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Hepatitis Delta Virus Mutant: Effect on RNA Editing

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During the replication cycle of hepatitis delta virus (HDV), RNA editing occurs at position 1012 on the 1679-nucleotide RNA genome. This changes an A to G in the amber termination codon, UAG, of the small form of the delta antigen (δAg). The resultant UGG codon, tryptophan, allows the translation of a larger form of the δAg with a 19-amino-acid C-terminal extension. Using HDV cDNA-transfected cells, we examined the editing potential of HDV RNA mutated from G to A at 1011 on the antigenome, adjacent to normal editing site at 1012. Four procedures were used to study not only the editing of the A at 1012, but also that of the new A at 1011: (i) nucleotide sequencing, (ii) a PCR-based RNA-editing assay, (iii) immunoblot assays, and (iv) immunofluorescence. Five findings are reported. (i) Even after the mutation at 1011, editing still occurred at 1012. (ii) Site 1011 itself now acted as a novel RNA-editing site. (iii) Sites 1011 and 1012 were edited independently. (iv) At later times, both sites became edited, thereby allowing the synthesis of the large form of the δAg (δAg-L). (v) Via immunofluorescence, such double editing became apparent as a stochastic event, in that groups of cells arose in which the changes had taken place. Evaluation of these findings and of those from previous studies of the stability of the HDV genomic sequence (H. J. Netter et al., J. Virol. 69:1687–1692, 1995) supports both the recent reevaluation of HDV RNA editing as occurring on antigenomic RNA (Casey and Gerin, personal communication) and the interpretation that editing occurs via the RNA-modifying enzyme known as DRADA.

RNA editing of hepatitis delta virus (HDV) RNA is essential during viral infection to create an open reading frame for one of the two proteins that are essential for virus replication (4, 20, 28). In particular, editing allows the translation of a second form of the delta antigen (δAg), the only protein encoded by HDV (27). This larger form, δAg-L, has an additional 19 amino acids (aa) at the C terminus (Fig. 1B) relative to the small form, δAg-S (Fig. 1A). While the original δAg-S is essential for genome replication (15), δAg-L is also biologically relevant, in that it is needed for virus assembly (7, 24). δAg-L is isoprenylated at a unique cysteine 4 aa in from the novel C-terminus region (Fig. 1B) (11). If δAg-L is mutated so that isoprenylation cannot occur, δAg-L will not function in the assembly of virion particles (19). It has been demonstrated that δAg-L is also a dominant negative inhibitor of genome replication as supported by δAg-S (8), but it is not established whether such inhibition is an essential part of the virus life cycle.

An RNA-editing site is unquestionably located at position 1012, but there may be other editing positions as well (22). The immediate target for the editing at position 1012 has been reported to be the genomic strand (3, 28), although more recent studies argue, to the contrary, that the target is the antienome (2). The mechanism of HDV RNA editing remains unclear. As will be explained, it could be carried out by the recently cloned RNA-modifying enzyme known as DRADA (14), which seems to be involved in the editing of the genomes of other RNA viruses (5) and also of certain cellular RNAs (6).

The following studies actually began out of an attempt to design a mutated HDV that could not make δAg-L even after the normal RNA-editing event at 1012. With this in mind, the G at 1011 on the antigenome was changed to A (Fig. 1C). Thus, editing at 1012 would not only replace the ochre termination codon, UAA, with an opal terminator, UGA. As documented here, we found that 1012 was still edited, and to our surprise, 1011 now became a new and independent editing site. Some HDV RNAs were even edited at both 1011 and 1012, thereby replacing the ochre terminator with tryptophan, UGG, and allowing synthesis of δAg-L (Fig. 1D).

For most of the studies reported here, we actually used not the single mutant (Fig. 1C) but a double mutant (Fig. 1E) with an additional mutation, a nearby deletion at position 979. This deletion was used as a nucleotide sequencing marker. For this double mutant, even if the δAg-L reading frame were opened by editing at 1011 and 1012, the 8 aa at the normal C terminus would be replaced by 9 aa in another reading frame, to produce a modified form of δAg-L, δAg-L′ (Fig. 1F). As reported here, we did in fact observe sequence changes consistent with editing at both 1011 and 1012. Therefore, to exclude any possible effect of this mutated C-terminal extension on editing, we repeated certain experiments, as indicated, with the single mutant, and the results were not significantly different.

MATERIALS AND METHODS

Plasmids. Plasmid pSVL(D3) was constructed by insertion of a trimer of unit-length cloned HDV cDNA (wild type) (16) into expression vector pSVL (Pharmacia), as described previously (15). A single mutation at nucleotide (nt) 1011 (Fig. 1C) and a double mutation at nt 1011 and 979 (Fig. 1E), were introduced via synthetic oligonucleotides. The single mutant was inserted as a partial dimer into the pSVL vector pDL538 (18). For the double mutant, a dimer of the modified monomer, permuted at the unique *Eco*RI site (16), was inserted into the pSVL vector.

Antibodies. Antibody specific for both δAg-S and δAg-L was raised in rabbits by inoculation of δAg prepared in *Escherichia coli*. Rabbit antibody rab-LP3 was derived against the peptide DILFPADPPFSPQSC, representing the carboxy terminus of δAg-L (residues 197 to 211) and was shown to recognize δAg-L but not δAg-S (26). Mouse monoclonal antibody 3G3, recognizing both forms of δAg, was a generous gift from Michael Lai (13).

Transfection procedure. Huh7 cells (21) were seeded in plastic 35-mm-diameter tissue culture wells (Costar) with or without an inserted 12-mm-diameter glass coverslip. Lipofectamine (Bethesda Research Laboratories) was used to transfect subconfluent cells in the presence of Opti-MEM (Bethesda Research Laboratories), as recommended by the manufacturer (12). Briefly, 12 μl of Lipofectamine at 2 mg/ml and 2 μg of plasmid DNA were each diluted into 0.5 ml of Opti-MEM. These two dilutions were combined, incubated for 30 min at

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A.  Wild type = δAg-S
 1012  1011                          979
CCAUAGGAUAUACUCUUCCCAGCCGAUCCGCCCUUUUCUCCCCAGAGUUGUCGACCCCAGUGAAUAAAGCGG
ProEnd

B.  Wild type, after 1012(A-G) = δAg-L
CCAUGGGAUAUACUCUUCCCAGCCGAUCCGCCCUUUUCUCCCCAGAGUUGUCGACCCCAGUGAAUAAAGCGG
ProTrpAspIleLeuPheProAlaAspProProPheSerProGlnSerCysArgProGlnEnd

C.  Single-mutant [1011(G-A] = δAg-S
CCAUAAGAUAUACUCUUCCCAGCCGAUCCGCCCUUUUCUCCCCAGAGUUGUCGACCCCAGUGAAUAAAGCGG
ProEnd

D.  Single-mutant, after 1011(A-G) and 1012(A-G) = δAg-L
CCAUGGGAUAUACUCUUCCCAGCCGAUCCGCCCUUUUCUCCCCAGAGUUGUCGACCCCAGUGAAUAAAGCGG
ProTrpAspIleLeuPheProAlaAspProProPheSerProGlnSerCysArgProGlnEnd

E.  Double-mutant [1011(G-A) and 979 deletion] = δAg-S
CCAUAAGAUAUACUCUUCCCAGCCGAUCCGCCCUUUU-UCCCCAGAGUUGUCGACCCCAGUGAAUAAAGCGG
ProEnd

F.  Double-mutant, after 1011(A-G) and 1012(A-G) = δAg-L'
CCAUGGGAUAUACUCUUCCCAGCCGAUCCGCCCUUUU-UCCCCAGAGUUGUCGACCCCAGUGAAUAAAGCGG
ProTrpAspIleLeuPheProAlaAspProProPhe  PheProArgValValAspProSerGluEnd
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FIG. 1. Changes on antigenomic HDV RNA as a result of mutagenesis and editing. Shown are the nucleotide and derived amino acid sequences for the 3′-terminal region (nt 1016 to 945) of the open reading frames on antigenomic HDV RNAs. (A and B) Wild type before and after editing at 1012. (C and D) Single mutant at 1011 before and after editing at both 1011 and 1012. (E and F) Double mutant, changed at 1011 and deleted at 979, both before and after editing at 1011 and 1012. In panel F, the deletion at nt 979 creates a frameshift and hence an altered amino acid sequence relative to panels B and D.

room temperature, and applied to cells that had been washed once with Opti-MEM. After 5 h of incubation at 37°C, the transfection mixture was removed and the cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

RNA extraction and Northern (RNA) analysis. As previously described (9), total RNA was extracted from transfected cells and, after glyoxalation, was submitted to electrophoresis in 1.5% agarose gels, followed by RNA blotting to detect genomic HDV RNA sequences. Genomic full-length HDV RNA probe was synthesized in vitro in the presence of [α-^{32}P]UTP and used as previously described (9). Quantitation and image processing were achieved with a Fuji imager (Fujix BAS1000).

Reverse transcription. For reverse transcription, we used a primer that bound to genomic RNA (nt 1301 to 1267, according to the nucleotide sequence of Kuo et al. (16). The RNA template (0.3 μg) was copied, by using avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.), for 1.5 h at 42°C. The product was incubated with RNase A (160 μg/ml, 20 min, 37°C) and then treated with pronase and sodium dodecyl sulfate, followed by extraction with phenol and then twice with ether, followed by precipitation with ethanol.

PCR and cDNA sequencing. The product of reverse transcription was subjected to PCR amplification with *Taq* polymerase and a Techne dry-block thermal cycler (20). The primers spanned nt 1217 to 1183 and 701 to 735 (16), and the reaction was carried out with 37 cycles of amplification. The PCR products were then gel purified and cloned with a pGEM-T vector system (Promega). The positive colonies were selected by a blue-white screening system and Perfectprep plasmid DNA kit (5 Prime 3 Prime) was used for preparation of plasmid DNA. The DNA sequence was determined by automatic sequencing (Applied Biosystems).

PCR and editing assay. As described elsewhere (10), the product of reverse transcription was subjected to nested PCR amplification with Vent polymerase (New England Biolabs) and an Idaho Technology thermal cycler. One of the final pair of primers was labeled at the 5′ end with biotin so that the product could be bound to superparamagnetic beads, denatured, and reconverted to double-stranded DNA with reverse transcriptase and a primer 5′ labeled with ^{32}P. The product released from the beads by digestion with *Sal*I was tested for susceptibility to further digestion with *Nco*I and *Nla*III. As shown in Table 2, susceptibility to these enzymes was specific for editing at positions 1011 and/or 1012 on the HDV genome. The digestions were assayed by electrophoresis of the products into an acrylamide gel followed by quantitation of radioactivity with a Fuji imager.

Immunoblot analysis. Transfected cells were lysed, and protein samples were subjected to electrophoresis in 15% polyacrylamide gels by the method of Laemmli (17). After electrotransfer of proteins to a nitrocellulose filter, δAgs were detected by using rabbit anti-δ antibodies and ^{125}I-labeled protein A (DuPont). Quantitation and image processing were achieved with a Fuji imager.

Immunofluorescence. Monolayer cultures on glass coverslips were fixed and stained, as previously described (1). Triple staining of cells was achieved by using a mixture of mouse monoclonal anti-δ antibody 3G3 and rabbit anti-peptide antibody rab-LP3, specific for δAg-L, as primary antibodies and by addition of 2 μg of 4′,6-diamidino-2-phenylindole (DAPI [Sigma]) per ml to stain the cellular DNA. Goat anti-rabbit immunoglobulin G polyclonal antibody conjugated with rhodamine (Boehringer) and goat anti-mouse immunoglobulin G plus immunoglobulin M conjugated with fluorescein (Sigma) were used as secondary antibodies. After being mounted, the samples were viewed with a Zeiss Axiophot microscope with a ×40 objective and specific filter blocks.

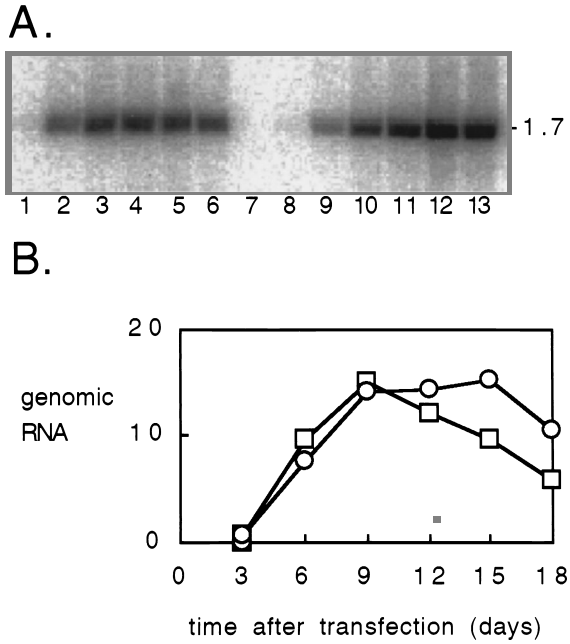

FIG. 2. Northern analysis of genomic HDV RNA in transfected cells. Cells were transfected with wild-type HDV cDNA (lanes 1 to 6), mock transfected (lane 7), or transfected with mutated HDV cDNA (lanes 8 to 13). Samples were taken at days 3 (lanes 1 and 8), 6 (lanes 2 and 9), 9 (lanes 3 and 10), 12 (lanes 4 and 11), 15 (lanes 5 and 12), and 18 (lanes 6 and 13) after transfection and were subjected to Northern analysis. (A) Radioactivity as detected with a Fuji imager. The mobility of the 1.7-kb genomic RNA is indicated at the right side. (B) Summary of the quantitation of the HDV RNA in transfected cells, expressed in arbitrary units per constant number of cells. Superimposed are the data for the wild-type (squares) and mutated (circles) HDV genomes.

RESULTS

Replication of the mutant HDV genome. As presented in the introduction, the following studies involved examining the consequences for RNA editing at position 1012 on HDV of altering the adjacent nucleotide at 1011. We compared the wild-type genome (Fig. 1A) with what we call the single mutant, which was modified at 1011 (Fig. 1C), or the double mutant, which was both modified at 1011 and deleted at 979 (Fig. 1E).

At the outset, it was necessary to determine the effect of the mutations on the ability of the genome to replicate. Figure 2A shows a detailed comparison of replication for the double mutant relative to the wild type. Cultures of Huh7 cells were transfected with either wild-type or double mutant HDV cDNAs, and at regular intervals thereafter, RNA was extracted and examined by Northern analysis for HDV genomic RNA sequences (Fig. 2A). Quantitation of these data (Fig. 2B) indicated that replication of the wild type reached a maximum at 9 days after transfection and then progressively declined. The extent of replication of the double mutant was as good as or better than that of the wild type. Similar results were obtained with the single mutant (data not shown).

Nucleotide sequence analysis. We next used reverse transcription-PCR, cloning, and sequencing to determine the actual sequence changes that occurred during replication of both the wild type and the double mutant. We used HDV RNAs extracted from cells at various times after transfection. In particular, we studied region 1016 to 945 of the antigenome, as indicated in Fig. 1.

A total of 92 clones were sequenced, with results as summarized in Table 1. Table 1 shows that for the wild type, the only changes detected were at position 1012. Consistent with nor-

TABLE 1. Sequence changes during HDV replication, as detected by nucleotide sequencing[a]

Sequence of antigenome at nt 1013–1011[b]	% of molecules with this sequence at given no. of days posttransfection[c]		
	9	18	35
Wild type			
UAG = amber terminator = parent	90 (9/10)	50 (5/10)	64 (7/11)
UGG = tryptophan = 1012(A→G)	10 (1/10)	50 (5/10)	36 (4/11)
Double mutant			
UAA = ochre terminator = mutated parent	64 (7/11)	75 (15/20)	3 (1/30)
UAG = amber terminator = 1011(A→G) = wild type	18 (2/11)	10 (2/20)	30 (9/30)
UGA = opal terminator = 1012(A→G)	18 (2/11)	15 (3/20)	37 (11/30)
UGG = tryptophan = 1011(A→G) + 1012(A→G)	<9 (0/11)	<5 (0/20)	30 (9/30)

[a] Cells were transfected as described in the legend to Fig. 2 with either the wild-type or double-mutant genome. At the indicated times after transfection, RNA-PCR, cloning, and sequencing of individual clones were performed.

[b] The table shows only sequences for antigenomic sequence 1013 to 1011, the position of the normal termination codon for δAg. Changes in other regions, with the exception of nt 979 on the double mutant, were not detected.

[c] Shown is the percentage of RNA molecules with the indicated sequence. In parentheses is the number of clones of this sequence/total number of clones examined.

mal HDV editing, these changes were of A→G on the antigenome, and the frequency of changes increased with time after transfection. The wild-type sequences progressively thus changed from the amber termination codon to the tryptophan codon, thereby allowing the synthesis of δAg-L.

However, for the double mutant, as summarized in Table 1, we detected not only changes at 1012 but also changes at 1011. These latter changes corresponded to A→G on the antigenome. Therefore, such changes occurred as if we had created a second site of editing adjacent to the normal one. It is important to note that at the early times, by this sequencing assay, we only detected changes at either location but not at both; that is, the two sites were independently susceptible to the nucleotide change. The initial sequence was the ochre termination codon, UAA. With time, some sequences changed A to G at 1012 or 1011, to create the opal or amber terminator, respectively. Later, by 35 days, we even found that 30% of the sequences were changed at both locations, so as to create the tryptophan codon, thereby allowing the synthesis of the modified form of δAg-L, as predicted in Fig. 1F.

RNA-editing assay. A limitation of the nucleotide sequencing method described above was sensitivity; for example, to detect nucleotide changes less frequent than 10%, it would be necessary to sequence much more than 10 clones. To solve this problem, we made use of a more sensitive editing assay (10). PCR amplification of RNAs edited at 1011 and/or 1012 can be detected via the creation of sites for the restriction enzymes *Nco*I and *Nla*III. As summarized in Table 2, we readily detected editing at 1012 on the wild-type genome. For the double mutant, it was now possible to detect single and double editing as early as days 9 and 18. While these assays were more sensitive than those obtained by the sequencing, for the double mutant they were unable to distinguish between unedited UAA and singly edited UAG. Overall, the results of the two methods were either complementary or in agreement. The biggest discrepancy between the two methods was at day 9 for the double mutant being converted to UGA; sequencing of 11 clones indicated 18% (Table 1), while the value deduced by the subtraction of two sets of restriction enzyme data was 1% (Table 2). Our interpretation is that this apparent discrepancy is consistent with the experimental errors of the two methods.

Immunoblot analysis of δAg. We next used immunoblot analysis in order to detect and quantitate the forms of δAg synthesized in the transfected cells. For transfections with the wild type (Fig. 3, lanes 1 to 6) we detected both δAg-S and δAg-L. As expected, the amount of δAg-L increased with time and became equal to that of δAg-S at day 18.

For the transfections with the double mutant (lanes 8 to 13), we also detected δAg-S, but in addition, there was a species with the mobility of δAg-L. On the basis of the initial sequence (Fig. 1E), the nucleotide sequencing data (Table 1), and the RNA-editing assays (Table 2), we made the preliminary interpretation that this band was δAg-L′, just as predicted in Fig. 1F. Consistent with this interpretation, this band and δAg-L, but not δAg-S, were detected with antibody rab-LP3, a polyclonal antibody specific for a peptide sequence unique to the C-terminal extension of δAg-L but also partially present in the predicted extension of the putative protein δAg-L′ (data not shown).

The kinetics of appearance of these δAg species for the transfections with the wild type and double mutant are shown in Fig. 3B and C, respectively. The levels of δAg-S showed an initial increase for 9 days, followed by a decline. Such results were very similar to those observed for the levels of RNA replication (Fig. 2B). The differences arise in terms of δAg-L

TABLE 2. Sequence changes during the HDV replication, as detected by editing assays[a]

Sequence of antigenome at 1013–1011	% of molecules with this sequence at given no. of days posttransfection[b]		
	9	18	35
Wild type			
UAG = amber terminator = parent	79	42	58
UGG = tryptophan = 1012(A→G)	21	58	42
Double mutant			
UAA = ochre terminator = mutated parent and UAG = amber terminator = 1011(A→G) = wild-type	96	71	30
UGA = opal terminator = 1012(A→G)	1	14	47
UGG = tryptophan = 1011(A→G) + 1012(A→G)	3	5	23

[a] Cells were transfected as for Fig. 2 and Table 1, with either the wild-type or double-mutant genome.

[b] At the indicated times posttransfection, RNA-PCR, followed by two editing assays, was used to determine the fraction of RNA molecules with a given sequence. The first assay was to determine the percentage of molecules with a newly created *Nco*I site (CCATGG), consistent with UGG at 1011 to 1013. For the double mutant, the second assay was for an *Nla*III site (NCATGN), consistent with both UGA and UGG. Thus, subtraction of the result of the first assay (%UGG) from that of the second (%UGA + UGG) yields %UGA. Also, subtraction of the result of the second assay from 100% yields %UAA + UAG.

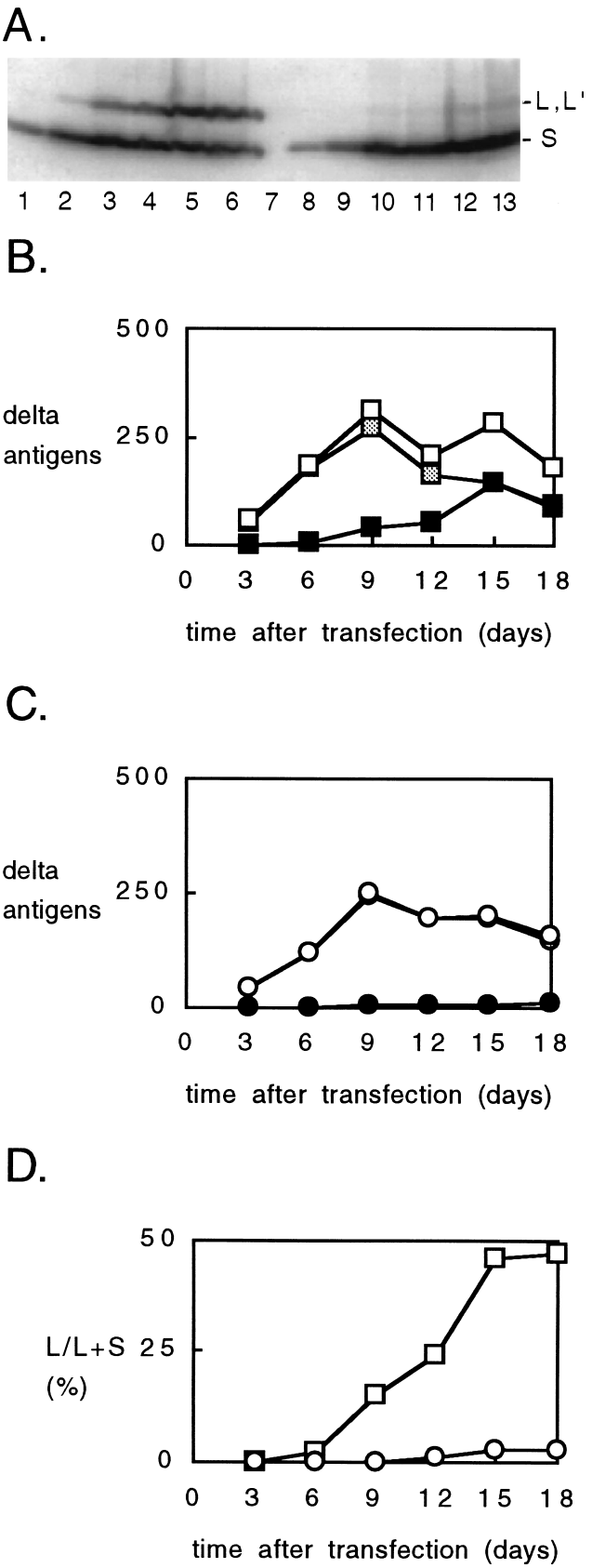

FIG. 3. Immunoblot analysis to detect δAgs in transfected cells. Cells were transfected exactly as described for Fig. 2. Samples were subjected to immunoblot analysis, and are indicated as in Fig. 2. (A) Radioactivity as detected with a Fuji imager. The mobility of the δAg-S (S) and δAg-L (L, L′) forms of the δAg are indicated on the right. (B and C) Summary of quantitation, in arbitrary units per constant number of cells, of total δAg (open symbols), δAg-S (shaded symbols), and δAg-L and δAg-L′ forms (solid symbols). (B and C) Transfections with wild-type (squares) and mutated (circles) HDV genomes, respectively. (D) Fraction (percentage) of the large forms (δAg-L and δAg-L′) relative to the total δAg. Data are superimposed for transfections with wild-type (squares) and mutated (circles) HDV genomes.

and δAg-L′. These differences can be seen in absolute terms (Fig. 3B and C) but more clearly in terms of the percentage of total δAg that is either δAg-L or δAg-L′ (Fig. 3D). For the wild type, this percentage increased, and by day 15, it reached a plateau of about 50%. In contrast, for the double mutant, the percentage progressively increased, but even by day 18 had not even reached 3%. A comparable value was obtained for the single mutant (data not shown).

Immunofluorescent detection of δAg. The appearance of δAg-L and δAg-L′ was further studied by immunofluorescence of transfected cells. Figure 4 shows the data at 9 days, which corresponds to the peak of genome replication and δAg-S expression. It is important to note that at this time, the wild-type and double-mutant genomes have replicated to approximately the same extent (Fig. 2B). Coverslips were triple stained: with DAPI to stain nuclear DNA, with an antibody against δAg to detect total δAg, and with an antipeptide antibody (rab-LP3) to detect only large forms of δAg (either δAg-L or δAg-L′).

For transfection with wild-type cDNA (Fig. 4A to C), about 40% of the DAPI-staining cells (Fig. 4A) were positive for both total δAg (Fig. 4B) and δAg-L (Fig. 4C). Positive cells were detected as small colonies. In these cells, the distribution of total antigen was nuclear. More specifically, there was diffuse nucleoplasmic staining, along with a variable number of discrete dots. δAg-L and δAg-L′ showed staining of the same dots, but the amount of diffuse nucleoplasmic staining was relatively smaller.

Cells transfected with the double-mutant cDNA gave a similar staining pattern, with one important exception. Only a fraction, about 30%, of the cells in a clone that were positive for total antigen (Fig. 4E) were also positive for the large form (Fig. 4F). This observation was made under conditions in which the sensitivity of the assay for the large form was increased relative to that for the total—that is, when the positive signals in panel F were stronger than those in panel E. It appears, therefore, that only in a subpopulation of mutant-transfected cells do the mutations occur that are needed to produce δAg-L′. From an examination of other colonies of positive cells, the same heterogeneity was found to be the predominant situation. Less abundant were two other types of HDV-positive colonies. The first, was one in which each cell was positive for both total antigen and for the large form. The second, was one in which none of the cells that were positive for total antigen were positive for the large form. Since nuclear dots were detected in these cells by using antibody to total antigen, it means that the large form was not necessary for accumulation of antigen into such dots.

The data presented above were collected on day 9. At earlier times, the large forms were more difficult to detect, especially for the single and double mutants. With the wild type, the large form was detectable by day 3, while with the mutants, detection was first possible at day 6. Even though immunofluorescence lacks quantitation, the method can have a greater sensitivity relative to an immunoblot; the large δAgs were detected 3 days earlier with immunofluorescence than with an immunoblot. At day 9, there was no detectable difference between the double mutant and the single mutant (data not shown).

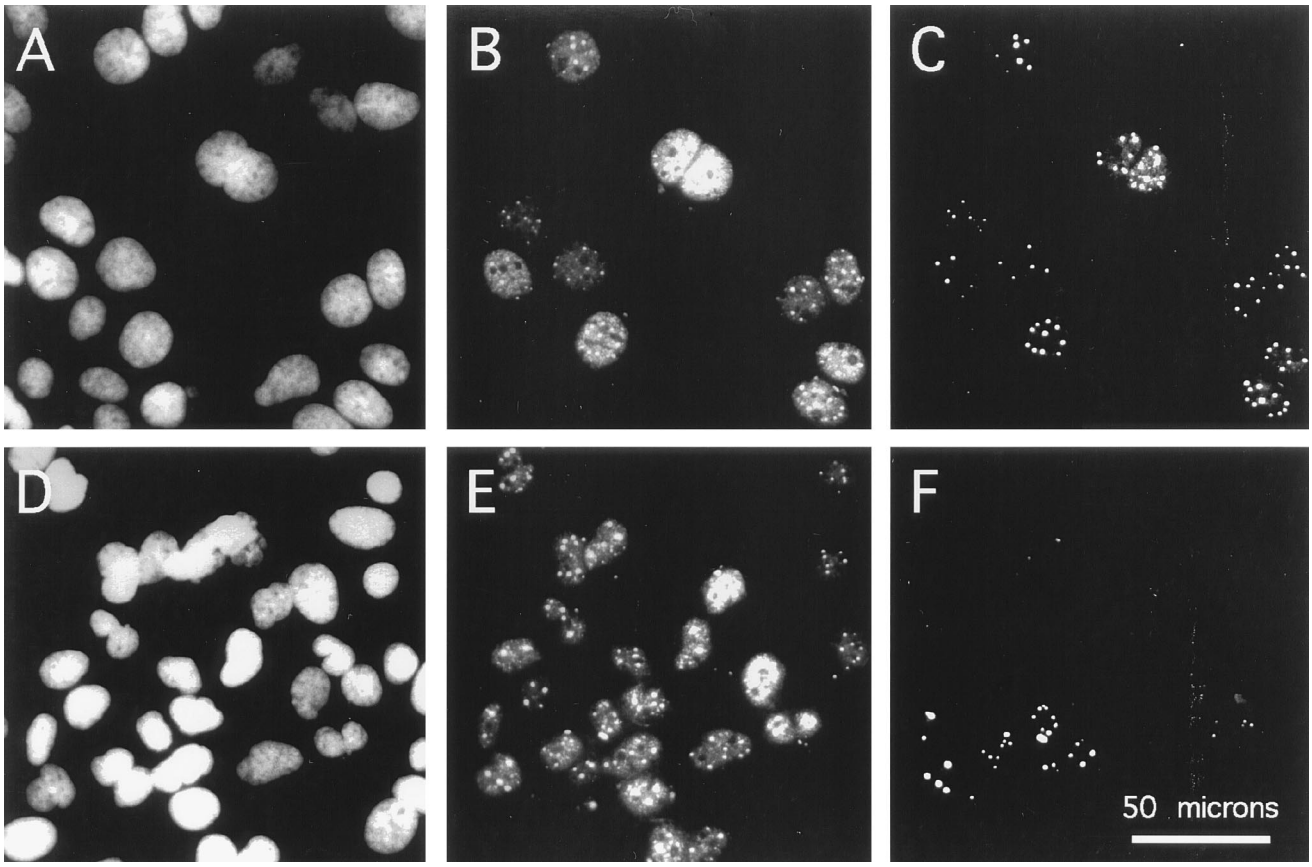

FIG. 4. Immunofluorescent detection of δAgs in transfected cells. At 9 days after transfection with wild-type (A to C) or mutated (D to F) HDV cDNA, coverslip cultures were assayed for DAPI staining (A and D), total δ proteins (B and E), and δAg-L and δAg-L′ (panels C and F). The scale is indicated in panel F. Panels A to C are assays of one field of cells; panels D to F are of another. Images were digitized with a Umax scanner and processed with Adobe Photoshop software.

DISCUSSION

The present study was begun as an attempt to interfere with the consequences of HDV RNA editing at position 1012. We made a base change of G-A on the antigenome at the adjacent nucleotide, 1011. The expectation was that editing at 1012, even if it occurred on such a mutated genome, would still not allow the synthesis of the large form of the δAg (Fig. 1C). We found that the mutated genome not only replicated as well as the wild type (Fig. 2), but it revealed the following important new information about HDV editing. (i) Editing of A→G still occurred at 1012 (Tables 1 and 2). (ii) The novel A at 1011 was also changed to G, as if it became a new target for editing. (iii) Editing occurred independently at both 1011 and 1012. (iv) At later times after transfection, molecules edited at both sites were detected. Consistent with this double editing, we detected by immunoblotting the appearance of species of δAg elongated at the C terminus (Fig. 3), just as predicted from the mutant sequence (Fig. 1D and F). (v) Finally, by immunofluorescence for this C-terminal extension, we detected novel species in transfected cells (Fig. 4). It was as if cells transfected with the initial sequence were going through cell divisions and progressively accumulating replication-competent HDV genomes that were doubly edited.

Our study has to be compared with a previous report by Casey et al. who studied the effects of a number of single-base mutations on the ability of HDV RNA to undergo editing at position 1012 (3). One such mutant was identical to the single mutant studied here (Fig. 1C). At 13 days after cDNA transfection, they examined the δAg species by immunoblotting and used an RNA-editing assay to detect changes at 1012. They did not detect the large δAg-L; this is not surprising in that we found the amount of δAg-L to be <2% relative to δAg-S (Fig. 3B). Also, they used *Nla*III to detect editing at 1012 and concluded the level was <30% of that achieved for the wild type. Our data agree with this in that we detected 4% and 19% editing at days 9 and 18, respectively (Table 2). However, our results differ in two important ways. First, when we assayed editing at even longer times, 35 days, the editing was as much as 70%. Second, we found that the mutated 1011 created a new site for editing.

For some time it has been known that during replication of HDV RNA, the change, referred to as RNA editing, takes place at position 1012. Casey et al. from an analysis of many replication-competent genomes, subjected to prior single-base mutations at and around the editing site, concluded that the substrate for editing was the genomic RNA (3). Zheng et al. subsequently came to the same conclusion by a different approach; they studied editing on nonreplicating HDV RNAs as expressed in cells and also as incubated in various nuclear extracts (28). It has not been possible to reproduce the results of Zheng et al. (data not shown), and furthermore, Casey and Gerin have more recently obtained better evidence to support the interpretation that editing occurs on the antigenomic RNA (2). That is, the change is equivalent to an A-G. Their new data have reopened the possibility (20) that HDV is edited post-transcriptionally by a recently cloned RNA-modifying activity, currently named DRADA, for double-stranded RNA-activated adenosine deaminase (14). DRADA is a nuclear activity

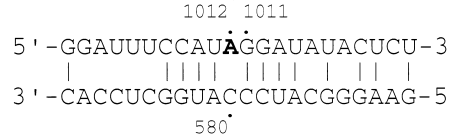

FIG. 5. Predicted rodlike folding of HDV antigenomic sequences at and around the editing site at 1012. The sequence, predicted base pairing, and numbering are as described by Kuo et al. (16). Note that the edited A at 1012, shown in boldface, is predicted to be adjacent to nt 580 on the other side of the rodlike structure. 1012 is thus not predicted to be base paired. Also, if 1011 is mutated to A, it is not predicted to be paired.

present in all animal cells (25). It acts on regions of complete or partial double-stranded RNA (6) and can convert adenosine to inosine (5). It is known to act on certain cellular RNAs and even viral RNAs (5, 6). In the case of a replicating RNA like HDV, this could explain the observed replacement of A-G. As represented in Fig. 5, the antigenome of wild-type HDV at and around the adenosine at 1012 can be predicted to fold into a region with some base pairing. Recently, Polson and Bass have reported in vitro studies of the RNA substrate requirements for optimal DRADA activity (23). They have examined not only the extent of base pairing but also the importance of nucleotides adjacent to the edited adenosine. The single striking rule that emerged concerns the 5′ neighbor: the frequency of editing is highest when this neighbor is A or U, much lower for C, and lowest for G. If we refer to 5′-A or 5′-U as satisfying the DRADA rule, then, as now summarized, the following three changes that occur on HDV RNA satisfy this rule: (i) the 1012 site of wild-type HDV, (ii) the 1012 site of HDV mutated at 1011, (iii) the 1011 site of mutated HDV. There are even additional examples. (iv) In a recent study of the stability of HDV during six consecutive passages in woodchucks, Netter et al. (22) changes corresponding to A→G on the antigenome not only at 1012 but also at 1005 and 1007, which were changes of at least comparable frequency. The latter two sites, which can be seen in Fig. 5, also fit the rule. (In Fig. 5, the three other A's, at 1009, 1014, and 1020, do not fit the rule and were not detected as changing). (v) Finally, Netter et al. detected a total of 40 single-base changes. Of these, 32 (80%) were consistent with an A→G change on either the genome or the antigenome, and 24 satisfy the 5′-neighbor rule (22).

In summary, the studies reported here for HDV replication in transfected cells and previous studies of HDV replication in animals are consistent with DRADA being the enzyme responsible for most of the nucleotide changes on replicating HDV RNA.

Is there competition between the replication of the edited and the unedited RNAs? Such competition could occur at two levels. First, it certainly may occur when HDV RNA is assembled into particles; in a subsequent infection of susceptible cells, genomes changed at 1012 would be unable to synthesize the δAg-S needed for replication. Second, from the study of Bichko et al. (1), we now know that even in the absence of assembly, HDV replication can continue after multiple rounds of cell division. Thus, at any time, a major feature in determining whether an HDV RNA species predominates could be replication competence. Some species, either because of the location or extent of editing, could be at a disadvantage to other edited RNAs and could certainly be at a disadvantage relative to the wild type.

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