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Genetics

Dimeric structure of a human apolipoprotein B mRNA editing protein and cloning and chromosomal localization of its gene

(chromosome 12/epitope tagging/protein dimerization)

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ABSTRACT **Apolipoprotein B (apoB) mRNA editing consists of a posttranscriptional C → U conversion involving the first base of the codon CAA encoding glutamine-2153 to UAA, a stop codon, in apoB mRNA. Using a cloned rat cDNA as a probe, we cloned the cDNA and genomic sequences of the gene for a human apoB mRNA editing protein. Expression of the cDNA in HepG2 cells results in editing of the intracellular apoB mRNA. By fluorescence *in situ* hybridization, we localized the gene for the editing protein to chromosome band 12p13.1-p13.2. By Northern blot analysis, it was shown that the human editing protein mRNA is expressed exclusively in the small intestine. The cDNA sequence predicts a translation product of 236-aa residues. By attaching an epitope tag sequence to the C terminus of the editing protein, we examined the polymerization state of the editing protein synthesized *in vitro*. We found that the editing protein undergoes spontaneous polymerization. The migration of the human apoB mRNA editing protein on an HPLC column and the stoichiometry of polymeric epitope-tagged to untagged protein indicate that the protein exists as a dimer. Dimerization does not require glycosylation of a consensus N-linked glycosylation sequence present in the protein and is not mediated by disulfide bridge formation. The human apoB mRNA editing protein is a cytidine deaminase showing structural homology to some known mammalian and bacteriophage deoxycytidylate deaminases. The latter enzymes exist as homopolymers. The fact that the apoB mRNA editing protein also exists as a homodimer has important implications for the mechanism of apoB mRNA editing in humans.**

Apolipoprotein B (apoB) is a major protein component of the circulating plasma lipoproteins: chylomicrons, very low density lipoproteins, intermediate density lipoproteins, low density lipoproteins, and a special lipoprotein called lipoprotein (a) (1). apoB exists in two forms, apoB-100 and apoB-48. In humans, apoB-100 is synthesized in the liver and is present in very low density lipoproteins and their metabolic products, intermediate density lipoproteins and low density lipoproteins. ApoB-48 is synthesized in the small intestine and is the form of apoB present in chylomicrons and chylomicron remnants. The biogenesis of apoB-48 is unique in that apoB-100 and apoB-48 are the products of the same gene. apoB-48 mRNA is produced from apoB-100 mRNA by RNA editing, which involves a C → U conversion of the first base of codon 2153, which encodes glutamine, changing it from CAA to UAA, an in-frame stop codon (refs. 2 and 3; reviewed in refs. 4 and 5). apoB-48 contains 2152 residues compared to 4536 residues in apoB-100.

In the rat, unlike in humans, apoB mRNA editing occurs in the liver as well as in the small intestine (6–8). Recently, the cDNA for the catalytic subunit of a rat apoB mRNA editing complex was cloned by Teng *et al.* (9). It predicts a M_r 27,000 protein whose mRNA appears to be widely distributed. In this communication, we report the cloning and chromosomal localization of the gene for the human apoB mRNA editing protein[§] and demonstrate that the protein exists as a homodimer. The conclusion on the dimer formation is based both on the migration position of the native editing protein on an HPLC column and on a method independent of its migration behavior. Expression of the cDNA in stably transfected HepG2 cells results in editing of the intracellular apoB mRNA. The mRNA for the 28-kDa human apoB mRNA editing protein is expressed exclusively in the small intestine, suggesting that this protein is essential for apoB mRNA editing; its absence in the liver explains why, unlike rodents, the human liver produces only apoB-100.

MATERIALS AND METHODS

Human Small Intestine cDNA Cloning and Northern Blot Analysis. Rat liver poly(A)-RNA was used as a template to clone the rat cDNA by reverse transcription–PCR based on the published cDNA sequence (9). This rat cDNA clone was used to probe a λgt11 human small intestinal cDNA library (3, 10). A total of 1.5×10^6 plaque-forming units were screened; 10 clones were identified, 4 of which were further characterized after subcloning into pGEM-3Z (Promega).

Multiple tissue Northern blots (Clontech) were probed by a nick-translated ^{32}P-labeled human cDNA probe. The prehybridization and hybridization solutions contained 5× SSPE, 10× Denhardt's solution, sheared, denatured *Escherichia coli* DNA at 100 μg/ml, 50% formamide, and 2% SDS; the filters were incubated in each solution for 16 h. Filters were then washed sequentially with 2× SSC, 0.05% SDS, 2× SSC, and 0.1% SDS and finally with 0.1× SSC/0.1% SDS at 55°C for 30 min. The filters were exposed to x-ray film with two intensifying screens at −80°C for 24–48 h.

Expression of Human apoB mRNA Editing Activity. The phagemid pBK-CMV (Stratagene) was used to subclone the entire coding sequence of human editing protein cDNA by PCR subcloning, and the nucleotide sequence was verified by double-stranded sequencing. The phagemid clone was transfected into HepG2 cells by electroporation. The transfected cells were selected with G418 at 1 mg/ml. Total RNA from individual clones was extracted by the guanidinium isothiocyanate/CsCl method (11). apoB mRNA was amplified by reverse transcription–PCR with primers spanning the editing site to generate a 200-bp product. The proportion of edited sequence in the purified 200-mer was assayed by primer extension in the presence of ddGTP as described (12).

Human Genomic DNA Cloning and Chromosomal Localization by Fluorescence *in Situ* Hybridization. A total of 2×10^6 plaque-forming units of a λEMBL3 human genomic library (Clontech) were plated and screened with a ^{32}P-labeled

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Abbreviation: apoB, apolipoprotein B.

[§]The sequence reported in this paper has been deposited in the GenBank data base (accession no. L26234).

nick-translated human apoB mRNA editing protein cDNA probe. Hybridization was performed in 6× SSC/10× Denhardt's solution/*E. coli* DNA (30 μg/ml) at 68°C. Filters were washed with 6× SSC/0.1% SDS, then 2× SSC/0.1% SDS for 1 h, and finally 1× SSC/0.1% SDS for 1 h. Two positive clones were subcloned into pGEM-3Z. Two P1 genomic clones (1197 and 1198) were obtained by screening a P1 human genomic library (Genome Systems, St. Louis) with two pairs of cDNA PCR primers: pair 1 (nucleotides 152–173 and 328–307, taking codon 1 ATG as the first base), 5′ primer (CGAAGCTCAGGCAAAAACACC) and 3′ primer (ACCAGGGTGCCGACTCAGAAA); pair 2 (nucleotides 664–687 and 857–834), 5′ primer (ATCCTTTTAGCTACAGGGCTGATA) and 3′ primer (GCCAGAGGTAATACATATTTATTG).

Alu PCR (13) products from P1 clones 1197 and 1198 were ethanol precipitated, and 1 μg of DNA was labeled by nick-translation using biotin-14-dATP (GIBCO/BRL). Hybridization to standard chromosome spreads from a male donor was performed (13, 14). Biotin-labeled DNA was detected using fluorescein isothiocyanate-conjugated avidin DCS (5 μg/ml) (Vector Laboratories). Chromosome identification was performed by simultaneous 4′,6-diamidino-2-phenylindole staining, which produces a Q-banding pattern. Digital images were obtained using an epifluorescence microscope (Zeiss) equipped with a computer-driven cooled charge-coupled device camera (Photometrics, Tucson, AZ) and computer software developed in the laboratory of A.B. (14).

Epitope Tagging of Editing Protein and Translation of Tagged and Untagged mRNA. We added a 3-alanine residue spacer immediately followed by a 9-amino acid *Haemophilus influenzae* hemagglutinin epitope (HA-1), YPYDVPDYA (15), to the C terminus of the editing protein by attaching the following oligonucleotide sequence to the cloned cDNA immediately 5′ to the stop codon: GCTGCTGCTTACCCATACGATGTTCCAGATTACGCA, by PCR subcloning. The sequence of the tagged cDNA was verified by double-stranded sequencing.

RNA was transcribed from tagged or untagged cDNA and capped with T3 RNA polymerase by using a Riboprobe kit (Promega). It was translated in nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (Amersham) for 1 h at 30°C. Immunoprecipitation was performed on ice for 1 h in the presence of polyclonal antibody HA.11 (Babco Emeryville, CA), which is specific for the HA-1 epitope. Fifty microliters of reaction mixture was allowed to react with 1 μl of HA.11 for 1 h. Fifty microliters of protein G beads (GammaBind G Sepharose; Pharmacia) was then added. Incubation on ice was continued for 1 h with intermittent mixing. The entire mixture was layered over a 0.3-ml sucrose cushion (1 M sucrose in TCBS) and centrifuged. The beads were washed with TCBS (0.05% Tween 20/20 mM citrate/0.5 M NaCl, pH 5.5) and then boiled for 10 min in an SDS/mercaptoethanol loading buffer and loaded on an SDS/12% PAGE gel (16). Gels were fixed and soaked in EN^{3}HANCE (New England Nuclear) and dried before they were exposed to x-ray film at −80°C for 14 h. The relative intensities of the fluorographic images of the gel bands were determined with a computerized, photometric image scanner (BioImage, Ann Arbor, MI).

RESULTS AND DISCUSSION

cDNA Cloning and Expression of a Human apoB mRNA Editing Protein. We isolated cloned cDNAs for the human apoB mRNA editing protein from a human small intestine λgt11 cDNA library by using a cloned rat editing protein cDNA as hybridization probe. The human cDNA sequence contains a 24-nt 5′ untranslated region, 711-nt open reading frame, a 152-nt 3′ untranslated region, and 12 adenylate residues (GenBank accession no. L26234). The open reading frame encodes a 236-aa sequence, which can be readily aligned with the rat editing protein sequence (Fig. 1). Except for a 7-residue C-terminal extension in the human editing enzyme, there are no insertions or deletions.

We expressed the cDNA in a human hepatoma cell line, HepG2, which has no editing activity and contains 100% unedited apoB mRNA. In eight independent HepG2 cell lines expressing the stably transfected cloned cDNA, the endogenous apoB mRNA contained 30.6% ± 9.6% edited (apoB-48) mRNA compared to 0% in nontransfected controls and those transfected with the vector only (data not shown). Therefore, the expression of the human apoB mRNA editing protein in HepG2 cells is sufficient to produce editing of the endogenous human apoB mRNA. Recently, Giannoni *et al.* (18) showed that expression of the rat cDNA also results in editing of human apoB mRNA in HepG2 cells.

Chromosomal Localization of the Gene for the Human apoB mRNA Editing Protein. We isolated four genomic clones for the human apoB mRNA editing protein: two from a λEMBL3 human genomic library by screening with the cloned cDNA probe and two from a P1 human genomic library by screening the library by PCR. Sequencing of the entire coding region of the exons revealed complete identity with the cloned cDNA sequences. We used the cloned genomic sequences as probes to localize the gene for the apoB mRNA editing protein in chromosomal spreads by fluorescence *in situ* hybridization. Three different genomic fragments individually gave identical results. At least 10 metaphases were analyzed per experiment. Hybridization signals from all three clones were observed in 90–95% of chromosome 12s and were located in the interval 12p13.1-p13.2 (data not shown). No hybridization signal was observed in other chromosomes.

Tissue-Specific Expression of Human apoB mRNA Editing Protein mRNA. apoB is produced only in the liver and small intestine in humans. However, it is unclear whether editing activity occurs in multiple organs because non-apoB mRNAs in other organs could potentially serve as substrates for the same editing activity. We performed Northern blot analysis of 16 different human tissues and found that the mRNA for the editing protein is present in the form of an ≈1.1-kb band in the small intestine only (Fig. 2). No clear hybridization signal was detected in the other tissues examined, indicating

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HUM  MTSEKGPSTGDPTLRRRIEPWEFDVFYDPRELRKEACLLYEIKWGMSRKIWRSSGKNTTN  60
RAT  *S**T**VAV**********H**E**F********T******N**GRHS***HTSQ**NK

HUM  HVEVNFIKKFTSERDFHPSISCSITWFLSWSPCWECSQAIREFLSRHPGVTLVIYVARLF  120
RAT  *******E***T**Y*C*NTR***********G***R**T******Y*H***F**I***Y

HUM  WHMDQQNRQGLRDLVNSGVTIQIMRASEYYHCWRNFVNYPPGDEAHWPQYPPLWMMLYAL  180
RAT  H*A*PR********IS********TEQ*SGY********S*SN*****R**H**VR**V*

HUM  ELHCIILSLPPCLKISRRWQNHLTFFRLHLQNCHYQTIPPHILLATGLIHPSVAWR      236
RAT  **Y****G*****N*L**K*PQ****TIA**S****RL*****W****K             229
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FIG. 1. Sequence alignment of human (HUM) and rat apoB mRNA editing protein. Overlined residues represent the conserved blocks in mammalian and bacteriophage cytidine and deoxycytidine deaminases. His-61, Cys-93, and Cys-96, marked by arrowheads, are thought to be important residues for interaction with zinc (17). A consensus tripeptide sequence that signals N-linked glycosylation at residue 57 is indicated by a brace (residues 57–59). Identical residues are represented by stars.

that the mRNA is either absent or present at a very low concentration in these tissues.

Structure of the Human apoB mRNA Editing Protein. The human and rat apoB mRNA editing proteins share 69% sequence identity (excluding the 7-residue C-terminal extension in the human sequence) (Fig. 1). Three oligopeptide regions are homologous to the highly conserved blocks found in cytidine and deoxycytidylate deaminases from both mammals and bacteriophages (17). Residues His-61, Cys-93, and Cys-96 are conserved in both the human and rat enzymes. These residues were thought to be important for interaction with zinc at the active site of T4 bacteriophage dCMP deaminase (19). Comparison of potentially functional sequence motifs in the rat and human editing enzymes shows some interesting similarities as well as differences. The rat enzyme (9) contains consensus phosphorylation sites of cAMP-dependent kinase (residue 36), protein kinase C (residues 13, 58, and 72), and casein kinase (residue 145). The human enzyme also contains sites for cAMP-dependent kinase (residue 71), protein kinase C (residues 13, 47, 54, 72, and 196), and casein kinase (residue 7). In addition, an N-linked glycosylation site (marked by a brace) is present in the human but not the rat enzyme. An interesting feature of the rat editing enzyme is the presence of a leucine-zipper-like sequence spanning residues 173–210. This sequence motif is well conserved in the human apoB mRNA editing protein, except that one of the leucine residues (no. 196) is replaced by a serine residue.

The Human apoB mRNA Editing Protein Is a Dimer. We translated a synthetic mRNA for this protein in reticulocyte lysate *in vitro*. Analysis of the translation product under denaturing conditions revealed an ^{35}S-labeled M_r ≈28,000 protein (Fig. 3*A Left*, lane 2), very similar to an estimated M_r of 28,177 predicted from its cDNA sequence. We further analyzed the ^{35}S-labeled editing enzyme on an HPLC column and found that the protein migrated with an apparent M_r of ≈56,000 (Fig. 4), suggesting that, in its native form, the human apoB mRNA editing protein may be a homodimer. We tested whether the human apoB mRNA editing protein exists as a dimer using a method independent of its migration behavior.

The design of the experiment is to use an antibody to specifically immunoprecipitate an epitope-tagged apoB mRNA editing protein and examine whether it coprecipitates

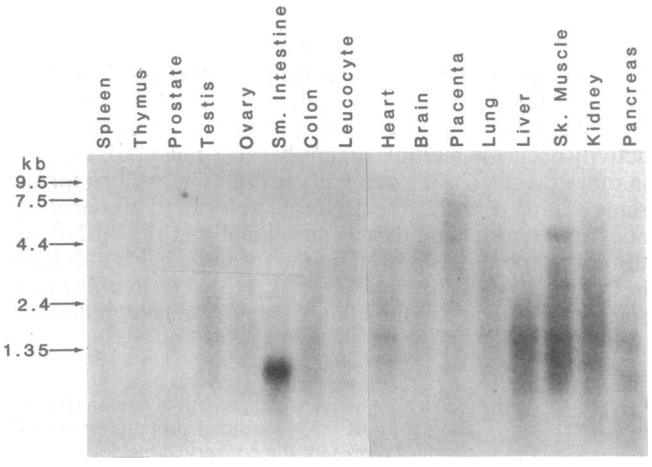

FIG. 2. Northern blot of RNAs from various human tissues using the human small intestinal apoB mRNA editing enzyme cDNA as hybridization probe. Each lane contains 2 μg of poly(A)-RNA [human multiple tissue Northern (MTN) blot and human MTN blot II from Clontech]. The nature of the background smear in the lanes for liver, skeletal muscle, and kidney is unclear. Sm., small; Sk., skeletal.

the untagged protein bound to the tagged protein in the form of a heterodimer. We modified the coding region of a synthetic editing protein mRNA by adding on a 12-codon 3′ extension containing a spacer region encoding three alanine residues, and nine codons corresponding to the epitope sequence for *H. influenzae* hemagglutinin HA-1. Translation of the synthetic mRNA for the epitope-tagged editing protein in rabbit reticulocyte lysate produced a protein with a M_r of 29,500 (Fig. 3*A Left*, lane 3). A monospecific antibody against the HA-1 epitope precipitated the epitope-tagged protein (lane 6) but not the untagged protein (lane 5) or a control protein (luciferase, lane 4) synthesized *in vitro*.

The translation reaction was also performed in the presence of dog pancreatic microsomal membranes (Fig. 3*A Right*). For a control glycoprotein, α factor, there was a marked shift in M_r with the inclusion of microsomal membranes in the translation reaction (lanes 1 and 2), indicating that the protein was efficiently glycosylated in this system. When the human apoB mRNA editing protein mRNA was translated *in vitro*, the newly synthesized products were identical in size in the absence or presence of dog pancreatic microsomal membranes (lanes 3 and 4). These results suggest that the consensus N-linked glycosylation site is not utilized.

To test whether the editing enzyme undergoes spontaneous polymerization, we translated a mixture of epitope-tagged and untagged editing protein mRNA *in vitro* in the presence of dog pancreatic microsomal membranes (Fig. 3*A Right*). On SDS/PAGE analysis, the two bands for the tagged and untagged translation products were well separated (lane 5). On immunoprecipitation using an antibody against HA-1, *both* bands were precipitated (lane 7), although the antibody was reactive only against the epitope-tagged protein (see lanes 2, 3, 5, and 6 in Fig. 3*A Left*). Therefore, the tagged and untagged editing proteins spontaneously formed heteropolymers, which were dissociated into the respective monomers under the denaturing conditions of SDS/PAGE.

Stoichiometry and Characteristics of the Dimerization State. We next translated the two mRNAs in the absence of dog pancreatic microsomal membranes (Fig. 3*B*). As expected, the anti-HA-1 antibody was found to be specific for the epitope-tagged editing protein (lanes 4 and 9) and failed to immunoprecipitate the untagged native editing protein (lanes 5 and 10). Again, when a mixture of the two mRNAs was translated in the same reaction, the epitope-specific antibody precipitated both products (lanes 1–3 and 6–8), indicating that the tagged and untagged unglycosylated editing proteins are bound to each other in the form of a heteropolymer and that N-linked glycosylation is not required for polymerization.

Disulfide bridge formation is a common mechanism for spontaneous dimerization of newly synthesized proteins. This possible mechanism was examined as follows: after translation *in vitro*, we incubated the ^{35}S-labeled products in the reducing agent dithiothreitol before they were immunoprecipitated by the anti-HA-1 antibody and analyzed on SDS/PAGE. As shown in Fig. 3*C*, the heteropolymer complex was stable and not disrupted in the presence of dithiothreitol, which indicates that disulfide bridge formation between the tagged and untagged apoB mRNA editing proteins was not the mechanism for dimerization.

The apparent M_r (≈56,000) of the native human apoB mRNA editing protein (Fig. 4) suggests that the protein exists as a dimer. We confirmed the stoichiometry of the polymerization state as follows: by producing *in vitro* increasing amounts of the untagged protein in the presence of a constant amount of the tagged protein, we could drive essentially all of the epitope-tagged editing protein into a heteropolymer. Therefore, with an excess of the untagged protein, the ratio of untagged to tagged protein approaches 1.0 for a dimer, 2.0 for a trimer, and so on. At saturating amounts of the untagged

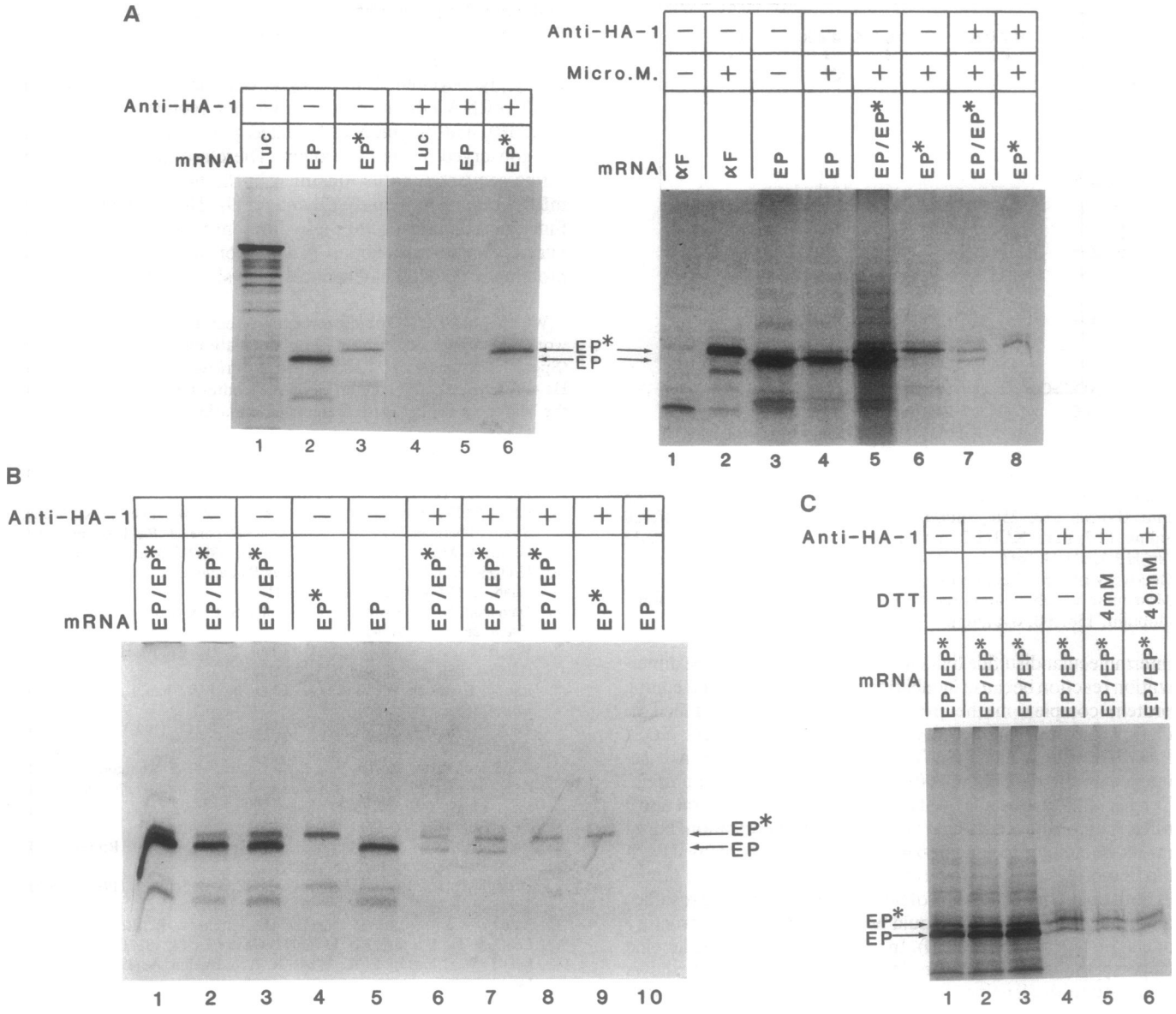

FIG. 3. Translation of human apoB mRNA editing protein mRNA. In all cases, EP and EP* represent the untagged and epitope-tagged human apoB mRNA editing proteins, respectively. (*A*) Synthetic mRNAs were translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. Translation products were analyzed on SDS/12% PAGE and fluorography (16). (*Left*) In lanes 1–3, products were directly analyzed; in lanes 4–6, products were analyzed after immunoprecipitation with a rabbit antibody specific for the HA-1 epitope. Luc, luciferase. (*Right*) Effect of inclusion of dog microsomal membranes (Micro. M.) in the *in vitro* translation. αF, α factor. (*B*) Translation of mRNA mixtures in the absence of dog microsomal membranes. (*C*) Effect of preincubation in dithiothreitol on dimer formation. Incubation was performed in 4 mM and 40 mM dithiothreitol (DTT) for 5 min at room temperature after completion of translation and before immunoprecipitation.

protein, we found that the ratio approached 1.0 (Fig. 5), indicating that the heteropolymer contains the untagged and tagged proteins in a 1:1 ratio (i.e., the human apoB mRNA editing protein exists as a dimer).

The finding of a homodimeric human apoB mRNA editing protein is interesting. The homologous enzyme deoxycytidylate deaminases from T4 bacteriophage (20), T2 bacteriophage (21, 22), donkey spleen (23), and HeLa cells (24, 25) are all homohexamers, and deoxycytidylate deaminase isolated from human spleen (26) appears to be a homodimer. The apoB mRNA editing protein shows both structural (Fig. 1 and ref. 17) and functional homology to the deoxycytidylate deaminases; both are zinc-requiring deaminases with one histidine and two cysteine residues in the active site. Given that all bacteriophage and mammalian deoxycytidylate deaminases studied to date are homopolymeric enzymes, the spontaneous homodimerization of the human editing protein may well be expected because of the similarities in structure and function. In this study, we have determined that the native apoB mRNA editing protein has an apparent M_r of ≈56,000 (Figs. 4 and 5), as expected for a dimeric enzyme. This is in contrast to previous estimates of 125,000 [by gel filtration for baboon editing activity (27)] and 240,000 [by glycerol gradient sedimentation for rat editing activity (28)] for the editing activity of mammalian small intestinal extracts. These apparent discrepancies can be explained by the presence of accessory proteins bound to the editing protein that are present in the tissue extracts. It is also possible that some of these anomalously migrating or sedimenting activities might represent nonspecific aggregates. We observed a very small amount of aggregated material with a $M_r > 600,000$ on the HPLC column (Fig. 4).

The mechanism for the homodimerization of the human apoB mRNA editing protein is unknown. There are eight cysteine residues (including the two putative active-site cysteine residues) in the protein. However, we have excluded an

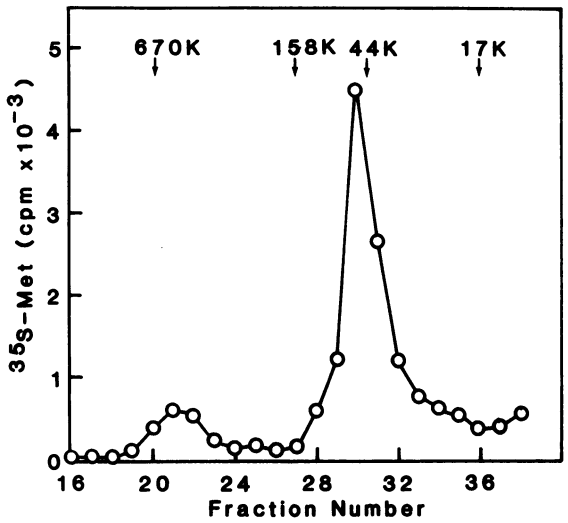

FIG. 4. HPLC analysis of ^{35}S-labeled human apoB mRNA editing protein. The *in vitro* translated editing protein was put on a 300 × 7.8 mm Bio-Sil SEC-250 column in Dulbecco's phosphate-buffered saline (2.7 mM KCl/1.2 mM KH_2PO_4/138 mM NaCl/8.1 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.1). Half-minute fractions were collected. Recovery of ^{35}S-labeled editing protein from this column was 75%. Molecular weight standards were thyroglobulin, 670,000; gamma globulin, 158,000; ovalbumin, 44,000; myoglobin, 17,000.

intermolecular disulfide linkage as the mechanism for the dimerization reaction because incubating the tagged–untagged editing protein complex in the presence of dithiothreitol failed to disrupt the heterodimer revealed by the subsequent SDS/PAGE analysis (Fig. 3*C*). There is a leucine-zipper-like sequence motif spanning residues 173–210 of the editing protein. Teng *et al.* (9) speculated that a similar sequence in the rat apoB mRNA editing protein might be involved in dimerization, based on its homology to leucine-zipper sequences in many DNA-binding proteins (29). However, the leucine-zipper-like motifs in both the human and rat proteins contain two proline residues (nos. 190 and 191), which would break the coiled-coil structure of classic leucine zippers (30). In addition to the classic leucine zippers, the editing protein leucine-rich region also shows similarity to the leucine-rich sequences found in "leucine-rich glycoproteins," which are typically involved in protein–protein interaction or cell–matrix interaction (reviewed in ref. 31). The leucine-rich glycoprotein sequences also contain a relatively high proline content. It is conceivable that the leucine-rich motif

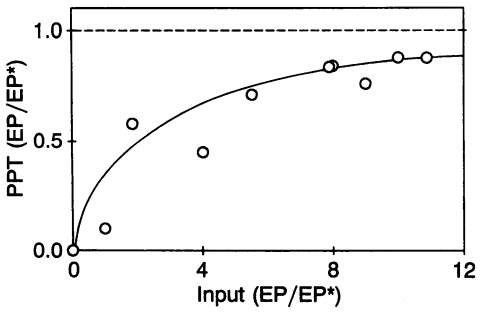

FIG. 5. Stoichiometry of dimerization reaction. Multiple translations of mixtures of untagged (EP) and tagged (EP*) apoB mRNA editing protein mRNA were analyzed (by providing increasing amounts of capped mRNA for the untagged protein). The total input and HA.11 antibody-immunoprecipitated ^{35}S-labeled untagged and tagged apoB mRNA editing protein bands on the fluorographs were quantified by an image scanner (see *Materials and Methods*) and the ratios of untagged to tagged apoB mRNA editing protein were calculated. PPT, immunoprecipitated.

in the apoB mRNA editing protein may be involved in interaction with other protein components of the editing enzyme complex. As their name implies, leucine-rich glycoproteins are highly glycosylated. Interestingly, dimerization of the human apoB mRNA editing protein does not require glycosylation (Fig. 3*B*), and the putative N-linked glycosylation sequence is not conserved in the rat protein. There are many other interesting sequence motifs identified in the human and rat apoB mRNA editing protein (Fig. 1 and ref. 9). The elucidation of the functional significance of these different motifs must await the purification (or production *in vitro*) of large amounts of the protein for detailed biochemical analysis.

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