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Molecular Cloning of Hepatitis Delta Virus RNA from an Infected Woodchuck Liver: Sequence, Structure, and Applications

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cDNA prepared from the single-stranded circular RNA genome of hepatitis delta virus was cloned in lambda gt11 by using RNA from the liver of an infected woodchuck. From the sequence of overlapping clones, we assembled the full sequence of 1,679 nucleotides. The sequence indicated an exceptional ability for intramolecular base pairing, yielding a rod structure with at least 70% of the bases paired and a predicted free energy of −805 kcal (−3, 368 kJ)/mol. Three of the lambda clones contained sequences that were not only expressed as fusion proteins with beta-galactosidase but were recognized by human hepatitis delta virus-specific antibody. These clones were sequenced so as to establish the reading frame of the delta antigen on the antigenomic strand. The fusion protein produced by one clone was purified by immunoaffinity chromatography and then was used to raise rabbit antibodies specific for the delta antigen.

Hepatitis delta virus (HDV) was first discovered by Rizzetto et al. (18) as a novel antigen in certain patients chronically infected with hepatitis B virus (HBV). This new virus has been implicated in a substantial number of cases of both fulminant hepatitis and chronic active hepatitis with increased liver damage (22). HDV can be experimentally transmitted to chimpanzees and woodchucks (16, 19). In each case, the HDV replication is dependent on concomitant hepadnavirus infection, be it HBV or woodchuck hepatitis virus. Moreover, the membranous coat of the resultant HDV is substituted by the surface antigen of the coinfecting hepadnavirus (4, 16, 19). The time course of infection of a woodchuck by HDV is similar to that observed in human and chimpanzee infections. Also, the HDV antigen produced in woodchuck infections is similar both serologically and biophysically to antigen obtained from infected primates (16).

We have carried out experiments to understand the ability of HDV to replicate in woodchucks. We have previously reported a characterization of HDV-related RNAs in the livers of infected woodchucks (5). Also, we have recently reported the successful growth of HDV in primary cultures of woodchuck hepatocytes (24). In this article we report the molecular cloning and sequencing of the whole genome of HDV as isolated from the liver of an infected woodchuck, together with production of immunogenic fusion proteins of the delta antigen to beta-galactosidase.

We and investigators at two other laboratories (11, 27) have shown that the genome of HDV is not only a single-stranded RNA but that, unlike any other known animal virus, it also has a circular conformation. At this time, five groups have reported the molecular cloning of either part or the whole of the HDV genome (7, 11, 14, 20, 27). All these studies have made use of RNA obtained from virions in the sera of infected primates. Two of these reports determined the complete nucleotide sequence as either 1,679 (26, 27) or 1,683 (14) nucleotides. We have obtained similar information by using not virion RNA but RNA from the liver of an infected woodchuck.

The delta antigen was initially detected in the liver by in situ immunofluorescence staining with antibody (18). Subsequent studies, with Western immunoblots, have revealed that the antigen has more than one electrophoretic form. Generally speaking, two proteins with molecular masses of about 24 to 26 and 26 to 29 kilodaltons (kDa) have been reported (2, 3). Several lines of evidence indicate that the delta antigen is encoded by an RNA, the antigenomic strand, that is complementary to the genomic strand present in virions. From the nucleotide sequence Wang et al. have predicted an open reading frame (ORF), designated ORF 5, that would encode the delta antigen (27). In their original report, from the 1,678-base sequence they predicted ORF 5 as 215 amino acids. Subsequently, they reported an additional base in ORF 5, which increased the length of the genome to 1,679 bases; because of this and the ambiguity arising from clonal heterogeneity, they now predict two possible forms of ORF 5, 214 and 195 amino acids long, respectively (26). As part of our study we have obtained a sequence for the HDV genome that predicts only the 195-amino-acid species. In addition, we have selected and characterized three fusion proteins with beta-galactosidase that express the N-terminal region of the predicted delta protein. One of these fusion proteins has been purified to homogeneity by immunoaffinity chromatography and inoculated into a rabbit to successfully produce antibody reactive to delta antigen.

MATERIALS AND METHODS

cDNA cloning and screening. Total RNA from the liver of an infected woodchuck was isolated by extraction with guanidine isothiocyanate and purified by centrifugation to equilibrium in cesium chloride (6). The RNA was further purified by rate zonal centrifugation in a sucrose gradient to obtain a size class containing full-length HDV RNA. Briefly, this RNA was converted with reverse transcriptase and random oligonucleotide primers (25) to double-stranded cDNA and sized; then *Eco*RI linker was added, and the product was inserted at the *Eco*RI site of lambda gt11. The library of clones obtained was initially screened with the subgenomic-length delta clone pkD3 (7). Clone C2, 670 bases long, was obtained as previously described (5). Clone Z1 was

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obtained by screening with oligonucleotide 23 (5'-CTT CGGGCCGGAGTGCTCTCCAGATCTGGAGGTTG-3'), as predicted from the sequence of pDeltaI (11). Clones X3, X7, W3, and W9 were similarly obtained by screening with oligonucleotide 33 (5'-GGGAGCCCCCTCTCCATCCTTA TCCTTCTTTCCGA-3'), as predicted from Wang et al. (27). Clone U1 was obtained by screening with both the nick-translated 432-base-pair *Pst*I fragment of clone C2 and oligonucleotide 33. Clones S4 and T4 were selected by immunoscreening as previously described (29, 30), using a human anti-delta polyclonal antibody. Clone W3 was initially selected by screening with oligonucleotide 33 and then by rescreening with antibody.

Preparation of *Escherichia coli* extracts. We followed the procedures of Young and Davis (29, 30). Briefly, lambda gt11 clones S4, T4, and W3 were used to make lysogens in *E. coli* Y1089. After being tested, the lysogens were grown and induced by heat shock and isopropyl-β-D-thiogalactopyranoside. The cells were collected and frozen with liquid nitrogen. Subsequently, the cells were disrupted by being thawed and sonicated, followed by clarification.

Immunoaffinity purification of fusion protein. Purification was achieved by a single passage of the clarified extract over a 1-ml column of monoclonal antibody to beta-galactosidase bound to Sepharose. The column was obtained from Promega, Madison, Wis., and used according to their instructions.

Preparation of rabbit anti-delta antibody. Each injection contained about 100 μg of purified S4 fusion protein; half was denatured with 0.01% sodium dodecyl sulfate and injected at a separate site. The first subcutaneous injections contained complete Freund adjuvant and were followed 2 and 4 weeks later by injections with incomplete Freund adjuvant. The immune response of the rabbit was subsequently boosted with intraperitoneal injections of antigen (without adjuvant), and the animal was bled 10 days later.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Samples were analyzed on gels of 8 or 12.5% polyacrylamide by the Laemmli method (13). After electrophoresis, the proteins were electroblotted onto nitrocellulose membranes and stained with India ink (10). The membranes were then blocked, incubated with the appropriate antibody, and washed to remove unbound antibody. Bound antibody was detected by incubation in the presence of ^{125}I-labeled *Staphylococcus aureus* protein A. After appropriate washes, the membrane was subjected to autoradiography by using an intensifying screen.

DNA sequencing and analysis. The nucleotide sequencing reactions were done by the dideoxy-chain termination method (21), with modifications by Zagursky et al. (31) and Tabor et al. (23). Areas of uncertainty were confirmed by use of the modified Maxam-Gilbert method (1). Computer-based analysis of the sequence information was carried out by using the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (8).

RESULTS

Assembly of cDNA clones to obtain the complete nucleotide sequence of HDV. As described previously (5) and more completely in the Materials and Methods, we created in lambda gt11 a cDNA library derived by the reverse transcription of RNA isolated from the liver of an infected woodchuck. We screened and obtained the clones with HDV-related sequences. A subset of the clones in this library that contained delta sequences was selected for subcloning into M13 vectors (mp10, mp18, and mp19) and Gemini vectors (pGem3 and pGem4-blue). Seven of the subcloned sequences that were subjected to nucleic acid sequencing are summarized (Fig. 1) along with the strategy used to obtain the complete nucleotide sequence (Fig. 2).

The complete sequence of the circular genome was 1,679 nucleotides, exactly the same as the modified sequence obtained by Wang et al. (26). Therefore, we have used their numbering scheme. Figure 2 also shows that our sequence agrees with Wang et al. at 1,670 of the 1,679 positions. This

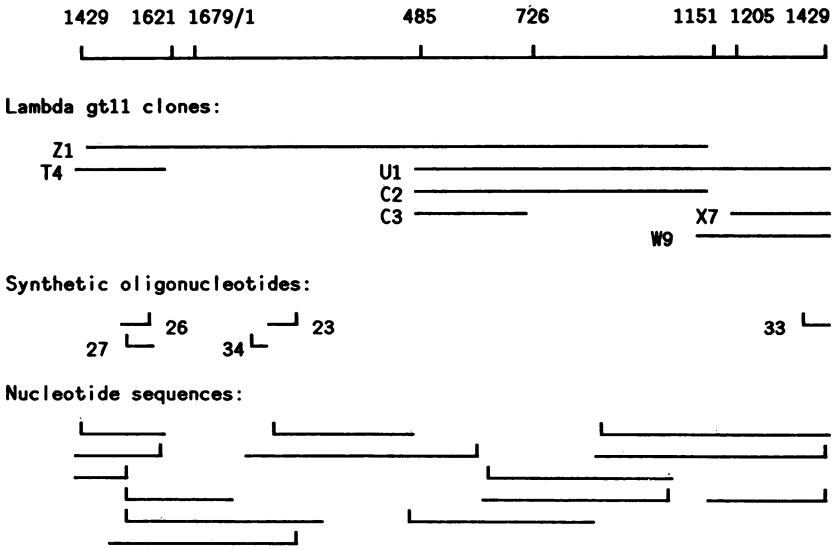


FIG. 1. Strategies used for cloning and sequencing the HDV genome. The inserts of cDNA clones in lambda gt11 are shown relative to the 1,679-nucleotide full-length HDV genome. The notation is the same as that of Wang et al. (26, 27), in which the first nucleotide corresponds to a unique *Hin*dIII site (actually absent from our sequence). The indicated synthetic oligonucleotides were used for library screening and nucleotide sequencing. The sequencing procedures are described in the Materials and Methods. The bottom part of the diagram summarizes the actual regions sequenced and combined to create the full sequence.

FIG. 2. The complete nucleotide sequence of HDV. The notation is as described in the legend to Fig. 1, with the nine differences relative to Wang et al. (26, 27) indicated beneath the main sequence in uppercase letters. The lowercase letters indicate the ambiguities in the sequence of Wang et al., using the convention y = C or T, r = A or G, and w = A or T.

represents an upper limit to the extent of agreement between the two sequences because the Wang et al. sequence actually contains an ambiguity arising from clonal heterogeneity of single nucleotides at 15 different positions, as represented in Fig. 2. (That is, they found such differences between independent clones spanning the same region of the HDV genome.) The remaining nine discrepancies between the sequences are indicated in Fig. 2. Note that they represent only single-base changes. We found neither insertions nor deletions.

In the earlier study by Wang et al. (27), it was shown that the HDV RNA could be folded on itself as a rod, with 61% of the bases paired. We have obtained a similar result. In addition, we used the computer analysis procedure of Zuker and Stiegler (32), as implemented by the Wisconsin Genetics Computer Group, using the Turner stacking and loop-destabilizing energies (9), to calculate the optimum folding of our sequence. One method of presenting these results is given (Fig. 3). An unbranched rod structure is the predicted shape with 70% of the nucleotides base paired. The free energy deduced for this structure was −804.6 kcal (−3,366 kJ)/mol. Similar analyses for the genomic sequences of Wang et al. (26, 27) and Makino et al. (14) yielded −771.5 and −784.1 kcal (−3,228 and −3,280 kJ)/mol, respectively. Also, analysis of the folding of our antigenomic strand yielded the most stable value, namely −828.3 kcal (−3,465 kJ)/mol. By comparison, the well-studied rod structure of the plant agent potato spindle tuber viroid (PSTV) was calculated to be −149.8 kcal (−626.8 kJ)/mol. This value, comparable to the published value of Riesner et al. (17) of −143.4 kcal (−600.1 kJ)/mol, is severalfold lower than that of these three HDV genomic sequences. Even when normalized for the length of the molecule, the HDV RNA was predicted to be −479 cal (−2,004 J) per nucleotide relative to −417 cal (−1,745 J) per nucleotide for PSTV.

Our complete sequence, both genomic and antigenomic, was analyzed for potential open reading frames (ORFs), with the results as shown (Fig. 4). These frames are similar but not identical to those of Wang et al. (27). Those authors predicted that the delta antigen is encoded on the antigenomic strand in what they designated as ORF 5, and they demonstrated that nucleotide sequences from this region when expressed as a fusion protein were recognized by human antibodies reactive to delta antigen. However, because of the above-mentioned heterogeneity in their sequence, they predicted not one but two sizes for ORF 5, namely 195 and 214 amino acids, which correspond to proteins of 22 and 24 kDa, respectively. In contrast we predict only the 195-amino-acid species and Makino et al. (14) predict only the 214-amino-acid species. To add to this confusion, certain laboratories have detected more than one, usually two, electrophoretic forms of delta antigen (2, 3). In addition, there has been disagreement as to the sizes of these proteins (2, 3). Therefore, as related below, we have carried out further experiments to understand the relationship between the observed and the predicted sequence(s) of the delta protein(s).

Isolation of fusion proteins and rabbit anti-delta antibody.

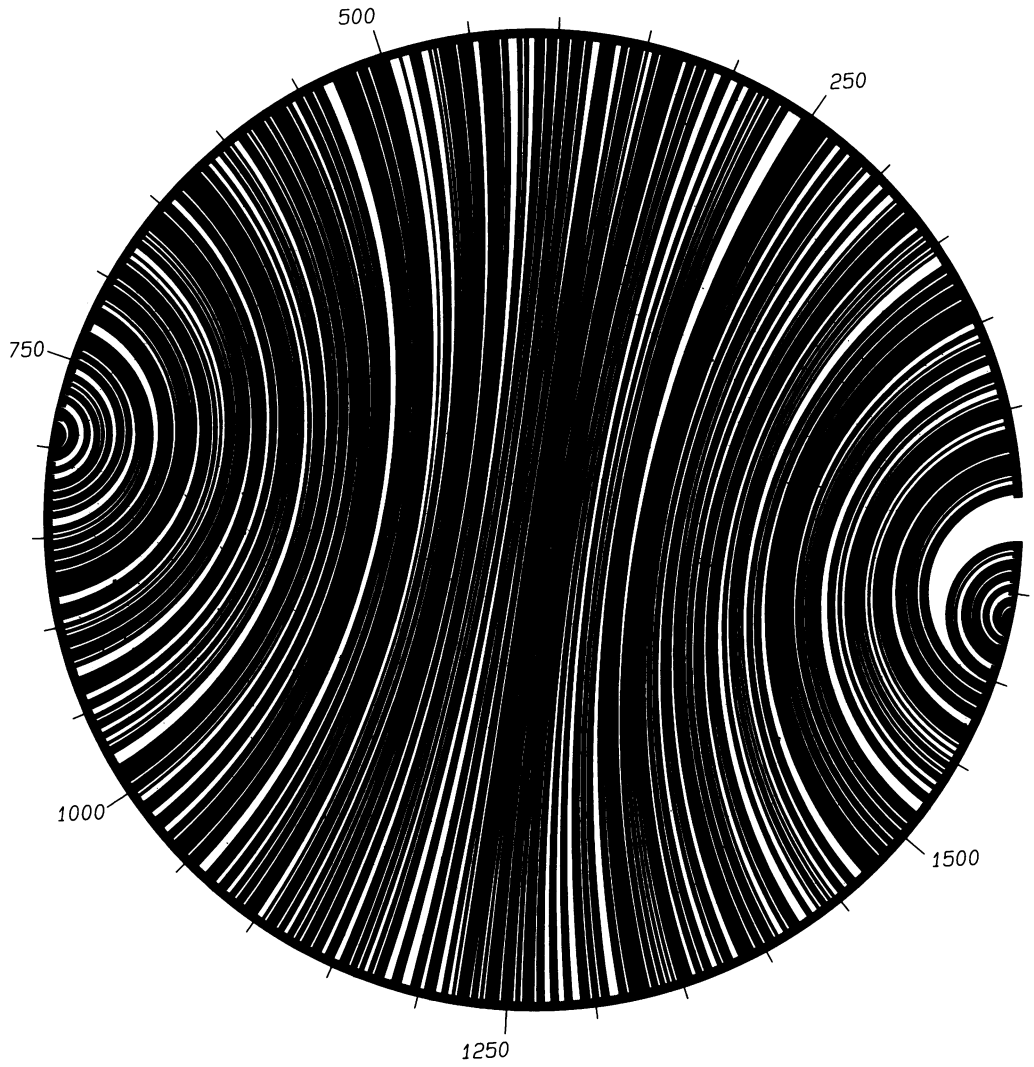


FIG. 3. Computer representation of the predicted lowest energy state of HDV genomic RNA. The plot is as used by Zucker and Stiegler (32) and modified by W. Winsborough of the University of Wisconsin Genetics Computer Group (8). Briefly, the full sequence is shown on the circumference of the circle and each pair of nucleotides that is predicted to be paired is joined by a line.

In order to identify the actual ORF used for the synthesis of delta antigen, we screened our lambda gt11 cDNA library with anti-delta serum from a patient chronically infected with HDV (2). Three immunoreactive clones (S4, T4, W3) were obtained.

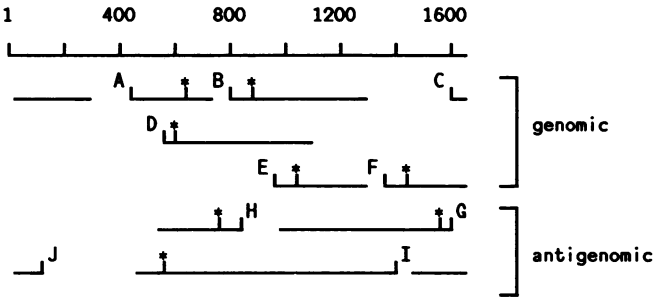
The cDNA inserts in these clones were then sequenced to determine their relationship to the beta-galactosidase gene of the lambda gt11 vector. The unmodified beta-galactosidase protein is known to be about 114 kDa, with the vector insertion site very close to the C terminus (30). For all three of our clones we found that the N terminus of the beta-galactosidase gene was fused to delta sequences in frame with the predicted N terminus of ORF G (Fig. 5). However, in only one of the three clones, namely S4, did the predicted reading frame correctly reenter the reading frame of the small C terminal fragment of the beta-galactosidase. The S4 protein contains 59 of the predicted 195 amino acids of ORF G.

In order to produce significant amounts of the three fusion proteins for further characterization, we first established the three recombinant bacteriophage as lysogens in *E. coli* Y1089. We then attempted to grow and purify the fusion proteins according to the procedures of Young and Davis (30). S4 protein was more stable than the other two and was the only one that could bind to an immunoaffinity column consisting of a monoclonal antibody to beta-galactosidase linked to Sepharose. As shown by the Western analysis in Fig. 6 (panel A, lanes 3 and 4), a single passage over the column purified the S4 fusion protein to virtual homogeneity.

The purified fusion protein S4 was used to raise antibody in a rabbit. As shown by the Western analysis in Fig. 7, the rabbit antibody was qualitatively the same as a human HDV-specific antibody in detecting delta-related antigens in the proteins of an infected human liver. Both sera reacted predominantly with a single protein. Relative to known markers, the molecular mass was deduced to be about 25 kDa.

DISCUSSION

Rizzetto et al. (19) and Ponzetto et al. (16) have previously shown that HDV can be experimentally transmitted not only



frame	range	length in AA's	first AUG	range from AUG	length from AUG in AA's
A	435-782	116	684	684-782	33
B	786-1253	156	891	891-1253	121
C	1608-288	120	-	-	-
D	539-1033	165	578	578-1033	152
E	937-1293	119	1036	1036-1293	86
F	1297-1641	115	1438	1438-1634	68
G	1619-1014	202	1598	1598-1014	195
H	821-510	104	731	731-510	74
I	1410-478	311	579	579-478	34
J	91-1414	119	-	-	-

FIG. 4. Predicted open reading frames on genomic and antigenomic HDV RNA. The ORFs are defined as the sequences between two translational terminators; only 10 ORFs larger than 100 amino acids were found and are indicated as A to J. The first in-frame AUG, if any, for each ORF, is indicated with an asterisk. The bottom portion of the figure provides detailed information regarding the 10 ORFs.

from human to chimpanzee but also from chimpanzee to woodchuck. We report the sequence of such a woodchuck-adapted HDV genome. The woodchuck HDV that we have sequenced is experimentally related to the chimpanzee HDV sequenced by Wang et al. (26, 27) in the following manner. Both virus stocks began with experimental transmission, using serum from the same HDV-infected individual. Wang et al. used the serum HDV after five serial transmissions in chimpanzees. The virus used to infect the woodchucks in our experiments was first submitted to two of the above serial transmissions in chimpanzees. On the basis of the work of Rizzetto et al. (19) and Ponzetto et al. (16), we would expect that our woodchuck-adapted HDV has a woodchuck hepatitis virus coat, whereas the chimpanzee HDV has an HBV coat. Nevertheless, our sequencing results show only minor changes in the HDV genomic sequence relative to that obtained by Wang et al. (26). Certainly this supports the hypothesis that the woodchuck is a good model system for studying HDV replication (24) since no substantial adaptation occurs during interspecies transfer. Also, when we have assembled our subgenomic HDV clones into a single molecule of HDV DNA we will be able to test the infectivity of recombinant HDV DNA and RNA and begin to attack two major questions: first, whether the HDV has the ability to replicate its genome in the absence of a hepadnavirus and second, the role, if any, of the HDV-coded protein in genome replication.

Other laboratories have sequenced independent sources of HDV. Kos et al. (11) obtained a 381-base partial sequence for a chimpanzee-adapted HDV; our sequence differed at only six positions (1.5%), with the differences being limited to single-base changes. Two laboratories have reported sequences of HDV RNA as obtained directly from human sera, without any experimental transmission. One, a complete sequence by Makino et al. (14), contains 1,683 nucleotides rather than our 1,679; compared with our sequence, the overall homology is 89%, which includes not only single-base changes but also three insertions and seven deletions. The other human sequence by Saldanha et al. (20) is only partial, 380 nucleotides long, and relative to our sequence the homology is 83%, including four insertions and seven deletions. It should be noted that these two human HDV sequences have only 93% homology relative to each other, again including insertions and deletions, whereas the two independent chimpanzee sequences and our woodchuck HDV sequence have more than 98.5% homology, with no insertions or deletions. Thus, the extent of sequence differences between laboratories is probably predominantly due to different strains of HDV rather than to the consequences of serial experimental transmission.

A feature of the HDV genome that is almost as striking as the circular conformation is the finding that the genome has the ability to undergo intramolecular base pairing so as to form a rod structure. Such a rod structure has been detected by electron microscopic examination under nondenaturing conditions (11). It is consistent with observations on the resistance of the genomic RNA to nucleases (5) and of more

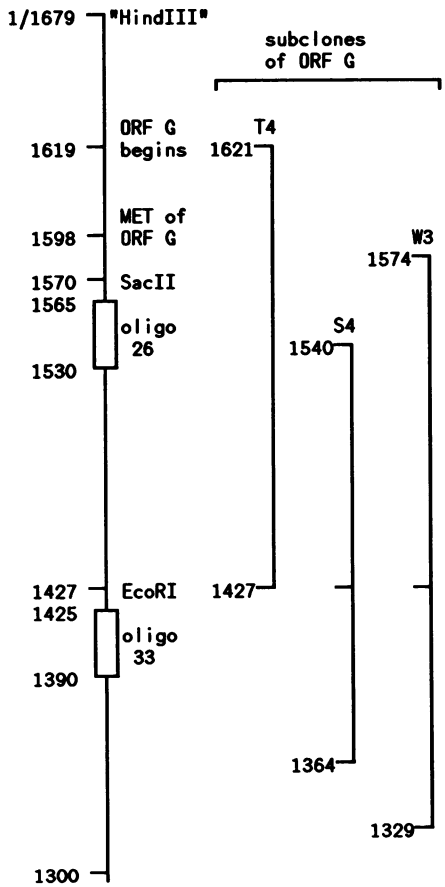


FIG. 5. Characteristics of three lambda gt11 clones that express fusion proteins reactive with HDV-specific human antibodies. The HDV genome is shown at the left. The sequences of T4 and S4 were obtained by direct sequencing of purified fragments spanning the insert, whereas the W3 insert was first subcloned and then sequenced. The sequences of the three fusion proteins are notated as in Fig. 2, with ORF G as detailed in Fig. 4. The two oligonucleotides used to help in screening are detailed in Fig. 1 and Materials and Methods.

significance, the rod structure has been predicted by Wang et al. (27) on the basis of an examination of the nucleotide sequence. We used a more sophisticated computer analysis to examine the foldings and made two important advances. First, we found a greater extent of base pairing, 70% (versus 61%), together with an additional 4.6% (versus 6%) due to G-U pairs. These values are similar to those for viroids such as PSTV, with 62% base pairing and an additional 8.4% due to G-U pairing. Second, we have obtained predicted changes in free energy that would accompany base pairing. By this criterion our HDV sequence, with a predicted value of −804.6 kcal (−3,366 kJ)/mol, is significantly more stable than that of even a viroid such as PSTV, which computes as only −149.8 kcal (−626.8 kJ)/mol. Even though it remains possible that within the cell or even within the HDV particle proteins may bind to the RNA so as to produce a different conformation, the predicted unbranched rod structure probably has significance for the life cycle of HDV and also for that of the viroids and virusoids. For example, it may confer nuclease resistance and hence stability, it may facilitate RNA polymerase binding, and it may trigger a response in the infected host.

Our studies with the three delta-specific beta-galactosidase fusion proteins established that the N-terminal third of the predicted ORF G of the antigenomic strand is actually utilized in the natural synthesis of delta antigen. We do not yet know whether the predicted 22-kDa protein of ORF G (Fig. 4) is the same as the 25-kDa protein detected by Western analysis of infected human liver (Fig. 7). Actually, as in other laboratories (2, 3), we have examined other infected liver samples and found more than one form of delta protein, sometimes two and even three species. An hypothesis consistent with this and the clonal heterogeneity observed by Wang et al. (27) is that there are variant forms of HDV encoding related but different forms of the delta antigen. However, the story will not be completed until the actual N and C termini of the natural proteins have been determined. Also, there is a need to determine which antigenomic RNA species actually acts as mRNA for the natural translation of this protein. The study of Kozak (12) argues against the utilization of a circular RNA species as an mRNA; also, the previously described 800-base poly(A)-containing RNA (5, 23a), although a better candidate for the delta mRNA, has not yet been successfully translated in vitro (23a).

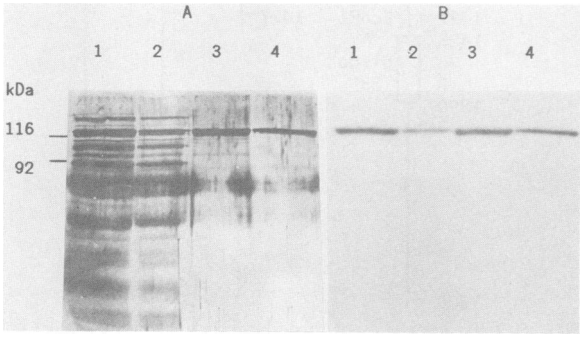


FIG. 6. Characterization and immunoaffinity purification of the HDV-reactive fusion protein S4. *E. coli* cell extracts both before (lanes 1 and 2) and after (lanes 3 and 4) purification were submitted to Western analysis by using a gel of 8% polyacrylamide. Lanes 1 and 3 contain twice as much material as lanes 2 and 4. (A) India ink staining. (B) The result of detection with human antibody to HDV followed by binding of ^{125}I-labeled protein A. Molecular mass markers (Bio-Rad Laboratories) are indicated at the left.

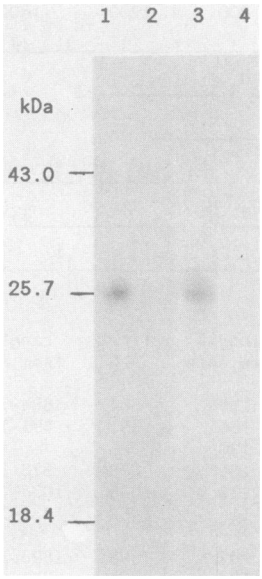


FIG. 7. Characterization of rabbit antibody raised against HDV-specific S4 fusion protein. Protein samples were subjected to Western analysis by using a gel of 12.5% polyacrylamide. In lanes 1 and 3 the protein was from the liver of an HDV-infected human, whereas lanes 2 and 4 were from an uninfected individual. The HDV-related proteins were detected with antibody followed by binding of ^{125}I-labeled protein A. In lanes 1 and 2 the antibody used was rabbit antibody (diluted 1:1,000) raised to the S4 protein, whereas in lanes 3 and 4 the antibody (diluted 1:1,000) was from an HDV-infected individual and the autoradiogram exposure was six times less. ^{14}C-labeled molecular mass markers (Bethesda Research Laboratories, Inc.) are indicated at the left.

Finally, we envisage that the purified delta-specific fusion protein and the corresponding rabbit antibody will be useful in the diagnosis, prophylaxis, and design of therapeutic strategies for treating HDV infections of humans. This antibody is better than those obtained from convalescent patients or infected animals, not only because it is free of HDV, HBV, and even human immunodeficiency virus (HIV) (sometimes found in these sera), but also because of the exclusion of HBV-specific immunoglobulins. The rabbit antibody is comparable to a recently described human monoclonal antibody of Pohl et al. (15) but can be more conveniently produced.

There is considered to be much overlap in the mechanisms of transmission of HIV, HBV, and HDV (28). Therefore, we suggest that the HDV-specific fusion protein we have described (maybe together with HBV-specific proteins) could be profitably incorporated into the Western assays currently being used to screen patients for antibody to HIV. This would then provide simultaneously diagnostic and epidemiological information on these diseases.

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