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A genotype of hepatitis D virus that occurs in northern South America

JOHN L. CASEY*, THOMAS L. BROWN*, ERNESTO J. COLAN†, F. STEPHEN WIGNALL†‡, AND JOHN L. GERIN*

*Division of Molecular Virology and Immunology, Department of Microbiology, Georgetown University School of Medicine, 5640 Fishers Lane, Rockville, MD 20852; and †Naval Medical Research Institute Detachment, Unit 3800, APO AA 34031

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ABSTRACT **Hepatitis D virus (HDV) is the cause of an unusually severe form of liver disease with distinct histologic features (morula cell) that occurs throughout northern South America and certain other areas of the world. Clinical studies of HDV disease worldwide indicate that there is, in fact, a wide variation in pathogenesis, and the reasons for these differences are presently unknown. One possible explanation is that factors associated with the viral genotype are determinants of HDV pathogenesis. In this study, nucleic acid sequences were determined for three different northern South American HDV isolates which were obtained from individuals with severe disease or a family history of severe disease, in areas that are hyperendemic for this disease pattern. The sequences of these three isolates are very similar to one another but only distantly related to other published HDV sequences. Comparison of the sequence of a semiconserved region from a total of 14 isolates indicates that there are at least three HDV genotypes. Most published HDV sequences, including those from North America, Europe, the Middle East, the South Pacific, and Asia, belong to a single genotype which may have some geographically based subtypes. A single Japanese isolate is the sole representative of a second HDV genotype. The South American sequences reported here constitute a third genotype. The association of a particular genotype with the severe form of type D hepatitis that occurs in northern South America supports the hypothesis that HDV genetic factors are important determinants in the pathogenesis of type D hepatitis.**

Hepatitis D virus (HDV) is a subviral agent that requires a preexisting or concurrent infection with hepatitis B virus (HBV), which provides the coat protein for the HDV virion (1, 2). Compared with infection with HBV alone, coinfection of HDV with HBV is associated with a higher rate of fulminant hepatitis in an acute infection; and superinfection with HDV of individuals with chronic HBV infection can lead to both acute and more progressive, chronic liver disease (3). The HDV genome is a 1.68-kb single-stranded circular RNA which is similar to the RNA genomes of unusual pathogens of higher plants (reviewed in ref. 4). HDV produces one known protein, the hepatitis D antigen (HDAg), which is encoded on the antigenomic strand (5) and has two forms (6) that differ in structure by 19 amino acids at the C terminus (5, 7). These two forms also differ functionally: the short form (HDAg-p24) is required for RNA replication (8); the long form (HDAg-p27) suppresses viral RNA replication (9, 10) and is required for packaging of the HDV genome with hepatitis B surface antigen (HBsAg) (11). HDAg-p27 arises via a specific RNA editing process that occurs during HDV replication (12) and that is dependent on a specific structure in the HDV RNA (13).

Viral hepatitis due to HDV infection, type D hepatitis, is found worldwide, and the complete nucleic acid sequence has been obtained for eight isolates from many locations: North America (14), Italy (5, 7) (after passage in chimpanzees and woodchucks), France (15), Lebanon (16), Taiwan (17), Japan (18), and the South Pacific island of Nauru (19). The pathogenesis of HDV infection varies in these areas from fulminant hepatitis to asymptomatic chronic liver disease. In certain regions of South America, such as Venezuela, the Amazon basin, and northern Colombia, HDV infections have been associated with hepatitis outbreaks of particularly high morbidity and mortality (20–27). Some of the disease patterns in these areas, such as Labrea black fever in the Amazon basin in Brazil (23), and Hepatitis de Sierra Nevada de Santa Marta in northern Colombia (25), have existed for at least 50 years. The typically more severe course of disease in northern South America is associated with a characteristic histologic lesion termed the morula cell, which exhibits a pattern of microvesicular steatosis and alteration of hepatocytes and granular eosinophilic degeneration (25).

To attempt to understand viral genotypic factors which might be responsible for the highly pathogenic infections in South America and to expand our understanding of the geography and genetics of HDV, we have cloned and obtained the sequences§ of two HDV isolates from outbreaks of severe hepatitis in the Amazon basin in Peru which occurred in 1986 and one isolate from an individual with a strong family history of severe hepatitis who was a resident of a village in the Santa Marta range in northern Colombia in which prior outbreaks of type D hepatitis had been reported (27).

MATERIALS AND METHODS

HDV Isolates. Peru-1 was obtained from an 18-year-old man in the Peruvian military who was stationed on the Yavari River near the Peruvian–Brazilian border. He developed a severe acute hepatitis which required transfer to a military hospital. Serologic evaluation indicated acute hepatitis B and D [$HBsAg^+$, IgM anti-hepatitis B core antigen-positive (anti-HBc^+), anti-HD^+, HDV RNA^+]. Peru-2 was obtained from another 18-year-old man in the Peruvian military who was also stationed in the Amazon basin, at a site on the Curaray river ≈150 miles to the east; he also developed severe acute hepatitis with a serological profile similar to Peru-1. The isolates were obtained in 1986 as part of an investigation of a continuing outbreak of severe hepatitis that was occurring in military recruits stationed at jungle outposts (24). The isolate Colombia was obtained from a 10-year-old girl from the town of Julio Zawady in northern Colombia and was part of a previous study (27); she was $HBsAg^+$ and anti-HD^+ and the records indicate a strong family history of severe hepatitis which was fatal in two of five siblings. US-2 and US-3 were

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Abbreviations: HDV, hepatitis D virus; HDAg, hepatitis D antigen; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.

‡Present address: NAMRU-2, Naval Medical Research Institute Detachment, Unit 8132, APO AP 96520-8132.

§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L22061–L22066).

obtained from American men with chronic type D hepatitis (isolates kindly provided by J. Hoofnagle, National Institutes of Health). Japan-3 was obtained from a 9-year-old Japanese girl with chronic HDV infection contracted from her mother, who had developed HDV infection from a blood transfusion (5 units) from Japanese donors. Both the mother and the daughter were HBsAg/*adr* carriers and both developed acute type D hepatitis with subsequent progression to chronic liver disease in the follow-up period (isolates kindly provided by K. Mitamura, Tokyo University). Sequences of other isolates were obtained from the published literature.

Molecular Cloning and Analysis. RNA was prepared from the serum of HDV-infected individuals (28). cDNA was prepared and amplified essentially as described (13). RNA equivalent to 5 μl of serum was reverse-transcribed with random hexamers and Moloney murine leukemia virus reverse transcriptase (BRL). cDNA was amplified by the polymerase chain reaction (PCR) with *Taq* polymerase (Perkin–Elmer) using the following thermal program: 2 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, then 7 min at 72°C. For cloning of the entire HDV genome, two different primer sets (Table 1) were used to amplify overlapping segments corresponding to the two ends of the HDV RNA rod structure. For analysis of a subgenomic segment, the fragment corresponding to positions 883–1288 [numbering according to Wang *et al.* (5, 7)] was amplified with primers E and A (Table 1). PCR products were purified (BRL GlassMAX) after agarose gel electrophoresis and cloned with the T-vector system (Novagen) or, in some instances, sequenced directly by thermal cycling with *Taq* polymerase (GIBCO/BRL) or by dideoxy chain termination with T7 polymerase (United States Biochemical) after asymmetric PCR amplification. For obtaining the sequence of the entire genome, clones of PCR-amplified material were selected for sequence analysis by the dideoxy chain-termination method. Sequence analysis was performed with the programs in the Wisconsin Genetics Computer Group package (29).

RESULTS

The isolate Peru-1 was obtained from a member of the Peruvian military who was stationed in the Amazon basin and developed severe acute hepatitis; the serological profile indicated coinfection with HBV and HDV. The scheme employed for cloning Peru-1 is shown in Fig. 1. The primer pair A/B was used to amplify a 0.9-kb cDNA fragment that corresponds to the end of the HDV RNA rod that includes the cleavage domains. Based on sequence obtained from clones of this segment, primers C and D were synthesized and used to amplify the other end of the HDV genome, with about 200 bp of overlap between the two sets of clones. For each amplified fragment, sequence was obtained from at least three clones from separate PCR amplifications to reduce the risk of misinterpreting errors introduced by the amplification process. The 12 discrepancies among the clones were resolved in favor of the most common representative. The isolate Peru-1 is 1677 nt in length, similar to the size reported for other isolates of HDV.

Table 1. Oligonucleotide primers used for PCR amplification and sequencing of HDV RNA

Primer	Sequence (5′ → 3′)	Location
A	GAAGGAAGGCCCTCGAGAACAAGA	1290–1267
B	CTCCAGAGGACCCCTTCAGCGAAC	307–330
C	CACCCGGTGGCTAGCCCCGTTG	445–424
D	GAACTCCCTGCAGGCTGGGCAC	1078–1099
E	GAGATGCCATGCCGACCCGAAGAG	887–910

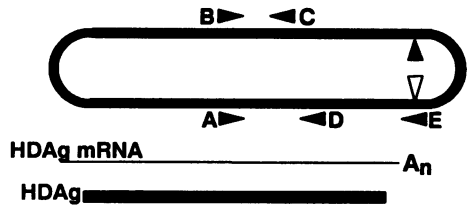

FIG. 1. Schematic diagram of HDV RNA, showing locations of primers used for PCR amplification of HDV cDNA. Filled and open arrowheads indicate the location of the genomic (*ca.* nucleotide 686) and antigenomic cleavage sites (*ca.* nucleotide 900) (30), respectively. The HDAg mRNA is indicated by a thin line [A_n, poly(A)], and HDAg by a thick bar.

Comparison of the complete nucleic acid sequence of Peru-1 with published complete sequences of independent HDV isolates indicates that Peru-1 is only distantly related to previously characterized isolates. While the majority of HDV isolates are >82% identical to one another, Peru-1 is only 61–63% identical to HDV isolates from other continents.

To allow a more practical analysis of additional isolates, we limited the sequence comparison to the segment comprising nucleotides 911–1260 [numbering according to Wang *et al.* (5, 7)]. This region begins near the antigenomic cleavage site (30) and includes the polyadenylylation signal of the viral mRNA (31) and the sequences encoding the C termini of both forms of the viral protein (Fig. 1). Because it is flanked by the highly conserved antigenomic cleavage domain and a short conserved region in the middle of the coding region, it is possible to amplify this segment even from widely differing isolates. Over 85% of the 911–1260 segment is translated, compared with just 40% for the entire genome; reflecting this difference in coding capacity, all isolates are more similar to one another in this segment than over the entire genome. The dendrogram obtained from the comparison of isolates over the 911–1260 region (Fig. 2) is similar to that obtained for the comparison of the entire genome (data not shown). Thus, despite the

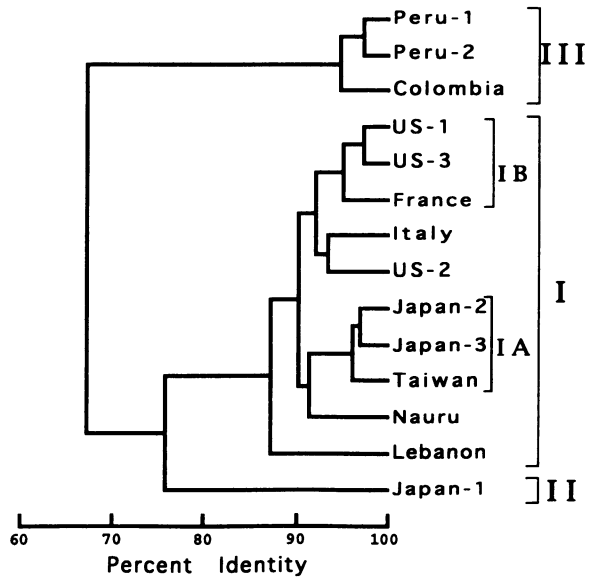

FIG. 2. Dendrogram showing relation of HDV isolates to one another. Nucleotides 911–1260 from different isolates were compared by the University of Wisconsin Genetics Computer Group program PILEUP (29), which performs a Needleman–Wunsch pairwise comparison of sequences (32). Isolates Peru-1, Peru-2, Colombia, Japan-3, and US-3 are from this study. Sources of other isolates are as indicated: US-1 (14), France (15), Italy (5, 7), US-2 (B. M. Baroudy, J.L.C., and J.L.G., unpublished data), Taiwan (17), Nauru (19), Lebanon (16), Japan-1 (18), and Japan-2 (33).

decreased sequence divergence, comparisons between different isolates in the 911–1260 region give a good representation of the comparison of the complete genome.

We obtained the sequence for the 911–1260 region of additional isolates from South America, Japan, and the United States. Comparison of these sequences indicates that there are three main genotypes of HDV (Fig. 2). Most HDV isolates examined to date belong to genotype I, which includes sequences from such geographically diverse regions as Asia, North America, and Europe. Genotype II has a single representative, Japan-1. All three isolates from South America belong to genotype III; these isolates are closely related to one another (>94% identity in the region analyzed) and are very different from other isolates (about 69% identity). Within genotype I there are at least two subgroups, IA and IB. Subgroup IA might represent an Asian subtype; it includes two Japanese isolates, Japan-2 and Japan-3, and the Taiwan isolate. The significance of the other subgroup is unclear, as it includes isolates from the west (US-1) and east coasts (US-3) of the United States and from France, and the sources of HDV exposure in these cases are uncertain.

The alignment of the Peru-1 sequence with sequences representative of the other genotypes is shown in Fig. 3. As noted in previous comparisons of sequences within genotype I (16, 17, 19), there is a high degree of sequence conservation in some regions of the genome. The most conserved regions, nucleotides 682–738 and nucleotides 859–907, include the genomic and antigenomic cleavage domains (30). Among all isolates there are only three segments of >20 consecutive conserved nucleotides; all three of these segments are within the cleavage domains. The region of the genome which encodes the viral protein also exhibits higher than average conservation (see above); however, the extent of conservation is lower among the three isolates from different genotypes compared in Fig. 3 than in previous comparisons within genotype I (17, 19). The most conserved segment in the coding region is 1262–1292, in which 29 of 31 positions are identical among all isolates. This region might encode part of the RNA-binding domain of HDAg (34). Nucleotide positions in the noncoding portion of the genome which are base-paired to the conserved sequences in the coding segment are conserved to a lesser degree than the coding sequences, but enough so that both the genomic and antigenomic RNAs are capable of forming the unbranched rod structure characteristic of HDV and viroid RNAs (5).

The predicted amino acid sequence of HDAg for the Peru-1 isolate is compared with the isolates Italy (genotype I) and Japan-1 (genotype II) in Fig. 4. In this comparison Peru-1 is about 65% identical to Italy and Japan-1, while Italy and Japan-1 are 73% identical. Allowing for semiconservative substitutions, HDAg of Peru-1 is 72% similar to Italy and Japan-1; and HDAg of Italy and Japan-1 are 78% similar (semiconservative identities are defined as follows: R and K; D and E; I, L, and V). Within genotype I the identities are 87–92%. Thus, at both the amino acid and nucleic acid levels, genotype III, as represented by Peru-1, is the most distantly related HDV genotype.

Among the three genotypes, there are several notable regions in the amino acid sequence of HDAg which exhibit

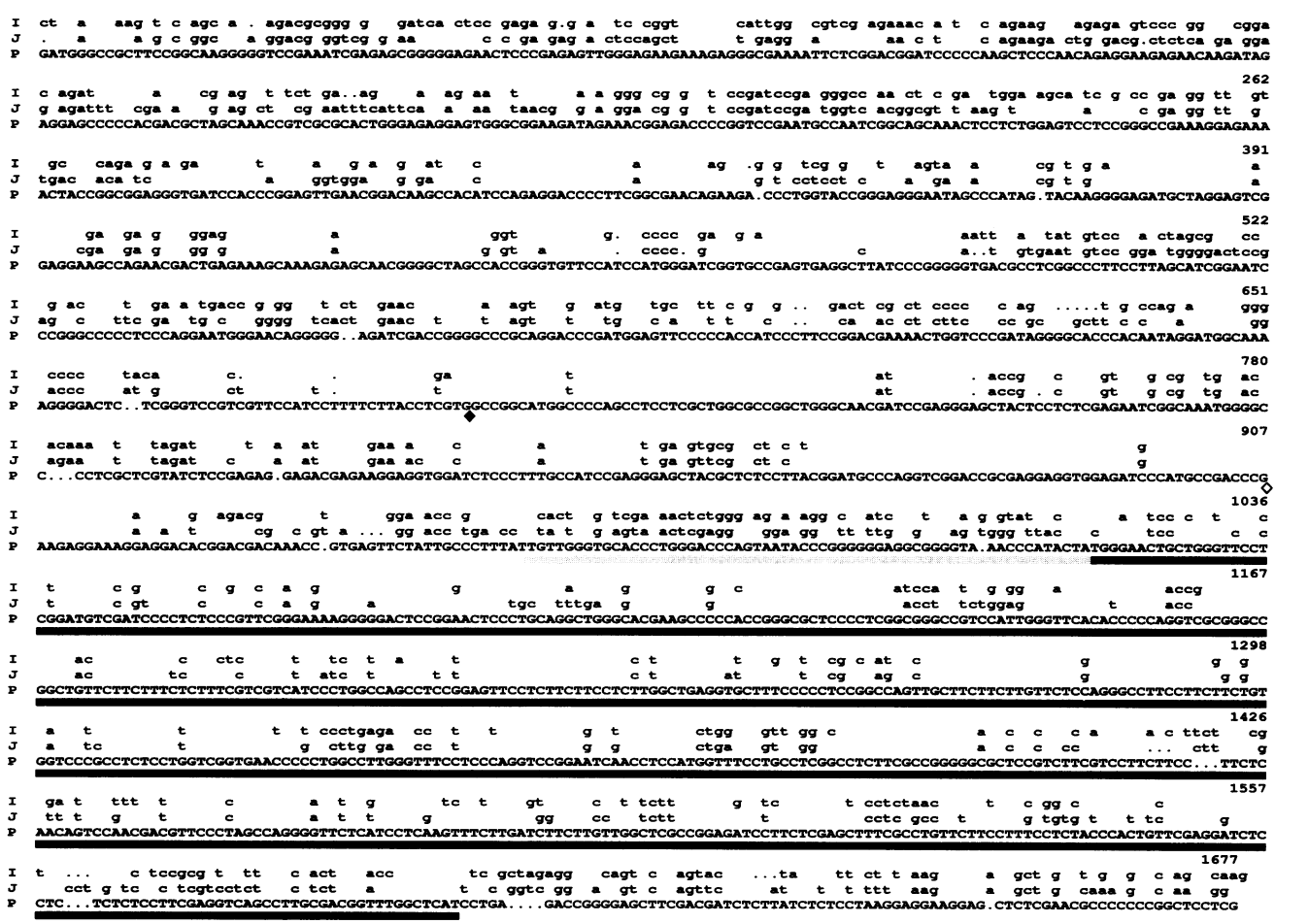

FIG. 3. Alignment of nucleic acid sequences of Peru-1, Italy, and Japan-1 HDV isolates. Alignment was obtained with the program PILEUP. The entire sequence of Peru-1 (P) is given, with dots indicating gaps in the alignment. For the Japan-1 (J) and Italy (I) sequences, positions which are different from Peru-1 are shown in lowercase type. The coding region for HDAg is underlined; the black bar indicates the segment common to HDAg-p24 and HDAg-p27, the gray bar indicates the C-terminal region of HDAg-p27.

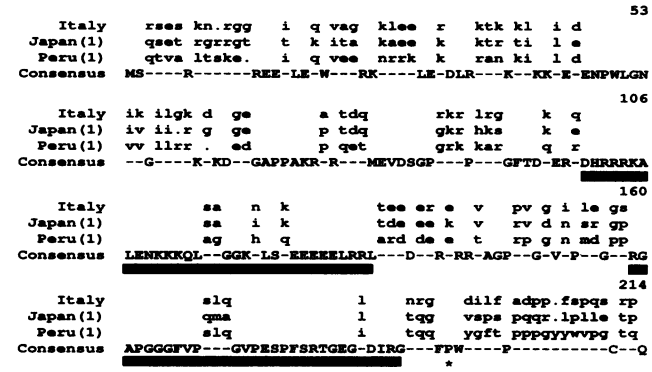

FIG. 4. Comparison of the amino acid sequences of the Peru-1, Italy, and Japan-1 isolates. Positions which are different from the consensus are shown in lowercase type. Gaps in the alignment are indicated by dots. A star indicates the C-terminal amino acid in HDAg-p24. Solid bars indicate highly conserved regions.

either high sequence conservation or extreme divergence. The most highly conserved regions are amino acids 100–132 and 159–184; these segments include part of the RNA-binding domain (34), a poly(glutamic acid) stretch following it, and a proline- and glycine-rich 36-amino acid segment near the C terminus of HDAg-p24. The least conserved regions among the three genotypes are the N terminus of both forms of HDAg and the C terminus of HDAg-p27. Within genotype I these two regions are highly conserved, particularly the 19-amino acid segment at the C terminus of HDAg-p27, in which there are only 3 deviations from the consensus among 11 isolates. Thus, the three genotypes exhibit distinct, extensive changes in particular segments of HDAg in addition to a gradual drift in sequence over the entire genome.

DISCUSSION

We report here the existence of an HDV genotype identified in two regions of northern South America. The two Peruvian isolates examined (Peru-1 and Peru-2) were from sites 150 miles apart in the Amazon basin, and the Colombian isolate was from a village in the Sierra Nevada de Santa Marta range in northern Colombia. Both regions have a high prevalence of HDV infection which has been associated with serious liver disease for many years. The severity of the disease is remarkable, and the acute hepatitis is associated with a characteristic histologic lesion (morula cell) which exhibits a pattern of microvesicular steatosis and eosinophilic necrosis that may be unique to this disease course (25). The Peruvian samples described here were obtained from individuals with acute hepatitis amid outbreaks characterized by severe acute hepatitis, and the Colombian sample was from an HDV-infected child with a strong family history of fatal hepatitis. Thus genotype III is truly a South American genotype and may be associated in some manner with the form of severe hepatitis which is characteristic of the region.

In most populations, HDV infection causes a particular type of hepatitis (type D hepatitis) which is more severe than that of the underlying HBV disease (35). Understanding the pathobiology of HDV is necessarily complicated by the reliance of HDV on helper function(s) of the underlying HBV infection, as well as genetic and environmental aspects of the host. The unusual histologic form of severe type D hepatitis found in northern South America is probably not dependent on the host genetic background, as it occurs in mixed racial populations and is not limited to the South Amerinds of the region. Pathogen adaptation to populations with limited genetic diversity (36), therefore, does not appear to be a determinant of this phenomenon. A similar disease course with the same histologic features has been reported in the Central African Republic (37), and an outbreak of comparable severity occurred in the South Kashmir region of India (38). It also seems unlikely that an environmental cofactor is the cause of this characteristic disease pattern, as suggested by Strauss (39), since the defining disease features are found in distinct geographic regions and in populations with different cultural backgrounds, as noted above. Likewise, there is no evidence to indicate that the endogenous HBV strains of this region are different in their pathogenic potential from those that occur in other hyperendemic regions, although the possibility that some genetic feature of an endogenous HBV strain might be pathogenic when combined with HDV in this setting cannot be excluded. Lastly, other studies indicate that there exists considerable variation in the disease course of HDV infection depending on geographic region (22, 40, 41). Accordingly, the pathogenesis of type D hepatitis may depend on certain properties of the HDV genome, and the data described here are consistent with that concept. Genotype analysis of additional geographic isolates and the association of HDV genetic structures with disease outcomes in well-defined studies will be required to prove this hypothesis.

The mechanisms of HDV-related liver disease are unknown and may involve both direct and indirect cytopathic mechanisms. The histologic pattern observed in northern South America is more suggestive of a direct cytopathic effect than it is of an immunological disease. It is not immediately obvious what viral genetic determinants might be responsible for the pathology associated with HDV infection in South America, but there are several interesting possibilities. Certainly, features of HDAg could influence viral pathogenesis by affecting viral replication, interactions with host factors in infected cells, or the host immune response to infection. The most dramatic differences among genotypes are in the C terminus of HDAg-p27 and the N terminus of both forms of HDAg. The C terminus of HDAg-p27 is required for packaging the HDV ribonucleoprotein with HBsAg (11) and inhibits further replication of HDV RNA (9, 10). Perhaps the differences among the genotypes in this region reflect variations in the ability to be packaged by different hepadnaviral subtypes, variable degrees of inhibition of HDV RNA replication by HDAg-p27, or different interactions with host factors, including the immune system.

Two additional notable features of genotype III which could be related to pathogenesis include a significantly altered RNA editing target structure and the possibility of a protein product from another open reading frame. While all genotype I isolates exhibit a particular base-paired structure which has been shown to be required for maximum RNA editing (13), the base pairing of this target structure is disrupted in Peru-1. Consistent with this alteration, the level of RNA editing in the Peru-1 isolate is lower than in any other isolate which we have examined by analysis of a PCR-amplified segment (13) containing this site. Although no evidence exists for protein products from other open reading frames in other HDV isolates, it remains a possibility that such products, though nonessential, could play a role in modifying HDV disease in some isolates. In Peru-1, in addition to the open reading frame encoding HDAg, there are four open reading frames of >100 amino acids; the longest of these is 262 amino acids long, is genomic-sense, and overlaps the previously described open reading frame 2 (5).

The occurrence of genotype III in association with the severe hepatitis in northern South America leads one to wonder about relationships between the other genotypes (and subtypes) and disease. For example, it would also be interesting to know the extent and histologic form of acute disease and geographic spread of genotype II, of which Japan-1 is the lone representative, particularly since Japan-1, like the isolate Peru-1, has a significantly altered RNA editing target. The clones of Peru-1 which we have obtained can be readily

joined to produce a complete infectious genome which will facilitate study of the biology and pathogenesis of genotype III in cell culture and animal model systems in which it is possible to control additional complicating factors such as the helper hepadnavirus and environmental conditions. It is hoped that molecular studies of additional pathogenic and nonpathogenic variants of HDV defined in clinical and epidemiological studies will provide a more complete understanding of the complex factors, including viral genes, that influence HDV disease.

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