined in analogous portions of total RNA and newly synthesized RNA fractions. Although the concentration of apoA-IV mRNA was 11-fold higher in 129 than in C57BL/6 hepatocytes, the amount synthesized in 1 h was only 1.5-fold higher in 129. These results indicate that the higher apoA-IV mRNA levels in strain 129 result from a combination of faster mRNA synthesis and slower mRNA degradation.

The genetic variation in apoA-IV mRNA levels involves both cis-acting elements linked to the apoA-IV gene, and a genetically distinct trans-acting factor(s). Using a sequence variation to distinguish between mRNA derived from 129 and C57BL/6 alleles in F1 mice, we determined that the 8-fold difference that occurs in the two strains is not maintained in the liver of F1 mice. This cis/trans test indicates that trans-acting genetic loci influence apoA-IV expression. Nevertheless, as 129 mRNA is present at a 3-fold higher concentration than C57BL/6 mRNA, cis-acting elements must also play a role. These results are in logical agreement with the molecular basis for the variation in mRNA levels, as the processes of both gene transcription and mRNA turnover involve the interaction of nucleic acid sequences (cis-acting elements) with proteins (trans-acting factors). Transcription requires the recognition of promoter or gene sequences by protein transcription factors, and mRNA turnover is mediated by the interaction of nucleases and other proteins with specific sequences or structures present in the mRNA. As described previously, 129 apoA-IV mRNA differs from that of strain C57BL/6 at four sites: single nucleotide changes at one site each in the 5'-untranslated, coding, and 3'-untranslated regions, and 12-nucleotide deletion within the coding region (11). It is possible that these sequence variations, or additional differences within apoA-IV gene regulatory or intronic sequences, represent cis-acting elements that influence apoA-IV expression levels. The nature of the trans-acting factor(s) that differs between C57BL/6 and 129 is not known; however, the availability of recombinant inbred strains derived from a cross between C57BL/6 and 129 may allow future genetic mapping of trans-acting factors that influence apoA-IV expression.

Genetic variations affecting other apolipoproteins have been described and characterized at the molecular level. These include variations in apoA-I expression in both humans and simians that are associated with sequence polymorphisms in the apoA-I gene promoter (36, 37). A genetic polymorphism occurring 76 bases upstream of the human apoA-I gene transcription start site leads to decreased promoter activity in vitro, and diminished apoA-I production rate in vivo (36). A divergence in gene regulatory sequences has also been implicated in the variation in apoA-I expression between African green and cynomolgus monkeys (37). A 2- to 3-fold higher abundance of apoA-I mRNA in African green monkeys correlates with a 2-fold higher apoA-I transcription rate, and increased promoter activity in vitro. Several points of sequence divergence exist in the apoA-I 5' regulatory region that could contribute to the variation in expression between the two monkey species. A genetic variation in mouse apoA-II expression has also been described (38). Inbred mouse strains exhibit a 2-fold variation in apoA-II protein levels and rates of synthesis, although mRNA levels are not discernibly different. It was shown that the two strains
differ in apoA-II translational efficiency, possibly related to nucleotide differences in apoA-II mRNA sequence. The variation in mouse apoA-IV mRNA expression represents a genetic polymorphism that affects gene expression at yet another level, mRNA turnover. Further characterization of these genetic variants may increase our understanding of the general mechanisms for gene expression and the molecular basis for individual variation.

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