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Hepatitis D Virus RNA Editing: Specific Modification of Adenosine in the Antigenomic RNA

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RNA editing plays a central role in the life cycle of hepatitis D virus (HDV), a subviral human pathogen. Previous studies (J. L. Casey, K. F. Bergmann, T. L. Brown, and J. L. Gerin, Proc. Natl. Acad. Sci USA 89:7149–7153, 1992; H. Zheng, T.-B. Fu, D. Lazinski, and J. Taylor, J. Virol. 66:4693–4697, 1992) had concluded that the genomic RNA of HDV was the target for RNA editing and that the editing reaction was a conversion of U to C. However, we show here that the antigenomic RNA of HDV is in fact the target for HDV RNA editing, which is therefore a conversion of A to G. This result is verified by using an assay specific for editing on the antigenomic RNA and by analyzing the editing of site-directed mutant RNAs in transfected cells and in cell extracts. Because editing occurs in the absence of viral antigens and the specificity for the HDV editing target site is present even in extracts from *Drosophila* cells, it is likely that HDV RNA is edited by one or more cellular factors that are conserved among higher eukaryotes. These results raise the likelihood that double-stranded RNA adenosine deaminase specifically edits HDV antigenomic RNA.

Hepatitis D virus (HDV) is a subviral human pathogen that is frequently found in patients with hepatitis B who have more severe liver disease (26). Hepatitis B virus infection is required for HDV propagation because hepatitis B virus provides the envelope for the HDV particle (27, 28). This particle is composed of HDV genomic RNA, hepatitis delta antigen (HDAg), which is the sole protein of HDV, and the viral envelope, hepatitis B surface antigen (2–5). The HDV RNA is circular and similar to the plant viroid agents, and it possesses intramolecular complementarity such that about 70% of the nucleotides can form base pairs in an unbranched rod structure (33). Consistent with a rolling-circle replication mechanism similar to that of the plant viroid agents, infected cells harbor a mixture of both genomic and antigenomic RNAs that includes monomeric circular species as well as monomeric, dimeric, and trimeric linear species (10; reviewed in reference 30).

RNA editing plays a central role in the HDV replication cycle (6, 21). The specific modification of position 1012 results in the production of two forms of the HDV protein, HDAg, that play opposed roles in the viral replication cycle. HDAg p24 is 195 amino acids (aa) long and is required for replication of the HDV RNA (16); HDAg p27 is 214 aa long, inhibits replication (16), and is required for packaging of HDV RNA with hepatitis B surface antigen (29, 34). These two forms of HDAg differ by the presence of an additional 19 or 20 aa at the C terminus of HDAg p27 (32). RNA editing at position 1012 abolishes the stop codon of HDAg p24, resulting in the translation of the additional C-terminal amino acids (6, 21, 35). The importance of the editing event in the HDV replication cycle is indicated by the strong conservation of a specific base-paired editing target structure among HDV genotype I isolates and by the tight regulation of the rate and extent of the editing reaction (6–8). Regulation of editing is important because the editing process yields RNAs that produce HDAg p27, which inhibits replication; the maximal extent of editing is typically around 30%.

Several attempts have been made to determine whether the genomic or the antigenomic RNA of HDV is the target for editing (6, 21, 36). Such a determination is complicated during analysis of replicating HDV RNA because of the rolling-circle mechanism of HDV RNA replication: both the genomic and antigenomic species are a mixture of edited and unedited RNAs. Editing of the genomic RNA would be a conversion of U to C; for the antigenomic RNA, the conversion would be from A to G. An initial attempt in which it was suggested that the antigenomic RNA is the target for editing was inconclusive and did not demonstrate specificity (21). Later studies indicated that the editing of HDV RNA occurs on the genomic strand and would thus be a conversion of U to C (6, 36). This conclusion was based on the analysis of the predicted effects of site-directed mutations on the essential base-paired structure required for editing of HDV genotype I RNAs (6) and on the direct observation of editing genomic RNA in transfected cells and in cell extracts (36).

In the course of developing methods to analyze editing in the absence of HDV RNA replication, it has appeared that the antigenomic RNA of HDV is in fact the substrate for RNA editing, contrary to the previous conclusions of U-to-C editing on the genomic RNA. In this report, the discrepancy is resolved through analysis of the editing activity of nonreplicating genomic and antigenomic HDV RNAs, a reverse transcription-mediated PCR (RT-PCR) assay specific for editing of antigenomic RNA, and editing of a panel of site-directed mutant RNAs both in cells transfected with nonreplicating constructs and in cells extracts.

MATERIALS AND METHODS

Plasmid constructs. The nonreplicating deletion construct pGDC-Δ1×1.2 was created by blunt-end ligation of the *Apa*I fragment, positions 1388 to 215 (numbering according to Wang et al. [33]), from pGDC1×1.2 (6) with *Sma*I-digested (positions 490 and 1110) pGDC1×1.2. In the resulting construct, about 550 bp of the 1,679-nucleotide HDV genome have been removed, such that most of the coding sequences for HDAg have been deleted, including functional elements necessary for viral replication such as the RNA binding domain (9, 17, 19). An approximately 1.5-kb *Hin*dIII-*Spe*I fragment from pGDC-Δ1×1.2 that contained

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1.2 copies of the HDV cDNA from this plasmid was inserted between the *Hin*dIII and *Xba*I sites of plasmids pCMV2 and pCMV3, which contain the cytomegalovirus (CMV) immediate-early promoter, the bacteriophage T7 promoter, and a polylinker. The orientation of the polylinker restriction enzyme sites is reversed in pCMV2 and pCMV3, so that pCMV2-DC-Δ1×1.2 and pCMV3-DC-Δ1×1.2 contain HDV sequences positioned relative to the CMV promoter so as to produce nonreplicating genomic and antigenomic HDV RNAs, respectively.

Constructs designed to yield monomeric genomic or antigenomic RNAs were created by insertion of the monomer-length *Xba*I fragment of pCMV2-DC-Δ1×1.2 into the *Xba*I site in the polylinker of pCMV2. Clones pCMV2-DC-Δ1×1 (G) and pCMV2-DC-Δ1×1 (A) contain the HDV cDNA oriented so as to produce genomic and antigenomic RNAs, respectively, upon transfection into cells and upon transcription with T7 polymerase in vitro. The orientation of the inserts was determined by restriction endonuclease digestion of plasmid DNA.

The nonreplicating deletion constructs pCMV2-DC-ΔApax1.2 and pCMV3-DC-ΔApax1.2, which are designed to produce genomic and antigenomic RNAs, respectively, upon transfection of cells or upon transcription with T7 polymerase in vitro, were created by *Apa*I digestion of the replication-competent parental plasmids pCMV2-DC1×1.2 and pCMV3-DC1×1.2. These parental plasmids were created similarly to plasmids pCMV2-DC-Δ1×1.2 and pCMV3-DC-Δ1×1.2, i.e., by insertion of a 2-kb *Hin*dIII-*Spe*I fragment from pGDC1×1.2 containing approximately 1.2 copies of HDV cDNA into the *Hin*dIII and *Xba*I sites of plasmids pCMV2 and pCMV3. Full-length defective constructs pCMV2-DC-ΔSstII×1.2 and pCMV3-DC-ΔSstII×1.2 were created by *Sst*II restriction digestion of pCMV2-DC1×1.2 and pCMV3-DC1×1.2, respectively, followed by end trimming with Klenow enzyme and ligation.

The 125-bp *Pst*-*Sal*I fragments containing the site-directed mutations 1011U/1012C (UGA), 1009C, and 1014C (6) were transferred from the parent plasmids into the *Pst*I-*Sal*I-digested plasmid pCMV3-DC-Δ1×1.2. For the site-directed mutations 580A, 578G, and 583G (6), a 297-bp *Bst*BI-*Bst*XI fragment was transferred. Cloning of mutations was verified by DNA sequencing.

Transfections. HuH-7 cells were transfected by the calcium phosphate method (11). Total RNA was harvested 6 days posttransfection, or as indicated, by using a sodium dodecyl sulfate lysis method (24).

In vitro RNA synthesis. pCMV2- and pCMV3-derived plasmids were linearized with *Bam*HI, which cuts beyond the inserted HDV sequences, relative to the T7 promoter. pGDC1 (6) was digested with *Pvu*II, which cuts the plasmid sequences in two locations. Transcription with T7 polymerase (Promega) was done according to the manufacturer's specifications; following transcription reactions, samples were treated with RQ1 DNase (Promega). RNAs were purified with spun columns (5Prime-3Prime) and quantified by agarose gel electrophoresis-ethidium bromide staining and A_{260}.

Editing in cell extracts. HDV RNAs were transcribed in vitro with T7 RNA polymerase from plasmids digested with either *Bam*HI or *Pvu*II. A 0.1-ng aliquot of RNA was incubated with 5 μg of HeLa nuclear extract (Life Technologies) or 5 μg of *Drosophila* embryo nuclear extract (Promega) overnight in buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6), 100 mM KCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1 U of the RNase inhibitor InhibitAce (5Prime-3Prime). After overnight incubation at the specified temperatures, RNA was purified by extraction with phenol-chloroform and ethanol precipitation.

Analysis of editing. Prior to amplification, all RNA preparations were treated with DNase (Life Technologies) to remove residual plasmid DNA. The effectiveness of this treatment was verified by the absence of PCR products on omission of the reverse transcription step. In most experiments, reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase (Life Technologies) at 37°C for 15 min and 42°C for 15 min, using random hexamers (Pharmacia) as primers. Samples were then amplified and radiolabeled by 30 cycles of PCR amplification with 5 to 10 μCi of [^{32}P]dCTP added per sample as described previously (6). Pipette tips containing filter barriers were used for all PCR procedures. The primer pairs for the PCR amplification were 5414 (5′-GAGATGCCATGCCGACCCGAAGAG-3′, positions 883 to 906) and 6657 (5′-CAGCAGTCTCCTCTTTACAGA-3′, positions 1658 to 1638) for pCMV-DC-Δ1×1.2 constructs and derivatives; for other constructs, primers 5414 and 5415 (5′-GAAGGAAGGCCCTCGAGAACAAGA-3′, positions 1288 to 1265) were used. Sample concentrations were adjusted such that the PCR product yield was not saturated after 30 cycles. Editing was analyzed by digestion of 5 μl of the radiolabeled PCR product with the restriction enzyme *Sty*I or *Dsa*I in a volume of 40 μl, followed by polyacrylamide gel electrophoresis and autoradiography. Where indicated, the PCR product was purified by agarose gel electrophoresis using GelAse (Epicenter Technologies) prior to restriction digestion analysis.

In the experiments presented in Table 1, reverse transcription was carried out either as described above or at 45°C for 30 min with the genomic-sense primer 6520A (5′-TTCCGATAGA-3′, positions 787 to 796). Following heat inactivation of the reverse transcriptase at 95°C, samples were cooled to 80°C, at which temperature the PCR components were added without allowing the temperature to drop. Thirty-five cycles of PCR were then performed as described above except that no radiolabeled nucleotide was added. Five microliters of the PCR mixture was then added to a 50-μl single-cycle PCR radiolabeling mixture (reaction carried out for 3 min at 95°C, 1 min at 55°C, and 3 min at 72°C) containing 10 μCi of [^{32}P]dCTP (3,000 Ci/mmol; Amersham) and 0.02 mM each deoxynucleoside triphosphate.

RESULTS

Plasmids used to produce nonreplicating HDV RNA in transfected cells are shown in Fig. 1. Because of the demonstrated requirement of base pairing for maximal editing activity (6), the deletions were constructed such that the RNA produced would be able to form the unbranched rod typical for HDV RNA. The 1.2-mer constructs would be expected to form circularized rods because they contain repeats of the genomic and antigenomic autocleavage and ligation sites (18). The parent construct pGDC-1×1.2 contains 1.2 copies of the complete DNA genome and thus produces large amounts of replicating genomic and antigenomic RNAs upon transfection of cells (6). The deletion constructs lack functional regions necessary for replication, including parts of the coding region for HDAg (9, 17, 19), and as yet undefined elements in the RNA (18). Upon transfection of HuH-7 cells, the deletion constructs did indeed produce exclusively nonreplicating RNAs of the anticipated sense, as demonstrated by the use of genomic- and antigenomic-sense hybridization probes on Northern (RNA) blots which included virion RNA as a control for the genomic sense (not shown). The lack of HDV RNA replication in cells transfected with these constructs is consistent with the results of Lazinski and Taylor (18), who showed that similar constructs failed to produce replicating HDV RNA even in cells expressing HDAg.

RNA was harvested from HuH-7 cells 6 days after transfection with the deletion constructs described in Fig. 1 and was analyzed for editing at position 1012 by *Sty*I digestion of the PCR-amplified cDNA (Fig. 2). At 6 days posttransfection, there was a balance between the effects of RNA degradation, which made detection of RNA increasingly difficult at longer times posttransfection, and editing levels, which were significantly higher at day 6 than at day 3. The enzyme *Sty*I discriminates between edited and unedited RNA templates because the editing reaction creates a *Sty*I restriction site that is not present in unedited genomes (6, 35). In other experiments, we have seen very good agreement between the extent of editing detected by this assay and either the amount of HDAg p27 produced or the amount of editing detected by sequencing of cloned PCR products. For all three pairs of deletion constructs, only cells transfected with the construct designed to synthesize antigenomic-sense RNA yielded RNA that was edited, as determined by the appearance of two appropriately sized *Sty*I digestion fragments (Fig. 2). The *Sty*I digestion products present in Fig. 2A, lanes 4 and 8, and Fig. 2C, lane 4, are due to digestion of the desired HDV RNA-derived PCR product: no additional bands of comparable intensity were present in the gel above the main band; gel purification of the main band prior to restriction enzyme digestion gave the same specific pattern of digestion (Fig. 2D); incubation of the PCR product with *Sal*I, for which there is a unique site in the amplified fragment, completely digested the DNA to yield products of the expected size; and sequencing of cloned PCR products showed that the presence of *Sty*I digestion products correlated with the presence of 1012C, and vice versa. Moreover, the use of different pairs of PCR primers yielded the same results. Digestion of PCR products derived from DNA containing 1012C verified that the sizes of the bands due to *Sty*I digestion were consistent with editing products and demonstrated the ability of *Sty*I to fully digest PCR products. Controls against potential contamination indicated that the observed

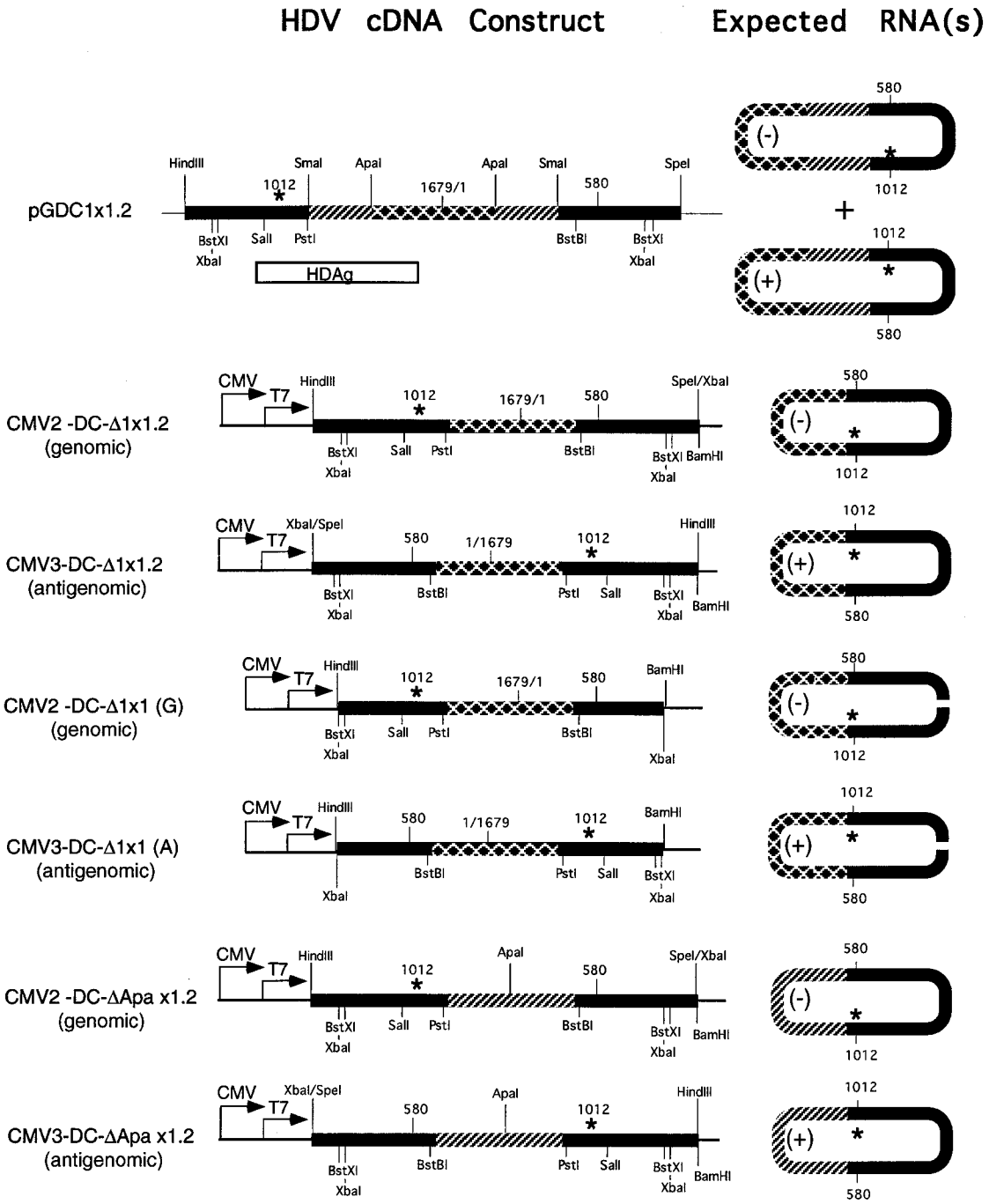

FIG. 1. Schematic diagram of constructs used to analyze editing of nonreplicating HDV RNA. Straight thick bars, HDV cDNA sequences; thin horizontal lines, plasmid sequences; circular thick lines, the RNA species produced; striped regions, sequences between the *Apa*I and *Sma*I sites which are deleted in the nonreplicating Δ1 constructs; cross-hatched regions, sequences between the two *Apa*I sites that are deleted in the nonreplicating Δ*Apa* constructs. For both sets of constructs, the deleted sequences comprise about one-third of the HDV RNA rod structure, but the ability of the RNA to form the base-paired rod structure is preserved. The orientations of the CMV and T7 promoters are indicated by arrows. Minus and plus signs within the circular RNA diagrams indicate genomic and antigenomic RNAs, respectively. An asterisk denotes the location of position 1012, the target for RNA editing; also indicated is position 580, which is apposed to position 1012 in the rod structure. Nucleotide numbering is according to Wang et al. (33) and refers to the genomic sequence. The diagram at the top corresponds to the parent replicating construct containing 1.2 copies of HDV cDNA, in which the genomic and antigenomic autocatalytic cleavage sites are duplicated.

PCR product was due to RNA produced in the transfected cells and not due to DNA contamination from either the transfected cells or laboratory sources. PCR amplification of DNase-treated samples without prior reverse transcription yielded no detectable PCR product, and PCR amplification of samples not treated with DNase (to amplify residual transfected plasmid DNA) yielded PCR products that were undigestible with *Sty*I (Fig. 2B).

Quantitation of the editing products in Fig. 2D, lane 4, by radioanalytic imaging (Ambis) indicated that about 20% of the

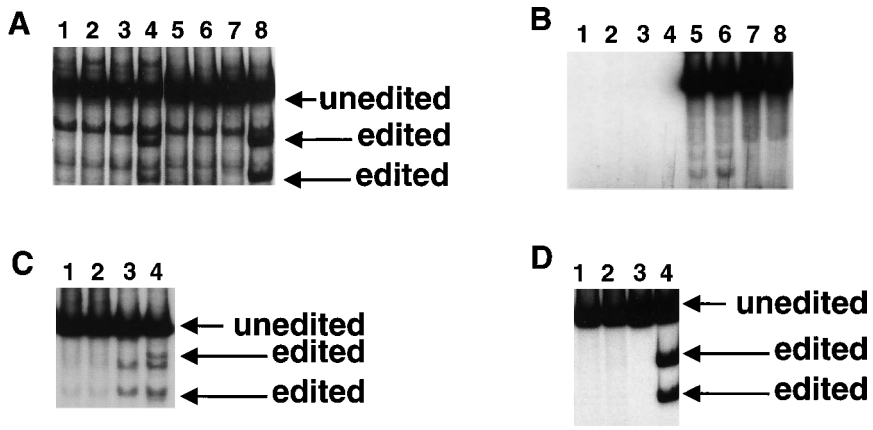

FIG. 2. HDV antigenomic RNA is edited in transfected cells. HuH-7 cells were transfected with nonreplicating constructs designed to synthesize either genomic or antigenomic HDV RNA. Editing of the HDV RNA was analyzed 6 days posttransfection as described in Materials and Methods. Smaller fragments produced by digestion with the restriction enzyme *Sty*I indicate editing of the RNA. (A) RNA from cells transfected with pCMV2-DC-Δ1×1.2 (lanes 1 and 2), pCMV3-DC-Δ1×1.2 (lanes 3 and 4), pCMV2-DC-Δ1×1 (G) (lanes 5 and 6), and pCMV2-DC-Δ1×1 (A) (lanes 7 and 8). pCMV2-DC-Δ1×1.2 and pCMV2-DC-Δ1×1 (G) are designed to produce genomic RNA in transfected cells; pCMV3-DC-Δ1×1.2 and pCMV2-DC-Δ1×1 (A) are designed to produce antigenomic RNA. Odd-numbered lanes, undigested PCR product; even-numbered lanes, PCR product digested with *Sty*I. (B) Control assays for samples analyzed in panel A. Lanes 1 to 4, PCR amplification without prior reverse transcription; lanes 5 to 8, PCR amplification without prior DNase treatment and without prior reverse transcription (to amplify residual transfected DNA). Lanes 1 and 5, pCMV2-DC-Δ1×1.2; lanes 2 and 6, pCMV3-DC-Δ1×1.2; lanes 3 and 7, pCMV2-DC-Δ1×1 (G); lanes 4 and 8, pCMV2-DC-Δ1×1 (A). (C) RNA from cells transfected wtih pCMV2-DC-ΔApax1.2 (lanes 1 and 2) and pCMV3-DC-ΔApax1.2 (lanes 3 and 4). pCMV2-DC-ΔApax1.2 is designed to produce genomic RNA in transfected cells; pCMV3-DC-ΔApax1.2 is designed to produce antigenomic RNA. Lanes 1 and 3, undigested PCR products; lanes 2 and 4, PCR products digested with *Sty*I. Panel D. The samples as are the same as those in lanes 5 to 8 of panel A except that the 495-base. PCR fragments were gel purified prior to *Sty*I digestion and gel analysis.

antigenomic RNA was edited 6 days after transfection; no editing of HDV genomic RNA was detectable. Preliminary analysis of editing in the RNAs derived from the different deletion constructs suggests that the extent of editing may depend on the type of deletion made. Constructs with the Δ1 deletion (Δ*Apa*I-*Sma*I) exhibited the highest levels of editing. Editing of RNA produced by a nonreplicating full-length 1.2-mer (pCMV3-DC-ΔSstII×1.2) was evident but difficult to detect (not shown). The significance of these observations is unclear at present but could be related to the ability of the different RNAs to fold into the correct target structure, interaction of the RNAs with cellular factors, or RNA stability.

Despite evidence that the constructs examined above are nonreplicating and produce HDV RNAs of the expected sense by means of transcription driven by the CMV promoter, it is possible that low levels of opposite-sense transcripts were produced either from endogenous promoters present in the transfected HDV cDNA (22) or from low levels of replication that are difficult to detect. In this event, low levels of genomic RNA could still be responsible for the editing observed, as suggested by previous studies (6, 36). Indeed, the assay used to measure editing in Fig. 2 does not attempt to distinguish between the presence of genomic and antigenomic RNAs. To verify that the observed editing is due to modification of the antigenomic RNA and not due to low levels of genomic RNA, we developed an RT-PCR assay to distinguish between editing on the genomic and antigenomic RNAs. The genomic-sense primer 6520A (see Materials and Methods) was used to reverse transcribe RNA at 45°C; this procedure was followed by PCR amplification, radiolabeling, and restriction enzyme digestion. The use of this primer decreased overall sensitivity of the RT-PCR assay but provided approximately 10-fold more sensitivity for antigenomic RNA than for genomic RNA. Experimentally prepared mixtures of RNAs transcribed in vitro were used to demonstrate that editing observed due to contaminating edited genomic RNA could be readily detected by comparing editing observed with random hexamers as primers with editing observed with primer 6520A, which is more sensitive for the antigenomic RNA. With about 12% of the total HDV RNA in a sample represented by genomic RNA with 1012C (i.e., edited) and either 10 or 50% of the total HDV RNA as genomic RNA, the editing assay with primer 6520A used in the reverse transcription reaction detected no edited RNA, while the assay with random hexamers detected about 12% for both samples (Table 1). Even if 90% of the total RNA in a solution was genomic sense and about 12% of the total was genomic RNA with 1012C, the editing assay with primer 6520A used in the reverse transcription reaction detected only 1% edited RNA, compared with the 11% detected by random hexamers. Thus, comparison of editing observed by using primer 6520A with that observed by using hexamers can readily detect an influence of contaminating edited genomic RNA on the assay even if the contaminating genomic RNA represents 90% of the total HDV RNA. RNAs from cells transfected with the antigenomic construct pCMV2-DC-Δ1-1 (A) (Fig. 1 and 2) were

TABLE 1. Comparison of editing detected by different reverse transcription primers

Sample[a]	Amt (%) of:		Amt (%) of editing detected with[b]:	
	Genomic RNA	Genomic RNA with 1012C	Random hexamers as primers	6520 A as RT primer
1	10	12	15.6	0
2	50	12	13.5	−0.2
3	90	12	11.8	1.0
4	?	?	22.5	19.5

[a] Samples 1, 2, and 3 contained various amounts of in vitro-transcribed antigenomic and genomic RNAs and approximately identical amounts of genomic RNA with 1012C (i.e., edited). Sample 4 was RNA from cells transfected with construct pCMV2-DC-Δ1×1 (A), designed to produce nonreplicating antigenomic RNA in transfected cells (Fig. 1 and 2).

[b] Determined by *Sty*I digestion of RT-PCR products, polyacrylamide gel electrophoresis, and radioanalytic imaging (Ambis) of the dried gel.

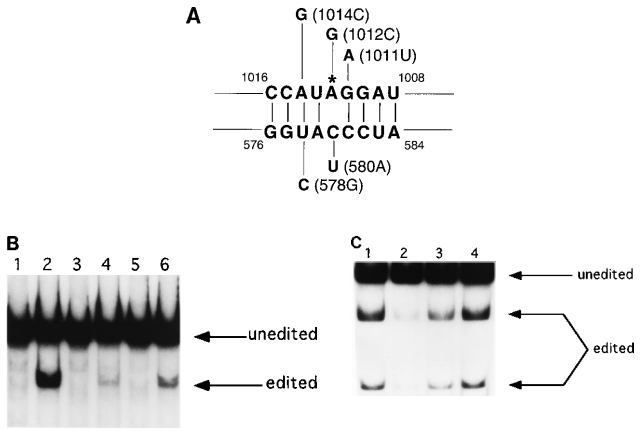

FIG. 3. Specificity of editing nonreplicating antigenomic HDV RNA in transfected Huh-7 cells. (A) The HDV RNA editing target site in the antigenomic RNA and constructs with the site-directed mutations used in this study. The mutations are named according to the nucleotide position and composition of the genomic sequence. (B and C) Site-directed mutations of the HDV RNA editing target were inserted in the nonreplicating construct pCMV3-DC-Δ1×1.2 and transfected into HuH-7 cells. Editing of the antigenomic RNA produced was analyzed 6 days posttransfection. (B) Lanes 1 and 2, wild type; lanes 3 and 4, mutant 1011C/1012U; lanes 5 and 6, mutant 580A; lanes 1, 3, and 5, undigested PCR product; lanes 2, 4, and 6, PCR product digested with *Sty*I. (C) Lane 1, wild type; lane 2, mutant 1014C; lane 3, mutant 578G; lane 4, mutant 578G/1014C. PCR products were gel purified and then digested with *Dsa*I, which recognizes the edited site in all of these mutants.

subjected to analysis of editing using either random hexamers or primer 6520A for the reverse transcription reaction. The absence of a difference between the amount of editing detected by the two assays (Table 1) indicated that the observed editing is indeed due to modification of the antigenomic RNA and confirms that the majority of HDV RNA present in these cells is antigenomic sense.

The finding that the antigenomic RNA of HDV is the target for editing is in contrast to the results of Zheng et al., who reported a similar experiment in which a nonreplicating HDV genomic RNA was edited in transfected cells (36). Therefore, to further confirm the validity of our finding, editing of the nonreplicating antigenomic HDV RNA was analyzed for specificity by using site-directed mutations. Previous studies have shown that editing of HDV RNA during replication is a highly specific process: a base-paired structure that is strongly conserved among genotype I isolates is essential for maximal levels of editing (6, 7). Thus, in replicating constructs, the efficiency of editing is reduced in constructs with site-directed mutations that disrupt this base-paired structure, in the construct with a mutation at position 580, which is apposed to position 1012 in the HDV RNA rod structure, and in the mutant 1011U/1012C (Fig. 3A). The results presented in Fig. 3B show that editing of nonreplicating antigenomic RNAs containing the site-directed mutations 580A and 1011U/1012C was strongly inhibited. While the 1011U/1012C mutation changes the immediate sequence context of the editing target, position 580 is more than 400 nucleotides away. The sharp reduction of editing in the 580A mutant provides strong support for the conclusion that the same specific editing target is recognized in both replicating and nonreplicating HDV antigenomic RNAs. It is therefore likely that the same cellular editing system is responsible for editing in both cases.

Further support for this conclusion is the pattern of editing exhibited by the nonreplicating site-directed mutations 578G, 1014C, and 578G/1014C. In replicating constructs, the 578G and 1014C mutations disrupt the base-paired structure of the RNA editing target and exhibit reduced levels of editing; the 578G/1014C double mutation restores both base pairing and editing efficiency (6). When these mutations were inserted into construct pCMV3-DC-Δ1×1.2, the same pattern of editing activity was observed for the nonreplicating HDV antigenomic RNA (Fig. 3C). Other mutations that affected the base-paired structure also gave the same relative amounts of editing in replicating and nonreplicating constructs (not shown). The set of 578G/1014C site-directed mutations is particularly interesting because the 578G/1014C covariation is found in naturally occurring genotype I HDV isolates and supports the model for the structure of the HDV genotype I editing target (6).

The results shown in Fig. 2 demonstrate that HDV antigenomic RNA is edited in the absence of HDV RNA replication and in the absence of the only known viral protein, HDAg. The editing activity must therefore be entirely of cellular origin. To examine the cellular components of the editing reaction, HDV antigenomic RNA transcribed in vitro was incubated with cell extracts. As shown in Fig. 4A, about 1% of the HDV antigenomic RNA was edited during an overnight incubation with a nuclear extract from HeLa cells. The activity was nearly undetectable at 30°C and maximal between 37 and 42°C. No HDV or HBV proteins were expressed in any of the extract preparations.

Editing activity was also examined in other cell extracts, particularly because the HeLa extracts possessed both high levels of RNase and factors that inhibit HDV RNA editing (5a). *Drosophila* nuclear extracts specifically edited HDV antigenomic RNA with greater activity than the HeLa extract (Fig. 4B). About 4% of the antigenomic RNA was edited at

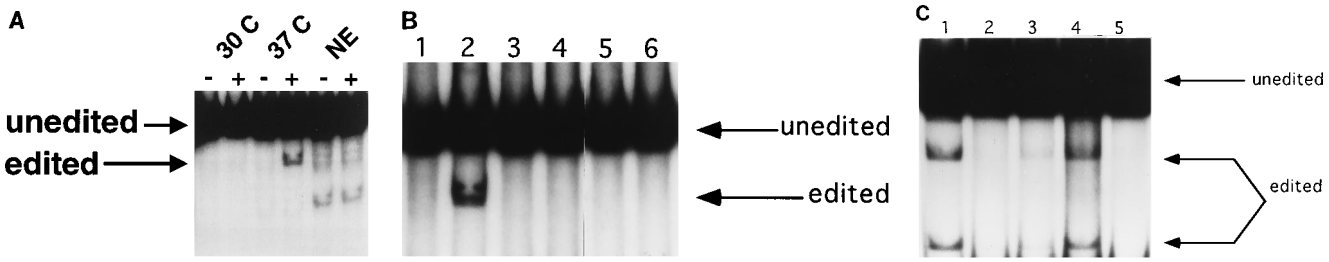

FIG. 4. Editing of HDV antigenomic RNA by nuclear extracts. A 0.1-ng aliquot of in vitro-synthesized HDV RNA was incubated overnight with 5 μg of the indicated nuclear extract. Editing was assayed as described in the text. (A) HDV antigenomic RNA incubated overnight with HeLa nuclear extract at the indicated temperatures. NE, no extract. PCR products were incubated with (+) or without (−) restriction enzyme *Sty*I. (B and C) HDV RNA incubated overnight at 30°C with *Drosophila* nuclear extract. (B) Lanes 1 and 2, wild-type HDV antigenomic RNA; lanes 3 and 4, wild-type HDV genomic RNA; lanes 5 and 6, HDV antigenomic RNA mutant 580A. Lanes 1, 3, and 5, undigested PCR product; lanes 2, 4, and 6, *Sty*I-digested PCR product. (C) Lane 1, wild-type antigenomic HDV; lane 2, mutant 1014C; lane 3, mutant 578G; lane 4, double mutant 578G/1014C; lane 5, mutant 1011U/1012C. PCR products were gel purified and then digested with *Dsa*I.

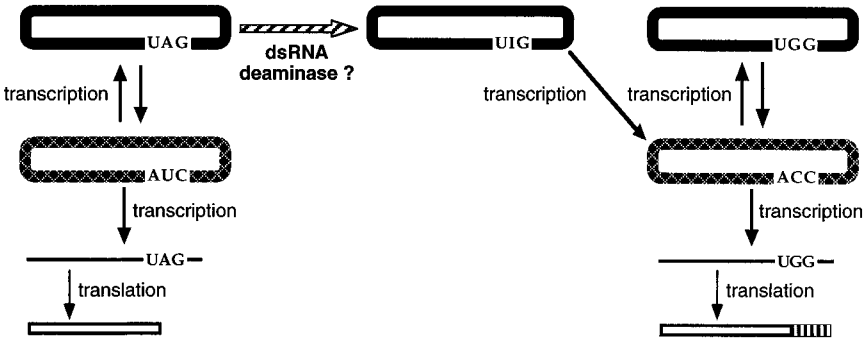

FIG. 5. Schematic of the HDV replication cycle, including the possible involvement of editing via dsRNA adenosine deaminase. Thick black lines, HDV antigenomic RNA; cross-hatched lines, genomic RNA; thin lines, HDAg mRNA; open rectangle, first 195 aa of HDAg; vertically striped rectangle, additional 19 aa present at the C terminus of HDAg p27 as a result of editing.

position 1012 after an overnight incubation with a *Drosophila* nuclear extract (Fig. 4B and C); no editing was detected at this position in the genomic transcript. This result further confirms the findings presented in Fig. 2: the antigenomic RNA of HDV is the target for editing. The same result was obtained with full-length (1,679-nucleotide) HDV RNAs for which the genomic and antigenomic senses were confirmed by hybridization with virion (genomic sense) RNA. To ascertain the specificity of the editing activity in the *Drosophila* extracts, the ability of each of the extracts to edit the site-directed mutants 580A, 1011U/1012C, 578G, 1014C, and 578G/1014C (Fig. 4B and C) was examined. In all cases, the relative levels of editing paralleled the activity seen in human HuH-7 cells transfected with either replicating (6) or nonreplicating (Fig. 3) HDV RNA constructs.

DISCUSSION

In this study, the ability of transfected cells and cell extracts to edit position 1012 in nonreplicating genomic and antigenomic HDV RNAs has been examined. The finding that position 1012 is edited only in the antigenomic RNA produced by nonreplicating constructs contradicts earlier conclusions (6) and the results of Zheng et al. (36) that the genomic RNA of HDV is the target for editing. The validity of the result presented in this study, that the antigenomic RNA of HDV is the editing target, is confirmed by the observation of identical editing levels in both an assay specific for antigenomic RNA and an assay that does not distinguish between genomic and antigenomic RNAs (Table 1). Further proof is provided by the finding that site-directed mutations result in the same relative editing activities in both replicating and nonreplicating constructs. The contradiction between the previous conclusion (6) and the present data suggests that the premise for that conclusion was incorrect; it had been assumed that A-C mismatches disrupt the essential base-paired structure more than G-U wobble pairs (6). While both the relative editing activities of site-directed mutants and the strict conservation of the base-paired structure among genotype I isolates consistently support the conclusion that base pairing is essential for maximal editing, the precise roles of conventional Watson-Crick base pairs, G-U wobble pairs, and base mismatches in forming the best editing target is not clear. Indeed, the observation that the proposed editing target structures for HDV genotype II and genotype III do not exhibit the same degree of base pairing as genotype I isolates (8) suggests the possibility that base pairing is just one of several factors that can influence the suitability of an editing target. Other data have shown that the nucleotide sequence can contribute significantly to the activity of the editing target (6, 7) and that sequence effects can even outweigh the effects of base pairing on editing. Thus, it is possible that the different activities of the site-directed mutants in the previous study could be explained by sequence and structural effects other than the relative effects of G-U wobble pairs and A-C mismatches on the stability of the base-paired editing target. In this regard, it might be worth noting that variations in the proximity of the mutations to the editing target site, position 1012, could equally well explain the effects of mutations on editing; in general, the least active mutations in the previous studies were closest to position 1012.

There is no obvious explanation for the discrepancy between the results presented in this study and those of Zheng et al. (36), who reported editing of nonreplicating HDV genomic RNA in cells and in cell extracts. In that study, the maximal extent of editing reported for nonreplicating RNAs was 2.1% in cells transfected with nonreplicating constructs, compared with about 20% editing of antigenomic RNAs produced by nonreplicating deletion constructs in this study. Perhaps in the study by Zheng et al. (36) low levels of antigenomic HDV RNA present in transfected cells were responsible for the editing observed; even assays which use primers designed to reverse transcribe only genomic-sense RNA might be capable of producing enough cDNA from antigenomic RNA to yield a PCR product derived from the antigenomic RNA. The failure to detect editing in cells transfected with antigenomic-sense transcripts might have been due to altered folding of the RNA; because of the design of the constructs used, some regions of the RNA transcribed would have had no region with which to form base pairs in the rod structure and thus could have interfered with the formation of the rod structure in the remainder of the RNA. The constructs examined in this study were designed such that a complete rod structure could be formed (Fig. 1).

The editing of position 1012 in the antigenomic RNA of HDV is a conversion of A to G. Because we observe this modification in the absence of replication, both in transfected cells and in cell extracts, the editing process is not the result of a misincorporation that occurs during replication but must come from a direct modification of the target nucleotide. Furthermore, both in transfected cells and in cell extracts, the viral protein, HDAg, is not required for editing to occur. Indeed, preliminary results indicate that HDAg can inhibit the editing reaction (unpublished data). The strong conservation of the specificity of the editing target from human cells to *Drosophila* extracts suggests a high degree of conservation of the editing system among higher eukaryotes. Perhaps the most likely

mechanism for editing is deamination of the target adenosine by the cellular enzyme which converts adenosines to inosines in double-stranded RNA (dsRNA) (1, 31), as was previously suggested (21). In the HDV replication cycle, the adenosine in position 1012 of the antigenomic RNA would be deaminated to inosine; subsequent transcription of the antigenomic RNA would yield cytidine at position 1012 in the genomic RNA, which would then be a template for the production of full-length antigenomic RNA with 1012G, as well as the antigenomic-sense HDAg mRNA with 1012G (Fig. 5). The antigenomic-sense HDAg mRNA itself is not a target for editing because it does not contain the sequences around position 580 which form the essential base-paired structure. In nonreplicating constructs, inosine in the RNA would be transcribed as G by the reverse transcriptase used in the RT-PCR assay (25), resulting in the appearance of the *Sty*I (or *Dsa*I) restriction site in the amplified cDNA (Fig. 2 and 3). Thus far, because of the very low level of modification observed in cell extracts, it has not been possible to directly detect whether adenosine in HDV RNA is converted to inosine (5a).

Several observations are consistent with the hypothesis that dsRNA deaminase could be responsible for HDV RNA editing: (i) HDV RNA, and in particular the editing target site, is partially double stranded; (ii) the specific editing of HDV RNA in a variety of cell types and even in *Drosophila* nuclear extracts is consistent with the ubiquitous nature of dsRNA deaminase activity; (iii) HDV RNA is localized in the nucleus of cells, as is dsRNA deaminase; and (iv) mutational analysis of the 5′ neighbor of the HDV editing site (UAG is a much better target than UGA) agrees with the observed 5′ neighbor preferences for maximal dsRNA deaminase activity (25). Because the target specificity of the deaminase appears limited to 5′ neighbor preferences in dsRNA, it might be expected that the partially double-stranded HDV RNA would be extensively modified by this enzyme; i.e., the deaminase might not possess sufficient specificity to be responsible for HDV RNA editing. However, the inability to detect conversion of adenosine to inosine in HDV RNA by cell extracts, under conditions such that 10% of adenosines in dsRNA are modified and about 5% of HDV RNA is edited at position 1012 (5a), suggests that the required specificity exists, in the form of either cellular factors or additional sequence and structural elements of HDV RNA that affect the target specificity.

The dsRNA adenosine deaminase has also been suggested as a likely candidate for the editing of mRNAs encoding subunits of glutamate-gated cation channels in mammalian brain (12, 20). Specific modifications of adenosine in these mRNAs at two positions, the Q/R and R/G sites, control the calcium permeability and kinetic properties of the channels, respectively, by changing codons in the protein coding region. The proposed target structures for editing of both sites contain a short base-paired region formed by the interaction between exonic and intronic sequences (12, 20). Comparison of the proposed editing target structure for the R/G site of GluR-B mRNA with that for HDV RNA shows that they are similar (Fig. 6); in both, the target adenosine is apposed to a cytidine in the midst of a larger base-paired structure. Moreover, analysis of site-directed mutations for both has shown that changing the apposed cytidine to uridine reduces editing to about one-third of wild-type levels (6, 20). Additionally, a site-directed mutation which changes the U-G wobble pair to the left of the R/G site to a U-A pair increases editing about threefold, whereas in HDV RNA, target mutation of the A-U pair at the same position to a G-U wobble pair (mutation 1014C) decreases editing about fivefold. Despite the similarities, HDV RNA and GluR-B mRNA are likely edited at very different

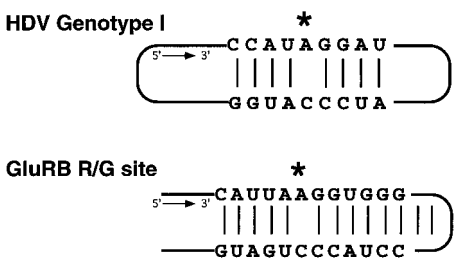

FIG. 6. Comparison of the editing targets in the HDV antigenomic RNA and the glutamate-gated channel mRNA R/G site (20). The adenosines targeted for modification are indicated by asterisks.

rates: only about 20% of nonreplicating HDV RNA is edited in the course of 6 days (less for a replicating RNA), while GluR-B mRNA can be nearly 100% edited at both the Q/R and R/G sites; presumably GluR-B editing occurs within minutes or hours, depending on the half-life of the unspliced mRNA (which is the substrate for editing). If indeed HDV and GluR-B RNAs are modified by dsRNA deaminase, the different activities could be explained by the effects of additional sequence and structural elements in the target RNA and by interactions with cellular and viral factors.

Examination of the components of the editing systems and the specificity requirements for their targets remains an important goal in our attempt to understand this important regulatory mechanism. The recent purification (13, 14, 23) and cloning (15) of the cDNA for dsRNA deaminase will likely permit a rapid evaluation of the role of dsRNA deaminase in HDV RNA editing. The ability to assess HDV RNA editing in the absence of replication will be important for studying both the components of the editing system and the mechanisms by which the process is regulated during the viral replication cycle.

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