Intracellular production of African horsesickness virus core-like particles by expression of the two major core proteins, VP3 and VP7, in insect cells

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To gain more insight into the structure of the African horsesickness virus (AHSV) core particle, we have cloned, partially characterized and expressed the two major core proteins, VP3 and VP7, of AHSV-9. VP7 was found to be highly conserved amongst different serotypes. The VP3 and VP7 genes were subsequently expressed in insect cells by means of recombinant baculoviruses. VP7 was synthesized to very high levels and aggregated into distinctive crystals. Co-expression of VP3 and VP7 resulted in the intracellular formation of core-like particles which structurally resembled empty AHSV cores.

African horsesickness is a highly infectious non-contagious disease of equines and in horses the mortality rate exceeds 90%. The aetiological agent, African horsesickness virus (AHSV), belongs to the genus Orbiviruses in the family Reoviridae. Like bluetongue virus (BTV), the prototype orbivirus, the AHSV virion is composed of seven structural proteins organized into a double-layered capsid containing the 10 double-stranded (ds) RNA segments of the viral genome (Bremer, 1976; Roy et al., 1994). The icosahedral core is composed of two major proteins, VP3 and VP7, and three minor structural proteins (VP1, 4 and 6). It has been shown that VP7 trimers constitute the surface of the BTV core: hence the prominent knob-like protrusions which overlay a scaffold of VP3 (Prasad et al., 1992; Grimes et al., 1995). The outer capsid layer is composed of the two major proteins, VP2 and VP5, of which VP2 is the serotype-specific antigen (Burrage et al., 1993; Vreede & Huismans, 1994; Martinez-Torrecuadrada & Casal, 1995).

Most of the structure–function related research on orbiviruses has been carried out with BTV (Roy, 1992). The baculovirus expression system has been exploited extensively over the past few years to investigate the assembly of the major BTV capsid proteins into core-like particles (CLPs) and virus-like particles (VLPs) via co-expression of the relevant genes in insect cells (French et al., 1990; French & Roy, 1990). It was shown that the formation of CLPs comprising VP3 and VP7 is self-primed. In addition, VP2 and VP5 can bind independently of each other to preformed cores to give rise to VLPs, but fail to assemble in the absence of this scaffold. No studies regarding the assembly of AHSV particles have been reported. Both the genes encoding the major core proteins of AHSV-4 have been cloned and sequenced (Roy et al., 1991; Iwata et al., 1992), but there have been no reports on the expression of VP3.

In this report, we describe the cloning and expression of the VP3 and VP7 genes of AHSV-9. We have found that their simultaneous expression in eukaryotic cells results in their spontaneous intracellular assembly into CLPs which structurally resemble empty authentic AHSV cores.

Complementary DNA (cDNA) copies of the AHSV-9 VP3 and VP7 genes were cloned into the Pst I site of pBR322 as described by Bremer et al. (1990) and then characterized by restriction enzyme analysis, dot-blot hybridization and terminal dideoxy sequencing. With respect to the AHSV-9 VP7 gene, a full-length clone, as well as truncated overlapping cDNA clones were obtained and sequenced. The gene was found to be 1167 bp in length with an open reading frame (ORF) of 1047 nucleotides. Comparative analysis with the cognate gene of AHSV-4 (Roy et al., 1991) revealed 96.2% conservation of nucleotides. The coding region of the AHSV-4 VP7 gene was found to be 12 nucleotides longer than that of the AHSV-9 VP7 gene because of two repeat sequences of 3 and 9 nucleotides in length. If the 4 amino acids encoded by these repeats are ignored, the only difference between VP7 of the two serotypes is a single non-conservative amino acid substitution at position 5. Wade-Evans et al. (1993) also cloned the AHSV-9 VP7 gene using a PCR technique with serotype 4
VP7 gene-specific terminal oligonucleotides as primers. There is a 97.7% conservation of nucleotides between the two sequences and the length of the coding region is the same. A cDNA clone containing the complete ORF of the AHSV-9 VP3 gene was obtained and characterized by restriction enzyme mapping and terminal sequencing. The sequence of the ORF termini (16% of the gene) indicated more than 94% identity with the VP3 gene of AHSV-4, confirming that VP3 is also highly conserved (Iwata et al., 1992).

In order to express the VP3 and VP7 genes of AHSV-9, the flanking homopolymeric G/C-tails were removed by a PCR technique with termini-specific primers. The following oligonucleotide primers were synthesized to modify and tailor the ends of the cloned cDNAs:

S3/1: 5′ GGAGATCTATGCAAGGGAATGAAAGAATAC 3′
S3/2: 5′ GGAGATCTGGCTGCTAAATCGTTGGTCG 3′
S7/1: 5′ CACAGATCTTTCGGTTAGGATGGACGCG 3′
S7/2: 5′ CACAGATCTGTAATGTATTCGGTATTGAC 3′

These primers incorporated BglII restriction recognition sites (underlined) at each terminus to facilitate subsequent DNA manipulations. In addition, the entire 5′ non-coding region of the VP3 gene was deleted whereas that of the VP7 gene was deleted up to 7 bp upstream from the initiation codon. Most of the 3′ non-coding region of the VP3 gene was also deleted. The modified PCR-tailored copy of the VP7 gene was fully sequenced to ensure that no mutations had been incorporated. In the case of the PCR-tailored VP3 cDNA, the major central region, constituting 71% of the gene (EcoRI–HindIII fragment), was replaced with the corresponding original cDNA region, thereby constructing a VP3 PCR-cDNA hybrid gene in pBR322.

To confirm that the respective VP3 and VP7 genes encoded full-length proteins, the modified VP3 and VP7 genes were each cloned into the BamHI site of the pBS transcription vector under control of the T7 promoter. The genes were transcribed using T7 polymerase and the mRNA transcripts were translated in vitro, in a cell-free rabbit reticulocyte system (Amersham). The [35S]methionine-labelled translation products were analysed by SDS–PAGE and autoradiography (Fig. 1a). Bands corresponding to the predicted size of VP3 (103 kDa) and VP7 (38 kDa) were observed. These bands co-migrated with the corresponding VP3 and VP7 proteins synthesized in AHSV-9-infected BHK cells. No similar bands were observed in the mock-infected BHK cell extract. The level of VP7 expression was much higher than that of VP3. Translation of VP3 transcripts also resulted in the synthesis of several peptides that were smaller than the expected size of VP3. This could perhaps be attributed to either translation of incomplete transcripts or premature termination of the translation (Liu et al., 1992).

The VP3 and VP7 genes were then individually expressed in insect cells using the BAC-to-BAC baculovirus expression system.
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system (Luckow et al., 1993) in which the recombinant baculovirus DNA is constructed in E. coli. Each gene was expressed under control of the polyhedron promoter. Analysis of Sf9 cells infected with the plaque-purified VP3 and VP7 recombinant baculoviruses, respectively, revealed that proteins corresponding to the estimated sizes of VP3 and VP7 were synthesized. No similar bands were observed in the wild-type or mock-infected cells (Fig. 1c). VP7 was synthesized to a much higher level (ca. 40 µg per 10⁶ cells) compared to VP3. This is in agreement with our in vitro expression results (Fig. 1a). This difference in expression levels may reflect an inherent property of each gene that is determined by the relative amount in which each protein is required. The ratio of VP7 to VP3 in authentic BTV cores is 13:2 (Burroughs et al., 1994).

Fig. 2. (a) Electron micrographic images of negatively stained purified AHSV CLPs produced in insect cells following purification. (b) Negative contrast electron micrograph of the purified CLPs bound to VP7 monoclonal antibodies. The bar marker represents 100 nm.
Accumulation of VP7 in the cytoplasm of infected cells led to the formation of a number of large, distinctive crystals in each cell (not shown). These crystals appeared to be similar to the crystalline structures found in AHSV-4 infected cells when observed under a light microscope (Chuma et al., 1992).

To establish whether AHSV VP3 and VP7 are able to assemble into CLPs, S9 cells were co-infected with the VP3 and VP7 recombinant baculoviruses at multiplicities of 5 p.f.u. per cell. The cells were harvested 48 h p.i., lysed at 4 °C in TNN buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 0.5% NP40) and the nuclei removed by low-speed centrifugation. The cytoplasmic fraction was analysed on a sucrose gradient (10–40%, w/v, in 0.2 M Tris–HCl, pH 8.0) by centrifugation for 2 h at 38 000 r.p.m. in an SW41 rotor. The gradient was VP3 and VP7 fractionated and the fractions were analysed by SDS–PAGE. A VP3–VP7 complex in the region of 225 ± 25 S was identified. This sedimentation value was approximately half of that of authentic AHSV cores (470 S) (Burroughs et al., 1994) and agreed with that of BTV CLPs (French & Roy, 1990). Fractions containing the VP3–VP7 complex were selected, concentrated (2 h at 42 000 r.p.m.) and analysed by electron microscopy. Samples were adsorbed to carbon-filmed copper 400-mesh grids, washed in 0.1 M Tris–HCl (pH 8.0) and negatively stained with 2% uranyl acetate. Electron micrographic images (Fig. 2a) indicated that the complexes largely resembled authentic empty AHSV core particles. Unlike the native AHSV cores, the empty synthetic particles were characterized by a dark central region with typical icosahedral symmetry. This was surrounded by a less-electron-dense layer with knobby morphological protrusions which extended outwards and conferred a fuzzy appearance to the CLPs. This outer layer is probably composed of VP7 trimers as for BTV (Grimes et al., 1995). The CLP diameter of approximately 72 nm is in agreement with the diameter of BTV CLPs (Hewat et al., 1992) obtained from cryo-electron microscopic images, which is slightly greater (4%) than the diameter of the authentic core (Burroughs et al., 1994). The identity of the CLPs was further verified by means of antibody decorating using VP7 monoclonal antibodies. Grids with pre-adsorbed material were incubated in different dilutions of VP7 monoclonal antibodies (Van Wyngaardt et al., 1992) for 30 min after which the grids were washed with 0.2 M Tris–HCl (pH 8.0) and negatively stained with 2% (w/v) uranyl acetate. Electron micrographs indicated that each particle of interest was masked by a dark shadow resulting from the specific interaction of VP7 monoclonal antibodies with the VP7 outer shell (Fig. 2b). Data obtained by Hewat et al. (1992) suggest that BTV CLPs differ from the authentic cores by the absence of VP7 spikes around the fivefold axis, thereby reducing the knobby appearance. Liu et al. (1992) reported that in vitro assembled CLPs displayed even less surface structure than BTV CLPs, perhaps due to incomplete VP7 incorporation. The AHSV CLPs also appeared to vary with respect to the quantity of knobby protrusions. This may be due to variable amounts of VP7 incorporated during intracellular assembly as a result of variation in the ratio of VP7 to VP3 expression in the insect cells co-infected with the two recombinant baculoviruses.

The fine structure of the AHSV CLPs remains to be investigated. We would also like to co-express the CLPs together with the outer capsid polypeptides of AHSV to produce VLPs. BTV VLPs were found to be highly immunogenic, even at low doses compared to VP2 alone or mixtures of VP2 and VP5 (Roy et al., 1992). It has been suggested that CLPs provide the necessary scaffold for the correct conformational presentation of the relevant epitopes on VP2. This has not been investigated in the case of AHSV. To accomplish this, there is a requirement for a dual recombinant vector expressing large amounts of VP3 and VP7 simultaneously.

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