Molecular characterization and expression of the S3 gene of muscovy duck reovirus strain 89026

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Although reovirus infection is one of the major virus diseases of muscovy ducks in France, no vaccine is available and nothing is known about the structure and function of the genes and proteins of the reovirus involved. The complete S3 genome segment of the muscovy duck reovirus strain 89026 has been cloned and the nucleotide and deduced amino acid sequences are reported here. The S3 genome segment is 1201 bp long and possesses the same terminal motifs (5’ GCTTTTT and TATTCATC 3’) as the S3 genome segment of known chicken reovirus strains. It contains one open reading frame that encodes a protein of 367 amino acids with a molecular mass of 40.8 kDa. The gene, encoding the σB major outer-capsid protein, was cloned into two different baculovirus transfer vectors and expressed in insect cells as a glutathione S-transferase fusion protein or a non-fused protein. The antigenicity of the two recombinant proteins was demonstrated by immunoblot assay. The potential immunogenic role of σB protein was studied in a protection assay against reovirus infection of specific-pathogen-free muscovy ducks. No antibodies could be detected by ELISA or immunoblot in ducks immunized with the recombinant proteins and no significant protection was noted after the challenge. However, whereas the weights of wild-type baculovirus-infected and challenge-control ducks were significantly lower than those of unchallenged ducks, the weights of male ducks previously immunized with the σB recombinant proteins did not differ significantly from males of either group. This work is the first to provide molecular data for a duck reovirus.

Introduction

Avian reoviruses have been recovered from several species of birds. They belong to the family Reoviridae, genus Orthoreovirus, together with the mammalian reoviruses, with which they share common physico-chemical properties and morphological characteristics. These include a double-stranded (ds) RNA genome consisting of ten segments packaged into a non-enveloped icosahedral double-capsid shell, 70–80 nm in diameter. The genomic segments can be separated on the basis of their electrophoretic mobility into three size classes, with three large (L1–L3), three medium (M1–M3) and four small (S1–S4) segments (Nick et al., 1975; Spandidos & Graham, 1976; Gouvea & Schnitzer, 1982; Wu et al., 1994; Heffels-Redmann et al., 1992). However, certain biological properties of the avian reoviruses differ from those of mammals, e.g. the lack of haemagglutination activity (Glass et al., 1976), the ability to induce fusion of cultured cells (Ni & Ramig, 1993) and their pathogenicity towards their natural hosts.

Little information is available on the molecular biology of avian reoviruses, notably on the structure and function of their genes and proteins, unlike those of mammalian reoviruses. Studies in this field have mainly been concerned with the chicken reoviruses. The protein coding assignments for the ten genomic segments of different strains of chicken reovirus have been studied by several authors but the results are conflicting (Schnitzer et al., 1982; Ni et al., 1993; Varela & Benavente, 1994; Martinez-Costas et al., 1997). At least ten structural polypeptides were identified in the latter study. The minor
outer-capssid polypeptide σC was shown to be the cell-attachment protein and to induce serotype-specific neutralizing antibodies (Schnitzer et al., 1982; Wickramasinghe et al., 1993; Theophilos et al., 1995). This protein was suggested to be structurally analogous to mammalian σ1 (Schnitzer et al., 1982; Shapouri et al., 1995). The major outer-capssid proteins μB and σB are related to the μ1C and σ3 proteins, respectively, of the mammalian reoviruses and are involved, in association or individually, in cell fusion (Ni & Ramig, 1993). Two studies identified μB and σB as the avian reovirus proteins involved in broad-specificity neutralization (Wickramasinghe et al., 1993; Shapouri et al., 1996).

The S3 genome segments of chicken reovirus strains S1133 (Yin et al., 1997) and 1733 (Vakharia et al., 1996) have been sequenced and expressed in vitro. These studies established that the σB protein encoded by the S3 gene is structurally related to the σ3 protein of mammalian reoviruses. However, Yin et al. (1997) did not find the motif reported in the σ3 protein that is responsible for dsRNA-binding activity. Vakharia et al. (1996) and Yin et al. (1997) demonstrated that the expressed σB protein reacted with an anti-reovirus polyclonal serum, but its role in protective immunity has not yet been evaluated.

Muscovy duck reovirus is the aetiological agent of a disease first described in South Africa in 1950 (Kaschula, 1950) and in France, where the virus was isolated in 1972 (Gaudry et al., 1972). The acute form of the disease is apparent in ducklings between 2 and 4 weeks of age, in which morbidity is high and the rate of mortality is 10% or more. Clinical signs include apathy accompanied by diarrhoea and difficulties in movement. Macroscopically, dead animals can show fibrinous pericarditis, marbled spleen and enlarged and friable liver (Gaudry et al., 1972; Malkinson et al., 1981; Marius-Jestin et al., 1988). At the microscopic level, the pericarditis consists of an infiltration of the serous membrane by different mononuclear inflammatory cells, associated with a fibrinous exudate. In the liver, there are either local necroses or small infiltrates of lymphocytes and plasmocytes, particularly in the portal region. In the spleen, reticulosis, depletion of lymphoid nodules and focal necrosis can be seen. The synovial sheath of the leg tendons shows an exudative inflammation (Marius-Jestin et al., 1988). The only molecular data available on muscovy duck reoviruses concern the electrophoretic patterns of the dsRNA genomes and proteins of two strains (Heffels-Redmann et al., 1992). These patterns were apparently similar to those of the chicken reovirus. However, V. Jestin (unpublished results) and Heffels-Redmann et al. (1992) demonstrated in cross-neutralization tests that the muscovy duck reovirus was antigenically different to the S1133 chicken reovirus, though the two strains share some epitopes, as shown by cross-immunodiffusion gel assay and ELISA (V. Jestin, unpublished results). In two successive trials carried out in our laboratory in 1982 and 1990, vaccination of muscovy ducks with chicken reovirus vaccine strain S1133 gave poor results (Marius, 1982; V. Jestin, unpublished results). Field attempts to protect with a duck reovirus inactivated vaccine were unsuccessful, despite good experimental results (Marius, 1983), and studies carried out in an attempt to obtain a duck reovirus attenuated vaccine have failed.

The aims of the present study were (i) to obtain new molecular data on the muscovy duck reovirus in order to extend our general knowledge of avian reoviruses and (ii) to analyse the antigenicity and possible immunogenicity of the muscovy duck reovirus σB protein. The σB protein of chicken reovirus induces broad-specificity neutralizing activity and could therefore be of interest in a vaccination strategy. The S3 genome segment of the muscovy duck reovirus strain 89026 was cloned and the nucleotide and deduced amino acid sequences were determined. The gene was then expressed in insect cells with a baculovirus expression system and the antigenicity of the recombinant protein was determined by immunoblotting. The potential immune response to the expressed protein and its role in protection against reovirus infection was studied in specific-pathogen-free (SPF) muscovy ducks.

Methods

- **Viruses and cells.** Reovirus strain 89026 was isolated in 1989 from the liver, spleen and heart of muscovy ducks with symptoms of disease, after several passages via the chorio-allantoic membrane (CAM) of SPF muscovy duck eggs, as described previously (Jestin et al., 1991). Reovirus strain 89330 was isolated in the same way from a sick muscovy duck from another farm. Both strains were characterized by immunofluorescence assay, as already described (Jestin et al., 1991), and cloned by limiting dilution.

Wild-type *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and recombinant baculoviruses (BaculoGold, PharMingen) were grown and plaque-assayed in monolayers of *Spodoptera frugiperda* (Sf9) insect cells, kindly provided by G. Devauchelle (INRA, CNRS UA 1184, St-Christol-les-Ales, France). Sf9 cells were cultured at 28°C in TC100 medium (Gibco BRL) supplemented with 5% foetal calf serum.

- **Reovirus purification and dsRNA extraction.** Virus strain 89026 was extracted from the CAM of infected eggs by fluorocarbon (1,1,2-trichlorotrifluoroethane) treatment. The resulting supernatants were collected, washed by dilution in 1,1,2-trichlorotrifluoroethane and subjected to ultracentrifugation at 200,000 g for 22 h (4°C). The fractions corresponding to densities of 1.34 to 1.37 g/cm³ were collected, washed by dilution in TNE buffer and ultracentrifuged (200,000 g, 90 min, 4°C). The pellets of the purified reovirus were then resuspended in 200 μl TE and stored at −70°C until use. The dsRNA was extracted as previously described (Le Gall-Reculé & Jestin, 1994), except that solutions were specific for RNA extraction. Purified dsRNA was stored at −70°C until use.

- **Cloning and sequencing of the S3 gene.** The method described by Lambden et al. (1992) for amplification of the human rotavirus dsRNA genome segments was used, with modifications, to clone the muscovy duck reovirus S3 gene. The oligonucleotide primer P1 (5' PO4-3'TTAACCGCCGAGGCTATA-NH2 3'), which we ligated to both 3'
ends of the dsRNA genome segments, was chemically modified to prevent self-ligation and subsequent concatenation during the RNA ligase reaction, as described by Lambden et al. (1992). Denaturation conditions (temperature and duration of denaturation) were optimized for reverse transcription of the reovirus dsRNA. P1-tailed dsRNA was denatured at 95 °C for 5 min, instead of 90 °C for 5 min, in the presence of primer P2 (complementary to P1). After reverse transcription, annealing and repair of cDNA strands was carried out, as described by Lambden et al. (1992), and amplification was accomplished by PCR with primer P2 only, consisting of a denaturation step at 95 °C for 5 min followed by 35 cycles of 30 s at 95 °C and 4 min at 68 °C and a final elongation step at 72 °C for 10 min. Amplified DNA products were separated on agarose gels and the 1250 bp fragment, which corresponded in size to that expected from the chicken reovirus data for the S3 gene plus primers, was excised. DNA was eluted from the agarose fragment overnight in 200 µl distilled water at 37 °C and an amplification by PCR was performed with 2 µl DNA solution. The PCR fragment was excised from the gel, agarose-purified (GeneClean II kit, Bio 101) and ligated into the Smal site of pUC18 with the SureClone ligation kit (Pharmacia Biotech). Epicurian Cofi XL-1 Blue competent cells (Stratagene) were transformed with recombinant plasmids and mini-preparations of plasmid DNA were obtained by the boiling method (Sambrook et al., 1989). Several plasmids containing an insert of the expected size were selected by restriction enzyme analysis, amplified and purified with the QIAgen kit) and the corresponding insert sequences were checked by DNA sequencing.

### Co-transfection and selection of recombinant baculoviruses.

For each construct, co-transfections were prepared with each of the two purified recombinant transfer vectors. Five µg recombinant plasmid DNA was co-transfected with 500 ng linearized BaculoGold baculovirus DNA (Pharmingen) with DOTAP transfection reagent (Boehringer Mannheim), according to the supplier’s instructions. Two recombinant baculoviruses from each co-transfection were purified and amplified.

### Expression and identification of recombinant σB proteins.

S9 cells were infected with either wild-type or recombinant baculoviruses at an m.o.i. of 10 p.f.u. per cell. The cells were then harvested and 72 h post-infection and the expressed proteins were analysed by immunoblot. Total proteins from 2 × 10⁶ pelleted cells were separated by 10% SDS–polyacrylamide gel before being transferred onto a nitrocellulose membrane (Bio-Rad). Proteins were identified by a reovirus-specific muscovy duck antiseraum and a peroxidase-labelled rabbit anti-duck IgG (H+L) conjugate (Nordic).

### Immunization of ducklings with wild-type and recombinant baculoviruses, and protection test.

S9 cells were infected at an m.o.i. of 2 p.f.u. per cell in a 250 ml spinner and harvested 72 h post-infection for each injection. Expression and antigenicity of recombinant σB protein were examined by immunoblot before each inoculation. SPF muscovy ducks (116 reovirus- and specific-antibody-free 2-week-old birds) were divided into five groups of homogenous weight according to sex and housed in filtered-air rooms. The absence of specific antibodies was checked before the assay. Ducks from three groups received two injections 3 weeks apart of either wild-type baculovirus-infected cells (WB group) or cells infected with one of the recombinant baculoviruses expressing non-fused or GST-fused S3 protein (S3RB and GST–S3RB groups). Before injection, the cells had been mixed with a water-in-oil adjuvant, routinely used for inactivated avian vaccines. Each duck from these three groups was inoculated subcutaneously with 1 × 10⁷ infected cells in a final volume of 1 ml for the first injection and 1.5 × 10⁷ cells in a final volume of 1.5 ml for the second injection. Three weeks later (when the ducks were 61 days old), all the ducks were weighed and subjected to the three groups inoculated with cell lysates and the challenge-control group (CC group) were challenged intramuscularly with 0.5 ml muscovy duck reovirus strain 89026 containing 10⁶.5 EID₅₀/ml (titre expressed as log₁₀ 50% embryo infective dose). This strain showed greater pathogenicity to ducks than 89026, despite its antigenic similarity in cross-neutralization tests (V. Jestin, unpublished results). In a last group (control group), ducks were not vaccinated and not challenged.

Sera were collected from five ducks from each group before the second immunization, and from ten ducks from each group before the virus challenge. During the assay, the ducks were observed for morbidity and mortality and any dead ones were examined for macroscopic lesions. At slaughter (3 weeks after the challenge, at 82 days old), all the ducks were weighed and examined for macroscopic lesions and their sera were collected. Samples of heart, liver, spleen and gastrocnemius tendon were collected and examined for microscopic lesions.

### Serological tests.

The above-described immunoblot assay was used for the detection of σB antibodies in sera from all the inoculated ducks. S9 cells expressing σB protein alone were subjected to electrophoresis, to avoid visualization of antibodies specific to the GST fragment from sera of ducks infected with the fusion protein. Since no duck reovirus ELISA is available, a chicken reovirus ELISA (Avian
reovirus antibody test kit, KPL), with the S1133 strain as antigen, was used for titration of $\sigma^B$ antibodies. The ELISA was adapted for the detection of duck reovirus antibodies by using an alkaline phosphatase-labelled rabbit anti-duck IgG (H+L) conjugate (Nordic) and reference positive and negative duck antisera.

Results

Analysis of nucleotide and deduced amino acid sequences of the S3 gene

The complete nucleotide sequence and deduced amino acid sequence of the S3 genome segment of muscovy duck reovirus strain 89026 is shown in Fig. 1. The nucleotide sequence was obtained from plasmids 3.12 and 3.25 and from partial sequences determined from other recombinant plasmids, and constitutes the consensus sequence for these different clones. The S3 gene is 1201 bp long and displays the terminal sequences $5'$ GCTTTTT and TATTCATC $3'$. A seven nucleotide inverted repeat adjacent to the terminal sequences was identified (TGAGCCC at nucleotide 7 and GGGCTCA at nucleotide 1186). The gene contains one ORF, which begins at nucleotide 31 and ends at nucleotide 1131. The start ATG codon is in a very favourable context for initiation of translation ($GCA^ATGG$), according to the consensus sequence ($A^GCC^ATGG$) established by Kozak (1986). The ORF encodes a protein of 367 amino acids with a molecular mass of 40.8 kDa. The $\sigma^B$ protein seems, both structurally and by analogy with the mammalian reovirus $\sigma^3$ protein, to possess a putative zinc-binding motif within the N terminus, from amino acids 51 to 75 (Fig. 2). Moreover, computer analysis revealed that the C terminus of the protein harbours a motif rich in basic amino acids (KKVSHYR, amino acids 287–293).

Alignment and comparison of the muscovy duck reovirus S3 gene sequence with other reoviruses

When the muscovy duck S3 gene sequence was compared, using the BLAST program, with available nucleotide sequences, the highest score was obtained with the chicken reovirus strain 1733 segment S3. The nucleotide sequence of the S3 gene of muscovy duck reovirus strain 89026 was compared with those of the avian reovirus strains S1133, 1733, 176 and 138. The percentage identities at the nucleotide level were 63–9, 64–1, 64–1 and 62–1%, respectively (data not shown). Since the amino acid sequences of the strains 1733 and 176 are similar, only the first was aligned and reported in Fig. 2. The muscovy duck reovirus $\sigma^B$ protein sequence was compared with those of the chicken reovirus strains S1133, 1733 and 176 and was 61, 60% and 60% identical, respectively (Fig. 2). The percentage identities with the mammalian reovirus (serotypes 1–3) $\sigma^3$ proteins each gave the same percentage of 26–6%, and only the Dearing strain (serotype 3) sequence is reported in Fig. 2.

Expression and characterization of $\sigma^B$ protein

The muscovy duck reovirus S3 gene was PCR-amplified from the 3.12 plasmid. The EcoRI-digested PCR product was

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**Fig. 1.** Complete nucleotide sequence (presented in the cDNA form) and deduced amino acid sequence of the S3 RNA segment of muscovy duck reovirus strain 89026 encoding $\sigma^B$ protein. The conserved $5'$- and $3'$-terminal nucleotide sequences are boxed with a grey background and the inverted repeats in italics are underlined. In the amino acid sequence, the putative zinc-finger motif (amino acids 51–75) is underlined and the basic stretch found in the C terminus (amino acids 287–293) is boxed. Nucleotide and amino acid positions are numbered on the right.
Fig. 2. Alignment of deduced amino acid sequences of σB proteins from muscovy duck reovirus strain 89026 (duck-89026) and chicken reovirus strains S1133 (chick-1133, GenBank accession no. AO20642), 1733 (chick-1733, AF004856) and 138 (chick-138, AF059721) and σ3 protein from mammalian reovirus Dearing strain (serotype 3) (Dearing, GenBank accession no. REOS4). Amino acid positions for each individual sequence are numbered on the right. Identical amino acids are indicated by an asterisk. The putative zinc-finger domain (CCHC form), identified by Mabrouk & Lemay (1994) within the mammalian reovirus σ3 protein, is boxed with a grey background and numbered 1 (amino acids 51–73) and the corresponding motif within the muscovy duck reovirus σB protein is boxed with a dotted line (amino acids 51–75). The repeated basic amino acid motif within the mammalian reovirus σ3 protein (Schiff et al., 1988; Miller & Samuel, 1992) is boxed with a grey background and numbered 2 (amino acids 234–240 and 291–297), whereas within the muscovy duck reovirus σB protein, the motif revealed by computer analysis to be rich in basic amino acids (H, R or K) is boxed (amino acids 287–293).

Fig. 3. Immunoblot analysis of muscovy duck reovirus strain 89026 σB protein expressed in two recombinant baculovirus systems and incubated with a reovirus-specific muscovy duck antiserum. (a) Total lysates of Sf9 cells infected with pAcGHLT-A recombinant baculovirus expressing a GST-fused σB protein (lane 1) or with wild-type baculovirus (lane 2) and a total lysate of non-infected Sf9 cells (lane 3). (b) Total lysates of Sf9 cells infected with wild-type baculovirus (lane 1) and with pVL1392 recombinant baculovirus expressing a non-fused σB protein (lane 2). Low molecular mass markers are shown in lanes M.

so they were then used to express σB recombinant proteins in a baculovirus expression system. Immunoblot analysis of proteins from Sf9 cell extracts infected with the two recombinant baculovirus types (transfected with pVL1392 and pAcGHLT-A recombinant vectors) showed single bands of 38.5 and 66 kDa, respectively (Fig. 3). The relative molecular masses were similar to those predicted for σB protein alone (40–8 kDa) or fused to GST (67–3 kDa). No proteins of these sizes could be detected in uninfected or wild-type baculovirus-infected Sf9 cells (Fig. 3). Thus, the σB protein expressed in a baculovirus expression system reacted with specific anti-reovirus duck serum and was shown to be antigenic.

Immunity induced by the recombinant baculoviruses

Antisera obtained from ducks before the second immunization and before the challenge were tested by ELISA against S1133 virus and by immunoblot against expressed σB protein, in order to detect antibody to the σB protein. Except for one serum (from the GST−S3RB group) collected before the second immunization and very slightly positive, no antibody could be detected in any of the sera tested by ELISA, whereas the reference positive duck serum gave a clear signal. Moreover, no band of the size expected for σB protein was revealed, irrespective of the serum used in the immunoblots successfully cloned into the two baculovirus transfer vectors pVL1392 and pAcGHLT-A. Sequence analysis of the inserts did not reveal any differences from the original insert sequence,
(except for the reference positive serum). After the challenge, ELISA analysis of the corresponding sera revealed the presence of reovirus antibodies in each, without any difference in titre and irrespective of the group under consideration.

Since no significant differences (t test) were detected between the CC and WB groups and between the S3RB and GST–S3RB groups, they were pooled in pairs for statistical analysis. No significant differences, based on morbidity, mortality and necropsy results, confirmed by histological data, could be found between ducks of the challenged groups, whatever the previous treatment (Table 1). However, analysis of body weights at slaughter revealed that the weights of male ducks inoculated with the two recombinant baculoviruses (S3RB and GST–S3RB groups) were not significantly different for either group, whereas the weights of the wild-type baculovirus-infected and challenge-control ducks (WB and CC groups) were significantly lower compared with the unchallenged ducks (Control group) (Table 2).

Discussion

In the present study, we describe the cloning and expression of the muscovy duck reovirus σB protein and demonstrate the antigenicity of the recombinant protein. The method previously described by Lambden et al. (1992) was adapted to clone the muscovy duck reovirus (strain 89026) small segment genes. These authors used this procedure, based on the use of a single amino-linked oligonucleotide primer followed by RT–PCR, to generate full-length cDNA clones of each dsRNA genome segment of a rotavirus without prior knowledge of the sequences. We obtained several recombinant clones containing different inserts.

The first sequencing results showed that the nucleotides at the 5′ and 3′ ends of the plus strand were identical to those of the S3 genome segment of the avian reoviruses already described. Moreover, for the first three nucleotides at the 5′ end and the last five nucleotides at the 3′ end, these sequences were identical to those of mammalian reoviruses (except for the 5′ end of the L1 segment in one mammalian strain). In fact, the extreme 5′- and 3′-terminal nucleotides were shown to be identical for all the dsRNA segments in the family Reoviridae and were conserved between virus isolates, but were different to those found in other viruses. These initial sequence data confirmed that we had succeeded in cloning a muscovy duck reovirus gene.

The complete sequence of this clone was then determined. By searching the nucleotide sequence databases for sequence similarity, we were able to confirm that the S3 gene of the muscovy duck reovirus strain 89026 had been cloned, as the highest degree of identity was obtained with the S3 segment sequence of an avian reovirus. As we had determined the sequence of a cloned PCR product, we then sequenced other recombinant plasmids (partially or fully), in order to establish the most probable sequence of the S3 gene. A non-representative gene could have been sequenced and PCR is also known to induce non-specific mutations. The sequence described in this paper constitutes the consensus sequence derived from all the available sequence data and we have recently confirmed the sequence of the σB-encoding cDNA fragment by direct sequencing of a PCR product (data not shown).

The degree of identity at the protein level between the deduced amino acid sequence of the reovirus strain 89026 S3 gene and the four chicken reovirus σB proteins was only about 61%, suggesting that the muscovy duck reovirus is very different. This result agrees with previous serological data, which demonstrated that the muscovy duck reovirus was antigenically different to strain S1133 of the chicken reovirus, as shown by cross-neutralization assays (V. Jestin, unpublished results; Heffels-Redmann et al., 1992). Comparison of the σB protein sequence with the σ3 sequence of the three mammalian reovirus strains revealed 26–6% identity, which was of the same order of magnitude as that between chicken reovirus strain 1733 and the mammalian reoviruses (27%, Vakharia et al., 1996).

In the mammalian reoviruses, two signal sequences located in the terminal regions of all ten genome segments, the conserved terminal sequences and adjacent inverted repeats, are believed to be important for specific functions required in replication and packaging. The conserved terminal sequences are expected to serve as a signal for the identification of viral RNAs (Antczak et al., 1982). The inverted repeats found in many viruses with a segmented genome are present in all the genome segments but their sequences are unique to each one (Gaillard et al., 1982; Anzola et al., 1987) and probably serve as a segment-specific signal, allowing only one copy of each segment to be packaged into each virion (Anzola et al., 1987). Ni et al. (1996) proved that in the S1 segment of chicken reovirus strain 81-5, 91–92 nucleotides from the 5′ end and 119–120 nucleotides from the 3′ end were necessary for replication and encapsidation and they identified the conserved terminal sequences and inverted repeats in these regions as being the likely signals. We also found these two types of sequence in the muscovy duck reovirus S3 genome segment and in the other cloned genome segments (data not shown), suggesting that this reovirus may share a similar replication mechanism.

The muscovy duck reovirus S3 gene possesses one ORF, which encodes a protein with a predicted molecular mass (40–8 kDa) similar to that of the chicken reovirus S3 gene product. We first confirmed that the ORF coded for σB protein by expressing it in Escherichia coli and analysing the recombinant cell lysates by SDS–PAGE. The results revealed the presence of an expressed protein of approximately 41 kDa that reacted specifically with antiserum from ducks vaccinated with strain 89026 reovirus (data not shown). In addition, computer analysis of the amino acid sequence showed two basic stretches located within the N and C termini of the protein that are...
similar to those of the mammalian reovirus σ3 protein, the counterpart of avian reovirus σB. The σ3 protein has been shown to have affinity for dsRNA (Huismans & Joklik, 1976) and to be composed of two independent functional domains, a zinc-finger motif and a dsRNA-binding region (Schiff et al., 1988). By analogy, the N-terminal basic motif observed in the zinc-finger motif and a dsRNA-binding region (Schiff et al., 1988) is structurally analogous to the major outer-capsid protein σ3 of mammalian reovirus.

The S3 gene was expressed in insect cells, in order to analyse the antigenicity and possible immunogenicity of the muscovy duck reovirus σB protein, by using a baculovirus expression system. We used this expression system because in a previous work we had obtained a major fraction of insoluble protein, probably due to incorrect folding, when σB protein was expressed in E. coli. Identical results had been obtained for bacterially expressed mammalian reovirus σ3 protein (Miller & Samuel, 1992; Wang et al., 1996) and by Yin et al. (1997) when they expressed chicken reovirus σB protein in E. coli. We chose to clone the S3 gene into two different baculovirus expression systems, one expressing a non-fused protein and the other a fused protein, in order to select the best model for the σB protein. The insert of the σB-recombinant plasmid 3.12 was cloned in the two baculovirus transfer vectors, as we had previously demonstrated the antigenic nature of this protein when expressed in E. coli.

Under our experimental conditions, we were unable to demonstrate any immune response against σB protein with two serological tests, ELISA and immunoblot. The ELISA employed used the S1133 strain as antigen and we demonstrated previously that it was sensitive enough to detect experimental infection with duck reovirus strains 89026 and 89330. In addition, immunoprecipitation data reported by Hefels-Redmann et al. (1992) show that reovirus strain S1133 antibodies are able to detect muscovy duck reovirus σB protein. The ELISA antigen used is a mixture of all viral proteins, of which the σB protein is supposed to constitute a
A significant proportion (30%, as reported by Schnitzer *et al.*, 1982). Thus, even if the S1133 antigen is not the most suitable and may result in lower sensitivity, it appears unlikely that the absence of an immune response is due to the poor applicability of the ELISA. Moreover, no response was observed following immunoblot analysis using the homologous σB protein as antigen.

We were also unable to demonstrate any significant protection against reovirus challenge. There are several possible explanations for this immunization failure. (i) Reovirus strain 89026 σB protein does not induce antibodies. Only the antigenicity of the chicken reovirus σB protein has been demonstrated (Vakharia *et al.*, 1996; Yin *et al.*, 1997) and its potential role in protection against reovirus infection has not yet been evaluated. Thus, our study constitutes the first assay *in vivo*. The 89026 strain of duck reovirus was also shown to elicit a lower level of neutralizing antibodies than other strains in ducks. (ii) The adjuvant used in the assay was not optimal for these expressed proteins. The adjuvant is known to play an important role in the success of immunization. Other adjuvants, particularly ones known to stimulate different immune mechanisms such as SBAS4, SBAS2 (Slaoui, 1998) and MF59 (Ott, 1998), will have to be tested. (iii) The 3.12 plasmid includes a mutation within an immunogen motif. An RT–PCR assay was recently designed to determine the genomic sequence of the S3 gene of the 89026 reovirus strain. We found that the sequence of plasmid 3.12 contained a single mutation at nucleotide 200 that resulted in a change in the corresponding amino acid (data not shown). We do not yet know if this sequence modification changes the conformation of an immunogen motif. (iv) The challenge dose may have been too severe. However, unvaccinated challenged ducks exhibited just 4% mortality and 28% morbidity, which corresponds to a moderate challenge.

Further studies are required to investigate the protective role of σB, notably by using a more immunogenic reovirus strain and testing other adjuvants.

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