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A Novel Form of Hepatitis Delta Antigen

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Hepatitis delta virus (HDV) is known to express a protein termed the small delta antigen, a structural protein which is also essential for genome replication. During replication, posttranscriptional RNA editing specifically modifies some of the HDV RNA, leading to the production of an elongated form of the delta antigen, the large form, which is essential for virus assembly. The present study showed that yet another form of HDV protein is expressed during genome replication. This novel form is not produced in all infected cells, but it arises during replication in transfected cells and in infected woodchucks, and as was previously reported, patients infected with HDV do make antibodies directed against it. These findings are an indicator of the complexity of gene expression during HDV infection and replication.

The genomes of most RNA viruses have been found to undergo rapid variation during replication (6). Several mechanisms have been proposed; the most common involves errors by the polymerase during transcription (5). After the changes occur there are also several levels of biological selection, for example, the ability to be replicated or transmitted. Some variants may produce novel proteins, which in turn may elicit antibodies; such novel proteins can even have a function, for example, in immune avoidance mechanisms.

Our studies are concerned with the open reading frames (ORFs) of hepatitis delta virus (HDV) and also with whether additional ORFs are created and expressed as a consequence of genome variation.

It is evident from sequence analysis that HDV has many ORFs, on both the genome and antigenome (18). Figure 1A summarizes the predictions based on the HDV sequence obtained by Kuo et al. (12). However, a study by Weiner et al. strongly suggested that only one ORF is actually translated into protein, the 195-amino-acid (aa) ORF for the small form of the delta antigen, δAg-S (19). A separate study of HDV mRNA supports this hypothesis (8). An important modification of this hypothesis has been made; the new hypothesis is that RNA editing of the amber termination codon for the delta antigen opens up a longer ORF to produce a related protein, the 214-aa large delta antigen, δAg-L (13). Subsequently, it was shown that δAg-S and δAg-L have essential but different roles in the life cycle of HDV (3, 4).

It is generally believed that ORFs besides those for δAg-S and δAg-L are not expressed during the replication of HDV. However, a study by Khudyakov and Makhov in 1990 (10), which included an examination of several HDV sequences available at the time, led to some speculations about the expression of an additional ORF. In order to test for expression of this ORF, Khudyakov et al. made a synthetic peptide corresponding to this region. They then developed an enzyme-linked immunosorbent assay to test for antibodies to this peptide in patient sera and found that 12.5% of 104 HDV-positive patients actually had serum antibodies specific for this peptide (9). The following studies were undertaken to further investigate the significance of this finding.

As shown in Fig. 1, there is a 63-aa sequence predicted that is encoded in a −1 reading frame relative to that of δAg-L and δAg-S. This region is designated ORF-K. Some of the HDV sequences studied by Khudyakov et al. were different not only in predicted amino acid sequence but also in that they each had an additional termination codon which reduced the size of ORF-K. Thus, they used only a 24-aa peptide, peptide K (Fig. 1).

For our experiments, we made use of a rabbit polyclonal antibody raised against peptide K. We found by immunofluorescence microscopy that this protein domain is expressed in infected hepatocytes in vivo and also in cultured cells transfected with HDV cDNA. Our findings also suggest how this expression occurred and indicate that HDV genome replication involves expression of more than just δAg-S and δAg-L.

Expression in liver. Animals chronically infected with woodchuck hepatitis virus were superinfected with HDV, and biopsies were performed either at the peak of the acute infection or later, during chronic disease, all as previously described (15, 16). We examined liver sections from a total of seven woodchucks chronically infected with woodchuck hepatitis virus, with or without superinfection with HDV. We used immunofluorescence microscopy as previously described (2, 15) to test whether the peptide K domain was expressed in HDV-infected hepatocytes. The 24-aa synthetic peptide CDLHRIYSSQPIR PFLPRVVDPSE, previously referred to as HF-1 (9) and designated here as peptide K (Fig. 1), was used to raise an antibody in a rabbit. Another antibody, specific for both the small and large forms of the delta antigen, was raised in a rabbit by inoculation with delta antigen which had been prepared in *Escherichia coli*. Rabbit antibody rab-LP3, provided by S. Lemon and J. Cullen, was raised against the 15-aa peptide DILFPADPPFSPQSC, representing the carboxy terminus of the large form of the delta antigen (residues 197 to 211), and was shown to recognize the large but not the small form of the delta antigen (17). Double staining of cells was achieved by using rabbit anti-peptide K antibody as the primary antibody, followed by goat anti-rabbit immunoglobulin G polyclonal antibody conjugated with rhodamine (Boehringer) and then by 2 μg of 4′,6-diamidino-2-phenylindole (DAPI; Sigma) per ml to stain the cellular DNA. Examples of the resulting immunofluorescence are shown in Fig. 2A and B. Figure 2A shows the immunofluorescence obtained with an antibody specific for

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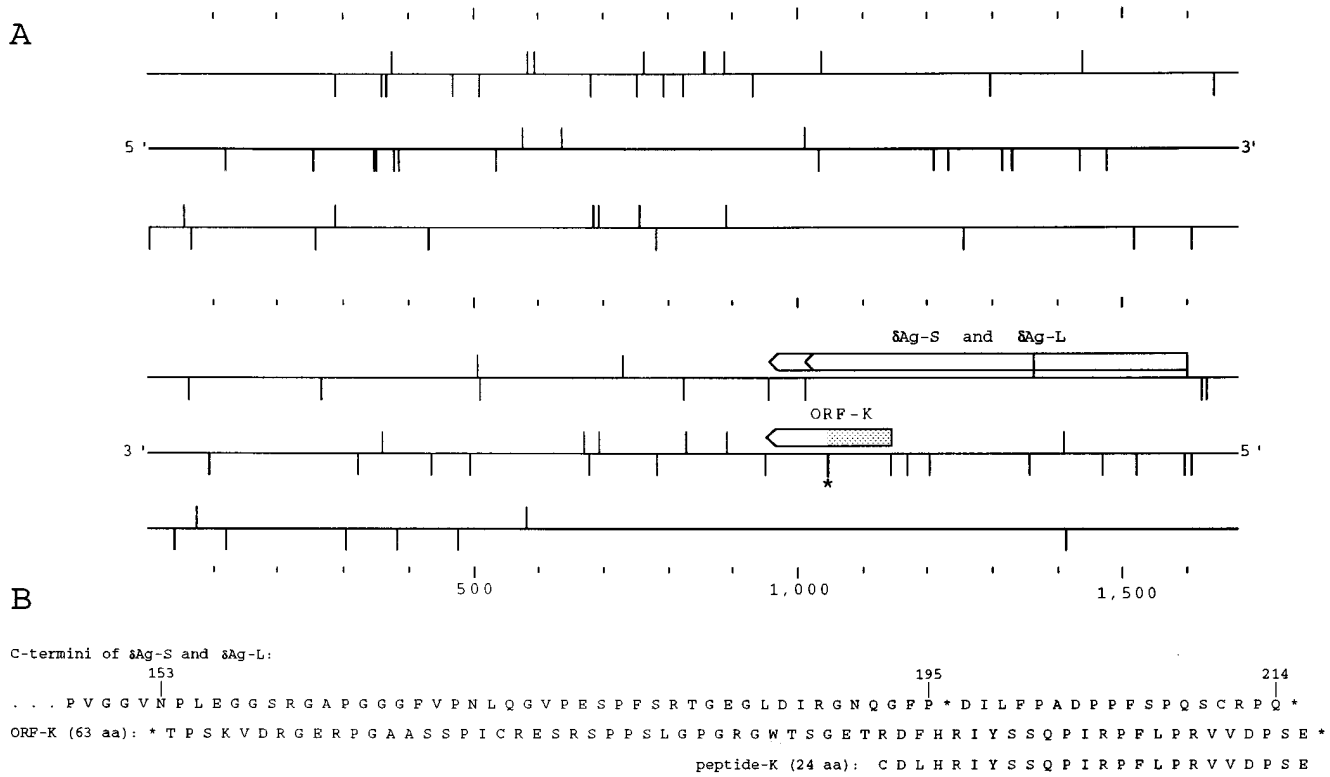

FIG. 1. Predicted ORFs of HDV. (A) Initiators (upward ticks) and terminators (downward ticks) for all possible reading frames on the HDV nucleotide sequence obtained by Kuo et al. (12). The ORFs for δAg-S and δAg-L are indicated on one frame of the antigenomic RNA. Overlapping these, on a separate reading frame, is ORF-K, part of which (unstippled) corresponds to peptide K, which is described in the text. A terminator absent from the sequence of Kuo et al. but present on some other reported HDV sequences is indicated with an asterisk. (B) Amino acid sequence for the C-terminal regions of the 195-aa δAg-S protein and the 214-aa δAg-L protein. Also shown are the sequences for ORF-K and peptide K, with their lengths indicated in parentheses. *, termination codons.

δAg-L. Figure 2B shows that a similar staining pattern was obtained with the antibody specific for peptide K; for convenience, we will subsequently refer to this antibody as domain K. Clearly the immunofluorescence signals for δAg-L and domain K were of comparable intensity and of comparable intracellular distribution, being nucleoplasmic with apparent exclusion of nucleolar regions. However, when we quantitated the cells positive for these two signals, we found a significant difference. Each cell that was positive for δAg-L was also positive for total delta antigen (total δAg), as assayed by double labeling with a human polyclonal antibody against delta antigens and vice versa (1). Each cell that was positive for domain K was also positive for total δAg by double labeling (1). In contrast, only a small fraction of the cells positive for total δAg were also positive for domain K (Table 1). This percentage was low but real for three of three animals with acute infections. In two of two animals with chronic infections, we were unable to detect cells that were positive for domain K. As can be seen from Table 1, this apparent inability was probably due to the fact that the liver sections of these animals sections contained far fewer cells that were positive for total δAg relative to those sections from acutely infected animals. Finally, the livers from two of two control animals not infected with HDV were negative for total δAg and domain K.

Expression in transfected cells. We next wondered whether a similar level of expression of domain K occurred when cultured cells were transfected with HDV cDNA. pSVL(D3), as described previously (11), was constructed by insertion of a trimer of unit-length cloned HDV cDNA (wild type) into expression vector pSVL (Pharmacia). Huh7 cells (14) were transfected by using Lipofectamine (Bethesda Research Laboratories) as recommended by the manufacturer (7), with modifications as previously described (20).

By immunofluorescence microscopy, we again found cells positive not only for δAg-L, as expected, but also for domain K, as shown in Fig. 2C and D, respectively. In both cases the signal was in the nucleoplasm. However, for the infected cells the signal was primarily found as a cluster of speckles, whereas for the liver sections it was diffusely distributed.

As with the liver sections, we quantitated the percentage of cells that were positive for domain K. The transfected cells, however, offered two advantages. First, we were able to conveniently monitor domain K expression as a function of time after transfection. The percentage of domain K-positive cells increased steadily with time, with the cells being first detected at day 6 and increasing to almost 2% at day 27 (Table 2). Second, we were able to monitor the progeny of transfected cells. The monolayer cultures were not split until day 12 after transfection; thus, we detected small groups of positive cells (up to 12 cells per group at day 12). These groups, which we interpret to be colonies, had qualitatively similar staining patterns.

Intracellular localization. In liver sections of infected woodchucks, the intracellular distributions of peptide K, δAg-L, and total δAg were indistinguishable (Fig. 2A and B and reference 1). However, this distribution was qualitatively different from what we detected in cDNA-transfected cells for peptide K (Fig. 2C) and δAg-L (Fig. 2D and reference 20), which both showed

FIG. 2. Results of immunofluorescence assays to detect δAg-L (A and C) and peptide K (B and D) in either liver sections of HDV-infected woodchucks (day 21) (A and B) or HDV cDNA-transfected Huh7 cells (day 9) (C and D). Such assays involved rhodamine labeling (red); DAPI was applied as a counterstain to all samples for detection of nuclear DNA (blue). Images were acquired with a charge-coupled device camera and processed by using Adobe Photoshop and Canvas software.

specific speckling patterns in the nucleoplasm and were actually colocalized (1). Total δAg was distributed not as clusters of speckles but more generally throughout the nucleoplasm (20). We did test whether the differences in intranuclear distributions between liver and cultured cells were due to differences in methods of fixation; typically, liver was formalin fixed, paraffin embedded, and then sectioned, while cultured cells were fixed with methanol and acetone. However, when we used comparable preparation procedures, the patterns were not changed (1). Other studies to characterize the speckles seen for δAg in cultured cells are now under way.

TABLE 1. Immunofluorescence detection of HDV proteins in woodchuck liver

HDV infection	Time after infection (days)	% Positive for domain K[a]	% Positive for total δAg[b]
Acute	21	0.22 (13/5,880)	20.3 (5,880/29,000)
Acute	21	0.28 (11/3,870)	16.8 (3,870/23,000)
Acute	21	0.07 (2/2,700)	13.2 (2,700/20,400)
Chronic	58	<0.38 (<1/260)	1.2 (260/21,000)
Chronic	62	<0.53 (<1/190)	0.6 (190/30,000)
None	NA[c]	NA	<0.003 (<1/30,000)
None	NA	NA	<0.004 (<1/28,000)

[a] Each value is the percentage of cells that were positive for total δAg that were also positive for domain K. In parentheses are the numbers of positive cells per total cells counted.

[b] Each value is the percentage of all cells, as quantitated by DAPI staining, that were positive for total δAg.

[c] NA, not applicable.

Possible mechanisms of expression. Our transfection studies were initiated with a cDNA of known sequence. Even our woodchuck experiments were initiated with virus assembled in cDNA-transfected cells (16). However, as shown in Fig. 1, peptide K is encoded in what we have designated as ORF-K, and the peptide could not be directly translated from this sequence because ORF-K lacks an in-frame initiator codon. Initiation at the δAg-S site followed by frameshifting is a possibility, as suggested by Khudyakov et al. (9). One mechanism of frameshifting is at the ribosomal level; the strongest argument against the involvement of this type of frameshifting is that it should occur in every infected cell, but we detected expression in only a small fraction of infected cells. Also, we did not detect expression in cells transfected with a nonreplicating cDNA construct that expresses δAg-S or δAg-L (1). An alternative mechanism of frameshifting would be at the level of

TABLE 2. Immunofluorescence detection of HDV proteins in transfected Huh7 cells

Time after transfection (days)	% Positive for domain K[a]
3	<0.005 (<1/20,000)
6	0.03 (5/20,000)
12	0.26 (29/11,000)
18	0.38 (34/8,900)
24	0.45 (19/4,200)
27	1.95 (16/820)

[a] Each value is the percentage of cells that were positive for total δAg that were also positive for domain K. In parentheses are the numbers of positive cells per total cells counted.

an insertion or deletion in the HDV genome. For example, a deletion of any one nucleotide between codons 153 and 206 in the δAg ORF would produce a protein detectable with the antibody (Fig. 1). (Addition of nine C-terminal amino acids of ORF-K to a delta antigen is sufficient for that protein to be recognized by the anti-peptide K antibody [1]). Three observations support but do not prove such a genetic change. First, not all infected cells express peptide K. Second, the fraction of positive cells increases with time after initiation of replication. Third, the positive cells are detected in clusters, which is consistent with clonal expansion and replication of a mutated genome.

It needs to be emphasized that our immunofluorescence data do not imply that peptide K is present on just one protein; since genetic changes consistent with frameshifting could occur in many ways and at many places, we may have a family of related proteins. Furthermore, we have not even proved that the proteins begin with the N-terminal region of δAg-S, although we think this is likely for two reasons. First, we know of only one mRNA that is involved in HDV replication (8). Second, the intracellular distribution of the expression of domain K was indistinguishable from that of δAg-L in both liver sections (Fig. 2A and B) and tissue culture cells (Fig. 2C and D).

Possible function. Finally, we need to address the possible function and significance of the expression of peptide K. A function in genome replication is unlikely since <2% of infected liver cells or transfected cells express this protein. However, expression does occur both early and reproducibly in liver and in cultured cells. It also occurs in patients, as evidenced by their production of specific antibody. Perhaps this and other variant proteins are routinely associated with HDV infections and the response of the host to the virus includes the response to such proteins.

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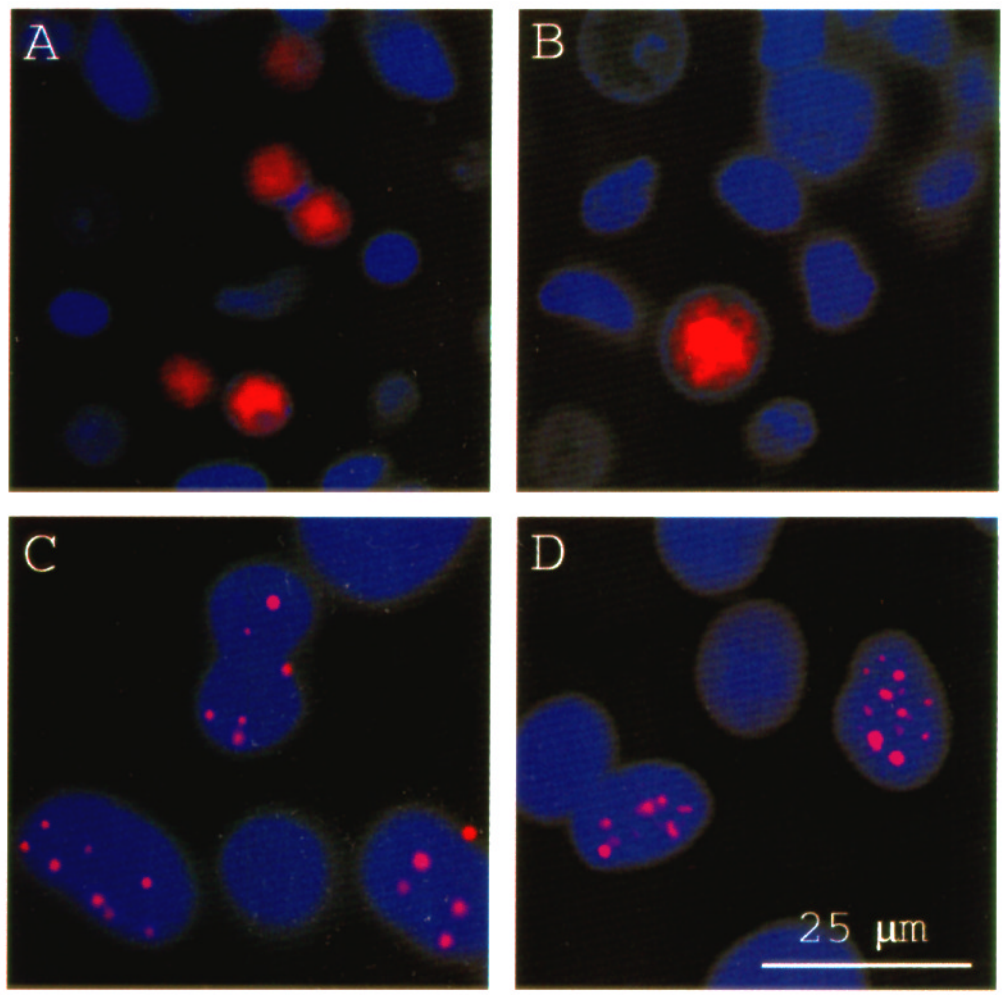
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