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Assembly of Hepatitis Delta Virus Particles

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Hepatitis delta virus (HDV) is a subviral satellite of hepatitis B virus (HBV). Since the RNA genome of HDV can replicate in cultured cells in the absence of HBV, it has been suggested that the only helper function of HBV is to supply HBV coat proteins in the assembly process of HDV particles. To examine the factors involved in such virion assembly, we transiently cotransfected cells with various hepadnavirus constructs and cDNAs of HDV and analyzed the particles released into the medium. We report that the HDV genomic RNA and the delta antigen can be packaged by coat proteins of either HBV or the related hepadnavirus woodchuck hepatitis virus (WHV). Among the three co-carboxy-terminal coat proteins of WHV, the smallest form was sufficient to package the HDV genome; even in the absence of HDV RNA, the delta antigen could be packaged by this WHV coat protein. Also, of the two co-amino-terminal forms of the delta antigen, only the larger form was essential for packaging.

Hepatitis delta virus (HDV) was discovered in 1977 in a study of liver biopsy specimens from patients already infected with hepatitis B virus (HBV) (30). From this and subsequent work by Rizzetto and coworkers it was made clear that HDV is a subviral agent which replicates in nature as a satellite of HBV and that its replication in the liver can significantly increase the amount of liver damage relative to that caused by an infection with HBV alone (30). Experimental transmission studies show that HDV not only can infect a chimpanzee (31) if HBV is present, but also can infect a woodchuck (28) if the corresponding woodchuck hepadnavirus, woodchuck hepatitis virus (WHV), is present.

The envelope of HDV is unquestionably provided by the helper hepadnavirus, be it HBV (31) or WHV (28). These two hepadnaviruses each make use of three forms of envelope or surface antigen (sAg). These three related proteins differ only in terms of their amino-terminal extensions and are described according to their size as large, middle, and small. In the infectious hepadnavirus particle, the Dane particle (11), these proteins are present in the ratio of 1:1:4, respectively (17). In serum, such Dane particles are always accompanied by an excess of noninfectious empty particles, both spherical and filamentous. The spherical particles are composed almost entirely of small sAg (17). In a similar study of partially purified HDV particles, the ratio was estimated to be about 1:5:95 (3). In this respect, HDV particles more closely resemble empty spherical HBV particles than they resemble Dane particles.

Inside the HDV particle there is the delta antigen, the only known protein of HDV, and the RNA genome. The single-stranded RNA genome of HDV is unusual in at least three ways (35): (i) it is very small, being about only 1,700 nucleotides in length; (ii) it is circular; and (iii) it has the ability to fold on itself, with about 70% of the bases paired, to form an unbranched rodlike structure (19, 37). Transfection studies (18) have established three important conclusions regarding the replication of the HDV genome: (i) it is independent of any sequence or function provided by the hepadnavirus; (ii) it is not even dependent upon the host cells being of liver origin; and (iii) it is strictly dependent upon the presence of delta antigen.

Delta antigen is encoded not by the genomic RNA, but by its complement, the so-called antigenomic RNA (10). This protein is present not only inside the HDV particles but also within the nucleus of the infected cell. This small (22-kDa) protein is highly basic and has been shown in vitro to have an RNA-binding ability specific for HDV RNA (7); more recently, it has been found that this binding depends, at least in part, upon the rodlike structure of the HDV RNAs (8).

There are two related forms of delta antigen (2, 37, 38): the small (S) form, of 195 amino acids, and the large (L) form, of 214 amino acids, which, relative to the S form, has a 19-amino-acid carboxy-terminal extension. It is the S form that is essential for the replication of the genome (18). During the course of genome replication, as initiated by using this S form, in the absence of the L form, there nevertheless soon appears a population of modified HDV genomes that encode the L form. The change occurs at a specific site; it corresponds to a change from A to G in the amber termination codon of the S form (24). There is no more than circumstantial evidence that this change is directed by a host-encoded RNA-modifying enzyme that acts preferentially on double-stranded RNAs (1). The L form not only fails to support genome replication but also acts as a dominant negative inhibitor of the ability of the S form to support such replication (9). In the studies described here, we investigated the process of virus assembly by using cells cotransfected with various combinations of recombinant DNAs encoding HDV RNA and proteins as well as hepadnavirus proteins. Our findings include evidence that the L form of the delta antigen plays an essential role in the assembly of HDV particles.

MATERIALS AND METHODS

Plasmids. In most studies we used the expression vector pSVL (Pharmacia). As previously described (18), cloned HDV cDNAs were inserted to create pSVL(D3), an infectious trimer of unit-length HDV, and pSVL(Ag-S), a cDNA of less than unit length that expressed antigenomic RNA that functioned as a mRNA for the synthesis of the S form of the delta antigen. pSVL(Ag-L) differed from the latter only in that the amber termination codon was changed, allowing the synthesis of the L form of the delta antigen. The infectious HBV construct pSV2A-Neo-$(HBV)_2$ was a gift of Chiaho Shih (34), and the infectious WHV clone pCMW82 was a gift of Christoph Seeger (33). Part of the latter clone, the 1.4-kb

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*Xho*II-*Xho*II fragment from positions 1593 to 3046 (19), was subcloned into pSVL to make pSVL(WHV-sAg). This clone expressed only the small sAg of WHV.

Transfection and analysis of released particles. A human hepatoma cell line, Huh7 (25), was transfected by using a calcium phosphate procedure (15) with the plasmids as indicated. Briefly, 4×10^6 cells were seeded onto a 100-mm-diameter dish 1 day prior to transfection with 25 μg of plasmid. The medium was harvested every 3 days from day 3 to day 12. It was clarified by low-speed centrifugation and then subjected to ultracentrifugation (Beckman SW40; 35,000 rpm for 16 h at 4°C) through a cushion of 20% isotonic sucrose in STE (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 0.1% bovine serum albumin.

Centrifugation to equilibrium on gradients of metrizamide. Particles collected as described above were resuspended and layered on a preformed gradient of 20 to 50% metrizamide (Sigma) in STE containing 10 mM vanadyl ribonucleosides (Bethesda Research Laboratories). The gradient was underlaid with 0.2 ml of 95% metrizamide and then subjected to ultracentrifugation (Beckman SW60; 42,000 rpm for 22 h at 4°C). Fractions were collected from the bottom. Aliquots of each fraction were analyzed for viral RNA and protein, as described below. Also, refractive index measurements were made to deduce the density of the fractions.

Immunoblot analysis. Protein samples were subjected to electrophoresis on 12% acrylamide gels by the method of Laemmli (20). The protein was then transferred electrophoretically to a nitrocellulose filter. As previously described (18), the delta antigen was detected by using a human polyclonal serum (a gift from John Gerin) followed by ^{125}I-labeled protein A (DuPont). After autoradiography, quantitation was performed via densitometry.

Radioimmunoassay. The Ausria II assay kit (Abbott) was used, as recommended by the manufacturer, to detect the WHV surface antigens. The data obtained were subsequently transformed to a linear scale by means of a calibration curve.

RNA analyses. Total nucleic acid was extracted both from sedimented particles and from the cells, as previously described (10), by a method involving sodium dodecyl sulfate and pronase. These nucleic acid samples were collected by precipitation with ethanol, treated with DNase I, and extracted once more. RNA samples were glyoxalated prior to electrophoresis into gels of 1.2% agarose. The RNA was then transferred electrophoretically to a nylon membrane and hybridized, as previously described (10), with specific RNA probes. The filter was subjected to autoradiography in the presence of two intensifying screens and then quantitated directly with a Radioanalytic Imaging System (AMBIS) to determine the amount of radioactivity in specific regions. An HDV genomic RNA standard was made in vitro with a pGem4Z (Promega) construct containing a unit length of HDV cDNA and was used to determine the number of HDV molecules per milliliter of tissue culture fluid.

Electron microscopy. Fractions from the metrizamide gradients were examined by negative staining. Samples of 2 μl were applied to a 300-mesh copper grid with a carbon-coated Formvar film. After 5 to 8 min, the liquid was blotted off and washed twice with double-distilled water. The grid was stained for 5 min on 0.5% uranyl acetate, blotted, and placed in the vacuum of a Philips 420 electron microscope. Micrographs were taken at ×37 to ×62,000 magnification. The particle dimensions were measured from magnified prints.

TABLE 1. Quantitation of HDV RNA released from cells, as particles, into the medium

Cotransfection[a]	% HDV RNA in particles[b]	No. of particles/ml[c]
None	<0.08	$<2.0 \times 10^6$
HBV	0.40	3.6×10^6
WHV	0.68	8.2×10^6
Small sAg of WHV	4.07	22.3×10^6

[a] All transfections contained pSVL(D3) to provide HDV genome replication. As indicated, the cotransfecting plasmids were to provide hepadnavirus sAg.

[b] Northern analyses were used on RNA both from the transfected cells at day 9 and from the RNA released as particles into the medium. Quantitation of the HDV RNA was done directly by using a radioanalytical imaging system to measure the radioactivity on the nylon membrane.

[c] In the Northern analyses we also used HDV RNA standards (1 and 0.1 ng of 1.7-kb genomic RNA) as synthesized in vitro. One nanogram is equivalent to 10^9 molecules.

RESULTS

Assembly of HDV RNA into particles. To determine the factors and the specificities involved in HDV virion assembly, we used transient cotransfection of cells with a series of hepadnavirus constructs and cDNA of HDV. The tissue culture medium was collected every 3 days up to 12 days. Then these fluids were clarified by low-speed centrifugation and sedimented through a cushion of 20% sucrose to collect viruslike particles. RNA was extracted from the pellet and examined by a Northern (RNA) assay for HDV RNA. In parallel, an aliquot of the RNA from the transfected cells was also examined.

The results from a series of such cotransfections are summarized in Table 1. When cells were transfected with an infectious cDNA of HDV, pSVL(D3), alone, HDV RNA genome replication was detected in the cells, as expected (18), and less than 0.08% of this RNA was detectable in the tissue culture medium. In contrast, when the cells were cotransfected with a plasmid that initiated replication and assembly of HBV particles, we detected 0.40% of the HDV RNA in particles. A similar amount, 0.68%, was obtained when the cells were cotransfected with a plasmid that initiated the replication of WHV. When cells were cotransfected with a plasmid that did not allow hepadnavirus replication but expressed only the small sAg of WHV, the efficiency of assembly was as high as 4.07%. It is important to note that in this latter case, the other two envelope proteins of WHV, large and middle sAg, were absent. It was both expected (23) and observed (32) that this construct, by itself, led to the assembly of empty noninfectious particles.

By using appropriate standards we were able to determine the number of HDV RNA-containing particles in the tissue culture fluid. For the particles assembled with only the small sAg of WHV, there were more than 2×10^7 particles per ml. Such a number is typical of virus titers in the serum of chronically infected animals (26), but still far short of reported values of 1×10^{12} particles per ml seen at the peak of an acute infection (29).

To determine whether the particles released into the medium were similar to HDV obtained from infected animals, we subjected the particles to equilibrium centrifugation on preformed gradients of metrizamide. After centrifugation, fractions were collected and each was assayed for HDV RNA, HDV antigen, WHV sAg, and density. Results are shown in Fig. 1A for the particles assembled by using only the small sAg of WHV. The delta antigen and RNA

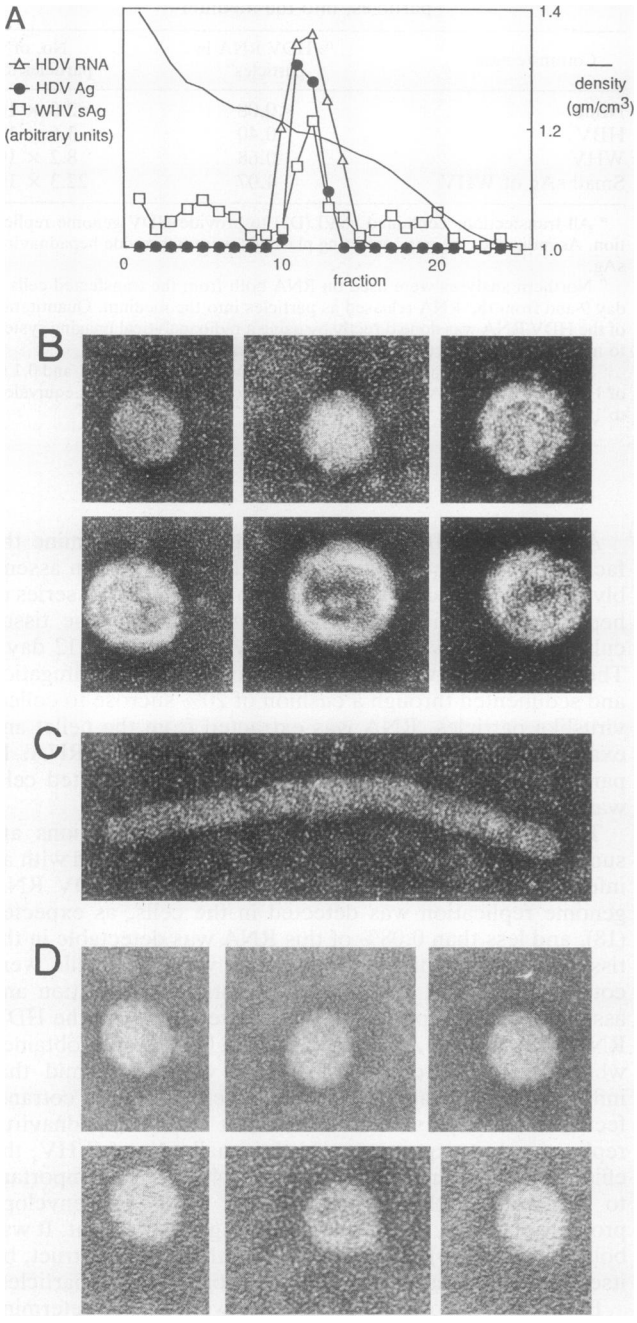

FIG. 1. Characterization of assembled HDV particles by equilibrium centrifugation on a metrizamide gradient. Huh7 cells were cotransfected with plasmids pSVL(D3) and pSVL(WHV-sAg). The fluids were collected and sedimented through a cushion of 20% sucrose and then centrifuged to equilibrium, as described in Materials and Methods, on a preformed gradient of metrizamide. Fractions were collected and assayed for HDV RNA (by Northern analysis), HDV antigen (by Western blot), WHV sAg (by radioimmunoassay), and density (by their refractive index relative to standard solutions). (A) Results of these determinations. (B) Electron-microscopic examination of HDV-like particles in fraction 12. (C) Empty filamentous form found in the same fraction as in panel B. (D) Similar examination of HDV particles, as purified above, but initially obtained from the serum of an infected woodchuck. Magnification, ×255,000 (panels B to D).

banded together, and the sAg was also detected through this region. The density of the HDV particles was deduced to be 1.192 g/cm^3. This value was in agreement with 1.198 g/cm^3, our independent measurements of density for HDV obtained from the serum of an infected woodchuck (32). Also, this density was intermediate between those of free HDV antigen and HDV RNA, which were determined to be 1.260 and 1.159 g/cm^3, respectively (32). Therefore we concluded that the assembled particles were not significantly contaminated with either free antigen or free RNA and that the majority of the released HDV particles were indistinguishable from particles obtained from infected animals.

Electron microscopy was also used to examine the assembled particles and compare them with those obtained from an infected woodchuck. As shown in Fig. 1B, the particles assembled with WHV small sAg ranged from a size similar to that reported by others (4) to larger particles (16) containing indications of a corelike structure. The mean diameter of assembled HDV-like particles was 42.6 ± 7.7 nm. Also seen was a comparable number of particles, with a smaller diameter, 24.3 ± 2.2 nm, both spherical and filamentous, which were probably empty filamentous forms. An example of the filamentous form is given in Fig. 1C. Others have been unable to see such filamentous particles released from cells transfected with the HBV small sAg (see reference 23 and references therein); the basis for this difference is not yet known.

As shown in Fig. 1D, when we examined HDV purified from the serum of an infected woodchuck, we obtained a similar mean diameter of 40.4 ± 2.4 nm for the particles.

Assembly of HDV antigen into particles in the absence of HDV RNA. The above studies showed that both delta antigen and HDV RNA were packaged into viruslike particles. We next tested whether the delta antigen alone, without HDV RNA, could assemble into particles. To do this the cotransfections were only slightly different; we replaced the pSVL(D3) with constructs that were insufficient to allow HDV genome replication, but expressed only a part of the antigenome, so as to allow expression of the delta antigen. We used various combinations of two such plasmids, pSVL (Ag-S) and pSVL(Ag-L), which express the S and L forms, respectively (9). As above, these combinations were cotransfected, this time with only the construct for the small sAg of WHV, since we have already shown that this protein alone is sufficient to make viruslike particles (Table 1). Again the tissue culture fluid from the cotransfected cells was collected and sedimented through a cushion of 20% sucrose. Aliquots of these pellet samples, along with aliquots of the cells, were examined by Western immunoblot to detect the delta antigens; the results are shown in Fig. 2. As expected, the delta antigens were detected in each of the transfected cell samples. Also, in the absence of WHV sAg (Fig. 2, lanes 1 to 3), no delta antigen was released into the medium. However, in the presence of the small WHV sAg (lanes 4 to 6), the results were striking. The S form of the delta antigen was not packaged (lane 4), whereas the L form was (lane 5). In fact, as much as 3.8% of the L form was packaged, in contrast to less than 0.2% of the S form. However, when cells were transfected with both the S and L forms of delta antigen (lane 6), both species were packaged. One obvious interpretation was that the S form must have made some prior interaction with the L form, so as to be packaged. It is important to emphasize that the HDV cDNAs used to transfect these cells did not contain the whole HDV sequence; thus there was neither replication of the genome nor even RNA of genomic polarity. The observed packaging was

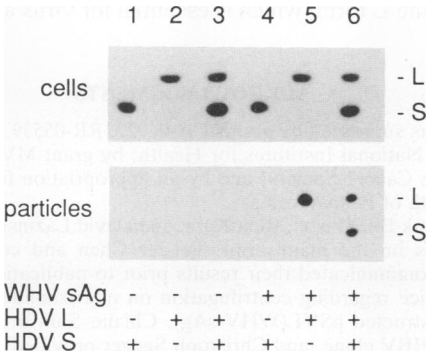

FIG. 2. Packaging of HDV antigens in the absence of HDV RNA, by the small sAg of WHV. A monolayer of Huh7 cells (60-mm dish) was cotransfected, and after 3 days the fluids and cells were harvested and examined by Western blot analysis. Each lane represents either 1/25 of the cell sample or 1/5 of the pellet sample. Six different combinations of plasmids were used in the cotransfections: lane 1, pSVL(Ag-S); lane 2, pSVL(Ag-L); lane 3, pSVL(Ag-S) + pSVL(Ag-L); 4 to 6, same as lanes 1 to 3 except that pSVL(WHV-sAg) was also present. On the right are indicated the positions of the two forms of the delta antigen (L and S).

therefore achieved without delta antigen first making an interaction with HDV genomic RNA.

To understand the prior interaction between the S and L forms that allowed the S form to be packaged, we carried out a series of similar cotransfections with different relative amounts of the plasmids expressing these two delta proteins. The absolute amount of pSVL(Ag-L) was kept constant, while the relative amounts of pSVL(Ag-S) were 0, 0.5, 1, 2, and 4. Then, as before, we used Western analyses to quantitate the species of delta antigen in both the transfected cells and the particles released into the medium. The results of this quantitation are summarized in Fig. 3. (As expected, the relative masses of plasmids used to for the transfection approximately determined the relative amounts of the two delta proteins produced within the cell.) The relative amounts within the particles were quite different from the values obtained for the cells from which these particles were released. As the S/L ratio in the cell was progressively

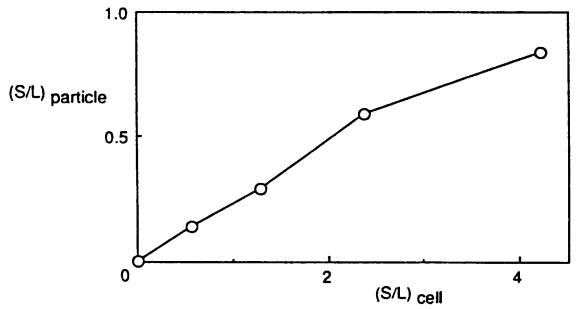

FIG. 3. Ratio of S to L forms of the delta antigen in particles released from cells, in the absence of HDV RNA. Transfections and assays were as for Fig. 2. Each transfection contained pSVL(WHV-sAg). Additionally present were various amounts of pSVL(Ag-S) and/or pSVL(Ag-L). Fluids were harvested at 3 days, and then the released particles were collected and analyzed, along with samples of the transfected cells, by Western blot to detect and quantitate the amounts of S and L forms of the delta antigen. The relative amount of the S form with respect to the L form is shown for the cells (horizontal axis) and for the released particles (vertical axis).

raised, the S/L ratio in the particles increased proportionally, but at about 25% of the rate. Our simple interpretation is based upon the observation that the S protein can get into these particles only if it is essentially chaperoned by one or more molecules of L protein.

The above model may be too simple, and further experiments are needed to determine the size of this essential prior assembly component, but the conclusion is still clear that the S form of the delta antigen can enter such particles only via an interaction with the L form, which in turn may interact with the WHV sAg.

DISCUSSION

By means of cotransfecting cells with various HDV and hepadnavirus constructs, we have demonstrated four key features about the assembly of delta antigen and RNA into particles: (i) the smallest hepadnavirus sAg is sufficient for packaging of HDV; (ii) the L form of the delta antigen is required for the assembly of such particles; (iii) the L form of the delta antigen can be assembled in the absence of HDV RNA; and, in contrast, (iv) the S form cannot be so assembled, unless it is aided by the presence of the L form.

Certain new questions arise from this work. They can be divided into three groups, insofar as they relate to HDV or hepadnaviruses or both. First, consider the implications for HDV. The data show that the S form of the delta antigen, even though it supports genome replication (18), is not sufficient for particle assembly. The L form is needed for this. These data confirm the prior finding of Chang et al. (6). We now have two potential explanations for the significance of the observation that during replication a fraction of HDV genomes encoding the S form are specifically changed so as to encode this L form (24). First, the appearance of the L form is a means of turning off genome replication (9). Second, this L form is an essential facilitator of virus assembly. Thus it would seem that the genome alteration that leads to the synthesis of the L form of the delta antigen is an essential part of the HDV life cycle.

The present data also show that the S form can be packaged if the L form is also present. One explanation is that during or, more probably, prior to packaging, there was an interaction between the available forms of the L and S forms of the antigen. This interaction occurred even in the absence of genomic HDV RNA. Also, the amount of the S form relative to the L form that was packaged was about four times smaller than the ratio present in the cell (Fig. 3). The two forms of the delta antigen each contain two domains analogous to the so-called leucine zipper (21), which in other contexts is known to facilitate protein dimerization (22). Also, cross-linking has been used to detect dimers and even tetramers of the delta antigen (21), and there is a report that even higher protein multimers have been found (14). However, our evidence for multimerization is only indirect and our experiments do not allow us to deduce the size of the putative multimer.

We encountered the following puzzling discrepancy in the relative abundance of the S and L forms between our two types of assembly experiments. When particles were assembled in the absence of HDV RNA, the S form was always less abundant than the L form, and yet in contrast, for particles assembled in the presence of HDV RNA, the S form was relatively more abundant. More specifically, for the RNA-containing particles shown in Fig. 1, the ratio was about 3.9 (32). The values reported by others for particles as seen in natural HDV infections, the ratios range from about

0.5 to 10 or even higher (2, 12, 38). Why, then, does the ratio not exceed 1 for the particles we have assembled in the absence of HDV RNA? One interpretation is that multimers of delta antigen assemble before or after binding to HDV RNA, and if many such multimers are packaged with RNA into particles, it is possible to obtain S/L ratios higher than 1.

We must also address the difference between the S and L forms that makes the S form but not the L form support genome replication and the L form but not the S form support packaging. The answer is probably more complicated than the suggestion that the 19-amino-acid C-terminal extension acts as some kind of unique signal per se. Support for this statement comes from a recent study by Glenn and White (13) in which several variants of the delta antigen, which differed only in terms of the length of this C-terminal extension, were constructed. Of these, some variants were, like the L form (9), able to inhibit the ability of the S form to support genome replication, whereas others were not. It would be interesting to know whether these variants support virus assembly. Probably the length variations of the delta antigen have some effects at the level of folding of the total protein, and these effects in turn modulate at least two different kinds of essential interactions: those needed for genome replication and those needed for packaging. Almost certainly these essential interactions include at least antigen-antigen interactions as well as interactions with the viral RNA.

The second area that should be discussed is that concerning the hepadnaviruses. We have shown here that only one of the three known hepadnavirus coat proteins is needed. This extends the findings of a previous report that with purified HDV, the amounts of large and middle sAg, relative to small sAg, were less than 5% (3).

Assembly of infectious hepadnavirus particles needs not only small sAg, but also large sAg (5) and possibly even middle sAg (36). Since there is evidence that it is the domain unique to large sAg (sometimes called the pre-S1 domain) which interacts with the host receptors (27), our assembly studies with only small sAg open the possibility that such HDV particles, if proven to be infectious, may make use of a receptor other than that used by the hepadnaviruses.

The results of our studies in which the coat of HBV was used to assemble HDV confirm those of Wu et al. (39). In addition, we have shown here that the coat of WHV packages just as well. This is not too surprising, because studies with experimental animals clearly show that both HBV and WHV can be used to package HDV (28, 31). It may be important that another hepadnavirus, duck hepatitis B virus, has not yet been demonstrated to act as a packaging agent for HDV, either in animals (32) or in packaging experiments such as reported here (32). Apparently, there are limits to the packaging specificity that is tolerated, even among hepadnaviruses.

The third and last area to be considered is that of actual interactions between HDV and the hepadnaviruses. We know from the works of others that the small sAg of the hepadnaviruses is very efficient in making empty particles (23), but we do not know how the L form of the delta antigen is able to interact with this protein and end up packaged inside the released particles or why the S form is unable to make this interaction. This story becomes more complex when we attempt to understand the additional interactions that must be made with the HDV RNA. Probably the HDV genomic RNA, as made in the nucleus, interacts with at least the S form of the delta antigen. Subsequently there will have to be some significant change in this ribonucleoprotein to include the L form, which is essential for virus assembly and release.

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