Baculovirus-directed high level expression of the hepatitis delta antigen in Spodoptera frugiperda cells

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The hepatitis delta antigen (HDAg) is a multifunctional protein. It forms the core-like structure of the hepatitis delta virus (HDV) but also enhances replication of HDV in the nucleus of the hepatocyte. A cDNA fragment encoding HDAg was inserted adjacent to the polyhedrin promoter of Autographa californica nuclear polyhedrosis virus present in the baculovirus transfer vector pVL941. After transfection of Spodoptera frugiperda (Sf9) cells a recombinant baculovirus Acφ1 was isolated and purified using filter hybridization techniques. Sf9 cells infected with Acφ1 express the HDAg as a non-fused, non-glycosylated protein with an abundance of up to 25% of the total cellular protein mass. Immunoblot analysis using a human polyclonal anti-HD conjugate identified a 22K and a 24K protein in the nucleus of Acφ1-infected Sf9 cells. Electron microscopic studies using immunogold labelling showed that the recombinant HDAg (recHDAg) was associated with the hetero-chromatin of the Sf9 cells. The recHDAg produced by Sf9 cells elicited anti-HD antibodies in chimpanzees when injected intramuscularly.

Introduction

Hepatitis delta virus (HDV) is a defective RNA virus causing a fulminant or chronic hepatitis in hepatitis B virus (HBV)-infected patients (Rizetto et al., 1977). HDV is defective and must utilize the hepatitis B surface antigen, which is supplied by HBV, as a coat protein. The HDV infection is not restricted to HBV-infected individuals. Species which can be infected with a hepadnavirus similar to HBV, such as the woodchuck and Pekin duck, are also susceptible to HDV (Ponzetto et al., 1987a, b).

HDV has a circular, single-stranded RNA genome (Kos et al., 1986; Wang et al., 1986) of 1679 to 1683 nucleotides (nt) which is highly conserved in the animal model (Kuo et al., 1988; Wang et al., 1986, 1987) but which displays considerable sequence variation in human-derived HDV isolates (Makino et al., 1987; Saldanha et al., 1990b). An 800 nt polyadenylated mRNA from the antigenomic HDV RNA strand which codes for the hepatitis delta antigen (HDAg) can be identified in the liver (Chen et al., 1986; Hsieh et al., 1990). The HDAg is a nucleoprotein with RNA-binding capacity (Chang et al., 1988), involved in HDV replication (Glenn et al., 1990). It bears no resemblance to the hepatitis B core antigen (Weiner et al., 1988) and lacks a capsid form (Roggendorf et al., 1987).

Polyclonal antibodies directed against HDAg identified two proteins with sizes between 22K and 30K (Wang et al., 1986; Roggendorf et al., 1987). The larger protein is synthesized due to a sequence heterogeneity in the RNA genome (Luo et al., 1990). The codon change also occurs after infection with a genomic HDV cDNA encoding only the smaller protein (Sureau et al., 1989). A glycosylation site has been predicted from the amino acid sequence of HDAg in HDV isolates from chimpanzees and woodchucks (Wang et al., 1986; Kuo et al., 1988). The HDV glycosylation site is conserved in all isolates except for one of the two human-derived HDV isolates (Makino et al., 1987; Saldanha et al., 1990b). The secondary structure of HDAg is highly conserved in humans and the animal model with sequential immunodominant epitopes covering 41% of the molecule with one additional epitope present in the 27K protein only (Bergmann et al., 1989; Wang et al., 1990).

The baculovirus-directed expression system in insect cells has been very successful in producing biologically active and antigenic eukaryotic proteins indicative of correct post-translational processing (Luckow & Summers, 1988). In this paper we report a baculovirus-directed high level expression of HDAg in Spodoptera frugiperda (Sf9) cells. The recombinant HDAg (recHDAg) was located in the nucleus of the Sf9 cells. Purified recHDAg from Sf9 cells induced an immune response in chimpanzees.
Methods

Cells and viruses. Sf9 cells were grown as monolayer cultures at 27 °C in TC100 medium (Gibco/BRL) supplemented with 10% heat-inactivated foetal calf serum (FCS) and 40 µg/ml gentamicin. Seeding of the cells, wild-type Autographa californica nuclear polyhedrosis virus (AcNPV E1) and recombinant virus infections were performed as described by Summers & Smith (1987).

DNA manipulations. Plasmid DNA manipulations and sequencing (T₇ polymerase kit, Pharmacia) were carried out essentially according to Maniatis et al. (1982) or to instructions of the manufacturer.

Construction of recombinant baculovirus transfer vector pVL61. A 783 bp HindIII/ScaI fragment encoding the HD Ag open reading frame (ORF) was isolated from a lambda gt10 phage purified from a HDV cDNA library. Sequence analysis of this fragment (T. Kos et al., unpublished results) showed that the ORF encoded a protein of 195 amino acids caused by a stop codon at nucleotide 1013 (notation of Wang et al., 1986). The HindIII site was filled in and the blunt end fragment was subcloned into the Smal site of pGEM3Zf[+] (Promega) resulting in pGEM617-3 (Fig. 1). The baculovirus transfer vector pVL941 contains the upstream sequence and the first 35 coding bp of the polyhedrin gene, but the polyhedrin ATG codon has been changed by homologous recombination replacing part of the polyhedrin gene containing 1.5 ml TC100 medium with supplements; 5 µg/ml tunicamycin was added at 24 h p.i. (Smith et al., 1983). After incubation at 27 °C for 24, 48 and 72 h respectively, the Sf9 cells were suspended in sample buffer and kept frozen at -70 °C until analysed.

Cotransfection and purification of the recombinant baculovirus. The DNA band on pVL61 was introduced into the AcNPV genome by homologous recombination replacing part of the polyhedrin gene according to Method 1 (Summers & Smith, 1987) with minor modifications. In short, Sf9 cells were seeded in a 25 cm² flask containing 1.5 ml TC100 medium with supplements (10% FCS, 40 µg/ml gentamicin) at a density of 2 x 10⁶/ml and cotransfected with 1 µg of wild-type AcNPV and 2 µg of transfer vector pVL61 DNA. After 7 days at 27 °C wild-type and recombinant viruses from the supernatant were diluted 10⁵-fold and used to infect 2 x 10⁶ Sf9 cells/well in 96-well plates (Pes et al., 1989). After 7 days the supernatant was stored, the infected Sf9 cells were lysed in 100 μl 0.2 M-NaOH (Fung et al., 1988) and spotted onto Hybond-N membranes (Amersham). The membrane was incubated with a 3²P-labelled BamHI/KpnI DNA fragment from pGEM617-3 encoding the HD Ag gene, thereby selecting for recombinant baculovirus. The HDV probe was removed by incubating the membrane in 0.1% SDS (5 min, 95 °C) and the DNA samples were rehybridized with the 3²P-labelled 535 bp AcNPV BamHI/KpnI fragment from pVL941. This fragment is present only in the wild-type baculovirus indicating a recombinant baculovirus supernatant contaminated with AcNPV when a hybridization signal was detected. The purification procedure was repeated with a HD Ag-positive recombinant virus from the first screening.

DNA and RNA isolation. Total DNA from baculovirus-infected S9 cells was isolated 64 h post-infection (p.i.) (Summers & Smith, 1987). Total cytoplasmic RNA was obtained according to Luckow & Summers (1988) with the modification that 0.1% diethylpyrocarbonate was added to all buffers before sterilization. Cells were lysed in 10 mM-Tris-HCl pH 7.4, 1 mM-EDTA, 1% SDS and proteinase K (1 mg/ml). After phenol/chloroform/isoamyl alcohol (25:24:1) extraction the RNA was ethanol-precipitated and resuspended in 10 mM-Tris-HCl pH 7-6, 1 mM-EDTA, 20 mM-vanadyl ribonuclease complex (Gibco/BRL).

PAGE and western blotting. HDV-containing serum from chimpanzee Jimmy (Kos et al., 1986) was incubated for 5 min at 95 °C in protein sample buffer (31 mM-Tris-HCl pH 6-8, 5% glycerol, 1% SDS, 2-5% 2-mercaptoethanol, 0.001% bromophenol blue), size-separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell) by electroblotting (45 min, 4 mA/cm²). Non-occupied binding sites on the membranes were saturated by 30 min of shaking at 37 °C in phosphate-buffered saline (PBS) pH 7-4, 5% bovine serum albumin (BSA). After washing five times in PBS/0.1% Tween 20 [5 min, room temperature (RT)] the membrane was incubated (1 h, RT) in a 1:10 diluted human polyclonal anti-HD peroxidase conjugate (Nocotech) which had been preadsorbed with 20% chimpanzee serum. Excess serum was removed by washing five times with PBS/0.1% Tween 20 and three times with PBS. Staining was performed for 5 min in the dark with 20 ml of PBS, 5 mg diaminobenzidine, 12 µl of 30% H₂O₂ followed by extensive washing with water.

N-linked glycosylation inhibition of recHD Ag. Sf9 cells were mock-infected or infected with Ac6I or AcNPV in a 24-well plate (3 x 10⁴ cells/well) at an m.o.i. of 10 and incubated for 2 h at 27 °C. The supernatant was replaced with 500 µl of TC100 medium with supplements; 5 µg/ml tunicamycin was added at 24 h p.i. (Smith et al., 1983). After incubation at 27 °C for 24, 48 and 72 h respectively, the Sf9 cells were suspended in sample buffer and kept frozen at -70 °C until analysed.

Immunoperoxidase staining. Sf9 cells were mock-infected or infected at an m.o.i. of 10 with either recombinant Ac6I virus or wild-type AcNPV virus. At 48 h p.i., the monolayers were rinsed twice with Grace's medium (Gibco/BRL) and harvested. The cells (6 x 10⁴) were allowed to attach to microwell cover slips (10 µl/well) at RT for 1 h in a humid atmosphere. The coverslips were washed once with PBS and fixed in 1:1 methanol/acetone (-20 °C) for 30 s, air-dried and stored at 4 °C. After rehydration in PBS for 30 min and incubation (2 h, 27 °C) in peroxidase-conjugated human polyclonal anti-HDV antibody, the Sf9 cells were washed three times (10 min each) in PBS. Immunostaining was completed by incubation in 10 ml PBS, 6 mg diaminobenzidine, 12 µl 30% H₂O₂ for 10 min in the dark, followed by extensive washing in PBS before examination.

Immunoelectron microscopic analysis. Sf9 cells, mock-infected or infected at an m.o.i. of 10 with recombinant baculovirus AcE1 or AcNPV were harvested (48 h.p.i.), fixed for 2 h at 4 °C in 4% paraformaldehyde, 0-1% glutaraldehyde in PBS and stored in 2% paraformaldehyde in PBS (16 h). Following washing in PBS and centrifugation (10 min at 900 g) the pellet was resuspended 1:1 in 20% gelatine at 37 °C, centrifuged (5 min at 900 g) and stored in 2% paraformaldehyde. Sf9 cells were then impregnated for 16 h in 2.3 M sucrose, frozen in liquid nitrogen and sectioned using a Reichert FC 4D Ultracut microscope. Preparation for electron microscopy was done according to Tokuyasu (1980). Immuno-gold labelling of Sf9 cells was carried out according to Slot et al. (1986) with a slight modification. Sections were incubated for 10 min in 0.05 M-glycine pH 7.4, washed in PBS, 0.5% BSA, 0.1% gelatine, 0.1% TWEEN 20 (PBS–BGT) and then with 10% normal goat serum to prevent non-specific IgG binding. Sf9 cells were incubated for 1 h with human polyclonal anti-HD serum or normal serum in control sections. After washing the sections were incubated in PBS–BGT containing Protein A conjugated with 10 nm gold particles diluted 1:50 (Janssen, Life Sciences), followed by washing in PBS and distilled and deionized water. The cells were stained for 10 min in uranyl acetate, and washed and stained in 2% uranyl-acid for 4 min. The grids were dipped five times in 1% tylol at 4 °C and dried overnight. Analysis was carried out on a Philips EM 410 transmission electron microscope.
Expression of hepatitis delta antigen

Fig. 1. Construction of baculovirus transfer vector pVL61. To create a cDNA fragment surrounded by a BamHI and a KpnI site (with the 5' end of the HDAg gene located near the BamHI site) a blunt end cDNA fragment encoding the HDAg gene was subcloned into the SmaI site of pGEM3Zf[-]. The BamHI/KpnI fragment was recloned in a site-directed way into pVL941 resulting in the recombinant baculovirus transfer vector pVL61.

Purification of recHDAg and immunization of chimpanzees. Twelve percent SDS-polyacrylamide (PAA) slices, containing 50 or 200 μg of recHDAg protein were dehydrated by a 16 h incubation in 96% ethanol, 0-1 M-potassium acetate at 4 °C. The dried slices were crushed, homogenized in 1.5 ml PBS and pressed through a 19-gauge needle connecting two syringes. Chimpanzees Barney (male, 9 years) and Randy (female, 7 years) were injected intramuscularly with recHDAg using the PAA as an adjuvant. Barney received two injections, first 50 μg of purified recHDAg followed by a second dose of 200 μg after 14 days. Randy received only one injection of 50 μg of recHDAg. Immune responses were determined by screening with an anti-HD kit (kindly provided by Dr J. Walsh, Noctech Ltd, Ireland).

Results

Isolation and purification of recombinant baculoviruses containing the HDAg gene

The cDNA fragment encoding the HDAg gene was subcloned into the baculovirus transfer vector pVL941 resulting in pVL61 (Fig. 1). The correct orientation of the HDV cDNA fragment in the polyhedrin gene from pVL941 was verified by sequence analysis of both DNA strands using two HDAg gene-specific primers, PR4 (position 1008 to 989; sequence notation of Wang et al., 1987) and PR5 (position 1519 to 1538; Wang et al., 1987), directed towards the 5' and 3' ends of the HDAg/polyhedrin junction. The purification of the recombinant baculovirus was carried out without any morphological screening of Sf9 cells. The cloning strategy resulted in the deletion of a 535 bp BamHI/KpnI fragment from the polyhedrin gene which was therefore absent in the recombinant baculovirus. This procedure enabled us to purify the recombinant baculovirus using only endpoint dilution and DNA dot-blot hybridization. As shown in Fig. 2(a), primary screening of the 105-fold diluted supernatant from the cotransfection experiment resulted in two positive signals with a HDAg [32P]cDNA probe (HDV*) in wells A4 and D11. In both wells contamina-
Fig. 2. Isolation and purification of recombinant baculovirus Ac61. (a) Supernatant containing recombinant baculovirus Ac61 was diluted 10^5-fold and used for infection of Sf9 cells in a 96-well plate (mock-infected Sf9 cells in wells G1 and H1). Cell monolayers were transferred to Hybond-N membranes and probed with a HDAg[32P]cDNA (top) or an AcNPV[32P]DNA fragment (bottom) absent in the recombinant baculovirus. (b) Supernatant from well A4 was used for the second plaque purification (mock-infected Sf9 cells in wells H1 and H2). Recombinant baculovirus Ac61 from well H6 was propagated for virus stock and production of recHDAg.

Supernatant from well A4 of the master plate was diluted from 10^4- to 10^7-fold for a second screening with the same probes (Fig. 2b). At the 10^5-fold dilution 55% of the recombinant baculovirus was free from wild-type virus with complete purification achieved at a 10^6 dilution. Supernatant from well H6 containing recombinant baculovirus Ac61 was used for a third screening but no wild-type baculovirus was found (result not shown).

**Analysis of recombinant viral DNA and RNA**

The recombinant baculovirus Ac61 and AcNPV were propagated in Sf9 cells, and viral DNA was extracted and hybridized with HDAg[32P]cDNA. Only DNA isolated from the Ac61-infected Sf9 cells hybridized with the probe resulting in a labelled fragment of 780 bp after digestion with BamHI/KpnI (Fig. 3a). This indicates that the complete HDAg gene had recombined into the genome of the baculovirus.

In order to determine the size of HDAg-specific mRNAs, total cellular RNA from Ac61-infected Sf9 cells was hybridized with a HDAg[32P]cDNA fragment. As shown in Fig. 3(b) an RNA molecule of 1400 nt was found which was identical to the expected size of the combined HDAg and polyhedrin mRNA.

**Expression and N-linked glycosylation of recHDAg in Sf9 cells**

Sf9 cells were mock-infected or infected (m.o.i. of 10) with either recombinant baculovirus Ac61 or wild-type AcNPV, lysed at 48 h p.i. and subjected to SDS-PAGE. Immunostaining with human polyclonal anti-HD serum showed that the recombinant virus Ac61 synthesizes a protein of 24K at high levels with a size comparable to...
Expression of hepatitis delta antigen

Fig. 3. Southern and Northern blots from AcΔ1- (lanes 1, 2 and 5) and AcNPV-infected (lanes 3, 4 and 6) Sf9 cells. DNA and RNA were hybridized with the 783 bp BamHI/KpnI [32P]cDNA fragment encoding HDAg. (a) Comparison of DNA patterns from AcΔ1- and AcNPV-infected Sf9 cells, undigested (lanes 1 and 3) or digested with BamHI and KpnI (lanes 2 and 4). (b) Total cytoplasmic RNA was isolated from Sf9 cells infected with AcΔ1 (lane 1) or AcNPV (lane 2) to determine the size of the HDAg/polyhedrin-specific mRNA. Cytoplasmic RNA was denatured for 10 min in 25 mM-CH3HgOH neutralized 1:1 with 40 mM-2-mercaptoethanol and size-separated on 1.2 % agarose with 2.2 M-formaldehyde. Markers (kb) were lambda DNA digested with HindIII (a) or the RNA ladder from Gibco/BRL denatured under similar conditions as the Sf9/baculovirus RNA (b).

Fig. 4. Western blot analysis of HDAg from serum (lane 1) and AcΔ1/Sf9-derived (lane 2) recHDAg. HDAg was isolated from 1 μl of serum from chimpanzee Jimmy (Kos et al., 1986) and recHDAg from Sf9 cells infected with AcΔ1 (m.o.i. of 10) 48 h p.i. Controls were AcNPV- (lane 3) and mock-infected (lane 4) Sf9 cells. Tunicamycin is a specific inhibitor of asparagine-linked glycosylation (Takatsuki et al., 1975). The 24K protein was found both in tunicamycin-treated Sf9 cells 24, 48 and 72 h after incubation as well as in untreated cells (Fig. 6). This indicates that the predicted glycosylation site at position 121 (Wang et al., 1986) is not used for asparagine-linked glycosylation in Sf9 cells. A 22K HDAg-specific protein, also unchanged in size in the presence of tunicamycin was detected 48 h and 72 h p.i. In AcNPV-infected Sf9 cells, a 64K baculovirus-specific protein known to be glycosylated (Stiles et al., 1983) shifted to a 58K position after addition of tunicamycin (result not shown) indicating successful inhibition of N-linked glycosylation.

Nuclear localization of recHDAg in Sf9 cells

An ELISA detecting HDAg (Noctech) and Western blotting showed that up to 64 h p.i. (m.o.i. of 10), HDAg-specific proteins were detected only inside transfected Sf9 cells, indicating that HDAg is not excreted into the...
Fig. 5. Expression levels of recHDAg. Sf9 cells washed with TC100 medium were seeded in 24-well plates (5 × 10⁵ cells/well). After attachment of the cells, medium was replaced by an inoculum of Acδ1 with m.o.i.s of 1 (lanes 1 to 5) or 10 (lanes 7 to 10) and incubated for 1 h at 27 °C. Supernatant was removed and the infected cells were incubated in TC100 medium with supplements. At 0 (lane 1), 16 (lanes 2 and 7), 24 (lanes 3 and 8), 48 (lanes 4 and 9), and 64 h (lanes 5 and 10) Sf9 cells from one well were washed, centrifuged and the pellets kept at −70 °C. After thawing, cells were resuspended in 100 μl protein sample buffer. Ten μl was heated (4 min at 95 °C), size-separated on a 12% PAA gel and stained with CBB. Mr markers are indicated (lane 6).

Fig. 6. N-linked glycosylation of recHDAg. Sf9 cells were seeded in a 24-well plate (3 × 10⁵ cells/well), infected with Acδ1 (lanes 1 to 6) or AcNPV (lanes 7 and 8) at an m.o.i. of 10 or mock-infected (result not shown). Twenty-four h p.i. the medium was removed, the cells were rinsed and incubated in TC100 medium with supplements without (lanes 1, 3, 5 and 7) or with 5 μg/ml tunicamycin (lanes 2, 4, 6 and 8). Samples taken 24 (lanes 1 and 2), 48 (lanes 3 and 4) and 72 h p.i. were size-separated by 12% SDS-PAGE, electroblotted and incubated with human polyclonal anti-HD serum.

medium. To localize the recHDAg, the Sf9 cells were infected with Acδ1 at an m.o.i. of 10. At 48 h p.i., cells were allowed to attach to coverslips and were prepared for immunohistology. A similar procedure was performed with AcNPV and mock-infected Sf9 cells. Under the light microscope, the nuclear matrix of the recombinant virus-infected cells showed intense granular immunostaining with the polyclonal anti-HD serum in addition to a faint staining of the cytoplasm (Fig. 7a). HDAg-related staining was not found in either the wild-
Expression of hepatitis delta antigen

Type AcNPV (Fig. 7b) or the mock-infected cells (Fig. 7c) although polyhedron structures in the AcNPV-infected cells indicate abundant expression of the polyhedrin gene.

Ultrathin cryosections of Acδ1-, AcNPV- and mock-infected Sf9 cells were prepared for electron microscopic analysis. As shown in Fig. 8(a), only in the AcNPV-infected Sf9 cells were polyhedra detected indicating a defective polyhedrin gene in Acδ1 (Fig. 8c). In both situations high levels of filamentous (F) structures were detected in the cytoplasm and the nucleus (Fig. 8a and c). To investigate whether the F structures in the Acδ1-infected cells were correlated with the high level expression of HDAG, immunostaining with Protein A-gold coupled to polyclonal anti-HD was carried out. The immunogold complex was exclusively attached to the heterochromatin of Acδ1-infected Sf9 cells (Fig. 8d). No immunogold label was detected in the F structures (Fig. 8c), the AcNPV- (Fig 8a and b) or mock-infected cells (result not shown). Replacing the polyclonal anti-HD serum with normal serum resulted in the absence of immunogold staining of the chromatin indicating the specificity of the polyclonal anti-HD serum (result not shown).

Immunization of chimpanzees with recHDAg

Chimpanzees Randy and Barney were inoculated with recHDAg to determine whether a baculovirus-expressed HDAg protein could induce an anti-HD response. As shown by competitive ELISA, Randy’s serum contained specific antibody after 32 days which lasted for a further 28 days by which time the anti-HD level had reached the cut-off value again (Fig. 9). The anti-HD levels in Barney were lower (Fig. 9) but they showed a plateau for up to 60 days. Comparison of this immune response with those of HDV-infected chimpanzees showed a 10^5-fold lower titre for the recHDAg-inoculated chimpanzee (result not shown).

Discussion

The HDAg is involved in the formation of the core-like protein structure of HDV and its replication. The protein could play a role in the regulation of the HDAg-specific mRNA synthesis (reviewed by Taylor, 1990) and it is also the only known protein causing an immune response after an HDV infection.

In this paper we report high level expression of the gene encoding the complete HDAg protein in a
baculovirus-directed expression system with production levels up to 120 mg/l in SF9 cells (2 × 10^6/ml). The baculovirus AcδI was isolated using a new cloning procedure for this expression system. By deletion of part of the polyhedrin gene we were able to perform a site-directed cloning in the baculovirus transfer vector and to purify the recombinant baculovirus without any morphological screening. Based on these results, the ratio of wild-type AcNPV and recombinant viruses (Fig. 2a) in the original supernatant could be determined; the recombination frequency approached 2.5%. The recHDAg was produced as a non-fused, intracellular 24K protein comparable to the smaller of the two HDAg-specific proteins present in the serum of our HDV-infected chimpanzee. In the sera and the liver of patients a heterogeneity in the HDV RNA encoding the HDAg was found (Xia et al., 1990). This heterogeneity also occurred after infection with an HDV cDNA sequence encoding only one ORF (Sureau et al., 1989). After infection of SF9 cells with the baculovirus AcδI, the smaller HDAg-specific protein was expressed indicating that this heterogeneity did not occur in the insect cells. In addition to the major 24K protein, a 22K HDAg-specific protein was found in SF9 cells. The 22K protein is probably a degraded 24K protein still retaining antigenic sites recognized by the polyclonal anti-HD serum. Several groups have presented data on HDAg-specific degradation products found in the liver (Bergmann & Gerin, 1986; Saldanha et al., 1990a), in bacteria expressing recombinant HDAg (Weiner et al., 1988) and in a hepatoma cell line (Macnaughton et al., 1990). They all found one or more degradation products which were reduced in size by 2K to 4K compared to the smaller HDAg protein found in serum. This indicates specific protease-sensitive sites. In the amino-terminal region of the 24K protein the sequence Gly-Arg-X is recognized twice by the serine protease factor Xa. Cleavage would result in a 21-5K degradation product comparable to the one we found in the SF9 cells. Further amino acid sequence analysis will be required to provide evidence for this hypothesis. Tunicamycin inhibition experiments showed that in SF9 cells both the 22K and 24K HDAg-specific proteins were expressed as non-glycosylated polypeptides similar to the HDAg in the HDV-infected hepatocyte (Zyzik et al., 1987).

The mRNA coding for HDAg present in the liver has an estimated length of 697 nt excluding the poly(A) tail (Hsieh et al., 1990). Our studies in the SF9 cells show that the HDAg-encoding cDNA fragment (nt 1624 to 819; Fig. 1) was transcribed as a 1400 nt mRNA. This indicates that the first seven non-coding nt of the HDAg mRNA are not essential for high level expression in SF9 cells when fused to the 5' end of the polyhedrin mRNA. Second, the HDV-specific transcription termination signal is not recognized by the RNA polymerase of the SF9 cells. Moreover, this mRNA contains the antigenomic autocleavage site (nt 900) surrounded by a maximum of 91 nt from HDV RNA. Based on the length of the mRNA transcribed from the baculovirus DNA we must conclude that the cleavage site is not recognized in SF9 cells. Sharmeen et al. (1988) showed autocleavage in a 179 bp antigenomic HDV RNA fragment (nt 785 to 964) which indicates either that sequences 785 to 819 are essential for autocleavage or that the site is not recognized at all in SF9 cells. The anti-genomic cleavage site was also present in the simian virus 40 (SV40)-based expression vectors used for producing recHDAg in COS7 cells (Chang et al., 1988; Kuo et al., 1989) and NIH3T3 fibroblasts (Glenn et al., 1990). Although both cell lines were not tested for the presence of this specific SV40 transcribed mRNA, both showed correct replication of the HDV RNA after introduction of the complete HDV genome.

The recHDAg accumulated only in the nucleus of SF9 cells which is comparable to the localization in hepatocytes. Functional transport of the protein obviously occurs from the cytoplasm to the nucleus independently from sequences of the HDV genome other than the HDAg coding region. Immunoelectron microscopic analysis revealed that the recHDAg did not precipitate in the SF9 cells and it was not associated with either the insoluble filamentous structures or the large vacuoles. The HDV-specific protein was located on the heterochromatin in the nucleus bound only to nucleic acids not involved in transcription and translation whereas no binding was detected with the euchromatin. This indicates that the ability of HDAg to bind to nucleic acids (Chang et al., 1988; Macnaughton et al., 1990) is still maintained in SF9 cells. A significant difference in the ratio of heterochromatin to euchromatin was detected in the AcδI- and AcNPV-infected cells with high levels of heterochromatin in the AcδI-infected cells possibly indicating an inhibitory effect of the recHDAg on the metabolism of the SF9 cell. In HDV-infected hepatocytes a c.p.e. is found which could be caused by interaction or cleavage with the 7S RNA due to sequence homology (Negro & Gerin, 1989b; Young & Hicke, 1990). This could indicate a new function for the HDAg in the still unsolved cytopathogenicity of HDV.

As soon as mass vaccination against HBV infection is performed, the occurrence of HDV will decrease because of the lack of a host supplying the coat protein for HDV. Presently there are about 280 million HBV carriers world-wide, each individual forming a potential HDV reservoir. This group cannot be protected against HDV infection by an HBV vaccine. The HDAg is the only known candidate protein for a vaccine although it is an internal HDV protein covered with HBsAg. However,
antibodies to HDAg protected chronic HBV carrier chimpanzees against a challenge with HDV (Rizetto et al., 1981), a protection that can last for several years (Negro et al., 1989a). Immunization studies were initiated by using the high level expression of recHDAg in SF9 cells. Chimpanzees injected with purified recHDAg acquired anti-HD antibody levels in the serum indicating the antigenicity of our recHDAg. Although antibodies were present, these levels were much lower than the antibody levels of a chimpanzee which had recovered from an acute HDV infection. Further experiments will determine whether these antibodies are neutralizing HDV infectivity. Also different adjuvants and immunization schemes will have to be explored with the aim of improving the response to recHDAg. These experiments must be carried out before clinical trials to protect HBV-infected individuals from an HDV infection can be initiated.

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