RNA Editing of Apolipoprotein B mRNA

SEQUENCE SPECIFICITY DETERMINED BY IN VITRO COUPLED TRANSCRIPTION EDITING

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Apolipoprotein (apo) B-48 mRNA is produced by in vivo RNA editing which involves a C→U conversion of the first base of the codon CAA for Gln-2153, changing it to UAA, an in-frame stop codon. We have reproduced the editing reaction in vitro using nuclear extracts. Efficient RNA editing was demonstrated by using apoB mRNA segments as substrate or in a coupled transcription-editing reaction using apoB minigenes as template. ApoB minigenes were constructed by ligating the adenovirus major late promoter to a fragment of apoB-100 DNA containing the editing site and used for the transcription-editing reaction. We defined the sequence specificity of the editing reaction using site-specific single and multiple mutant base changes constructed by the polymerase chain reaction. Among 22 different mutant apoB-100 minigene constructs containing mutations in the bases immediately flanking the edited C-6666, 20 were edited in the coupled transcription-editing reaction. The results suggest a relatively lax sequence specificity for apoB mRNA editing. Our observation may have important implications for apoB-48 biogenesis as well as for the editing process as a general biologic regulatory mechanism.

RNA editing is a molecular biological phenomenon whereby the primary structure of an RNA transcript is altered by mechanisms other than splicing (1-4). The first description of this process involved the addition of nongenomically encoded uridine (U) residues to mitochondrial mRNAs in the kinetoplastid protozoa (5). Subsequently, U residues have been found to be removed from some transcripts (6, 7).

Recently, Thomas et al. (8) described the addition of two coded uridine (U) residues to mitochondrial mRNAs in the kinetoplastid protozoa (5). Subsequently, U residues have been found to be removed from some transcripts (6, 7).

The only putative RNA editing described in mammals is the presence of dideoxy GTP was used as a rapid assay for the absence (i.e. unedited template) or presence (i.e. edited template) of primer-extended product beyond C-6666. An oligonucleotide with sequence 5'-AATCATGTAAATCATAACTATCTTTAATA-3' (34-mer) was used as primer. Primer extension was performed essentially as described by Driscoll et al. (22). The primer extension products were separated by electrophoresis on a 12% polyacrylamide sequencing gel and exposed to Kodak XAR-5 x-ray film for varying periods of time.

The mechanism behind the C→U conversion is unknown. In this study, we have reproduced the RNA editing in vitro using a nuclear extract and a template consisting of either a segment of apoB DNA (i.e. coupled transcription editing) or apoB mRNA (i.e. direct RNA editing). By altering the nucleotide sequence of the template by site-specific mutagenesis, we have defined the sequence specificity of the editing reaction. The sequence specificity of the reaction was found to be relatively lax, which has important implications for the potential role of RNA editing in the regulation of gene expression.

MATERIALS AND METHODS

Primer Extension Assay for RNA Editing—Primer extension in the presence of dideoxy GTP was used as a rapid assay for the presence (i.e. unedited template) or absence (i.e. edited template) of primer-extended product extending beyond C-6666. An oligonucleotide with sequence 5'-AACACGTGAAACATCAAATCTTCTTAATATTGC-3' (34-mer) was used as primer. Primer extension was performed essentially as described by Driscoll et al. (22). The primer extension products were separated by electrophoresis on a 12% polyacrylamide sequencing gel and exposed to Kodak XAR-5 x-ray film for varying periods of time.

Rat Liver Nuclear Extract Preparation—All manipulations were performed in the cold, and all solutions, tubes, and centrifuges were chilled to 0 °C. Rat liver nuclear extracts were prepared essentially as described by Gorki et al. (23). We normally obtained approximately 10-15 mg of nuclear protein/15 of adult rat liver. Nuclear extracts from HeLa and Hep3B cells were prepared exactly as described by Shapiro et al. (24).

Coupled in Vitro Transcription-editing Reactions—An apoB-100 minigene was constructed by ligating the adenovirus major late promoter to the 5' end of an apoB-100 DNA segment corresponding to different lengths of apoB mRNA identified in the legend to Table 1 (Fig. 9A). In vitro transcription reactions (50 μl) contained 3.6 μg of circular DNA template and 3-5 μg/ml nuclear protein extract in a buffer containing 25 mM Hepes (pH 7.6), 50 mM KCl, 6 mM MgCl2, 0.6 mM each of ATP, CTP, GTP, and UTP, 12% glycerol, and 1 μl of RNasin (40 units, Promega Biotech). After 1 h of incubation at 30 °C, the reactions were terminated by the addition of 380 μl of stop buffer (50 mM Tris-HCl, pH 7.5, 1% sodium dodecyl sulfate, and 5 mM EDTA). Ten μg of Proteinase K was then added, and the reaction was incubated at 65 °C for 30 min. After Proteinase K treatment, the RNA was extracted twice with equal volumes of phenol and chloroform and precipitated by ethanol with 10 μg/ml tRNA carrier.

The abbreviations used are: kb, kilobase(s); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction.
Coupled Transcription Editing of ApoB mRNA

Quantitation of B-Gln and B-Stop Sequences—Nucleic acids from the reaction mixture were treated with Sau3A1, and the enzyme was removed by Proteinase K treatment. The sample was phenol-extracted and the nucleic acids precipitated in ethanol. They were recovered and treated with RQ DNase I (Promega Biotech) (6 units/μg sample) in 40 mM Tris, pH 8.0, 5 mM EDTA, and 0.5% sodium dodecyl sulfate. The RNA was then extracted with phenol/chloroform and precipitated in ethanol (25).

PCR Cloning-Hybridization Assay—RNA samples were amplified by the polymerase chain reaction (26) (PCR), fractionated on 2% agarose gels, and the amplified products cut out from the gels. They were then cloned into the EcoRI/BamHI site of pGEM-3Z in Escherichia coli host JM109. Colony hybridization on nitrocellulose filters was performed directly on double-stranded DNA by the method of Sanger et al. (27).

RESULTS AND DISCUSSION

Editing of ApoB RNA in Vitro—In our initial testing of RNA editing in vitro, we incubated T7 transcripts of cloned apoB-100 cDNA segments containing C-6666 with a nuclear extract from rat liver, a tissue known to contain large amounts of apoB-100 RNA transcripts in the reaction, the primer extension failed to detect the editing of an apoB transcript when incubated with rat liver nuclear extracts. A 34-base oligonucleotide primer (apoB 6708-6675) was 5' end-labeled with 32P by polynucleotide kinase and [γ-32P]ATP and hybridized to the apoB mRNA. Primer extension was performed in the presence of high concentrations of deoxyGTP such that it terminated at the next C. Termination at C-6666 would produce a 43-mer and at C-6655 would produce a 54-mer. Lanes 3-4 and 4-5 represent different amounts of apoB-48 (B-Stop) and apoB-100 (B-Gln) RNA in the in vitro editing reaction, respectively. These lanes were exposed to a Kodak XAR-5 x-ray film for 16 h. Lanes 1' and 4'-6' were repeat exposures of the corresponding numbered lanes for 48 h in the presence of two intensifying screens. A 54-mer (B-Stop) band was readily identified in the 0.3 ng B-48 lane (1'); the lowest concentration of B-100 RNA that produces an identifiable band of the 54-mer in the B-100 RNA reaction is 10 ng (lane 5').

is in turn dependent on the total input RNA in the assay. Therefore, it is not an accurate assay for the extent of editing, and its sensitivity is much inferior to the PCR cloning-hybridization assay, which is independent of the input RNA concentration. Thus, the latter assay alone was used in all subsequent experiments.

Both nuclear extract and apoB mRNA were needed for the reaction. Incubation of apoB mRNA with buffer alone or with heat-inactivated nuclear extract failed to show any C→U conversion. The authenticity of the edited product was confirmed by direct sequencing. Of all the Cs in the B-mRNA, only one (C-6666 of the codon CAA for Gln-2153) was converted to a U; none of the other Cs were changed. Also, this base substitution is the only one observed; by direct sequencing, there is no other type of base changes in the edited RNA.

Coupled Transcription Editing of ApoB-100 Minigene—The nuclear extract (23) used for RNA editing had been found earlier to be active in transcribing a number of mammalian genes (including the albumin and serum amyloid A genes, data not shown). We tested its ability to direct the coupled transcription editing of an apoB-100 minigene.

An apoB-100 minigene was constructed by ligating the adenovirus major late promoter to the 5' end of an apoB-100 DNA segment corresponding to different lengths of apoB mRNA (Fig. 2A). The AdB-100 minigene was incubated with the nuclear extract. The RNA product was purified and assayed for the relative proportions of B-Stop versus B-Gln transcripts by the PCR cloning-hybridization assay. Authenticity of the products was further confirmed by direct sequencing. It is evident that the nuclear extract can transcribe the AdB-100 minigene constructs as well as edit the transcripts under these conditions (Table I, B). The nuclear extract was the capillary substrate to the 5' and of an apoB-100 DNA segment corresponding to different lengths of apoB mRNA (Fig. 2A). The AdB-100 minigene was incubated with the nuclear extract. The RNA product was purified and assayed for the relative proportions of B-Stop versus B-Gln transcripts by the PCR cloning-hybridization assay. Authenticity of the products was further confirmed by direct sequencing. It is evident that the nuclear extract can transcribe the AdB-100 minigene constructs as well as edit the transcripts under these conditions (Table I, B). The nuclear extract was somewhat more effective in the coupled transcription-RNA editing of AdB-100,000 than the two longer constructs. Furthermore, both the supercoiled and linearized minigenes were transcribed and edited although the efficiency may be slightly higher with the supercoiled substrate. As in the case of RNA substrates, the only base change detected in these coupled transcription-RNA editing reactions was C-6666.

FIG. 1. Primer extension assay on apoB-Gln and apoB-Stop RNAs incubated with rat liver nuclear extracts. A 34-base oligonucleotide primer (apoB 6708-6675) was 5' end-labeled with 32P by polynucleotide kinase and [γ-32P]ATP and hybridized to the apoB mRNA. Primer extension was performed in the presence of high concentrations of deoxyGTP such that it terminated at the next C. Termination at C-6666 would produce a 43-mer and at C-6655 would produce a 54-mer. Lanes 1-4 and 4-5 represent different amounts of apoB-48 (B-Stop) and apoB-100 (B-Gln) RNA in the in vitro editing reaction, respectively. These lanes were exposed to a Kodak XAR-5 x-ray film for 16 h. Lanes 1' and 4'-6' were repeat exposures of the corresponding numbered lanes for 48 h in the presence of two intensifying screens. A 54-mer (B-Stop) band is readily identifiable in the 0.3 ng B-48 lane (1'); the lowest concentration of B-100 RNA that produces an identifiable band of the 54-mer in the B-100 RNA reaction is 10 ng (lane 5').
TABLE I

Direct RNA editing and coupled transcription editing in vitro

| Reaction conditions* | % editing ((B-Stop/(B-Stop + B-Gln)) × 100) |
|----------------------|--------------------------------------------|
| **A. Direct RNA editing in vitro** | |
| Extract + B-mRNA<sub>100b</sub> | |
| 100 ng of mRNA | 0.76 (10/1320)<sup>b</sup> |
| 10 ng of mRNA | 1.71 (62/3607) |
| 1 ng of mRNA | 0.94 (15/1595) |
| Buffer + 10 ng of B-mRNA<sub>100b</sub> | 0 (0/8800) |
| Extract + buffer | NP* |
| **B. Coupled transcription-RNA editing in vitro** | |
| Supercoiled ADB-100<sub>100</sub> + rat liver extract | 1.47 (194/13394) |
| Supercoiled ADB-100<sub>100</sub> + rat liver extract | 0.72 (18/11263) |
| Linear ADB-100<sub>100</sub> + rat liver extract | 0.48 (49/10169) |
| Linear ADB-100<sub>100</sub> + rat liver extract | 0.84 (69/8205) |
| Linear ADB-100<sub>100</sub> + rat liver extract | 0.28 (24/8680) |
| Linear ADB-100<sub>100</sub> + rat liver extract | 0.51 (48/9460) |
| Supercoiled ADB-100<sub>100</sub> + buffer | NP* |
| Buffer + rat liver extract | NP* |
| Supercoiled ADB-100<sub>100</sub> + HeLa extract | 0 (0/5725) |
| Supercoiled ADB-100<sub>100</sub> + Hep3B extract | 0 (0/3431) |

*AdB-100<sub>100</sub> is the construct shown in Fig. 2A. It contains bases 6552–6815 of apoB-100 mRNA. ADB-100<sub>100</sub> contains bases 6552–7034, and ADB-100<sub>100</sub> contains 6552–7573. B-mRNA<sub>100</sub> is the in vitro T7 transcript from ADB-100<sub>100b</sub>. For direct RNA editing, a T7 transcript of linearized ADB-100<sub>100</sub> was used as substrate. For coupled transcription editing, either supercoiled or linear (i.e. cut by XbaI) DNA constructs were directly used as substrates.

* Actual number of clones hybridizing to B-Stop- and B-Gln-specific oligonucleotide probes are given in parentheses: B-Stop/(B-Stop + B-Gln).

* NP, no PCR product detected. In the coupled transcription-editing experiments, the treatment of the reaction mixture described under "Materials and Methods" completely removed any AdB-100 DNA that could be amplified by the PCR.

In both the direct RNA-editing and the coupled transcription-editing experiments, misincorporation in the PCR as the cause of the C→U substitution was excluded by the following: (i) in the direct editing experiments, incubation of the RNA in buffer alone did not produce any B-Stop sequences assayed by the PCR cloning-hybridization technique; furthermore, primer extension assay of the RNA products without PCR amplification confirmed the editing reaction; (ii) in the coupled transcription-editing experiments, the PCR cloning-hybridization assay performed directly on the DNA template (i.e. ADB-100<sub>100</sub>) showed 100% B-Gln sequences; and (iii) direct sequencing of multiple cloned B-Gln or B-Stop PCR products revealed no other base substitutions.

Comparison of the direct RNA-editing and coupled transcription-editing experiments indicates that the two reactions were comparable in efficiency. The fact that the editing reaction occurred under cell-free conditions using nuclear extracts suggests that RNA editing in vivo may occur in a similar manner, i.e. as a coupled reaction in the nucleus. Therefore, we used the coupled transcription editing of supercoiled ADB-100 minigenes to analyze the tissue and sequence specificity of the reaction.

In mammals, there is species- and tissue-specific variation in the efficiency of RNA editing. We tested the ability of nuclear extracts from three different tissues to perform the coupled transcription-RNA editing (Table I, B). All three extracts efficiently transcribed the apoB minigene. However, only nuclear extracts from rat liver edited the transcript. The activity of the extracts from HeLa or Hep3B cells was undetectable. This observation correlates with the presence of substantial amounts of apoB-48 mRNA in rat liver RNA and its absence in Hep3B RNA (data not shown); HeLa cells normally do not produce any apoB mRNA.

Sequence Specificity of ApoB mRNA Editing in Vitro—The sequence specificity of the coupled transcription-editing reaction was examined by site-directed mutagenesis of the ADB-100<sub>100</sub> DNA minigene (Fig. 2B). We constructed 22 different
mutant apoB-100 minigenes, including constructs that contain single or multiple base substitutions in the bases immediately flanking C-6666, as well as one construct that contains a single-base insertion, and another, a single base deletion. The efficiency of editing was quantified by the PCR cloning-hybridization assay and compared with the editing efficiency of wild type AdB-100260. The most obvious conclusion that can be drawn from these in vitro mutagenesis experiments is that the reaction is promiscuous with respect to the sequence of the RNA substrate; of the 22 mutant RNA sequences, all except two were edited by the nuclear extract in vitro (Table II). The results obtained in Table II were all confirmed by direct sequence analysis (data not shown).

In mutants a⁻→n, the three bases flanking the 5' and 3' sides of C-6666 were mutated individually, producing transition mutants (a⁻→f) or transversion mutants (g⁻→n). These constructs were edited in vitro with varying efficiency. For transition mutants, enhanced editing efficiency was observed for the mutant containing CGA instead of CAA (mutant a, which had a 3-fold increase in efficiency); mutant T-6669⁻→C (mutant c) was also edited somewhat more efficiently (~1.5-fold) than wild type. Mutant A-6665⁻→G (mutant d) was edited with normal efficiency. The two other constructs containing transition mutations (mutants e and f) were edited with an efficiency of 50% of wild type. Three of the transversion mutants, h, g, and k, were edited with markedly, moderately, and slightly reduced efficiency, respectively. The other transversion mutants were edited with the same (mutants i, j, and l) or higher efficiency (mutants m and n) compared with wild type. As noted below, introduction of single Cs in the proximity of C-6666 appears to stimulate editing in some instances.

**Table II**

RNA editing efficiency of ApoD-100 mutants

Coupled transcription editing was performed using adenovirus major late promoter-apoB-100, AdB-100260. The mutant minigenes were identical to the wild type with base substitutions shown. Asterisks represent identical residues. C-6666 is marked by an arrow. Following incubation, RNA was purified and analyzed by the PCR cloning-hybridization method as described under "Materials and Methods." Clones that hybridized to the B-Gln- or B-Stop-specific oligonucleotides were confirmed by direct DNA sequencing.

| Mutant | Sequence | Efficiency |
|--------|----------|------------|
| Wild type | ATA CAA TTT | ++++ |
| Transition mutants | | |
| a | * * * * G * * * | ++++ |
| b | * * * G * C * * | ++ |
| c | * * * G * * C * | ++++ |
| d | * C * * * * * | +++ |
| e | G * * * * * | +++ |
| f | G * * * * * | +++ |
| Transversion mutants | | |
| g | * * * T * * * | ++ |
| h | * * * * A * * | +++ |
| i | * A * * * * | +++ |
| j | T * * * * * | +++ |
| k | * * C * * * | +++ |
| l | * * * C * * | +++ |
| m | * * * C * * | +++ |
| Double mutants | | |
| Transition | | |
| o | * * G * G * * | ++ |
| p | * G G * * * | ++++ |
| Transversion | | |
| q | * T T * * * | 0 |
| r | * C C * * * | +++ |
| Deletion, s | * * * [ ] * | 0 |
| Insertion, t | * * * C * * | ++ |
| Multiple mutants | | |
| Transitions, u | G C G * * * C C C | ++ |
| Mixed, v | G C T C C | +++ |

*The estimated efficiencies of the mutant AdB-100 sequences are normalized to the wild type editing efficiency by the following formula: (% B-Stop in mutant/% B-Stop in wild type) X 100. Editing efficiencies are: 0%, 0; <25%, +; 25-50%, ++; 50-75%, +++; 75-125%, ++++; 125-200%, +++++; >200%, +++++++. By definition, wild type is taken as 100% or ++++. The actual percent B-Stop in wild type AdB-100260 transcripts varies from 0.6 to 2% in individual experiments. Each mutant was assayed in three separate experiments with consistent results. For each mutant, two separate oligonucleotide probes, one for the unedited C and one for the edited T, were used for specific hybridization to each C residue examined. In individual experiments, the total number of PCR-generated apoB clones was usually 4,000-10,000 with a range of 2,000-20,000. In the experiments where no editing was detected (mutants q and s) a total of over 20,000 colonies in three separate experiments was found to hybridize only to B-Gln oligonucleotides but not to the B-Stop oligonucleotides; in the same experiments editing was demonstrated with other constructs. Furthermore, the same B-Stop oligonucleotide probes produced strong hybridization signals to colonies of constructs containing a C-6666⁻→T mutation generated by site-directed mutagenesis.

*The multiple results in these constructs refer to the different Cs in close proximity to, and including C-6666, that were edited, starting with the 5'-most C.

**Simultaneous editing of the neighboring Cs.**

**Simultaneous editing of C-6666, C-6667, C-6668, C-6669, C-6670, and C-6671 in that order.**
Four double mutants tested were found to be edited with drastically different efficiencies. One, mutant p, which had CGG substituted for CAA, had a 2-3-fold increase in editing efficiency compared with wild type. Another, mutant r, which had CCC substituted for CAA, was edited at normal efficiency. One double transversion mutant (q) containing the mutated bases A-6665→T and A-6667→T was consistently not edited in vitro in three separate experiments. This complete inhibition of editing should be contrasted with the roughly 50% editing efficiency of the corresponding double transition mutant (o). Complete inhibition of editing was also observed for the single deletion mutant, s, where one of the two As following C-6666 was deleted. In the insertion mutant, t, where an extra C was inserted next to C-6666, both Cs were edited with reduced efficiency.

The fact that the vast majority of mutations do not seriously impair editing efficiency suggests that the sequence requirement of this reaction is not very stringent. To test this hypothesis, we constructed two 6-base substitution mutants where the two codons flanking the CAA are mutated, being replaced completely by transitional substitutions (mutant u) or five transitional substitutions and a single transversional substitution (mutant v). Transcripts from both constructs were edited with an efficiency approximately one-third (for mutant u) and two-thirds (for mutant v), respectively, of that of wild type.

In a number of the mutants that we studied, one or more Cs were introduced in the sequence in close proximity to C-6666. These constructs include mutants m, n, r, and t. Like C-6666, most of the neighboring Cs were also found to be edited in vitro. It is interesting that introduction of a single C immediately next to C-6666 stimulated editing when the relative position of C-6666 was not altered (mutant m). Both Cs in mutant m were simultaneously edited with high efficiency. In mutant t, where there was an insertion, there was moderate impairment of editing efficiency, the 3′ C being affected more than the 5′ C. In comparing the relative efficiency with which each C was edited in individual mutants, there appears to be some general pattern. In three instances where the relative position of C-6666 was not changed (mutants m, n, and r), the original C-6666 was edited much more efficiently than the Cs at the neighboring positions. Mutant r contains a substitution of the triplet CCC for CAA. In this case, the first C-6666 was found to be edited with normal efficiency, whereas the two 3′ Cs, C-6667 and C-6668, were edited poorly. Simultaneous editing of the two 5′ Cs was also observed, though infrequently. These results suggest that there may be a distance effect between the edited C and some flanking DNA sequence. Results with mutants m, n, and r suggest that a minimal distance between C-6666 and some 3′ sequence or a maximal distance between this base and some 5′ sequence is preferred for efficient editing. The slightly more efficient editing of the 5′ C of the insertion mutant t is difficult to interpret because there is significant impairment of editing of both Cs.

Implications of Sequence Specificity of ApoB mRNA Editing—We have found that a rat liver nuclear extract not only can edit synthetic apoB RNA in vitro (Table I, A), as was demonstrated recently by Driscoll et al. (22), but also could perform coupled transcription editing of a minigene construct in which the in vitro nascent apoB transcript was edited with fidelity (Table I, B). RNA editing is likely a nuclear event in vivo. The system described here may mimic the in vivo situation better than one that uses a synthetic RNA as substrate. We have tested synthetic RNAs corresponding to each of the mutant constructs in direct editing experiments. All of them, including mutants q and s, were edited in vitro, which suggests that coupled transcription editing may have a more stringent sequence requirement than direct RNA editing.

The ability to introduce multiple mutations to apoB RNA without seriously reducing the editing efficiency indicates that specific base pairing or hydrogen bonding involving the bases immediately flanking C-6666 is not required for recognition by the putative cytidine deaminase enzyme. The relative laxity of the structural requirement is also supported by the fact that when additional C residues were introduced to the vicinity of C-6666, they were edited often to the same extent as C-6666. On the other hand, since mutants q and s, which contained double transversions and a single deletion, respectively, were consistently not edited in the coupled transcription editing reaction, there is definitely a preferred RNA structure recognized by the enzyme.

Although most of the mutant apoB RNA sequences were edited with normal or reduced efficiency, two of the constructs (mutants a and p) were consistently edited at an efficiency approximately 3-fold that of wild type. Synthetic transcripts of both mutants that were fed into the nuclear extracts were also edited efficiently (data not shown). It is possible that both apoB RNA mutants are deleterious to the organism. Mutant a, which contains CGA in place of CAA, would be edited so efficiently in the liver that insufficient apoB-100, a physiologically important protein, would be produced. In contrast, editing of mutant p, which contains CGG instead of CAA, would produce an Arg→Trp substitution and not a stop codon, and no apoB-48 would be produced in the intestine. For these reasons, we speculate that both mutations, if they occurred, would be eliminated from the population.

Recently, an analogous RNA-editing mechanism involving C→U conversions has been described as a common phenomenon in plant mitochondria (30, 31). In our current analysis, the relatively loose stringency of the editing reaction suggests that other RNAs may also be edited in vivo. Thus, in mammals as in plant mitochondria, RNA editing may not be a unique biologic phenomenon confined to apoB.

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