An improved reverse transcription–polymerase chain reaction method to study apolipoprotein gene expression in Caco-2 cells

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Abstract We report a method for the detection and analysis of apolipoprotein B mRNA using the thermostable enzyme rTth to perform coupled reverse transcription–polymerase chain reaction (RT-PCR) amplification. This method, which is at least a 100-fold more sensitive than traditional RT-PCR, was used to examine elements of apolipoprotein B (apoB) gene expression in Caco-2 cells. A region of apoB mRNA spanning the edited site was amplified from pre- and postconfluent Caco-2 cells both under different growth conditions and following alterations in exogenous lipid flux to determine changes in posttranscriptional editing. Apolipoprotein A-IV (apoA-IV) mRNA levels were examined in the same samples. The results suggest that apoB mRNA editing increases in Caco-2 cells during growth but this response is more variable than previously reported. Additionally, evidence was found for differential editing of the 14 kb and 7 kb transcripts. By contrast, there was a consistent growth-related increase in apoA-IV mRNA abundance. Neither apoB mRNA editing nor apoA-IV mRNA abundance was modulated in postconfluent cells in response to different combinations of exogenous lipid. This method should facilitate the study of apolipoprotein gene expression in Caco-2 cells and other situations where the target RNA is limited either as a result of low abundance or limiting tissue sample size.—**Giannoni, F., F. J. Field, and N. O. Davidson.** An improved reverse transcription–polymerase chain reaction method to study apolipoprotein gene expression in Caco-2 cells. *J. Lipid Res.* 1994. **35:** 340–350.

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Studies of intestinal lipoprotein assembly and secretion have been facilitated by the availability of cell culture models, in particular the Caco-2 cell line which, though imperfect, capitulates many aspects of a mammalian enterocyte (1–6). Caco-2 is a human colon carcinoma cell line which differentiates in culture, forming a tight monolayer of polarized cells that develop an apical membrane with microvilli and express high levels of intestinal hydrolases (sucrase-isomaltase, aminopeptidases, alkaline phosphatase) (1). Cells maintained on semipermeable membrane support have been demonstrated to direct lipoprotein secretion across the basal-lateral membrane, providing direct evidence for functional polarization (3).

Interest in Caco-2 cells as a model system in which to study aspects of the molecular regulation of apoB gene expression has been heightened by the demonstration (3, 5) that these cells synthesize and secrete both the full-length form of apoB (apoB-100) and the truncated species (apoB-48), the latter arising as a result of posttranscriptional cytidine deamination which produces an in-frame stop codon in apoB-48 mRNA (7, 8). Recent studies have suggested that apoB mRNA editing may be modulated in Caco-2 cells in association with alterations in growth and differentiation (9, 10). This observation would make Caco-2 cells an appealing model in which to examine the developmental regulation of intestinal apoB mRNA editing, the molecular basis for which is unknown. Such a model would be of interest in view of the demonstration that human fetal small intestinal apoB mRNA undergoes a developmentally modulated increase in editing in association with corresponding changes in the synthesis and secretion of apoB-100 and apoB-48 (11). One important limitation in this regard has been the relatively low abundance of apoB mRNA, particularly in preconfluent Caco-2 cells, which has forced investigators to use large amounts, in some reports up to 40 μg (9), of cellular RNA as starting material from which to undertake studies of apoB gene expression. This RNA requirement imposes practical constraints upon the ability to compare, simultaneously, large numbers of different experimental conditions.

In order to facilitate studies of apoB gene expression in Caco-2 cells we have developed a new RT-PCR method that

Abbreviations: RT-PCR, reverse transcription–polymerase chain reaction; MMLV, Moloney murine leukemia virus reverse transcriptase; apo, apolipoprotein; β2m, β2 microglobulin; EGTA, [ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid].

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uses the thermostable DNA polymerase, rTth, which will perform reverse transcription in the presence of manganese and PCR amplification in the presence of magnesium (12). Thus, by chelating manganese following reverse transcription, the entire RT-PCR can be performed in the same tube. We have optimized this method for the detection and analysis of apolipoprotein B (apoB) and apolipoprotein A-IV (apoA-IV) mRNAs from both Caco-2 cells and human fetal colon. The method is at least a 100-fold more sensitive than previous RT-PCR protocols and has been used, in this report, to evaluate several aspects of apoB gene expression in Caco-2 cells, with particular emphasis on the modulation of apoB mRNA editing.

MATERIALS AND METHODS

Cell culture and RNA extraction

Caco-2 cells from passage number 34 to 57 were derived from stocks previously detailed (4, 13–15) and grown from subconfluent cultures on plastic tissue-culture flasks (T25, Corning). Other cells were plated onto 25-mm 6-well dishes containing polycarbonate filter inserts (Transwells, Costar) or filters coated with Matrigel diluted as recommended by the manufacturer (Collaborative Research). Cells were maintained in Dulbecco's minimal essential medium with high glucose (4.5 g/l), 20% fetal bovine serum (Hyclone Laboratories), 1% nonessential amino acids (Gibco), 1 mM Na pyruvate, 4 mM L-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2 μg/ml gentamicin. At the indicated times after plating, cells were either lysed directly in 4 M guanidinium thiocyanate (11) or, in the case of cells grown on Matrigel, collected after incubation for 2 h with dispase, 50 U/ml (Collaborative Research) and total RNA was extracted as previously detailed using the modified acid guanidinium-phenol method (11). Total RNA was similarly extracted from human fetal or adult small intestinal samples. For experiments in which alterations in lipid flux were examined (see Fig. 3D), three groups of filter-grown cells were studied, 15 days after plating. These conditions have been previously established to produce alterations in triglyceride and/or cholesterol flux in Caco-2 cells and have been extensively characterized in terms of their accompanying effects on lipoprotein secretion (4, 13–15): Group A (Fig. 3D), control (lanes 1, 2) containing M-199 medium alone in 10 mM HEPES, pH 7.4 (Medium-199/Earle's, Gibco, Grand Island, NY); 5 mM Na taurocholate, 30 μM monoolein (= micellar lipid) in M-199 medium (lanes 3, 4); micellar lipid plus 150 μM cholesterol in M-199 (lane 5); micellar lipid plus 1 μg/ml 25-hydroxycholesterol (lane 6) (ref. 13). Group B (Fig. 3D), control (lanes 7, 8) containing 0.288 mM bovine serum albumin in M-199 medium (BSA); 1 mM oleic acid in BSA (lanes 9, 10); 1 mM eicosapentaenoic acid in BSA (lanes 11, 12, refs. 4, 14, 15). Group C (Fig. 3D), control (lanes 13, 14) containing M-199 medium alone; 5 mM Na taurocholate in M-199 (lanes 15, 16); 5 mM Na taurocholate plus 10 mM mevalonate (lanes 17, 18); 10 mM mevalonate alone (lanes 19, 20); 5 mM Na taurocholate plus 150 μM cholesterol (lanes 21, 22); 5 mM Na taurocholate plus 1 μg/ml 25-hydroxycholesterol (lanes 23, 24, ref. 13).

Optimized apoB mRNA RT-PCR

Aliquots of total RNA (2–10 μg) were treated with 0.5 units of DNAse RQ1 (Promega, WI) at 37°C for 45 min in 50 μl 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl, 10 mM dithiothreitol, 20 U RNAse inhibitor (RNAsin, Promega). The RNA was then sequentially extracted with phenol–chloroform and chloroform, precipitated with ethanol, washed once (with 70% ethanol), and resuspended in 20–40 μl H_2O. A range of 50 to 500 ng was routinely used for RT-PCR. Reverse transcription was performed at 70°C for 15 min in a final volume of 20 μl RT buffer, containing 10 mM Tris-HCl, pH 8.3, 90 mM KCl, 1 mM $MnCl_2$, 200 μM each dNTP, 30–60 pmol PCR 10B, 5 U rTth (#N808-0097, Perkin-Elmer Cetus, CT), and the reaction was covered with 80 μl mineral oil. Following reverse transcription, 80 μl (final volume) of PCR buffer was added to each tube, containing 8 μl chelating buffer (50% v/v glycerol, 100 mM Tris-HCl, pH 8.3, 1 M KCl, 7.5 mM EGTA, 0.5% Tween 20), 6 μl 25 mM $MgCl_2$ (1.5 mM final concentration) and 30–60 pmol PCR 5 in 66 μl of H_2O. After 3 min at 95°C, PCR was performed for 30 cycles in a Perkin-Elmer thermocycler as follows: 30 sec at 95°C, 1 min at 55°C, 1.5 min at 72°C. A final 10-min extension was added after the last cycle. For each RNA sample a negative control was run to check for DNA contamination using either AmpliTaq (Perkin-Elmer Cetus) or rTth, leaving the sample on ice during reverse transcription. Additionally, each reaction contained a tube with all the above buffers and enzymes but without RNA to exclude PCR product contamination. One-tenth of the reaction material was analyzed by 2% agarose electrophoresis to confirm the expected amplification product. Conventional apoB mRNA RT-PCR was performed on pre- or postconfluent Caco-2 RNA samples using Moloney murine leukemia virus (MMLV) reverse transcriptase and Taq DNA polymerase under conditions as detailed by Perkin-Elmer Cetus (#N808-0017). This method is essentially as reported previously (9–11). Reaction products were assayed for editing by primer extension as detailed below.

14 and 7 kb RT-PCR

Selective amplification of the 14 kb and 7 kb apoB transcripts was conducted on total RNA from three different groups of postconfluent, filter-grown Caco-2 cells. The 14 kb transcript was reverse transcribed using PCR-14 with

rTth as above and PCR was performed using PCR5 as the upstream primer, to give a product of 775 bp. To amplify the 7 kb transcript, total RNA was reverse transcribed using 60 pmol oligo-dT (12–18 mer, Pharmacia # 00103788) to prime first strand cDNA synthesis as follows: a mix containing DNA-sed RNA, RT buffer, H_2O, and oligo-dT primers was heated at 75°C for 5 min to denature the RNA, cooled to room temperature, and annealed for 10 min at 42°C. Reverse transcription was performed at 60°C for 1.5 min after adding rTth, $MnCl_2$, and dNTPs as above. The cDNA thus generated was then amplified using PCR5 and PCR10B. Control reactions were reverse transcribed with oligo-dT and PCR amplified using PCR5 and PCR-14 to ensure that no 14 kb transcript had been reverse transcribed. For each RNA sample a PCR was run without reverse transcription using PCR5 and PCR10B to exclude genomic DNA or PCR product contamination. Reaction products were then purified and analyzed by primer extension as detailed below.

Primer extension

RT-PCR products were purified using PCR purification spin kit columns as detailed by the manufacturer (Qiagen, Studio City, CA). 30 ng of human apoB primer (BT-1) was radiolabeled with γ^{32}PATP (6000 Ci/mmol, 10 mCi/ml, DuPont-NEN) using T4 kinase (USB). A dilution of the purified PCR products was denatured for 5 min at 95°C and annealed under 20 μl mineral oil for 10 min at 70°C in 7 μl (final volume) containing 1 μl 10 × buffer (400 mM Tris-HCl, pH 7.5, 200 mM $MgCl_2$, 500 mM NaCl) and 3 × 10^4 cpm of primer. After annealing, the mixture was cooled to 42°C over 2 min and 1 μl 0.1 M DTT, 1 μl 1 mM each dATP, dCTP, dTTP, 0.5 μl 5 mM dideoxyGTP, and 1.6 U T7 DNA polymerase (USB Corp., OH) added in a final volume of 3 μl. Primer extension was performed for 10 min at 42°C. The reaction was stopped on ice, the products were precipitated, and extension products were resolved on an 8% denaturing polyacrylamide gel. The dried gel was exposed to XAR film at −80°C with enhancing screens. Autoradiographs at comparable exposures were scanned using a laser densitometer.

ApoA-IV/β_2 microglobulin (β_2m) RT-PCR

Reverse transcription was performed as for apoB mRNA using 30 pmol hAIVE3-R and 30 pmol hβ_2m/R in the same tube. For PCR, equal concentrations of hAIVE2/F and hβ_2m/F were added together with 0.3 μl ^{32}PdCTP (3000 Ci/mmol, 10 mCi/ml, DuPont). Deoxynucleotide and magnesium concentrations and the temperature settings and cycle times (25 cycles) for PCR were unchanged from the protocol above. Primers for both apoA-IV and β_2m were chosen to flank introns, giving a 255 bp product for apoA-IV mRNA and a 201 bp product for β_2m mRNA (16, 17). Twenty μl of reaction products was analyzed by 2% agarose–2% NuSieve gel electrophoresis and the bands corresponding to apoA-IV and β_2m were excised and subjected to liquid scintillation counting (Packard 1500 LS, Downers Grove, IL). All experimental conditions were assayed in triplicate. Background counts were assessed by counting the region of the gel between the two bands. In addition, every sample was subjected to PCR without reverse transcription to exclude PCR product contamination.

Oligonucleotides

PCR 10B (5'CACGGATATGATAGTGCTCAT3', 5' at 6786); PCR 5 (5'CTGAATTCATTCAATTGGGAGAGA CAAG3', 5' at 6504); PCR-14 (5'CTTGTTGTAGGACAT TGCTTAGCT3',5' at 7278); BT-1 (5'AATCATGTAAATC ATAACTATCTTTAATATACTGA3', 5' at 6708); hAIVE3-R (5'CCCGATCTTCTGGCTCACCTCATT3', 5' at 1622); hAIVE2/F (5'AATGCCAAGGAGGCCGTGGAACAT3', 5' at 591); hβ_2m/F (5'GTGGAGCATTCAGACTTGTCT TTCAGCA3', 5' at 1477); hβ_2m/R (5'TTCACTCAATCC AAATGCGGCATCTTC3', 5' at 3537).

RESULTS AND DISCUSSION

Optimization of RT-PCR conditions for apoB mRNA detection and quantitation of posttranscriptional editing

Thirty to sixty pmol of each primer and 1.5–2.0 mM Mg^{2+} produced optimal signal amplification (**Fig. 1A**). The optimized protocol yielded apoB PCR product reproducibly from as little as 50 pg of total RNA from human jejunum and 500 pg RNA from post-confluent, filter-grown Caco-2 cells (Fig. 1B). This protocol thus permits the amplification of apoB mRNA from extremely small quantities of total RNA. In order to establish the enhanced sensitivity of this method, RT-PCR was undertaken in parallel using PCR 10B (downstream primer) and MMLV reverse transcriptase to prime first strand cDNA synthesis followed by PCR amplification using Taq DNA polymerase. The results (Fig. 1C), demonstrate a reaction product from preconfluent Caco-2 cells with 5 ng RNA using the current method, while a product was barely detectable with 500 ng RNA using MMLV reverse transcriptase-Taq polymerase-directed RT-PCR (Fig. 1C, left panel). Studies undertaken in postconfluent Caco-2 cells demonstrate a reaction product with 500 pg RNA using the current method in comparison to 50 ng RNA using MMLV reverse transcriptase-Taq polymerase-directed RT-PCR (Fig. 1C, right panel). Thus, the current method is at least 100-fold more sensitive than other published methods (9–11) for the detection of apoB mRNA (see also below).

In order to examine the posttranscriptional editing of apoB mRNA, linearity was established for the primer extension reaction between 1% and 99% CAA using mix-

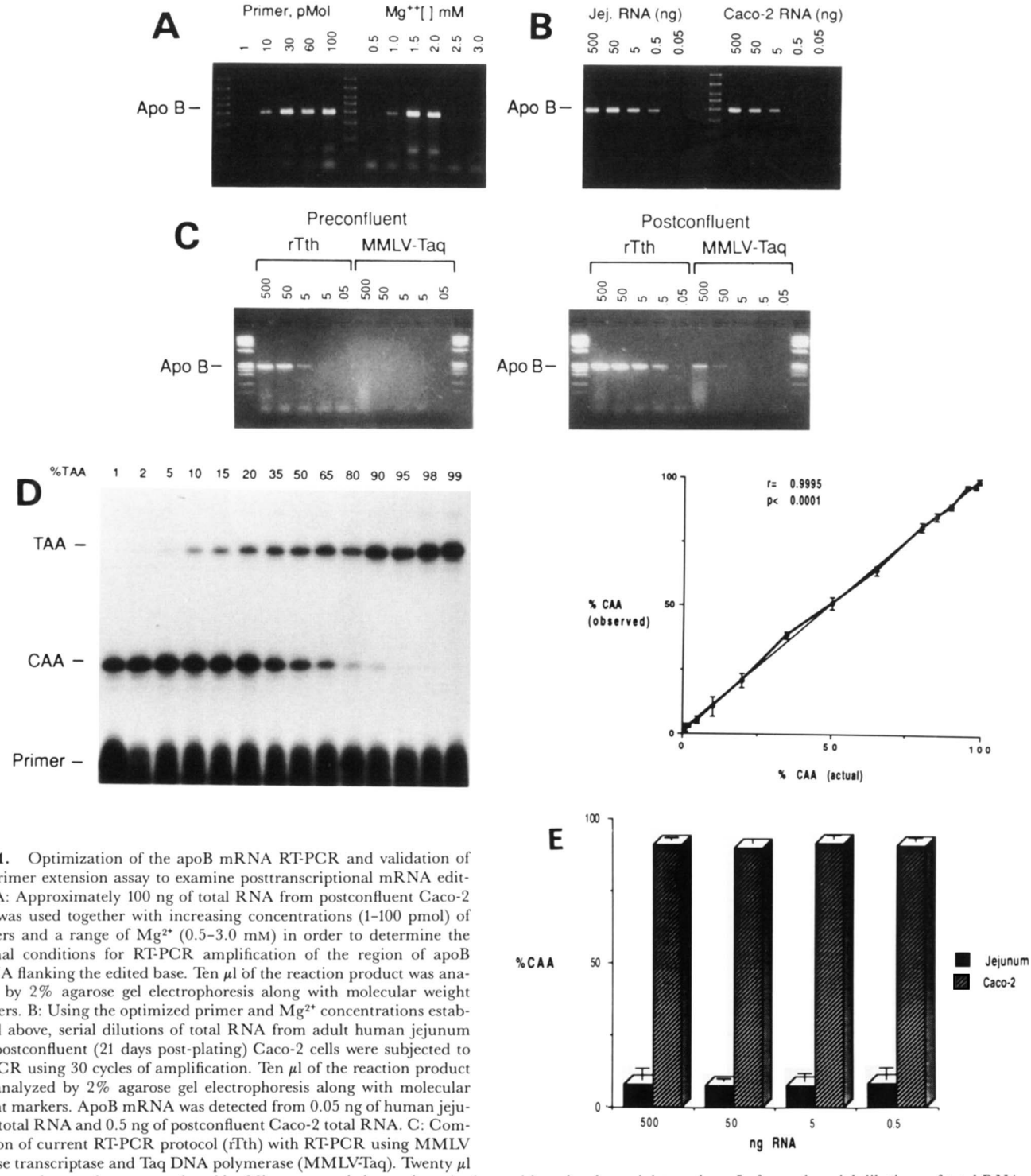

Fig. 1. Optimization of the apoB mRNA RT-PCR and validation of the primer extension assay to examine posttranscriptional mRNA editing. A: Approximately 100 ng of total RNA from postconfluent Caco-2 cells was used together with increasing concentrations (1–100 pmol) of primers and a range of Mg^{2+} (0.5–3.0 mM) in order to determine the optimal conditions for RT-PCR amplification of the region of apoB mRNA flanking the edited base. Ten μl of the reaction product was analyzed by 2% agarose gel electrophoresis along with molecular weight markers. B: Using the optimized primer and Mg^{2+} concentrations established above, serial dilutions of total RNA from adult human jejunum and postconfluent (21 days post-plating) Caco-2 cells were subjected to RT-PCR using 30 cycles of amplification. Ten μl of the reaction product was analyzed by 2% agarose gel electrophoresis along with molecular weight markers. ApoB mRNA was detected from 0.05 ng of human jejunum total RNA and 0.5 ng of postconfluent Caco-2 total RNA. C: Comparison of current RT-PCR protocol (rTth) with RT-PCR using MMLV reverse transcriptase and Taq DNA polymerase (MMLV-Taq). Twenty μl of the reaction product was analyzed by 2% agarose gel electrophoresis along with molecular weight markers. Left panel: serial dilutions of total RNA (ng) from preconfluent Caco-2 cells (2 days post-plating) demonstrating a product with 5 ng RNA using rTth method and barely detectable product with 500 ng RNA using MMLV-Taq. Right panel: serial dilutions (ng) of total RNA from postconfluent (20 days post-plating) Caco-2 cells demonstrating a product with 500 pg RNA using rTth method in contrast to 50 ng with MMLV-Taq. D: Standard curve of the primer extension assay. 829 nucleotide cRNAs containing the edited (UAA) and unedited (CAA) apoB RNA flanking the edited site were transcribed from a cloned human apoB cDNA. These cRNAs were reverse transcribed and PCR amplified using rTth with the primers PCR 5 and 10-B. Known amounts of the CAA and TAA containing products (actual %) were mixed and primer extension assays were run in triplicate. The primer extension products were analyzed by denaturing urea-acrylamide electrophoresis. The dried gels were subjected to autoradiography and scanned with a laser densitometer. Observed percentage of the CAA band was plotted as a function of the actual percentage of CAA. E: Serial dilutions of total RNA from adult human jejunum and postconfluent Caco-2 cells were subjected to RT-PCR as detailed above and the PCR products were analyzed by primer extension. The autoradiographs were scanned by laser densitometry and the observed ratios of edited and unedited apoB cDNAs were determined. The graph shows no change in the percentage of CAA after primer extension analysis of PCR products obtained from 1000-fold dilutions of RNA.

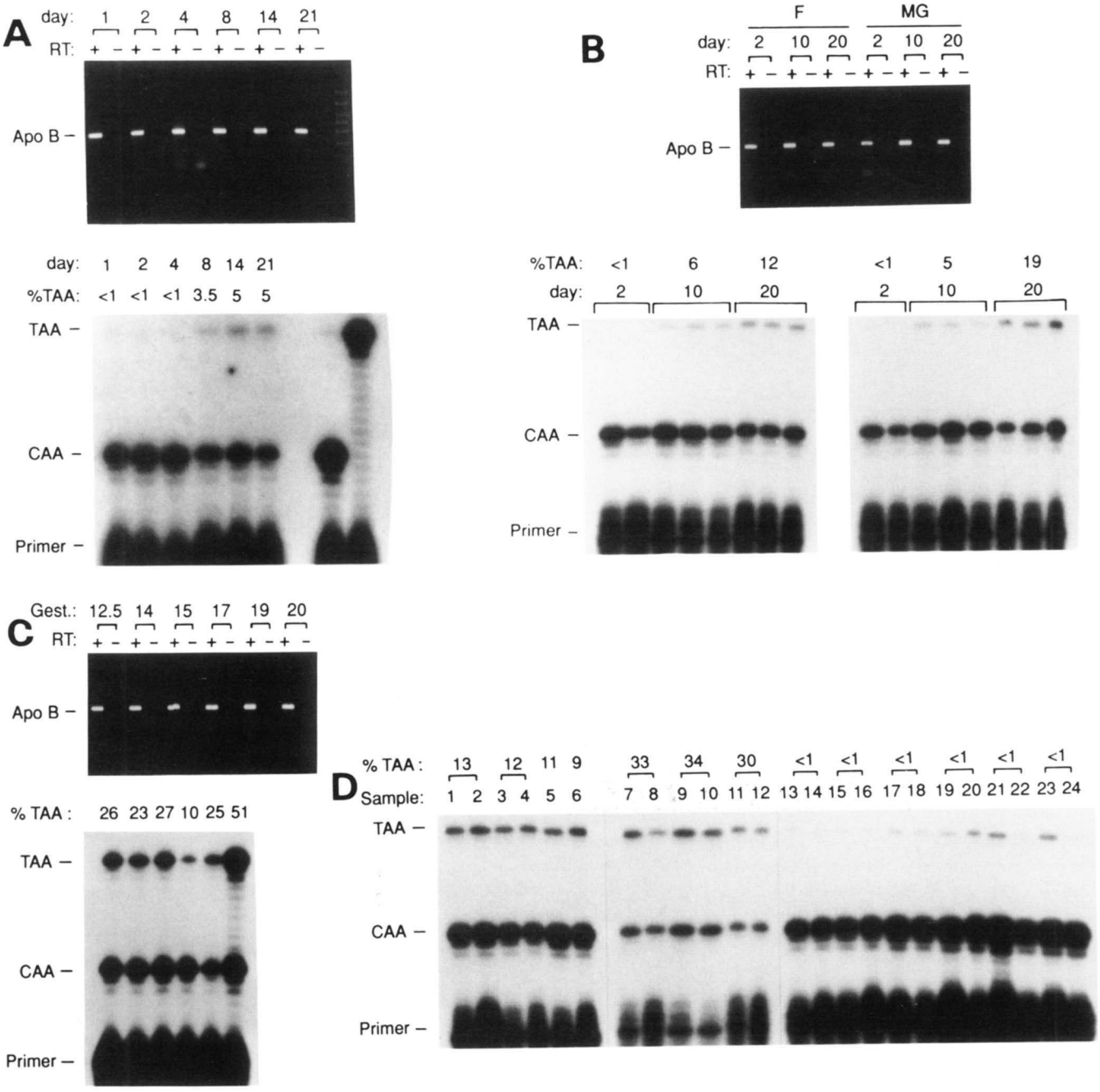

Fig. 2. RT-PCR of apoB mRNA and primer extension analysis of Caco-2 RNA and human fetal colon: effects of growth conditions and alterations in lipid metabolism on apoB mRNA editing in Caco-2 cells. A: RT-PCR and primer extension analysis of Caco-2 cells grown on plastic tissue-culture flasks. Total RNA was extracted from cells grown for different lengths of time and either 500 ng (days 1, 2, and 4) or 100 ng (days 8, 14, and 21) was used for RT-PCR as described in Methods. Paired incubations were analyzed from samples with (+) or without (−) reverse transcription (RT). Two percent agarose electrophoresis of 10 μl of the PCR products is shown. Primer extension analysis of the PCR products was undertaken as above, with each time point being assayed in duplicate. As shown in the lower half of the figure, apoB mRNA editing was undetectable at early stages, increasing after confluence (day 6) and reaching 5% TAA at 14 and 21 days after plating. The mobility of CAA and TAA standards is also shown. B: Time course of apoB mRNA editing in Caco-2 cells grown on uncoated, semipermeable filters (F) and filters coated with Matrigel (MG). One hundred to 500 ng total RNA was used to amplify apoB mRNA by means of RT-PCR. Paired incubations were analyzed from samples with (+) or without (−) reverse transcription (RT). Aliquots of the PCR products were electrophoresed through 2% agarose as above. Primer extension assay of the purified PCR products is shown in the lower half of the figure. C: ApoB mRNA editing in human fetal colon. Two hundred ng total RNA from various gestational age fetal colon samples was used for RT-PCR analysis as above. Paired incubations were analyzed from samples with (+) or without (−) reverse transcription (RT). Aliquots of the PCR products were electrophoresed through 2% agarose as above. Primer extension analysis of the purified products was conducted as above and the assay results are shown in the lower half of the figure. D: ApoB mRNA editing in filter-grown, postconfluent Caco-2 cells: effects of alterations in cellular lipid metabolism. Three groups of postconfluent Caco-2 cells (18 days post-plating) were studied. In each case 50 ng total RNA was used to perform RT-PCR amplification of apoB mRNA as described above. Group A: Control (lanes 1, 2) containing M-199 medium alone; 5 mM Na taurocholate–30 μM monoolein (= micellar lipid) in M-199 medium (lanes 3, 4); micellar lipid plus 150 μM cholesterol in

tures of PCR products containing both the unedited and edited base at nucleotide 6666 in predetermined proportions (Fig. 1D). Additionally, reproducibility of individual apoB mRNA editing values was established over a 1000-fold range of target RNA from both human jejunum (0.5 ng = 8.2% CAA; 5 ng = 7.6% CAA; 50 ng = 7.5% CAA; 500 ng = 7.9% CAA) and postconfluent Caco-2 cell RNA (0.5 ng = 90.4% CAA; 5 ng = 91.1% CAA; 50 ng = 89.7% CAA; 500 ng = 90.9% CAA) (Fig. 1E).

Due to its highly ordered secondary structure, this region of apoB mRNA has been difficult to amplify from samples expressing low levels of the gene such as preconfluent Caco-2 cells (11). The ability of rTth to reverse transcribe at 70°C enhances annealing of the downstream primer to its complementary sequence, which reduces mispriming and improves subsequent amplification (12). These characteristics also account for enhanced sensitivity of the method because the mRNA is denatured at that temperature and can be reverse transcribed with improved efficiency. This method can be used to study the extent of RNA editing in cells or tissues expressing very low levels of apoB mRNA (fetal tissues, adult kidney, adrenal, lung, testis) and eventually to study cell to cell differences in culture or in vivo. Studies using this method are now in progress.

ApoB mRNA editing in Caco-2 cells: modulation in response to differentiation and alterations in cellular lipid flux

The optimized RT-PCR method was applied to an analysis of apoB mRNA editing in preconfluent and differentiating Caco-2 cells. As shown by our own and other laboratories (11, 18), apoB mRNA abundance increases 10- to 20-fold during Caco-2 cell differentiation, which imposes different requirements for starting material for RT-PCR amplification. The current method yields a detectable amplification product from as little as 5 ng (Fig. 1C) and reliably from 500 ng preconfluent Caco-2 RNA extracted 1 day after plating on a plastic surface (**Fig. 2A**). These findings emphasize the increased sensitivity of the optimized RT-PCR protocol and suggest its application to the study of factors that may mediate the regulation of apoB gene transcription in Caco-2 cells (18).

Primer extension assay of the PCR products revealed undetectable apoB mRNA editing in cells harvested at early stages after plating, i.e., < 1% TAA, with a progressive increase after confluence, reaching 5% TAA in cells plated on plastic (Fig. 2A). Studies conducted in cells grown on either semipermeable filters or filters coated with Matrigel revealed a similar, progressive increase in apoB mRNA editing with time, but demonstrated more extensive editing, up to 12% TAA in filter-grown cells and 19% TAA in cells grown on Matrigel-coated filters (Fig. 2B). These findings indicate the feasibility of studying the factors that mediate this "differentiation"associated increase in posttranscriptional editing without the need for large numbers of cells.

Similar studies were conducted with samples of human fetal colon from 12–20 weeks gestational age. Two hundred ng total RNA was used to generate apoB cDNAs for primer extension assay, revealing a variable extent of posttranscriptional editing (10–27% TAA) with an increase (51% TAA) at 20 weeks gestation (Fig. 2C). These results, however, may reflect individual variability and should not be construed as evidence against developmental modulation in the fetal colon. Previous studies in human fetal small intestine demonstrated a progressive increase in apoB mRNA editing during the second trimester (11) and further studies will be required to evaluate the developmental and tissue-specific regulation of apoB mRNA editing.

Studies were next conducted in three groups of filter-grown, postconfluent Caco-2 cells exposed to different mixtures of exogenous lipid in order to determine the effects of these perturbations on apoB mRNA editing. As illustrated in Fig. 2D, two important conclusions emerged from this experiment. First, there was no effect of exogenous lipid administration (including fatty acids, bile salts, and cholesterol) on apoB mRNA editing in any sample when compared to its contemporaneous control. The conditions used have been previously shown to produce substantial alterations in cellular cholesterol and triglyceride metabolism in Caco-2 cells (4, 13–15), suggesting that apoB mRNA editing in these cells may not be sensitive to changes in lipid flux. Similar conclusions were reached by previous workers based on the ratio of apoB-100 and apoB-48 secretion from Caco-2 cells incubated with different forms of exogenous lipid (3, 5, 6, 15). Second, there was a wide range of apoB mRNA editing between different groups of postconfluent, filter-grown Caco-2 cells (from < 1% TAA to 34% TAA) despite the fact that they originated from the same stocks and were maintained and harvested under identical conditions. All experiments were conducted with cells from passage number 34 to 57 with no relationship between increasing passage and extent of apoB mRNA editing.

M-199 (lane 5); micellar lipid plus 1 μg/ml 25-hydroxycholesterol (lane 6). Group B: Control (lanes 7, 8) containing 0.288 mM bovine serum albumin in M-199 medium (BSA); 1 mM oleic acid in BSA (lanes 9, 10); 1 mM eicosapentaenoic acid in BSA (lanes 11, 12). Group C: Control (lanes 13, 14) containing M-199 medium alone; 5 mM Na taurocholate in M-199 (lanes 15, 16); 5 mM Na taurocholate plus 10 mM mevalonate (lanes 17, 18); mevalonate alone (lanes 19, 20); 5 mM Na taurocholate plus 150 μM cholesterol (lanes 21, 22); 5 mM Na taurocholate plus 1 μg/ml 25-hydroxycholesterol (lanes 23, 24). Primer extension assay was performed on the purified PCR products and the autoradiograph of the resulting gel is shown.

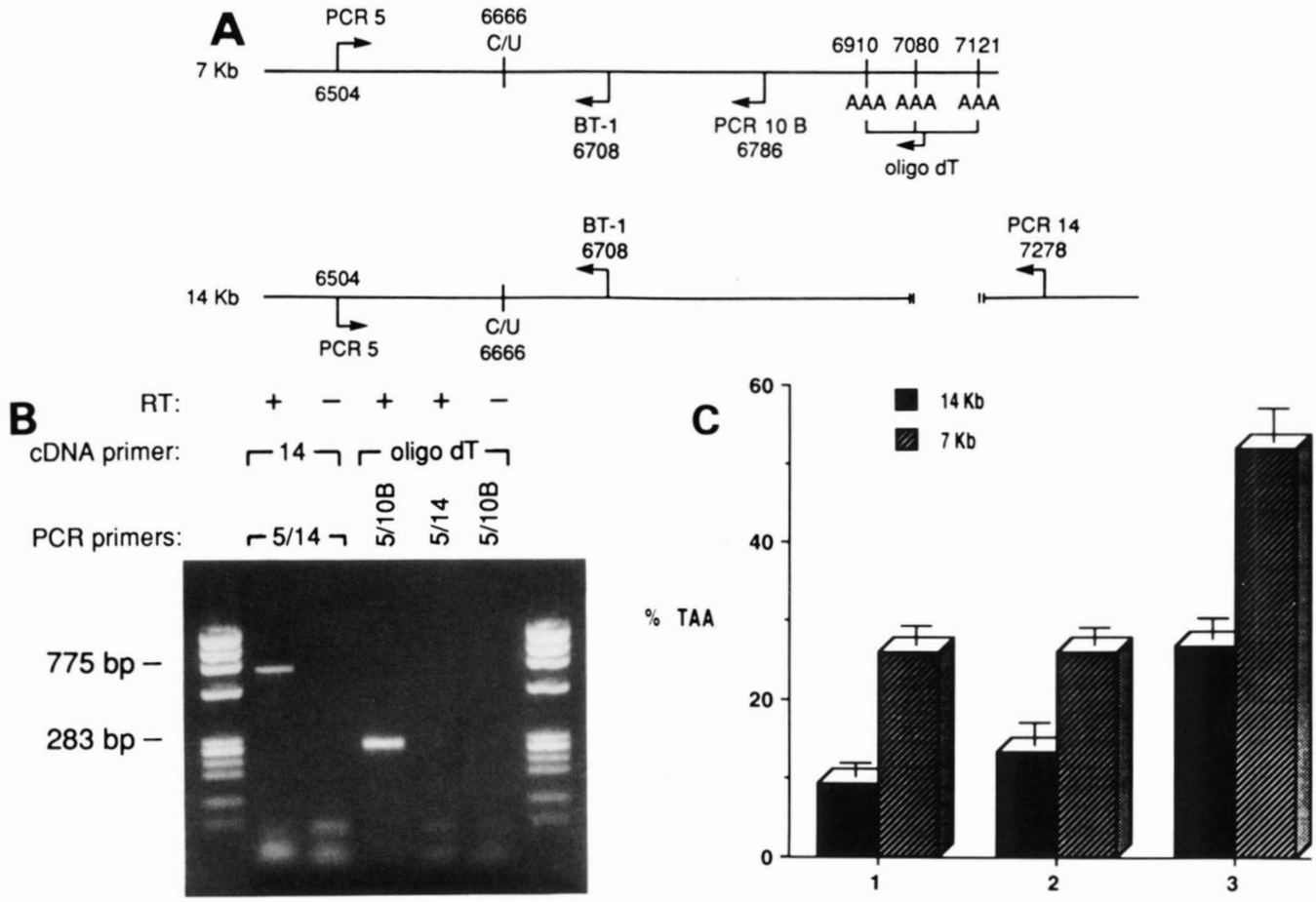

Fig. 3. Separate amplification of the 7 and 14 kilobase apoB transcripts. A: Location of the primers used for RT-PCR. The rationale for the use of oligo-dT to prime first strand DNA synthesis for selective amplification of the 7 kb transcript is based on previous descriptions of three potential polyadenylation sites downstream of the edited base (7). B: Paired incubations were analyzed from samples with (+) or without (−) reverse transcription (RT). Selective reverse transcription of the 7 kb form of apoB mRNA used a short reaction time (1.5 min) to minimize reverse transcription from the longer apoB mRNA. PCR amplification of apoB cDNA with PCR5 and 10B gave positive products while PCR using primers PCR5 and 14 was used to exclude contamination with the 14 kb mRNA. The longer apoB mRNA transcript was reverse transcribed using PCR-14 as downstream primer and amplified using primers PCR 14 and PCR 5. Ten μl aliquots of the PCR products were analyzed by 2% agarose electrophoresis. C: ApoB mRNA editing in the 7 and 14 kb transcripts was analyzed by primer extension in total RNA extracted from three groups of postconfluent Caco-2 cells, each group being assayed in triplicate. Samples 1 and 3 are from Caco-2 cells grown on uncoated filters. Sample 2 is from Caco-2 cells grown on filters coated with Matrigel. The mean percent TAA values in the 14 kb transcript are 9.3, 13.3, and 26.7 in samples 1, 2, and 3, respectively, while in the 7 kb transcript the corresponding values are 26, 25.9, and 51.9% TAA, respectively. All differences between TAA values for 7 and 14 kb species are significant, $P < 0.005$.

Previous work suggested that apoB mRNA editing increased in filter-grown, postconfluent Caco-2 cells (40% TAA) in comparison to postconfluent cells maintained on plastic (4% TAA) (9). However, a recent report from the same group, using methodology different from that used in the present report, demonstrated a differentiation-associated increase in apoB mRNA editing in cells maintained on plastic, with values increasing from 4% to 32% TAA in pre- versus postconfluent Caco-2 cells, respectively (10). Earlier descriptions of apoB synthesis and secretion from Caco-2 cells grown on filters alluded to a wide range of apoB-100 to B-48 ratios observed in the basal-lateral medium in different experiments conducted over 1 year (3). Although the question of parallel variation in apoB isoform ratios and apoB mRNA editing was not specifically addressed in the current studies, the data suggest substantial heterogeneity in the response of Caco-2 cells to the, as yet undefined, developmental cues that modulate apoB mRNA editing. The reasons underlying the variability noted are not apparent at present but may reflect either a transient mosaicism accompanying partial differentiation, as recently described (19) or true clonal heterogeneity. In this regard, others have demonstrated mosaics of expression of sucrase-isomaltase and membrane peptidases in different clones of Caco-2 cells and it may be that there is also a mosaic of apoB expression and posttranscriptional modification (20, 21). Until the basis for this variability is established, data concerning the differentiation-associated modulation of apoB mRNA editing in Caco-2 cells should be interpreted with caution.

Possible clonal variation in apoB gene expression in Caco-2 cells will be more readily identified using the current method as the reduced RNA requirements will permit screening of larger numbers of individual clones.

Differential modulation of apoB mRNA editing in Caco-2 cells: 14 kb versus 7 kb RNAs

Previous studies have established the presence of two transcripts for apoB as determined by Northern blot and solution hybridization analysis of both human small intestine and Caco-2 RNA, with the 14 kb species predominating in both instances (7, 8, 11). In the adult human small intestine, where apoB mRNA is substantially all edited, it is likely that both species of mRNA encode apoB-48 protein (7, 8, 11). The demonstration that Caco-2 cells synthesize and secrete both apoB-100 and apoB-48 proteins prompted an examination of whether both apoB species are edited to the same extent. The scheme for selective amplification is illustrated in **Fig. 3A.** Based on the earlier demonstration of potential polyadenylation sites at nucleotide positions 6910, 7080, and 7121 in apoB cDNA (7), selective amplification of the 7 kb transcript was undertaken using oligo-dT primed first strand synthesis and a brief (1.5 min) reverse-transcription (Methods). Subsequent PCR amplification using primers 5 and 10B produced a product of 283 bp (Fig. 3B). Validation of selective amplification of the 7 kb transcript was established by the absence of an amplification product following PCR amplification using primers 5 and 14 (Fig. 3B). The 14 kb transcript was amplified using a downstream-specific primer (PCR 14) and produced an amplification product of 775 bp (Fig. 3B). The respective products were purified and analyzed by primer extension, revealing the shorter transcript to be more extensively edited in every case (26.0 ± 2.4%, 25.9 ± 2.1% and 51.9 ± 4.2% TAA versus 9.3 ± 1.7%, 13.3 ± 2.9% and 26.7 ± 2.6% TAA, respectively, $P < 0.005$, Fig. 3C). The inter-sample variability in apoB mRNA editing is further emphasized in this examination of three separate preparations of Caco-2 cell RNA. Despite these individual differences, however, the 7 kb transcript is edited to a significantly greater extent than the 14 kb species (Fig. 3C). The current method will facilitate investigation of the differential editing of the 7 and 14 kb apoB mRNA species particularly in relation to other posttranscriptional events such as splicing and polyadenylation.

Optimization of RT-PCR conditions for apoA-IV mRNA quantitation

Conditions identical to those detailed for apoB were used to establish a sensitive RT-PCR assay for measuring apoA-IV mRNA abundance (**Fig. 4**). To establish the working range of amplification, serial, 2-fold dilutions of target RNA from 800 to 0.8 ng total RNA from (filter-

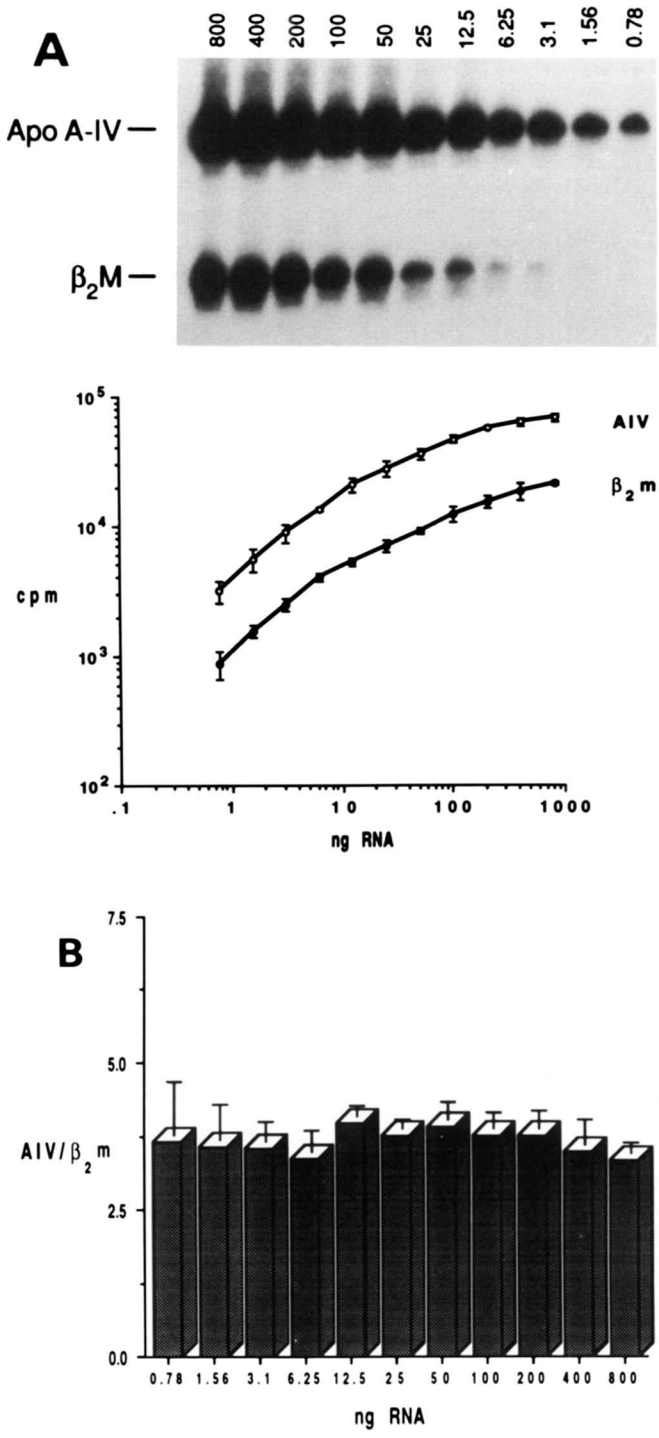

Fig. 4. Quantitation of apoA-IV mRNA levels by RT-PCR using β_2 microglobulin as an internal standard. A: Serial dilutions from 800 to 0.78 ng total RNA from postconfluent Caco-2 cells were reverse transcribed and PCR amplified for apoA-IV and β_2 microglobulin in the same reaction tube together with 3 μCi of ^{32}PdCTP. Twenty five cycles of PCR was used. Each sample was assayed in triplicate and aliquots of the PCR products were run on a 2% agarose–2% NuSieve gel. Bands corresponding to the predicted size were excised and counted in scintillation fluid. The amplification is exponential and parallel between the two mRNAs over a wide range of template concentration. B: The ratio between the apoA-IV and the β_2 microglobulin bands is not significantly different over a wide range of starting amount of mRNA template.

grown, postconfluent) Caco-2 cells were amplified using 25 cycles of PCR. Fig. 4A shows that the conditions chosen result in parallel, exponential amplification of both apoA-IV and β_2 microglobulin, the latter mRNA coamplified as an internal "housekeeping" control. A constant ratio of apoA-IV/β_2 microglobulin amplification products was demonstrated over at least a 250-fold range, from 0.78 to over 200 ng RNA, thereby establishing the utility of this method over a wide range of input RNA (Fig. 4B). The demonstration of a wide range of exponential amplification of both target transcripts using postconfluent Caco-2 total RNA is an important control as the increased expression of apoA-IV mRNA relative to that of β_2 microglobulin mRNA is potentially more likely to lead to unequal amplification. This possibility was excluded as evidenced by the data in Fig. 4A but will need to be validated for the individual mRNA of interest in the appropriate cell type in future studies. Using 100 ng of RNA from the same Caco-2 sample, RT-PCR amplification was conducted using 10, 15, 18, 20, 25, and 30 cycles of PCR. This revealed parallel, exponential amplification of both mRNA species between 15 and 25 cycles (data not shown). Subsequent studies were standardized to 25 cycles of PCR. This format for quantitation of apoA-IV mRNA abundance is similar to that detailed for examination of mdr-1 gene expression where the amplification of one transcript was normalized to the coamplification of another transcript from the same RNA sample and in the same tube (22, 23). This method could be easily adapted to provide more accurate quantitation of endogenous mRNA levels by the inclusion of an internal standard (24, 25) or the use of competitive PCR fragments (26, 27).

Regulation of apoA-IV mRNA abundance in Caco-2 cells

There was a consistent, 5-fold increase in apoA-IV mRNA abundance in Caco-2 cells over a 21-day period of growth and differentiation on plastic tissue culture flasks

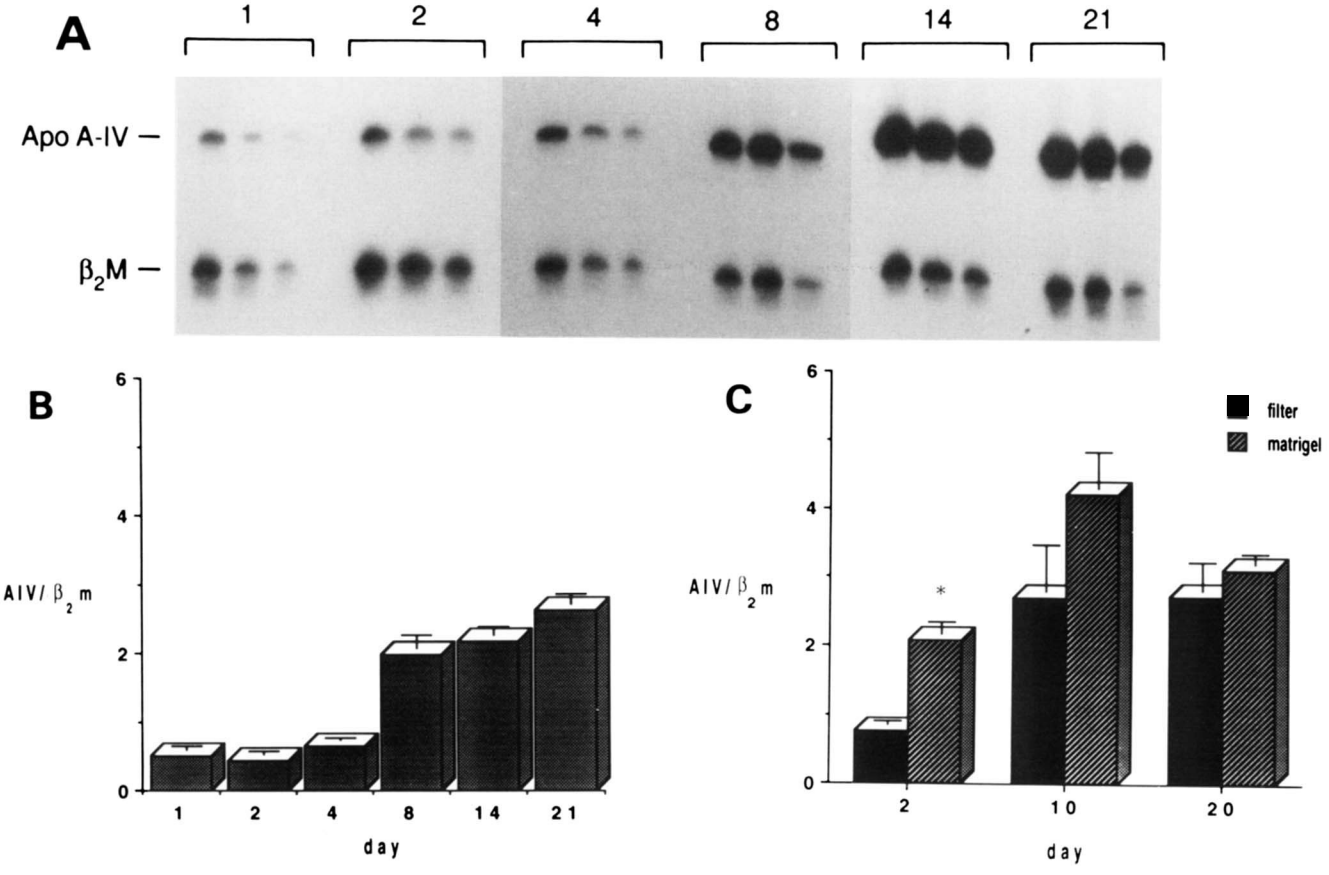

Fig. 5. ApoA-IV mRNA quantitation in Caco-2 cells grown on plastic, filters, and filters coated with Matrigel. A: Total RNA was extracted from Caco-2 cells grown on plastic from days 1, 2, 4, 8, 14, and 21. All samples were assayed in duplicate in three dilutions (200, 100, and 50 ng RNA) using 25 cycles of PCR. Four μl aliquots of RT-PCR products were run on a 6% non-denaturing polyacrylamide gel that was dried and subjected to autoradiography. B: Ratios were determined between apoA-IV and β_2 microglobulin in a time course of Caco-2 cells grown on plastic. Each time point was assayed in duplicate and each sample was run at three different dilutions (200, 100, and 50 ng RNA). The mean values are shown ± standard errors. ApoA-IV mRNA levels are constant in preconfluent cells and increase after confluence. C: Caco-2 grown on filters and filters coated with Matrigel. Each sample was run at three different dilutions (100, 50, and 25 ng RNA), n = 2 for day 2 and 10, n = 3 for day 20. The mean ± SE values are shown. At day 2 apoA-IV mRNA levels are significantly higher in the cells grown on Matrigel than in the cells on filters ($P < 0.005$). Under both conditions apoA-IV mRNA levels increase with time and are significantly higher at day 20 compared to day 2 ($P < 0.005$).

as shown in **Fig. 5A.** When normalized to the expression of β_2 microglobulin, the increase in apoA-IV mRNA abundance was detectable at day 8 after plating (A-IV/β_2 m ratio day 1 = 0.5; day 2 = 0.4; day 4 = 0.6; day 8 = 2:0; Fig. 5B). Thereafter, apoA-IV/β_2 microglobulin ratios were essentially unchanged (day 14 = 2.2, day 21 = 2.6, Fig. 5B). Caco-2 cells plated on filters demonstrated a profile of apoA-IV mRNA increase with time similar to that observed above (Fig. 5C). However, cells maintained on Matrigel-coated filters demonstrated a rise in apoA-IV mRNA abundance by 2 days after plating (A-IV/β_2 m ratio day 2 = 2.1; day 10 = 4.2; day 20 = 3.3; Fig. 5C). These changes in apoA-IV mRNA abundance were accompanied by a parallel increase in the secretion of apoA-IV protein into the basal-lateral medium of filter-grown cells as assessed by Western blotting using anti-human apoA-IV antiserum (data not shown). The consistent increase in apoA-IV mRNA abundance is contrasted with the variable modulation of apoB mRNA editing noted above in the same samples. Examination of the samples generated from the lipid flux experiments described above in Fig. 2 (see also Methods) demonstrated no alteration in apoA-IV mRNA abundance relative to β_2 m (data not shown). These data are consistent with previous studies showing that apoA-IV secretion is not modulated in filter-grown Caco-2 cells after exposure to oleate (3) but further studies will be required to establish whether other elements of cellular lipid flux are important in the regulation of apoA-IV gene expression.

In conclusion, the current method facilitates studies of regions of mRNA with a highly ordered secondary structure and may be used in the analysis of mRNA species expressed at low levels in cell culture or in vivo. As demonstrated above, this method is readily applicable to the study of intestinal apolipoprotein gene expression in Caco-2 cells.

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