Cloning and expression of an immunodominant region of the hepatitis delta antigen

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A cDNA clone prepared from hepatitis delta virus (HDV) RNA extracted from human serum was subcloned in the bacterial expression vector pPL31 to produce a fusion protein consisting of the first 98 amino acids of MS2 polymerase and of 64 amino acids from near the N-terminal region of hepatitis delta antigen (HDAg). The fusion protein was shown to be related to HDAg by a commercial sandwich immunoassay (Abbott) and immunoblotting with human anti-HDAg serum. Antiserum against the fusion protein was raised in rabbits and used to identify HDAg extracted from the serum and liver of an HDV-infected woodchuck and chimpanzee and from the serum of an HDV-infected human, by immunoblotting and immunohistology. A single, major polypeptide of 24K was detected in both serum and liver extracts, with a minor polypeptide of 26K sometimes present. Liver extracts also contained lower M, polypeptides thought to be degradation products, the major species being 22-5K. The same pattern of staining was obtained with human anti-HDAg serum. Absorption experiments with the expressed protein and cross-competition experiments with the rabbit antiserum suggest that a major immunodominant region of HDAg is present near the N-terminal end of the antigen, between positions 1561 and 1368 on the genome. Both the expressed protein and rabbit antiserum were shown to be good diagnostic reagents.

Hepatitis delta virus (HDV) is a defective human virus which requires hepatitis B virus (HBV) for infection. The virus particles are made up of a circular, ssRNA molecule, approximately 1700 nucleotides long, associated with a virus-encoded antigen, the hepatitis delta antigen (HDAg), and encapsidated in the HBV surface antigen (HBsAg). HDAg was first detected in liver cells by immunofluorescence (Rizzetto et al., 1977). Chronic HDV carriers develop high antibody titres against HDAg and such sera have been used to develop an HDAg radioimmunoassay (Rizzetto et al., 1980). Both the antigen and antibody have been used as markers of viral infection.

Characterization of HDAg by immunoblotting has shown the presence of two polypeptides, of 24K to 26K M, (P24) and of 26K to 29K M, (P27) (Bergmann & Gerin, 1986; Bonino et al., 1986; Pohl et al., 1987; Zyzik et al., 1987; Weiner et al., 1988). The number of polypeptides detected by immunoblotting appears to depend on the source of HDAg; antigen from serum shows the presence of P24 and P27, whereas liver-derived HDAg consists mainly of P24 plus, in some cases, several polypeptides of lower M, (Bergmann & Gerin, 1986; Kuo et al., 1988) considered to be degradation products. The complete sequence of HDV RNA has been reported by three groups, using RNA isolated from chimpanzee serum (Wang et al., 1986), human serum (Makino et al., 1987) or HDV-infected woodchuck livers (Kuo et al., 1988), and suggests that HDAg is encoded by an open reading frame (ORF) on
Short communication

MS2 polymerase gene coding for the first 98 amino acids

Cut with PstI

Cut with PstI

Ligate

AmpR

Cut with PstI

TetR

Fig. 2. Construction of plasmid D280E.

the antigenomic strand (ORF 5). The product of this ORF is a single polypeptide of 195 (Kuo et al., 1988) or 214 amino acids (Makino et al., 1987; Chang et al., 1988). Heterogeneity in the sequence of Wang et al. (1986, 1987) predicts two peptides of 195 and 214 amino acids. Expression of the whole chimpanzee-derived HDAg ORF in bacterial and yeast expression vectors yielded two proteins, 24K and 27K, shown to be identical to the natural P24 and P27 proteins (Weiner et al., 1988), whereas expression of the human-derived HDV ORF in COS 7 cells yielded a single polypeptide of Mr, 26K, which was phosphorylated (Chang et al., 1988). Kuo et al. (1988) expressed the N-terminal region of HDAg in a bacterial expression vector and showed that antibodies against this protein were found to react with HDAg species in immunoblotting experiments.

In order to characterize HDAg further and to establish a good source of antigen and antibody for further studies and as diagnostic reagents, we have subcloned the N-terminal region of the HDAg ORF in a bacterial expression vector using a clone, D280, from a cDNA library derived from human serum (Saldanha et al., 1987). This clone contained a cDNA insert, approximately, 250 nucleotides long, covering part of ORF 5 between positions 1561 and 1368 (Fig. 1). The insert was cut out of the plasmid with PstI and recloned into the PstI site of the expression vector pPL31 to generate clone D280E (Fig. 2). The inserted gene was under the control of the leftward promoter (pl) of phage λ and the

Fig. 3. Immunoblot analysis of D280E-expressed protein and E. coli proteins. The proteins were separated by 12% SDS–PAGE and blotted onto nitrocellulose filters. Filter (a) was stained with amido black. Filters (b) to (e) were incubated with antiserum (1:500 to 1:1000 dilution) and developed with Protein A–gold and in some cases enhanced with silver. The antisera used were human anti-HDAg serum (b), rabbit anti-D280E serum (c), human anti-HDAg serum pre-absorbed with D280E protein (d) and rabbit anti-D280E serum pre-absorbed with E. coli proteins (e). The protein samples were D280E-expressed protein (lanes 1) and E. coli proteins (lanes 2). Mr markers are indicated on the left.
Fig. 4. Immunoblot of proteins extracted from liver and sera. Liver tissue (1 g) was minced with scissors and homogenized in a Dounce homogenizer in 3 ml of 6 M-guanidine-HCl pH 6.6. After a 3 h incubation at 4 °C, the extracts were pelleted at 12000 x g for 10 min. The supernatant was dialysed extensively against PBS and clarified by low speed centrifugation. Serum samples (0.1 to 0.75 ml) were overlaid on 2 ml of 20 % (w/w) sucrose in PBS and centrifuged at 190000 x g for 5 h at 4 °C. The pellets were resuspended in 100 μl of 2% SDS, 0.05 M-Tris–HCl pH 6.8 and heated at 100 °C for 10 min. Filters (a) and (c) were incubated with rabbit anti-D280E serum, whereas filter B was incubated with human anti-HDAg serum. Proteins were extracted from HDV-infected woodchuck serum (lane 1 of a and b; lane 2 of c), HDV-infected woodchuck liver (a and b, lane 3; c, lane 4), HDV-infected chimpanzee liver (a and b, lane 4; c, lane 3), HDV-infected human serum (a and b, lane 2) and normal human serum (c, lane 1). Mr markers are indicated on the left. Arrowheads indicate the positions of the 26K, 24K and 22.5K major HDV-specific polypeptides.

expressed protein consisted of a fusion protein containing the first 98 amino acids of the MS2 replicase and 64 amino acids from HDAg. Escherichia coli C600 cells containing a temperature-sensitive gene for the λ repressor protein were transformed with plasmid D280E. The cells were grown at 28 °C overnight, then at 42 °C for 3 h to induce the protein. The cells were centrifuged and the protein was extracted by three cycles of sonication and centrifugation. The final pellet was resuspended in phosphate-buffered saline (PBS) containing 6 M-urea. E. coli proteins were extracted in a similar manner from C600 cells which had not been grown at 42 °C. Analysis of the proteins by SDS-PAGE showed three major polypeptides of Mr values 25K, 22K and 19K (Fig. 3a) (the predicted size of the fusion protein is 27K). The smaller polypeptides were probably breakdown products of the 25K protein, as their relative concentrations increased with the cells' incubation time for the induction of protein expression (M. Easton, personal communication). A commercial assay for HDAg (Noc-tech) showed that the expressed protein was related to HDAg and further confirmation was obtained by Western blot analysis. Incubation of immunoblots of the expressed protein with human anti-HDAg serum gave specific staining of the major D280E bands and a minor band corresponding to 17K (Fig. 3b, lane 1). These bands were not detected with E. coli proteins (Fig. 3b, lane 2). Prior incubation of human anti-HDAg serum with D280E inhibited antibody binding (Fig. 3d, lane 1), whereas no inhibition was observed when the human anti-HDAg serum was preabsorbed with E. coli protein.

Table 1. Anti-HDAg antibody assay (Abbott assay)*

<table>
<thead>
<tr>
<th>Sample</th>
<th>C.p.m.</th>
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<tbody>
<tr>
<td>HDV-positive human serum no. 128</td>
<td>2469</td>
</tr>
<tr>
<td>Serum no. 128 preabsorbed with D280E</td>
<td>7904</td>
</tr>
<tr>
<td>Serum no. 128 preabsorbed with E. coli proteins</td>
<td>2665</td>
</tr>
<tr>
<td>Negative control serum</td>
<td>24876</td>
</tr>
<tr>
<td>Positive control serum</td>
<td>464</td>
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* The Abbot assay is a competitive radioimmunoassay in which anti-HDAg antibody in serum competes with a constant amount of anti-HDAg antibody labelled with 125I for a limited number of binding sites on beads coated with HDAg. Cut-off point: (0-4 x c.p.m. negative control) + (0-6 x c.p.m. positive control) = (0-4 x 24876) + (0-6 x 464) = 10 228. Samples with c.p.m. < cut-off point are positive for anti-HDAg.
Fig. 6. Immunoperoxidase staining of liver sections. Sections were dewaxed in xylene and decreasing concentrations of alcohol. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 15 min. Slides were washed, incubated in normal swine serum diluted 1:50 in 0.05 M-Tris-buffered saline pH 7.0 (TBS) for 15 min followed by three washes in TBS. Following incubation with anti-HDAg serum (1:500 dilution) for 1.5 h the slides were incubated for 30 min with biotinylated swine anti-rabbit immunoglobulin diluted 1:300 (Dako), washed three times in TBS and incubated for 30 min with an avidin-peroxidase conjugate–biotin complex (1 μl of avidin–peroxidase conjugate and 1 μl of biotin in 125 μl TBS). The slides were then washed in TBS for 3 h and the peroxidase was developed with 0.01% 3,3′-diaminobenzidine for 2 to 3 min. Finally, the sections were counterstained with haematoxylin, dehydrated and mounted. Sections (b) and (c) were directly stained by incubation with peroxidase-conjugated human anti-HDAg serum (a gift from Professor M. Rizzetto). Liver sections from HDV-infected woodchucks were incubated with (a) rabbit anti-D280E serum, (b) human anti-HDAg serum, (c) human anti-HDAg serum following preincubation of the section with rabbit anti-D280E serum. Section (d) was an HBV-infected human liver stained with rabbit anti-D280E serum. Section (e) was an HBV-infected human liver stained with rabbit anti-D280E serum.
rabbit anti-D280E serum was analysed further by the immunostaining of HDAg-positive liver sections. Staining of sections from chronically infected humans, chimpanzees and woodchucks, with anti-D280E gave specific nuclear staining (Fig. 6a), identical to the staining pattern observed when sections were stained with horseradish peroxidase (HRP)-labelled human anti-HDAg serum (Fig. 6b). Preincubation of sections with anti-D280E serum resulted in blocking of the specific signal on subsequent staining with HRP-labelled human anti-HDAg serum (Fig. 6c). Anti-D280E serum did not stain sections from chronic HBV patients (Fig. 6d).

Analysis of the predicted amino acid sequences of this region of the ORF using the published sequences shows very few amino acid changes (Fig. 1). A recent study by Pohl et al. (1987), who used a MAb to HDAG in immunoblotting experiments, suggests that all the HDAG species detected in immunoblotting experiments share the same epitopes. Only one ORF on the antigenomic strand has been identified as the HDAG ORF (Wang et al., 1986, 1987; Makino et al., 1987; Kuo et al., 1988) and presumably encodes the major 24K polypeptide identified in our experiments. Expression of this ORF, or in vitro translation of antigenomic HDV RNA, results in a single 25K (Kuo et al., 1988) or 26K (Chang et al., 1988) polypeptide. The P27 species, which is a minor component in our experiments and in some liver extracts (Bergmann & Gerin, 1986; Pohl et al., 1987), may be generated by modification of the major polypeptide. Wang et al. (1986, 1987) suggested that P27 may be expressed as a result of heterogeneity in the virus sequence and in a later study Weiner et al. (1988) showed that expression of the ORF in a bacterial expression vector produced both P24 and P27. Although these polypeptides share the same epitopes, the relationship between them will be resolved only by sequencing the termini of the natural polypeptides. Further experiments on characterization of the complete ORF product are presently being done using eukaryotic expression systems in which post-translational modifications occur. Its putative role in the replication of the virus, recently suggested by Kuo et al. (1989), is also being investigated. Besides the HDAG ORF, several other ORFs have been identified both on the genomic and antigenomic strands. Although no antibodies to the gene products of these ORFs have been detected in sera from chronic HDV carriers (Wang et al., 1986), it is conceivable that the products of these ORFs may play a role in the replication and/or pathogenesis of the virus.

Finally, we have shown that antibodies to the expressed D280E are able to detect the major epitopes identified by human anti-HDAg serum indicating the presence of these epitopes within this region (positions 1368 to 1561 on the genome) of HDAG. The D280E protein and its polyclonal antibody are good diagnostic reagents for serum markers of HDV infection and immunodetection of HDV on Western blots and liver sections, and constitute specific, standardized and biologically safe reagents.

References


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