JOURNAL OF VIROLOGY, Aug. 1991, p. 4057–4062
0022-538X/91/084057-06$02.00/0

Vol. 65, No. 8

The Antigen of Hepatitis Delta Virus: Examination of In Vitro RNA-Binding Specificity

MEI CHAO, SEN-YUNG HSIEH, AND JOHN TAYLOR*

Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111

Received 12 February 1991/Accepted 24 April 1991

The only known protein of hepatitis delta virus (HDV), the delta antigen, is found both within virus particles and within the nucleus of the infected cell, where it has one or more roles essential for RNA genome replication. Others have demonstrated that the antigen has the ability, in vitro, to specifically bind HDV RNA species. We report a further examination of this phenomenon, using partially purified recombinant protein, expressed as a fusion with the staphylococcal protein A. From Northwestern (RNA-immunoblot) analyses with both complete and various subdomains of HDV genomic and antigenomic RNAs, we found that a necessary feature for specific binding was that the RNA be able to fold to some extent into the so-called rodlike structure; this structure is a predicted intramolecular partial base-pairing of the circular RNA, with about 70% of all bases involved, so as to produce an unbranched rodlike structure. Six different subregions of the HDV rodlike structure, three on the genomic RNA and three on its complement, the antigenomic RNA, were tested and found to be sufficient for antigen binding. However, features in addition to the rodlike structure may also be necessary for specific binding, because we found that a similar structure present in the RNA of the potato spindle tuber viroid did not allow binding.

The genome of hepatitis delta virus (HDV) is a single-stranded RNA molecule. This RNA is unique among the genomes of other known animal viruses: at about 1,700 nucleotides, it is the smallest; its conformation is not linear but circular; and from the known nucleotide sequence, it is expected that this RNA can fold on itself by base-pairing of about 70% of all the nucleotides to form what could be described as an unbranched rodlike structure (6, 15, 30, 31, 33). This genome is replicated in the nucleus of an infected cell (32) by RNA-directed RNA synthesis, involving the synthesis of a complementary RNA that is also circular and which has been referred to as the antigenome (6).

In nature, HDV replicates only in the liver of persons who are also infected with hepatitis B virus. From tissue culture experiments, we know that HBV is not needed for the replication of the HDV genome (17), but it does provide the envelope proteins, the so-called surface antigens, necessary for the packaging of progeny HDV virions (2). These virions contain not only the HDV genomic RNA but also the so-called delta antigen (1, 2).

The delta antigen is the only known protein expressed by HDV. It is encoded on the antigenomic RNA and is expressed in the infected cell by means of a noncircular cytoplasmic polyadenylated RNA of about 800 nucleotides (6, 10). In nature, there seem to be two main delta antigen proteins, a 195- and a 215-amino-acid species (1). These are translated from genetically different RNA species; the difference is that for some RNAs, a single base in the amber termination codon of the open reading frame of the smaller form of the delta antigen is changed, so as to allow synthesis of the protein that is 19 amino acids longer (33, 34). A puzzling observation is that even when replication is initiated from a single sequence, encoding only the smaller protein, the same change can occur on some HDV RNAs, so that both proteins are actually synthesized (21, 29). This change is apparently of some significance; the smaller protein has been shown to be essential for genome replication, while in contrast, the larger form is not only insufficient, but can act as a dominant negative mutation to suppress the replication facilitated by the smaller protein (5).

From the predicted amino acid sequence of the delta antigen, we expect it to be a highly basic protein (18). It is thus not surprising that both the small and large forms have, in vitro, an RNA-binding ability (3, 8, 20, 23). In addition, the binding ability is specific for HDV RNA (8, 20, 23). Lin et al. have further shown that the ability of the large delta antigen to bind RNA is dependent upon an internal domain of no more than 75 amino acids; this domain includes two arginine-rich regions which are similar to those found in certain other RNA-binding proteins (20). Since this internal domain is of course present in both forms of the delta antigen, it remains to be determined why only one form is able to support genome replication.

The studies described here were undertaken (i) to determine what feature(s) of the HDV RNA might be the basis for the shared binding ability of these two forms of the delta antigen and (ii) to see whether, in a side-by-side comparison, we might detect some difference between the RNA-binding abilities of the large and small delta antigens so as to explain the quite different functional roles of the two forms. We found that the ability of the HDV genomic and antigenomic RNAs to fold into the rodlike structure was an essential feature for specific RNA binding, and we were able to detect a functional difference in this respect between the two forms of the delta antigen.

MATERIALS AND METHODS

Plasmids. The HDV cDNA that was used was cloned and sequenced by Kuo et al. (18). Both full-length and subgenomic fragments of this DNA were subcloned into the transcription vector pGem4B (Promega). The subgenomic fragments were based on the restriction map presented in Fig. 1. Also subcloned were a region of the genome of Rous

* Corresponding author.

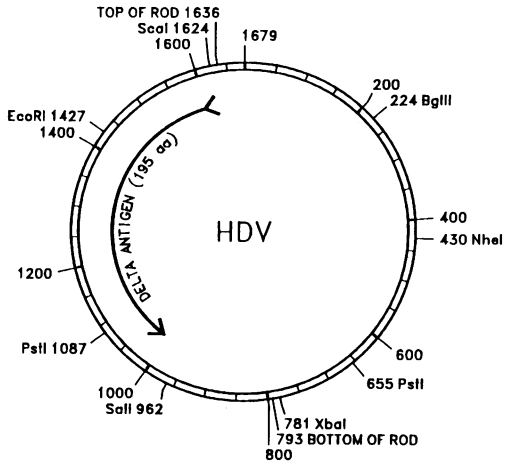

FIG. 1. Restriction map of HDV cDNA. The circle shows the 1,679-base sequence of HDV, as deduced by Kuo et al. (18), with the top and bottom of the rod and the open reading frame for the small form of the delta antigen indicated. Computer-based analysis of sequence information was done with the sequence analysis software package of the University of Wisconsin Genetics Computer Group (7). aa, amino acids.

sarcoma virus (22) and a dimer of the genome of potato spindle tuber viroid (PSTV) (26).

RNA syntheses. Subgenomic regions of genomic and antigenomic HDV RNA were transcribed from the pGem4B constructs, after appropriate cleavage of these clones, with the RNA polymerases of phage SP6 or T7 (Bethesda Research Laboratories [BRL]). In this way we were able to generate the 20 different RNA species that are diagrammed in Fig. 3. The method of RNA synthesis was that recommended by BRL with [α-^{32}P]UTP (800 Ci/mmol; Du Pont). For the experiment shown in Fig. 4B, we also added ITP during synthesis as an alternative to GTP (25) in the amounts indicated in the figure legend. The various labeled RNAs were extracted and tested for integrity by gel electrophoresis.

Fusion proteins. We followed the manufacturer's protocols for application of the vector pRIT2T (Pharmacia). The delta antigen open reading frame was inserted C-terminal to a 26-kDa domain of the staphylococcal protein A. Expression of the fusion protein in *Escherichia coli* was then heat induced, after which the protein was extracted and partially purified by a single passage over a Sepharose-immunoglobulin affinity column.

To obtain the initial recombinant, the antigenomic sequence from positions 1598 to 894 was amplified by polymerase chain reaction of a cDNA template with a *Pvu*II adaptor primer and a regular primer (11). This allowed the relevant sequence to be cleaved from the product with *Pvu*II and *Sal*I and then force-cloned into pRIT2T DNA that had been cut in its multiple cloning site with *Sma*I and *Sal*I. The resultant construct expressed the small form of the delta antigen. To obtain the larger form, we replaced the region between two *Pst*I sites, one at 1087 in HDV and the other in the multiple cloning site of pRIT2T, with a corresponding region from pSVL(Ag-L), which encodes the large antigen (5).

Gel analyses. Denaturing gels were made by the procedure of Laemmli (19) with 8% polyacrylamide. After this, the proteins were transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell). The protein was then located by staining with india ink (9), and the delta antigen-related material was detected by incubation with a specific human antibody, followed by incubation with ^{125}I-protein A of *Staphylococcus aureus* and autoradiography.

Alternatively, the membrane with the transferred protein was used to detect RNA-binding activity with a procedure based on the method of Krogstad and Champoux (16). Briefly, the protein on the filter was denatured by incubation in 7 M guanidine hydrochloride, after which it was washed briefly, incubated in a buffer containing glycerol, sodium chloride, and Nonidet P-40 for 1 day at 4°C, and then washed and incubated in HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid)-buffered 0.4% nonfat powdered milk at room temperature. This second incubation, as explained in the text, had to be at least 16 h long for us to detect specific binding in the subsequent steps. The filter was then incubated with about 5×10^5 cpm of [^{32}P]RNA per ml at room temperature for 30 min. In some cases, as noted in the text, not one but two RNAs were used in such incubations, and in these cases, it was necessary to hybridize these two RNAs to each other prior to the incubation step (27). After the incubation, the filter was rinsed for 30 min at room temperature and finally examined by autoradiography. From preliminary studies, we determined the amount of each fusion protein needed to give optimal binding specificity; with five times less than the chosen amount, the level of binding was not above the background level.

The gel retardation assays were carried out basically as described by Konarska and Sharp (14). Briefly, the partially purified fusion protein was incubated with 10^5 cpm (total) of labeled RNA in 10 μl of binding buffer (10 mM HEPES [pH 7.6], 0.3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 1 mM dithiothreitol, 40 U of RNasin [Promega], 1 mg of tRNA per ml). After 30 min at 30°C, the sample was mixed with dye and subjected to electrophoresis in a nondenaturing gel of 4% polyacrylamide. After this, the gel was dried and examined as described above.

RESULTS

Delta antigen fusion proteins in a Northwestern analysis. The delta antigen is present both inside virions obtained from serum and in infected liver. However, when we used such materials in a Northwestern (RNA-immunoblot) analysis, we were unable to detect specific RNA binding. Thus, we undertook to begin again, but with much larger amounts of antigen, as can be obtained with a partially purified recombinant protein. The small and large forms of the delta antigen were obtained this way by using a fusion with part of the staphylococcal protein A. These two partially purified fusion proteins were then subjected to acrylamide gel electrophoresis, electrotransferred to a nitrocellulose filter, and tested as follows.

We compared the total protein on the filter, as detected by staining with india ink (Fig. 2A), with the regions that responded to an antibody specific to the delta antigen (Fig. 2B). We identified the expected fusion proteins and the staphylococcal protein itself.

The next step was to test these proteins in a particular RNA-binding assay, a so-called Northwestern assay. The results are shown for labeled HDV RNA (Fig. 2C) and a single-stranded non-HDV RNA (Fig. 2D). More specifically, the HDV RNA was full-length genomic RNA, and the non-HDV RNA was that transcribed from the unmodified RNA transcription vector pGem4B. The results show specific

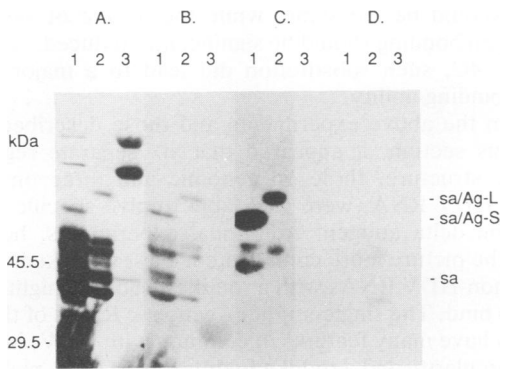

FIG. 2. Verification of fusion proteins. Three recombinant proteins were expressed in bacteria and partially purified by affinity chromatography prior to electrophoresis in a gel containing 8% polyacrylamide. Proteins: lane 1, staphylococcal protein A fused to the small form of the delta antigen (sa/Ag-S); lane 2, protein A fused to the large form of the delta antigen (sa/Ag-L); lane 3, unmodified protein A (sa). After electrophoresis and electrotransfer to nitrocellulose, the filter was examined four ways: (A) india ink staining; (B) Western immunoblot with antibody specific for the delta antigen; (C) Northwestern blot with labeled full-length genomic HDV RNA; (D) as in panel C, but with non-HDV RNA transcribed from unmodified pGem4B. The positions of prestained protein electrophoresis markers are indicated at the left.

binding of the HDV RNA to the fusion proteins of the small and large delta antigens. It can be noted that of the bands reactive with the antibody (Fig. 2B), only the largest bands were also able to react with HDV RNA (Fig. 2C).

To obtain such specific binding, we followed the protocol of Krogstad and Champoux (16). After electrotransfer, the protein on the filter was denatured fully by incubation with guanidine hydrochloride, and the filter was subjected to incubation at 4°C and then at room temperature prior to addition of the labeled RNA. The extent of this second incubation period was important. Without it, RNA binding was nonspecific, and as the length was increased from 1 to 3 to 16 h, we saw the appearance of specific binding. At the longest time, the specificity was deduced to be at least 100-fold (Fig. 2C and D). No further specificity was achieved when the incubation was extended to 48 h. Our interpretation is that during the incubation time, not only was blocking being achieved, but the protein was undergoing an appropriate renaturation. To pursue this further, we carried out an experiment to quantitate the effects of varying the length of the second incubation. The results as summarized in Table 1, showed that the binding ability of recombinant protein sa/Ag-S (staphylococcal protein A fused to small delta antigen) not only increased absolutely with time, but also increased maybe as much as three times more than that of recombinant protein sa/Ag-L (staphylococcal protein A fused to large delta antigen).

Regions of HDV sequence necessary for RNA binding. Our next question was to determine whether one or more specific regions on the HDV RNA were essential for binding. Figure 3 summarizes the results for 20 different RNAs that were tested, 10 genomic and 10 antigenomic. Each of the regions is indicated in relationship to the circular RNA, drawn in its predicted rodlike folding, and underneath is an indication of whether the RNA was able to bind to the delta antigen fusion protein. The experiments were performed for both the small and large fusion proteins, and the results were identical.

Consider first the genomic HDV RNAs shown in panels 1 to 10 of Fig. 3. The full-length RNA bound (panel 1), but five smaller regions (panels 2 to 6), which in total spanned the whole genome, were individually unable to bind. Even one side of the rod (panel 7) or the other (4) was unable to bind. However, we noted that when the region was able to fold intramolecularly into an end of the rodlike structure, as in panels 8 and 9, binding occurred. Not only did these two end regions bind; when we assembled a middle region, as shown in panel 10, binding also occurred. In panel 10, the assembly was intermolecular, that is, between two RNA species, and as will be subsequently made clearer, it was necessary, in

TABLE 1. Effect of renaturation time on signal strength obtained in Northwestern analyses

Renaturaton time (days)[a]	Signal bound[b]		Signal ratio, sa/Ag-S to sa/Ag-L
	sa/Ag-S	sa/Ag-L	
0	100	80	1.25
0.7	60	40	1.5
2	210	60	3.5
4	380	120	3.2
6	410	95	4.3

[a] As in Fig. 2, multiple samples of the two recombinant proteins, sa/Ag-S and sa/Ag-L, were transferred electrophoretically from the gel to the filter. The filter was divided into equivalent strips, treated with guanidine hydrochloride, and incubated at 4°C, first in buffer containing glycerol, sodium chloride, and Nonidet P-40 and then in buffer containing HEPES and 0.4% nonfat powdered milk. The total of these incubation times was 7 days, and the time indicated is that of the second incubation.

[b] The filter strips were incubated together with [^{32}P]RNA transcribed as described for panel 8 in the legend to Fig. 3. The resulting autoradiogram was quantitated by densitometry.

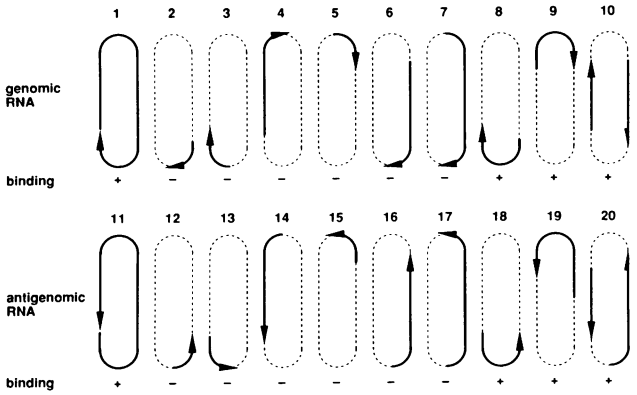

FIG. 3. Regions of HDV RNA necessary for binding to delta antigen. Northwestern analyses of the delta antigen fusion protein were carried out as described in the legend for Fig. 2C except that 20 different labeled HDV RNA species were tested individually. Panels 1 to 10 contained genomic RNA, and panels 11 to 20 contained antigenomic RNA. The approximate locations of these RNAs on the rodlike structure of HDV are indicated in the figure. The solid line represents the unlabeled RNA, with the arrowhead being the 3′ end of that RNA; the broken line represents the remainder of the rodlike structure. RNA-binding ability is indicated beneath each panel. The precise locations can be deduced from the restriction map in Fig. 1, along with the following descriptions of the cDNA fragments that were transcribed from the pGem4B constructs: 1, positions 962 to 961; 2, 655 to 781; 3, 781 to 1087; 4, 1087 to 1624; 5, 1624 to 224; 6, 224 to 781; 7, 1624 to 781; 8, 655 to 1087; 9, 1427 to 224; 10, 224 to 655 and 1087 to 1427; 11 to 18 are complements of 1 to 8, respectively; 19, 430 to 1427; 20, 781 to 224 and 1427 to 1087.

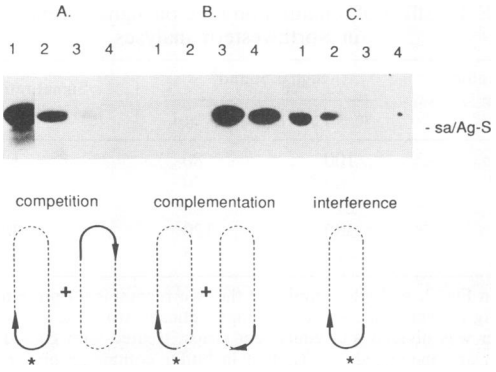

FIG. 4. Evidence that HDV rodlike structure is necessary for RNA-binding specificity. Northwestern analyses were carried out with the small delta antigen fusion protein but with three different labeling procedures. The representation of HDV RNAs used in binding is as described in the legend to Fig. 3, except that the labeled species are indicated beneath with an asterisk. (A) Competition between one labeled rodlike HDV RNA and increasing amounts of a different unlabeled rodlike HDV RNA. Labeled/unlabeled RNA ratios: lane 1, 1:0; lane 2, 1:10; lane 3, 1:30; lane 4, 1:90. (B) Complementation experiment with one labeled nonrod HDV RNA and increasing amounts of unlabeled RNA from the opposite side of the rod. Labeled/unlabeled RNA ratios: lane 1, 1:0; lane 2, 1:0.25; lane 3, 1:1; lane 4, 1:4. (C) Interference experiment in which the labeled HDV rodlike RNA was synthesized with increasing amounts of ITP relative to GTP. ITP/GTP ratios: lane 1: 0:1; lane 2, 0.2:0.8; lane 3, 0.5:0.5; lane 4, 0.8:0.2.

order for binding to occur, that the two RNAs first be hybridized to each other.

When these experiments were repeated with antigenomic HDV RNAs (panels 11 to 20), we obtained virtually the same correlation; that is, binding was observed only if a part of the rodlike structure could be formed.

Further evidence that rodlike structure is necessary for HDV RNA binding. Figure 4 presents three additional experiments undertaken to test the interpretation that the rodlike structure of HDV RNAs was necessary for specific binding to the delta antigens.

The first experiments were to test whether the binding could be inhibited. As summarized in Fig. 4A, the binding ability of a region of labeled rodlike genomic HDV RNA could be competed with by the presence, during incubation, of an increasing excess of unlabeled rodlike RNA from a different region of the genome. Such competition could also be achieved with unlabeled RNA from the same region, but not with a subdomain of HDV genomic RNA that was unable to fold into the rodlike structure (4).

The second experiments were to see whether a labeled RNA from a region of the genome that could not bind could ultimately be made able to bind by hybridization to an unlabeled RNA from the other side of the rod structure. As summarized in Fig. 4B, this complementation did indeed occur. The inverse experiment, in which the labeled strand was replaced by an unlabeled strand and vice versa, gave an identical result (4).

The third experiments were to see whether labeled RNA from a region capable of intramolecular folding into the rodlike structure might lose its binding ability if it were deprived of its folding ability. To do this, we synthesized the labeled RNA with increasing amounts of ITP. This substrate can substitute for GTP (25), and should lead to a product in which the degree of base-pairing, of the Watson and Crick type, should be the same, while the degree of associated hydrogen bonding should be significantly reduced. As shown in Fig. 4C, such substitution did lead to a major loss of RNA-binding ability.

From the above experiments and those described in the previous section, it appeared that six separate regions of rodlike structure, three on genomic and three on antigenomic HDV RNA, were each able to give specific binding with the delta antigen. Additional experiments, however, made the picture more complicated. First we asked whether other non-HDV RNAs with a rodlike structure might also be able to bind. The single-stranded genomic RNAs of the plant viroids have many features in common with HDV, including both circularity and a rodlike folding (30, 31). We picked one such viroid, PSTV. From a dimer of cDNA inserted into pGem4B, we transcribed labeled RNA and tested it for binding. Relative to HDV rodlike RNA, the PSTV RNA, which could have folded into a rod, failed to bind to delta antigen (4). (The PSTV RNA species used was a dimer rather than a monomer. We believe this does not weaken our conclusion because trimers of HDV, as well as monomers, still bind efficiently [4].) The second level of complication came when we tested 100% double-stranded RNAs. In each case, we used a pGem4B construct to synthesize a pair of complementary RNAs, prehybridized these, and tested them for binding to the delta antigen. We tested three such hybrids: (i) a nonrod region on the HDV genome; (ii) a 400-base sequence from the genome of Rous sarcoma virus (22); and (iii) the dimer of PSTV RNA. All of these double-stranded species gave efficient binding (4). In contrast to this, both double- and single-stranded DNAs failed to bind (4).

From the above observations of RNA binding, we were concerned with the possibility that rodlike RNAs or 100% double-stranded RNAs might not bind more efficiently, but preferentially escape some RNase activity during incubation with the nitrocellulose filter. Three arguments can be made against this possibility. First, the results of the incubations were unchanged by the presence or absence of a relatively large amount of unlabeled tRNA. Second, we tested the labeled RNAs both before and after incubation by glyoxal gel electrophoresis (24) and were unable to detect any degradation. Third, the PSTV RNA was expected to be a rodlike structure, and it failed to bind.

Gel retardation assays of HDV RNA binding. The studies described above were carried out with a single assay procedure, the Northwestern. As described below, we undertook to confirm the results with a second procedure, a gel retardation assay. We used the method of Konarska and Sharp (14), in which the labeled RNA is incubated with the fusion protein before being loaded onto a nondenaturing acrylamide gel. Typical results are shown in Fig. 5 for both an HDV rodlike RNA and a non-HDV non-rodlike RNA. It can be seen that as the amount of fusion protein was adjusted, some specificity, as measured by the amount of retarded HDV RNA relative to that for non-HDV RNA, could be achieved (lane 3), but even this specificity was lost when more fusion protein was used (lane 4). (The complexity of the retarded band was reproducible but remains unexplained. It could be because of alternative conformations of the labeled RNA. It may also be due to lack of size homogeneity for the antigen [see Fig. 2A].) Direct quantitation of the signals in panels A and B, lanes 3, indicated about a 10-fold greater level of binding to the HDV RNA. We first optimized certain other annealing variables, such as the

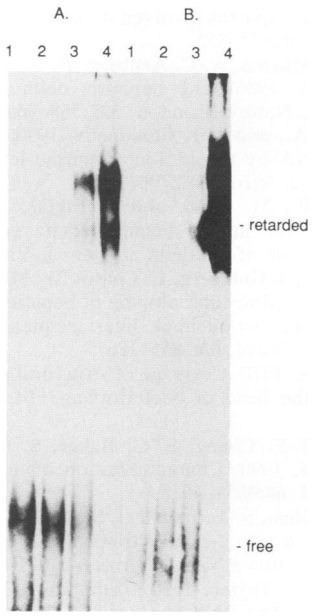

FIG. 5. Gel retardation assay of RNA binding to antigen. (A) Aliquots of labeled HDV rodlike RNA were incubated with increasing amounts (0, 0.4, 1, and 2 μl in lanes 1 to 4, respectively) of a concentrated solution of the partially purified fusion protein with the small antigen prior to electrophoresis on a nondenaturing gel. (B) Similar analysis except that the labeled RNA was replaced by that made from unmodified pGem4B.

presence of a competitor RNA, the concentration of magnesium ion, and the temperature, in order to get this result.

In summary, only after careful adjustment of several variables could even a limited amount of HDV-specific RNA binding be demonstrated by this gel retardation assay. Thus, the evidence of specificity for the rodlike structure comes only from the Northwestern analysis.

DISCUSSION

Our results on the binding of the two forms of delta antigen to HDV RNA species are consistent with those of two previous studies, which showed that certain HDV RNA species bound more efficiently than non-HDV RNAs (8, 20, 23). However, our data appear to be the first evidence for a role of HDV RNA structure. The most abundant naturally occurring species of genomic and antigenomic HDV RNAs are circular and are predicted to be able to fold by intramolecular base-pairing into a rodlike structure. We have actually tested a total of six different rodlike regions on the genome and antigenome and found that they all give antigen-specific binding in a Northwestern assay. However, certain qualifications must be made regarding the interpretation of our results.

(i) Our evidence for specificity was from the Northwestern assays, not from the gel retardation assays. The success of the former results could be a consequence of the ability to allow a renaturation of the recombinant protein prior to the binding assay.

(ii) It may be relevant that we did not use natural delta antigens. They were not only protein fusions; because they were grown in *E. coli*, they may have lacked some critical posttranslational modifications, such as phosphorylation at serines (3), that could be necessary for full function. One can state, however, that Macnaughton et al. (23) did use the small form of the delta antigen as grown in animal cells and in the absence of any fusion, but nevertheless were unable to achieve as much specificity as reported here.

(iii) It might seem inconsistent that the rodlike RNA of PSTV, unlike that of HDV, did not also bind. It is unlikely that this was due simply to a difference in the extent of intramolecular base-pairing; PSTV has 70% base-pairing (26), versus 74% for the HDV sequence of Kuo et al. (18). The quality of pairing may be relevant; crudely, PSTV has 54% G+C, while the HDV sequence of Kuo et al. has 65% G+C. This interpretation might fit with our data on the effects of ITP substitution; when the G+C of the rodlike HDV RNA was reduced from 65% to no less than 52%, there was a dramatic loss of binding ability (Fig. 4C, lane 2). Another explanation is that it is not the rod per se but something within or associated with the rod that is necessary for binding. After all, PSTV replicates without the need for any encoded protein; thus, both PSTV and HDV RNAs may have a basic rod structure so as to facilitate RNA-directed RNA synthesis. Proceeding further with this logic, we speculate that the HDV genome might have evolved further, so as to be able not only to code for a protein, but to have multiple sites within its rodlike structure for the binding of that protein to allow certain nonviroid functions, especially packaging.

(iv) We are puzzled that 100% double-stranded RNAs, both HDV and non-HDV, were able to bind as efficiently as rodlike HDV sequences. However, it must be noted that the regions of such undisrupted strictly double-stranded RNA in the predicted rodlike structure of Kuo et al. (18) are short; the longest is only 11 bp. Also, we need to question the relevance of 100% double-stranded RNA to the in vivo situation; we know that such sequences are avoided by animal cells, presumably because if unchecked, they can lead to induction of interferons (28).

(v) Our observations that both the large and small forms of the delta antigen will bind HDV RNA are consistent with the studies of Lin et al. (20); they have defined, within the coding region shared by both proteins, a domain essential for RNA binding. Our results are also consistent with our previously reported in vivo assays in that the two proteins differ fundamentally in their ability to support genome replication; actually, the large antigen acts as a potent dominant negative mutation on the essential small antigen (5, 17). Such studies indicate that binding to HDV RNAs involves multiple copies of both forms of the antigen, with only the small form supporting genome replication, while in the presence of even relatively few copies of the large antigen, the replication is suppressed. Thus, RNA binding, as it occurs in vivo, may involve significant protein-protein interactions; there may be antigen-antigen interactions, and it could even be that one or more cellular proteins participate to achieve additional specificity.

(vi) In terms of virion assembly, our results are consistent with the observation that both forms of the delta antigen can be packaged (1). We were unable to see any difference between the binding of genomic and antigenomic RNA; this result is consistent with the role of the antigen in supporting the synthesis of both genomic and antigenomic RNAs, but we still have an incomplete picture of why HDV virions package almost exclusively genomic RNA with very little antigenomic RNA.

(vii) Our finding that there are multiple regions on the HDV genomic and antigenomic RNAs that support antigen binding may mean that there is no equivalent of a single

"origin-of-assembly sequence," as has been found on many other RNA viruses, such as tobacco mosaic virus (13) and even the HBV pregenomic RNA (12). Of course, HDV is different from other animal viruses in many ways, and this might be just one more difference; alternatively, such a sequence may actually exist but has not been detected as such by our assays.

ACKNOWLEDGMENTS

J.T. was supported by grants CA-06927, RR-05539, and AI-26522 from the National Institutes of Health, grant MV-7M from the American Cancer Society, and an appropriation from the Commonwealth of Pennsylvania.

We thank Susan Weiss for valuable discussions and also for the coronavirus nucleocapsid RNA-binding protein used in preliminary studies. We also thank William Mason, Christoph Seeger, and Wang-Shick Ryu for critical reading of the manuscript. Laura Coates provided technical assistance in some of the protein purifications. We thank Karen Trush for computer graphics, Tony Yeung for oligonucleotide synthesis, Sam Sorof for ^{125}I-protein A, John Gerin for antibody to the delta antigen, and Robert Owens for the PSTV clone.

REFERENCES

1. **Bergmann, K., and J. Gerin.** 1986. Antigen of hepatitis delta virus in the liver and serum of humans and animals. J. Infect. Dis. **154**:702–706.
2. **Bonino, F., H. M. Rizzetto, and W. H. Gerlich.** 1986. Hepatitis delta virus protein composition of delta antigen and its hepatitis B virus-derived envelope. J. Virol. **58**:945–950.
3. **Chang, M.-F., S. C. Baker, and M. M. C. Lai.** 1988. Human hepatitis delta antigen is a nuclear phosphoprotein with RNA-binding activity. J. Virol. **62**:2403–2410.
4. **Chao, M., S.-Y. Hsieh, and J. Taylor.** Unpublished data.
5. **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.
6. **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.
7. **Devereaux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence programs for the VAX. Nucleic Acids Res. **12**:387–395.
8. **Gowans, E. J., T. B. Macnaughton, A. R. Jilbert, and C. J. Burrell.** 1991. Cell culture model systems to study HDV expression, replication, and pathogenesis, p. 299–308. *In* J. L. Gerin, R. H. Purcell, and M. Rizzetto (ed.), The hepatitis delta virus. Wiley-Liss, New York.
9. **Hancock, K., and V. C. W. Tsang.** 1983. India ink staining of proteins on nitrocellulose paper. Anal. Biochem. **133**:157–162.
10. **Hsieh, S.-Y., M. Chao, L. Coates, and J. Taylor.** 1990. Hepatitis delta virus genome replication: a polyadenylated mRNA for the delta antigen. J. Virol. **64**:3192–3198.
11. **Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White.** 1990. PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
12. **Junker-Niepmann, M., R. Bartenschlager, and H. Schaller.** 1990. A short *cis*-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J. **9**:3389–3396.
13. **Klug, A.** 1979. The assembly of tobacco mosaic virus: structure and specificity. Harvey Lectures **74**:141–172.
14. **Konarska, M. M., and P. A. Sharp.** 1986. Electrophoretic separation of complexes involved in the splicing of precursors to mRNAs. Cell **46**:845–855.
15. **Kos, A., R. Dijkema, A. C. Arnberg, P. H. van der Meide, and H. Schelekens.** 1986. The hepatitis delta virus possesses a circular RNA. Nature (London) **322**:558–560.
16. **Krogstad, P. A., and J. J. Champoux.** 1990. Sequence-specific binding of DNA by the Moloney murine leukemia virus integrase protein. J. Virol. **64**:2796–2801.
17. **Kuo, M. Y.-P., M. Chao, and J. Taylor.** 1989. Initiation of replication of the human hepatitis delta virus genome from cloned DNA: role of the delta antigen. J. Virol. **63**:1945–1950.
18. **Kuo, M. Y.-P., J. Goldberg, L. Coates, W. Mason, J. Gerin, and J. Taylor.** 1988. Molecular cloning of hepatitis delta virus RNA from an infected woodchuck liver: sequence, structure, and applications. J. Virol. **62**:1855–1861.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Lin, J.-H., M.-F. Chang, S. C. Baker, S. Govindarajan, and M. M. C. Lai.** 1990. Characterization of hepatitis delta virus RNA. J. Virol. **64**:4051–4058.
21. **Luo, G., M. Chao, S.-Y. Hsieh, C. Sureau, K. Nishikura, and J. Taylor.** 1990. A specific base transition occurs on replicating hepatitis delta virus RNA. J. Virol. **64**:1021–1027.
22. **Luo, G., and J. Taylor.** 1990. Template switching by reverse transcriptase during DNA synthesis. J. Virol. **64**:4321–4328.
23. **Macnaughton, T. B., E. J. Gowans, B. Reinboth, A. R. Jilbert, and C. J. Burrell.** 1990. Stable expression of hepatitis delta virus antigen in a eukaryotic cell line. J. Gen. Virol. **71**:1339–1345.
24. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74**:4835–4838.
25. **Mills, D. R., and F. R. Kramer.** 1979. Structure-independent nucleotide sequence analysis. Proc. Natl. Acad. Sci. USA **76**:2232–2235.
26. **Owens, R. A., and D. E. Cress.** 1980. Molecular cloning and characterization of potato spindle tuber viroid cDNA sequences. Proc. Natl. Acad. Sci. USA **77**:5302–5306.
27. **Sharmeen, L., M. Y.-P. Kuo, and J. Taylor.** 1989. Self-ligating RNA sequences on the antigenome of human hepatitis delta virus. J. Virol. **63**:1428–1430.
28. **Stanton, G. J., and S. Baron.** 1984. Interferon and viral pathogenesis, p. 3–10. *In* A. L. Notkins and M. B. A. Oldstone (ed.), Concepts in viral pathogenesis. Springer-Verlag, New York.
29. **Sureau, C., J. Taylor, M. Chao, J. Eichberg, and R. Lanford.** 1989. A cloned DNA copy of hepatitis delta virus is infectious in the chimpanzee. J. Virol. **63**:4292–4297.
30. **Taylor, J.** 1990. Hepatitis delta virus *cis* and *trans* functions needed for replication. Cell **61**:371–373.
31. **Taylor, J.** 1990. Structure and replication of hepatitis delta virus. Semin. Virol. **1**:135–141.
32. **Taylor, J., W. Mason, J. Summers, J. Goldberg, C. Aldrich, L. Coates, J. Gerin, and E. Gowans.** 1987. Replication of human hepatitis delta virus in primary cultures of woodchuck hepatocytes. J. Virol. **61**:2891–2895.
33. **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**:508–513.
34. **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$^{\delta}$ and p27$^{\delta}$. J. Virol. **62**:594–599.