papers and notes on methodology

An improved method for precise quantitation of cellular and tissue apolipoprotein A-I mRNA levels by use of an internal standard

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Abstract We have developed a method for the quantitation of apolipoprotein A-I (apoA-I) mRNA by using a variation of traditional S1 nuclease analysis. This method uses an internal standard RNA that allows a level of precision not obtainable with traditional S1 nuclease analysis. The internal standard RNA is synthesized in vitro using the T7 promoter from the transcription vector, pApoAI, which contains the full length apoA-I cDNA. This RNA molecule is identical to authentic apoA-I mRNA except for the addition of 48 bases at the 5'-end which are derived from the vector. A labeled ssDNA probe is produced from pApoAI in such a way that solution hybridization of the probe to a mixture of total RNA and internal standard RNA followed by S1 nuclease digestion results in the protection of DNA fragments from authentic and internal standard RNA, which differ in size by 48 bases. The DNA fragments can be resolved by gel electrophoresis and quantitated. The addition of internal standard RNA to each hybridization reaction allows for correction of variations in hybridization and other sources of experimental error. Using this method we demonstrate a sevenfold increase in precision (the 90% confidence interval was reduced from ±186% to ±26% of the mean value) when compared to traditional S1 nuclease analysis of apoA-I mRNA in liver biopsies and hepatocytes in culture. The internal standard/S1 nuclease method can be adapted to the analysis of any mRNA. —**Pape, M. E., K. R. Marotti, and G. W. Melchior.** An improved method for precise quantitation of cellular and tissue apolipoprotein A-I mRNA levels by use of an internal standard. *J. Lipid Res.* 1990. **31:** 727–733.

Supplementary key word S1 nuclease

Apolipoprotein A-I (apoA-I) is the major protein constituent of high density lipoproteins (HDL). Since HDL is believed to serve a protective role in the development of atherosclerosis (1), there is heightened interest in the metabolism of that protein. Recent studies have focused on factors that control apoA-I mRNA metabolism (2–7) primarily because alterations in cellular apoA-I mRNA levels could result in significant alterations in plasma apoA-I amount and thus, HDL levels. Those studies demonstrated that the cellular levels of apoA-I mRNA varied in different developmental, dietary, hormonal, and genetic states; but, those differences were relatively small compared to changes that have been reported in the mRNA levels of genes encoding proteins essential for cell function. The mRNAs encoding the cytokines, for example, can change several-fold under different conditions (8 and references therein), whereas it is rare to see changes of more than two- to threefold in apoA-I mRNA levels, even in the most extreme circumstances (2–7). As a result, relatively precise measurements are required for reliable quantitation of apoA-I mRNA amount.

There are several methods that are currently used to measure the prevalence of a specific RNA species. These methods can be broadly categorized into two types: those in which total RNA or poly (A+) RNA is transferred to a solid support (such as nitrocellulose) and incubated with solution containing complementary DNA or RNA that has been radiolabeled; or those in which the RNA and complementary probes are allowed to hybridize in solution, and nonduplex nucleic acids (including nonhybridized probe) are digested by nuclease. We have found RNA quantitation using solid support matrices (Northern and RNA dot-blot analysis) unreliable for quantitation of the small changes in apoA-I mRNA because of the imprecision inherent in those methods. That is particularly true when tissues such as the liver, which contain high amounts of polysaccharides, cholesterol, or other lipids are used.

The solution hybridization method, on the other hand, overcomes many of the problems encountered in solid support analysis of RNA prevalence, and numerous derivations of this method have been published (9–13). Sorci-Thomas et al. (5) and Williams et al. (14), for exam-

Abbreviations: HDL, high density lipoproteins; TCA, trichloroacetic acid; DEPC, diethyl pyrocarbonate.

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ple, utilized S1 nuclease analysis to reliably quantitate apoA-I mRNA. However, to correct for variations in hybridization conditions and other sources of experimental error, each RNA sample had to be analyzed several times. We report here an alternative method that substantially increases the precision of the S1 nuclease analysis by inclusion of an internal standard in each hybridization reaction.

MATERIALS AND METHODS

Materials

Commercial materials were obtained from the following sources: RNAzol (Cinna/Biotecx); in vitro transcription kit (Stratagene); Klenow fragment (Bethesda Research Laboratories); Pvu II, T4 polynucleotide kinase (New England Biolabs), deoxyribonucleotides (Pharmacia); Formamide (Clonetech); S1 nuclease (US Biochemical); molecular biology grade glycogen (Boehringer Mannheim); (γ-^{32}P)-ATP (>5000 Ci/mmol, Amersham).

Isolation of total RNA

Total RNA was isolated from cynomolgus monkey hepatocytes in culture or from liver biopsies of cynomolgus monkeys. Typically, 1-2 × 10^6 cells (containing 20-30 μg of RNA) were lysed in RNAzol solution and processed as suggested by the manufacturer except that an additional phenol-chloroform extraction was performed before the final precipitation. Total RNA was quantitated by absorbance at 260 nm and the purity was assessed by the 260 nm/280 nm ratio. All ratios were 2.0 or higher. When liver biopsies were analyzed, an additional TCA precipitation was performed before quantitating the RNA (14). This improved the 260 nm/280 nm ratio from about 2.00 to 2.20.

In a direct comparison of the RNAzol method with the guanidine isothiocyanate/CsCl method of Chirgwin et al. (15), we found that the RNAzol method yields approximately three times more RNA than the latter method, and degradation of that RNA was significantly reduced as assessed by gel electrophoresis.

Synthesis of the internal standard

The full length cDNA for cynomolgus monkey apoA-I (16) was subcloned into the Pst I site of the pBS transcription vector (Stratagene) and termed pApoAI. To generate the sense RNA strand, pApoAI was digested with Hind III and used as the template in the in vitro transcription reaction. The transcription reaction was performed using the Stratagene in vitro transcription kit as suggested by the manufacturer, except that the reaction ingredients were increased fivefold (5 μg of digested plasmid) and allowed to proceed for 60 min at 37°C. Following digestion of plasmid with DNase, the RNA was extracted with phenol-chloroform, precipitated, and resuspended in DEPC-treated water. An aliquot of the extension product was run on a denaturing agarose gel containing formaldehyde. Only a single RNA species of the correct molecular length was evident upon staining with ethidium bromide. There were no detectable plasmid sequences present as evidenced by the absence of higher molecular length species. The synthesized internal standard RNA was quantitated by absorbance at 260 nm and the typical 40 μg/ml = 1 O.D. unit value was used to calculate the concentration of the internal standard RNA. Under these conditions, we generated 6-7 μg of RNA per μg of plasmid template.

Synthesis and isolation of ssDNA probe

The 36-base oligonucleotide 5'-CAGGAGCTTTAGGT TTAGCTGTTTTCCCAAGGCGGA-3', which is complementary to bases 217-252 in the cynomolgus monkey cDNA sequence (16), was synthesized. Three hundred nanograms of that oligonucleotide were end-labeled in a reaction containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, 400 μCi (γ-^{32}P)-ATP (>5000 Ci/mmol), and 10 units of T4 polynucleotide kinase at 37°C for 30 min. The reaction was stopped by the addition of EDTA (20 mM final concentration) followed by phenol-chloroform extraction. The oligonucleotide was thrice precipitated in the presence of 20 μg of glycogen and stored in DEPC-treated water at −20°C. An aliquot of that suspension was precipitated with TCA (17) to determine the specific activity of the probe. Typically, a specific activity of 6-8 × 10^8 cpm/μg was obtained.

The labeled 36-mer was used as a primer on denatured pApoAI to synthesize a ssDNA probe. Eighteen micrograms of CsCl banded pApoAI was denatured by heating at 100°C for 5 min in a 100 μl solution containing 0.2 N NaOH and 0.2 mM EDTA. The solution was neutralized by the addition of 150 μl of 1.5 M ammonium acetate, pH 4.4, followed by ethanol precipitation. The denatured plasmid was resuspended in 100 μl of a solution containing 7 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 50 mM NaCl, and 2-3 × 10^7 cpm of labeled primer (20-30 ng). This solution was incubated at 40°C for 15 min to allow the radiolabeled oligonucleotide to anneal to the complementary plasmid strand. The primer was extended on the template by the addition of dNTPs and DTT to final concentrations of 430 μM and 6 mM, respectively, as well as the addition of 10 units of Klenow fragment. The extension reaction was performed at 37°C for 60 min and stopped by phenol-chloroform extraction. After precipitation, the extended DNA/plasmid hybrids were digested with Pvu II to generate a ssDNA probe of known length and sequence. The Pvu II digestion products were separated by denaturing urea-polyacrylamide gel electrophoresis. After electrophoresis, the gel was exposed to film for

2 min to visualize the ssDNA probe. The ssDNA probe was eluted from the gel by two successive runs of the gel slice in the IBI Unidirectional Electroeluter. The eluted DNA was precipitated in the presence of 20 μg of glycogen as carrier and resuspended in 100 μl of DEPC-treated water; at this stage it was ready to use in the hybridization reaction. We recovered greater than 80% of the DNA from the gel by this procedure. The specific activity of the ssDNA probe was 7.5–9.5 × 10^7 cpm/μg.

Approximately 25% of the original 36-mer was converted to the extended product under these reaction conditions. This procedure generated enough ssDNA probe for 14 assays (see below).

Although this procedure can easily be scaled-up (we often produce enough material for 56 assays), we have observed that increasing the amount of end-labeled 36-mer by 10-fold in the extension reaction produces probe with extremely high background after S1 nuclease analysis and little improvement in yield of the extended product. Therefore, we suggest scaling up the procedure by running multiple annealing and extension reactions in separate tubes.

Hybridization and S1 nuclease analysis

RNA and 5–10 × 10^4 cpm of ssDNA probe were coprecipitated with 20 μg of glycogen as carrier. The pellet was resuspended in 10 μl of 5 × hybridization buffer (2 M NaCl, 500 mM EDTA, 200 mM PIPES, pH 6.5) followed by the addition of 40 μl of formamide. The samples were heated at 95°C for 4 min and placed at 37°C. Solution hybridization was performed in 1.5-ml microfuge tubes that were wrapped with Parafilm and fully submerged in the water bath. After hybridization, S1 nuclease digestion was performed by the addition of 250 μl of S1 digestion solution which contained 1.2 × S1 buffer (5 × 1.4 M NaCl, 0.25 M sodium acetate, pH 4.5, 25 mM $ZnCl_2$), 30 μg/ml sonicated calf thymus DNA, and 20 units S1 nuclease. The digestion reaction proceeded for 60 min at 30°C and was stopped by the addition of 80 μl S1 stop solution which contained 4 M ammonium acetate, 20 mM EDTA, and 10 μg glycogen. The protected DNA fragments were precipitated by the addition of 1 ml ethanol and the pellet was resuspended in 5 μl of sequence loading solution (90% formamide, 20 mM EDTA, 0.3% bromophenol blue, and 0.3% Xylene cyanol). The samples were heated at 90°C for 3 min and 4 μl of sample was run on a 6% sequencing gel. After electrophoresis the gel was exposed to Kodak XAR5 film at −70°C.

Quantitation of apoA-I mRNA

The gel was also analyzed in the Ambis Radioanalytic Analyzer to determine the radioactivity content of the bands corresponding to both the internal standard and authentic apoA-I mRNA. The amount of apoA-I mRNA in the sample was determined by dividing the cpm in the band corresponding to authentic apoA-I mRNA by the cpm in the internal standard band, and then multiplying that ratio by the mass of internal standard added. The mass figure so obtained was corrected for the slight differences in molecular weight of the internal standard and apoA-I mRNA to obtain the actual apoA-I mRNA mass.

RESULTS AND DISCUSSION

The experimental design is shown in **Fig. 1**. A critical element in that pathway was the synthesis of the ssDNA

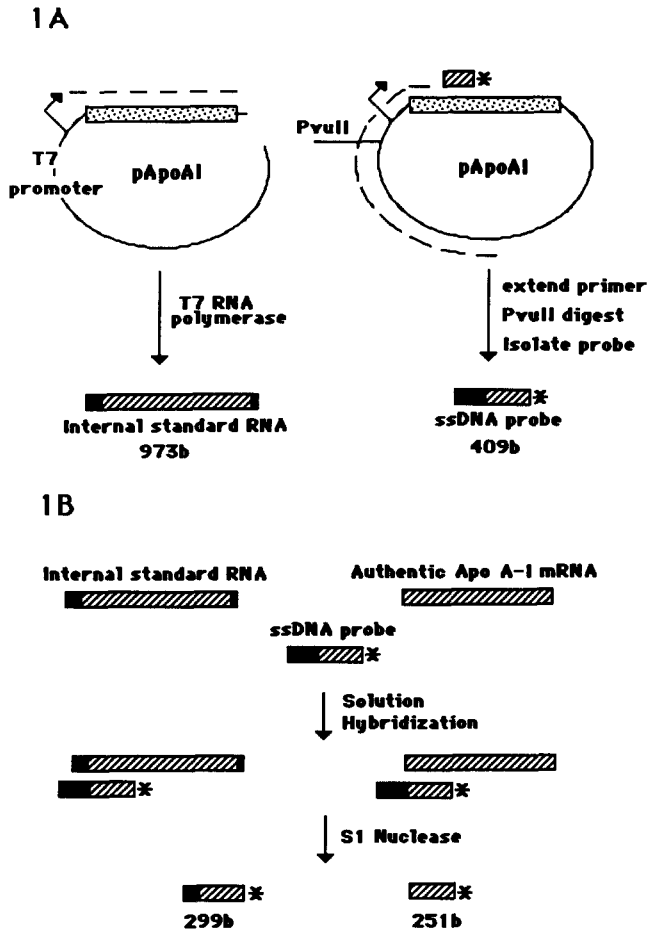

Fig. 1. Experimental design of internal standard/S1 nuclease method. A: The synthesis of both the internal standard RNA (left panel) and ssDNA probe (right panel) are diagrammed. pApoAI in the left panel is diagrammed after having been linearized with Hind III. The arrow on pApoAI represents the approximate location of the transcription start site for T7 RNA polymerase. Stippled boxes, apoA-I cDNA; black boxes, vector sequences; hatched boxes, apoA-I mRNA sequences. The black and hatched boxes for the ssDNA probe represent the complementary sequences for the vector and apoA-I mRNA, respectively. The dashed lines represent the extended products for the RNA and DNA polymerases. The asterisk represents the labeled 5'-end of the ssDNA probe. B: The three nucleic acid elements of the assay are diagrammed. During solution hybridization the ssDNA probe hybridizes to both apoA-I RNA species. After S1 digestion of single-stranded nucleic acids, the sizes of the protected DNA probe fragments from the internal standard RNA and authentic apoA-I mRNA differ as indicated.

probe (1A). It was necessary that the site at which the restriction enzyme cleaved the DNA after its extension on the plasmid template lie outside of the transcription initiation site of the T7 promoter. That generated a probe that contained more vector sequences than the RNA synthesized from this promoter, and ensured that there would be a distinct separation after S1 analysis and gel electrophoresis of undigested (or S1 resistant) probe from in vitro synthesized RNA. The same principle was used to distinguish the in vitro synthesized RNA from authentic apoA-I mRNA (Fig. 1B).

An autoradiograph showing the separation of the three nucleic acid elements used in the assay is shown in **Fig. 2**. Note that a band corresponding to S1 resistant probe is evident near the top of the gel, which is probably due to the carry over (during the isolation of the labeled ssDNA) of some plasmid sequences complementary to the probe. That material would create substantial background in an S1 analysis where the protected fragments were simply counted following TCA precipitation. However, since this procedure resolves the protected fragments by gel electrophoresis, that problem is eliminated.

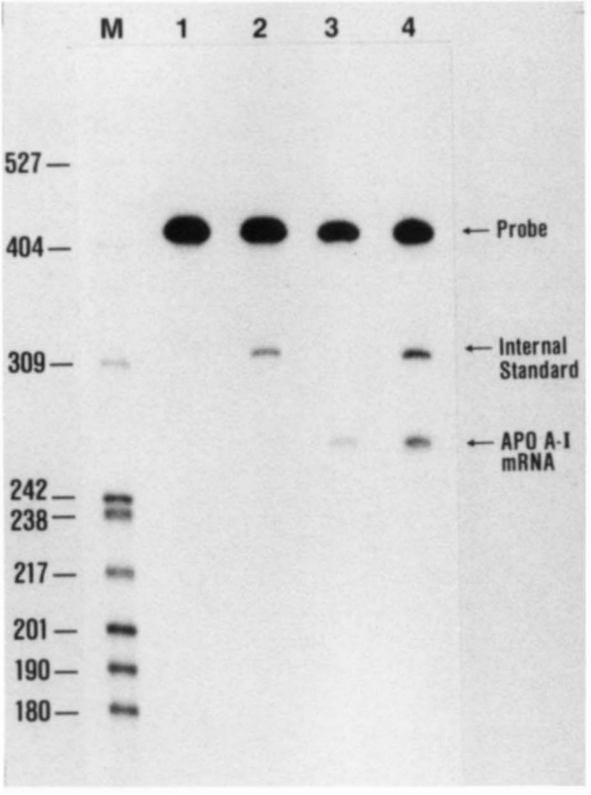

Fig. 2. Autoradiograph of the three nucleic acid elements involved in the internal standard/S1 nuclease method. Labeled ssDNA probe was hybridized with either 5 μg tRNA (lane 1), 300 pg internal standard RNA (lane 2), 5 μg total RNA from cultured monkey hepatocytes (lane 3), or 300 pg internal standard and 5 μg total RNA from monkey hepatocytes (lane 4) and then subjected to S1 nuclease. The protected DNA fragments were resolved on a 6% sequencing gel. The molecular weight markers are Hpa II fragments from pBR322.

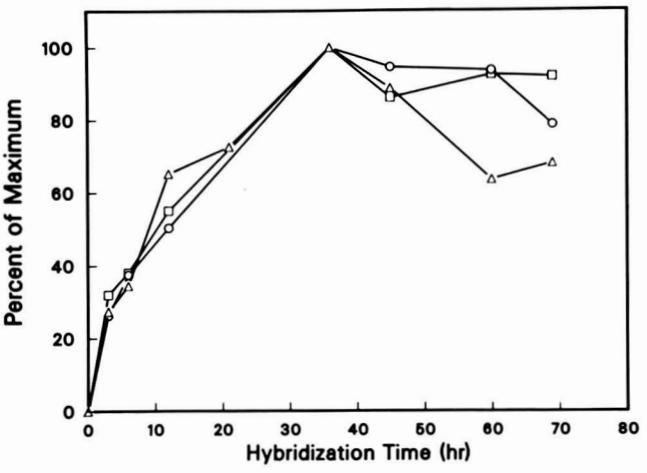

Fig. 3. Determination of the optimal time of hybridization. One hundred pg of internal standard RNA was hybridized to ssDNA probe for various periods of time either in the absence (△) or presence (□) of 3.5 μg of total RNA from monkey hepatocytes. After S1 nuclease digestion and gel electrophoresis the radioactive bands corresponding to internal standard RNA were quantitated. The hybridization signal for authentic apoA-I mRNA in the internal standard/total RNA sample is also shown (○).

Determination of optimal hybridization time and the linear range of the assay

To determine the optimal hybridization time as well as whether the internal standard hybridized with the same kinetics as authentic apoA-I mRNA, 100 pg of internal standard RNA or a mixture of 100 pg internal standard RNA and 3.5 μg of total hepatocyte RNA were assayed for the amount of S1 resistant counts for each RNA species at various times of hybridization. **Fig. 3** shows that the optimal time for hybridization for all samples and RNA species is 36 h. The decrease in S1 resistant counts evident after 36 h may reflect degradation of the RNAs under these conditions. Nonetheless, we chose to use a hybridization time of 36–40 h in the standard assay.

The data in Fig. 3 also show that the hybridization kinetics of authentic apoA-I mRNA and internal standard RNA are the same whether the internal standard is hybridized alone or in the presence of cellular RNA. This indicates that the use of the internal standard RNA to quantitate authentic apoA-I mRNA is valid. In addition, we were also able to estimate the experimental error of the method by determining the ratio of authentic apoA-I mRNA counts to internal standard counts at each time point. We would predict that regardless of the hybridization time, the ratio of authentic apoA-I mRNA hybridized to internal standard RNA would be the same. Indeed, we find a ratio of 3.02 ± 0.31 throughout the course of hybridization, indicating only about a 10% error. This demonstrates that with this procedure it is not necessary to drive the hybridization to completion in order to accurately quantitate apoA-I mRNA. We chose to allow the

hybridization to go to completion to increase the sensitivity of the assay.

To determine the linear range of the assay, 50–1000 pg of internal standard RNA was analyzed. **Fig. 4** demonstrates that up to 1000 pg of apoA-I RNA can be analyzed under these conditions and still be in the linear range of the assay. We chose to use a total apoA-I RNA content range of 300–700 pg in the standard assay.

We set the limit of detection with this method at 50 pg although a much higher level of sensitivity could be obtained by using a uniformly labeled ssDNA probe. We initially used an end-labeled probe because of primer extension experiments that were in progress in the laboratory. We find that an end-labeled probe is adequate for analysis for apoA-I mRNA amount because of its abundance. To use the internal standard method for the analysis of mRNAs present in smaller amounts, or for greater sensitivity in general, a uniformly labeled ssDNA probe can be synthesized by simply adapting the extension reaction. The "nibbling" effect of S1 nuclease on the labeled 5'-end of the ssDNA probe has not proven to be a problem in the analysis of apoA-I mRNA but may prove to be in the analysis of other mRNAs. Careful titration of the S1 nuclease activity should alleviate this problem.

Analysis of apoA-I mRNA in cultured cells and tissue samples

As indicated in the introduction, we have found it extremely difficult to quantitate apoA-I mRNA by RNA slot-blot analysis when the samples consisted of liver biopsies from cynomolgus monkeys that had been consuming a high fat–high cholesterol diet. To test the ability of this method to measure apoA-I mRNA in samples from a fatty liver, we used an RNA sample that we had been consistently unable to analyze by RNA slot-blot (presumably because of the high content of polysaccharides and other cellular material). Those results are shown in **Fig. 5**. We added increasing amounts of total RNA to 300 pg of internal standard RNA and performed the standard assay in duplicate at each total RNA concentration to measure apoA-I mRNA amount in each sample. Those data indicate that a sample that cannot be analyzed by RNA slot-blot analysis can be analyzed for apoA-I mRNA amount by the internal standard/S1 nuclease method.

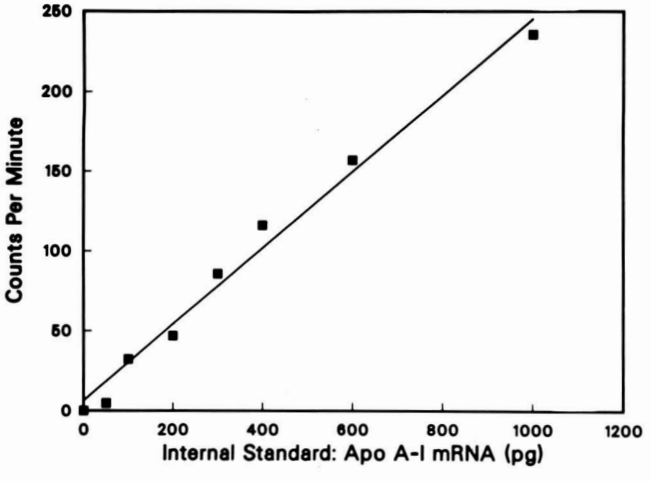

Fig. 4. Determination of the linear range of the internal standard/S1 nuclease method. Increasing amounts of internal standard RNA were hybridized with a constant amount of ssDNA probe for 38 h as described in Materials and Methods. After S1 nuclease digestion and gel electrophoresis the radioactive bands corresponding to internal standard RNA were quantitated.

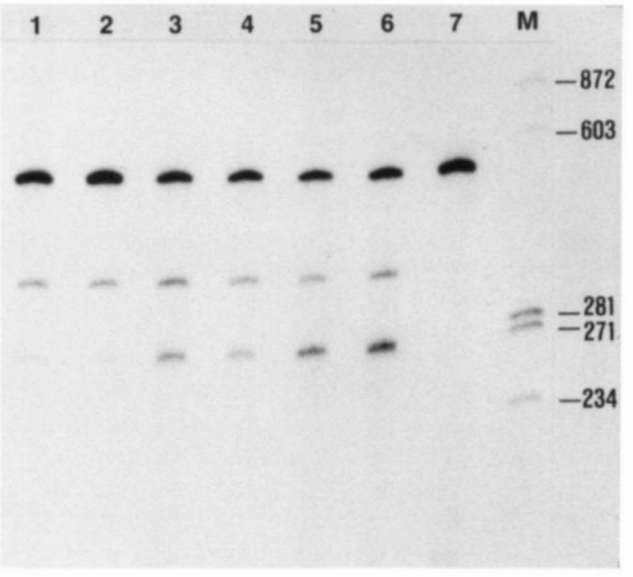

Fig. 5. Autoradiograph of total RNA from a fatty liver biopsy analyzed for apoA-I mRNA by the internal standard/S1 nuclease method. Increasing amounts of total RNA from a liver biopsy were hybridized with ssDNA probe. Each hybridization reaction contained 300 pg of internal standard RNA. The standard assay was performed in duplicate as described in Materials and Methods and the protected DNA fragments were resolved on a 6% sequencing gel. Lanes 1 and 2, 2.5 μg; lanes 3 and 4, 5 μg; lanes 5 and 6, 10 μg; lane 7, probe alone. The molecular weight markers are Hae III fragments from ϕX174.

These data also allowed us to compare the precision of the traditional S1 nuclease method with this method. **Fig. 6A** shows a graph of apoA-I mRNA amount in each total RNA sample without normalization, using data derived from the gel shown in Fig. 5. Note the large scatter in the measurements from the non-normalized data as evidenced by an r^2 value of 0.81 (Fig. 6A). However, when these data points are normalized to the internal standard the scatter is reduced significantly to an r^2 value of 0.99 (Fig. 6B).

Another measure of precision is to determine the boundaries of the line at the 90% confidence limit. That analysis allows one to determine the accuracy of a measurement at any point along the line. For example, we find that from a single analysis of 5 μg of total RNA which is not normalized, that the determined value is ± 186%

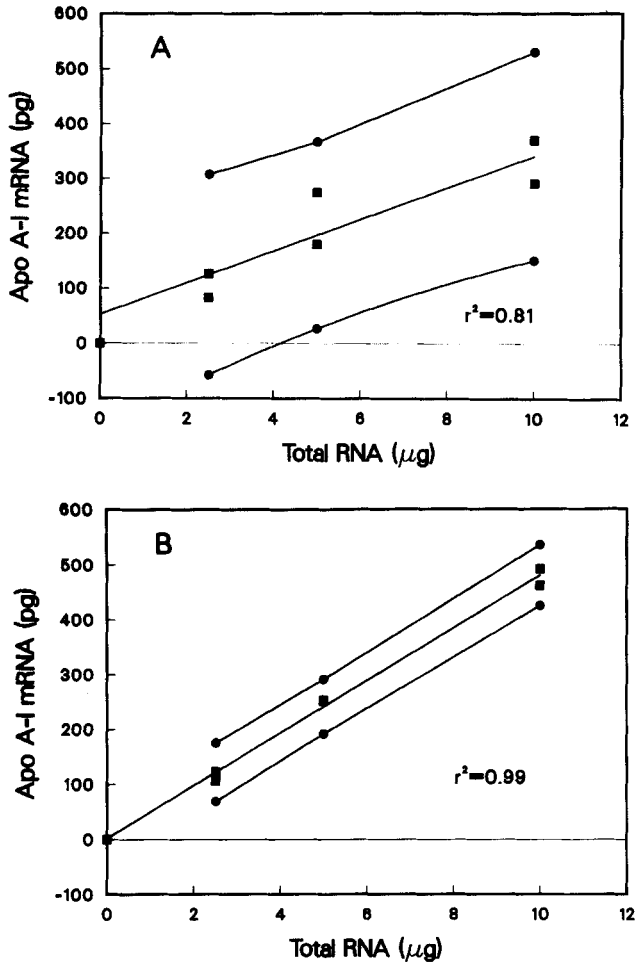

Fig. 6. Comparison of the precision of traditional S1 nuclease analysis and the internal standard/S1 nuclease method. The radioactive bands from Fig. 5 corresponding to the internal standard RNA and authentic apoA-I mRNA were quantitated and the data were put in graphic form. A: Data from the authentic apoA-I mRNA bands that were not normalized to the internal standard. B: Data from the authentic apoA-I mRNA bands that were normalized to the internal standard. The duplicate data points for each total RNA amount analyzed are shown (■). In addition, the boundaries of the line at the 90% confidence limit are shown (●).

from the mean value (value from the line) at the 90% confidence limit (Fig. 6A). However, after normalization of the data, the level of accuracy improves markedly to where a single analysis of apoA-I mRNA amount in 5 μg of total RNA is ± 26% from the mean value at the 90% confidence limit (Fig. 6B). Thus, the level of precision can be increased at least sevenfold by use of the internal standard.

The slopes of the lines in Fig. 6 are a measure of the abundance of apoA-I mRNA in the liver sample. The amount of apoA-I mRNA determined from the non-normalized data and normalized data is 28.0 pg and 47.9 pg per microgram of total RNA, respectively. Thus, the non-normalized method would significantly underestimate the amount of apoA-I mRNA. Regardless of these differences, the values compare favorably with the value of 35.2 pg per microgram of total liver RNA reported for cynomolgus monkeys by Sorci-Thomas et al. (5).

The concept of the internal standard/S1 nuclease method can be easily applied to the analysis of any RNA species. Since many cDNA fragments are already subcloned into transcription vectors, all that is required is the synthesis of a primer complementary to sequences within the cDNA. In some instances simple extension of the primer on a targeted RNA species and an internal standard RNA would extend the concept to an internal standard/primer extension method to quantitate specific RNAs. Although this was our original intent, premature termination of the extension reaction apparently due to the falling-off of reverse transcriptase limited the usefulness of the approach to quantitate apoA-I mRNA. Thus, we were led to develop an assay based on S1 nuclease digestion. Although the synthesis and purification of the probe can be somewhat time consuming, we feel that it is offset by the resulting increase in the level of precision one obtains using this method. Finally, although we use a radioanalytic analyzer to quantitate the radioactivity on the gel, the same results can be obtained by cutting out the various bands and counting them individually in a scintillation counter.

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REFERENCES

1. Maciejko, J. J., D. R. Holmes, B. A. Kottke, A. R. Zinsmeister, D. M. Dinh, and S. T. T. Mao. 1983. Apolipoprotein A-I as a marker of angiographically assessed coronary-artery disease. *N. Engl. J. Med.* **309:** 385-389.
2. Apostolopoulos, J. J., G. J. Howlett, and N. Fidge. 1987. Effects of dietary cholesterol and hypothyroidism on rat apolipoprotein mRNA metabolism. *J. Lipid Res.* **28:** 642-648.
3. Haddad, I. A., J. M. Ordovas, T. Fitzpatrick, and S. K. Karathanasis. 1986. Linkage, evolution, and expression of the rat apolipoprotein A-I, C-III, and A-IV genes. *J. Biol. Chem.* **261:** 13268-13277.
4. Tam, S-P., T. K. Archer, and R. G. Deeley. 1986. Biphasic effects of estrogen on apolipoprotein synthesis in human hepatoma cells: mechanism of antagonism by testosterone. *Proc. Natl. Acad. Sci. USA.* **83:** 3111-3115.
5. Sorci-Thomas, M., M. M. Prack, N. Dashti, F. Johnson, L. L. Rudel, and D. L. Williams. 1988. Apolipoprotein (apo) A-I production and mRNA abundance explain plasma apoA-I and high density lipoprotein differences between two nonhuman primate species with high and low susceptibilities to diet-induced hypercholesterolemia. *J. Biol. Chem.* **263:** 5183-5189.
6. Elshourbagy, N. A., M. S. Boguski, W. S. L. Liao, L. S. Jefferson, J. I. Gordon, and J. M. Taylor. 1985. Expression of rat apolipoprotein A-IV and A-I genes: mRNA induction

during development and in response to glucocorticoids and insulin. *Proc. Natl. Acad. Sci. USA.* **82:** 8242–8246.
7. Ertel Miller, J. C., R. K. Barth, P. H. Shaw, R. W. Elliot, and N. D. Hastie. 1983. Identification of a cDNA clone for mouse apoprotein A-I (apoA-I) and its use in characterization of apoA-I mRNA expression in liver and small intestine. *Proc. Natl. Acad. Sci. USA.* **80:** 1511–1515.
8. Dinarello, C. A. 1989. Interleukin-1 and its biologically related cytokines. *Adv. Immunol.* **44:** 153–205.
9. Durnam, D. M., and R. D. Palmiter. 1983. A practical approach for quantitating specific mRNAs by solution hybridization. *Anal. Biochem.* **131:** 385–393.
10. Dutton, F. L., and A. Chovnick. 1987. Quantitation of genes and transcripts by saturation hybridization in urea solutions using strand-selected probes. *Anal. Biochem.* **164:** 227–235.
11. Thompson, J., and D. Gillespie. 1987. Molecular hybridization with RNA probes in concentrated solutions of guanidine thiocyanate. *Anal. Biochem.* **163:** 281–291.
12. Tsuda, M., M. Yagi, Y. Akizawa, Y. Nagao, and T. Tsuchiya. 1988. Quantitation of specific mRNA by RNA-RNA hybridization kinetics with single stranded riboprobes. *J. Biochem.* **104:** 595–599.
13. Toscani, A., D. R. Soprano, S. C. Cosenza, T. A. Owen, and K. J. Soprano. 1987. Normalization of multiple RNA samples using an in vitro synthesized external standard cRNA. *Anal. Biochem.* **165:** 309–319.
14. Williams, D. L., T. C. Newman, G. S. Shelness, and D. A. Gordon. 1985. Measurement of apolipoprotein mRNA by DNA-excess solution hybridization with single-stranded probes. *Methods Enzymol.* **128:** 671–689.
15. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active nucleic acid from sources enriched in ribonuclease. *Biochemistry.* **24:** 5294–5299.
16. Polites, H. G., G. W. Melchior, C. K. Castle, and K. R. Marotti. 1986. The primary structure of cynomolgus monkey apolipoprotein A-I deduced from the cDNA sequence: comparison to the human sequence. *Gene.* **49:** 103–110.
17. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.