Ribonucleoprotein Complexes of Hepatitis Delta Virus

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Human hepatitis delta virus (HDV) is a subviral satellite agent of hepatitis B virus (HBV). The envelope proteins of HDV are provided by the helper virus, HBV, but very little is known about the internal structure of HDV. The particles contain multiple copies of the delta antigen and an unusual RNA genome that is small, about 1,700 nucleotides in length, single stranded, and circular. By using UV cross-linking, equilibrium density centrifugation, and immunoprecipitation, we obtained evidence consistent with the interpretation that delta antigen and genomic RNA form a stable ribonucleoprotein (RNP) complex within the virion. Furthermore, electron-microscopic examination of the purified viral RNP revealed a roughly spherical core-like structure with a diameter of 18.7 ± 2.5 nm. We also isolated HDV-specific RNP structures from the nuclei of cells undergoing HDV genome replication; both the genome and antigenome (a complement of the genome) of HDV were found to be in such complexes. From the equilibrium density analyses of the viral and nuclear RNPs, we were able to deduce the number of molecules of delta antigen per molecule of HDV RNA. For virions, this number was predominantly ca. 70, which was larger than for the nuclear RNPs, which were more heterogeneous, with an average value of ca. 30.

Human hepatitis delta virus (HDV) is a subviral satellite which receives its envelope from its helper virus, hepatitis B virus (HBV) (25). It can also be experimentally transmitted to woodchucks chronically infected with woodchuck hepatitis virus (WHV) (23). It has been shown that inside the HDV particle there is the delta antigen, the only protein that HDV is known to encode (1, 30, 31), along with the single-stranded RNA genome. This RNA is only about 1,700 nucleotides in length, is circular, and can fold on itself, with about 70% of the nucleotides paired, to form an unbranched rod-like structure (10, 29, 30). Inside the nucleus of an infected cell, this genomic RNA replicates by RNA-directed RNA synthesis, via a complementary intermediate referred to as the antigenome (10).

The delta antigen is found not only inside virus particles but also in the nucleus of infected cells. Actually, there are two related forms of the delta antigen, which differ by a 19-amino-acid carboxy-terminal extension. These species are 195 and 214 amino acids in length and are referred to as the small and large forms of the delta antigen, respectively (1, 30, 31). The formation of these two species is mediated by specific RNA editing (3, 33), but what is relevant here is that the small form is needed for genome replication (17) whereas the large form is needed for particle assembly (5, 26).

In vitro, both forms have been shown to possess HDV-specific RNA-binding ability (18), and this binding depends on the ability of HDV RNA to form the above-mentioned rod-like structure (8). This being the case, one might expect to readily isolate specific HDV RNPs from both virions and infected cells; however, to date this has not been the situation. Earlier electron-microscopic studies of HDV had failed to demonstrate any form of HDV core structure, even in undisrupted virions (2). More recently, in a study of assembled virions, we could detect what appeared to be a core in about 15% of undisrupted particles (26). Also, Lin et al. (18) obtained some evidence for a ribonucleoprotein (RNP) in virions; with an antibody against the delta antigen, they were able to precipitate at least some of the HDV RNA

from disrupted virions. Given this situation, we undertook a systematic study of HDV RNPs. By using four different procedures, we were able to isolate and characterize such RNPs both from virions and from the nuclei of cells in which the HDV genome was replicating.

MATERIALS AND METHODS

Viruses. HDV was obtained from a woodchuck that had been experimentally infected with HDV. The animal's serum contained about 0.4×10^{12} particles of HDV per ml. To purify the virus, the serum was clarified by low-speed centrifugation, pelleted by centrifugation (Beckman SW40 rotor; 32 krpm for 16 h at 4°C) through a cushion of 20% sucrose, and then resuspended in STE buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Centrifugation to equilibrium on gradients of metrizamide. Preformed gradients of metrizamide (Sigma) in STE buffer were prepared as previously described (26). Samples were layered and subjected to equilibrium centrifugation (Beckman SW60 rotor; 42 krpm for 22 h at 4°C). Fractions were collected from the bottom of the tube. Aliquots were analyzed for refractive index and, as indicated, for various viral markers. The Ausria II assay kit (Abbott) was used to detect the WHV surface antigens as recommended by the manufacturer. The data obtained were subsequently transformed to a linear scale by means of a calibration curve.

UV cross-linking procedures. HDV particles in serum were diluted into 100 μ l of standard cross-linking buffer (50 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA), incubated for 5 min on ice, and then transferred to a quartz cuvette with lid. The cuvette was placed horizontally on ice inside a Stratalinker (Stratagene) and exposed to 254-nm UV irradiation. As described below, two different assays were used to determine the extent of cross-linking.

To assay for HDV RNA species that were cross-linked to protein, aliquots of the exposed samples were subjected to two extractions with phenol followed by one extraction with ether and then collected by precipitation with ethanol. As a control, prior to the phenol extraction, some aliquots were treated for 1 h at 37°C with pronase in a buffer consisting of

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10 mM EDTA, 10 mM Tris-HCl (pH 8.0), 0.5% sodium dodecyl sulfate (SDS), and pronase (1 mg/ml). The recovery of HDV RNA species was assayed by Northern (RNA) analysis (10) followed by direct quantitation with a Radioanalytic Imaging System (AMBIS).

We also examined the extent of cross-linking of the delta antigen. Immediately following exposure to UV, aliquots were collected by precipitation with 4 volumes of ethanol and examined by a Western immunoblot procedure, as previously described (17). The delta antigen was detected with a rabbit anti-delta antigen antibody followed by incubation with 125 I-labeled protein A (DuPont). After autoradiography, quantitation was carried out with the optical system of the Radioanalytic Imaging System.

Electron microscopy. Aliquots from the metrizamide gradients were examined by negative staining. Samples of 2 μ l were applied to 300-mesh copper grid with a carbon-coated Formvar film. After 5 to 8 min, the liquid was blotted off and the grid was washed twice with double-distilled water. The grid was stained for 5 min with 0.5% uranyl acetate, blotted, and examined with a Philips 420 electron microscope. Micrographs were taken at a $\times 51,000$ magnification. The particle dimensions were obtained from magnified prints.

Transfection and isolation of nucleoplasm. A human hepatoma cell line, Huh7 (20), was transfected, by a calcium phosphate procedure (14), with the eukaryotic expression plasmid pSVL(D3), which can express a trimer of unit-length HDV RNA via the simian virus 40 late promoter (17). The nucleoplasmic fraction was prepared essentially by the method of Choi and Dreyfuss (11). To isolate HDV-specific RNPs, at 11 days after transfection the cell monolayers were washed twice with cold phosphate-buffered saline. The cells were then scraped with a rubber policeman into 1 ml per 10-cm plate of cold lysis buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2.5 mM $MgCl_2$) containing 0.5% Triton X-100 and incubated on ice for 10 min. The nuclei were separated by a brief spin (3 krpm; Sorvall HS-4 rotor), resuspended in 0.5 ml of cold lysis buffer, and sonicated for 30 s on ice to break the nuclei. The suspension was layered on a 30% sucrose cushion in cold lysis buffer and centrifuged (5 krpm for 15 min; Sorvall HS-4 rotor) to remove chromatin and nucleoli. The supernatant fraction was used for isolation of HDV-specific nuclear RNPs.

Immunoprecipitation. A 20- μ l portion of protein G-Sepharose (Sigma) was washed three times, resuspended in 100 μ l of NTE-NDS buffer (100 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS), and incubated with 2 μ l of rabbit anti-delta antibody at room temperature for 1 h with rocking. The protein G-Sepharose was washed three times, resuspended in 100 μ l of NTE-NDS buffer containing 1 mM dithiothreitol and 80 U of RNasin (Promega), and incubated with 5 μ l of viral RNP or nuclear RNP for 5 h at 4°C with rocking. The pellet and supernatant were assayed by Northern or Western analysis.

RESULTS

UV cross-linking of HDV RNP in virions. We used a sensitive UV cross-linking assay to identify HDV-specific RNPs within HDV virions, since previous attempts to demonstrate such RNPs were not successful (2, 27). UV irradiation can create a covalent cross-link between protein and RNA molecules if they are in direct contact (15). As an assay to detect cross-linking, we used a strategy previously used to demonstrate that a protein is covalently attached to the

FIG. 1. UV cross-linking of HDV particles. (A) UV cross-linking of HDV RNA to protein. Aliquots of HDV were exposed to the indicated doses of UV. Samples were analyzed without ($\blacktriangle$) or with ($\triangle$) pronase treatment. The HDV RNA, not cross-linked to protein, was then recovered after two phenol extractions and assayed by Northern analysis. (B) UV cross-linking of delta antigen. Aliquots of HDV were exposed to the indicated doses of UV. The fraction of free delta antigen, not cross-linked, was assayed by Western analysis.

nucleic acids of hepadnaviruses (12). The principle is that if a nucleic acid molecule contains as little as one molecule of covalently bound protein, after two phenol extractions most of that nucleic acid will have been removed from the aqueous phase. This will not happen to a naked nucleic acid or to one that has been treated with a protease prior to the phenol extraction.

Aliquots of HDV were exposed to a series of doses of UV and subjected to phenol extractions, and the fraction of HDV RNA remaining in the aqueous phase was quantitated by Northern analysis. As shown in Fig. 1A, it appeared that with increasing doses of UV (up to 10 mJ/cm²), we could drive most of the HDV RNA into a form cross-linked to protein. Consistent with this interpretation, we found that when aliquots of the UV-treated virions were digested with pronase prior to the phenol extractions, virtually all of the RNA remained in the aqueous phase. The efficient recovery of full-length RNA after pronase digestion, as demonstrated by its normal electrophoretic mobility (27), indicated that direct damage to RNA could not explain the loss of signal.

The above assay measured the ability of UV to cross-link an unidentified protein to HDV RNA. This protein is presumed to be the delta antigen because previous studies have demonstrated that the delta antigen is an RNA-binding protein that can make specific interactions with HDV RNA

in vitro (8, 18). To test whether the cross-linked protein really was the delta antigen, the amount of delta antigen remaining free after UV cross-linking was quantitated by Western blot. Delta antigens cross-linked to RNA would not be scored as free protein. As shown in Fig. 1B, even after a dose of 10 mJ/cm², as much as 42.5% $\pm$ 3.5% of the delta antigen still migrated as free delta antigen. Therefore, if it is assumed that the delta antigen is the protein that can be cross-linked to HDV RNA, virions must contain a molecular excess of delta antigen relative to HDV RNA.

Using the data from Fig. 1, we estimated the number of protein molecules available for cross-linking to HDV RNA by assuming that (i) the delta antigen was the only protein that could cross-link to the HDV RNA, (ii) all delta antigens cross-link to RNA independently, and (iii) no cross-linking occurs between delta antigens (see the Appendix). With these assumptions, we deduced that the average HDV particle contains at least 22 $\pm$ 2 molecules of delta antigen per HDV RNA which are in direct contact with HDV RNA (average of two experiments).

To determine whether all the delta antigen could, in fact, be cross-linked, we used even higher doses of UV irradiation. As shown in Fig. 1B, after 50 mJ/cm² no free delta antigen was detectable. This result showed that all the delta antigen was cross-linkable but did not prove that this cross-linking was exclusively to HDV RNA. In fact, when we treated the complexes with RNase A prior to electrophoresis, we were unable to release monomers of delta antigen (27). This result suggested that after such high doses of UV irradiation, some of the delta antigen that was lost because of cross-linking could include protein-protein cross-linking rather than just protein-RNA cross-linking. If this is the case, the above deduction of 22 $\pm$ 2 molecules per HDV RNA represents a lower limit to the number of molecules of delta antigen per virion.

The studies described above support but do not establish the interpretation that the delta antigen, a known HDV-specific RNA-binding protein (8, 18), exists within virions in an RNP structure. As a prelude to isolating this putative RNP, we made further use of the UV cross-linking assay to test whether one or more of the buffer conditions used in such in vitro studies (for instance, divalent cations) might stabilize the naturally occurring RNP. For this study, aliquots of virions were resuspended in several different buffers, exposed to 0.35 mJ of UV per cm², and then assayed (as in the experiment whose results are shown in Fig. 1) for the fraction of HDV RNA that was free of protein. As expected, after exposure to this dose in our standard buffer (50 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA), about half of the RNA was still protein free (27). Our standard buffer is actually identical to that used by Lin et al. in their in vitro binding studies (18). When another buffer (40 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.6], 0.3 mM MgCl₂) was tested, as used in previous in vitro studies by Chao et al. (8), virtually the same result was obtained (27). There was no indication that divalent ions were needed to stabilize the RNP interaction. As expected, after addition of 0.1% SDS to our standard buffer, essentially no cross-linking was detectable (27).

Analysis of the virion RNP structure by equilibrium density centrifugation. Others have used nonionic detergent to disrupt the outer envelope of HDV and then tried to demonstrate an RNP by equilibrium centrifugation on CsCl. These studies were without success, in that the delta antigen and HDV RNA separated (2). Therefore, we chose metrizamide as an alternative medium for equilibrium density analysis.

Metrizamide is an iodinated monosaccharide that, owing to its nonionic nature and chemical inertness (24), has certain advantages over CsCl for the analysis of RNP complexes. Metrizamide has been used successfully by others for RNP analysis of other RNA viruses (32).

HDV particles derived from an experimentally infected woodchuck were subjected to equilibrium centrifugation on a 20 to 50% preformed gradient of metrizamide. Each fraction was analyzed for HDV genomic RNA by Northern blot and for delta antigen by Western blot. HDV banded with a density of 1.198 g/cm³ (Fig. 2A). The WHV surface antigens, as detected with an Ausria II kit, banded at about the same density as HDV. Our interpretation, on the basis of electron microscopy (see Fig. 3B), is that in undisrupted virus the major species of particles were not HDV but 24-nm particles, presumably empty surface antigen particles (2).

To isolate the putative virion RNP structure, the virions purified from serum as described in Materials and Methods were disrupted by Nonidet P-40 (NP-40) and dithiothreitol in the presence of 10 mM vanadyl ribonucleoside complex (VRC) (Bethesda Research Laboratories, Inc.) and banded in metrizamide buffered with STE buffer and containing 10 mM VRC. As shown in Fig. 2B, delta antigen and genomic RNA banded separately in this gradient, with densities of 1.255 and 1.157 g/cm³, which were consistent with free protein and free RNA, respectively (Fig. 2B). Although VRC was included here to prevent RNA from being degraded by serum nucleases, our interpretation, in retrospect, was that VRC also disrupted the putative HDV RNP. This result was consistent with previous findings in which VRC disrupted other RNP complexes such as the 27S RNP of the editosome (28) and also interfered with interactions between HDV RNA and delta antigen in Northwestern (RNA-protein) assays (9). Optimistically, this gradient served as a negative control and provided density values for free delta antigen and free HDV RNA.

We next examined virions disrupted with NP-40 and dithiothreitol in the presence of RNasin. As shown in Fig. 2C, we found that HDV genomic RNA and delta antigen banded together at a density of 1.245 g/cm³. This value was intermediate between those of free RNA and free protein. Virtually all the HDV RNA was in this complex. These data were interpreted as evidence for the existence of a stable RNP complex inside the HDV particles.

It should be noted that following disruption of virus with NP-40 and dithiothreitol, the ability to detect WHV envelope proteins (or even HBV surface proteins) by the Ausria II system was lost, presumably because the assay was conformation dependent (27).

The density of an RNP complex can be used to determine the proportion of protein and RNA in that complex (22). To this end, we subjected the disrupted virus to another metrizamide analysis, this time with a more shallow gradient of 45 to 55%, to get better resolution of the RNP complex (Fig. 2D). Again, the genomic RNA and delta antigen were cofractionated. A calculation was then made to determine for this virion RNP the number of molecules of delta antigen per molecule of HDV RNA. This was done by using the experimentally determined densities of the RNP, the free RNA, and the free antigen in that shallow gradient, together with a previously reported formula (22). With the assumption that the viral RNP contains predominantly just two components, the HDV RNA and the delta antigen, we deduced that there are 35 to 95 molecules of delta antigen per genome, with a peak incidence of ca. 70 molecules.

Aliquots of the RNP complex as isolated on metrizamide

FIG. 2. Equilibrium density gradient analysis of HDV-specific RNPs isolated from virion particles. Samples were centrifuged to equilibrium on preformed gradients of metrizamide. For panels A to C, 20 to 50% preformed gradients were used. For panel D, a 45 to 55% preformed gradient was used. (A) HDV particles were banded in STE buffer; (B) HDV particles were treated with 0.5% NP-40 in the presence of RNasin and 10 mM dithiothreitol and then banded in STE buffer containing 10 mM VRC; (C) HDV particles were treated with 0.5% NP-40 in the presence of RNasin and 10 mM dithiothreitol and then banded in STE buffer; (D) same as panel C but with a shallow (45 to 55% metrizamide) gradient.

FIG. 3. Electron-microscopic examination of HDV RNPs. HDV-specific core RNP particles were revealed by negative staining. (A) HDV core particles after NP-40 and dithiothreitol treatment were obtained from the peak fraction of the gradient that was similar to the one shown in Fig. 2C. (B) HDV particles before NP-40 and dithiothreitol treatment, obtained from the peak fraction of the gradient that was similar to the one shown in Fig. 2A. This fraction contained primarily spherical and filamentous WHV surface antigen particles and relatively few HDV particles. Magnification, $\times 255,000$.

from the HDV particles were negatively stained and then examined by electron microscopy. As shown in Fig. 3A, we observed particles that were approximately spherical, with an average diameter of 18.7 ± 2.5 nm ($n = 102$). As a control, we show in Fig. 3B the undisrupted serum particles. This demonstrates a small fraction of relatively large particles, as on the upper left side, with a diameter of 40.4 ± 2.4 nm, which, from our previous study (26), are identified as HDV particles. Most of the particles, however, are smaller, being either spherical or filamentous, with diameters of 23.9 ± 2.1 nm ($n = 102$) and 24.0 ± 2.1 nm ($n = 69$), respectively. These smaller particles are identified as empty WHV surface antigen particles.

Immunoprecipitation of virion core RNP. The results described above indicated that virions contain HDV RNA in a stable RNP complex; however, the results did not show that HDV RNA and delta antigen were present in the same RNP complex. To test this, HDV particles disrupted with NP-40 were subjected to immunoprecipitation with anti-delta antigen antibodies, as described in Materials and Methods. As shown in Fig. 4A, HDV genomic RNA was immunoprecipitated by anti-delta antigen antibodies but not by a preim-

FIG. 4. Immunoprecipitation by anti-delta antigen antibody of HDV RNPs isolated from HDV virion particles (A) and from nuclei of transfected cells (B). After immunoprecipitation with either preimmune (-) or anti-delta antigen sera (+), both pellet (P) and supernatant (S) were assayed for HDV genomic and antigenomic RNAs by Northern analyses and for delta antigen by Western analysis. The percentages of signal in the pellet are indicated below the lanes.

mune serum. Also, after a prior VRC treatment which disrupts the RNP, even anti-delta antigen antibody failed to precipitate the RNA. However, the delta antigen was immunoprecipitable, even after such treatment with VRC. These results therefore provided evidence that the HDV RNA and delta antigen were present in the same RNP complex.

Isolation of HDV-specific RNPs from nuclei. During HDV replication in infected cells, both HDV genomic RNA and delta antigen are localized in the nucleus (6). Since delta antigen has the ability to bind HDV-specific RNA in vitro (8, 18), it was expected that HDV RNA and delta antigen might form an RNP complex in the nuclei of infected cells.

FIG. 5. Equilibrium density gradient analysis of HDV-specific RNPs isolated from nuclei of transfected cells. Samples, isolated as described in Materials and Methods, were centrifuged to equilibrium on 20 to 50% preformed gradients of metrizamide.

Therefore, we tested such nuclei for the presence of such putative HDV-specific RNPs by using equilibrium density centrifugation.

Huh7 cells were transfected with an infectious clone of HDV cDNA, pSVL(D3). After 11 days, the nucleoplasm was isolated, as described in Materials and Methods, and subjected to equilibrium centrifugation on a 20 to 50% preformed gradient of metrizamide. Each fraction was analyzed for HDV RNA and delta antigen. As shown in Fig. 5, it was found that HDV genomic RNA and delta antigen banded together at the same density, quite distinct from that of free delta antigen or free HDV RNA. This result suggested that HDV genomic RNA and delta antigen formed an RNP complex in the nuclei during HDV genome replication. Almost all of the HDV RNA and delta antigen existed in this RNP complex rather than in a free form. In addition, the antigenomic RNA, a complement of genome, was found in same density fraction. Therefore, both antigenomic RNA and genomic RNA were associated with the HDV-specific RNP complex. The density of the nuclear RNP was 1.215 g/cm³, significantly lower than that of virion RNP (1.245 g/cm³) in a similar gradient (Fig. 2C). Similar analysis was done to deduce a number of delta antigen molecules associated with the RNP, as before. Such analysis indicated that nuclear RNPs contain a broad range distribution, from 0 to 85 molecules of delta antigen per RNA, with a peak incidence of about 30 molecules.

To test whether HDV RNA and delta antigen are present in the same RNP complex, we performed immunoprecipitation with nucleoplasm isolated from transfected cells. As shown in Fig. 4B, HDV genomic RNA was specifically immunoprecipitated by anti-delta antigen antibodies but not by preimmune serum. Furthermore, antigenomic RNA was also immunoprecipitated. These results indicated that both antigenomic RNA and genomic RNA were involved in the formation of similar HDV-specific nuclear RNP complexes. After VRC pretreatment, anti-delta antigen antibody did not precipitate the RNA but did precipitate delta antigen. It was noted that relative to the small form of delta antigen, the large form was hardly detectable (less than 5%). This result was consistent with the notion that actively replicating HDV-specific nuclear RNPs should not contain more than 5% of the large form of delta antigen (7).

DISCUSSION

Enveloped viruses usually contain an internal core structure whose roles include the protection of the viral genome and the delivery of that genome to the correct site for genome replication (16). It has been known for some time that the HDV envelope contains both the viral RNA genome and the delta antigen, a known HDV RNA-specific binding protein. Nevertheless, until this study, little was known about the expected internal core structure. Here we have presented evidence for HDV-specific RNP structures, as isolated not only from inside HDV particles but also from the site of HDV genome replication (i.e., the nucleus).

The RNPs as isolated from virions were purified on metrizamide gradients and examined under the electron microscope. By using various assumptions, we were able to deduce, both by the metrizamide gradient analysis and by the UV cross-linking analysis, the number of molecules of delta antigen per molecule of HDV genomic RNA. Analysis of UV cross-linking data was consistent with the interpretation that at least 22 molecules of delta antigen are in contact with RNA (Fig. 1), whereas the metrizamide gradient analysis indicated ca. 70 antigens per RNA. These numbers are significantly smaller than the 180 molecules of the 22-kDa core protein of a hepadnavirus core (21). However, our number for HDV is reasonable when one remembers that the diameter of a hepadnavirus core is 27 nm whereas the diameter of the HDV core is only 18.7 ± 2.5 nm; that is, the volume should be about three times smaller. Further studies are needed to determine whether the roughly spherical HDV cores are in fact icosahedral. If they are, it is relevant to note that 60 subunits is the minimum number consistent with icosahedral symmetry (4).

We were also able to demonstrate HDV-specific RNP complexes in the nuclei of transfected cells by using similar equilibrium density gradients of metrizamide (Fig. 5). Almost all of the nuclear HDV RNA was detected in this RNP complex; very little free HDV RNA was detected. Furthermore, no free delta antigen was detected on the density gradient. Using various assumptions, we deduced the number of molecules of delta antigen per HDV genomic RNA. Such analysis indicated that the nuclear RNP contained a broad distribution with a peak around 30 molecules of delta antigen per HDV genomic RNA. This number was in approximate agreement with an earlier independent estimate of 23 ± 2 molecules, made by Chao et al. (7), on the basis of an observed potent *trans*-dominant negative effect on HDV genome replication exerted by the large form of the delta antigen. However, the value we obtained was significantly smaller than that obtained for the virion RNP. It must also be made clear that the nuclear RNP could have contained proteins other than just the delta antigen; for example, if this RNP is the template for RNA-directed RNA synthesis by host RNA polymerase II, the complex may even contain one or more molecules of this polymerase and even additional transcription factors. If this were true, the nuclear RNP would contain even fewer copies of delta antigen per RNA.

Inside an HDV-infected cell, genome replication and virion assembly take place in two different sites separated by the nuclear membrane, just as in infections by influenza virus (19). Early in infection RNPs either enter the nucleus or are reassembled there. Later, newly made nuclear RNPs are exported to the cytoplasm for virion assembly. The nuclear RNP may follow some kind of maturation pathway, for example, by means of the progressive association of more copies of the large form of the delta antigen, which is

known to function as a dominant negative inhibitor of this replication (7, 13, 17). There must also be interactions of the RNP with the hepadnavirus surface antigens at the endoplasmic reticulum and, finally, the assembly and release of progeny virions. These later steps are somehow also facilitated by the large form of the delta antigen, since its presence is essential for virion assembly (5, 17). An additional relevant question concerns the antigenomic RNA. Both genomic RNA and antigenomic RNA were detected within nuclear RNP complexes (Fig. 5), and yet less than 1% of the RNA in virions is antigenomic (10). Since the copy number of genomic RNA is typically 10-fold higher than that of antigenomic RNA in infected cells (10), it could be that antigenomic RNA is a 10-fold-better template for HDV RNA synthesis. If this were true, it might be that antigenomic RNP is sequestered into the transcription process and is thus relatively unavailable for the assembly pathway.

APPENDIX

We have determined the decrease of both free RNA and protein as a function of UV dose. At least at very low UV doses, cross-linking is a rare event and thus is adequately described as a Poisson process. We do not require this to be true for high UV doses. Let $-\gamma$ be the initial slope of $\log [\text{RNA}]$ versus dose, and let $-\delta$ correspond in the same way to the protein. Then, for small doses, $R = e^{-\gamma d}$ and $P = e^{-\delta d}$ represent the fraction of RNA and protein surviving a dose d without being cross-linked. We assume here that all cross-links are formed between RNA and protein and do not quantitatively account for protein-protein bonds formed. With this assumption, we identify $P = e^{-\delta d}$ not only as the surviving fraction of non-cross-linked protein but also as the probability that each individual protein molecule remains free at dose d . Furthermore, we assume that each of the n protein molecules within the viral capsid becomes cross-linked to the RNA independently of all the others. This being the case, the chance that all n protein molecules are free is $(e^{-\delta d})^n = e^{-n\delta d}$. This, however, is just the condition required for the RNA to be free (not cross-linked). The RNA is free if and only if all n protein molecules are free. Thus, at dose d the chance that the RNA is free, $e^{-\gamma d} = e^{-n\delta d}$. This chance is also the fraction of total RNA that is free. Therefore, $\gamma = n\delta$, and, finally, $n = \gamma/\delta$. Using data from Fig. 1, we estimate for γ , $\log [0.385] = -\gamma d$, where $d = 0.5$, and for δ , $\log [0.425 \pm 0.035] = -\delta d$, where $d = 10$. Therefore, $n = 22 \pm 2$.

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REFERENCES

1. Bergmann, K. F., and J. L. Gerin. 1986. Antigens of hepatitis delta virus in the liver and serum of humans and animals. *J. Infect. Dis.* **154**:702-706.
2. Bonino, F., B. Hoyer, J. W.-K. Shih, M. Rizzetto, R. H. Purcell, and J. L. Gerin. 1984. Delta hepatitis agent: structural and antigenic properties of the delta-associated particles. *Infect. Immun.* **43**:1000-1005.
3. Casey, J. L., K. F. Bergmann, T. L. Brown, and J. L. Gerin. 1992. Structural requirements for RNA editing in hepatitis delta virus: evidence for a uridine-to-cytidine editing mechanism. *Proc. Natl. Acad. Sci. USA* **89**:7149-7153.
4. Casper, D. L. D., and A. Klug. 1962. Physical principles in the

- construction of regular viruses. Cold Spring Harbor Symp. Quant. Biol. 24:1-24.
5. Chang, F.-L., P.-J. Chen, S.-J. Tu, C.-J. Wang, and D.-S. Chen. 1991. The large form of hepatitis delta antigen is crucial for the assembly of hepatitis delta virus. *Proc. Natl. Acad. Sci. USA* 88:8490-8494.
 6. Chang, M.-F., S. C. Baker, L. H. Soe, T. Kamahora, J. G. Keck, S. Makino, S. Govindarajan, and M. M. C. Lai. 1988. Human hepatitis delta antigen is a nuclear phosphoprotein with RNA-binding activity. *J. Virol.* 62:2403-2410.
 7. Chao, M., S.-Y. Hsieh, and J. Taylor. 1990. Role of two forms of the hepatitis delta virus antigen: evidence for a mechanism of self-limiting genome replication. *J. Virol.* 64:5066-5069.
 8. Chao, M., S.-Y. Hsieh, and J. Taylor. 1991. The antigen of hepatitis delta virus: an examination of in vitro RNA-binding activity. *J. Virol.* 65:4057-4062.
 9. Chao, M., and J. Taylor. Unpublished observation.
 10. Chen, P.-J., G. Kalpana, J. Goldberg, W. Mason, B. Werner, J. Gerin, and J. Taylor. 1986. Structure and replication of the genome of hepatitis delta virus. *Proc. Natl. Acad. Sci. USA* 83:8774-8778.
 11. Choi, Y.-D., and G. Dreyfuss. 1984. Isolation of the heterogeneous nuclear RNA-ribonucleoprotein complex (hnRNP): a unique supramolecular assembly. *Proc. Natl. Acad. Sci. USA* 81:7471-7475.
 12. Gerlich, W. H., and W. S. Robinson. 1980. Hepatitis B virus contains protein attached to the 5' terminus of its complete DNA strand. *Cell* 21:801-809.
 13. Glenn, J. S., and J. M. White. 1991. *trans*-Dominant inhibition of human hepatitis delta virus genome replication. *J. Virol.* 65:2357-2361.
 14. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
 15. Greenberg, J. R. 1979. Ultraviolet light-induced cross-linking of mRNA to proteins. *Nucleic Acids Res.* 6:715-732.
 16. Helenius, A. 1992. Unpacking the incoming influenza virus. *Cell* 69:577-578.
 17. Kuo, M. Y., M. Chao, and J. Taylor. 1989. Initiation of replication of the human hepatitis delta virus genome from cloned DNA: role of delta antigen. *J. Virol.* 63:1945-1950.
 18. Lin, J.-H., M.-F. Chang, S. C. Baker, S. Govindarajan, and M. M. C. Lai. 1990. Characterization of hepatitis delta antigen: specific binding to hepatitis delta virus RNA. *J. Virol.* 64:4051-4058.
 19. Martin, K., and A. Helenius. 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell* 67:117-130.
 20. Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato. 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. *Cancer Res.* 42:3858-3863.
 21. Onodera, S., H. Otori, M. Yamaki, and N. Ishida. 1982. Electron-microscopy of human hepatitis B virus cores by negative staining-carbon film technique. *J. Med. Virol.* 10:147-155.
 22. Perry, R. P., and D. E. Kelly. 1966. Buoyant density of cytoplasmic ribonucleoprotein particles of mammalian cells: distinctive character of ribosome subunits and the rapidly labeled components. *J. Mol. Biol.* 16:255-268.
 23. Ponzetto, A., P. J. Cote, H. Popper, B. H. Hoyer, W. T. London, E. C. Ford, F. Bonino, R. H. Purcell, and J. L. Gerin. 1984. Transmission of the hepatitis B virus-associated delta agent to the eastern woodchuck. *Proc. Natl. Acad. Sci. USA* 81:2208-2212.
 24. Rickwood, D., and G. D. Birnie. 1975. Metrizamide, a new density gradient media. *FEBS Lett.* 50:102-110.
 25. Rizzetto, M., B. Hoyer, M. G. Canese, J. W. K. Shih, R. H. Purcell, and J. L. Gerin. 1980. Delta agent: association of delta antigen with hepatitis B surface antigen and RNA in serum of delta-infected chimpanzees. *Proc. Natl. Acad. Sci. USA* 77:6124-6128.
 26. Ryu, W.-S., M. Bayer, and J. Taylor. 1992. Assembly of hepatitis delta virus particles. *J. Virol.* 66:2310-2315.
 27. Ryu, W.-S., H. J. Netter, M. Bayer, and J. Taylor. Unpublished observation.
 28. Smith, H. C., S.-R. Kuo, J. W. Backus, S. G. Harris, C. E. Sparks, and J. D. Sparks. 1991. In vitro apolipoprotein B mRNA editing: identification of a 27S editing complex. *Proc. Natl. Acad. Sci. USA* 88:1489-1493.
 29. Taylor, J. 1992. The structure and replication of hepatitis delta virus. *Annu. Rev. Microbiol.* 46:253-276.
 30. Wang, K.-S., Q.-L. Choo, A. J. Weiner, J.-H. Ou, R. C. Najarian, R. M. Thayer, G. T. Mullenbach, K. J. Denniston, J. L. Gerin, and M. Houghton. 1986. Structure, sequence and expression of the hepatitis delta viral genome. *Nature (London)* 323:508-514.
 31. Weiner, A. J., Q.-L. Choo, K.-S. Wang, S. Govindarajan, A. G. Redeker, J. L. Gerin, and M. Houghton. 1988. A single antigenomic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides p24^δ and p27^δ. *J. Virol.* 62:594-599.
 32. Wunner, W. H., R. M. L. Buller, and C. R. Pringle. 1976. Separation of viruses in metrizamide density gradients, p. 159-173. In D. Rickwood (ed.), *Biological separations in iodinated density gradient media*. IRL Press, Oxford.
 33. Zheng, H., T.-B. Fu, D. Lazinski, and J. Taylor. 1992. Editing on the genomic RNA of human hepatitis delta virus. *J. Virol.* 66:4693-4697.