A solution hybridization/RNase protection assay with riboprobes to determine absolute levels of apoB, A-I, and E mRNA in human hepatoma cell lines

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Summary A solution hybridization/RNase protection assay with riboprobes was developed to quantitate apolipoprotein mRNA concentrations. Previously, radiolabeled DNA probes have been used in solution hybridization/S1 nuclease protection assays for this purpose. The new assay requires less time for probe preparation and hybridization compared to previous assays. In addition, the vector used for riboprobe preparation can also be used to conveniently produce cRNA required to generate the standard curve to quantitate absolute apolipoprotein mRNA levels. The solution hybridization RNase protection assay was used to quantitate apoB, A-I, and E mRNA levels in four human hepatoma cell lines, HepG2, Hep3B, WRL-68, SK-Hep2. HepG2 and Hep3B, but not WRL-68 and SK-Hep2 cells had concentrations of all three apolipoprotein mRNAs comparable to liver in vivo. These data suggest that HepG2 and Hep3B are suitable models to study liver specific apolipoprotein gene expression. —**Azrolan, N., and J. L. Breslow.** A solution hybridization/RNase protection assay with riboprobes to determine absolute levels of apoB, A-I, and E mRNA in human hepatoma cell lines. *J. Lipid Res.* 1990. **31:** 1141–1146.

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The quantitation of apolipoprotein mRNA in tissues and cell cultures is an important part of many studies designed to understand the role of apolipoprotein synthesis in lipoprotein metabolism. Current methods for performing such quantitation include the use of radiolabeled cDNA or genomic double-stranded DNA probes for Northern or slot blotting analysis or single-stranded DNA probes for solution hybridization followed by S1 nuclease digestion (1, 2). Each of these methods has limitations. The Northern and slot blotting techniques require long hybridization times, are insensitive and only semiquantitative, and provide relative not absolute quantitation of mRNA levels. The solution hybridization S1 nuclease digestion technique requires lengthy probe preparation, long hybridization times, and for quantitation utilizes a standard curve of M13 DNA that has different hybridization kinetics than mRNA (2, 3).

In the current study, we report the development of a solution hybridization/RNase protection assay using riboprobes containing sequences corresponding to apoB, A-I, and E coding regions. This assay involves short probe preparation time, fast hybridization, and provides sensitive and specific quantitation of absolute apolipoprotein mRNA levels. In addition, the same vector can be used to prepare a standard cRNA. We have applied this method to determine the absolute levels of apoB, A-I, and E mRNA in four human hepatoma cells lines.

METHODS

Cell cultures

Human hepatoma cell lines HepG2, Hep3B, SK-Hep1, and WRL-68 were obtained from the American Type Culture Collection (Rockville, MD) and grown in Eagle's MEM supplemented with 2 mM glutamine and 10% fetal bovine serum. Stock cells were subcultured weekly in 75-cm^2 flasks. All cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2. HepG2 cells were subcultured at a 1:4 ratio; Hep3B, SK-Hep1, and WRL-68 cells at a 1:6 ratio. Stock cultures were supplied with fresh medium twice weekly.

Cellular RNA isolation and Northern blotting analysis

Cells were grown to 90% confluence in 100-cm^2 Falcon plastic Petri dishes. Six dishes of each cell type were used for RNA isolation. The cells were washed three times with cold phosphate-buffered saline, scraped into 6 M guanidinium thiocyanate, and RNA was isolated by serial precipitations in guanidinium–HCl/ethanol according to Chirgwin et al. (4). These RNA preparations were stored as precipitates at −70°C in the presence of 200 mM Na acetate and 72% ethanol.

For Northern analysis, 20 μg of total cellular RNA was electrophoresed in formaldehyde-containing gels (1). Comparable sample loading in each lane was verified by ethidium staining. The electrophoresed RNA was transferred to nylon membranes (Genescreen; NEN) via capillary blotting. The filters were baked in vacuo at 80°C for 2 h and then utilized immediately for hybridization to ^{32}P-labeled riboprobes. Riboprobe hybridizations were performed at 65°C for 18 h in the presence of 300 μg/ml salmon sperm DNA, 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, 10 mM EDTA), 0.2% Blotto, 0.5% SDS, 50% formamide, and 10^6 cpm/ml probe. Filters were washed at 68°C in 0.1×SSC (1×SSC is 0.15 M NaCl plus 0.015 M Na citrate) and 0.2% SDS. The filters were dried and then exposed to Kodak XAR film at −70°C with dual intensifying screens. After development, signal intensity was determined using an LKB ultroscan XL laser densitometer.

Riboprobes and cRNA preparation

A 296nt portion of the apoB gene in the coding sequence, nt 4650–4946 (5) was isolated by a SalI/BamHI

Abbreviations: apo, apolipoprotein; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate.

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digestion of an apoB gene cosmid (Cos B1(6)) and cloned into a Sal I/Bam HI-digested pGem1 vector (Promega). A 476nt portion of apoA-I cDNA, nt390–866 (7), was isolated by a BamHI/PstI digestion of an apoA-I cDNA clone (pAI 113(8)) and ligated into a BamHI/PstI-digested pGem1 vector. A 157nt sequence of apoE cDNA, nt514–671 (9), was isolated from a PstI digestion of an apoE cDNA clone (pE301(10)) and ligated into a PstI-digested pGem1 vector. A 230nt segment of γ-actin cDNA was isolated by HindIII/XbaI digestion of a γ-actin cDNA clone (PHF1(11)), and ligated into a HindIII/XbaI-digested pGem1 vector.

The pGem1 vectors with the cDNA inserts were linearized by restriction enzyme digestion 5' to the insert and riboprobes corresponding to the antisense DNA strand were synthesized with SP6 RNA polymerase in the case of apoB and T7 RNA polymerase for apoA-I, apoE, and γ-actin. ^{32}P-labeled apoB, A-I, and E riboprobe synthesis was performed in a 50-μl reaction volume in 1.5-ml microfuge tubes. The reaction mixture contained: 10 μl linearized plasmid (0.8–1.0 μg), 10 μl of 5× RNA polymerase transcription buffer (BRL), 8 μl of ribonucleotide solution (containing 3.3 mM each of ATP, CTP, and GTP, pH 7.0), 2 μl 100 mM DTT, 2 μl RNasin (Promega), 8 μl α ^{32}P-UTP (400 mCi/mmol) and 9 μl DEPC-treated water. Riboprobe synthesis was initiated by adding 1 μl containing 10 units of either SP6 or T7 RNA polymerase. The reaction tubes were incubated in a water bath at 37°C. After 45 min, 2 μl DNase I (RNase-free, Boehringer Mannheim Biochemicals) and 2 μl RNasin were added and the tubes were incubated an additional 15 min. To end the reaction, 5 μl containing 50 μg of yeast tRNA and 55 μl of 4 M ammonium acetate was added to the tube, and the riboprobes were precipitated with 280 μl of cold ethanol (95%). The pellets were washed three times with cold 70% ethanol and resuspended in 100 μl TE (10 mM Tris, pH 7.5, 0.1 mM EDTA). The incorporation of radioactivity into each riboprobe was determined by TCA precipitation of 1 μl of the TE solution, collection of precipitated riboprobe on G F/L glass fiber filters (Whatman), and scintillation counting. In typical apolipoprotein probe preparations 6–10 × 10^5 dpm/μl were incorporated. ^{3}H-labeled γ-actin riboprobe synthesis was carried out according to the scheme just described, except that 5,6-^{3}H UTP, GTP, ATP, and CTP (100 μ Ci; 40 mCi/mmol) were added in lieu of the unlabeled nucleotides and [^{32}P]UTP. In a typical γ-actin probe preparation, 1 × 10^6 dpm/μl were incorporated. It is important to note that due to high specific activity, the ^{32}P- and ^{3}H-labeled riboprobes are kept at 4°C, and must be utilized within 16 h after synthesis to avoid the complication of probe degeneration.

Unlabeled cRNA corresponding to the sense DNA strand was prepared from each of pGem1 vectors containing apolipoprotein and γ-actin sequences. The plasmids were linearized 3' to the DNA insert and cRNA complimentary to each riboprobe was synthesized using either SP6 or T7 RNA polymerase according to Melton et al. (12). The plasmid template was eliminated by digestion with DNase I. The cRNA was then purified, solubilized in TE, quantified spectrophotometrically at 260 nm, and frozen in aliquots at −70°C. Prior to use as a hybridization standard, aliquots were thawed on ice and the concentration was adjusted to 10 pg/μl.

Solution hybridization/RNase-protection/filter counting assay procedure

One to 20 μg of total cellular RNA precipitate is centrifuged in a 1.5-ml polypropylene microfuge tube for 30 min. The supernatant is then carefully decanted. ^{32}P-labeled apolipoprotein riboprobe (2 × 10^5 dpm per 100nt probe length) plus ^{3}H-labeled γ-actin riboprobe (2 × 10^6 dpm) are then added with 25 μl hybridization solution (80% formamide, 40 mM HEPES, pH 6.7, 0.4 M NaCl, 1 mM EDTA) and overlayered with 25 μl of paraffin oil. The tubes are then incubated at 63°C in a water bath for 3.5 h. Three hundred μl of ice-cold RNase solution (0.3 M NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, with 24 μg RNase A and 3 units T_1 RNase per μg total RNA assayed) is then added below the paraffin oil layer with a pipet and the tubes are immediately removed to a 34°C bath. After a 40-min incubation, 15 μl (150 μg) of yeast tRNA plus 400 μl of cold 20% TCA are added, and the tubes are kept on ice for 15 min. The samples are then filtered through a G F/L glass fiber filter (Whatman). The filters are washed extensively with cold 7% TCA, dried, and counted in 10 ml scintillation fluid (Readysafe; Beckman) in a Beckman model LS5000 TD scintillation counter calibrated for ^{3}H/^{32}P dpm. It was determined that it is not necessary to solubilize the RNA from the filter prior to counting.

RESULTS

Riboprobes detect apolipoprotein mRNA in human hepatoma cell lines by Northern analysis

Riboprobes to apoB, A-I, and E mRNA were used to survey four human hepatoma cell lines by Northern analysis and the results are shown in **Fig. 1.** HepG2 and Hep3B contained all three messages, whereas SK-Hep1 contained only a small amount of apoB mRNA and WRL-68 contained none of them. The RNA preparation from all four cell lines hybridized to a γ-actin riboprobe control. Laser densitometric scanning of the Northern blot signals indicated that HepG2 when compared to Hep3B contained equal amounts of apoB and E mRNA

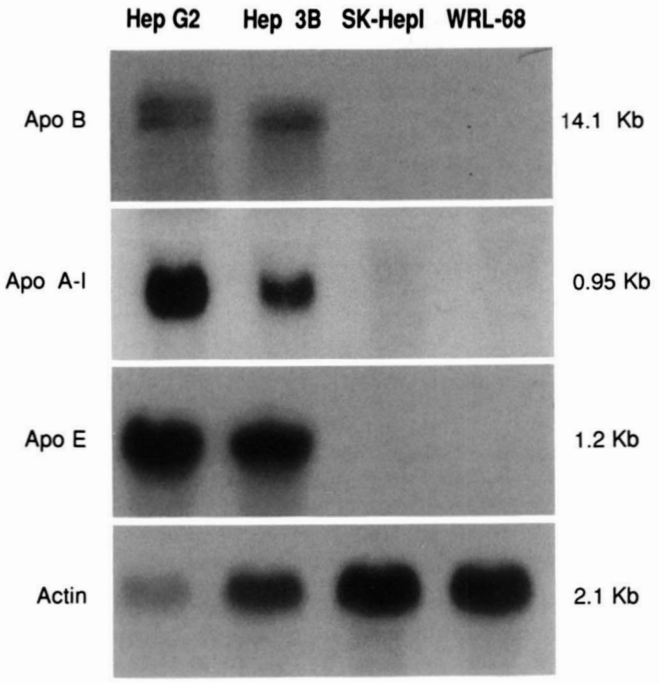

Fig. 1. Northern analysis of apolipoprotein mRNA levels in human liver cell lines. Total RNA was isolated from cultured cells as described in Methods. Twenty μg of each sample was analyzed by the Northern blot using formaldehyde-containing gels (1). RNA integrity and input equivalence were verified by ethidium bromide staining prior to blotting. The figure was assembled from strips cut from autoradiograms of the blots. The size (in nucleotides) of each band is shown on the right. The probe used for each strip is shown on the left.

and 3.2 times as much apoA-I mRNA. The γ-actin signal was least in HepG2 cells, more in Hep3B, and most in SK-Hepl and WRL-68, suggesting an inverse relationship with apolipoprotein mRNA concentrations.

Riboprobes are specifically protected from RNase digestion by hepatoma cell line RNA

To verify the specificity of each riboprobe for its respective mRNA, labeled riboprobes were hybridized in solution to HepG2 cell RNA. After treatment with RNase A and T_1, the protected fragments were resolved by gel electrophoresis and the autoradiogram is shown in **Fig. 2.** The apoB riboprobe (319nt) resulted in an RNase-resistant protected fragment of 296nt. The apoA-I riboprobe (550nt) resulted in a protected fragment of 476nt and the apoE riboprobe (241nt) one of 157nt. All of the protected fragments would be predicted by a complete match of each of the riboprobes with their respective mRNAs. In addition, none of the riboprobes showed detectable RNase-resistant bands after hybridization with yeast tRNA.

Apolipoprotein mRNA quantitation by an RNase protection solution hybridization/filter counting assay

To generate a standard curve, apolipoprotein cRNAs were hybridized in solution to uniformly labeled apoB, A-I, or E riboprobes. After RNase digestion, protected radiolabeled probe was precipitated, collected on a filter, and counted. When yeast tRNA was used instead of cRNA, the background was typically less than 0.08% of the total cpm in each reaction mixture. As shown in the left hand side of each panel of **Fig. 3,** hybridization was linear for each riboprobe up to 100 pg of cRNA. The specificity of the RNase protection/filter counting assay was confirmed by the observation that 35 pg of γ-actin cRNA in each hybridization had no effect on the linearity of the standard curve with each of the apolipoprotein cRNAs from 0 to 100 pg. It was also determined that increasing amounts of apolipoprotein cRNA from 0 to 100 pg per assay had no effect on the level of the γ-actin cRNA hybridization signal.

The expression of apolipoprotein mRNA in hepatoma cell lines was next quantified by the solution hybridization assay. As shown in the right hand side of each panel of Fig. 3, the hybridization signal for each riboprobe was linear to 10 μg of HepG2 RNA. The hybridization of 5 μg of RNA from all four hepatoma cell lines with each of the riboprobes is shown in **Table 1.** Multiple measurements were shown to be quite reproducible with standard deviations at 10% or less of the mean. In each assay a ^{3}H-labeled γ-actin riboprobe was simultaneously present to also quantitate γ-actin mRNA concentrations. Table 1 also shows that the γ-actin measurement had the same

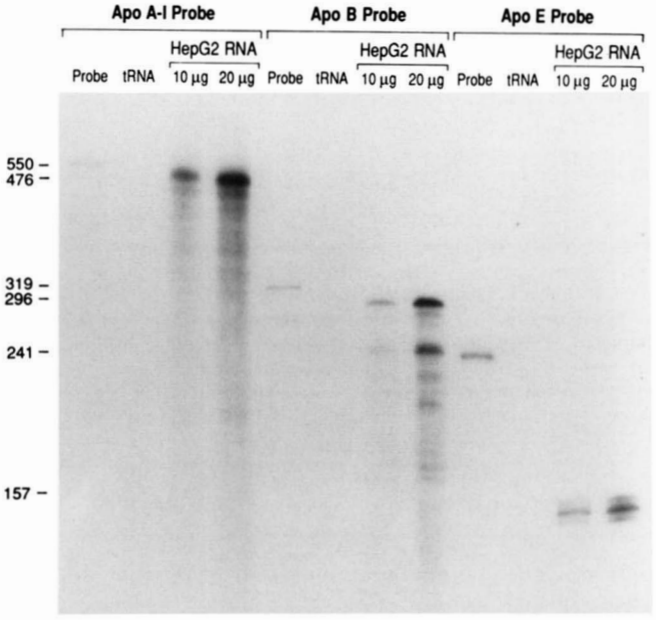

Fig. 2. RNase-protected fragments of riboprobe-hybridized apolipoprotein mRNA. Apolipoprotein mRNA-specific riboprobes were hybridized in solution to 20 μg of yeast tRNA or 10 and 20 μg HepG2 RNA. The samples were digested with both RNase "A" and "T_1" and subjected to electrophoresis through an 8 M urea, 8% polyacrylamide gel. The sizes of gel bands were determined by direct comparison to ^{32}P-labeled MspI-cut pBR322 restriction fragment and are provided at the left side of the figure.

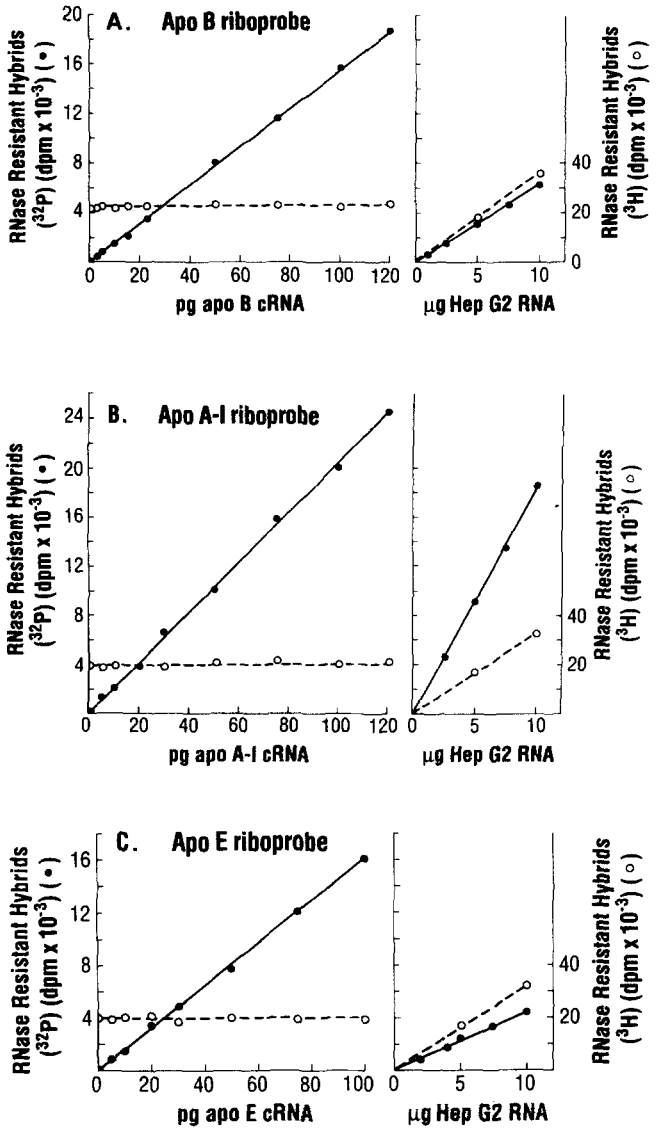

Fig. 3. Measurement of human apolipoprotein mRNA. Sense-strand cRNA hybridization standards were synthesized from cDNA in vitro as described in Methods and were utilized in standard curves (left). Actin cRNA (35 pg) was added to each apolipoprotein solution hybridization standard. ^{3}H-Labeled γ-actin (○---○) and ^{32}P-labeled apolipoprotein (●—●) B(A), A-I(B), or E(C) probes were used together in protection assays to generate the curves. RNA isolated from HepG2 cells was hybridized in solution to both actin (^{3}H; ○---○) and apoB(A), A-I(B), or E(C) riboprobes (^{32}P; ●—●) (right).

reproducibility as the apolipoprotein measurements and was identical no matter which apolipoprotein riboprobe was also present. It can be seen that the degree of RNase protections for each riboprobe differed greatly between cell lines. The HepG2 and Hep3B RNA produced high levels of protection for all three apolipoprotein riboprobes, whereas SK-Hep1 and WRL-68 produced little to no protection. With regard to the γ-actin riboprobe, the opposite was observed with HepG2 and Hep3B RNA producing less protection than SK-Hep1 or WRL-68.

TABLE 1. RNase resistance of riboprobe-protected RNA

Cell Line	RNase-Resistant Hybrids		
	ApoB	ApoA-I	ApoE
	dpm		
HepG2	3081 ± 275	9152 ± 728	3127 ± 236
γ-Actin	17394 ± 1658	17126 ± 1586	17423 ± 1751
Hep3B	3195 ± 267	3756 ± 357	2025 ± 194
γ-Actin	45182 ± 4112	46977 ± 3847	44360 ± 4081
SK-Hep1	230 ± 27	ND	ND
γ-Actin	100735 ± 10538	99557 ± 9196	100476 ± 8402
WRL-68	ND	ND	ND
γ-Actin	80034 ± 6035	83243 ± 6851	80272 ± 7583

Apolipoprotein and γ-actin riboprobes were hybridized in solution with 5 μg RNA isolated from the indicated cell line, digested with RNase, and quantified by scintillation counting as described in Methods. Each value represents the mean ± SD of six independent determinations; ND, not detectable.

Utilizing the standard hybridization curves generated with a known amount of apolipoprotein cRNA (see left-hand side of each panel of Fig. 3), apolipoprotein mRNA concentrations in four different hepatoma cell lines were calculated with the RNase resistance data presented in Table 1. These results are shown in **Table 2** and reveal that HepG2 and Hep3B have similar levels of apoB mRNA (190 pg/μg total cellular RNA). In addition, HepG2 had 2.4- and 1.5-fold higher levels of apoA-I and apoE mRNA, respectively, than Hep3B with the HepG2 levels for apoA-I mRNA equal to 21 and apoE mRNA equal to 21 pg/μg total cellular RNA.

TABLE 2. Apolipoproteins mRNA abundance in human tissue culture cells

Cell Line	ApoB	ApoA-I	ApoE
	pg mRNA/μg total RNA		
HepG2	187 ± 17	21 ± 3	21 ± 2
Hep3B	194 ± 20	9 ± 1	13 ± 1
SK-Hep1	14 ± 3	ND	ND
WRL-68	ND	ND	ND

Apolipoprotein B, A-I, and E mRNA were measured in total RNA extracted from the indicated cell line as described in Methods. Each value is derived from the data presented in Table 1 and is calculated from the respective hybridization standard curve, protected probe length, and the mRNA size. For example, as shown in Table 1 and Fig. 3A, 5 μg of HepG2 RNA hybridized to 3081 dpm of apoB mRNA-specific riboprobe; when compared to the apoB cRNA standard (Fig. 3A, left), this is equivalent to 20 pg apoB riboprobe. Upon correction for the size of the protected probe (296 bases) compared to apoB mRNA (14,100 bases), it was determined that apoB mRNA is present at 934 pg/5 μg or 187 pg per μg HepG2 RNA. Each value represents the mean ± SD of six independent measurements read on the same standard curve generated under sample assay conditions and performed simultaneously as sample hybridization; ND, not detectable.

DISCUSSION

This report describes a solution hybridization/RNase protection assay with riboprobes and the application of this technique to determine absolute levels of apoB, A-I, and E mRNA in human hepatoma cell lines. The method is accomplished with a simple probe preparation of less than 2 h that does not require chromatography, and a rapid hybridization time of only 3.5 h. The plasmid used to prepare the probe can also be used to prepare a sense cRNA standard that allows the determination of absolute levels of mRNA. The assay is sensitive to 5 pg of mRNA and linear to 120 pg of mRNA. This makes it easily usable with 5 μg of total RNA, which corresponds to the RNA from approximately 300,000 HepG2 cells or 1 mg of liver tissue (2). The use of a non-^{32}P-labeled probe as an internal hybridization control, such as the [^{3}H]actin riboprobe described here, allows for correction of sample replicates for aliquoting errors as well as more accurate comparisons between apolipoprotein mRNA levels in the same sample when different aliquots are reacted with different riboprobes. The assay is specific and not interfered with by exogenously added γ-actin cRNA or γ-actin riboprobe. Finally, the background is quite low, typically less than 0.08% of the total cpm in each reaction mixture.

This assay has several advantages over the only other method used to quantify apolipoprotein mRNA levels, the solution hybridization/S1 nuclease protection assay (2). The latter requires either M13-derived or double-stranded DNA-derived single-stranded probes. Compared to the pGem1 vectors used in our assay, the M13 vector is more difficult to grow. In addition, it is tedious and relatively difficult to prepare single-stranded probes by chromatography of denatured double-stranded DNA. In the solution hybridization/S1 nuclease protection assay probe, preparation typically takes 1.5 to 2 days. Furthermore, the lesser affinity of DNA probes for mRNA, compared to riboprobes, also requires a much longer hybridization time for the S1 nuclease assay, typically 30–60 h (2). The S1 nuclease assay has comparable specificity, but the background is often much higher (approx. 0.2% probe input) than that seen with the RNase protection assay and this can adversely effect the sensitivity. Finally, for absolute quantitation in the S1 nuclease assay, a standard curve is routinely prepared from M13 DNA (2). Since the kinetics of annealing differ between DNA/RNA and DNA/DNA hybridization (3), the accuracy and usefulness of the S1 standard curve might be compromised unless complete hybridization occurs.

The solution hybridization/RNase protection assay with riboprobes was used to quantify the absolute mRNA levels of apoB, A-I, and E in three human hepatoma (HepG2, Hep3B, SK-Hep1) cell lines and one human embryonic liver-derived cell line (WRL-68). We found that HepG2 compared to Hep3B had comparable levels of apoB mRNA, 2.5-fold more apoA-I mRNA, and 1.5-fold more apoE mRNA. The SK-Hep1 had low levels of apoB mRNA (approximately 7% of HepG2 and Hep3B) and undetectable levels of apoA-I and apoE mRNA. All three apolipoprotein mRNAs were undetectable in the WRL-68 cell line. Previous studies have shown apolipoprotein synthesis by HepG2 and Hep3B (13–15), but this is the first comparison of absolute levels of apolipoprotein mRNA levels between these cells. Recently, the rate of accumulation of apolipoproteins in the medium of HepG2 and Hep3B cells was studied. It was found that medium from HepG2 and Hep3B accumulated apoB at the same rate, whereas HepG2 accumulated apoA-I 2.3 times faster, and apoE 19 times less than Hep3B (15). These results are compatible with our mRNA quantitation for apoB and apoA-I, but suggest that apoE mRNA levels are not at all related to secretion rate. It is of interest that mRNA concentration and tissue culture medium accumulation rates of apoB are the same in HepG2 and Hep3B cell lines, yet it is known that there is no correlation in vivo between hepatic apoB mRNA and plasma apoB concentration (16, 17). With regard to apoA-I, evidence is accumulating suggesting a correlation between hepatic apoA-I mRNA levels, apoA-I secretion, and plasma apoA-I concentration (18, 19), whereas hepatic apoE mRNA levels do not correlate with plasma apoE concentrations (16).

Lastly, we can now assess whether HepG2 and Hep3B apolipoprotein mRNA levels are comparable to liver in vivo. Data on human liver apolipoprotein mRNA levels are not in the literature, presumably due to the general unavailability of healthy human liver samples. However, in the course of metabolic studies, nonhuman primates have been killed and apolipoprotein levels have been quantified. With diets modified to elicit similar levels of total plasma cholesterol of cynomolgus and African green monkeys, Sorci-Thomas et al. (19) used the S1 nuclease protection assay to determine that levels of hepatic apoA-I mRNA were 35 and 70 pg/μg RNA, respectively, and levels of hepatic apoE mRNA were 34 and 48 pg/μg RNA, respectively. Recently, using the RNase protection assay, we determined in cynomolgus monkeys that hepatic apoB mRNA levels were between 150 and 220 pg/mg RNA in different animals. As shown in Table 2, both HepG2 and Hep3B contain apolipoprotein mRNA levels comparable to liver in vivo and from this point of view, these cell lines are a suitable model for the study of apolipoprotein gene expression.

In summary, the solution hybridization/RNase protection assay with riboprobes to quantify apolipoprotein mRNA concentrations is superior to previous methods. This assay should facilitate future studies of the effects of apolipoprotein gene expression, as represented by mRNA levels, on lipoprotein metabolism.

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