A 40 kilodalton rat liver nuclear protein binds specifically to apolipoprotein B mRNA around the RNA editing site

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ABSTRACT

Apolipoprotein (apo) B-48 mRNA is the product of RNA editing which consists of a C→U conversion changing a CAA codon encoding Gln-2153 in apoB-100 mRNA to a UAA stop codon in apoB-48 mRNA. In the adult rat, RNA editing occurs both in the small intestine and the liver. We have studied the ability of rat liver nuclear extracts to bind to synthetic apoB mRNA segments spanning the editing site. Using an RNA gel mobility shift assay, we found the sequence-specific binding of a protein(s) to a 65-nucleotide apoB-100 mRNA. UV crosslinking followed by T1 ribonuclease digestion and SDS-polyacrylamide gel electrophoresis demonstrated the formation of a 40 kDa protein-RNA complex when ^{32}P-labeled apoB-100 mRNA was incubated with a rat liver nuclear extract but not with HeLa nuclear extract. Binding was specific for the sense strand of apoB mRNA, and was not demonstrated with single-stranded apoB DNA, or antisense apoB RNA. The complex also failed to form if SDS was present during the UV light exposure. Binding experiments using synthetic apoB mRNAs indicate that the 40 kDa protein would also bind to apoB-48 mRNA but not apoA-I, apoA-IV, apoC-II or apoE mRNA. Experiments using deletion mutants of apoB-100 mRNA indicate efficient binding of wildtype 65-nucleotide (W65), 40-nucleotide (W40) and 26-nucleotide (W26) apoB-100 mRNA segments, but not 10-nucleotide (or smaller) segments of apoB-100 mRNA to the 40 kDa protein. In contrast, two other regions of apoB-100 mRNA, B-5′ (bases 1128 – 3003) and B-3′ (bases 11310 – 11390), failed to bind to the protein. The 40 kDa sequence-specific binding protein in rat liver nuclear extract may play a role in apoB-100 mRNA editing.

INTRODUCTION

The tissue-specific editing of apolipoprotein (apo) B mRNA results from a C→U conversion which changes a CAA codon encoding Gln-2153 in apoB-100 mRNA to a UAA stop codon in apoB-48 mRNA (1,2). Apart from their drastically different sizes, apoB-100 (containing 4536 residues) and apoB-48 (containing 2152 residues) have very different properties both functionally and metabolically (3). ApoB-100 is produced in the liver and is a physiologic ligand for the low density lipoprotein receptor (4). The putative receptor binding domain(s) of apoB-100 has been localized to the COOH-terminal half of the molecule (5,6), which is missing in apoB-48. In adult humans, the latter protein is produced almost exclusively in the small intestine; being required for proper fat absorption, apoB-48 plays a structural role in intestinal chylomicrons. In contrast to other nonrodent mammalian species, the adult rat liver produces significant amounts of apoB-48 as well as apoB-100 mRNA (7), although as in other mammals the rat small intestine produces almost exclusively apoB-48. Like alternate RNA splicing which can generate more than one protein from the same transcription unit, the editing of apoB mRNA allows the production of two functionally divergent proteins from the same gene.

Recently, the sequence-specific editing of apoB mRNA segments was demonstrated in cell-free systems derived from cultured rat hepatoma cells (8) or normal rat liver (9). Both synthetic apoB-100 mRNA segments (i.e., direct RNA editing, refs. 8,9) and apoB-100 minigenes (i.e., coupled transcription editing, ref. 9) were edited in these systems. The mechanism for the C→U change is most likely a deamination of the C in the 4 position, converting it to a U residue. The putative cytidine deaminase activity in the cell-free systems was abolished by proteinase K treatment (8,9), suggesting that a protein component was required for RNA editing. In this study, we show that a rat liver nuclear extract that contains apoB mRNA editing activity also contains a 40 kDa protein that binds specifically to apoB mRNA sequences around the editing site.

EXPERIMENTAL PROCEDURES

Construction of ApoB mRNA Segments

Human apoB DNA constructs of various lengths were produced by direct synthesis of the two complementary strands on an Applied Biosystems Inc. 380A DNA Synthesizer, with artificial EcoRI and BamHI restriction sites attached in the 5′ and 3′ ends. The double-stranded DNAs were subcloned in pGEM-3Z, and the corresponding synthetic RNA transcript was produced by T7 RNA polymerase in the presence of ^{32}P-UTP according to the supplier's instructions (Promega). The ^{32}P-labeled RNAs were purified on 8% sequencing polyacrylamide gel and used for binding experiments. For production of unlabeled apoB-mRNA, the ^{32}P-UTP was omitted from the reaction.

Preparation of Rat Liver Nuclear Extract

Sprague-Dawley rat liver nuclear extracts were prepared with minor modifications as described by Gorski it al (10). All manipulations were performed in the cold, and all solutions, tubes and centrifuges were chilled to 0°C. Minced rat liver (15 g) was homogenized in 30 ml of Buffer A (10 mM Hepes, pH 7.6, 25 mM KCl, 0.15 mM Spermine, 0.5 mM Spermidine, 1 mM EDTA, 2 M sucrose, and 10% glycerol) with a motor-driven Teflon-glass homogenizer. The homogenate was checked under the microscope to ensure that greater than 90% of the cells were broken before it was filtered through a layer of cheese cloth. The filtered homogenate was diluted to 85 ml with Buffer A, layered in three 27 ml portions over three 10 ml cushions of the same buffers and then centrifuged at 24,000 rpm for 30 minutes at 0°C in an SW28 rotor. The combined nuclear pellets were resuspended with 50 ml of an 8:1:1 (V/V/V) mixture of Buffer A, glycerol, and Buffer B (Buffer A without sucrose and glycerol) with a loose-fitting Teflon-glass homogenizer. This nuclear suspension was layered over two 10 ml cushions as described above, and centrifuged at 24,000 rpm for 60 minutes at 0°C. The pelleted nuclei were resuspended in 20 ml of nuclear lysis buffer (10 mM Hepes, pH. 7.6, 100 mM KCl, 3 mM MgC12, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol) using a Dounce homogenizer. The nuclear suspension was diluted to 80 ml and was extracted with $(NH_4)_2S0_4$. One-tenth volume of 4 M $(NH_4)_2S0_4$ was added dropwise, and the extract was gently stirred for 60 minutes. The viscous lysate was then centrifuged at 45,000 rpm for 60 minutes in a Ti50 rotor to pellet the chromatin. The supernatant volume was measured and powdered $(NH_4)_2S0_4$ (0.4 g/ml) was added and slowly dissolved. After the $(NH_4)_2S0_4$ had completely dissolved, the solution was gently stirred for an additional 30 minutes. The precipitated proteins were sedimented by centrifugation at 20,000 rpm for 30 minutes in an SW28 rotor. The protein pellet was then dissolved with 0.5 ml of buffer (20 mM Hepes, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol) and dialyzed against the same buffer for 4 hours with one change of buffer. Protein precipitates formed during dialysis were removed by centrifugation in a microcentrifuge at the end of dialysis. The dialyzed protein extract was used for RNA editing or RNA binding experiments. Both activities were stable at 4°C for at least 2 months. We normally obtained approximately 10 to 15 mg of nuclear protein per 15 g adult rat liver.

In vitro Binding of ^{32}P-labeled ApoB mRNA to Rat Liver Nuclear Extracts

RNA gel mobility shift assay and SDS-polyacrylamide gel analysis of UV-crosslinked protein-RNA complex were performed by the method of Moore et al. (11). 200 ng of ^{32}P-labeled RNA were incubated with 15 μg of nuclear extract at room temperature for 30 min. in a 20 μl reaction containing 5 μg unlabeled yeast RNA (Sigma), 10 U RNasin, 5 μg Leupeptin (Sigma), in 10 mM Hepes (pH 7.6), 20 mM KCl, 25 mM EDTA, 0.5 mM DTT and 10% glycerol. For competition studies, 10 fold molar excess of cold competitor RNA were preincubated for 5 min. prior to the addition of radioactive labeled probe. 10 μl of the reaction mixture were loaded on a 5% nondenaturing polyacrylamide gel in 50 mM Tris-glycine (pH 7.9) buffer. Electrophoresis was performed at 4°C for 2 hrs. The other 10 μl were exposed to UV light for 30 min. on ice with a short-wave UV lamp (Ultraviolet products, San Gabriel, California) held at 1 cm from the samples. Similar results were obtained with 10 min., 15 min., 30 min., or 60 min. exposure to the UV lamp. 10 U of T1 ribonuclease (Pharmacia) were added to digest the UV-crosslinked samples for 30 min., followed in some cases by the addition of heparin (5 mg/ml Sigma). The samples were boiled in SDS, dithiothreitol loading buffer for 5 min. and analyzed on a 12.5% SDS polyacrylamide gel.

RESULTS

ApoB mRNA Segment Binds to a Specific Protein in Rat Liver Nuclear Extract

Cell-free preparations from the rat liver contain an RNA-editing activity that specifically converts C-6666 in synthetic human apoB mRNA to a U residue (8,9). To see whether the editing activity is associated with a protein in the nuclear extracts that binds specifically to the RNA substrate, we analyzed for the presence of RNA-binding proteins by an RNA gel mobility shift assay. A ^{32}P-labeled 65-nucleotide human apoB-100 mRNA (W65, bases 6632−6696) synthesized in vitro was incubated with rat liver nuclear extract prepared by a modification of the method of Gorski et al. (10); the same extract had been shown to be active in in vitro apoB mRNA editing (9). The mixture was fractionated on a 5% nondenaturing polyacrylamide gel (Figure 1). Preincubation of the ^{32}P-apoB-100 mRNA segment with the nuclear extract retarded the mobility of the labeled RNA on the

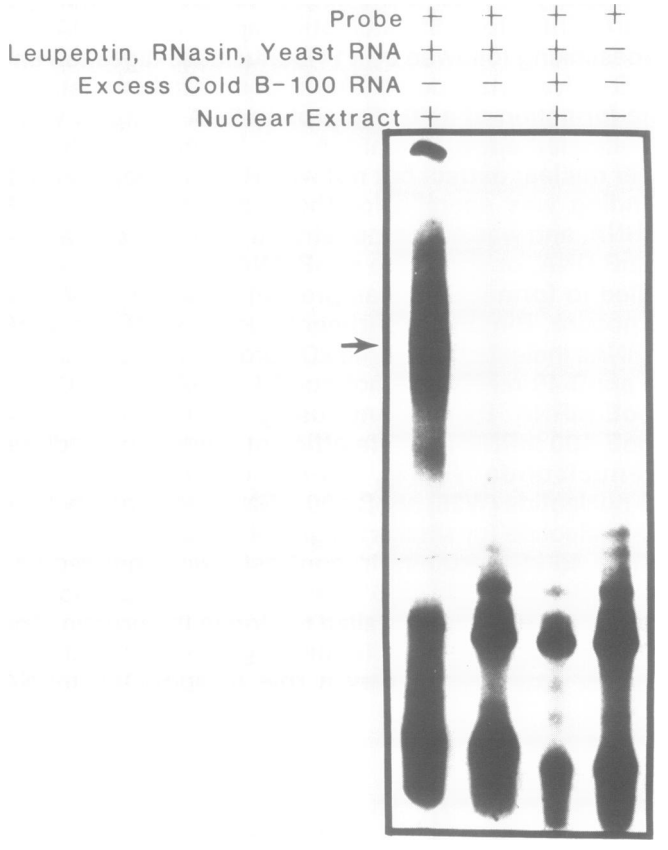

Figure 1. RNA gel mobility shift assay. Rat liver nuclear extracts were incubated with ^{32}P-labeled synthetic 65-nucleotide apoB-100 mRNA in the presence and absence of 10-fold excess unlabeled apoB-100 mRNA for 30 min. The mixture was digested with T1 ribonuclease and analyzed on 5% nondenaturing polyacrylamide gel as described in Experimental Procedures. The arrow indicates the position of a ^{32}P-labeled protein-RNA complex formed in the presence of rat liver nuclear extract but in the absence of excess unlabeled apoB-100 mRNA.

gel, indicating the formation of a protein-RNA complex. The production of the slow-migrating complex was dependent on the presence of the nuclear extract. When the RNase inhibitor, RNasin, and the protease inhibitor, Leupeptin, were incubated with the labeled apoB-100 mRNA in the absence of nuclear extract, no complex was formed. Addition of excess unlabeled synthetic apoB-100 mRNA but not yeast RNA completely abolished the radiolabeled complex.

To further examine the protein-RNA complex formed during the incubation, we exposed the mixture of ^{32}P-labeled apoB mRNA and rat liver nuclear extract to ultraviolet (UV) light to effect crosslinking, digested the mixture with T1 ribonuclease and analyzed the product by SDS-polyacrylamide gel electrophoresis (Figure 2). A 40 kDa radiolabeled band was detected on the gel only if nuclear extract was present during the UV crosslinking. In the absence of nuclear extract, only fast-migrating short ^{32}P-labeled oligoribonucleotides which were T1 digestion products were seen on the gel. Furthermore, the 40 kDa band was completely abolished by the inclusion of excess, unlabeled synthetic apoB-100 mRNA during the incubation and UV crosslinking.

We investigated the conditions required for the formation of the protein-RNA complex (Figure 3). The presence of SDS during the incubation and UV crosslinking abolished the band. The presence of heparin, in contrast, had no apparent effect on the formation of the complex. In other experiments, varying the amounts of dithiothreitol (0.5 mM−10 mM) during the incubation had no effect on the formation of the 40 kDa protein-RNA complex (data not shown). The 40 kDa protein-RNA complex was formed with rat liver nuclear extract but not with HeLa cell nuclear extract (Figure 3).

The 40 kDa Protein Binds to Both ApoB-100 and ApoB-48 mRNA But Not to ApoB-100 DNA or Antisense RNA

The UV crosslinking experiments above demonstrated that the substrate of an RNA editing reaction, apoB-100 mRNA, bound to a 40 kDa protein in rat liver nuclear extract. We next tested whether the reaction product, apoB-48 mRNA, would also bind

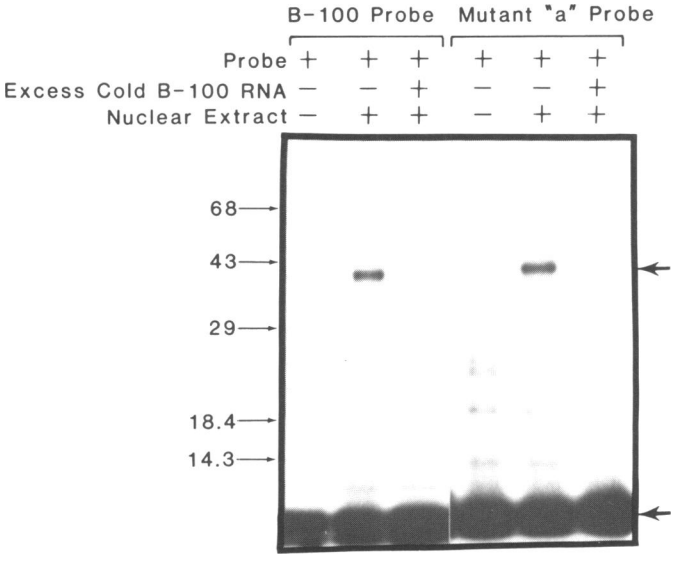

Figure 2. UV crosslinking and SDS gel electrophoresis of rat liver nuclear extract incubated with ^{32}P-labeled wildtype or mutant apoB-100 mRNA. A 65-nucleotide ^{32}P-labeled wildtype apoB-100 mRNA (bases 6632−6696) or a mutant sequence (mutant *a* that contains an A-6667 → G substitution) was used as probe. Lower arrow: T1 digested products of the ^{32}P-labeled probe. Upper arrow: ^{32}P-labeled 40 kDa protein-RNA complex. Molecular weight standards were: bovine serum albumin, 68 kDa; chicken ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa; and lysozyme, 14.3 kDa.

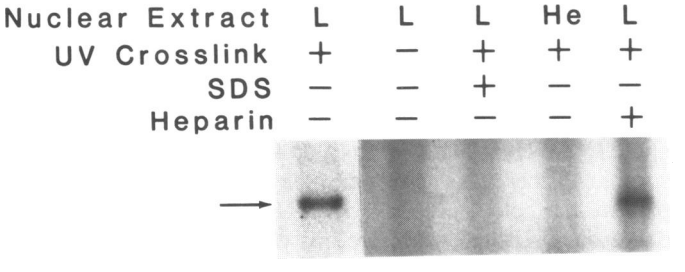

Figure 3. Effect of SDS, heparin and the use of HeLa cell nuclear extract on the formation of the 40 kDa protein-RNA complex. L, rat liver nuclear extract; He, HeLa cell nuclear extract. The arrow indicates the position of the 40 kDa protein-RNA complex.

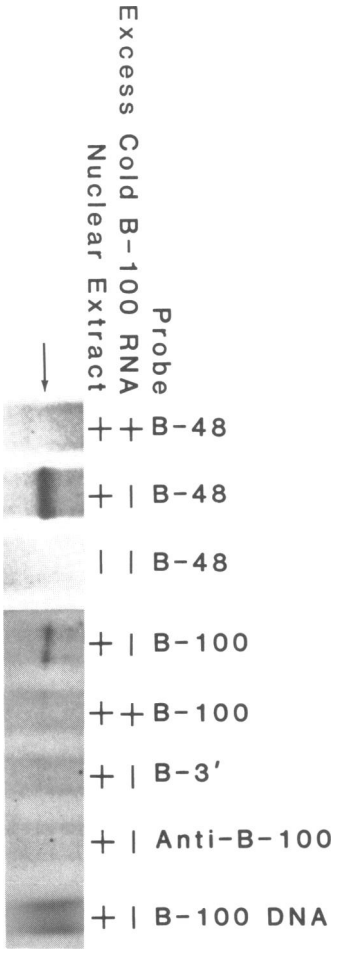

Figure 4. Use of different ^{32}P-labeled nucleic acid probes on the formation of the 40 kDa protein-RNA complex. The ^{32}P-labeled probes used were: B-48, 65-nucleotide apoB-48 mRNA (bases 6632−6696); B-100, 65-nucleotide apoB-100 mRNA (bases 6632−6696); anti-B-100, antisense apoB-100 RNA spanning the same region as the 65-nucleotide apoB-100 probe; B-100 DNA, single-stranded ^{32}P-labeled 40-nucleotide apoB-100 DNA spanning bases 6658−6697; B-3′, 81-nucleotide apoB-100 RNA (bases 11260−11340). The arrow indicates the position of the ^{32}P-labeled 40 kDa protein-RNA complex. The difference in intensity in the apoB-48 and apoB-100 samples is not seen in other experiments.

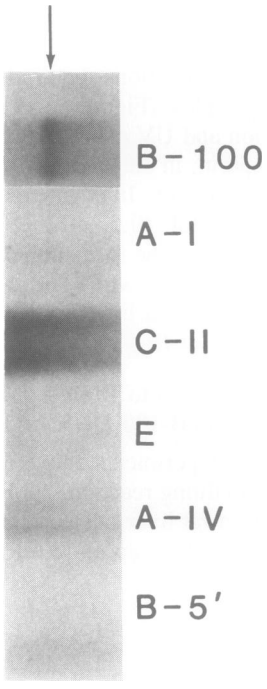

Figure 5. Binding of different ^{32}P-labeled apolipoprotein mRNA probes to the 40 kDa protein. The synthetic apolipoprotein RNA probes were synthesized on previously published cloned cDNAs (A-I, ref. 13; A-IV, ref. 14; C-II, ref. 15; E, ref. 16). The apoB-100 5′ (B-5′) mRNA sequence corresponds to nucleotides 1128–3003.

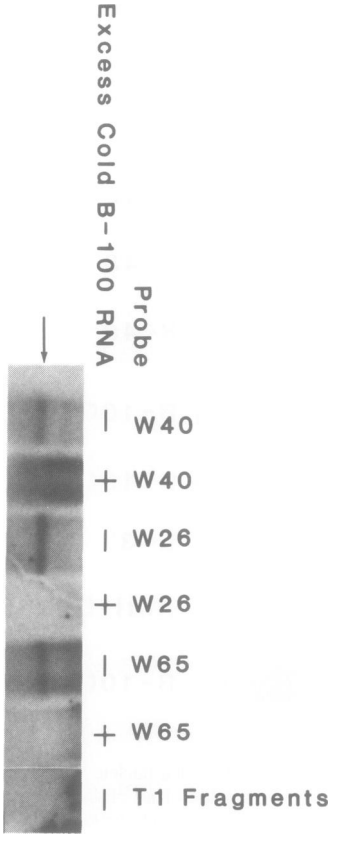

Figure 6. Binding of apoB-100 mRNA of various lengths to rat liver nuclear extract. The probes used were: W40, 40-nucleotide apoB-100 mRNA (bases 6658–6697); W26, 26-nucleotide apoB-100 mRNA (bases 6661–6686); T1 fragments, T1 ribonuclease-digested RNA fragments of W40. The arrow indicates the position of the ^{32}P-labeled 40 kDa protein-RNA complex.

to the same protein (Figure 4). When ^{32}P-labeled apoB-48 mRNA (bases 6632–6696) was used in the UV crosslinking experiment, a 40 kDa band was again formed on SDS-polyacrylamide gel analysis. An excess of unlabeled apoB-48 mRNA or apoB-100 mRNA completely abolished the band, indicating that the same protein was crosslinked to apoB-48 as apoB-100 mRNA. In contrast, the use of a ^{32}P-labeled apoB-100 DNA or ^{32}P-antisense apoB-100 RNA probe spanning the same region did not result in the formation of a protein-nucleic acid complex.

The 40 kDa Protein Binds Specifically to ApoB mRNA

In order to establish the sequence specificity of the 40 kDa binding protein, we have studied its binding to other mRNA sequences. A mutant apoB-100 mRNA sequence, mutant *a*, also binds to this protein with specificity (Figure 2). (Mutant *a* contains a single base mutation (A-6667→G) and is edited efficiently in vitro (9).) In addition, we also tested synthetic mRNA segments corresponding to human apoA-I, apoA-IV, apoC-II, and apoE mRNA for binding. None of these apolipoprotein mRNAs displayed any binding to the 40 kDa protein (Figure 5).

ApoB-100 mRNA Sequence Required for Specific Binding to 40 kDa Protein

We showed above that a 65-nucleotide apoB-100 mRNA (W65) would bind to the 40 kDa protein. We investigated the effect of progressively shortening the apoB-100 mRNA sequence on protein binding. We first tested the binding of a 40-nucleotide sequence (W40, bases 6658–6697) and a 26-nucleotide sequence (W26, bases 6661–6686) that span the editing site. As shown in Figure 6, both of these shorter apoB-100 mRNA sequences could be specifically UV-crosslinked to the 40 kDa protein. In contrast, a combination of short pieces of apoB-100 mRNA covering the W40 sequence that were produced by T1 digestion of the latter, which included two 10-nucleotide RNAs (bases 6663–6672 and bases 6678–6687) and four shorter oligoribonucleotides (sequences of these T1 fragments are shown in Figure 7) failed to form the protein-RNA complex (Figure 6). We tested one apoB-100 mRNA construct (B-5′) containing sequences 5′ (Figure 5, bases 1128–3003) and another construct (B-3′) containing sequences 3′ (Figure 4, bases 11310–11390) to the editing site. Neither construct would bind to the 40 kDa protein.

DISCUSSION

ApoB-48 mRNA biogenesis results from the conversion of C-6666 in apoB-100 mRNA to a U residue. The mechanism which allows the selective editing of this residue among the numerous C residues in a 14-kilobase mRNA is unknown. It is likely, however, that a sequence-specific RNA-binding protein, which may or may not be the editing enzyme itself, is involved in 'tagging' the editing site among the over 14,000 bases in apoB-100 mRNA, allowing RNA editing to occur in a sequence-specific manner. This communication describes an RNA-binding protein that potentially plays such a role.

We demonstrated that there is a protein in rat liver nuclear extracts that bound to apoB-100 mRNA. Binding appeared to be noncovalent and was inhibited by the addition of SDS. By competition experiments, it was found to be sequence-specific and binding was not affected by the presence of heparin. The apparent molecular mass of the protein-RNA complex was

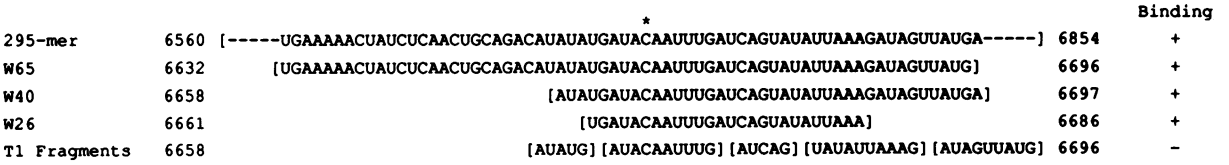

Figure 7. Sequences of synthetic apoB-100 mRNAs used for binding experiments. The edited C is marked by an asterisk.

approximately 40 kDa. Ideally, one would like to test whether mutant apoB-100 mRNA segments that fail to be edited would bind to this protein. This is, however, not currently feasible because all apoB-100 mRNA variants containing single-base mutations close to the editing site are edited in vitro (9). One such mutant, *a*, which contains an A-6667→G substitution also binds specifically to the 40 kDa protein. The sequence specificity of the 40 kDa protein binding is further reflected by its failure to bind to the mRNA sequences for a number of apolipoproteins including apoA-I, apoA-IV, apoC-II and apoE (Figure 4). Using different lengths of apoB-100 mRNAs that span the editing site, we observed specific binding among synthetic RNAs that varied in length from 295 nucleotides (bases 6560−6854, data not shown) to 65, 40, and down to 26 nucleotides (W65, W40 and W26, Figures 4 & 6). When we used T1 fragments (including two 10-nucleotide and four smaller fragments) that made up W40 for binding, the 40 kDa protein-RNA complex was not formed, indicating that the minimal length of RNA required for binding was somewhere between the 10-nucleotide fragments and W26. Furthermore, two other regions of apoB-100 mRNA, B-5′ (bases 1128−3003) and B-3′ (bases 11310−11390), also failed to bind to the 40 kDa protein (Figures 4 and 5).

Two recently published studies indicate that rat liver cell extracts can specifically convert C-6666 in synthetic apoB-100 mRNA to a uridine residue in vitro (8,9). In transfection studies, the shortest apoB-100 mRNA that was efficiently edited in a rat hepatoma cell line contained the same 26 nucleotides as W-26 (12), which is also the shortest apoB-100 mRNA segment that was successfully edited by the rat liver nuclear extract in vitro (data not shown, Ref. 9). The 40 kDa sequence-specific binding protein characterized in this communication may play a role in apoB-100 mRNA editing in rat liver cells in vitro and in vivo. We are currently engaged in experiments to purify this protein and to further characterize its interaction with apoB-100 mRNA in detail.

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