Expression of muscovy duck parvovirus capsid proteins (VP2 and VP3) in a baculovirus expression system and demonstration of immunity induced by the recombinant proteins

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The gene encoding the muscovy duck parvovirus (DPV)-strain 89384 capsid proteins VP2 and VP3 was cloned in a baculovirus expression system and expressed in insect cells. The recombinant proteins were found to react with specific anti-DPV serum by Western blotting and to be located in the nucleus of insect cells (Sf9) as shown by immunofluorescence. Empty virus-like particles (VLPs) identical in size and appearance to DPV virions were observed by electron microscopy. The antigenicity and immunogenicity of the recombinant proteins were evaluated by ELISA and seroneutralization. Immunization of 3-week-old muscovy ducklings induced anti-DPV antibodies; neutralizing antibody titres were consistent with those observed in ducklings inoculated with a commercial inactivated vaccine. The way to develop these promising results is discussed.

The muscovy duck parvovirus (DPV) is the causative agent (Jestin et al., 1991a; Fournier & Gaudry, 1992) of a disease which appeared in France in 1989 (Jestin, 1990; Drouin et al., 1991) that is characterized by high mortality in very young muscovy ducklings, stunting, markedly reduced body weights, and delay in the process of feathering in older ones (Jestin, 1990). In addition, nervous signs and associated microscopic lesions have been reported (Dalibart et al., 1993). In the field, only attenuated live and inactivated vaccines are available (Fournier & Gaudry, 1992; Jestin et al., 1993). They are intended (i) to give high antibody levels in breeders in order to obtain good maternal immunity (ii) to actively immunize ducklings that become vulnerable after the drop in maternal antibodies. For that purpose, the vaccination schedule is expensive and time consuming since several injections are required. Moreover, the quality of protection of 2- to 3-week-old ducklings is questionable (Jestin et al., 1993). This can account for chronic cases of the disease that are still observed later on and are responsible for significant economic losses. Therefore, there is the need for further improvement of the vaccination strategy.

DPV has a linear single-stranded DNA genome of about 5300 bases with terminal palindromic hairpins (Le Gall-Reculé & Jestin, 1994). The right open reading frame (ORF) of the DPV genome encodes three capsid proteins VP1, VP2 and VP3 (G. Le Gall-Reculé & V. Jestin, unpublished results). The corresponding nucleic sequence was established for the 89384-DPV strain (accession number Z68272 in nucleotide sequence databases), and comparison of its predicted amino acid sequence with those of mammalian parvoviruses showed a higher homology level with the defective parvovirus, the adeno-associated virus type 2 (AAV2) (G. Le Gall-Reculé & V. Jestin, unpublished results). By analogy with the AAV2 capsid proteins gene organization (Becerra et al., 1988; Cassinotti et al., 1988; Trempe & Carter, 1988), and by reference to the observed molecular masses of DPV VP2 and VP3 proteins (Le Gall-Reculé & Jestin, 1994), we determined the most favourable initiation codon positions of the two genes.

The baculovirus expression system has been used to efficiently produce large amounts of viral antigens (Luckow & Summers, 1988). When expressed using such a system, parvovirus capsid proteins self-assemble and form empty virus-like particles (VLPs). In most cases, the recombinant particles were shown to be antigenically and immunologically identical to native parvovirus virions (Kajigaya et al., 1991; Lopez de Turiso et al., 1992; Martinez et al., 1992; Saliki et al., 1992; Christensen et al., 1994). The purpose of this paper is (i) to describe the cloning and the expression in baculovirus of the...
DPV gene encoding VP2 and VP3, and (ii) to demonstrate the antigenicity and the immunogenicity of the expressed proteins.

The 89384-DPV strain was isolated from liver, heart and spleen of a muscovy duck showing symptoms of the disease (Jestin et al., 1991a). DPV virions were purified by banding in CsCl and the DPV DNA was extracted as previously described (Le Gall-Reculé & Jestin, 1994, 1995). We cloned inserts containing the entire right ORF of the DPV genome and the recombinant plasmid pDPV2 was selected for the recombinant transfer vector construction. We chose to express both DPV VP2 and VP3 by construction of a recombinant baculovirus containing the genomic segment coding for the two genes. Indeed, Ruffing et al. (1992) showed that empty capsid-like structures were observed only when AVA2 VP2 was coexpressed with any other capsid protein. Moreover, some authors succeeded in expressing both Aleutian mink disease parvovirus VP1 and VP2 structural proteins, using a recombinant baculovirus generated from a single cDNA containing the start ATGs for the two polypeptides (Christensen et al., 1993). The gene segment coding for VP2 and VP3 was amplified from purified pDPV2 by using the polymerase chain reaction (PCR) technique. The two primers DPV-U 5' CCCCATGGATGGCTCCTGCTAA 3' for the upper one and DPV-L3 5' CCCCATGGAAAAATCAAAAGA3' for the lower one, correspond in their DPV-specific sequence to the nucleotides 670-683 and 2455-2443 respectively, according to the DPV sequence in the nucleotide sequence databases. This procedure permitted us to mutate the VP2 ACG codon to an ATG one (bold T in the upper primer) corresponding to a more favourable context for the initiation of translation, and to introduce a NcoI restriction endonuclease site allowing cloning into the NcoI site of the pGmAc3MR baculovirus transfer vector. This vector contains essential parts of the polyhedrin gene of Autographa californica nuclear polyhedrosis virus (AcNPV) which were cloned into the pUC8 vector. Kmpl and NcoI sites constitute the multiple cloning site region. Since the DPV capsid protein gene contains a Kmpl site (Le Gall-Reculé & Jestin, 1994), we had to clone into the NcoI one. The amplification was performed as previously detailed (Jestin et al., 1995) except that the denaturation step at 95 °C for 5 min was followed by 5 cycles of 20 s at 95 °C, 30 s at 50 °C, 30 s at 75 °C, then 30 further cycles of 20 s at 95 °C, 30 s at 55-5 °C and 30 s at 75 °C. The amplification was terminated with an elongation step at 75 °C for 6 min. The purified PCR product was digested by NcoI and successfully cloned into the transfer vector. Recombinant plasmids containing a single insert in the correct orientation were selected by restriction enzyme analysis. One recombinant plasmid (p114) was amplified and the insert sequence was checked by DNA sequencing. Five micrograms of p114 plasmid DNA was cotransfected with 500 ng of purified wild-type AcNPV DNA into Spodoptera frugiperda (Sf9) insect cells by using DOTAP transfection reagent (Boehringer), according to the supplier's instructions. Recombinant baculoviruses were purified and one (AcDPV114) was used to express DPV recombinant proteins.

Sf9 cells were infected at an m.o.i. of 10 p.f.u./cell either with wild-type or AcDPV114 recombinant baculoviruses. Cells were harvested 48 h post-infection and total proteins from 2 × 10⁶ pelleted cells were separated by 10% SDS-PAGE before being transferred onto a nitrocellulose membrane. The proteins were identified with a specific DPV duck antiserum and a peroxidase-labelled rabbit anti-duck IgG (H + L) con-
jugate (Nordic). The antiserum was prepared by inoculating DPV virions into specific pathogen free (SPF) muscovy ducks. Western blot analysis showed two bands of approximately equal intensity that corresponded to two expressed proteins of approximately 73 and 59 kDa (Fig. 1a). Their relative molecular masses were similar to those of the DPV VP2 and VP3 proteins (74 and 58 kDa, respectively) established by Western blotting in the same conditions (Fig. 1b). No bands of these sizes were detected in wild-type baculovirus-infected Sf9 cells (Fig. 1a). To localize the subcellular distribution of the expressed proteins in Sf9 cells, we performed an indirect immunofluorescence analysis. Infected Sf9 cells were prepared as previously described and 2 x 10⁴ cells were dispensed per well. After being fixed, the slides were incubated with a specific DPV duck antiserum or a SPF duck serum and stained with an FITC-conjugated rabbit anti-duck immunoglobulin serum (Nordic). Observation of the slides containing recombinant baculovirus-infected Sf9 cells incubated with DPV duck serum, confirmed the expression of DPV capsid proteins and showed that they were localized in the nucleus of the cells (data not shown). No reaction was observed either with these cells following incubation with the SPF duck serum, or with wild-type baculovirus-infected Sf9 cells incubated with the DPV antiserum. Nuclear location was described previously for baculovirus expression of both VP1 and VP2 structural proteins of Aleutian mink disease parvovirus (Christensen et al., 1993) and of recombinant AAV2 proteins, when VP1 or VP2 with VP3 were coexpressed (Ruffing et al., 1992).

We examined whether the recombinant proteins could self-assemble into VLPs. For this purpose, Sf9 cells infected by recombinant baculoviruses as described above were pelleted by centrifugation, washed with cold PBS, and resuspended in a buffer containing 1% deoxycholate, 0.1% SDS, 0.1 mM-EDTA, 10 mM-Tris–HCl pH 8.0. Cells were incubated on ice for 30 min and centrifuged (12000 g, 10 min, 4 °C). The supernatant was layered onto a double cushion of 50 and 30% (w/v) sucrose and ultracentrifuged at 100000 g for 2 h (4 °C). The interface was diluted in PBS, centrifuged at 200 000 g for 2 h (4 °C) and the pellet was resuspended in PBS. A fraction was examined by electron microscopy. For morphology comparison with wild-type DPV, virions were CsCl gradient-purified as previously described (Le Gall-Reculé & Jestin, 1994) and examined at the same magnification. Electron micrographs showed some aggregates of virus-like particles with a morphology very similar to that of native DPV virions (Fig. 2). The diameter of the particles was estimated to be about 22 nm, which is consistent with the size of DPV virions (Le Gall-Reculé & Jestin, 1994). However, few particles only were detected in our purified preparations although we used a combination of 0.1% SDS and 1% deoxycholate in case they were present as hardly soluble aggregates in Sf9 cells. These results revealed that recombinant capsid proteins VP2 and VP3

Table 1. DPV antibodies in muscovy ducks inoculated with recombinant baculovirus expressing VP2 and VP3 capsid proteins

<table>
<thead>
<tr>
<th>Duck no.</th>
<th>Before the third inoculation</th>
<th>2 weeks after the third inoculation</th>
<th>3 weeks after the third inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA*</td>
<td>log₂ SN†</td>
<td>ELISA</td>
</tr>
<tr>
<td>1</td>
<td>8.8 (0.5)</td>
<td>7.0</td>
<td>9.0 (0.0)</td>
</tr>
<tr>
<td>2</td>
<td>3.7 (0.6)</td>
<td>6.3</td>
<td>3.0 (0.0)</td>
</tr>
<tr>
<td>3</td>
<td>5.7 (0.6)</td>
<td>8.3</td>
<td>8.0 (0.0)</td>
</tr>
<tr>
<td>4</td>
<td>8.4 (1.1)</td>
<td>9.6</td>
<td>7.0 (0.0)</td>
</tr>
<tr>
<td>5</td>
<td>5.3 (0.6)</td>
<td>7.0</td>
<td>3.3 (0.0)</td>
</tr>
<tr>
<td>6</td>
<td>7.6 (1.8)</td>
<td>8.3</td>
<td>3.7 (0.0)</td>
</tr>
<tr>
<td>7</td>
<td>8.6 (0.6)</td>
<td>8.3</td>
<td>8.0 (0.0)</td>
</tr>
<tr>
<td>8‡</td>
<td>5.0 (0.0)</td>
<td>7.0</td>
<td>10.0 (0.0)</td>
</tr>
<tr>
<td>Mean ratio of the group</td>
<td>6.6</td>
<td>7.8</td>
<td>6.8</td>
</tr>
</tbody>
</table>

* Mean of at least three repetitions/serum (standard deviation).
† Statistical mean of three repetitions.
‡ Duck no. 8 received 0.2 dose, 0.25 dose and 0.75 dose at first, second and third inoculation.
were able to self-assemble into DPV-like empty capsids, demonstrating that the presence of DPV VP1 was not necessary for capsid formation. Similar observations have been reported for the formation of VLPs of AAV2 (Ruffing et al., 1992) and some autonomous paroviruses (Kajigaya et al., 1991; Martinez et al., 1992; Saliki et al., 1992; Christensen et al., 1993, 1994).

For evaluating the immunity induced by the expressed DPV capsid proteins, SF9 cells were infected either with recombinant baculovirus (RB) or with wild-type baculovirus (WB) at an m.o.i. of 10 to 20 and harvested 48–60 h post-infection. Nineteen 3-week-old SPF muscovy ducks were separated into three groups. Eight and five ducks received VLPs plus adjuvant in a final volume of 1 ml, at 2 week intervals. The adjuvant was identical to the one included in the Parvol vaccine (Rhône-Mérieux, France) against muscovy duck parvo-virus. Six more ducks were put in close contact with the latter ones. Thereafter, the absence of specific antibodies having been checked before the first injection, sera were collected from all birds before the third injection, then 2 and 3 weeks later. For titration of DPV antibodies, the GM ELISA test was used as described (Jestin et al., 1992). This test employs the DPV GM strain that had been shown to be very closely related to strain 89384 (Jestin et al., 1991b). Each serum sample was at least tested three times. Neutralizing antibodies were titrated in decomplemented sera as previously described (Jestin et al., 1991a) with the exception that Eagle’s medium modified McPherson-Stocker BHK21 (Eurobio) was used. Irrespective of the time of serum sampling, no antibodies were detected in ducks from group WB or contact ducks. On the contrary, all the RB ducks exhibited high to medium antibody ELISA titres and medium serum neutralizing antibody (SN) titres (Table 1). Two weeks after the third inoculation, no booster effect was noticed except for the duck no. 8 that received lower doses at each inoculation. One week later, antibody ELISA titres were decreasing whereas SN titres were more stable. Thus, we demonstrated that the expressed parovirus capsid proteins induced specific duck parovirus antibodies. Our results are very consistent with those obtained by testing the French commercial bivalent inactivated oil vaccine (designed to stimulate maternal immunity) on 2-week-old muscovy ducklings. In fact, 2 and 3 weeks after the second injection mean ELISA titres reached 3·4 then 4·3, respectively, and mean seroneutralization titres (log2) 6·3 then 7·6 (V. Jestin, unpublished results). Thus, for breeder immunization, purified VLPs plus adjuvant could be used.

In the present study, we have described for the first time the expression of VP2 and VP3 capsid proteins of the muscovy duck parovirus in insect cells infected by recombinant baculoviruses and we have demonstrated that immunization with VLPs induced neutralizing antibodies against DPV in ducks. A recombinant vaccine would be highly desirable. For this purpose further improvements are required, notably the optimization of VLP expression levels, and the construction of new recombinant baculoviruses is currently under investigation.

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References


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