Characterization of the nucleic acid-binding activity of the avian reovirus non-structural protein σNS

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The avian reovirus non-structural protein σNS has previously been shown to bind single-stranded (ss) RNA in vitro in a sequence-independent manner. The results of the present study further reveal that σNS binds poly(A), poly(U) and ssDNA, but not poly(C), poly(G) or duplex nucleic acids, suggesting that σNS has some nucleotide-sequence specificity for ssRNA binding. The current findings also show that σNS is present in large ribonucleoprotein complexes in the cytoplasm of avian reovirus-infected cells, indicating that it exists in intimate association with ssRNAs in vivo. Removal of RNA from the complexes generates a σNS protein form that sediments between 4.5 and 7 S, suggesting that RNA-free σNS associates into small oligomers. Expression and purification of recombinant σNS in insect cells allowed us to generate specific antibodies and to perform a variety of assays. The results of these assays revealed that: (i) RNA-free σNS exists as homodimers and homotrimers; (ii) the minimum RNA size for σNS binding is between 10 and 20 nt; (iii) σNS does not have a preference for viral mRNA sequences; and (iv) its RNA-binding activity is conformation-dependent. Baculovirus expression of point and deletion σNS mutants in insect cells showed that the five conserved basic amino acids that are important for RNA binding and ribonucleoprotein-complex formation are dispersed throughout the entire σNS sequence, suggesting that this protein binds ssRNA through conformational domains. Finally, the properties of the avian reovirus protein σNS are compared with those of its mammalian reovirus counterpart.

INTRODUCTION

Avian reoviruses, which belong to the genus Orthoreovirus of the family Reoviridae, are involved in a variety of disease conditions that cause major economic losses in the poultry industry (reviewed by Jones, 2000; Robertson & Wilcox, 1986; van der Heide, 2000). These cytoplasmic-replicating viruses have a genome formed by 10 segments of double-stranded RNA (dsRNA) encased by a non-enveloped, double-protein capsid shell (Spandidos & Graham, 1976). Their genome encodes at least eight structural proteins and four non-structural proteins (Bodelón et al., 2001; Schnitzer, 1985; Varela & Benavente, 1994).

Avian reovirus genes are transcribed by a core-associated RNA polymerase to produce mRNAs that are identical to the positive strands of the dsRNA segments, possessing a type 1 cap at their 5’ ends and lacking a 3’ poly(A) tail (Martínez-Costas et al., 1995). Reoviral mRNAs are used as templates for the synthesis of viral proteins and minus-strand RNAs. Avian reovirus replication and morphogenesis occur within globular viral factories that are not microtubule-associated (Tourís-Otero et al., 2004a).

The 367 aa avian reovirus σNS protein, which is encoded by the S4 gene (Chiu & Lee, 1997; Schnitzer, 1985; Varela & Benavente, 1994), binds single-stranded RNA (ssRNA) in a sequence-independent fashion (Yin & Lee, 1998, 2000) and is present in cytoplasmic globular inclusions in infected cells through an association with the non-structural protein μNS (Tourís-Otero et al., 2004b). In this study, we have further characterized the nucleic acid-binding activity of avian reovirus σNS.

METHODS

Cells and viruses. Primary cultures of chicken embryo fibroblasts (CEF) were grown in monolayers in medium 199 (Invitrogen) supplemented with 10% tryptose phosphate broth and 5% calf serum. The S97 insect cell line was grown in suspension culture in serum-free SF-900 II medium (Invitrogen) at 27°C. Conditions for avian reovirus 1733 propagation, titration and purification have been described previously (Grande & Benavente, 2000). Propagation of baculoviruses in S97 cells has also been described previously (Hsiao et al., 2002).

Cloning and generation of recombinant baculoviruses. Cloning and sequencing of the recombinant plasmid pCR2.1-S4,
containing the 1733 eNS-encoding S4 gene, has been reported previously (Toursi-Otero et al., 2004b). To express a recombinant eNS (rNS) protein in insect cells, the S4-coding sequence of the pCR2.1-S4 plasmid was PCR-amplified by using the forward primer 5'-GGAGATCCCATGGAACACCCGTGC-3' (EcoRI site underlined) and the reverse primer 5'-GGCTCTAGATCCTACGATCC- AGCTGG-3' (XhoI site underlined). The PCR product was digested and cloned into the EcoRI and XbaI sites of pFastBac1 (Bac-to-Bac system; Invitrogen) to generate pFastBac1-S4, which was then used to produce the baculovirus Bac-eNS according to the supplier's protocol. This baculovirus expresses the S4 gene under the control of the polyhedrin promoter.

To generate recombinant baculoviruses expressing deleted eNS mutants, PCR amplification was performed with the following primers: to express the N-terminal mutant rNS ΔN11, the forward primer was 5'-GGAGATCCATGGAACACCCGTGC-3' (EcoRI site underlined) and the reverse primer was 5'-GGCTCTAGATCCTACGATCC-AGCTGG-3' (XhoI site underlined). To express C-terminal mutants, the forward primer was 5'-GGAGATCCGAGCAGAACAACCGTG-3' and the reverse primers were 5'-GGCTCTAGATCCTACGATCC-AGCTGG-3' for ΔC16, 5'-GGCTCTAGATCCTACGATCC-AGCTGG-3' for ΔC50 and 5'-GGCTCTAGATCCTACGATCC-AGCTGG-3' for ΔC100 (XbaI sites underlined). To express point eNS mutants, we performed site-directed mutagenesis of pFastBac1-S4 by using a QuickChange site-directed mutagenesis kit (Strategene) according to the manufacturer's instructions. Mutations were performed to change to leucine those lysine and arginine residues indicated in Table 1. The mutant pFastBac1-S4 sequences were verified by restriction analysis and nucleotide sequencing. These mutants were used to generate recombinant baculoviruses as described above.

Viral infection, protein radiolabelling, preparation of cell extracts and protein analysis. Mock-infected and avian reovirus-infected CEF monolayers (5 p.f.u. per cell) were radiolabelled metabolically with 500 μCi (18.5 MBq) [35S]methionine/cysteine ml⁻¹ (Amersham Biosciences) for 1 h at 16 h post-infection. The cells were lysed in STE buffer [10 mM Tris/HCl (pH 7-4), 1 mM EDTA, 0.5 % Triton X-100 and 200 mM NaCl], the extract was centrifuged at 10000 g for 10 min and the supernatant was considered the soluble S10 fraction.

For velocity-sedimentation analysis, S10 fractions were left untreated or treated with either 50 μg RNase A ml⁻¹ (Sigma-Aldrich) or 50 U RNase V1 ml⁻¹ (Ambion) for 15 min at 37 °C. An aliquot of the RNase-treated sample was adjusted to 1 M NaCl and incubated for 15 min at 37 °C. These samples, as well as a reticulocyte lysate that had been programmed with total RNA isolated from avian reovirus-infected cells, were loaded onto 10–40 % sucrose gradients in STE buffer. After centrifugation at 150000 g for 16 h at 4 °C in a Beckman SW50.1 rotor, 15 fractions of 300 μl were collected from the top of the tubes. These fractions and the pellets, as well as aliquots of both the original extract and purified avian reovirions, were boiled in Laemmli sample buffer and analysed by 12 % SDS-PAGE and autoradiography. Protein standards of known molecular masses and sedimentation coefficients were all from Sigma: BSA (66 kDa, 4.3S), gamma-globulin (156 kDa, 7S), catalase (250 kDa, 11-3S) and thyroglobulin (670 kDa, 19S). The standards were run on identical sucrose gradients, collected from the top and detected by measurement of A260.

For Sepharose bead-binding assays, the radioactive S10 fraction was subjected to ultracentrifugation (10000 g for 2 h). Aliquots of the supernatant (100 μl) were supplemented with different NaCl concentrations and mixed with 50 μl of each of the various Sepharose beads indicated in Fig. 3 (Sigma). After incubation for 30 min at 4 °C in STE buffer, the mixtures were centrifuged for 30 s and subjected to five rounds of washing (with 1 ml STE buffer) and centrifugation. The pelleted beads were washed with STE buffer containing increasing salt concentrations (0-4–2-0 M NaCl) and centrifuged. The original extracts, the different washes and the final pellets were boiled in Laemmli sample buffer and analysed by 12 % SDS-PAGE and autoradiography. For competition assays, the supernatants of ultracentrifuged S10 extracts were incubated for 15 min at 4 °C in STE buffer with the indicated amounts of GTP or competitor soluble nucleic acids (0-5 mg ml⁻¹) prior to the binding assays.

Expression and purification of rNS and generation of polyclonal antibodies. Insect S9 cells were infected with 5 p.f.u. Bac-eNS per cell and incubated at 27 °C for 72 h. The cells were then pelleted by low-speed centrifugation and resuspended in cold lysis buffer [10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 10 mM NaCl and 0.5 % Triton X-100] at 2 x 10⁶ cells ml⁻¹. The extract was incubated for 10 min at 4 °C and then centrifuged for 30 min at 2500 g, the pellet was discarded and the supernatant was centrifuged at 4 °C for 1 h at 28000 r.p.m. in a SW40 rotor (Beckman). The supernatant was discarded and the rNS-enriched pellet was resuspended in lysis buffer containing 1 M NaCl, incubated for 15 min at 4 °C and passed through a 22-gauge needle. The mixture was centrifuged again at 4 °C for 1 h at 30000 r.p.m. in a SW40 rotor, the pellet was discarded and the resulting supernatant was diluted five times with lysis buffer and subjected to poly(A)–Sepharose affinity chromatography. The flow-through fraction was discarded and the column was washed with buffer containing increasing salt concentrations. Protein rNS was eluted with buffer containing 0-8 M NaCl and concentrated by centrifugation using a Centricon YM-10 (Millipore). Purified rNS was used as immunogen to raise a rabbit polyclonal antiserum, as described elsewhere (Bodelón et al., 2001).

Immunoprecipitation, immunoblotting, chemical cross-linking and in vitro translation. Immunoprecipitation and immunoblotting were performed as described previously (Toursi-Otero et al., 2004a). For chemical cross-linking, glutaraldehyde (Sigma) was diluted to the working concentration in PBS and incubated with purified rNS for 30 min at room temperature. The reactions were stopped by adding Tris/HCl (pH 7-5) to a final concentration of 50 mM, followed by a 15 min incubation. Isolation of total RNA from avian reovirus-infected cells and its translation in rabbit reticulocyte lysates have been described previously (Varela & Benavente, 1994).

Gel mobility-shift assays. Uncapped and polyadenylated luciferase mRNA was purchased from Promega and poly(A) from Sigma. All other ssRNA probes were generated by in vitro transcription using the T7 RiboMAX Express RNA-production system (Promega). The DNA template for avian reovirus s1 mRNA transcription was generated by PCR amplification of S1 sequences contained within the pBsect-S1 plasmid (Bodelón et al., 2001) with the forward primer 5'-GGCTTTATAACTGCTACTAGCGTCTTTCATCCTCTTCT-3' (T7 promoter sequence underlined) and the reverse primer 5'-GATGAGAACACCTAGAGTAC-3'. The template for the synthesis of the 10 nt RNA was generated by annealing the oligonucleotides 5'-GGCTTTATAACTGCTACTAGG-3' and 5'-GGCTTTATAACTGCTACTAGG-3'. The template for the synthesis of the 20 nt RNA was generated by annealing the oligonucleotides 5'-GGCTTTATAACTGCTACTAGG-3' and 5'-GGCTTTATAACTGCTACTAGG-3'. The latter primer was also used as a 40 nt ssDNA probe. Annealing of this primer to its complement produced a 40 bp dsDNA probe. A 20 bp dsRNA was generated by annealing the 20 nt RNA with the RNA synthesized by T7 run-off transcription of NotI-predigested plasmid pCl-Neo.

The 5' end of the probes (20 pmol) was dephosphorylated by incubation with 20 U alkaline phosphatase (Roche Diagnostics) and then radiolabelled by incubation for 1 h at 37 °C with 50 μCi
(1.85 MBq) [γ-32P]ATP (ICN) and 5 U T4 polynucleotide kinase (Promega) in 70 mM Tris/HCl (pH 7.6), 10 mM MgCl₂ and 5 mM dithiothreitol. Unincorporated nucleotides were removed by Sepharose G50 chromatography.

Gel mobility-shift assays were performed by incubating 75 ng to 2 µg purified rαNS in PBS with 10 000 c.p.m. of each radiolabelled probe in 20 µL STE buffer containing 10 U RNasin for 15 min at 4°C. The samples were mixed with gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in H₂O) and subjected to electrophoresis in 10% polyacrylamide native gels in TBE buffer. At the end of the runs, the gels were dried and exposed to X-ray films. For competition assays, 1 µg purified rαNS was preincubated with 0–2 µg non-radioactive RNA prior to the addition of 50 ng radiolabelled s1 mRNA probe.

Membrane-filter assays. North-Western blot assays were performed with extracts from insect cells that had been infected for 72 h with wild-type baculovirus or with recombinant baculovirus Bac-αNS, as described by González & Ortín (1999). Proteins were separated in 12% SDS-PAGE gels and transferred onto nitrocellulose membranes in 25 mM Tris, 192 mM glycine (pH 8.3). Membranes were incubated for 16 h at 4°C in renaturing buffer [50 mM NaCl, 1 mM EDTA, 0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.1% Triton X-100, 10 mM Tris/HCl (pH 7.5)], then incubated for 2 h at room temperature in renaturing buffer containing 10% c.p.m. 32P-radiolabelled s1 mRNA, washed four times (1 h each) with renaturing buffer and finally dried and exposed to X-ray film.

For membrane-filter assays, serial threefold dilutions of purified rαNS, ranging from 0.1 to 9 µg, were prepared in STE buffer. BSA was then added to equalize the protein content of each sample to 9 µg and the final volume to 150 µL. Half of each sample was incubated with 3.5 M urea at 50°C for 15 min and the other half was mock-incubated. Aliquots containing one-third of each sample were deposited onto three different 0.2 µm nitrocellulose membranes (Bio-Rad) by using a dot-blot apparatus (Millipore). The first membrane was incubated with Ponceau S solution (0.1% Ponceau S in 5% acetic acid), Sigma, the second membrane with anti-rαNS antibodies and the third membrane first for 1 h in STE buffer containing 0.5 mg BSA ml⁻¹ and then an additional hour in the same buffer containing 100 000 c.p.m. 32P-radiolabelled s1 mRNA. The membrane was finally washed five times in STE buffer containing 0.5 mg BSA ml⁻¹, then dried and exposed to X-ray film.

RESULTS

Protein αNS is found in large ribonucleoprotein complexes in avian reovirus-infected cells

To evaluate the capacity of avian reovirus αNS to associate with nucleic acids in vivo, both a reticulocyte lysate programmed with viral RNA and a virus-free cytoplasmic extract from avian reovirus-infected cells were subjected to velocity-sedimentation analysis through 10–40% sucrose gradients. The 35S-radiolabelled proteins present in the original extracts, in the gradient fractions and in the pellets, as well as in purified avian reovirions, were analysed by SDS-PAGE and autoradiography (Fig. 1). Most of the in vitro-translated αNS sedimented in fractions 4 and 5, a narrow zone between the 4:3S and 7S protein markers (Fig. 1a), although faster than expected for a globular monomer of 40 kDa (3:5S), suggesting that it is an oligomer. In contrast, most of the αNS present in extracts of infected cells was detected in the pellet fraction (lane P in Fig. 1b), indicating that αNS is present in large complexes. To assess whether αNS is associated with RNA within the complexes, the extract from infected cells was treated with either RNase V1 or RNase A before centrifugation. The RNase V1 treatment did not alter the sedimentation profile of αNS, suggesting that αNS is not associated with dsRNA (Fig. 1c). In contrast, the RNase A treatment caused a shift in the αNS distribution to slower-sedimenting fractions (Fig. 1d), suggesting both that ssRNA is present in the αNS-containing complexes and that αNS is associated with ssRNA within the complexes. The RNase A treatment did not, however, generate a homogeneous αNS population, as the protein sedimented in a broad zone between fractions 4 and 15, with a large αNS fraction still present in the pellet (Fig. 1d). Subsequent incubation of the RNase A-treated extract with 1 M NaCl caused αNS to sediment in the same fractions as the in vitro-translated protein, suggesting that the combined treatment caused total RNA removal from the complexes (Fig. 1e). Collectively, our results demonstrate that αNS is associated with ssRNA within large ribonucleoprotein complexes in infected cells.

Expression of recombinant αNS in insect cells, purification and antibody generation

To obtain large amounts of protein αNS for antibody production and biochemical characterization, we generated the recombinant baculovirus Bac-αNS, which contains the avian reovirus S4 gene under the control of the polyhedrin promoter. Expression of high levels of a 40 kDa protein, designated rαNS, was observed upon infection of Sf9 cells with Bac-αNS, but not with wild-type baculovirus (Fig. 2a, compare lanes 1 and 2). The authenticity of the rαNS protein synthesized in insect cells was confirmed in different ways. Firstly, rαNS and αNS from avian reovirus-infected cells have identical electrophoretic mobilities (Fig. 2a, Fig. 2b, c).

Maximal accumulation of rαNS occurred 72 h after infection of Sf9 cells with 5 p.f.u. Bac-αNS per cell (data not shown) and this time was therefore chosen for large-scale rαNS production. Based on its ability to form large ribonucleoprotein complexes and to bind ssRNA, we devised a simple and highly efficient scheme for rαNS purification, as described in Methods (Fig. 2a). Virtually pure rαNS was eluted from a poly(A)–Sepharose column with buffer containing 0.8 M NaCl, as judged by Coomassie blue staining of an SDS-PAGE gel (lane 10). A sample of αNS similarly purified from avian reovirus-infected cells is shown in lane 11. We generally obtained 15 mg pure rαNS from 11 Bac-αNS-infected Sf9 cells. The purified
protein was used as immunogen to generate polyclonal anti-\(\sigma\)NS antibodies in rabbits.

Cross-linking with glutaraldehyde was performed next, to evaluate the oligomeric state of purified \(\sigma\)NS. The immunoblot shown in Fig. 2(d) revealed that, whereas the anti-\(\sigma\)NS antibodies only recognized a 40 kDa protein band before cross-linking (lane 1), two additional bands of 80 and 110 kDa showed up after incubation with glutaraldehyde (lanes 2 and 3), suggesting that they correspond to \(\sigma\)NS homodimers and homotrimers.

### Binding to immobilized nucleic acids

To further characterize the \textit{in vitro} nucleic acid-binding activity of \(\sigma\)NS, a cytoplasmic extract from \(\text{\(^{35}\)S}\)-amino acid-labelled avian reovirus-infected cells was ultracentrifuged and incubated with different resin-immobilized nucleic acids; the beads were subsequently washed with buffer containing increasing salt concentrations (Fig. 3a). The radioactive proteins in the original extracts (lane 8), in the flow-through fractions (lane 1) and in the different washes (lanes 2–6), as well as those that remained attached to the matrix after the final wash (lane 7), were resolved by SDS-PAGE and visualized by autoradiography. Only the viral proteins \(\sigma\)A and \(\sigma\)NS displayed binding activity for the nucleic acids used in these assays, but not for control Sepharose beads (upper panel). Protein \(\sigma\)A bound very tightly to poly(1: C), as it remained resin-bound even in the presence of 2 M NaCl and was able to bind poly(1: C)–Sepharose in the presence of 2.5 M NaCl (Fig. 3b). On the other hand, \(\sigma\)NS bound with moderate affinity to poly(A), poly(U) and ssDNA, but not to poly(G), poly(C), poly(1: C) or dsDNA. Based on the salt concentration required for elution, we conclude that \(\sigma\)NS exhibits a higher affinity for poly(A) than for poly(U), and for poly(U) than for ssDNA (Fig. 3a). A similar conclusion was reached when the poly(A)-binding assays were performed with \textit{in vitro}-translated \(\sigma\)NS (data not shown), or when monitoring \(\sigma\)NS attachment to, rather than elution from, the affinity beads at different salt concentrations (Fig. 3b).

To further characterize the nucleic acid-binding activities of \(\sigma\)A and \(\sigma\)NS, competition-binding assays with soluble, non-radioactive nucleic acids were performed. As shown in Fig. 3(c), the binding of \(\sigma\)A to poly(1: C) could only
be inhibited by soluble viral dsRNA and not by ssRNA, ssDNA or dsDNA, confirming previous observations that σA binds exclusively to dsRNA in a sequence-independent manner (Martínez-Costas et al., 2000; Yin et al., 2000). The binding of σNS to poly(A) and poly(U) could only be competed by soluble poly(A) or poly(U), not by the other nucleic acids tested.

It has been reported that GTP concentrations over 0.5 mM outcompete the binding of mammalian reovirus non-structural protein σNS (mrσNS) to poly(A) and poly(U) (Richardson & Furutachi, 1985). To assess whether a similar situation holds true for avian reovirus σNS (areσNS), radiolabelled cytoplasmic extracts of both avian and mammalian reovirus-infected cells were supplemented with different GTP concentrations before incubation with poly(A)–Sepharose beads. After washing the beads with binding buffer, the attached proteins were eluted by boiling in Laemmli sample buffer and analysed by SDS-PAGE and autoradiography (Fig. 3d). Whilst GTP concentrations over 1 mM reduced the binding affinity of mrσNS to poly(A) (compare lanes 11 and 12), they apparently enhanced the affinity of areσNS for poly(A) (compare lanes 5 and 6), suggesting that GTP has opposing effects on the RNA-binding activity of the two σNS proteins.

**Gel mobility-shift assays**

Consistent with the results of the Sepharose-immobilized nucleic acids, our gel-shift assays revealed that σNS forms complexes with radiolabelled ssRNA and ssDNA, but not with dsRNA or dsDNA (Fig. 4a), and that the intensity of the ssRNA–σNS band was directly dependent upon the amount of σNS used in the assays (Fig. 4b). We further found that purified σNS also forms complexes with poly(A) and luciferase mRNA (data not shown). When fixed amounts of σNS were mixed with increasing amounts of unlabelled s1 mRNA or luciferase mRNA before the addition of the radiolabelled avian reovirus s1 mRNA probe, the two unlabelled mRNAs competed with similar efficiencies with radiolabelled s1 mRNA for σNS binding (Fig. 4c). A similar competition efficiency was also exhibited by poly(A) (data not shown). These results suggest that σNS does not have a preference for avian reovirus sequences.

Finally, to determine the minimum RNA size required for σNS binding, fixed amounts of 32P-labelled ssRNAs of different sizes were incubated with increasing concentrations of σNS and subjected to gel-shift analysis. The results revealed that, whilst σNS formed complexes readily with a 20 nt RNA, it did not do so with a 10 nt RNA (Fig. 4d), indicating that the minimum RNA size required for σNS binding is between 10 and 20 nt.

**Filter-binding assays**

The RNA-binding activity of σNS was also analysed by filter-binding assays. For this, the proteins present in extracts from both Bac-σNS- and wild-type baculovirus-infected S9 cells were resolved by SDS-PAGE and transferred onto nitrocellulose filters, and the filters were then probed with 32P-labelled s1 mRNA (Fig. 5a). Whilst RNA binding by a cellular or baculovirus 30 kDa protein was evident in extracts from baculovirus-infected cells (lanes 3
Fig. 3. Binding to Sepharose-bound synthetic nucleic acids. (a) A virus-free cytoplasmic extract from [35S]methionine/cysteine-labelled avian reovirus-infected cells (10 p.f.u. per cell) (lane 8), prepared as described in Methods, was incubated with the various Sepharose beads indicated on the left. Lanes: 1, flow-through fraction; 2–6, fractions eluted with binding buffer containing the NaCl concentrations indicated above; 7, proteins eluted by boiling the final pelleted beads in Laemmli sample buffer. Samples were analysed by 12% SDS-PAGE and autoradiography. Positions of the viral proteins are indicated on the right. The bottom five autoradiograms show viral proteins of the σ class only. (b) Aliquots of the extract from infected cells (E) were supplemented with the salt concentrations indicated above prior to incubation with the indicated Sepharose beads. The beads were centrifuged and washed twice with the corresponding buffer, and the final pellets were boiled in Laemmli sample buffer and analysed as for (a). The positions of σA and σNS are indicated on the left and right, respectively. (c) Aliquots of the extract from infected cells (E) were preincubated with water or with the soluble nucleic acids shown above (0·1 mg ml⁻¹) prior to the addition of the Sepharose beads indicated on the left. Samples were then analysed as in (b). The positions of σA and σNS are indicated on the right. (d) Cytoplasmic extracts from avian reovirus-infected CEF cells (AR-CEF) and from mammalian reovirus-infected mouse L cells (MR-L) were preincubated with the GTP concentrations indicated above prior to the addition of poly(A)–Sepharose beads. Samples were then analysed as for (b). Positions of arσNS and of mrsNS are indicated on the left and right, respectively.

and 4), no RNA binding by a protein migrating in the position of rσNS (40 kDa) could be observed (lane 4). These results suggest that filter-immobilized σNS monomers do not bind ssRNA. Further RNA-binding assays were performed with purified rσNS spotted directly onto membranes, either before or after denaturation with urea (Fig. 5b). Ponceau S staining of one membrane revealed that all wells contained amounts of similar protein (top panel). Incubation of the second membrane with anti-σNS antibodies demonstrated that both native and denatured rσNS were retained by the membrane with similar efficiencies (medium panel). Probing the third membrane with 32P-labelled avian reovirus s1 mRNA revealed that only native rσNS displayed RNA-binding activity (bottom panel).

Effect of σNS mutagenesis on RNA binding and complex formation

To identify regions of σNS that might be important for RNA binding and complex formation, we initially assayed the complex-forming and RNA-binding capacities of rσNS versions lacking end sequences. We first generated the recombinant baculovirus Bac-σNS ΔN11, which encodes a σNS version lacking the 11 most N-terminal residues, including two arginines at positions 6 and 11. Both rσNS ΔN11 and full-length rσNS were detected in soluble extracts from baculovirus-infected cells. These extracts were subsequently subjected to poly(A)–Sepharose-binding and velocity-sedimentation analysis, and the resulting fractions were subsequently analysed by Western blot
Fig. 4. Gel-retardation assays. (a) Samples containing no rNS (–) or purified rNS (+) were incubated with the following 32P-labelled probes: avian reovirus s1 mRNA (ssRNA), a 40 nt-long ssDNA (ssDNA), a 20 bp dsRNA (dsRNA) or a 40 bp dsDNA (dsDNA). Generation and labelling of these probes are described in detail in Methods. (b) Samples containing 0–75 ng to 2 μg rNS were incubated with a fixed amount of 32P-labelled s1 mRNA. (c) Samples containing 1 μg rNS were incubated with 0–2 μg of the mRNAs indicated above, prior to incubation with 50 ng 32P-labelled s1 mRNA. (d) Samples containing 75 ng to 2 μg rNS were incubated with a fixed amount of two 32P-labelled ssRNAs of 10 and 20 nt. All samples were analysed on non-denaturing 10% PAGE gels followed by autoradiography. Positions of the free probes and of the rNS-probe complexes are indicated.

using anti-rσNS antibodies (Fig. 6a). As with σNS from avian reovirus-infected cells, full-length rσNS bound poly(A) (left top panel) and formed large complexes (centre top panel), which could be partially disassembled by preincubation with RNase A (right top panel), suggesting that no other avian reovirus factors are required for the σNS activities. In contrast, rσNS ΔN11 did not bind poly(A) or form large complexes (Fig. 6a, bottom panels), indicating that the region comprising the first 11 σNS residues is important for RNA binding and complex formation.

We next generated recombinant baculoviruses that expressed proteins lacking the C-terminal 16, 50 and 100 residues from the C terminus of σNS. Unfortunately, none of the C-terminally truncated proteins expressed in insect cells were found in the soluble fractions, which precluded their use for further analysis (Fig. 6b). These results, however, suggest that the σNS C-terminal sequences are important for stability and/or solubility of the viral protein.

Site-directed mutagenesis was performed next to identify specific residues that are important for the activities of σNS. An alignment of the avian reovirus 1733 and S1133 σNS sequences with those of their counterparts from muscovy duck reovirus, Nelson Bay virus and mammalian reovirus type 3 revealed that the only conserved basic residues in all four proteins were those at avian reovirus σNS positions 6, 11, 29, 67, 80, 223, 234, 251, 262, 287, 310 and 365 (indicated by triangles in Fig. 7). Recombinant baculoviruses expressing mutated σNS in which each of these basic residues was changed to leucines were generated and used to infect insect cells. All mutants were detected in the soluble fractions (Fig. 6c), which allowed us to test...
their capacity to bind poly(A) and to form complexes. The results, summarized in Table 1, revealed that mutation of the arginines at the σNS positions 6, 11, 234, 287 and 365 (indicated by filled triangles in Fig. 7) prevented RNA binding and complex formation, whereas mutation of the other conserved basic residues did not (indicated by empty triangles in Fig. 7). Furthermore, all mutants that did not bind RNA exhibited the same electrophoretic mobility as non-mutated σNS, indicating that they did not undergo degradation (Fig. 6c).

**DISCUSSION**

Identification of the 40 kDa product of the avian reovirus S4 genome segment, σNS, as a major non-structural, ssRNA-binding protein that accumulates in viral factories of avian reovirus-infected cells (Yin & Lee, 1998; Tourís-Otero et al., 2004b) identifies σNS as a good candidate for playing key roles in RNA packaging and replication. It was therefore of interest to characterize the nucleic acid-binding properties of this protein.

Our results reveal that σNS functions as an RNA-binding protein in infected cells and that it possesses ssRNA- and ssDNA-binding activity in vitro. Our observation that avian reovirus σNS binds poly(A) and poly(U), but not poly(C) or poly(G), suggests some specificity in the σNS–RNA interaction. Although the significance of this specificity is not clear, it could be related to viral mRNA recognition, as all avian reovirus mRNAs sequenced so far contain 5′-GCUUUUU-3′ and 5′-UUAAUUCAC-3′ at their 5′ and 3′ ends, respectively, and these U-rich sequences are predicted to adopt a single-stranded conformation (data not shown). However, this hypothesis is not supported by our findings that σNS does not have a preference for viral sequences in gel-shift assays and that σNS associates with RNA in insect
Table 1. Effect of rNS mutations on its capacities to bind poly(A) and to form ribonucleoprotein complexes

Assays to determine the capacity of rNS mutants to bind poly(A) and to form ribonucleoprotein complexes were performed as described in the legend to Fig. 6(a).

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<th>rNS mutant</th>
<th>Poly(A)-binding capacity and complex formation</th>
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<td>Wild-type</td>
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The results of our RNA filter-binding assays suggest that native rNS conformation is essential for RNA binding. One likely explanation is that rNS RNA-binding activity is dependent on multimerization, as has been reported to occur with rotavirus NSP2 (Boyle & Holmes, 1986; Taraporewala et al., 1999). Consistent with this hypothesis, native rNS spotted onto a membrane filter binds RNA (Fig. 5b, bottom panel), whereas the protein subjected to SDS-PAGE and renatured does not (Fig. 5a, lane 4). These cells. Furthermore, the fact that poly(A), luciferase mRNA and s1 mRNA compete for the binding of rNS to the s1 mRNA with similar efficiencies suggests that rNS lacks sequence specificity and has no preference for capped and/or polyadenylated RNAs.

The possibility exists that viral cofactors enhance the RNA-binding specificity of rNS within infected cells; a good candidate would be μNS, which associates with rNS and mediates its recruitment into viral factories (Tourís-Otero et al., 2004b). Another possibility is that cellular mRNAs are excluded from the factories, obviating the need for inclusion-associated rNS to have a preference for viral sequences. In this scenario, sequence-independent RNA-binding activity would still allow rNS to concentrate and retain viral mRNAs within the factories, to provide them with RNase protection, to destabilize duplex regions and/or to improve their template efficiency for minus-strand synthesis. Sequestration of viral mRNAs within factories would direct them towards replication and would preclude their release for cytosolic translation.

Fig. 7. Comparison of deduced amino acid sequences of rNS proteins from different reoviruses. Amino acids are designated by single-letter codes. Identical residues are indicated by (*) and similar residues by (:), depending on the threshold. Amino acids considered to be similar are A, S and T; D and E; N and Q; R and K; I, L, M and V; F, Y and W. Gaps in the sequence are indicated by dashes (–). The conserved basic amino acids whose mutation prevented RNA binding are indicated by © and those whose mutation did not interfere with the binding activity of rNS are indicated by ▼. The sequences shown in the figure correspond to avian reovirus 1733 (1733; GenBank accession no. AY303992), avian reovirus S113 (Avian; accession no. U95952), muscovy duck reovirus (Muscovy; accession no. AJ133122), Nelson Bay reovirus (Nelson; accession no. AF059726) and mammalian reovirus type 3 Dearing (Mammalian; accession no. NC_004283).
findings appear to suggest that oligomeric σNS, but not monomeric σNS, possesses RNA-binding activity. However, we cannot rule out the possibility that a properly folded σNS monomer binds RNA as well, and that the lack of RNA-binding activity shown by ρσNS in the North-Western blot assay is due to inability of the renaturing conditions to induce correct σNS folding.

The fact that five conserved arginine residues, dispersed widely along the primary σNS sequence, are important for poly(A) binding and complex formation suggests that σNS interacts with RNA through conformational domains and not through a linear RNA recognition motif, like the linear motifs found in various nucleic-acid binding proteins (Burd & Dreyfuss, 1994; Kenan et al., 1991; Kochan et al., 2003; Lazinski et al., 1989; Merrill et al., 1988; Query et al., 1989). One possibility is that σNS interacts with RNA through various domains located at different positions in the primary σNS sequence, as occurs with several other RNA-binding proteins (Cordingley et al., 1990; Fillmore et al., 2002; González & Ortín, 1999; Rould et al., 1989). Another possibility is that the five basic residues that are important for RNA binding may cluster together on native σNS to create a conformational RNA-binding motif. These basic residues are likely to be surface-exposed and to make direct contacts with RNA. Alternatively, they could be important for maintaining the native σNS conformation, although this possibility is unlikely, as the mutated ρσNS versions that do not bind RNA still interact with μNS and are recruited to μNS inclusions (unpublished data). Furthermore, all point mutants and the N-terminal σNS truncation used in this study are present in the soluble fraction, whereas the σNS C-terminal truncations are insoluble. Collectively, these results suggest both that the C-terminal sequences of σNS are important for its conformational stability and that the point mutants and the N-terminal truncation are not misfolded.

The avian and mammalian reovirus σNS proteins have similar characteristics. Both are proteins of ~40 kDa that bind DNA and RNA non-specifically in vitro, that assemble into ribonucleoprotein complexes and that accumulate into viral factories through an association with μNS (Gillian & Nibert, 1998; Gillian et al., 2000; Gomatos et al., 1981; Huismans & Joklik, 1976; Miller et al., 2003; Richardson & Furuichi, 1985; Tourís-Otero et al., 2004b; Yin & Lee, 1998). However, the results of this study also revealed specific differences between the two proteins. Firstly, whereas the 4:3–7S RNA-free form of arσNS exists as dimers and trimers, the RNA-free form of mrσNS appears to contain more monomeric subunits, as it sediments between 7 and 9 S (Gillian & Nibert, 1998; Gillian et al., 2000). Secondly, whilst mrσNS binds equally well to all four ribopolynucleotides at low salt concentrations and has a slight preference for poly(U) at 0.4 M NaCl (Huismans & Joklik, 1976), arσNS does not bind poly(C) or poly(G) and exhibits a slight preference for poly(A) over poly(U). Thirdly, GTP exerts opposite effects on the binding affinity of mrσNS and arσNS to poly(A)–Sepharose (Richardson & Furuichi, 1985). Fourthly, mrσNS has been detected in the nucleus and cytoplasm of infected and transfected cells and has been reported to bind native and denatured DNA (Miller et al., 2003; Shelton et al., 1981), whereas arσNS is not present within the nucleus and has no affinity for dsDNA (Tourís-Otero et al., 2004b). Finally, the N-terminal 118 aa of mrσNS have been reported to be sufficient for RNA binding and complex formation (Gillan & Nibert, 1998), whereas basic residues located at both the N and C termini of arσNS are required for these activities.

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