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Genetics

In vitro apolipoprotein B mRNA editing: Identification of a 27S editing complex

(cytidine/uridine "editosome"/mooring sequence)

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ABSTRACT **Specific apolipoprotein B (apoB) mRNA editing can be performed *in vitro* on apoB RNA substrates. Native gels and glycerol gradient sedimentation have been used to determine the physical properties of the *in vitro* editing activity in rat liver cytosolic S100 extracts. ApoB RNA substrates were progressively assembled as 27S complexes for 3 hr with similar kinetics as seen for the accumulation of edited RNA. Assembly was not observed on RNAs from apoB deletion constructs that did not support editing. The 27S complex contained both edited and unedited RNA sequences. Inhibition of 27S complex assembly by vanadyl–ribonucleoside complexes was accompanied by inhibition of editing. Based on these data, we propose that the 27S complex is the *in vitro* "editosome." A "mooring sequence" model for RNA recognition and editosome assembly has been proposed involving RNA sequences flanking the edited nucleotide.**

Apolipoprotein B (apoB) is translated from a 14-kilobase (kb) mRNA that is transcribed from a single-copy gene on human chromosome 2 (1, 2). In rats (3–6) and humans (7), apoB exists as a high molecular weight form ($apoB_H$ or apoB100) and a low molecular weight form ($apoB_L$ or apoB48). Although $apoB_L$ is synthesized from the same primary transcript as $apoB_H$, its mRNA undergoes an unusual form of RNA processing referred to here as "C/U RNA editing" wherein a cytidine at nucleotide 6666 is converted to a uridine (8–11). This transition alters the sense of codon 2153 from glutamine (C̲AA) to a translation stop (U̲AA). The contribution of $apoB_H$ very low density lipoprotein (VLDL) to low density lipoprotein (LDL; an atherogenic risk factor) through metabolic conversion in the plasma has focused attention on the various metabolic levels where the $apoB_H/apoB_L$ ratio can be regulated.

Development of intestine induces the capacity to synthesize and secrete $apoB_L$ and virtually eliminates all traces of $apoB_H$ production and secretion (1, 8–12). In rat liver, $apoB_L$ production and secretion are also developmentally regulated, while the $apoB_H$ phenotype is maintained (1, 8–13). In adult rat liver, 40–50% of the total apoB mRNA is edited (13, 14).

Hepatic production of $apoB_L$ is reduced by fasting, whereas $apoB_H$ is not (15, 16). This is accompanied by a ≥2-fold reduction in edited RNA compared with that of control rats (17). Forty-eight hours of refeeding rats with a glucose-rich diet results in a 2-fold increase in apoB mRNA abundance and a ≥9-fold increase in edited RNA. Hormonal regulation of apoB mRNA editing can also be demonstrated in rats following multiple doses of thyroid hormone (T3) *in vivo* (18, 19). Under these conditions, 90% or more of the total hepatic apoB mRNA is edited. Hepatic $apoB_H$ synthesis is correspondingly reduced to below detectable levels, while $apoB_L$ synthesis remains relatively unaltered.

To a first approximation, apoB mRNA editing appears similar to plant mitochondrial editing (20–22), which primarily involves cytidine-to-uridine conversions under conditions where the reading frame is maintained. It is believed that C/U editing might be catalyzed by a form of cytidine deaminase (23, 24), although other possibilities have not been ruled out. Hemoflagellate mitochondrial mRNA editing differs from apoB mRNA editing in that it involves insertion and deletion of single or multiple uridine residues at single and multiple sites (25–27). This form of editing results in extensive modification of mRNAs and their reading frames. Paramyxovirus mRNA editing involves a different mechanism, wherein single guanidine nucleotides are inserted at select sites within a subset of mRNAs to produce major changes in the reading frame (28).

Point mutant constructs of the apoB editing site suggest a lax sequence requirement within the immediate vicinity of the edited nucleotide (23). Mutants which place additional cytidines adjacent to the editing site were edited at these additional cytidine residues. The paradox of the editing activity having absolute specificity for a single nucleotide yet lacking selectivity when confronted with multiple cytidine residues within the region suggests that editing specificity is achieved by the positioning of the editing activity over the correct nucleotide. A role for more distal sequences in this process is suggested by deletion-mutant studies (24, 29, 30). ApoB mRNA deletion constructs ranging in size from 2.4 kb to 26 base pairs (bp) were both expressed and edited in transient expression assays with McArdle 7777 cells (29). In contrast, transcripts from these subclones are all edited *in vitro* by McArdle cell cytosolic S100 extracts except the 26-base transcript (30). The lack of editing on the shortest probe suggests that there are different factors *in vitro* governing the efficiency of the probe's utilization that are either not important or not rate-limiting *in vivo*. The data presented here demonstrate sequence-specific *in vitro* assembly of 27S macromolecular complexes, or "editosomes," on apoB RNA substrates in rat liver S100 cytosolic extracts.

METHODS

Preparation of Liver Extract. Livers of Sprague–Dawley male rats (250–280 g) were perfused *in situ* via the hepatic portal vein with ice-cold STOP buffer (0.25 M sucrose/50 mM triethanolamine, pH 8/5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride/0.5 μg of aprotinin per ml/5 μg of

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Abbreviations: apoB, apolipoprotein B; VLDL, very low density lipoprotein; LDL, low density lipoprotein; $apoB_H$, apoB of higher molecular weight; $apoB_L$, apoB of lower molecular weight; VRC, vanadyl–ribonucleoside complex.

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leupeptin per ml/20 units of soybean trypsin inhibitor per ml). After teflon-to-glass homogenization, nuclei were removed by centrifugation at 1500 × *g* for 10 min, and the resultant supernatant was recentrifuged at 100,000 × *g* for 1 hr to obtain the S100 supernatant.

***In Vitro* Editing Reactions.** RNA substrates were transcribed and capped with T7 RNA polymerase (or with T3 RNA polymerase for anti-sense RNA substrates) from an apoB pGEM-4 cDNA subclone (pRSA13) (30). Sequencing of the RSA13 apoB insert showed that it consisted of 448 nucleotides of apoB cDNA corresponding to nucleotides 6413–6860 with 48 and 2 nucleotides of polylinker sequences 5′ and 3′, respectively. ApoB constructs pBS55 contained 55 nucleotides of apoB sequence (6649–6703) with 40 and 4 nucleotides of polylinker sequence 5′ and 3′, respectively. ApoB construct pBS26 contained 26 nucleotides of apoB sequence (6662–6687) with 38 and 2 nucleotides of polylinker sequence 5′ and 3′, respectively. Some assembly experiments used a 456-nucleotide adenovirus RNA splicing precursor containing the first and second exon of the late transcription unit separated by a shortened intron (31).

Sixty micrograms of S100 extract protein and 60 ng of RNA substrate were combined in a final volume of 50 μl of editing buffer (10 mM Hepes, pH 7.9/10% (vol/vol) glycerol/50 mM KCl/50 mM EDTA/0.25 mM dithiothreitol (30) containing 2 units of RNasin (Promega) and were incubated at 30°C. At the indicated times, reactions were terminated by quick-freezing in liquid nitrogen.

The occurrence and extent of editing were determined by reverse transcriptase extension of end-labeled deoxyoligonucleotide primer DD3 (5′-AATCATGTAAATCATAATTATCTTTAATATACTGA-3′, 5′ end at 6708) for pRSA13 and pBS55 or primer DD5 (5′-CGATATCAAGCTTTAATATACTGA-3′, 5′ end at 6687) for pBS26, with excess dideoxy GTP (ddGTP; "poisoned priming") to ensure termination at position 6666 (unedited cytidine) or at position 6655 (the next cytidine 5′ of 6666 in edited RNA) (30) (see Fig. 1*A*). Products of the sequencing reactions were resolved on denaturing 10% polyacrylamide gels and autoradiographed.

Editosome Analysis. Editing reactions were carried out with [α-^{32}P]CTP-labeled RNA substrates, and one-fifth of each reaction was resolved on 4% acrylamide/0.5% agarose "native gels" containing 50 mM Tris, 50 mM glycine, and 10 mM EDTA (pH 8.8) as gel buffer and running buffer (32, 33). Gels were prechilled and electrophoresed at 7°C (180 V for 30 min followed by 250 V for 3.5 hr) (33, 34).

The size of the *in vitro* complexes from 3-hr editing reactions was estimated by sedimentation analysis on 10–50% glycerol gradients in editing reaction buffer. Gradients were centrifuged at 100,000 × *g* for 5 hr at 7°C (the reduced temperature inhibits further assembly and editing) and were sampled in 0.7-ml fractions from the top. Catalase (11S) and *in vitro* assembled 60S spliceosomes (32–37) served as sedimentation markers on parallel gradients.

RNAs were extracted and purified from *in vitro* complexes after transferring native gels to DEAE paper and eluting complexes with 1 M NaCl/10 mM EDTA; this was followed by extraction with phenol and precipitation with ethanol. Deoxyoligonucleotide PCR12 (5′-AACCAAATGTAGATCATGG-3′, with 5′ end at 6823) was used to prime cDNA synthesis, which subsequently was amplified by the polymerase chain reaction (PCR) using primers PCR12 and PCR5 (5′-CTGAATTCATTCAATTGGGAGAGACAAG-3′, with 5′ end at 6504). *Thermus aquaticus* (*Taq*) polymerase (Promega) reaction conditions and thermal cycler (Hybaid/National Labnet) parameters were as described by Driscoll *et al.* (30). PCR products were sequenced by the poisoned priming method.

RESULTS

Rat liver cytosolic S100 extracts showed near linear kinetics of editing for up to 3 hr at 30°C on RNA substrates containing 448 nucleotides of apoB sequence (Fig. 1). Editing was determined by primer extension sequencing on RNA substrates as described. The editing activity in rat liver cytosolic S100 extracts was similar to that of McArdle cells (30) in its kinetics, efficiency, optimal activity in the presence of 50 mM EDTA, lack of an ATP-regenerating system requirement, and absolute dependence on extract protein (data not shown). Approximately 2–5% of input RNA was edited after 3 hr of reaction, as calculated by direct scintillation counting of excised CAA and TAA gel bands.

The physical properties of the editing activity were determined by electrophoretic separation of reaction components on high-porosity native gels (32, 33). Two distinct complexes referred to as B and B′ appeared in a time- and temperature-dependent manner (Fig. 2*A*). These were clearly distinguishable from A and A′, which were observed with RNA alone (data not shown) and with extract on ice (Fig. 2*A*, lane 0 min). A and A′ are therefore not protein–RNA complexes. Complexes with slower electrophoretic migration than A′ and extending up from A′ were observed within 15 min of incubation at 30°C. These initial complexes showed linear accumulation into complexes with even slower migration (B complexes) for at least 3 hr. At this time, B complexes involved ≈15% of the input RNA. The broad distribution of the B complexes results from the level of resolution in the native gel systems (32–35) as well as the heterogeneity in the B complexes resulting from the progressive nature of their assembly (see also Fig. 6). The B′ complex (a portion of which will enter the gel with higher voltage and longer

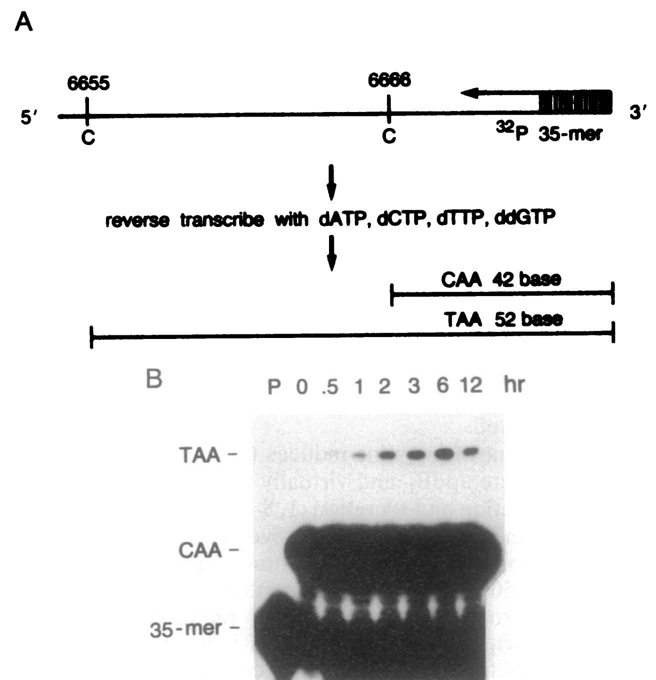

FIG. 1. Kinetics of *in vitro* editing. (*A*) *In vitro* editing of pRSA13 was detected by poisoned primer extension of DD3 as described. Chain termination occurred at nucleotide 6666 on unedited RNAs and at 6655 on edited RNAs. (*B*) pRSA13 apoB RNA substrates were incubated with rat liver cytosolic S100 extracts under editing conditions for the lengths of time in hr indicated at the top of each lane, and the RNA substrates from each reaction were primer-extended as described. CAA and TAA correspond to primer-extension product termination on unedited and edited RNAs. Lane P shows the absence of primer-extension products on RNAs of rat liver S100 cytosolic extract alone.

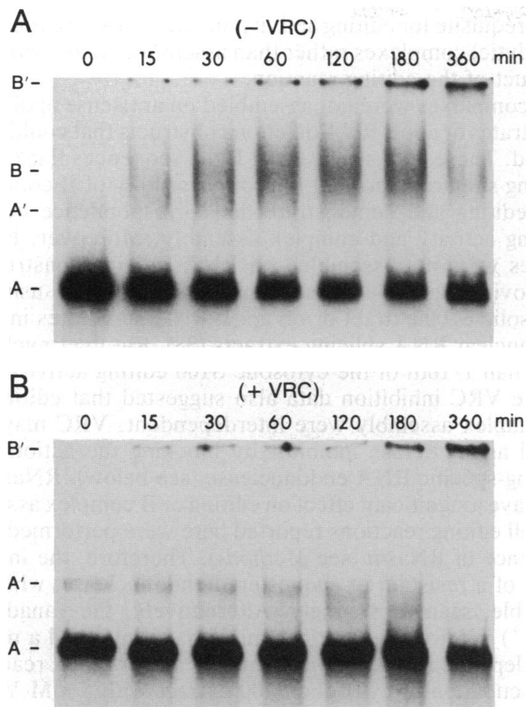

FIG. 2. Kinetics of complex assembly. (*A*) Aliquots from editing reactions incubated for the indicated times in min (top of each lane) were resolved on native gels and autoradiographed as described. (*B*) S100 extracts were preincubated on ice for 30 min with 50 mM vanadyl–ribonucleoside complex (VRC) and then diluted 1:9 into editing reaction buffer, incubated under editing conditions for the indicated times, and resolved on native gels.

electrophoresis; see below) has slower kinetics of assembly but can accumulate up to 25% of the input RNA. When cytosolic S100 extracts were preincubated with 50 mM VRC, A and A′ were detected, B′ complexes became apparent after a kinetic lag, but B complexes were not seen (Fig. 2*B*). Primer extension on RNA substrates from the reactions showed that VRC inhibited editing (Fig. 3).

If the B complex is the *in vitro* editosome, then it should contain edited apoB RNA substrates. To evaluate this possibility, complexes from a 3-hr reaction were resolved on native gels, transferred to DEAE paper, and eluted as described. The RNA in the A and A′ and the B and B′ complexes was extracted, purified, PCR-amplified, and sequenced by the ddGTP poisoned priming method. Edited RNA was only detected in the B complexes (Fig. 4).

The correlation between B-complex assembly and editing could also be demonstrated on RNA substrates transcribed from shorter apoB constructs. RNA substrates containing 448 nucleotides of apoB sequence are edited more efficiently (2- to 3-fold) than substrates containing 55 nucleotides of apoB sequence when compared on the basis of equal mol of RNA ends (Fig. 5*A*, lanes 4 and 5). RNA substrates containing 26 nucleotides of apoB sequence were not edited (Fig. 5*A*, lane 6). B complexes were assembled on 55 nucleotides of apoB sequence but with lower efficiency (Fig. 5*B*, lane 2). B complex assembly could be increased by adding 5-fold more RNA to the reactions (Fig. 5*B*, lane 3). The 26-nucleotide apoB construct did not assemble B complexes, and the bulk of the RNA appeared either as B′ complexes (Fig. 5*B*, lane 4) or was degraded and migrated with the dye front (not shown). Nonediting RNA substrates such as the adenovirus late leader RNA did not assemble B complexes (Fig. 5*B*, lane

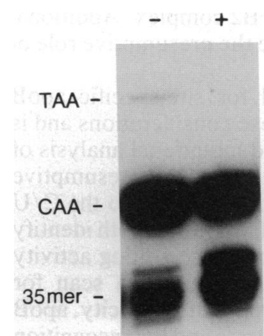

FIG. 3. VRC inhibits editing. Primer extension sequencing of RNA substrates from 3-hr editing reactions of control (lane –) and VRC-treated (lane +) extracts was as described in Fig. 1.

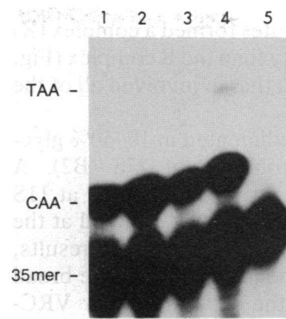

FIG. 4. The B complex contains edited RNA. Native gel complexes were transferred to DEAE paper, the complexes were localized by autoradiography and eluted, and the corresponding RNAs were extracted and primer-extended by using the poison priming method. Lanes 1–5 correspond to primer-extension products from complexes A′, A, B′, and B and from DD3 primer alone, respectively.

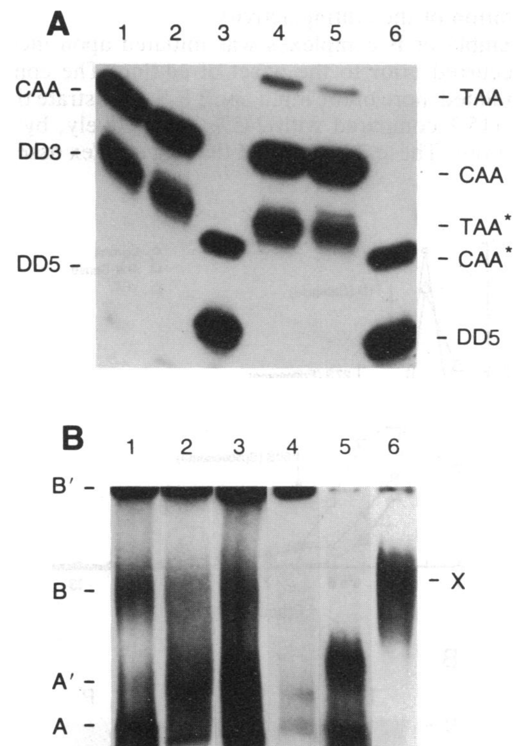

FIG. 5. B-complex assembly is specific for RNA substrates that edit. (*A*) Deletion apoB RNA substrates were compared to pRSA13 for editing activity by using the poisoned primer-extension assay. Lanes 1–3 show primer-extension products on pRSA13, pBS55, and pBS26 RNA substrates before incubation in the editing reaction. Lanes 4–6 show primer-extension products on pRSA13, pBS55, and pBS26 RNA substrates after a 3-hr editing reaction. CAA and TAA extension products and the location of primer DD3 (for pRSA13 and pBS55) and primer DD5 (for pBS26) are indicated to the left and right. The positions of CAA and TAA primer-extension products from DD5 are indicated as CAA* and TAA*. (*B*) Complexes assembled on deletion apoB RNA substrates and nonediting RNA substrates were compared by native gel electrophoresis. Lanes 1–6 correspond to complexes assembled: 10 ng (60 fmol) of pRSA13 RNA, 2 ng (60 fmol) of pBS55 RNA, 10 ng (300 fmol) of pBS55 RNA, 1 ng (60 fmol) of pBS26 RNA, 10 ng of intervening sequence 1 (IVS1) splicing precursor RNA, and 10 ng of antisense pRSA13 RNA. The migrations of A, A′, B, and B′ complexes are shown to the left. The antisense complex, "X," is indicated to the right.

5), and antisense apoB RNA substrates formed a complex (X) with slower electrophoretic mobility than the B complex (Fig. 5, lane 6). X also differed from B in that in involved all of the input RNA.

Fully assembled B complexes sedimented in 10–50% glycerol gradients with a mean distribution of 27S (B2). A presumptive B2 precursor complex (B1) sedimented at 11S (Fig. 6). A and A′ complexes were primarily retained at the top of the gradient. Consistent with the native gel results, most of the anti-sense RNA sedimented with a single broad peak of 8–27S. Virtually all of the input RNA in VRC-inhibited reactions remained at the top of the gradient as A and A′.

DISCUSSION

The ability of factors within cytosolic S100 extracts from rat liver to recognize and accurately edit cytidine-6666 to uridine in human apoB RNA substrates has made possible the identification of an unusual macromolecular assembly, which we propose to be an editosome responsible for the editing activity. The cytosolic S100 is an operationally defined cellular fraction and does not necessarily reflect the *in situ* localization of the editing activity.

Assembly of B complexes was initiated upon incubation and occurred prior to the onset of editing. The complexes accumulated more of the input apoB RNA substrate than was edited (15% compared with 2–5%, respectively, by 3 hr of incubation). These data suggest that B-complex assembly is a prerequisite for editing activity and that these are functional (catalytic) complexes rather than assemblies that form on the product of the editing reaction.

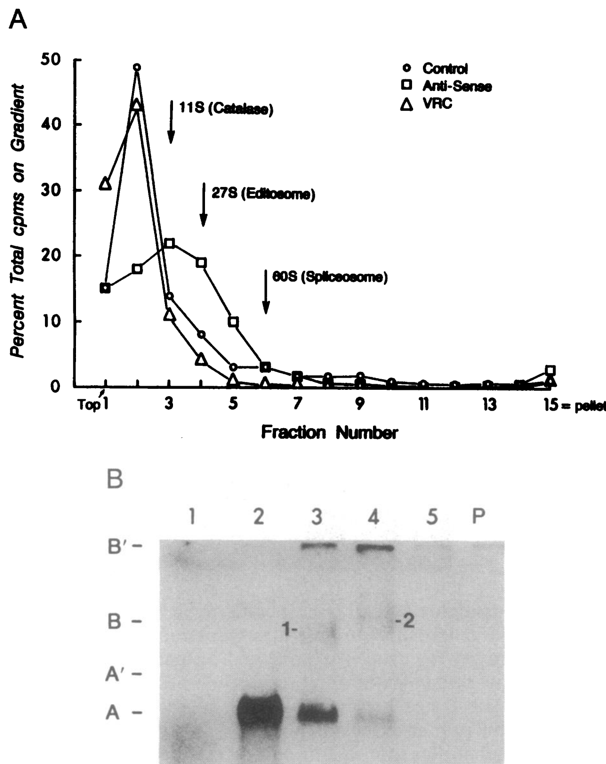

FIG. 6. B complexes are 27S. (*A*) Control, VRC-inhibited, and antisense editing reaction products were sedimented through parallel 10–50% glycerol gradients, fractionated (0.7 ml per fraction), and scintillation-counted. The peak sedimentation of catalase (11S) and *in vitro* assembled 60S spliceosomes served as sedimentation standards in parallel gradients. Antisense complexes sedimented broadly with a peak at 11S. (*B*) Select control gradient fractions (identified at top of lanes) were resolved by native gel electrophoresis. Fully assembled B complexes (B2) were in fraction 4 (27S), and a complex with faster electrophoretic mobility (B1), proposed to be an assembly precursor (Fig. 2), was detected in fraction 3 (11S). P, pellet fraction.

B complexes were not assembled on antisense apoB RNA substrates or apoB RNA deletion constructs that could not be edited. These data suggest that RNA sequences flanking the editing site are important for both assembly of B complexes and editing and demonstrate the correspondence between editing activity and complex assembly. Moreover, B complexes were not assembled on RNA splicing constructs of adenovirus late leader sequence (31–37) when using the cytosolic S100 extract or on apoB RNA substrates in HeLa cell nuclear RNA splicing extracts (38). Rat liver nuclei had less than 1/10th of the cytosolic S100 editing activity.

The VRC inhibition data also suggested that editing and B-complex assembly were interdependent. VRC may have acted as an RNase inhibitor by blocking the action of an editing-specific RNA endonuclease (see below). RNasin did not have a significant effect on editing or B complex assembly and all editing reactions reported here were performed in the presence of RNasin (see *Methods*). Therefore, the involvement of a resistant or sequestered endonuclease, while still possible, seems less likely. Alternatively, the vanadyl ion (VO^{2+}) in VRC preparations might have inhibited a nucleotide-dependent enzyme (i.e., an NTPase or redox reaction). Preincubation of cytosolic S100 extract with 5 mM $VOSO_4$ did not inhibit editing or B-complex formation (data not shown), whereas 5 mM VRC (which contains 5 mM VO^{2+}) totally inhibited both processes. A third possibility is that VO^{2+}–nucleoside complexes are able to mimic cytidine monophosphate (CMP) as a competitive inhibitor of cytidine deaminase (39, 40). A 5 mM solution of VRC is 1.25 mM in adenine, cytosine, guanine, and uracil (40), which is similar to the K_i of cytidine deaminase for CMP (0.45–1.22 mM; ref. 39).

Human apoB mRNA is 13,692 nucleotides in length, contains 3135 cytidines and 375 CAA codons of which 100 are in-frame glutamine codons (41). As only one CAA codon is modified, selection of the correct CAA for editing (codon 2153) must be a nonrandom process. Data presented here suggest that protein–RNA and/or RNA–RNA interactions in the form of a ribonucleoprotein complex play a major role in this process. Both native gel electrophoresis and glycerol gradient sedimentation analysis suggest that the apoB RNA substrate interacts with extract factors to form 11S complexes (B1), which are then competent for assembly into the 27S editosome (B2 complexes).

The presence of B1 and B2 complexes after 3 hr of reaction is evident from the electrophoretic heterogeneity of the B complexes on native gels and suggests that editosome assembly *in vitro* is a slow but continuous process. Longer reaction times of up to 6 hr revealed that edited RNA substrates (Fig. 1) and B complexes (Fig. 2) were no longer accumulating. The electrophoretic mobility of the 6-hr complexes was similar to that of the 11S B1 complexes, suggesting that B2 disassembly released B1 complexes. These data support the possibilities that the B1 complex is both a precursor to and a component of the B2 complex. Additional proof will be required to demonstrate the presumptive role of B1 as an editosome precursor.

The "mooring sequence" model for site-specific apoB mRNA C/U editing incorporates these considerations and is based on the data presented here and mutational analysis of Chen *et al.* (23) and Driscoll *et al.* (30). The presumptive mooring sequences would be flanking and distal to the C/U editing site and interact with cellular factors that both identify the specific site for editing and "moor" the editing activity within the vicinity of nucleotides 6665–6668 to scan for cytidine residues (Fig. 7). For the purpose of simplicity, apoB mRNA recognition as an editing substrate and the recognition

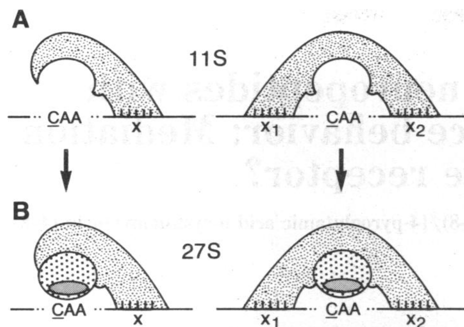

FIG. 7. The mooring sequence model for the editosome. Genetic analyses (23, 29, 30) suggest that the recognition and binding sequence(s) are distal to and different from the sequences in the immediate vicinity of the editing site. These sequences are proposed to facilitate site-specific editing by localizing the editing activity to a limited stretch of sequence. The term "mooring sequences" is proposed for these distal sequences. (*A*) Recognition complexes of 11S form through the interaction of cellular factors with the mooring sequence (X), which might be a specific primary sequence or a unique secondary structure of apoB mRNA. Two forms of the 11S complex are depicted that have either single (X) or multiple (X_1 and X_2) mooring sequences. (*B*) The one possibility shown proposes that the catalytically active editosome forms when the presumptive cytidine deaminase binds to factors of the 11S complex to form the 27S complex.

of the site to be edited are both presumed to be mediated by one set of factors that recognize and bind to the mooring sequences. These interactions are presumed to be the basis for the assembly of the 11S complexes (Fig. 7*A*). The lax sequence specificity of the presumptive cytidine deaminase involved in apolipoprotein mRNA C/U editing (23) suggests that it does not have the "capacity" to recognize and bind to mRNA in a sequence-specific manner, and therefore it has not been proposed as a component of the 11S complex. To a first approximation, deletional analysis (32) places parts of the mooring sequences within nucleotides 6649–6661 ("X_1"), 6688–6703 ("X_2"), or both. The data presented here show that 55 nucleotides of apoB RNA substrate are not used as efficiently as substrates containing 442 nucleotides of apoB sequence. The 55-nucleotide probe must therefore contain the information for site-specific editing, but less than what is necessary for efficient and/or stable editosome assembly.

A single mooring sequence (X) and recognition complex vs. a bipartite mooring sequence (X_1 and X_2) and a recognition complex composed of half sites are shown as two potential editosome configurations. It is speculated that assembly of the functional 27S editosome involves binding of an activity such as cytidine deaminase to the recognition complex (Fig. 7*B*). Through these specific interactions, apoB site-specific mRNA C/U editing is imposed upon the presumptive cytidine deaminase activity by mooring. The activity then scans for cytidines within the nucleotide triplet (the CAA codon) and edits as many as are in "reach."

In conclusion, native gel B complexes are proposed as the editosome complex based on similar criteria used in analyses of spliceosomal complexes (reviewed in ref. 36). These criteria were: (*i*) editing did not occur in the absence of B complex assembly, (*ii*) only RNAs with the correct sequences assembled B complexes, and (*iii*) only B complexes contained edited as well as unedited apoB RNA.

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