Effects of Conserved RNA Secondary Structures on Hepatitis Delta Virus Genotype I RNA Editing, Replication, and Virus Production

Geetha C. Jayan and John L. Casey*

Department of Microbiology and Immunology, Georgetown University Medical Center, Washington, D.C.

Received 14 March 2005/Accepted 6 June 2005

RNA editing of the hepatitis delta virus (HDV) antigenome at the amber/W site by the host RNA adenosine deaminase ADAR1 is a critical step in the HDV replication cycle. Editing is required for production of the viral protein hepatitis delta antigen long form (HDAG-L), which is necessary for viral particle production but can inhibit HDV RNA replication. The RNA secondary structural features in ADAR1 substrates are not completely defined, but base pairing in the 20-nucleotide (nt) region 3′ of editing sites is thought to be important. The 25-nt region 3′ of the HDV amber/W site in HDV genotype I RNA consists of a conserved secondary structure that is mostly base paired but also has asymmetric internal loops and single-base bulges. To understand the effect of this 3′ region on the HDV replication cycle, mutations that either increase or decrease base pairing in this region were created and the effects of these changes on amber/W site editing, RNA replication, and virus production were studied. Increased base pairing, particularly in the region 15 to 25 nt 3′ of the editing site, significantly increased editing; disruption of base pairing in this region had little effect. Increased editing resulted in a dramatic inhibition of HDV RNA synthesis, mostly due to excess HDAG-L production. Although virus production at early times was unaffected by this reduced RNA replication, at later times it was significantly reduced. Therefore, it appears that the conserved RNA secondary structure around the HDV genotype I amber/W site has been selected not for the highest editing efficiency but for optimal viral replication and secretion.

Hepatitis delta virus (HDV) causes acute severe and chronic liver disease in humans. HDV encodes just one protein, hepatitis delta antigen (HDAG), and relies heavily on host functions and the structure of its RNA for replicative functions. One of these functions is RNA editing, which plays a central role in the HDV replication cycle. During HDV replication, the host RNA adenosine deaminase ADAR1 deaminates, or edits, the adenosine in the UAG (amber) stop codon for the short form of HDAG (HDAG-S) to inosine (11, 27, 31). As a result of editing this adenosine, referred to as the amber/W site (27), the HDAG reading frame is extended by an additional 19 to 20 amino acids to encode the long form of HDAG (HDAG-L). Editing is critical for HDV because HDAG-S is required for viral RNA replication and HDAG-L is required for packaging (6, 7). HDV must modulate the extent of editing for at least three reasons. First, both edited and unedited genomes are packaged, but only unedited genomes are likely to be infectious because HDAG-L does not support RNA replication; second, insufficient HDAG-L production will decrease virus secretion (11); and third, excessive and premature production of HDAG-L can inhibit RNA replication (10, 29), although the significance of this last point has been questioned (20).

Like other substrates for editing by ADAR1, editing at the HDV amber/W site requires specific structures in the immediate vicinity of the target adenosine (3, 11, 27, 31). However, the role of sequences and structures further removed has not been clearly defined. Analysis of editing on double-stranded RNA (dsRNA) templates in vitro has led to a model in which ADAR1 interacts with a base-paired region extending about 20 nucleotides (nt) to the 3′ (downstream) side of edited adenosines (14, 26); this interaction most likely occurs via the dsRNA binding domains of ADAR1 (17, 19). Small disruptions of base pairing in this 20-nt region could be tolerated in dsRNA substrates, but the presence of a 6-nt internal loop strongly diminished editing (14, 26).

The RNA secondary structure downstream of the HDV amber/W site in HDV genotype I contains base-paired segments as well as several internal loops and bulges. In light of the model for ADAR1 substrate activity, these latter features raise questions about the functional role of this region in editing at the amber/W site. Indeed, Sato et al. (30) were able to detect editing on substrates from which all but 5 nt 3′ of the editing site were removed. On the other hand, a recent study indicated that RNA secondary structure between 20 and 36 nt downstream of the HDV amber/W site can affect editing efficiency (29, 30), but the roles of specific base-paired and unpaired elements in this region have not been carefully examined. In this report, we examine the effects on editing at the amber/W site of predicted RNA secondary structures—both paired and unpaired—in the region from 8 to about 50 nt downstream of the amber/W site; we also analyzed the effects of the resultant editing variations on both RNA replication and virus production. We conclude that the structural features downstream of the HDV genotype I amber/W site are selected not for maximal editing activity but for maximal virus replication and particle secretion; that is, the editing site is the result of a compromise between the competing needs of viral RNA replication and virus secretion.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Georgetown University Medical Center, 3900 Reservoir Rd., NW, Washington, DC 20007. Phone: (202) 687-1052. Fax: (202) 687-1800. E-mail: caseyj@georgetown.edu.
MATERIALS AND METHODS

Plasmids. Constructs pHDV-NR, pHDV-I(+), pHDV-I(-)/Ag(+), and pCMVAg-S have been described previously (4, 28). Upon transfection of cultured HuH-7 cells, pHDV-NR expresses a nonreplicating, non-HDAg-producing genotype I HDV RNA that serves as a substrate for editing at the amber/W site; pHDV-I(+)/Ag(-) expresses replicating antigenomic HDV genotype I RNA; and pHDV-I(-)/Ag(-) expresses a replication-competent antigenomic HDV genotype I RNA that does not express HDAg. pCMVAg-S expresses HDAg-S. Mutations were created by site-directed mutagenesis (3). Sequences of cloned fragments were verified for the presence of the desired mutation and the absence of unintended mutations by sequence analysis (MWG Biotech, High Point, NC). The mutant 578G was reported previously (5, 27); in this construct, the A-U base pair 2 positions upstream of the amber/W site is changed to an A-C mismatch pair and editing is strongly reduced (3, 5). The HDAg-S expression plasmid pCDNAneo-HDAg-S was created by inserting the HindIII-XbaI fragment from pCMVAg-S (4) into the pCDNAneo expression vector (Invitrogen, Carlsbad, CA).

Cell culture and transfections. Human hepatoma cells, HuH-7, were cultured in six-well dishes using Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 1 mM glutamine. Cells were transfected using the LipofectAMINE Plus Reagent (Invitrogen), following the manufacturer's protocol. Transfections were done in duplicate. In order to normalize for efficiency of transfection, we included 0.1 μg of the construct pSEAP2Control (Clontech, Palo Alto, CA) in all transfections (8). The cell line HuH-7/Ag-S was created by transfecting HuH-7 cells with pCDNAneo-HDAg-S; cells were grown in medium containing 500 μg/ml G418 (Invitrogen) to select for HDAg-S-producing cells. For RNA analysis following transient transfections, total RNA samples were prepared from cells at indicated times posttransfection using an RNeasy Mini Kit (QIAGEN), following the manufacturer's protocol.

Analysis of amber/W editing in HDV RNA. Editing assays were performed as described previously (5, 27). Briefly, RNA samples were treated with DNase (Invitrogen) to remove traces of transfected plasmid DNA and then subjected to reverse transcription (RT)-PCR using PCR primers 5414 and 5415 (23). The effectiveness of DNase treatment was verified by the absence of PCR products after PCR amplification without prior RT. PCR products were analyzed for amber/W editing by single-cycle labeling with [32P]dCTP, followed by restriction digestion with Styl. Amber/W editing was indicated by the appearance of a Styl site in the amplification product (3, 27). Editing was quantified by electrophoresis, followed by radioanalytic imaging with either a Packard InstantImager or a Molecular Dynamics Storm PhosphorImager. Previous analysis of HDV RNA in transfected cells by direct sequencing of RT-PCR products or by sequencing numerous cloned RT-PCR products indicated that no detectable specific editing occurs at other adenosines and that the restriction digestion assay is highly accurate (28).

Northern blot analyses for HDV RNA replication. RNA was electrophoresed through 1.5% agarose gels containing 2 M formaldehyde, transferred to positively charged nylon membranes, and hybridized with an antigenomic-sense 32P-labeled probe as described previously (5). The hybridization temperature was 60°C, and the posthybridization wash temperature was 70°C. The integrity of the RNA samples and equivalency of loading were assessed by visualization of ethidium bromide-stained rRNA bands. Relative levels of HDV RNA were determined by radioanalytic scanning of blots with either a Packard InstantImager or a Molecular Dynamics Storm PhosphorImager.

Immunoblot analysis. Transfected cells were rinsed twice with cold 1× phosphate-buffered saline and harvested at the indicated times. Cell lysates were obtained by treatment with 2% sodium dodecyl sulfate (SDS)–0.2 M Tris-HCl (pH 7.5)–1 mM EDTA and analyzed for HDAg by SDS-polyacrylamide gel electrophoresis (12% acrylamide gels) and immunoblotting with human monoclonal anti-HD-AgT (Gibco BRL, Gaithersburg, MD) and a chemiluminescence assay kit (LumiGLO; KPL). Immunoblot detection was by horseradish peroxidase-conjugated goat anti-human immunoglobulin G (H+L; KPL, Gaithersburg, MD) and a chemiluminescence assay kit (LumiGLO; KPL).

Analysis of viral RNA. Viral RNA was harvested from the medium of transfected cells 6 and 12 days posttransfection as previously described (23); for samples harvested on day 12, the medium was replaced on day 6. Briefly, medium was collected from two 35-mm wells and centrifuged at 17,000 × g for 10 min in a Beckman SW41Ti rotor to remove particulate matter and debris. The supernatant was loaded onto a 3-mL cushion of 20% sucrose–0.2 M HEPES (pH 7.4)–0.01 M MgCl2–0.1% bovine serum albumin and centrifuged at 200,000 × g for 6 h. Pelleted virions were suspended in lysis buffer (0.05 M HEPES, pH 7.2, 0.05 M EDTA, 0.2 M NaCl, 2% SDS) containing 1 mg/ml proteinase K and incubated at 37°C overnight. RNA was purified by phenol-chloroform extraction and ethanol precipitation and analyzed by gel electrophoresis-blot hybridization as described for viral RNAs harvested from cells.

RESULTS

Editing at the amber/W in HDV genotype I has been shown to require a highly conserved sequence and secondary structure in the immediate vicinity of the site (3, 27). The four positions on either side of the target adenosine form Watson-Crick base pairs with positions from the noncoding side of the circular genome, and the site itself forms an A-C mismatch pair (Fig. 1). Disruption of these base pairs or alteration of the C in the mismatch pair decreases editing substantially. Further downstream of the amber/W site, the four Watson-Crick base pairs are followed by an asymmetric internal loop, 8 bp that typically include one to three G-U wobble pairs, and then another asymmetric internal loop. Beyond this second internal loop, consecutive base pairs are limited to between two and seven and are flanked by single unpaired bases or small internal loops or bulges.

The presence of the conserved internal loops and short disrupted base-paired segments downstream of the amber/W site is curious in light of the model for ADAR1 activity on dsRNAs (14, 26), which suggests that base pairing within 20 nt downstream of an adenosine is important for editing to occur. We attempted to address the roles of these conserved secondary structure features in editing at the amber/W site by either increasing or decreasing the base pairing in the ca. 50-nt region downstream of the site. Thus, mutations M1 to M5 were created to increase predicted base pairing by either removing unpaired bases or inserting additional bases that could pair with positions that form bulge loops in the predicted wild-type structure (Fig. 1). These mutations were introduced into the expression construct pHDV-NR, which upon transfection of cultured cells produces a nonreplicating HDV antigenic RNA that neither replicates nor produces HDAg but serves as a substrate for editing (28). Total RNAs were harvested 3 days posttransfection, and editing at the amber/W site was quantified by Styl restriction digestion of RT-PCR amplification products (5, 10, 27). Because editing levels for the nonreplicating RNA in transfected Huh-7 cells are near maximum—overexpression of ADAR1 or ADAR2 by cotransfection of ADAR1 and ADAR2 expression constructs with an HDV RNA expression construct increased amber/W site editing from around 40% to a level of only about 60%—we transfected the mutant constructs into CHO-K1 cells, in which editing at the amber/W site is much lower than in Huh-7 cells (ca. 4% versus 30%; Fig. 2).

Mutations M1 to M4 exhibited substantial increases in editing at the amber/W site. These mutations increased base pairing between 8 and 30 nt downstream of the amber/W site by removing internal loops and bulges as far as 27 nt downstream. The largest effect (a three- to fourfold increase in editing) was observed for the M3 mutant, which contained 18 consecutive base pairs from 8 to 25 nt downstream of the amber/W site. On the other hand, the M5 mutation, which created 16 consecutive base pairs further from the amber/W site (between 33 and 48 nt downstream), did not increase editing; rather, editing decreased about twofold for this mutant. These results are consistent with the model for ADAR1.
binding downstream of editing sites via interactions between the dsRNA binding domains of ADAR1 and dsRNA segments of the substrate (14, 26). In light of this model, these results suggest that the conserved asymmetric internal loops and single-nucleotide bulges located 15 to 25 nt downstream of the wild-type amber/W site could reduce the optimal editing activity of the site, perhaps by diminishing the ability of the dsRNA binding domains of ADAR1 to bind the RNA in this region.

We therefore wondered whether ADAR1 requires the limited dsRNA structure downstream of the editing site. Mutations M6 and M7 were created to disrupt predicted base pairing in the 8-bp segment downstream of the amber/W site by introducing 6-nt internal loops; in dsRNA substrates, such loops have been shown to prevent editing by ADAR1 within a 20-nt region (14). The combination of these two mutations restores base pairing. Mutation 578G (3) served to illustrate the magnitude of the effect of disrupting base pairing in the immediate vicinity of the amber/W site. Although the increased editing observed for mutations M1 to M4 indicated that increased base pairing can increase editing at the amber/W site, mutations M6 and M7, which disrupt the conserved 8-bp segment from 8 to 15 nt downstream of the amber/W site by introducing 6-nt internal loops, had no apparent effect on editing (Fig. 2B). Taken together, the effects of the mutants analyzed in Fig. 2 are consistent with a model in which ADAR1 does not make use of the limited base pairing downstream of the wild-type HDV genotype I amber/W site.

We previously showed that overexpression of ADAR1 increased editing by approximately 10-fold and strongly inhibited HDV RNA replication (10). Sato et al. found that a mutation that increased editing by at least fourfold resulted in dramatically reduced levels of RNA replication (29). We wondered...
how the increased base pairing in the nonreplicating mutants in Fig. 2 would affect editing in replicating RNAs and whether the relatively more modest increases in amber/W site editing would also inhibit HDV RNA replication. Mutations M2 and M3 were inserted into the HDV expression construct pHDV*1(+3) containing the indicated mutations. RNA and protein were harvested 6 days posttransfection. Analysis of amber/W site (23) indicated no detectable editing at sites other than excessive HDAg-L production, most likely due to the disruption of structural features required for replication, or the creation of structures that could trigger cellular responses to dsRNA because of the increased number of consecutive base pairs. We took two approaches to determine the extent to which the decreases in replication observed in Fig. 3 were due to decreased editing at the amber/W site. For these experiments, we chose to analyze just the M2 mutation, which had the smallest number of consecutive base pairs within the 25-nt region downstream of the amber/W site yet substantially increased editing.

It was previously shown that changing the editing site from UAG to UGA results in very poor editing and HDAg-L production (3, 32). The low editing is most likely due to the fact that the editing site in this context has G immediately 5' of the editing site (26). We therefore created a combined M2/UGA mutant HDV expression construct and compared genomic RNA synthesis and amber/W site editing for this construct, the UGA parent, the M2 mutant construct, and the wild type (Fig. 4A). As previously found, editing levels are very low (ca. 1.5%) for the UGA construct compared with the wild type (7%). Similar to the 578G mutation (Fig. 3), this decreased editing had no detectable effect on HDV RNA replication: the UGA constructs with the wild-type sequence and structure downstream of the editing site yielded levels of genomic RNA essentially identical to those observed for the wild type (Fig. 4A). Combination of the UGA editing site with the M2 mutant led to levels of genomic RNA synthesis 17-fold higher than observed for the M2 mutant alone (34% of the wild type versus 2%; Fig. 4A). This combined mutant construct also produced higher levels of editing (7%) than observed for the UGA mutation alone, again demonstrating the effect of increased base pairing downstream of the editing site; however, these increased editing levels were no higher than for the wild-type (UAG) construct. Sequence analysis of RT-PCR products derived from a 450-nt region of the RNA that includes the amber/W site (23) indicated no detectable editing at sites other than amber/W for any of these constructs. Thus, the relatively high level of genomic RNA synthesis observed for the combined M2 UGA construct is consistent with the interpretation that the majority of the approximately 50-fold reduction in genomic RNA synthesis for the M2 mutant is due to increased amber/W site editing.
Our second approach to separate the effects of sequences affecting replication through effects on editing from other mechanisms was to analyze genomic RNA synthesis in cells transfected with wild-type and M2 versions of the antigen-deficient HDV RNA expression construct pHDVI(H11001/Ag(H11002) (4), in which the reading frame for HDAg is disrupted. Cells were cotransfected with a pHDAg-S expression construct to support RNA replication (12). Under these conditions, no HDAg-L is made because the replicating RNA, which is a substrate for editing, does not produce HDAg, and the mRNA produced from the HDAg expression construct is not a substrate for amber/W site editing. Replication of HDAg-deficient M2 mutant RNA was 33% of the Ag(H11002) construct with wild-type sequence in the vicinity of the amber/W site (Fig. 4B). Thus, the relative level of replication for the HDAg-deficient M2 construct is very similar to that observed for the M2 UGA construct (Fig. 4B).

From the results presented in Fig. 3 and 4, we conclude that the M2 mutation, which increases base pairing 3’ of the amber/W site, affects genomic RNA synthesis primarily by increasing editing at the amber/W site. Increased editing leads to increased synthesis of HDAg-L, which inhibits genomic RNA synthesis. That we did not observe full recovery of replication levels in the absence of HDAg-L synthesis (Fig. 4B) or under conditions where editing was restricted (Fig. 4A) suggests that the increased base pairing does affect replication independent of its effect on editing. Whether this effect is due to the disruption of sequences and structures required for replication or to the creation of excessive dsRNA character that induces cellular antiviral responses remains to be determined.

The dramatic negative effects on genomic RNA replication by the mutations that increase editing could explain the conservation of the predicted RNA secondary structure downstream of the amber/W site. However, since levels of virus production may be just as important or more important for virus viability than levels of intracellular replication, and since HDAg-L produced as a result of editing is required for virus production, we analyzed the effects of these mutations on virus production 6 days and 12 days after cotransfection with an HBV expression construct. Remarkably, 6 days posttransfection the level of HDV virion RNA in the medium was considerably higher for the highly edited mutant constructs than might have been expected from the levels of intracellular RNA alone (Fig. 5A and B). Levels of virus production were closely correlated with levels of HDAg-L synthesis (compare Fig. 5B and 3B), consistent with the conclusion that HDAg-L is a

FIG. 4. HDV genomic RNA synthesis is decreased in the M4 mutant primarily because of increased editing at the amber/W site. Blot hybridization analysis of HDV genomic RNA harvested 6 days following transfection of Huh-7 cells with different wild-type (wt) and M4 mutant antigenomic RNA expression constructs. (A) Cells were transfected with pHDV•I(+) containing either wild-type or M2 mutant sequences 3’ of the amber/W site (Fig. 1) along with the wild-type UAG HDAg-S stop codon or the poorly edited mutant UGA stop codon, as indicated. Values beneath the lanes are average levels of expression from two experiments, relative to the wild-type UAG construct. (B) Cells were cotransfected with pHDAg-S and pHDV•I(+)Ag(–) containing wild-type, 578G, or M2 sequences, as indicated (filled bars). For comparison, cells were also transfected with pHDV•I(+) containing either wild-type or indicated mutant sequences (open bars). Within a group, values are normalized to wild-type expression levels. All values represent the average of two experiments.

FIG. 5. Effects of editing site mutations on HDV virion secretion. Huh7 cells were cotransfected with wild-type (wt) and mutant versions of the expression construct pHDV•I(+) as indicated, and the hepatitis B surface (HBs) antigen expression construct pGEM3-HBV(BspEI) (2). Total cellular RNA and viral RNA were harvested 6 and 12 days posttransfection and analyzed for HDV RNA replication and virion secretion by Northern blot hybridization as previously described (2). Panels: A and C, HDV genomic RNA in transfected cells 6 and 12 days posttransfection, respectively; B and D, HDV genomic RNA in virus particles harvested between days 1 and 6 (B) or between days 7 and 12 (D).
levels of virus secretion. Between days 6 and 12, however, the level of virus production for most of the mutant constructs had dropped considerably, reflecting the sharply reduced level of intracellular RNA (Fig. 5 C and D) and HDAg (not shown). Thus, over an extended period of time, the wild type exhibited not only superior intracellular replication levels but also higher levels of virus secretion.

**DISCUSSION**

Editing at the amber/W site plays a critical role in the HDV replication cycle. Here we report that the extent of editing at the HDV genotype I amber/W site is sensitive to increased, but not decreased, base pairing within a 27-nt region downstream of the amber/W site. Increased base pairing in this region increased editing, and this increase correlated with increased HDAg-L production and decreased HDV RNA replication. The sensitivity of replication to increased editing was remarkable—a 2.5-fold increase in editing efficiency was correlated with a 50-fold decrease in replication (Fig. 3). These results are consistent with the effects observed for two site-directed mutants that increased base pairing over a wider region downstream of the editing site (29). The decrease in replication that accompanied increased editing in this study and in previous studies (10, 29) clearly demonstrates that excess HDAg-L production can diminish HDV replication even when produced during the replication cycle.

Remarkably, the dramatic inhibitory effect of increased editing on RNA replication was not apparent when virus production was analyzed over the first 6 days posttransfection. The most likely explanation for the lack of correlation is that the limiting HDV component for virus production is HDAg-L, not HDV RNA. This conclusion emphasizes the potential of HDV antiviral therapy directed against HDAg-L function (1). When viral replication was strongly inhibited over 12 days, and intracellular RNA became undetectable, virus production declined. This result suggests that the conserved structural features of the 27-nt region downstream of the amber/W site have been selected on the basis of virus production after the first week of virus replication.

Our results indicate that HDV genotype I uses an editing site that is optimized for virus production rather than suitability as a substrate for ADAR1. This conclusion is similar to that in a recent study (29), which found that increasing base pairing over a ca. 30-nt region downstream of the amber/W site increased editing and decreased HDV RNA replication levels. A consequence of decreased editing activity could be decreased editing specificity, which would be deleterious to the virus (10). How does HDV achieve adequate specificity while using a suboptimal structure for editing at the amber/W site? Although the structural requirements for editing are not fully understood, it is clear that adenosines flanked on the 5’ side by guanosines are poor editing substrates (13, 26). Analysis of the HDV genome indicates that the dinucleotide pair GA and its complement, UC, are by far the most common and occur at more than twice the frequency of AA and UU, the next most common NA and UN pairs. This bias may be due, in part, to selection for sequences that place non-amber/W adenosines in contexts that are less likely to be edited.

According to a proposed model for ADAR1 editing (14, 26), the enzyme binds to dsRNA segments downstream of editing sites. Lehmann and Bass (14) found that introduction of a 6-nt symmetric internal loop prevented editing within 20 nt of the loop. The region downstream of the HDV genotype I amber/W site contains several internal loops and bulges. Consistent with the idea that ADAR1 binds substrates via its dsRNA binding domains, we found that removing the unpaired segments up to 25 nt away from the amber/W site increased editing by as much as fourfold; increased base pairing further from the site did not increase editing (Fig. 2). Thus, it appears that ADAR1 can bind near the amber/W site more efficiently when the region downstream of the site has more dsRNA character.

Somewhat surprisingly, for two mutations in which the base pairing downstream of the HDV genotype I amber/W site was disrupted by 6-nt internal loops, there was no discernible decrease in editing (Fig. 2). This result raises questions about the nature of the interaction between the dsRNA binding domains of ADAR1 and the imperfectly base-paired region downstream of the HDV genotype I amber/W site. The dsRNA binding domains of ADAR1 are required for editing of other substrates (15, 16, 18, 19). However, compared with most other substrates for site-specific editing by ADAR1 and ADAR2, the ca. 25-bp region downstream of the HDV amber/W site contains fewer consecutive base pairs and is predicted to be less energetically stable. It will thus be interesting to determine the nature of the involvement of the dsRNA binding domains of ADAR1 in HDV amber/W site editing.

Expression of the longer, p150, isoform of ADAR1 is induced by interferon (24, 25), which has been used with limited success to treat HDV infection (22). Recently, Hartwig et al. (9) demonstrated that treatment of transfected cells with interferon doubled the level of editing at the amber/W site in replicating HDV RNA. Although levels of HDV RNA and HDAg were not reported, our results (Fig. 3) suggest that doubling the level of editing would result in a substantial decline in intracellular levels of HDV RNA and levels of virus production, at least in the long term (Fig. 5). As such effects could certainly influence the course of HDV infection, the effects of interferon on HDV replication and virus production warrant further study.

**ACKNOWLEDGMENTS**

This work was supported by grant R01-AI42324 from the National Institutes of Health.

We thank Quifang Cheng for assistance with immunoblots and both Quifang Cheng and Ruxiang Chen for comments and suggestions on the manuscript.

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