Expression of Hepatitis Delta Virus RNA Deletions: *cis* and *trans* Requirements for Self-Cleavage, Ligation, and RNA Packaging

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The hepatitis delta virus (HDV) genome is a circular, single-stranded, rod-shaped, 1.7-kb RNA that replicates via a rolling-circle mechanism. Viral ribozymes function to cleave replication intermediates which are then ligated to generate the circular product. HDV expresses two forms of a single protein, the small and large delta antigens (δ Ag-S and δ Ag-L), which associate with viral RNA in a ribonucleoprotein (RNP) structure. While δ Ag-S is required for RNA replication, δ Ag-L inhibits this process but promotes the assembly of the RNP into mature virions. In this study, we have expressed full-length and deleted HDV RNA inside cells to determine the minimal RNA sequences required for self-cleavage, ligation, RNP packaging, and virion assembly and to assess the role of either delta antigen in each of these processes. We report the following findings. (i) The cleavage and ligation reactions did not require either delta antigen and were not inhibited in their presence. (ii) δ Ag-L, in the absence of δ Ag-S, formed an RNP with HDV RNA which could be assembled into secreted virus-like particles. (iii) Full-length HDV RNAs were stabilized in the presence of either delta antigen and accumulated to much higher levels than in their absence. (iv) As few as 348 nucleotides of HDV RNA were competent for circle formation, RNP assembly, and incorporation into virus-like particles. (v) An HDV RNA incapable of folding into the rod-like structure was not packaged by δ Ag-L.

Hepatitis delta virus (HDV) is a subviral human pathogen that propagates only in the presence of its helper, hepatitis B virus (HBV) (28). Although HDV genome replication can occur in the absence of an hepadnavirus, HBV provides envelope proteins (small, medium, and large surface antigens [sAg-S, sAg-M, and sAg-L]) required for the assembly and release of infectious virions (4, 15, 25, 28, 29, 34). The HDV genome is a single-stranded, circular, 1.7-kb RNA that folds into an unbranched rod-like structure in which 70% of its nucleotides are paired (36). This genomic RNA exists both within virions and in infected nuclei as a ribonucleoprotein RNP that contains the only protein that HDV is known to encode, the delta antigen (30). The antigenome, a circular product of replication whose sequence is the complement of the genome, is similarly complexed with the delta antigen (30). RNA replication occurs in the nucleus, and recent evidence suggest that the host RNA polymerase II (traditionally thought to accept only DNA as its template) may be responsible for this RNA-directed transcriptional event (12, 23). A rolling-circle model for HDV replication in which transcription proceeds around the circular template has been proposed (35). HDV-encoded ribozymes cleave the nascent RNA at a specific site so as to generate unit-length monomers (17, 32). It is believed that as this linear RNA folds into the predicted rod-like structure, its 5' and 3' ends are brought into apposition promoting an autocatalytic ligation reaction (33). The resulting circular antigenome acts as the template for genome synthesis via an analogous mechanism.

Two forms of the delta antigen are observed during infection. The small delta antigen (δ Ag-S), a 22-kDa nuclear phosphoprotein encoded by the virus, is synthesized early and

is required for replication to occur (5, 15). Later in infection, a specific RNA-editing event leads to the mutation of the δ Ag-S termination codon and the synthesis of the large antigen (δ Ag-L), which contains an additional 19 amino acids at its C terminus (3, 22, 38). δ Ag-L not only is incapable of supporting genome replication but also acts as a potent dominant inhibitor of this process (7). In addition, the large antigen, when present in combination with δ Ag-S, has been shown to promote the assembly of the HDV RNP into HBV envelope particles (4, 29).

The mechanisms by which δ Ag-S promotes and δ Ag-L inhibits replication have not been elucidated. One could imagine that they might effect the initiation of transcription, δ Ag-S acting as a transcriptional transactivator and δ Ag-L acting as a repressor. Alternatively, the delta antigen may function posttranscriptionally, modulating either the rate at which HDV RNA self-cleaves and ligates or the stability and/or intracellular location of processed HDV RNA. Although RNA fragments containing the ribozyme have been studied extensively in protein-free reactions, there has been almost no investigation of the activity of these sequences within their natural context (i.e., the rod-like structure) inside cells. As a consequence, nothing is known about how the association of nascent HDV RNA with the delta antigens might affect the processing reactions. In particular, the ligation reaction has been speculated to be stimulated by δ Ag-S (20), based on the observation that δ Ag-S binds to HDV rod-like RNA and this same structure is required for ligation to occur (8, 33).

HDV RNA binding activity has been established *in vitro* for both forms of the delta antigen (5, 8, 21). δ Ag-S and δ Ag-L bind to both genomic and antigenomic RNA with similar affinities, and each recognizes only HDV RNA which is capable of folding into the rod structure (8). However, these studies used denatured recombinant fusion proteins immobilized on nitrocellulose, and thus the actual process of multi-

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FIG. 1. Expected pathway for the generation of circular genomic and antigenomic truncated HDV RNAs from cDNA vectors.

meric RNP assembly could not be studied. Therefore, it is not known whether $\delta\text{Ag-S}$ and $\delta\text{Ag-L}$ bind RNA equivalently within the RNP or even whether $\delta\text{Ag-L}$, in the absence of $\delta\text{Ag-S}$, is competent for RNP assembly. Similarly, the minimal RNA sequences necessary for RNP assembly have not been defined, and it is not known, for instance, whether the packaging reaction imposes any size restrictions on the RNA.

Although the precise sequences required for the cleavage reaction have been defined in protein-free reactions, much less is known about the requirements for the ligation reaction (17, 24, 32, 33, 37). Prior to this report, there had been no effort to delineate the sequences needed so that both processes can occur to generate the circular product.

With these points in mind, we set out to investigate the following issues. What HDV RNA sequences are needed within the cell to promote both cleavage and ligation, and what function, if any, do the delta antigens provide in these reactions? Is $\delta\text{Ag-L}$, in the absence of $\delta\text{Ag-S}$, competent for RNP assembly, and if so, can such an RNP be incorporated into HBV-enveloped particles? How much of the HDV genome is needed for interaction with the delta antigens, and is there a lower limit on the size of RNA which can be packaged? We addressed these questions by delivering both unit-length replication-competent and deleted replication-incompetent HDV RNAs to cells in either the absence or presence of the delta antigens. RNAs from within cells, as well as those secreted in the presence of HBV sAg-S, were then evaluated by Northern analysis to assess their ability to undergo self-cleavage, ligation, and interaction with the delta antigens.

MATERIALS AND METHODS

Plasmid constructions. The HDV RNA-expressing constructs used for this study are diagrammed in Fig. 1. The plasmids that express $\delta\text{Ag-S}$, $\delta\text{Ag-L}$, HBV sAg-S, and full-length antigenome ($\alpha\text{G-FL}$), pDL444, pDL445, pMSVgptSM, and pDL481, respectively, have been described previously (19). The $\alpha\text{G-}\Delta\text{Apa}$ expression vector, pDL480, resulted from the digestion of pDL481 with *Apa*I followed by religation; the $\alpha\text{G-}\Delta\text{Nco}$ expression vector, pDL459, resulted from the removal of HDV sequences between the *Nco*I sites at positions 576 and 1011 according to the sequence of Kuo et al. (16). Purification of the vector-containing fragment of pDL481 after digestion with *Apa*I and bacterial alkaline phosphatase followed by ligation with the smaller *Apa*I fragment of the same vector yielded the $\alpha\text{G/G-}\Delta\text{Apa}$ expression plasmid, pDL482.

The full-length genome (G-FL)-producing plasmid, pDL542, contains the same vector backbone as pDL481, with the simian virus 40 (SV40) late promoter on one side of the polylinker and the T7 RNA polymerase promoter in opposite orientation on the other side of the polylinker. Within the polylinker, a *Sty*I-*Sty*I-*Xba*I HDV fragment (positions 624 through 1679/1 to and beyond 624 to position 785) has been inserted so that genomic RNA is generated by the SV40 late promoter. $\text{G-}\Delta\text{Apa}$, $\text{G-}\Delta\text{Sma}$, $\text{G-}\Delta\text{Sal/Sty}$, and $\text{G-}\Delta\text{Xba/Sty}$ were expressed by pDL539, pDL540, pDL541, and pDL564, respectively, each of which was generated from pDL542 by excision of the indicated HDV sequences. pDL541 digested with *Sst*I and transcribed with T7 RNA polymerase in the presence of [^{32}P]UTP was used to make probes for the

detection of genomic RNA, while pDL459 digested with the same enzyme was used to make probes when antigenomic RNA was detected.

DNA transfections and determination of transfection efficiency. For all experiments, the human hepatoma cell line Huh7 was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected at near confluence with calcium-precipitated DNA (14). In all experiments, 5% of the transfected DNA included a vector that expressed secreted alkaline phosphatase (SEAP), which was monitored as an indicator of transfection efficiency (1). Two days posttransfection, 100 μ l of medium was assayed for SEAP activity, using standard reaction conditions (1) except that incubation was at room temperature. A TiterTek 96-well plate spectrophotometer was programmed so as to measure A_{405} every 20 s for a total of 10 min. The slope that resulted from the linear regression fit of these 30 data points was used as a measure of transfection efficiency. RNA samples were then diluted so as to normalize for SEAP activity. In each experiment, there was less than a twofold variation in the range of SEAP activities obtained from individual transfections.

For Fig. 2 to 5, 35-mm-diameter tissue culture plates were transfected with 2 μ g of RNA expression vector and 2 μ g of protein expression vector; pSVL was substituted for the protein expression vector when protein was not expressed. Approximately one-fifth of the total RNA isolated (after minor corrections for SEAP activity) was loaded in each lane. For Fig. 6, 100-mm-diameter tissue culture plates were transfected with 10 μ g of RNA expression vector, 10 μ g of delta antigen expression vector, and 10 μ g of HBV sAg-S expression vector. Approximately 1/40 of the total cell RNA and 1/4 of the total medium RNA (after minor corrections for SEAP activity) was loaded in each lane.

RNA isolation and Northern analysis. RNAs were purified from cells as well as from isolated virus-like particles, using a modification of the acid guanidine-thiocyanate-phenol-chloroform method (10). Virus-like particles were isolated from media by centrifugation through sucrose as previously described (29). RNA samples were dissolved in water in a manner so as to normalize for SEAP activity. For Fig. 2 to 4 and 6, samples were loaded onto a horizontal gel of 1.5% agarose containing $1 \times$ Tris-borate-EDTA (TBE) and 10 μ g of ethidium bromide per ml. The gels were run submerged in $1 \times$ TBE containing 1 μ g of ethidium bromide per ml at 10 V/cm until the bromophenol blue dye front reached the bottom of the gel. For the gels in Fig. 5, samples were first diluted with formamide to a final concentration of 70% and heated at 95°C for 2 min in the presence of 1 mM EDTA immediately prior to loading. Samples were run in 12.5-cm-long, 1.5-mm-thick vertical gels containing 4 or 6% polyacrylamide (acrylamide/bisacrylamide, 19/1), 10 M urea, and $1 \times$ TBE at 300 V for 2 and 3.3 h, respectively. Phage ϕ X174 DNA digested with *Hae*III and labeled with 32 P by using T4 DNA kinase served as the marker in Fig. 2 to 6. All gels were transferred electrophoretically onto a charged nylon membrane (Zetaprobe), cross-linked with UV light, and then hybridized and washed as previously described (9).

RESULTS

Experimental design. Two series of expression vectors were specifically designed and constructed to express exclusively either genomic or antigenomic circular RNAs from primary transcripts driven by the SV40 late promoter. Since the HDV ligation reaction requires that both the 5' and 3' termini be properly processed by the endogenous ribozyme, it was neces-

sary to include two copies of the self-cleavage site and associated ribozyme in each cDNA construct. Figure 1 depicts these constructs as well as the expected pathway by which the linear primary transcript would be converted to circular RNA.

A vector was designed to exclusively express G-FL RNA, starting at nucleotide (nt) 623 upstream of the genomic self-cleavage site (between nt 685 and 686) and continuing in genomic orientation around the entire genome, terminating at nt 785 (Fig. 1A). Within the delta antigen open reading frame, a 2-bp deletion that results in a frameshift was introduced so that the vector could not express delta antigen (15). This plasmid contains two genomic self-cleavage sites but only one antigenomic site so that only unit-length genomic RNA could arise as a consequence of DNA-directed synthesis. Thus, the appearance of unit-length antigenomic product is predicted to be indicative of RNA-directed synthesis from the circular genomic template, i.e., HDV RNA replication.

An analogous vector that expresses α G-FL RNA was constructed previously and shown to be competent in initiating HDV replication provided that δ Ag-S is supplied in *trans* (19). δ Ag-S must be supplied in *trans* because this vector, like the G-FL expression construct, contains a 2-bp deletion within the delta antigen open reading frame. This construct contains the entire HDV sequence starting before the antigenomic self-cleavage site (between nt 900 and 901) at nt 967 and continuing in the antigenomic direction to and beyond nt 967, terminating at nt 781. It contains two self-cleavage sites in the antigenomic orientation but only one in the genomic direction.

Since the two sides of the HDV rod-like structure display roughly 70% complementary, a number of restriction sites that appear on the cDNA from one side of the rod also reside in the complementary region of cDNA from the opposing side of the rod. Such sites were used to generate mutant HDV truncations which lacked various amounts from the top of the rod but still preserved the general rod-like structure as well as the sequences located at the bottom of the rod, thought to be required for self-cleavage and ligation (17, 32, 33). In addition, one mutant, G- Δ Xba/Sty, was designed to completely lack rod-like structure. The restriction sites used to construct these vectors along with the predicted RNA products are depicted in Fig. 1.

Truncated genomic RNAs were competent for self-cleavage but not replication. RNAs isolated from Huh7 cells transiently transfected with DNAs that expressed either unit-length or truncated genomic RNA were loaded onto nondenaturing agarose gels and then subjected to Northern analysis with probes specific for the bottom of either the genomic or antigenomic strand of HDV. In all experiments, a reporter construct that expresses SEAP was included so that differences in transfection efficiency could be measured and corrected for (1). An advantage in using native gels for this purpose is that under these conditions, both linear and circular molecules comigrate, simplifying the interpretation of data. In addition, the hydrodynamic shape of native rod-like RNAs has been found to be very similar to that of double-stranded DNA of comparable size (31), indicating that during nondenaturing electrophoresis, the two species should display similar mobilities.

When the genomic strand RNAs that resulted from DNA directed synthesis were visualized (Fig. 2A), products of different sizes were observed with each construct. The vectors that encode these RNAs contain more than 1 kb of SV40 sequence upstream of the HDV cDNA and no known polyadenylation or termination signals downstream. Thus, the unprocessed primary transcripts that originate from these vectors are expected to be very large. However, the genomic RNAs

FIG. 2. Northern analysis of the products produced after transfection with DNAs expressing genomic strand RNA. RNA samples were harvested 4 days posttransfection, subjected to native electrophoresis on duplicate gels of 1.5% agarose, and probed with radiolabeled RNA complementary to the bottom 348 nt of the HDV genomic strand (A) or antigenomic strand (B). G-FL was loaded in lanes 1 and 2, G- Δ Apa was loaded in lanes 3 and 4, G- Δ Sma was loaded in lanes 5 and 6, and G- Δ Sty/Sal was loaded in lanes 7 and 8. Where indicated, δ Ag-S was coexpressed with genomic RNA expression vectors. Lane M includes DNA markers of ϕ X174 digested with *Hae*III. The length of each marker fragment is indicated at the left side in nucleotides.

detected within Huh7 cells appeared to have been processed at both self-cleavage sites, as judged by their mobilities compared with double-stranded DNA markers. For instance, the predominant RNA in Fig. 2A, lane 1, is replicating G-FL with a known size of 1677 nt. Under these electrophoretic conditions, it migrated slightly faster than the 1,744-nt (872-bp) DNA marker. A second band of less intensity in the same lane represents HDV dimeric RNA (3,354 nt), and it migrated somewhat more slowly than the 2,706-nt (1,353-bp) DNA. Similarly, G- Δ Apa, with a predicted size of 1,165 nt, ran very slightly ahead of the 1,206-nt (603-bp) marker, while G- Δ Sma, with a predicted size of 627 nt, ran slightly more slowly than the 620-nt (310-bp) marker (Fig. 2A, lanes 3 to 6). It should be noted that the smallest RNA, G- Δ Sty/Sal, ran somewhat slower than the DNA marker of corresponding size (Fig. 2A, lanes 7 and 8). Additional confirmation that this transcript did, in fact, undergo two self-cleavage events, using denaturing polyacrylamide electrophoresis is presented in Fig. 5.

In Fig. 2B, the antigenomic products, which should be diagnostic for HDV replication, were visualized. When the DNA that expressed G-FL was cotransfected with a δ Ag-S expression vector, an intense signal was detected; however, when the same vector was transfected in the absence of the delta antigen, no signal was detected (compare lane 1 with lane 2 in Fig. 2B). This was expected since the G-FL expression vector contained a frameshift mutation in the δ Ag-S open reading frame which was previously shown to prevent replication unless δ Ag-S was provided in *trans* (15). Since the G-FL expression vector was unable to direct the synthesis of unit-length antigenomic RNA in the absence of δ Ag-S, the presence of such species when δ Ag-S was provided was an indica-

tion that HDV replication had occurred. No antigenomic signal could be detected when any of the truncated genomic constructs were tested (Fig. 2B, lanes 3 to 8). Thus, even in the presence of δ Ag-S, G- Δ Apa, G- Δ Sma, and G- Δ Sal/Sty were incompetent for replication.

The accumulation of larger RNAs was enhanced in the presence of the delta antigens. In the absence of δ Ag-S, the amount of a truncated RNA that was detected was inversely related to its size; i.e., large RNAs were less abundant than small RNAs (Fig. 2A, lanes 3, 5, and 7). However, in the presence of δ Ag-S, the RNAs accumulated to similar levels regardless of their sizes (Fig. 2A, lanes 4, 6, and 8). Results obtained through radioanalytic imaging (AMBIS) indicated that the 348-nt truncation accumulated to less than a twofold-greater level when expressed in the presence of δ Ag-S compared with in its absence. Thus, if δ Ag-S stimulated self-cleavage of the RNA at all, it did so by less than a factor of 2. However, the larger genomic mutants showed a greater dependence on the presence of δ Ag-S for their stable accumulation. The level of the 627-nt species was increased fourfold by δ Ag-S, and the 1,165-nt RNA level was increased ninefold.

Since the stimulation of HDV RNAs by δ Ag-S was size dependent, it seemed unlikely that δ Ag-S was increasing the rate of transcription. To formally rule out such a possibility, the SEAP reporter gene was fused to the SV40 late promoter, and after transfection with this vector, SEAP activities were measured in either the absence or presence of δ Ag-S. The two values obtained differed by less than 30%, indicating that δ Ag-S did not increase the rate of transcription (18). Thus, δ Ag-S does not generally enhance SV40 late promoter transcription, nor does it stimulate the level of a heterologous

FIG. 3. Northern analysis of time course of G- Δ Apa expressed in the absence and presence of the delta antigens. Samples were harvested 2, 4, and 6 days posttransfection, subjected to native electrophoresis on 1.5% agarose gels, and probed with radiolabeled RNA complementary to the bottom 348 nt of the HDV genome. Where indicated, δ Ag-S and δ Ag-L were coexpressed with G- Δ Apa. Lane M is as in Fig. 2.

RNA, i.e., SEAP mRNA. A likely explanation to account for the stimulation of HDV RNA is that in the absence of δ Ag-S, the genomic RNAs were susceptible to nucleolytic degradation, and simply by virtue of their size, larger RNAs were more likely to undergo attack than smaller ones. However, if δ Ag-S was able to form RNPs with these genomic truncations, then they might be protected from nucleolytic attack and thereby accumulate to comparable levels.

A second line of evidence to establish that the genomic truncations were unable to replicate was obtained by subjecting the largest mutant, G- Δ Apa, to a time course analysis. Previously it was observed that in cDNA-transfected cells, the level of replicating HDV increases with time, reaching a plateau after 9 to 12 days (6). Consistent with this finding, in an analysis of RNA harvested from cells 3, 6, and 9 days after transfection with the G-FL and δ Ag-S expression vectors, maximal genomic signal strength was observed with the 9-day time point (18). In contrast, the level of nonreplicating SEAP mRNA expressed by transient transfection reached a maximum (as assessed by SEAP activity) within 3 days and steadily declined in daily assays from 4 to 9 days (18). Figure 3 shows a time course in which G- Δ Apa-transfected samples were harvested either 2, 4, or 6 days posttransfection. As expected, the intensity of the G- Δ Apa signal was greatest at day 2 and continued to decline thereafter (compare lanes 1, 5, and 9). When δ Ag-S was coexpressed, again the signal was increased approximately ninefold compared with in its absence at each time point (compare lanes 1 with 2, 5 with 6, and 9 with 10).

However, consistent with a lack of replication, the G- Δ Apa signal in the presence of δ Ag-S decreased with time (lanes 2, 6, and 10).

We next tested whether the large form of the delta antigen was similarly capable of stimulating the accumulation of the 1,165-nt truncation. Lanes 3, 7, and 11 show that G- Δ Apa accumulated to very similar levels in the presence of δ Ag-L versus δ Ag-S. Comparable levels were also observed when an equimolar mixture of δ Ag-S and δ Ag-L was supplied (lanes 4, 8, and 12). This result provided at least circumstantial evidence that δ Ag-L was competent for intracellular RNP assembly in the absence of δ Ag-S.

Nonreplicating antigenomic RNAs were also competent for self-cleavage. Figure 1B depicts three mutant antigenomic RNAs constructed for this study. In two, the top of the rod-like structure was removed to generate RNAs with predicted lengths of 1,165 and 435 nt, while the third mutant had no deletion. In this last case, sequences at the top of the rod structure were precisely exchanged to generate a unit-length antigenomic/genomic hybrid (α G/G- Δ Apa). In Fig. 4A, antigenomic signals for α G-FL and each of the three mutants were observed, and the sizes of these RNAs were consistent with what would be expected if the primary transcripts had undergone two self-cleavage events (lanes 5, 8, and 11). From Fig. 4B, it is clear that only the wild-type construct was competent for replication (lanes 1, 5, 8, and 11). As was the case for the nonreplicating genomic constructs, in the absence of the delta antigens, the antigenomic RNAs accumulated in a size-dependent manner; although the 435-nt RNA was abundant, the 1,677-nt α G/G- Δ Apa RNA could barely be detected (Fig. 4a, lanes 4 and 10). Similarly, when either δ Ag-S or δ Ag-L was coexpressed, the level of the larger antigenomic species was greatly increased. For example, the 1,677-nt α G-FL and α G/G- Δ Apa RNAs both accumulated to a 15-fold-higher level in the presence of δ Ag-L compared with in its absence despite the lack of replication in both cases.

The 348-nt genomic truncation was competent for ligation. We next examined whether the HDV truncated RNAs were competent for ligation and, hence, circle formation. This was achieved by subjecting the smallest mutant, G- Δ Sal/Sty, to Northern analysis under denaturing conditions. It has long been established that during electrophoresis, denatured circular RNAs exhibit aberrantly slow mobility compared with linear markers (2). The mobility is further retarded as the concentration of polyacrylamide within the gel is increased. For this reason, RNA isolated from cells that had been transfected with the G- Δ Sal/Sty expression vector was run on both 4 and 6% polyacrylamide gels that contained 10 M urea.

Although G- Δ Sal/Sty migrated as a single band during native electrophoresis in Fig. 2A, lane 7, under denaturing conditions, it was resolved into two bands (Fig. 5A and B, lanes 1). The more rapidly migrating and less abundant species ran in an equivalent position relative to the linear markers in either gel, which indicated that it had a linear conformation. If this RNA represented the linear product of two self-cleavage events, then it would be predicted to be 348 nt in length. Consistent with this, on either gel it ran somewhat above the 310-nt linear single-stranded DNA marker (Fig. 5A and B, lanes 1). In contrast, the slower and more abundant species ran aberrantly relative to the linear markers on the two gel systems; it migrated more rapidly than the 603-nt marker on the 4% gel but more slowly than the 1,078-nt marker on the 6% gel. Given that this species and the 348-nt linear RNA comigrated under native conditions, we concluded that it was a 348-nt RNA of circular conformation.

Quantitation of the signal strength of the two species

FIG. 4. Northern analysis of the products produced after transfection with DNAs expressing antigenomic strand RNA. Samples were harvested 3 days posttransfection, subjected to native electrophoresis on 1.5% agarose gels, and probed with radiolabeled RNA complementary to the bottom 435 nt of the HDV antigenomic strand (A) or genomic strand (B). α G-FL was loaded in lanes 1 to 3, α G/G-*Apa* was loaded in lanes 4 to 6, α G- Δ *Apa* was loaded in lanes 7 to 9, and α G- Δ *Nco* was loaded in lanes 10 to 12. Where indicated, δ Ag-S and δ Ag-L were coexpressed with antigenomic RNAs. Lane M is as in Fig. 2.

revealed that 89% of the total G- Δ *Sal/Sty* RNA was circular. Since ligation had occurred efficiently inside cells in the absence of either form of the delta antigen, neither protein played an obligatory role in this process. When G- Δ *Sal/Sty* was cotransfected with either δ Ag-S or δ Ag-L, the total signal was increased by between two- and threefold, consistent with what

was observed in Fig. 2A, lanes 7 and 8. In addition, when G- Δ *Sal/Sty* was expressed in the presence δ Ag-S and especially δ Ag-L, up to eightfold more linear product was observed (Fig. 5; compare the linear bands of lanes 1 to 3). Linear RNAs are believed to be less stable than circular RNAs (26), possibly because they are susceptible to attack by a hypothesized 5'

FIG. 5. Northern analysis of G- Δ *Sty/Sal* under denaturing conditions. Samples were harvested 3 days posttransfection, subjected to electrophoresis under denaturing conditions on polyacrylamide gels of 4% (A) and 6% (B), and probed with radiolabeled RNA complementary to the bottom 348 nt of the HDV genome. Where indicated, δ Ag-S and δ Ag-L were coexpressed with G- Δ *Sty/Sal*. Lane M includes the same marker as appears in Fig. 2 except that in this case, the double-stranded DNA has been denatured.

FIG. 6. Release of nonreplicating HDV RNA into virus-like particles. Samples were harvested 4 days posttransfection, subjected to native electrophoresis on 1.5% agarose gels, and probed with radiolabeled RNA complementary to the bottom 348 nt of the HDV genome (A) or the bottom 435 nt of the antigenome (B). For panel C, samples were probed with radiolabeled RNA complementary to the entire HDV genome; thus, longer HDV mutants displayed stronger signal strength than smaller mutants. In each panel, RNA samples were isolated from both transfected cells (lanes 1 to 4) and from the corresponding tissue culture medium after centrifugation through sucrose (lanes 5 to 8). In panel A, lanes 1 and 5, cells were cotransfected with G-FL, δ Ag-L, and HBV sAg-S. In the remaining samples, G- Δ Sty/Sal was coexpressed with δ Ag-L and HBV sAg-S (lanes 2 and 6), δ Ag-S and HBV sAg-S (lanes 3 and 7), and δ Ag-L alone (lanes 4 and 8). In panel B, lanes 1 and 5, cells were cotransfected with α G-FL, δ Ag-L, and HBV sAg-S. In the remaining samples, α G- Δ Nco was coexpressed with δ Ag-L and HBV sAg-S (lanes 2 and 6), δ Ag-S and HBV sAg-S (lanes 3 and 7), and δ Ag-L alone (lanes 4 and 8). In panel C, samples were obtained from cells transfected with both δ Ag-L and HBV sAg-S as well as G- Δ Apa (lanes 1 and 5), G- Δ Sma (lanes 2 and 6), G- Δ Xba/Sal (lanes 3 and 7), and both G- Δ Sty/Sal and G- Δ Xba/Sal (lanes 4 and 8). Samples from lanes 1, 2, 5, and 6 were transfected and harvested at a different time from those in lanes 3, 4, 7, and 8. Lane M is as in Fig. 2.

exonuclease. Perhaps most of the G- Δ Sal/Sty linear product was destroyed in the absence of the delta antigens but was stabilized by RNP formation in their presence.

The 348-nt truncation was packaged and secreted in the presence of δ Ag-L and HBV sAg-S. Previously, it was found that δ Ag-L is required for the assembly of full-length replicating HDV RNPs into secreted HBV sAg-S particles (4, 29). Since δ Ag-S was also present in these studies, it was not clear whether the large antigen bound directly to HDV RNA or indirectly via a coiled-coil interaction with small delta antigen in the RNP. Here we found that δ Ag-L could increase the yield of large processed HDV RNAs (Fig. 3 and 4), indicating that it might be competent for RNP assembly. Since RNP assembly is a necessary prerequisite for the inclusion of HDV RNA into secreted HBV sAg-S particles (30), we tested the ability of δ Ag-L to form an RNP by investigating whether genomic RNA could be packaged and secreted in the absence of δ Ag-S.

G-FL was cotransfected with δ Ag-L and HBV sAg-S, and the RNAs isolated both from within the cells and from the tissue culture medium after centrifugation through sucrose were visualized by Northern analysis. As shown in lane 5 of Fig.

6A, G-FL was liberated into the medium. The gel from Fig. 6A had been stained with ethidium bromide prior to transfer. Intense staining of the cellular RNA was observed; however, no staining of the medium samples could be detected. From RNA concentration determinations using UV spectroscopy at 260 nm, it was found that the medium RNA sample contained at least 60-fold less total RNA than the intracellular RNA sample did (Fig. 6A, lanes 5 and lane 1). Given that the G-FL signals in these two lanes were nearly equal, we concluded that G-FL was purified at least 50-fold relative to total RNA in the medium sample. This specific enrichment indicated that the appearance of G-FL in the medium did not occur through some trivial mechanism such as cell lysis. In this experiment, 1/4 of the total media RNA was loaded, compared with 1/40 of the cellular RNA. Quantitation of the signal strength of G-FL inside and outside of cells, after correction for loading differences, indicated that 9% of the total G-FL was released to the medium.

We then addressed whether the packaging and secretion reactions have a rigid RNA size requirement by testing whether the smallest genomic construct, G- Δ Sal/Sty, could

similarly be packaged. Data from Fig. 6A, lanes 2 and 6, indicate that, indeed, 13% of this 348-nt truncation was released into the medium. Since authentic HDV packaging and secretion is absolutely dependent on the presence of both δ Ag-L and HBV sAg-S (4, 29), we tested whether the secretion of G- Δ Sal/Sty displayed similar requirements. As shown in lanes 7 and 8 of Fig. 6A, omission of either of these proteins completely abolished secretion.

Full-length and truncated antigenomic RNAs were packaged and secreted. Since δ Ag-L could also stabilize antigenomic HDV RNA, it seemed that it may be possible to package and secrete antigenomic RNA in a manner analogous to what was done with genomic RNA in Fig. 6A. As can be seen in Fig. 6B, this was indeed the case, as both α G-FL and α G- Δ Nco were efficiently liberated into the medium (15 and 8%, respectively; lanes 5 and 6). Again, there was a strict requirement for both δ Ag-L and HBV sAg-S (lanes 7 and 8). Based on RNA concentration determinations of the samples from lanes 1 and 5, α G-FL was enriched at least 100-fold relative to cell RNA in the secreted sample compared with the intracellular RNA.

HDV RNA packaging requires the presence of the rod structure. Previously, it was shown that in vitro, δ Ag-L can bind only to HDV RNA fragments which contain rod-like structure (8). We tested whether such a requirement exists in vivo by examining whether two additional rod-structured mutants, G- Δ Apa and G- Δ Sma, could be preferentially packaged and secreted compared with a mutant which lacks rod-like structure, G- Δ Xba/Sty (see Fig. 1A for descriptions). As shown in Fig. 6C, both G- Δ Apa and G- Δ Sma were efficiently packaged (12 and 5%, respectively; lanes 5 and 6), while no detectable levels of G- Δ Xba/Sty were observed in the medium RNA sample (lane 7). As further proof for the requirement of rod structure, both G- Δ Xba/Sty and G- Δ Sal/Sty were cotransfected into the same cells. Although G- Δ Xba/Sty was actually somewhat more abundant than G- Δ Sal/Sty within the cells (Fig. 6C, lane 4), only G- Δ Sal/Sty was released to the medium (lane 8).

DISCUSSION

By transiently expressing both full-length and truncated genomes and antigenomes in the presence or absence of the delta antigens, we were able to determine some of the molecular requirements involved in the processes of HDV RNA replication, self-cleavage, and ligation, RNP assembly, and sAg-mediated secretion. Three independent lines of evidence established that none of the mutant constructs reported here were competent for replication. (i) The RNA-expressing vectors were designed to produce properly processed products of only one specific strand. Thus, the appearance of a processed product of the opposing strand would indicate that RNA-directed RNA replication had occurred. Although the full-length constructs could direct the synthesis of both strands, only the strand which could be synthesized as a primary transcription product from DNA was observed with the truncated mutants. (ii) Replicating HDV RNA reached a maximum at least 9 days following transfection, while nonreplicating SEAP mRNA reached a peak within 3 days. Consistent with a lack of replication, G- Δ Apa levels were higher at 2 days posttransfection than at 4 or 6 days despite the coexpression of δ Ag-S. (iii) It was previously established that δ Ag-L potently inhibits HDV replication (7, 13). Consistent with this observation, when δ Ag-L was coexpressed with the full-length replication-competent RNAs, the signal of the strand diagnostic for replication was completely abolished, while the signal from the

strand which could arise from either RNA or DNA was greatly reduced compared with when δ Ag-S was supplied. In contrast, when expressed with δ Ag-S, the truncation mutants displayed no signal when probed for the strand diagnostic for replication and much less signal than full-length replicating HDV RNA when probed for the strand that could arise from either DNA or RNA. Furthermore, unlike the situation with full-length replicating HDV, this signal was not reduced when δ Ag-L was substituted for δ Ag-S.

In addition to the mutants described here, we had made other mutants which preserved both the top and bottom of the rod-like structure but contained various internal deletions; e.g., sequences between the *Apa*I and *Sma*I sites on each side of the rod-like structure were excised. None of these mutants were competent for replication even when δ Ag-S was provided (18). From these negative results, therefore, we could only conclude that HDV replication did not tolerate such drastic deletions and alterations.

Despite their failure to replicate, the deletion mutants were quite informative in other respects. All antigenomic and genomic mutants tested were competent for self-cleavage, and as judged by their sizes, the products resulting from the cDNA-directed transcripts were cleaved at both self-cleavage sites. In addition, the majority of these cleaved products were observed to be ligated into a circular conformation. The formation of circular species was further confirmation that self-cleavage had occurred at both sites, since the HDV ligation reaction requires properly processed termini (33). No more than 348 nt originating from the bottom of the HDV rod structure were needed for both self-cleavage and ligation. Furthermore, both the self-cleavage and ligation reactions occurred with similar efficiencies in the absence or presence of the delta antigens.

Although δ Ag-S and δ Ag-L had no significant effect on the processing reactions, two independent lines of evidence established that they could interact with processed mutant HDV RNAs. These were the observations that the delta antigens can increase the yield of larger rod-shaped HDV RNAs and that δ Ag-L could direct the packaging and assembly of truncated HDV RNAs into HBV sAg-S particles.

In the absence of the delta antigens, smaller rod-shaped RNAs accumulated to much higher levels, on a molar basis, than larger ones. However, if larger RNAs were synthesized in the presence of either δ Ag-S or δ Ag-L, then their resulting yield was increased by as much as 15-fold and was similar to that observed with the smaller RNAs. One interpretation of these results is that the delta antigens bound to and stabilized these RNAs by forming an RNP structure. It is possible that HDV RNA not bound with the delta antigens was more susceptible to nucleolytic attack. Larger RNAs would be more likely to encounter an RNase molecule and would thus be intrinsically less stable than smaller RNAs. We speculate that when complexed with either delta antigen as an RNP, RNase access to the RNA is significantly reduced.

Whatever mechanism accounts for the manner by which the delta antigens stabilize HDV RNA, the phenomenon must be considered an important and newly recognized role of these proteins. This stabilization property alone, however, does not explain how δ Ag-S promotes replication and δ Ag-L inhibits it, since we observed that both proteins could stabilize HDV RNA to an equivalent level. It was previously hypothesized that the opposing effects on replication that the two delta antigens display may result as a consequence of possible differences in the intracellular locations of the two RNPs (19). Specifically, δ Ag-L was postulated to move the RNP out of the nucleus, rendering it incompetent for replication but now

competent for interaction with HBV sAg. A careful analysis of the intracellular location of nonreplicating HDV RNA mutants complexed with either δ Ag-S and δ Ag-L could be a means by which to test the validity of this hypothesis.

Although the large HDV RNAs were unstable unless the delta antigens were coexpressed, the 348-nt genomic truncation was intrinsically stable in their absence. This is of interest given that the size of G- Δ Sty/Sal is very similar to that of the plant viroids. These RNAs range in size from 246 to 375 nt and are similar to HDV in that they are single-stranded and circular and assume an unbranched rod-like structure due to extensive intramolecular pairing (11). Viroids, similarly, are thought to recruit the host RNA polymerase II for their synthesis and replicate via a rolling-circle mechanism (27). A major difference between HDV and the viroids is that the latter do not express proteins. It is tempting to speculate, from results obtained with the HDV truncations, that the conservation of the small size among the different plant viroids may indicate an evolutionary constraint imposed by RNA stability. Perhaps viroids of 500 to 2,000 nt in length have not evolved because, in the absence of a viral core protein, such RNAs would be too rapidly degraded by the host.

Experiments in which HDV RNA was assembled into HBV sAg-S particles provided a second and more direct form of evidence to establish the interaction of HDV truncated mutants with the delta antigens. From such experiments, we concluded that δ Ag-L could form an RNP that was competent in its interaction with HBV sAg-S. Thus, inside the cell, δ Ag-L can bind HDV RNA even in the absence of δ Ag-S. The specificity of the packaging reaction was established by four observations. (i) It was dependent on the presence of HBV sAg-S. (ii) δ Ag-L was required for HDV RNA secretion, and δ Ag-S could not be substituted. (iii) The proportion of HDV RNA to total RNA was increased at least 50-fold in the secreted material compared with that inside the cell. Thus, δ Ag-L displayed more than a 50-fold preference in binding to HDV RNA relative to host RNA. (iv) It was previously observed *in vitro* that δ Ag-L is unable to bind to HDV RNAs that lack the rod structure (8). Consistent with this finding, we observed that δ Ag-L could not promote the secretion of an HDV RNA which lacked rod-like structure.

We also observed that both full-length and truncated antigenomic RNAs could be packaged and secreted in the presence of δ Ag-L and HBV sAg-S. Again the reaction absolutely required each of these proteins, and the secreted HDV antigenomic RNA was enriched relative to cellular RNA by more than 100-fold. This packaging of antigenomic RNA by δ Ag-L is consistent with two previously reported results, namely, that the antigenome exist within the nucleus as an RNP complexed with both δ Ag-S and δ Ag-L and that δ Ag-L can bind antigenomic RNA in an RNA-immunoblot (Northwestern) assay (8, 30). It is also consistent with the observation reported here that δ Ag-L can bind to and stabilize antigenomic RNA *in vivo*. Nevertheless, the ability of δ Ag-L to promote the packaging and secretion of antigenomic RNA is surprising in light of what is observed during a natural infection. The sera from HDV-infected woodchucks contain roughly 10,000-fold more genome than antigenome, indicating that there is some mechanism whereby the antigenome is effectively excluded from the secretory pathway (23a).

There are a number of differences between the tissue culture system presented here and the woodchuck model. In an infected woodchuck, HDV replication is occurring, the genome is expressed in a 10-fold excess relative to the antigenome, and both δ Ag-L and δ Ag-S as well as all three woodchuck hepatitis virus envelope proteins are present (9). In

the experiments described here, antigenomic RNA is expressed in the absence of both the genome and replication, and δ Ag-S, HBV sAg-M, and HBV sAg-L are not present. It will be interesting to determine which of these differences enables antigenomic packaging to occur in tissue culture as well as to elucidate the mechanism by which the antigenome is excluded from the assembly pathway in an infected animal.

We do not know whether HDV RNA contains an origin of assembly, i.e., a specific site of nucleation at which the delta antigens first bind. However, since we observed that G-FL and G- Δ Sty/Sal were packaged and secreted with similar efficiencies, then if such an origin does exist, it must reside within the bottom 348 nt of the rod-like structure. Alternatively, it remains possible that there are multiple delta antigen binding sites scattered throughout the HDV genome. Such a model would be consistent with results obtained in which recombinant protein was used in a Northwestern assay (8). Secretion experiments using wild-type δ Ag-L inside Huh7 cells, in combination with RNA fragments originating from various regions within the HDV rod structure, are currently under way to distinguish between these two models.

It is also not known whether HDV RNA provides the backbone around which a multimeric delta antigen complex assembles or whether such a protein complex preassembles prior to the encapsidation of RNA. The former model predicts that the size of the RNA dictates the number of protein constituents in the RNP; in the latter case, this number would remain constant regardless of the size of the RNA to be packaged. Thus, an accurate assessment of the protein stoichiometry in the G-FL and G- Δ Sty/Sal RNPs could resolve this issue.

The intracellular stabilization of HDV RNA by the delta antigens and the assembly of nonreplicating HDV RNA into secreted surface antigen particles represent two new assays within cells that use wild-type and posttranslationally modified proteins in their natural environment to assess the competence of delta antigen-RNA interactions. We anticipate that these assays will be useful not only in resolving some of the remaining issues just mentioned concerning RNP assembly but also, when used with specific mutations in the delta antigen, in determining the protein sequences and domains involved in RNA interaction and RNP assembly. We have also described a stable and readily detectable 348-nt circular RNA which is capable of excising itself from a heterologous primary transcript. This RNA could be used as a reporter cassette. When inserted into various regions within a transcription unit of interest, the amount of 348-nt RNA obtained would indicate whether transcription had terminated prior to or elongated through the site of insertion. In addition, given the intrinsic stability that the G- Δ Sty/Sal circular RNA displayed, it may prove to be a useful vehicle for the expression of foreign inserted sequences such as antisense RNA, RNA decoys, and targeted ribozymes.

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