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Determinants involved in regulating the proportion of edited apolipoprotein B RNAs

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ABSTRACT

Editing of apolipoprotein B (apoB) RNA involves deamination of cytidine by the catalytic subunit, APOBEC-1, as a component of an editosome. A tripartite sequence (editing motif) is essential for editosome assembly and site-specific editing. Current theory for the regulation of apoB RNA editing proposes that APOBEC-1 is rate limiting in cells and determines the proportion of edited apoB mRNAs. An evaluation of how the overexpression of APOBEC-1 increased the proportion of edited RNAs has led to the discovery of a paradox. McArdle cells edit a constant proportion of apoB RNA regardless of the total number of apoB RNAs expressed. Despite virtually identical editing motifs, apoB RNA transcripts from the endogenous gene and transfected, exogenous cDNA were edited with characteristic, but different efficiencies. This suggested that these RNAs were interacting with the editing machinery as distinct and noncompeting populations. We evaluated whether the presence of introns in the endogenous transcript may have distinguished it as a distinct population having reduced editing efficiency. The editing efficiency of chimeric splicing-editing RNA substrates was highest on fully processed RNA. Increased exon length improved utilization of the editing motif in these chimeric constructs. Taken together, the data suggest that the close proximity of introns can reduce apoB RNA editing efficiency. A population "gating" hypothesis is proposed wherein the proportion of edited RNAs in a population is determined by multiple *cis*- and *trans*-acting factors as RNAs pass through a nuclear restriction point.

Keywords: gating hypothesis; RNA abundance; RNA editing; RNA splicing

INTRODUCTION

The apolipoprotein B (apoB) gene spans 43 kb, contains 29 exons, and encodes a 14-kb mRNA (Ludwig et al., 1987). ApoB serves an essential structural role in the assembly and secretion of triglyceride-rich lipoproteins from small intestine and liver and also functions in their uptake by peripheral body tissues (Sparks & Sparks, 1994). Two major variants of apoB (apoB100 and apoB48) are expressed in a tissue-specific manner and differ markedly in their role in lipoprotein secretion and uptake (Davidson, 1993). The proportion of total apoB occurring as each variant is largely determined by the proportion of total cellular apoB mRNA that is edited (Chan, 1992; Greeve et al., 1993; Teng et al., 1994). This proportion varies among different tissues and different species from 0% to nearly 100% (Chen et al., 1987; Powell et al., 1987; Backus et al., 1990; Greeve et al., 1993). Alterations in these proportions have been observed throughout tissue development (Jiao et al., 1990; Teng et al., 1990a, 1990b; Wu et al., 1990; Higuchi et al., 1992; Funahashi et al., 1995) and following metabolic perturbation (Davidson et al., 1988; Baum et al., 1990; Inui et al., 1994; Thorngate et al., 1994; Funahashi et al., 1995). Due to the larger variant's correlation with risk of acquiring atherogenic diseases, several studies have focused on the *cis*- and *trans*-acting factors involved in regulating apoB RNA editing.

ApoB RNA editing involves the deamination of cytidine at nucleotide 6666 (C6666), thereby converting a CAA glutamine codon to a UAA translation STOP codon (Chen et al., 1987; Powell et al., 1987). Site-specific cytidine deamination results from interactions of *cis*- and *trans*-acting factors in the assembly of editing complexes, or "editosomes" (Smith et al., 1991). A tripartite

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editing motif has been described that consists of a mooring sequence, a spacer region, and a regulator region (Smith, 1993). The mooring sequence is both necessary and sufficient for editosome assembly and site-specific editing in a variety of native and chimeric RNA contexts (Boström et al., 1989; Backus & Smith, 1991, 1992; Driscoll et al., 1993; Harris et al., 1993; Backus et al., 1994).

RNA sequences flanking the editing site contribute an ill-defined, bulk RNA "context" that can stimulate editing efficiency in direct proportion to its length and AT-content (Boström et al., 1989; Davies et al., 1989; Driscoll et al., 1989; Backus & Smith, 1991, 1994; Backus et al., 1994). This appears not to be due to a specific RNA secondary structure because mutagenesis targeted at creating or eliminating RNA secondary structure at the editing site has failed to demonstrate such a requirement (Shah et al., 1991; Backus & Smith, 1994; Backus et al., 1994). With regard to potential RNA secondary structure, editing of apoB nuclear RNA precursors containing intron 25 had a sevenfold lower editing efficiency of cytidine 6666, within exon 26, compared to spliced RNA (Lau et al., 1991). Moreover, nonpolyadenylated, unspliced, apoB nuclear precursors were edited 33-fold less efficiently than polyadenylated, fully spliced nuclear mRNA. These data appear to be in contrast with the clear role for RNA secondary structure in editing of RNAs encoding the glutamate receptor subunits (Higuchi et al., 1993; Egebjerg et al., 1994). In this system, site-specific adenosine deamination is carried out by a mammalian double-stranded RNA adenosine deaminase (O'Connell et al., 1994; Dabiri et al., 1996), which binds to RNA secondary structure formed between a unique RNA sequence flanking the editing site in exon 11 and an exon-complementary sequence (ECS) within the downstream intron.

The catalytic subunit of the editosome, APOBEC-1, (Teng et al., 1993; Hadjiagapiou et al., 1994; Yamanaka et al., 1994; Davidson et al., 1995) is a zinc-dependent cytidine deaminase (Barnes & Smith, 1993; Navaratnam et al., 1993a; Yamanaka et al., 1994) whose amino acid sequence shares homology with other known cytidine deaminases (Bhattacharya et al., 1994). In hepatocytes, APOBEC-1 expression levels correlate directly with the proportion of apoB RNA edited (Giannoni et al., 1994a; Inui et al., 1994; Teng et al., 1994; Sowden et al., 1996). In other tissues, or during early stages of tissue development, editing activity can be regulated without changing APOBEC-1 abundance (Funahashi et al., 1995).

APOBEC-1 has a weak and nonsequence-specific binding affinity for RNA (Anant et al., 1995; MacGinnitie et al., 1995; Navaratnam et al., 1995) and cannot edit apoB RNA in the absence of other cellular proteins, "auxiliary factors." These auxiliary proteins are present in extracts isolated from a variety of tissues obtained from a number of different species (Teng & Davidson, 1992; Navaratnam et al., 1993a; Teng et al., 1993; Driscoll & Zhang, 1994; Yamanaka et al., 1994). Proteins with molecular masses of 66 kDa and 44 kDa, which bind selectively to RNAs containing the apoB editing site (Lau et al., 1990; Driscoll et al., 1993; Harris et al., 1993; Navaratnam et al., 1993b), have been implicated as the presumptive auxiliary factors responsible for editing site-specific interactions during editosome assembly (Harris et al., 1993).

We have examined the role of *cis*-acting elements, RNA abundance, and APOBEC-1 abundance in the regulation of apoB editing. In transfected cells, the proportion of edited apoB RNAs was a function of: (1) *cis*-acting sequence within the tripartite editing motif; (2) the amount of distal bulk RNA flanking the editing site; (3) the presence of 5′ or 3′ introns; and (4) the abundance of APOBEC-1. The proportion of edited molecules in an RNA population remained relatively constant over a range of RNA abundance despite the co-expression of potentially competing RNA editing substrates and the apparent rate-limiting quantities of APOBEC-1. Regulation of this nature suggested that the proportion of edited RNAs must be determined during RNA biogenesis/processing and not at the level of the cytoplasmic mRNA pool. A population "gating" hypothesis has been proposed wherein the proportion of edited RNAs is determined by *cis*-acting elements and *trans*-acting factor abundance at a nuclear restriction point, temporally and/or spatially close to the time when premRNA is undergoing splicing.

RESULTS

ApoB RNA editing is mooring-sequence dependent in transfected cells

To evaluate the mooring-sequence dependence of apoB RNA editing, wild-type (WT) and mutant human apoB RNA constructs used previously to define the role of the mooring sequence in vitro (Backus & Smith, 1991) were subcloned into the eukaryotic expression vector pRc/CMV and transiently transfected in duplicate into McArdle RH7777, rat hepatoma cells. McArdle cells were selected for this study because they transcribe and edit apoB RNA, and secrete lipoprotein particles containing large and small apoB variants (Davies et al., 1989). Editing of RNA from the transfected human cDNAs (exogenous RNA) was assayed on reverse transcriptase-PCR (RT-PCR) products synthesized with pRc/CMV-specific amplimers (see the Materials and methods).

Previous in vitro editing analyses demonstrated that translocation of the mooring sequence to a position 3′ of an otherwise unedited cytidine (at nt 6434) in apoB RNA (referred to as the translocation construct, TL) resulted in editing of that cytidine. In transfected McArdle cells, WT human apoB exogenous RNA supported 25% editing (SEM = 1.2%; $n = 10$) (Fig. 1A, lane 2). The

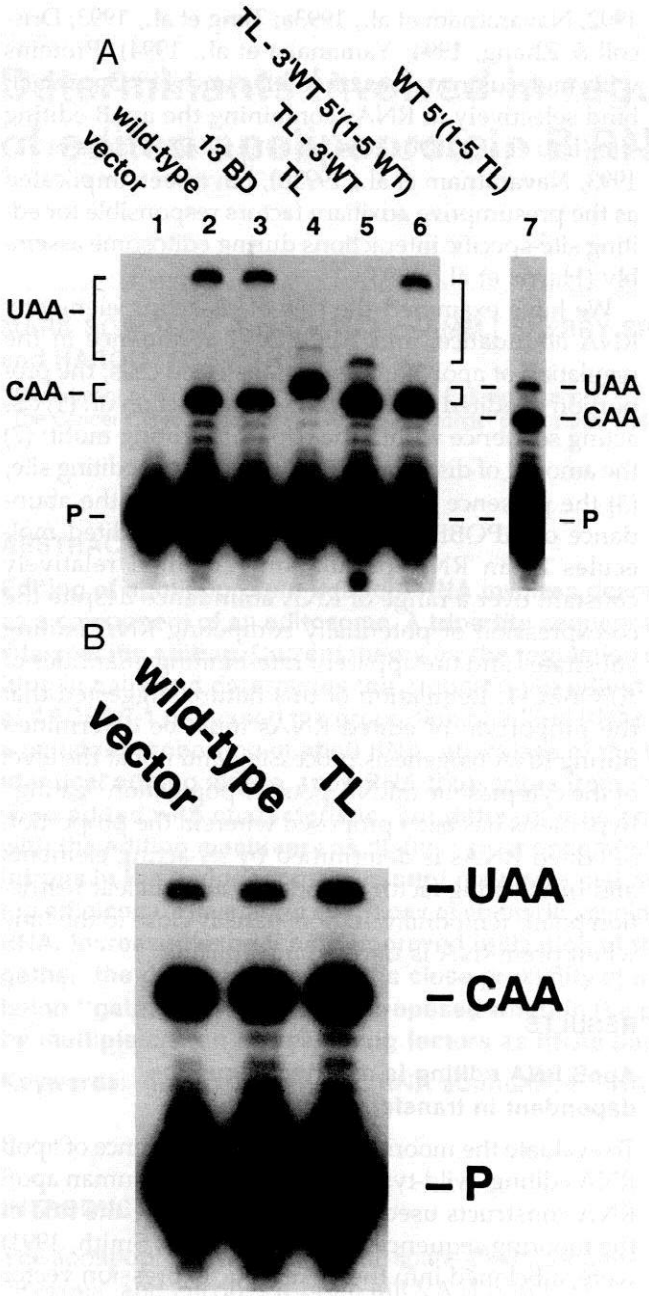

FIGURE 1. Mooring sequence-dependent apoB RNA editing in McArdle cells. **A:** Primer extension analyses performed upon RT-PCR-amplified human apoB RNA substrates transiently expressed in McArdle cells (see the Materials and methods). Positions of the primer (P), DD3, and the extension products generated from unedited (CAA) and edited (UAA) RNAs are indicated. Lane 1 (vector), control primer extension on RNA isolated from cells transfected with pRc/CMV alone. Lane 3 (13-BD), parental construct for the subsequent mutant RNAs (lanes 4–7) wherein the *Bcl* I restriction site at nt 6440 has been destroyed (Backus & Smith, 1991). **B:** Primer extension analyses performed upon RT-PCR amplified endogenous McArdle-cell apoB RNA from the same cDNA population amplified in A, but with ND1 and ND2 amplimers.

importance of the mooring sequence in McArdle cell editing was supported by the ability of the TL construct to edit at low levels (3%, SEM = 0.5%; $n = 6$) (lane 4). Consistent with previous studies (Backus & Smith, 1991), the editing efficiency of TL constructs increased when the spacer region in TL was shortened to that found in WT apoB RNA (AAUAU to AAUU; 6.2%, SEM = 0.3% ; $n = 4$) (lane 5, TL-3'WT) and the 5' regulator region of TL was corrected to that found in WT apoB RNA (CACAU to UGAUA; 19%, SEM = 1.3% ; $n = 5$) (lane 6, TL-3'WT 5'(1-5 WT)). Importantly, reverse mutations that substituted the TL regulator sequence for that of the WT RNA's regulator reduced editing efficiency fourfold (5.4%, SEM = 0.5%; $n = 3$) (lane 7, WT 5'(1-5 TL)). These data demonstrate that the rules of the tripartite editing motif established in vitro are operational in transfected cells and influence the efficiency at which an RNA will be edited.

ApoB RNA abundance does not affect editing efficiency

Mutating TL to TL-3'WT 5'(1-5 WT) may have improved editing efficiency of the exogenous RNA by enhancing its ability to compete with the endogenous apoB gene's transcript (endogenous RNA) for rate-limiting *trans*-acting factors. To evaluate this possibility, the specific editing efficiency of the endogenous cellular apoB RNA, in the presence of transfected WT or TL human apoB RNA substrates, was evaluated by poisoned primer extension analysis. Editing of the endogenous McArdle apoB RNA remained constant (11%, SEM = 1%; $n = 16$) regardless of which exogenous RNA was expressed (Fig. 1B). The data presented in Figure 1 suggest the paradox that the editing efficiency of the endogenous substrate is limited to low values due to saturation of RNA substrate, whilst an increase in RNA abundance and the presence of competing exogenous RNA substrates does not affect editing efficiency; a situation that is presumably only possible when the system is not at saturation.

To evaluate the effect of apoB RNA abundance on McArdle cell editing efficiency, increasing quantities (1–20 μg) of either WT or TL cDNA expression constructs were transfected into McArdle cells, in duplicate, (using the parental pRc/CMV plasmid to normalize the total DNA input in each transfection). The resultant increase in exogenous apoB RNA expression was verified by RT-PCR of identical amounts of total cellular RNA from each transfection 60 h after DNA addition (see quantification in Fig. 4) using amplimers specific for the endogenous and exogenous apoB RNAs (Fig. 2). The absence of RT-PCR products when AMV reverse transcriptase had been omitted from the first strand cDNA reaction (−) demonstrated the absence of apoB plasmid or genomic DNA contamination of the RNA samples. The amount of RT-PCR product corresponding to human wt (544 bp) and human TL (313 bp) apoB RNA expressed in each transfection suggested increased RNA abundance in proportion to the amount of input plas-

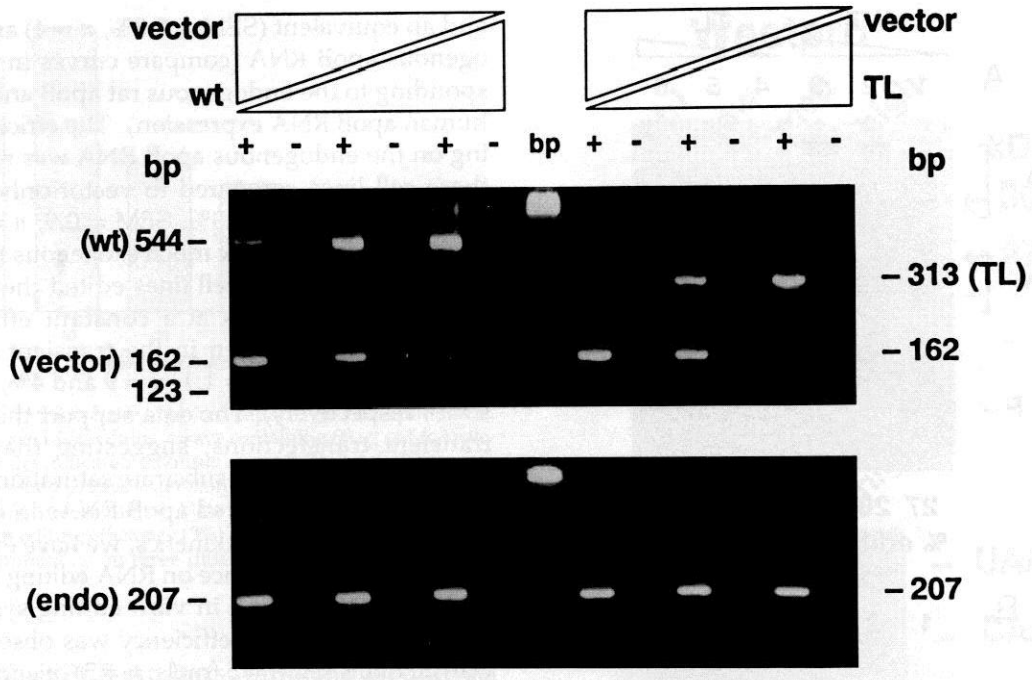

FIGURE 2. Transient overexpression of exogenous human apoB RNA. Ethidium bromide-stained 3:1 NuSieveGTG:agarose gel demonstrating (top panel) the amount of WT (544 bp) and TL (313 bp) human apoB RNAs expressed in each transfection. The 162-bp band corresponds to RNAs produced from the polylinker region of the parental pRc/CMV vector used to normalize DNA loads in transfections. Bottom panel demonstrates the amount of endogenous (endo) McArdle cell apoB RNA from the same transfectant shown directly above in the top panel. ± indicates the presence or absence of AMV reverse transcriptase in the first-strand cDNA synthesis reaction. bp indicates 123-bp DNA size markers (Gibco). The transfection efficiency of McArdle cells was determined to be greater than 50% by the use of a beta-galactosidase-expressing plasmid (pCMVβ-gal, M. Sowden, unpubl.) transfected in parallel dishes.

mid (Fig. 2, top panel). The amount of 207-bp RT-PCR product from the endogenous rat apoB RNA expressed in transfected cells appeared not to have changed over the range of the transfection experiment (Fig. 2, bottom panel).

Quantitative RT-PCR was performed by the simultaneous amplification of the endogenous rat and exogenous human WT RNAs (see the Materials and methods) (Fig. 3). Comparison of the curves for the endogenous rat apoB to that of the transiently expressed human apoB established that a 20% increase in the total apoB RNA population occurred at the highest level of transient transfection (corresponding to lane 3 in Fig. 4A,B).

Having quantified the increase in RNA abundance, an equal volume of each RT-PCR reaction described in Figure 2 was analyzed by poisoned primer extension assay to determine RNA editing efficiency. The proportion of WT and TL human apoB exogenous RNAs edited remained constant over the range of RNA abundance examined (Fig. 4A). The editing efficiency of the endogenous rat apoB RNA also remained constant despite the additional editing that occurred on the exogenous RNA substrate (Fig. 4B).

Transient expression of exogenous RNA substrates may not reflect accurately the proportions of edited RNAs that would be achieved eventually at steady state.

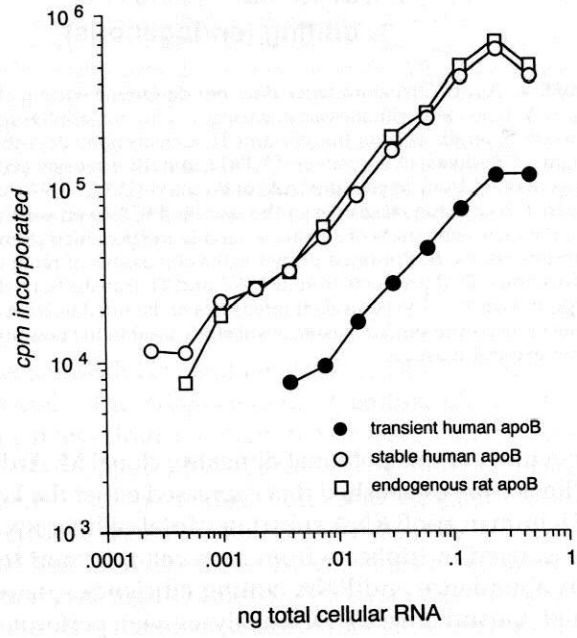

FIGURE 3. Quantification of apoB RNA expression. Exogenous human apoB, expressed in a stable cell line or as a transient transfection, was compared to the endogenous rat apoB RNA by quantitative RT-PCR (see the Materials and methods). The data shown are from the highest level of expression observed in either stable or transient transfectants. Other stable cell lines assayed expressed 54% and 60% of the levels of the endogenous rat apoB RNA.

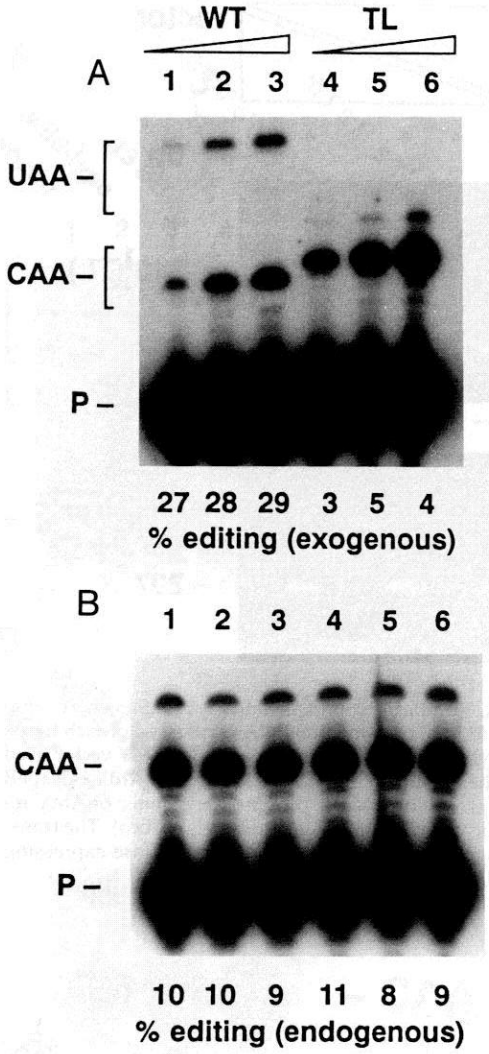

FIGURE 4. ApoB RNA abundance does not determine editing efficiency. **A:** Poisoned primer extension assays of human apoB (exogenous) PCR products from the WT and TL transfections described in Figure 2. Positions of the primer (P), DD3, and the extension products generated from unedited (CAA) and edited (UAA) RNAs are indicated. Increasing intensities of the unedited (CAA) product reflects the increasing mass of substrate used in the poisoned primer extension assays. **B:** Poisoned primer extension assays of rat apoB (endogenous) PCR products from the WT and TL transfections described in Figure 2. The equivalent intensities of the unedited (CAA) product reflects the similar masses of substrate used in the poisoned primer extension assays.

To circumvent this potential difficulty, clonal McArdle cell lines were established that expressed either the WT or TL human apoB RNA substrate. Total cellular RNA was isolated in triplicate from each cell line, and the RNA abundance and RNA editing efficiencies determined. Quantitative RT-PCR analyses were performed to determine the relative abundance of the exogenous WT and endogenous apoB RNAs expressed in each of three stable McArdle cell lines (Fig. 3). These analyses established that, relative to the expression level of endogenous apoB RNA, the stable cell lines expressed 54% (SEM = 2.5%, $n = 4$), 60% (SEM = 6.5%; $n = 4$), and an equivalent (SEM = 3.8%, $n = 4$) amount of exogenous apoB RNA (compare curves in Fig. 3 corresponding to the endogenous rat apoB and exogenous human apoB RNA expression). The efficiency of editing on the endogenous apoB RNA was not altered in these cell lines compared to vector-only transfected control cell lines (11–13%, SEM = 0.9; $n = 12$). Moreover, regardless of how much exogenous RNA was expressed, each of the cell lines edited the WT and TL exogenous substrates at a constant efficiency and equivalent to that seen in the transient transfection analyses (28%, SEM = 1.1; $n = 9$ and 4%, SEM = 0.6; $n = 9$, respectively). The data support those from the transient transfections, suggesting that editing in McArdle cells is not at substrate saturation despite the low proportion of edited apoB RNAs in these cells.

To model saturation kinetics, we have evaluated the effect of RNA abundance on RNA editing efficiency in a McArdle cell extract in vitro editing system. Maximum in vitro editing efficiency was observed by 3 h with 20 fmols (SEM = 5 fmols; $n = 5$) of apoB RNA substrate and 60 μg of McArdle cell extract protein (Fig. 5). These findings are consistent with kinetic measurements of editing activity in extracts that indicated saturation kinetics at low RNA concentrations and a K_m of 0.4–2.0 nM (1–20 fmols of RNA) (Boström et al., 1990; Greeve et al., 1991; Teng & Davidson, 1992; Inui et al., 1994). Increased input RNA substrate resulted in progressive reduction in editing efficiency and suggested competition for rate-limiting *trans*-acting factors. The capacity to maintain a constant proportion of edited RNAs as the size of the RNA population increased was therefore not observed in cell extracts and was unique to editing within intact cells.

Relationship of APOBEC-1 abundance to the proportion of edited RNAs

An alternate explanation for how cells can maintain a constant proportion of edited RNAs within an expanding RNA population is that APOBEC-1 expression may have undergone a commensurate increase. The amount of APOBEC-1 protein could not be determined accurately because its expression in WT McArdle cells is below the detection limit of our peptide-specific polyclonal antibody (Fig. 6, control lane in the top panel), as has also been observed with antibodies prepared by other laboratories (Lau et al., 1994; Teng et al., 1994). A quantitative assessment of APOBEC-1 induction at the transcription level was therefore performed on *apobec-1* mRNA. Approximately 1 μg of RNA from cells expressing the lowest and the highest level of transiently transfected exogenous human apoB RNA was analyzed by quantitative PCR as described above.

Conditions were established that enabled separate but simultaneous amplification of RNAs encoding APOBEC-1 and β2-microglobulin ($\beta 2M$) and the quan-

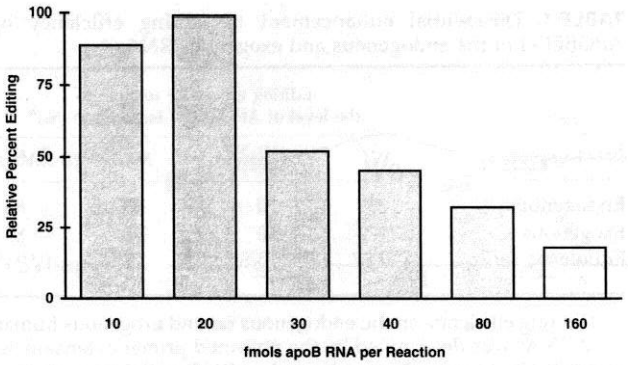

FIGURE 5. Cell extracts have a limited capacity to edit apoB RNA. Products of in vitro RNA editing assays containing 60 μg of McArdle-cell extract and the indicated amounts of apoB RNA substrate were assayed by poisoned primer extension and quantified as described (see the Materials and methods). Data is expressed as a percentage of the maximum editing efficiency (3%) and is representative of three separate determinations on three different extract preparations.

tification of their PCR products within the exponential range of amplification. *β2M* was used as an internal standard reference because it is an RNA that is expressed widely and whose abundance is refractory to most metabolic alterations (Giannoni et al., 1994b). Figure 7 indicates parallel amplification of *β2M* and *apobec-1* following serial twofold dilutions of first-strand cDNAs, between 0.5 pg and 1 ng. The data indicated that *apobec-1* was expressed at levels significantly lower than *β2M* in McArdle cells and, importantly, demonstrated that this level was relatively invariant under conditions of low and high exogenous apoB RNA expression. Furthermore, the superimposition of the *β2M* curves indicated that an equivalent mass of RNA from the low and high transfections was used for these analyses as well for as those in Figure 2. These data suggest strongly that APOBEC-1 abundance did not change in response to an increased size of the apoB RNA population. We cannot rule out, however, the possibility of translational control mechanisms for APOBEC-1 or that other proteins of the editosome may have been regulated.

To evaluate directly the effect of an increase in APOBEC-1 expression on the proportion of edited RNAs in the endogenous and exogenous RNA populations, cell lines stably expressing different levels of APOBEC-1 were cloned. Cells were ranked for APOBEC-1 expression according to the editing efficiency on the endogenous rat apoB RNA as low (20–40%), medium (45–65%), and high (70+%) (Fig. 6, lower panel). Western blotting of whole-cell extracts with APOBEC-1, peptide-specific polyclonal antibody revealed an eightfold increase in APOBEC-1 protein from the low to high expressing cell line (Fig. 6, top panel), demonstrating that a greater proportion of apoB RNA can be edited when APOBEC-1 abundance is increased.

As expected, the editing efficiency of transiently transfected human exogenous apoB RNA also increased with elevated levels of APOBEC-1, but, interestingly, the enhancement of editing efficiency was much less dramatic than that observed on the endogenous rat apoB RNA (Table 1). The ratio of editing efficiency in the endogenous and exogenous RNAs increased from 0.57 for wt McArdle cells to 1.0 in the APOBEC-1 overexpressing cell lines designated medium and high (Table 1). The data suggest that, had there been a commensurate induction of APOBEC-1 due to an increase in size of the apoB RNA population, then it would have been apparent as an elevated ratio of editing efficiency of endogenous to exogenous RNAs. This was not observed in our experiments involving wt McArdle cells transfected with apoB cDNAs. These data strongly support the role of APOBEC-1 abundance

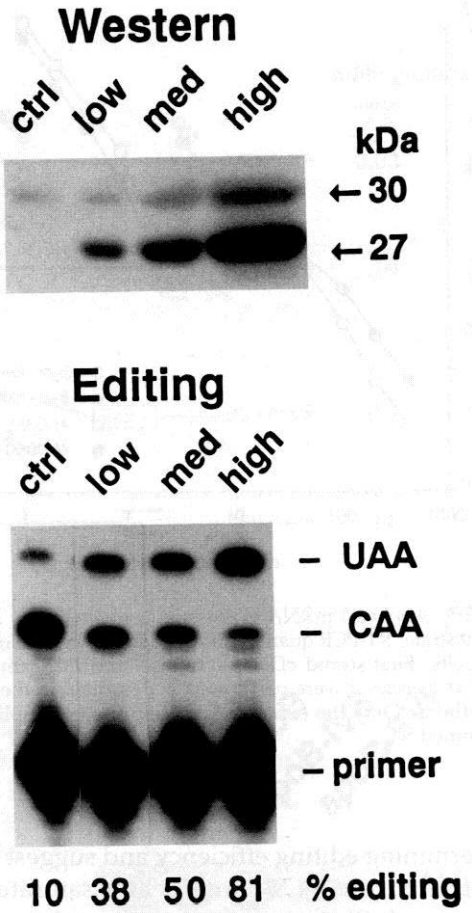

FIGURE 6. Editing efficiency increases with elevated APOBEC-1 abundance. Western: Total cellular protein extracts from select stable *apobec*-1 transfected McArdle cell lines were western blotted and reacted with an APOBEC-1, peptide-specific antibody, and visualized using an I^{125}-labeled secondary anti-rabbit IgG as described in the Materials and methods. Locations of APOBEC-1 (27 kDa) and of an uncharacterized but possibly nonspecific 30-kDa protein are indicated. Ctrl indicates stable McArdle cell line only transfected with vector. Editing: Poisoned primer extension assays for editing of endogenous apoB RNA from the corresponding control McArdle (ctrl) and APOBEC-1 overexpressing McArdle cell lines (shown above) were performed and quantified by phosphorimaging as described in the Materials and methods.

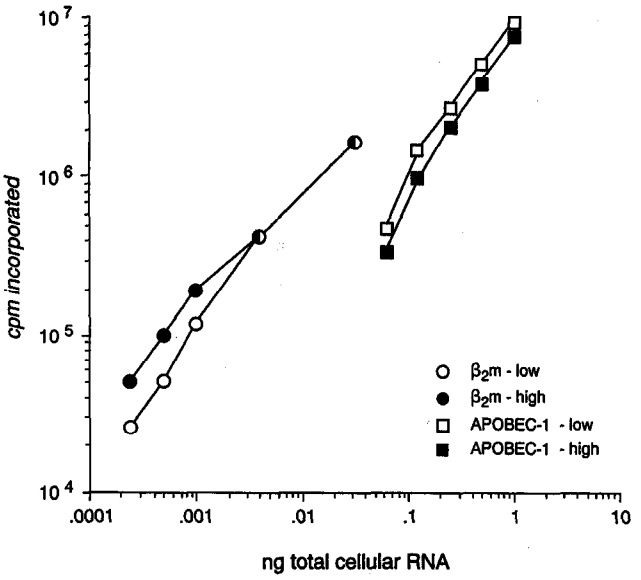

FIGURE 7. Apobec-1 mRNA expression is unaltered by increasing RNA substrate. RT-PCR quantification of *apobec-1* expression in transfected cells. First-strand cDNA synthesis and PCR amplification of *β2M* and *apobec-1* were performed as described in the Materials and methods. Only the exponential phase of each amplification is diagrammed.

TABLE 1. Differential enhancement of editing efficiency by APOBEC-1 of the endogenous and exogenous RNAs.[a]

	Editing efficiency relative to the level of APOBEC-1 expression (%)[b]			
RNA substrate	WT	Low	Med	High
Endogenous[c]	12[d]	20	55	85
Exogenous	21	30	55	85
Endo/exog ratio	0.57	0.67	1[d]	1[d]

[a] Editing efficiency on the endogenous rat and exogenous human apoB RNAs was determined by the poisoned primer extension assay in duplicate upon three independent RNA isolations from each cell as described in the Materials and methods. Cell lines used were control McArdle cells (transfected with vector alone) and stable cell lines expressing increasing amounts of APOBEC-1 and editing activity (see Fig. 6).

[b] The relative abundance of APOBEC-1 protein is determined from the western blots of Figure 6, where wt is below the detection limit of the antibody. High and Med are 8 and 4 arbitrary units normalized as a relative western blot signal where Low has been given an arbitrary unit of 1.

[c] Endogenous and exogenous refer to the RNA transcripts from the endogenous rat apoB gene and from the human apoB partial cDNA described in the Materials and methods.

[d] Each value represents the average of six independent determinations, where the range of the standard error of the mean across the whole study was 0.7–1.8%.

in determining editing efficiency and suggest that the editing activity in wt McArdle cells is saturated at the existing apoB RNA substrate concentration.

The presence of introns suppresses editing efficiency

We inferred from the low editing efficiency of apoB nuclear RNA precursors in the study of Lau et al. (1991) that premRNA may not be the preferred substrate of the editing apparatus and that introns in the endogenous RNA may have contributed to its lower editing efficiency relative to that of the exogenous RNA in our study. This also suggests the possibility that the presence of introns may have distinguished the endogenous and exogenous RNA as unique RNA populations with distinct editing efficiencies. To test this possibility, we constructed chimeric splicing/editing RNA substrates (composed of the adenovirus-spliced late-leader sequence and the WT, human apoB RNA) and expressed them as stable McArdle cell transfectants. The adenovirus-spliced late-leader sequence positioned in the antisense orientation 5′ or 3′ to the apoB RNA containing the editing site served as same-length controls (Fig. 8A, SVI-apoB and apoB-SVI, respectively). cDNAs from unspliced IVS-apoB and apoB-IVS RNAs were amplified selectively from three separate clonal cell lines using the MS amplimer pair (Fig. 8A). Total cellular chimeric RNA was amplified with apoB exon-specific amplimers PCR5 and PCR12 (Fig. 8A). Alternatively, two RT-PCR products corresponding to unspliced and spliced RNAs were generated using SP6 and T7 as amplimer pair on RNA isolated from cells transfected with either IVS-apoB or apoB-IVS chimeric constructs (data not shown). This method produced equivalent results to those described below. Densitometric quantification of these PCR products indicated that the unspliced RNA was present at less than one-fiftieth of the abundance of the spliced population.

The editing efficiency of the unspliced IVS-apoB RNA was below the quantification limits of the primer extension assay, but could be determined accurately as 1.7% on apoB exon-specific RT-PCR products (Fig. 8B, first pair of lanes, intron and apoB respectively). The low abundance of unspliced chimera and the lack of editing on intron-containing precursor suggested that 1.7% must correspond to the editing efficiency of the processed chimera. Editing of the same length control SVI-apoB was markedly elevated (56% and 52%) when assayed on chimera-specific or apoB-specific RT-PCR products, respectively (Fig. 8B, second pair of lanes, SVI and apoB, respectively). The approximate doubling in editing efficiency of SVI-apoB compared to that described above for the WT human apoB RNA construct is consistent with the nonspecific stimulatory effect of bulk RNA 5′ of the editing site when bulk apoB RNA lies 3′ of the editing site (Backus & Smith, 1994) and the general stimulatory effect that increased RNA length has on editing efficiency (1,121 nt compared to 701 nt for SVI-apoB and WT, respectively) (Boström et al., 1989; Davies et al., 1989; Driscoll et al., 1989; Smith et al., 1991; Backus & Smith, 1994). IVS-apoB is

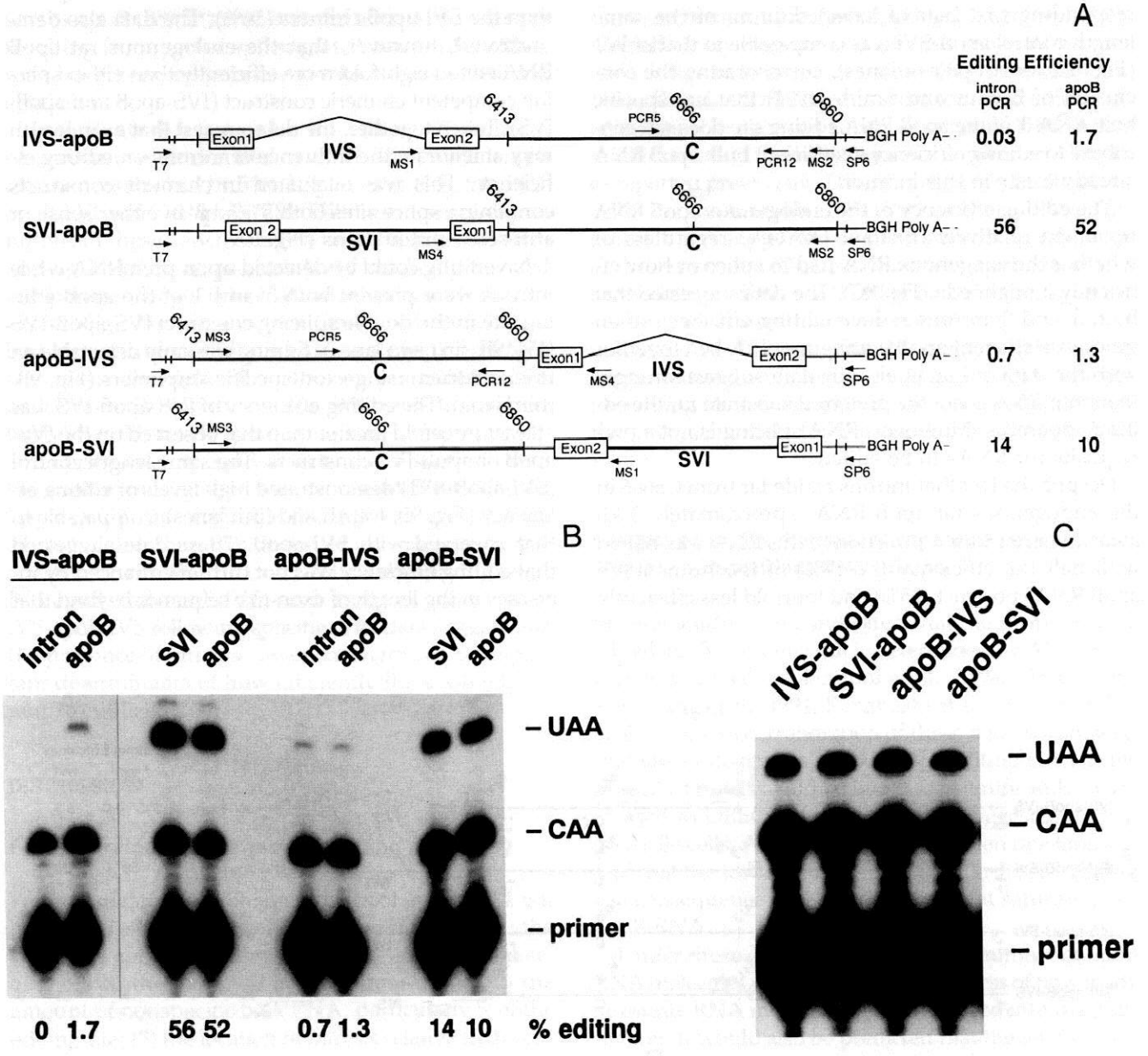

FIGURE 8. Effect on 5′ or 3′ introns on editing efficiency. **A:** Diagram of the chimeric apoB-intron RNA expression constructs. Coordinates of the human apoB RNA sequence are given (Knott et al., 1986) and the location of PCR amplimers shown. PCR5 and PCR12 were used to amplify total apoB sequences, and the construct-specific MS amplimer pairs used to amplify intron sequence containing RNAs from the same first-strand cDNA reaction. "BGH PolyA" indicates the vector-derived bovine growth hormone polyadenylation signals. **B:** Poisoned primer extension assays of chimeric apoB-intron RNAs. Stable clonal McArdle cell lines that express each RNA construct (designated at the top of each pair of lanes) were selected and editing efficiencies (% editing) determined in triplicate from three separate lines of each chimeric RNA construct. "intron" and "SVI" refer to RT-PCR products specific to RNA containing an intron in the sense (IVS) or anti-sense (SVI) orientation, respectively. "ApoB" refers to RT-PCR products specific for the apoB exon. **C:** Poisoned primer extension assays of endogenous McArdle cell apoB RNAs analyzed from the same first-strand cDNA synthesis reactions analyzed in B as indicated by construct designation at the top of each lane. Average editing efficiencies were determined as 12%, 12.5%, 14%, and 13.5% ($n = 9$) for IVS-apoB through apoB-SVI, respectively.

the same length as SVI-apoB prior to splicing and is 889 nt after splicing. The marked reduction in editing efficiency of IVS-apoB chimeric RNA cannot be attributed to changes in length alone and appears, therefore, to be directly attributable to the fact that this RNA must be spliced.

The effect of introns located 3′ of the editing site on RNA editing efficiency was also evaluated. Editing efficiency was low, but detectable on intron-containing precursor apoB-IVS (0.7%) and only marginally more efficient after splicing (1.3%) (Fig. 8B, compare third pair of lanes, intron and apoB, respectively, to the cor-

responding first pair of lanes). Editing of the same length control apoB-SVI was comparable to that of WT (Fig. 8B, fourth pair of lanes), corroborating the conclusion of Backus and Smith (1994) that nonspecific bulk RNA 3′ of the apoB RNA editing site does not contribute to editing efficiency if sufficient bulk apoB RNA already exists in this location.

The editing efficiency of the endogenous apoB RNA remained relatively constant (12–14%) regardless of whether the exogenous RNA had to splice or how efficiently it might edit (Fig. 8C). The data suggested that both 5′ and 3′ introns reduce editing efficiency of an otherwise efficient apoB editing motif. Taken together with the data of Lau et al., our data suggest strongly that premRNA is not the preferred substrate for the editing apparatus. Moreover, RNA splicing is not a prerequisite for RNAs to be edited.

Despite the fact that introns reside far from C6666 in the endogenous rat apoB RNA (approximately 3 kb away in either 5′ or 3′ directions), this RNA was edited with half the efficiency (12–14%) of the human WT apoB RNA construct (25%) and fourfold less efficiently than the SVI-apoB chimera (56%). The data also demonstrated, however, that the endogenous rat apoB RNA edited eightfold more efficiently than either splicing competent chimeric construct (IVS-apoB and apoB-IVS). Taken together, the data suggest that exon length may ameliorate the influence of introns on editing efficiency. This was evaluated in chimeric constructs containing splice sites both 5′ and 3′ in either sense or anti-sense orientations (Fig. 9A).

No editing could be detected upon premRNA while introns were present both 5′ and 3′ of the apoB editing site in the double splicing construct IVS-apoB-IVS (Fig. 9B, first two lanes). Editing was only detectable on this construct using exon-specific amplimers (Fig. 9B, third lane). The editing efficiency of IVS-apoB-IVS was at least threefold greater than that observed on the IVS-apoB or apoB-IVS constructs. The same length control (SVI-apoB-SVI) demonstrated high levels of editing efficiency (Fig. 9B, fourth and fifth lanes), comparable to that observed with SVI-apoB. These data suggested that editing efficiency was not further enhanced by increases in the length of exon-like sequence beyond that

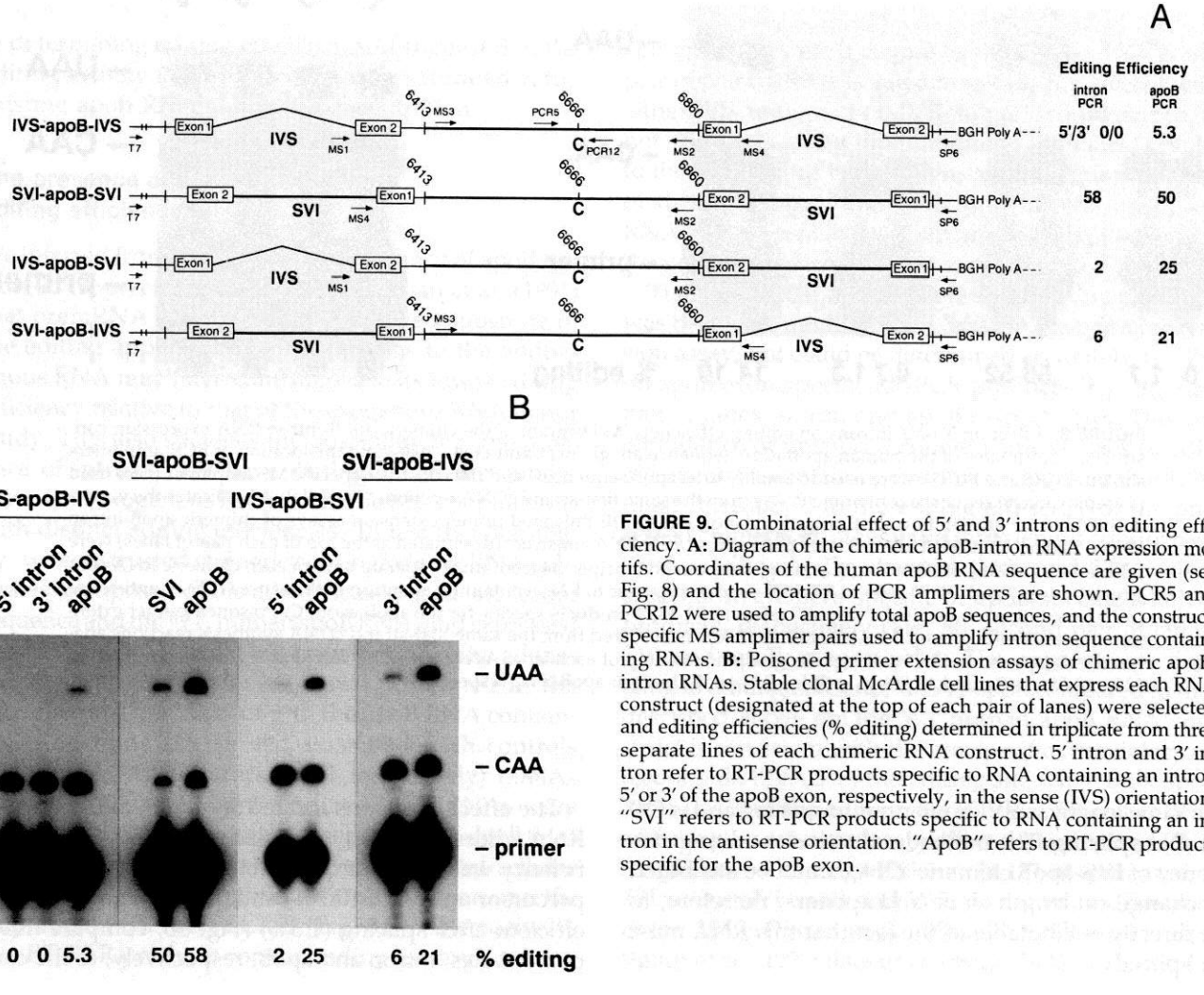

FIGURE 9. Combinatorial effect of 5′ and 3′ introns on editing efficiency. **A:** Diagram of the chimeric apoB-intron RNA expression motifs. Coordinates of the human apoB RNA sequence are given (see Fig. 8) and the location of PCR amplimers are shown. PCR5 and PCR12 were used to amplify total apoB sequences, and the construct-specific MS amplimer pairs used to amplify intron sequence containing RNAs. **B:** Poisoned primer extension assays of chimeric apoB-intron RNAs. Stable clonal McArdle cell lines that express each RNA construct (designated at the top of each pair of lanes) were selected and editing efficiencies (% editing) determined in triplicate from three separate lines of each chimeric RNA construct. 5′ intron and 3′ intron refer to RT-PCR products specific to RNA containing an intron 5′ or 3′ of the apoB exon, respectively, in the sense (IVS) orientation. "SVI" refers to RT-PCR products specific to RNA containing an intron in the antisense orientation. "ApoB" refers to RT-PCR products specific for the apoB exon.

of SVI-apoB (889 nt) and that the added exon length resulting from partial splicing of IVS-apoB-IVS RNA may have been responsible for its enhanced editing efficiency.

To evaluate this last point, the editing efficiency of constructs was determined in which an optimal length of exon was flanked by 5′ or 3′ splice sites (Fig. 9A, IVS-apoB-SVI and SVI-apoB-IVS). Editing efficiency of premRNA in these constructs (2% and 6%, [Fig. 9B, sixth and eighth lanes, respectively]) was markedly elevated compared to single splice site constructs with shorter exons (Fig. 9B, first and fifth lanes, respectively). In both constructs, the editing efficiency of spliced RNAs was markedly higher than that on premRNAs (25% and 21% for IVS-apoB-SVI and SVI-apoB-IVS, respectively [Fig. 9B, seventh and ninth lanes, respectively]), and approximately equivalent to that observed when the apoB exon segment was expressed alone (Fig. 4). Importantly, this editing efficiency was greater than 14-fold higher than that observed on single splice site constructs with shorter exons following splicing (e.g., IVS-apoB and apoB-IVS) and fourfold greater than IVS-apoB-IVS following splicing. The data suggest that the presence of introns and exon length can be important determinants of how efficiently the apoB editing motif is utilized.

DISCUSSION

Four characteristics affecting editing efficiency

We investigated four characteristics of apoB RNA editing that influenced its efficiency within cells: (1) the degree of conformity in the editing-site proximal sequences to the tripartite apoB editing motif; (2) the amount of nonspecific bulk RNA, particularly 5′ of the editing site; (3) the location of introns relative to the editing site; and (4) the level of APOBEC-1 expression. Our data demonstrate that the three *cis*-acting characteristics serve to define an RNA's intrinsic ability to be identified by the editing apparatus as a substrate. Expression of apoB WT and mutant cDNA constructs in transfected cells demonstrated that apoB RNA editing in McArdle cells was mooring-sequence dependent and utilized a tripartite editing motif established from mutagenesis and in vitro editing assays (Smith, 1993). These data corroborate and extend those reported by Driscoll et al. (1993) to include spacer and regulator element requirements.

This is the first analysis of the effect of RNA abundance on apoB RNA editing efficiency in cells that express endogenous and exogenous RNA editing substrates stably and simultaneously. Our data demonstrate that the endogenous and exogenous RNAs were processed in McArdle cells as separate populations of RNAs with distinct editing efficiencies. The ability of the exogenous substrate to support a higher level of editing efficiency than the endogenous substrate has not been observed in previous studies (Davies et al., 1989; Driscoll et al., 1993). The different reported editing efficiencies of exogenous substrates may be attributable to differences in flanking sequence content and length as reported previously (Davies et al., 1989; Smith et al., 1991; Backus & Smith, 1994; Backus et al., 1994). This possibility is supported by the high editing efficiency of the exogenous RNA substrate SVI-apoB (56%) and a low editing efficiency (3–4%) of a 207-nt rat apoB exogenous RNA substrate (data not shown). The important aspect of our data is that they demonstrate that the exogenous and endogenous substrates do not compete for editing factors as might have been predicted from the current models for the regulation of apoB RNA editing.

Paradoxes in the theories of regulation of mooring sequence-dependent RNA editing

Several studies have indicated that the proportion of edited apoB RNA in a cell is determined by APOBEC-1 expression levels (Giannoni et al., 1994a; Inui et al., 1994; Teng et al., 1994; Funahashi et al., 1995; Sowden et al., 1996). In corroboration of these data, we showed that low endogenous apoB mRNA editing efficiencies in McArdle cells could be increased significantly by increased APOBEC-1 abundance. This suggested that, in McArdle cells, APOBEC-1 may have been rate limiting, and that the low efficiency of WT McArdle cell editing is a consequence of the system being at saturation for apoB RNA.

Under these conditions, a constant number of apoB RNA molecules would be edited regardless of how many substrate RNA molecules are introduced into the population. It would also be predicted that the relative proportion of edited RNAs in the population would decrease as the number of substrate RNAs in the population increased. Specifically, if the McArdle cell's editing pathway was operating at saturation kinetics, it would be anticipated that the exogenous RNA should reduce the proportion of edited endogenous RNA in a direct relationship to its abundance. We have shown, however, that the proportion of edited RNA in either the endogenous or exogenous apoB RNA populations remained unaltered regardless of the type or quantity of editing substrate expressed. These data suggest the paradox that the proportion of edited molecules in a population appears to be determined by rate-limiting levels of APOBEC-1 (characteristic of a system at saturation for RNA substrate), but that this proportion is not affected by the actual number of RNAs in the population (characteristic of a system not at saturation). We are unaware of any other RNA processing system that exhibits this paradox.

The "population gating" hypothesis

We propose a "population gating" hypothesis to explain the paradox in the mechanism by which *cis*- and *trans*-acting factors interact to determine the unique editing efficiency of distinctly different RNAs within coexisting RNA populations. The essential premise of this hypothesis is that regulation of the proportion of edited RNA in a population can only be achieved from a control point through which all RNAs must traverse during synthesis and/or processing (a restriction point or "gate"). This mechanism of regulation would be more efficient than one that might be envisioned in an alternative model, where editosomes diffuse through cells and edit RNAs in a dispersed and preexisting population. The mechanism also accounts readily for why the proportion of edited apoB RNA is not 100%, i.e., the gate has limited access to RNAs or a "window of opportunity" to edit.

We propose that the gate must have the capacity for evaluating the occurrence of editing sites in RNA by "recognizing" mooring sequences and other *cis*-acting characteristics. Presumably at the same time, the context of the editing motif (the AT-content of bulk RNA 5′ and 3′ of the editing motif and the proximity or absence of introns) would be assessed. Each RNA passing through the gate would have a statistical probability of being edited (based on its *cis*-acting RNA characteristics and APOBEC-1 abundance), resulting in a unique proportion of edited RNAs in any given population independent of the ultimate size of that population or the occurrence of potentially competing populations of RNAs.

Ultimately, this sensing must be translated to the efficiency with which catalytically active editosomes assemble on each RNA passing through the gate. In this regard, the population gating hypothesis incorporates current theory for apoB RNA editing regulation by proposing that APOBEC-1 is part of the gate. Elevated levels of APOBEC-1 would result in an increased proportion of edited RNAs by increasing the number of catalytically competent enzymes available for editosome assembly at the gate.

An alternative explanation for these data would be if APOBEC-1 had a catalytic cycle involving regulation of the active site or the sites of interactions with auxiliary factors (e.g., through phosphorylation). The proportion of edited RNAs in a population would be a function of the number of enzyme complexes in the catalytically competent phase of the enzyme cycle. More APOBEC-1 would increase the probability of RNA interactions with active enzyme complexes and thereby raise the proportion of edited RNAs in the population. This explanation is supported by the enhancement in editing efficiency when APOBEC-1 is overexpressed. It cannot account adequately for the ability of McArdle cells to edit the endogenous RNA and exogenous RNAs, such as SVI-apoB-SVI, as noncompeting populations with distinct editing efficiencies. The duration of the enzyme cycle should be a property of the editing activity (the editosome) and should not vary depending on which RNA is being edited. Importantly, the observation that a constant proportion of RNAs could be edited regardless of the number of substrate RNAs introduced into the system suggests that the "catalytic cycle" of APOBEC-1 is probably not solely responsible for the observed regulation.

The effect of splice sites on mooring sequence-dependent RNA editing

Our data indicated that there was a profound effect of RNA splice sites on editing efficiency. The average length for internal exons of eukaryotic genes is only 200 nt (Hawkins, 1988). ApoB exon 26, which contains the editing site, is more than 7.5 kb in length and, according to the exon definition model (Robberson et al., 1990), would be proposed to be spliced inefficiently. However, more recent work (Chen & Chasin, 1994) has shown that exons up to 1.4 kb are spliced efficiently. The apoB mRNA editing site is flanked by more than 3 kb of RNA sequence on either side. Our data on the effect of long RNAs (SVI-ApoB-SVI construct, 1.65 kb) and long exons (IVS-apoB-SVI and SVI-apoB-IVS, 889 nt) on editing efficiency suggest that the endogenous rat apoB RNA editing efficiency should be much higher. The multiple splicing events that this premRNA must undergo might have influenced its editing efficiency.

In contrast, adenosine to inosine conversion resulting from editing of RNA encoding neural calcium-gated glutamate receptors (Sommer et al., 1991) involved a base paired sequence comprised of a 10-nt sequence within the 3′ intron and a complementary sequence in the exon, centered on the edited base. Base conversion may arise from the action of a ubiquitous nuclear double-stranded RNA adenosine deaminase (DRADA) with RNA-binding and catalytic activities (Higuchi et al., 1993; Dabiri et al., 1996). Unlike APOBEC-1 editing of apoB RNA, double-stranded RNA adenosine deaminase renders nearly 100% of the GluR-B RNAs edited (Higuchi et al., 1993). The characteristics of this system clearly indicate that this enzyme requires the secondary structure provided by GluR-B intron-exon interactions for RNA editing activity and therefore must occur before RNA splicing (O'Connell et al., 1994; Dabiri et al., 1996).

The mooring sequence-dependent editing mechanism was impaired by the proximity of introns in all constructs that were tested and must therefore be distinct from that involved in GluR-B editing. The effect of splice sites on mooring sequence-dependent editing efficiency, particularly the ability of exon length to ameliorate the affect of introns, suggested the possibility that spliceosomes may physically impede the function

or block the assembly of the C/U editosome. This possibility is further supported by the observed higher editing efficiency of spliced chimeric RNA relative to intron-containing chimeras.

We concur with the conclusions of Lau et al. (1991) in so far as we place the "gate," and hence RNA editing, within the cell nucleus. This must be true or else the presence of introns could not have impaired editing efficiency. The conclusion from our data differs from that of Lau et al. in that our data does not suggest that editing is coincident with premRNA splicing. Our data suggest that editing can take place on RNAs that do not have to splice and that premRNA is not the preferred substrate of the editosome.

To account for this relationship, we propose that one possible physical location of the "gate" may be in close proximity with nuclear structures involved in premRNA splicing (Smith, 1992; Spector, 1993; Mattaj, 1994). RNAs would transiently associate with these structures (restriction points) and become spliced and subsequently edited or (in the case of RNAs lacking introns) only become edited. As suggested earlier, the "gating" mechanism has a temporal aspect in that RNAs only have a limited opportunity to be edited. Once through the gate, editing may no longer occur. This suggests that, if RNA editing is restricted to the cell nucleus, then RNA editing might not occur normally on cytoplasmic RNA.

MATERIALS AND METHODS

Plasmid constructions

Expression vectors for WT and mutant apoB RNA substrates were generated by subcloning human apoB cDNA described previously (Backus & Smith, 1991, 1992) as *Hind* III/*Kpn* I fragments into *Hind* III/*Xba* I linearized pRc/CMV (Invitrogen, California). *Kpn* I and *Xba* I restriction ends were made blunt by the use of T4 DNA polymerase (Gibco Laboratories, New York) and Klenow fragment of DNA Polymerase I (Promega, Wisconsin) according to the manufacturer's recommendations. 5′ splice constructs were generated by the blunt-ended insertion of a 421-bp adenovirus late spliced leader motif into the unique *Hind* III site of WT. The adenovirus sequence consists of partial first and second exons of the adenovirus late spliced leader separated by the natural but shortened intron (Smith et al., 1989). This sequence was excised from its parental plasmid, pIVPX, as a *Bam*HI/*Hind* III fragment and made blunt ended by the use of Klenow enzyme as described above. 3′ splice constructs were generated by the blunt-ended insertion of the same adenovirus sequence into a unique *Apa* I site within the 3′ end of WT apoB motif. Chimeric adenovirus-apoB clones containing a 5′ splice site in the forward (IVS-apoB) or reverse (SVI-apoB) orientation and a 3′ splice site in the forward (apoB-IVS) or reverse (apoB-SVI) orientation were also constructed and confirmed by dideoxy sequencing (Sequenase V2.0, Amersham Life Sciences).

apobec-1 (Teng et al., 1993) cDNA was produced from oligo (dT)-primed, rat small intestine, total RNA using AMV reverse transcriptase (Promega) and amplified with *Pfu* DNA Polymerase (Stratagene, California) according to the manufacturer's recommendations in a PCR reaction using REPR-5′ and REPR-3′ as amplimers. Thermal cycling conditions were: 1 cycle at 94 °C for 3 min; 5 cycles at 94 °C for 45 s, 52 °C for 1.5 min, 72 °C for 1.5 min; and 30 cycles at 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min. The PCR product was subcloned into pSP73 (Promega) through the *Pst* I and *Eco*R I restriction sites in the amplimers, verified by dideoxy DNA sequencing, and subcloned into the *Hind* III and *Eco*R V sites of pRc/CMV.

Cell culture

The rat liver hepatoma cell line McArdle RH7777 was obtained from ATCC (Rockville, Maryland) and maintained in DMEM containing 10% horse serum and 10% fetal bovine serum. Cells were transfected according to the method of Chen and Okayama (1988); 48–72 h post DNA addition, cells were either harvested for RNA isolation (transient transfections) or placed under 500 μg/mL Geneticin™ (Gibco) selection for the establishment of stable transfected cells. Clonal cell lines were obtained by limiting dilution under Geneticin™ selection. Whole-cell protein was prepared for western blotting from selected lines by hypotonic lysis and shearing through a 26-gauge needle in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg aprotinin/mL (Sigma Chemical Co., Missouri), and 0.5 μg leupeptin/mL (Sigma).

RNA isolations

Total cellular RNA was isolated from approximately 2×10^6 cells by the use of Tri-Reagent (Molecular Research Center [MRC], Ohio) according to the manufacturer's recommendation. After isopropanol precipitation, RNAs were reprecipitated from 0.3 M sodium acetate in ethanol at −20 °C. RNA preparations were digested with RQ-DNase I (Promega), according to the manufacturer's recommendations, for 30 min. Buffer conditions were modified appropriately and an additional 30-min digestion with a restriction enzyme(s) having a recognition site between the PCR primer annealing sites of target substrates was performed. These were *Eco*R I and *Rsa* I for human and rat apoB RNA substrates, respectively. RNAs were phenol extracted, precipitated, and quantified by spectrophotometry.

Editing assays

The proportion of editing upon apoB RNAs synthesized in transfected cells was determined by RT-PCR methodology. First-strand cDNA was generated from 1 μg of oligo-dT primed total RNA using AMV reverse transcriptase (Promega) according to the manufacturer's recommendations. Differential PCR amplification of the endogenous rat apoB sequences encompassing the editing site and those from the transfected human apoB cDNA was performed from the same first-strand cDNA reaction using amplimers ND1/ND2 (Driscoll et al., 1989) and SP6/T7, PCR5/PCR12 (Driscoll et al., 1989), or MS1/2/3/4 combinations, respectively. PCR was performed with *Taq* DNA Polymerase (Promega) in the presence of 2.5 mM $MgCl_2$ according to the manufacturer's recom-

mendations. Thermal cycling conditions were: 1 cycle at 94 °C for 2 min; 5 cycles at 94 °C for 45 s, 52 °C for 1.5 min, 72 °C for 1.5 min; and 30 cycles at 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min. The absence of contaminating genomic or plasmid DNA from all RNA samples was confirmed by failure to generate a PCR product without prior reverse transcriptase synthesis of first-strand cDNA. PCR products were gel isolated (Qiaex, Qiagen, Inc., California) and quantified by gel electrophoresis and comparison to a DNA mass ladder (Gibco).

Editing efficiency was evaluated by the poisoned primer-extension analysis described previously (Backus & Smith, 1992) using [γ-^{32}P] ATP (6,000 mCi/mmol; NEN) end-labeled DD3 to prime both rat and human apoB products (Driscoll et al., 1989). Primer-extension products were resolved on a 10% denaturing polyacrylamide gel and quantified by scintillation counting of excised gel bands or by laser densitometric scanning (Phosphorimager Model 425E, Molecular Dynamics). The editing assay was linear between 50 pg and 10 ng of input 207-bp rat apoB PCR substrate. Quantification was performed in triplicate upon a titration of input PCR products between 0.5 and 10 ng. The fidelity of the assay was verified by the generation of wt apoB PCR products from an in vitro T7 RNA polymerase-transcribed WT-edited apoB RNA using *Pvu* II linearized pRc/CMV/WT as template. Poisoned primer extension analysis of the products revealed an edited substrate at less than 0.02% as determined by phosphorimager analysis.

In vitro editing assays containing 20 fmols of a 448-nt human apoB RNA substrate and 60 μg of protein as McArdle rat liver hepatoma cell extract were performed as described (Smith et al., 1991). Purified RNA was subjected to poisoned primer-extension analysis as described above. McArdle editing extracts were prepared by rinsing monolayer cells once with PBS followed by scraping into 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM dithiothreitol, 1.5 mM $MgCl_2$, 1 mM PMSF, 20 units soybean trypsin inhibitor/mL (Sigma), 0.5 μg aprotinin/mL, and 0.5μg leupeptin/mL. Cells were cleared by centrifugation (300 × *g*, 10 min), resuspended in five packed cell volumes of the buffer described above (diluted to 1/10 strength), and allowed to swell on ice for 30–40 min. Cells were centrifuged (2,500 × *g*, 10 min), resuspended in two packed cell volumes in the same buffer, and sheared sequentially through 18-, 20-, and 22-gauge needles at high pressure. Extracts were brought to 200 mM KCl, allowed to incubate on ice for 15 min, and cleared at 13,000 × *g*, 30 min. Total cellular protein was quantified using the BioRad protein reagent (BioRad Laboratories, Inc., California). Extract preparation was performed at 4 °C with sterile, diethyl pyrocarbonate-treated buffers. Extracts were stored at −20 °C.

RNA quantification by RT-PCR

Reverse transcription was performed upon RNAs as described above. First-strand cDNAs were diluted serially and 10-mL aliquots assayed separately but simultaneously by PCR for beta-2-microglobulin (β2M) and *apobec*-1 expression in a method similar to those described recently (Murphy et al., 1990; Giannoni et al., 1994b). All PCR reactions were performed in the presence of 2.5 mM $MgCl_2$, 1 mM of each primer, 1 mM deoxynucleotides, and 0.3 mL [α^{32}P] dCTP (3,000 Ci/mmol; NEN). Cycling conditions were 1 cycle at 94 °C for 2 min; 5 cycles at 94 °C for 45 s, 54 °C for 1.5 min, 72 °C for 1.5 min; and 94 °C for 45 s, 54 °C for 1 min, 72 °C for 1 min for 20 cycles or 35 cycles for b2M and *apobec*-1, respectively. Primers for both β2M (β2M 5′ [127–145], b2M 3′ [364–383]; Cole et al., 1989) and *apobec*-1 (p27X [528–548], p27Y [622–644]; Teng et al, 1993) were chosen to flank introns (M. Sowden & H. Smith, unpubl. data) and yield a 257-bp and 117-bp product for β2M and *apobec*-1, respectively. Ten microliters of the reaction products were resolved on a 3:1 NuSieveGTG:agarose gel, the gels dried, and quantified by laser densitometric scanning.

Determination of the relative amounts of endogenous rat and transfected human apoB substrates in McArdle cell lines was performed upon serially diluted first-strand cDNAs. PCR reactions were performed in the presence of 2.5 mM $MgCl_2$, 1 mM of each primer, and 1 mM deoxynucleotides under the following thermal cycling conditions: 1 cycle at 94 °C for 2 min; 5 cycles at 94 °C for 45 s, 55 °C for 1.5 min, 72 °C for 1.5 min; and 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min for 20 cycles. Primers for rat (ND1, ND2) and transfected human (SP6, T7) apoB RNAs yield 207-bp and 544-bp products, respectively. Each PCR reaction contained in addition 1 × 10^6 cpm (approximately 0.1 mM) of each of ^{32}P end-labeled SP6 and ND2 primers. Reaction products were quantified as described above.

Miscellaneous procedures

APOBEC-1-specific polyclonal antibodies were raised in rabbits against a peptide corresponding to an N-terminal region of APOBEC-1 (amino acids 22–36) using the services of Genosys, Inc. (Texas). Proteins were resolved on 10.5% SDS PAGE, transferred to nitrocellulose, and reacted with the polyclonal antibody under blotting conditions described previously (Smith et al., 1986).

Deoxyoligonucleotides

Deoxyoligonucleotides use in this study were:

REPR-5′	5′ CTCCTGCAGCAAGATGAGTTCCGAGA CAG 3′
REPR-3′	5′ CTCGAATTCCCAGAAGTCATTTCA ACCCTG 3′
ND1	5′ ATCTGACTGGGAGAGACAAGTAG 3′
ND2	5′ GTTCTTTTTAAGTCCTGTGCATC 3′
SP6	5′ GCTCTAGCATTTAGGTGACACTATAG 3′
T7	5′ TAATACGACTCACTATAGGG 3′
DD3	5′ AATCATGTAAATCATAACTATCTTTAATA TACTGA3′
β2M 5′	5′ CAACTTCCTCAACTGCTAC 3′
β2M 3′	5′ GCTCCATAGAGCTTGATTAC 3′
p27X	5′ AATGAAGCTCATTGGCCAAGG 3′
p27Y	5′ CTTCTTAAAATATTTAAACAGGG 3′
MS1	5′ CCTTGATGATGTCATACTTATCC 3′
MS2	5′ GTACTTCCACTTTTGTTAAAATC 3′
MS3	5′ GAAAATACAGAGCAGCCCTG 3′
MS4	5′ AGAAGTCATGCCGCTTTTGAGA 3′
PCR5	5′ CTGAATTCATTCAATTGGGAGAGA CAAG 3′
PCR12	5′ AACAAATGTAGATCATGG 3′.

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