Role of Two Forms of Hepatitis Delta Virus Antigen: Evidence for a Mechanism of Self-Limiting Genome Replication

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The replication of the RNA genome of hepatitis delta virus is greatly facilitated by the presence of the only known virus-coded protein, the delta antigen. Most, if not all, infections are characterized by the presence of two electrophoretic forms of the delta antigen. These forms correspond to polypeptide lengths of 195 and 214 amino acids which are encoded by genomes with different nucleotide sequences. We used cDNA transfections to investigate the functions of these two forms of the delta antigen. We found that only the small form of delta antigen supported hepatitis delta virus genome replication and that the large form acted as a dominant negative repressor of such replication. This inhibition was potent. For example, the amount of genome replication was reduced eightfold when as little as 10% of the delta antigen was present as the large form. One interpretation of our results is that the delta antigen normally functions as part of a multimeric structure. In addition, our data suggest that synthesis of the large form, either during genome replication in cultured cells or even during infection in animals, may suppress delta replication, possibly leading to a self-limiting infection.

Hepatitis delta virus (HDV) is a subviral satellite of hepatitis B virus. The envelope of HDV is composed of the surface proteins of hepatitis B virus (2). Inside the particle are the HDV RNA genome and related forms of the one protein that HDV is known to encode, the delta antigen (1, 2, 4, 20). The RNA genome is a single-stranded RNA, about 1,700 bases in length; it is exceptional (relative to the genomes of other RNA viruses of animals) in that it is in the form of a covalently closed circular species and also because this species has the ability to fold on itself by base pairing to form an unbranched rodlike structure (4, 12).

HDV replication takes place through RNA-directed RNA synthesis (4, 13, 17). Therefore, an early step in infection is the transcription of genomic RNA into a complementary RNA, referred to as the antigenome (4), which serves as a template for subsequent synthesis of genomic RNA. cDNA clones of the HDV RNA can be used to transfect animal cells and initiate the replication of the RNA genome either from a DNA-directed genomic RNA transcript (6, 11, 18) or from an antigenomic transcript (unpublished data). Transfection studies have established that HDV genome replication is independent of hepatitis B virus sequences or functions but is dependent on the presence of the delta antigen (6, 11).

Immunoblot analyses show that for most natural HDV infections, both serum and liver samples contain two electrophoretic forms of the delta antigen. The corresponding nucleotide sequence information has shown that both forms of the delta antigen are encoded by the antigenomic RNA; infected animals contain HDV sequence variants corresponding to a 195- and a 214-amino-acid species, with the difference in the proteins being restricted to a 19-amino-acid carboxy-terminal extension on the larger form (1, 2, 15, 21; M. Chao, S.-Y. Hsieh, G. Luo, and J. Taylor, *in J. L. Gerin, R. H. Purcell, and M. Rizzetto, ed., The Hepatitis Delta Virus*, in press).

An intriguing aspect of the two forms of delta antigen was recently revealed by cDNA transfection studies (11, 15). It was found that a cDNA clone containing the coding region

for the small form of delta antigen not only initiates genome replication but also ultimately leads to the accumulation of genomic variants that code for the large form of delta antigen. It is not yet known how such variants arise, although there is circumstantial evidence for the involvement of a cellular RNA-modifying activity (15). We now present results which suggest why such variants might accumulate during the course of genome replication. By cotransfection of cells with DNAs encoding the large and small forms of the delta antigen, we found that the large form was not only unable to support genome replication but was also a strong inhibitor of the *trans*-acting activity of the small form, as evidenced by an inhibition of genome replication by the large form. Our data are consistent with the interpretation that the essential role of the delta antigen in genome replication is dependent on the formation of multimers of this protein.

MATERIALS AND METHODS

Vectors and recombinant clones. Most of the transfection studies were carried out with clones of HDV cDNA inserted into the eucaryotic expression vector pSVL (Pharmacia, Inc.). The clones pSVL(D3) and pSVL(D2M) were as previously described (11). pSVL(D2M) contains two copies in tandem of the 1,679-base HDV genome, as permuted at the unique *EcoRI* site at position 1427 according to the sequence of Kuo et al. (11). Unlike pSVL(D3), this clone has a deletion of two bases, 1434 to 1435, relative to the normal sequence within the coding region for the delta antigen and is oriented relative to the simian virus 40 late promoter in pSVL such that genomic HDV RNA can be transcribed. Another clone, pSVL(Ag-S), uses the simian virus 40 promoter to express a fragment of the antigenomic sequence, from the *BglII* (base 224) to the *XbaI* (base 781) site, that encodes the delta antigen. This clone expresses the small, 195-amino-acid form of the delta antigen. A mutant of this clone, pSVL(Ag-L), contains a G rather than an A at position 1012 within the termination codon for the small antigen. This mutation leads to the synthesis of a longer, 214-amino-acid form of the delta antigen.

Radioactive RNA probes were used for Northern (RNA)

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analyses. For maximum sensitivity, we used a probe to the full length of the HDV genome, as previously described (11). However, to detect antigenomic RNA, a fragment of HDV cDNA, from *Bgl*III (base 224) to *Xba*I (base 781), was inserted into a transcription vector, pGem4blue (Promega Biotec) and used to make an RNA probe. This probe did not share any sequences with those present in pSVL(Ag-S) and pSVL(Ag-L).

Transfections. Monolayer cultures of COS7 cells were prepared by seeding 16-mm wells with 200,000 cells. After 1 day, the cultures were transfected by using DEAE-dextran followed by a dimethyl sulfoxide shock (5). The amount of pSVL(D3) or pSVL(D2M) used was 250 ng. For the cotransfections, we also used 250 ng of pSVL(Ag-S) and the indicated amounts of pSVL(Ag-L). In each experiment, salmon sperm DNA was used to keep the total amount of DNA per transfection at a constant value.

RNA extraction and Northern analyses. As previously described (9, 11), the total nucleic acid was first extracted from the transfected cells by using pronase in the presence of sodium dodecyl sulfate, which was followed by phenol-chloroform and then two ether extractions. The nucleic acids were collected by precipitation with ethanol and were further treated with RQ DNase I (Promega) to remove any residual plasmid DNA; these steps were followed by an additional extraction. These samples were collected and denatured by using glyoxal in the presence of heat and dimethyl sulfoxide before being electrophoresed into 1.5% agarose gels. After electrophoresis, the RNA was electrophoretically transferred to a nylon membrane (ZetaProbe; Bio-Rad Laboratories), linked by exposure to UV light (Stratolinker; Stratagene), and then hybridized with region- and strand-specific HDV probes, as described above.

Immunoblot analyses. After transfection, cell cultures were solubilized in the buffer described by Laemmli (14) and separated by electrophoresis into a 15% polyacrylamide gel. Proteins related to the delta antigen were then detected by a standard immunoblot procedure with an antibody to delta antigen, by incubation with ¹²⁵I-staphylococcal protein A, and finally by autoradiography.

RESULTS

The following experiments were undertaken to determine the roles of the large and small forms of the delta antigen, especially in terms of their abilities to support the replication of the HDV RNA genome. The strategy was to use DNA transfections to determine how HDV genome replication depended upon the relative amounts of large and small forms of the delta antigen.

A potential complication for such a study is that during the replication of the HDV genome, a cytoplasmic mRNA species which acts as the template for the translation of the delta antigen is also synthesized (9). Thus, there is normally a linkage between the amount of genome replication and the synthesis of delta antigen. Therefore, we decided to obviate this linkage by initiating genome replication through cotransfections with two and even three distinct plasmids. As a source of an RNA genome for subsequent replication, we transfected a DNA construct that contained a mutation in the open reading frame for the delta antigen. We then provided the two forms of delta antigen by cotransfection with separate expression plasmids encoding the forms of the delta antigen. In this way, the ratio of large to small delta antigen could be controlled independently of genome replication, since the sole source of the delta mRNA would be by transcription of the transfected DNA templates.

A head-to-tail dimer of the mutant genome, pSVL(D2M), was therefore cotransfected into COS7 cells along with different amounts of expression plasmid for small delta antigen, pSVL(Ag-S), or large delta antigen, pSVL(Ag-L). At day 6 after transfection, the replication of the HDV genome was examined by a Northern analysis. The mutant genome gave a result indistinguishable from those of untransfected cells (Fig. 1A, lanes 1 and 12). The presence of small antigen supported genome replication (Fig. 1A, lane 2). The large antigen was not only unable to support replication (Fig. 1A, lane 3) but in fact was able to inhibit the ability of the small antigen to support replication (Fig. 1A, lanes 4 through 7). [Even the replication of a nonmutated genome, pSVL(D3), could be inhibited by the presence of antigen produced by pSVL(Ag-L) (Fig. 1A, lanes 8 through 11).] When the Northern hybridization was made not with the probe for genomic RNA but with a region-specific antigenomic probe, we obtained essentially similar results except that the amount of HDV-specific signal was about ten times less (data not shown).

To demonstrate that these results were coincident with delta antigen formation, we used an immunoblot analysis to determine the ratio of large to small delta antigen in the transfected cells. These results are shown in Fig. 1B.

Both the Northern hybridization and immunoblot data were subjected to quantitation, and the results are summarized in Table 1. The immunoblot data showed that there was only a small discrepancy between the observed amounts of large and small antigen and the amounts predicted on the basis of the relative inputs of the two plasmids used in the cotransfections. We therefore examined the relationship between the amount of genome replication observed and the actual amounts of large antigen relative to those of small antigen present per culture. Some of the results from Table 1 are shown graphically in Fig. 1C. Clearly, genome replication was inhibited by relatively low amounts of the large antigen. In order to see whether these levels of inhibition might be a function of the time after transfection, we compared the results at earlier (day 4) and later (day 8) time points. The results were essentially the same (data not shown). From both Table 1 and Fig. 1C, it can be seen, for example, that with as little as 11% of large delta antigen relative to amounts of small delta antigen, genome replication was inhibited by 88%.

DISCUSSION

We have studied, under controlled and well-defined conditions, the abilities of the two forms of the delta antigen to support the replication of the HDV genome. We found that the small but not the large form of the antigen was competent in this respect. In addition, we found that when both species were present within a cell, the large antigen was able to suppress the action of the small antigen. This is what Herskowitz has called a dominant negative mutation (7). Furthermore, the inhibitory effect was potent. For example, even 1 part in 10 of large antigen relative to the small antigen was enough to reduce genome replication to 12% of the control value achieved with small antigen alone. The possible explanations for how the large antigen inhibits depend on the mechanism, as yet unknown, by which the small antigen acts to support genome replication. Consider the following three possibilities. (i) If the small antigen functions as a monomer, then inhibition by the large antigen could be as a competitive inhibitor. However, this is unlikely, unless the large antigen has a considerably greater affinity than the

FIG. 1. Replication of HDV, as controlled by the two forms of the delta antigen. COS7 cells were transfected with one or more specific HDV clones and then examined after 6 days for HDV genome replication by either Northern analysis (A) or immunoblot analysis (B). These data were quantitated as summarized in footnotes *c* and *d* of Table 1. (A) cDNA transfections were as follows. Lanes: 1, pSVL(D2M); 2, pSVL(D2M) + pSVL(Ag-S); 3, pSVL(D2M) + pSVL(Ag-L); 4, pSVL(D2M) + pSVL(Ag-S) + 0.1 equivalents of pSVL(Ag-L); 5, pSVL(D2M) + pSVL(Ag-S) + 0.3 equivalents of pSVL(Ag-L); 6, pSVL(D2M) + pSVL(Ag-S) + 1 equivalent of pSVL(Ag-L); 7, pSVL(D2M) + pSVL(Ag-S) + 3 equivalents of pSVL(Ag-L); 8, pSVL(D3); 9, pSVL(D3) + 0.1 equivalents of pSVL(Ag-L); 10, pSVL(D3) + 0.3 equivalents of pSVL(Ag-L); 11, pSVL(D3) + 1 equivalent of pSVL(Ag-L); 12, nontransfected control. The RNA samples were separated on a 1.5% agarose gel, and HDV genomic sequences were detected with a strand-specific probe. Lane M, End-labeled *Hind*III fragments of bacteriophage lambda DNA. Position of the 1,700-base RNA genome is indicated on the right. (B) Similar transfected samples examined by immunoblot analysis to detect delta antigen-related proteins. The mobilities of the large and small delta antigens are indicated at the right as L and S. (C) The relationship between genome replication and the amount of the large form relative to the small form of the delta antigen. The data shown in panels A and B were used to make quantitations as in Table 1.

small antigen for the substrate that is acted on. (ii) If the antigen normally acts as a dimer, it is possible that the presence of the large antigen leads to the formation of nonfunctional dimers. In this respect, it is relevant to point out that the delta antigen does have one (or even two)

TABLE 1. Quantitation of genome replication and synthesis of delta antigens in transfected cells

Transfection ^a	Expected abundance of large to small antigens ^b	Observed abundance of large to small antigens ^c	Observed genome replication ^d
2	0	0	100
4	0.1	0.03	45 ± 4
5	0.3	0.11	12 ± 3
6	1	0.67	<1
7	3	1.8	<1
3	∞	∞	<1

^a The transfections are numbered as described in the legend to Fig. 1.

^b This expectation was based on the relative masses of pSVL(Ag-L) and pSVL(Ag-S) used in transfection.

^c This was obtained by quantitation of the immunoblots by densitometric analysis of the autoradiogram.

^d This was obtained by quantitation of the Northern analyses by direct counting of the nylon membrane with an AMBIS radioanalytic imaging system. The standard deviations were deduced from four separate transfections.

potential leucine zipper structures (M. M. C. Lai, Y.-C. Chao, M.-F. Chang, J.-H. Lin, and I. Gust, in J. L. Gerin, R. H. Purcell, and M. Rizzetto, ed., *The Hepatitis Delta Virus*, in press), which have been shown by others to facilitate dimer interactions (10). Again, this model would not explain the observed potency of inhibition. (iii) Finally, what if the delta antigen functions as a multimer? Consider some possible precedents. With the hepadnaviruses, the presence of a relatively small amount of defective core protein acts as a potent inhibitor of the assembly of normal core protein into multimeric core particles (8). Similarly, for the retrovirus human immunodeficiency virus type 1, defective *gag* protein interferes with core assembly (19). If the large delta antigen were acting in such a way, we could estimate the size of the multimer. For example, if the multimer contains *n* subunits of either small or large antigen, but the presence of just one large antigen renders the multimer nonfunctional, then our data, as summarized in Fig. 1C and Table 1, are consistent with $n = 23 \pm 2$. There are, of course, numerous variables that could render this estimate inaccurate (e.g., cooperativity in binding). It is important to mention that the multimer structure could be assembled on another molecule, such as the viral RNA. Such a possibility would be consistent with the reported HDV-specific RNA-binding ability of both forms of the delta antigen (3; E. J. Gowans, T. B. Macnaughton, A. R. Jilbert, and C. J. Burrell, in J. L. Gerin, R. H. Purcell, and M. Rizzetto, ed., *The Hepatitis Delta Virus*, in press). This interpretation is also consistent with what is known for an infectious RNA that is present in certain strains of yeast. This RNA, known according to its sedimentation value as 20S RNA, has recently been shown to be HDV-like in several ways, including circularity (Y. Matsumoto, R. Esteban, R. Fishel, and R. B. Wickner, in J. L. Gerin, R. H. Purcell, and M. Rizzetto, ed., *The Hepatitis Delta Virus*, in press). What is relevant here is that in infected cells, the 20S RNA is found in a ribonuclease-resistant ribonucleoprotein particle that has been estimated by rate-zonal and equilibrium-density analyses to contain 18 to 20 identical subunits of a 23-kilodalton protein (22).

Although the large form of the delta antigen suppressed genome replication, it may still have relevance to other aspects of the HDV life cycle. As mentioned above, this large antigen has RNA-binding activities *in vitro* (3). Also, it should not be forgotten that in an infected host, both small

and large forms of the antigen are packaged into new virions (1, 2). It is tempting to speculate that the specific HDV sequence alteration that occurs during genome replication and leads to the appearance of the large antigen, as has been documented by Luo et al. (15), represents a mechanism to suppress further genome replication and begin packaging of genomic RNA into new virus particles. In such a model, only those packaged genomes that encode the small form of the delta antigen might be able to go on and successfully infect another cell.

A suppression of genome replication, as documented here in transfected cells, might also occur in the liver of patients with chronic infections. Most patients appear to express both forms of the delta antigen (1). Also, the relative amount of the large antigen is such that we might predict that in the liver, genome replication has already been largely suppressed.

A related argument might also apply. That is, when a patient goes through a peak of acute infection, it must be then that the large form appears to rescue the patient. It must be a very potent inhibitor of continued replication, because the acute phase lasts less than a week and then the appearance of HDV RNA drops off dramatically. If the change does not occur in time, that acute infection may rapidly go on to fulminant hepatitis and lead to death (16).

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