Reovirus Protein σNS Binds in Multiple Copies to Single-Stranded RNA and Shares Properties with Single-Stranded DNA Binding Proteins

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Reovirus nonstructural protein σNS interacts with reovirus plus-strand RNAs in infected cells, but little is known about the nature of those interactions or their roles in viral replication. In this study, a recombinant form of σNS was analyzed for in vitro binding to nucleic acids using gel mobility shift assays. Multiple units of σNS bound to single-stranded RNA molecules with positive cooperativity and with each unit covering about 25 nucleotides at saturation. The σNS protein did not bind preferentially to reovirus RNA over nonreovirus RNA in competition experiments but did bind preferentially to single-stranded over double-stranded nucleic acids and with a slight preference for RNA over DNA. In addition, σNS bound to single-stranded RNA to which a 19-base DNA oligonucleotide was hybridized at either end or near the middle. When present in saturative amounts, σNS displaced this oligonucleotide from the partial duplex. The strand displacement activity did not require ATP hydrolysis and was inhibited by MgCl2, distinguishing it from a classical ATP-dependent helicase. These properties of σNS are similar to those of single-stranded DNA binding proteins that are known to participate in genomic DNA replication, suggesting a related role for σNS in replication of the reovirus RNA genome.

Mammalian orthoreoviruses (reoviruses) encode three nonstructural proteins, μNS, σNS, and σ3, whose roles during viral infection remain poorly understood. This report concerns σNS, which is known to be essential for reovirus replication based on the phenotype of a conditionally lethal (temperature-sensitive) mutant with its lesion in the σNS-encoding S3 gene segment (9, 27). The σNS protein comprises 366 amino acids and has a molecular mass of 41 kDa. Interaction of σNS with the viral plus-strand RNAs in infected cells is well documented (3, 14). Moreover, when σNS from infected cells is used to bind those RNAs in vitro, it protects 20- to 40-nucleotide fragments of the RNAs from RNase T1 digestion (34). Since the protected fragments include the 3’ ends of at least some of the plus-strand RNAs, it was proposed that σNS binds specifically to those regions (34). In addition, σNS and two other reovirus proteins, μNS and σ3, are found to associate with the viral plus-strand RNAs shortly after they are transcribed in infected cells and before minus-strand synthesis converts them into the double-stranded RNA (dsRNA) genome segments (3). These findings have led to a hypothesis that σNS plays a role in selecting or condensing the viral plus-strand RNAs for packaging during early stages of particle morphogenesis (3, 14, 28, 34). A role for σNS in translation of proteins from the reovirus plus-strand RNAs has also been suggested (10, 14).

Evidence for a direct role of σNS in minus-strand synthesis is limited. The temperature-sensitive mutant whose lesion maps to the S3 gene segment (27) produces little or no dsRNA at restrictive temperatures (9, 15), but this indicates only that σNS provides a required function at or before minus-strand synthesis in the replication cycle. Other previous work demonstrated that σNS-containing ribonucleoprotein complexes isolated from reovirus-infected cells display a poly(C)-dependent poly(G) polymerase activity (12, 13). However, recombinant σNS expressed in Escherichia coli or insect cells does not display this activity (10, 28), whereas recombinant reovirus protein λ3 obtained from mammalian or insect cells does (35; D. L. Farsetta and M. L. Nibert, unpublished data). These findings suggest that the σNS-containing complexes characterized by Gomatos et al. (12, 13) also contained λ3, which is a known component of the reovirus RNA polymerase (22, 35). Thus, despite not having polymerase activity itself, σNS might play a role in minus-strand synthesis within these λ3-containing complexes.

In the present study, we performed additional characterizations of the RNA-binding properties of σNS to learn more about its roles in reovirus replication. In initial experiments, we analyzed purified, baculovirus-expressed σNS (10) for its capacity to bind single-stranded RNA (ssRNA) molecules in gel mobility shift assays. The results indicated that multiple units of σNS bind to single molecules of ssRNA with positive cooperativity and in numbers dependent on RNA length such that each unit of σNS covered approximately 25 nucleotides at saturation. Competition experiments with various nucleic acids addressed the specificity of σNS binding. These studies revealed a preference for σNS to bind single-stranded over double-stranded nucleic acids, with a slight preference for RNA over DNA, but they showed no preference for σNS to bind reovirus over nonreovirus RNA. We also characterized the capacity of σNS to bind ssRNA to which short DNA oligonucleotides were hybridized. Displacement of these oligonucleotides from the partial duplexes was observed, and further characterizations demonstrated that this strand displacement activity of σNS is distinct from that of a classical helicase. The observed activities of σNS are similar to those of several well-characterized single-stranded DNA (ssDNA) helicases such as the dimeric 30-kDa human replication protein A (21) and the 42-kDa human B. subtilis DNA helicase.

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binding proteins, which are known to be involved in genomic double-stranded DNA (dsDNA) replication, suggesting that NS may play a related role in replication of the reovirus dsRNA genome.

**MATERIALS AND METHODS**

**Overexpression and purification of NS.** NS was overexpressed in insect cells infected with a recombinant baculovirus containing the ns-encoding reovirus type 1 Lang S3 gene and purified as described previously (10). Although RNase A was used in the purification procedure, no nuclease activity was detected in the final purified preparations of NS. As noted in the previous study (10), the ns protein obtained by this protocol was ≥95% pure according to results with Coomassie-stained sodium dodecyl sulfate-polyacrylamide gels and appeared to be relatively homogeneous in that it migrated between 7- and 98-kDa in 5 to 20% sucrose gradients in the absence of RNA. Nonetheless, whether these complexes represent one discrete type of ns oligomer (e.g., tetramer or hexamer) or a mixture and whether or not they can self-associate under some conditions to form higher-order multimers remain to be determined.

**Construction of the plasmids pGEM-DS4 and pGEM-3DS4.** The reovirus type 3 Dearing S4 gene was amplified from a linearized plasmid (19) by PCR (29) with Vent DNA polymerase (New England Biolabs, Beverly, Mass.) and primers (Integrated DNA Technologies, Coralville, Iowa) representing the 5' and 3' ends of S4 plus EcoRI and PstI sites, respectively. The purified PCR product was digested with EcoRI and PstI, again purified, and ligated into those sites in the pGEM-4 plasmid (Promega, Madison, Wis.). A PCR with other primers was used to amplify nucleotides 738 to 1196 of the S4 gene from the new plasmid as well as the majority of pGEM-4 sequences between the PstI and EcoRI sites. Another PCR was used to amplify nucleotides 1 to 536 of the S4 gene. A final PCR was used to join and amplify the purified products from the first two reactions (8). The purified product from the final reaction was digested with SpeI and Xhol and ligated to the purified SpeI-Xhol fragment of the cloned S4 gene to generate new plasmid pGEM-DS4, with the entire S4 gene positioned immediately 3' to the SpeI RNA polymerase promoter such that the first nucleotide of the S6 transcript was S4 nucleotide 1 (23). For subcloning the 3' end of S4 from pGEM-DS4, we performed PCR with two primers (Integrated DNA Technologies) and Deep Vent DNA polymerase (New England Biolabs). One primer represented the plus strand of pGEM-DS4 from S4 nucleotides 1061 to 1096, except for two nucleotide substitutions that generated a new EcoRI site at S4 nucleotides 1082 to 1087, and the second primer represented the minus strand of pGEM-DS4 in vector sequences 3' to the S4 gene and an intervening HindIII site. The amplified product was digested with EcoRI and HindIII and ligated into those sites in the pGEM-4Z vector to generate plasmid pGEM-3DS4. The first 6 nucleotides of the S6 transcript from this plasmid were from pGEM-4Z, the next 6 were the EcoRI site, and the next 109 were from the S4 3' end, beginning with S4 nucleotide 1088 and ending with the final S4 nucleotide, 1196.

**Generation of RNA probes and competitors by in vitro runoff transcription.** To synthesize linear DNA templates with the desired termini for in vitro runoff transcription, we performed PCR with different primer pairs (Integrated DNA Technologies) and Deep Vent DNA polymerase (New England Biolabs) to make templates of full-length (5' S4-211, 5' S4-271, and 5' S4-121) S4 plus-strand transcripts, one primer represented the plus strand of pGEM-DS4 or pGEM-3DS4 in vector sequences 5' to the SP6 promoter and the second primer represented the minus strand of pGEM-DS4 or pGEM-3DS4 in S4 sequences beginning either at the 3' end (relative to the plus strand) or the indicated (Table 1) internal position of S4. For making the 121-nucleotide vector transcript (pGEM-4Z), the primer represented the plus strand of pGEM-4Z in sequences 5' to the SP6 promoter and the second primer represented the minus strand of pGEM-4Z in the appropriate downstream sequences. The fragments were isolated from 1% agarose gels and purified using the Qiagen II kit (Qiagen, Valencia, Calif.).

**TABLE 1. DNA primers used for PCR to generate linear DNA templates for in vitro transcription**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Plasmid</th>
<th>Upstream</th>
<th>Downstream</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4 full length</td>
<td>pGEM-DS4</td>
<td>2787-GGCTTGACATATAGTTGCTG-2804</td>
<td>1196-GATGAACTAGAGCCTGGTCC-1179</td>
</tr>
<tr>
<td>5'S4-121</td>
<td>pGEM-DS4</td>
<td>2787-GGCTTGACATATAGTTGCTG-2804</td>
<td>121-TCTTGCGGCTGTATGGTGATACAG-96</td>
</tr>
<tr>
<td>5'S4-151</td>
<td>pGEM-DS4</td>
<td>2787-GGCTTGACATATAGTTGCTG-2804</td>
<td>151-GGCTTGCTGATGGTTTGGTCC-126</td>
</tr>
<tr>
<td>5'S4-181</td>
<td>pGEM-DS4</td>
<td>2787-GGCTTGACATATAGTTGCTG-2804</td>
<td>181-AGGACCGGGCCACCATCACC-161</td>
</tr>
<tr>
<td>5'S4-211</td>
<td>pGEM-DS4</td>
<td>2787-GGCTTGACATATAGTTGCTG-2804</td>
<td>211-GATCATACGTACCAAGATAG-188</td>
</tr>
<tr>
<td>5'S4-271</td>
<td>pGEM-DS4</td>
<td>2787-GGCTTGACATATAGTTGCTG-2804</td>
<td>271-TGATGAGGCTCTGTAGATCATTAG-242</td>
</tr>
<tr>
<td>3'S4-121</td>
<td>pGEM-3DS4</td>
<td>2654-GCTGAAAGGCGATATAGTTGCTG-2674</td>
<td>1196-GATGAACTAGAGCCTGGTCC-1179</td>
</tr>
<tr>
<td>3'S4-151</td>
<td>pGEM-3DS4</td>
<td>2654-GCTGAAAGGCGATATAGTTGCTG-2674</td>
<td>151-GGCTTGCTGATGGTTTGGTCC-126</td>
</tr>
<tr>
<td>3'S4-181</td>
<td>pGEM-3DS4</td>
<td>2654-GCTGAAAGGCGATATAGTTGCTG-2674</td>
<td>181-AGGACCGGGCCACCATCACC-161</td>
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<tr>
<td>3'S4-271</td>
<td>pGEM-3DS4</td>
<td>2654-GCTGAAAGGCGATATAGTTGCTG-2674</td>
<td>271-TGATGAGGCTCTGTAGATCATTAG-242</td>
</tr>
<tr>
<td>T7-17</td>
<td>pGEM-3DS4</td>
<td>349-TGCATGGAGGCGATATAGTTGCTG-359</td>
<td>52-ATGAAAGTATGCTGGTCC-70</td>
</tr>
<tr>
<td>SP6-146</td>
<td>pGEM-3DS4</td>
<td>2654-GCTGAAAGGCGATATAGTTGCTG-2674</td>
<td>71-AGGACCGGGCCACCATCACC-94</td>
</tr>
</tbody>
</table>

* a RNA transcripts generated by in vitro runoff transcription from PCR-amplified DNA fragments. Each was generated using SP6 RNA polymerase except for T7-17, which was generated using T7 RNA polymerase.

* b Plasmid DNA used as the template for PCR.

* c Sequence numbers are those designated for pGEM-4Z in the Promega Life Science catalog unless otherwise indicated (see footnote d).

* d Sequence numbers are those for the type 3 Dearing S4 plus strand.

* e RNA transcripts generated by in vitro runoff transcription from PCR-amplified DNA fragments. Each was generated using SP6 RNA polymerase except for T7-17, which was generated using T7 RNA polymerase.

* f Plasmid DNA used as the template for PCR.

* g Sequence numbers are those designated for pGEM-4Z in the Promega Life Science catalog unless otherwise indicated (see footnote d).

* h Sequence numbers are those for the type 3 Dearing S4 plus strand.
TABLE 2. Oligonucleotides used in forming partial duplexes, RNA-DNA or RNA-RNA

<table>
<thead>
<tr>
<th>Type of oligonucleotide</th>
<th>Region of longer RNA strand to which oligonucleotide was hybridized&lt;sup&gt;c&lt;/sup&gt;</th>
<th>T&lt;sub&gt;c&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA 121-TCTTGGGCCTGTAGATTGTT-103</td>
<td>3&lt;sup&gt;d&lt;/sup&gt; end</td>
<td>43.1</td>
</tr>
<tr>
<td>DNA 66-CACGACCTGTAGACCGTGGT-50</td>
<td>Middle</td>
<td>44.1</td>
</tr>
<tr>
<td>DNA 19-GGGAGAACGGCAAAATACGC-1</td>
<td>5&lt;sup&gt;d&lt;/sup&gt; end</td>
<td>35.5</td>
</tr>
<tr>
<td>RNA 70-GGGAGAACGCCCTGTAGAT-54</td>
<td>3&lt;sup&gt;d&lt;/sup&gt; end</td>
<td>48.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> For DNA oligonucleotides, sequence numbers are those for the type 3 Dearing S4 plus strand. For the RNA oligonucleotide, sequence numbers are those for pGEM-4Z as designated in the Promega Life Science catalog. All sequences are written in 5′-to-3′ orientation. Sequence numbers decrease from left to right since the sequences represent minus strands (SP6 transcripts are defined as plus strands).

<sup>b</sup> DNA oligonucleotides were individually hybridized to RNA 5′-S4-121. The RNA oligonucleotide was hybridized to RNA SP6-146.

<sup>c</sup> Melting temperatures calculated for RNA-DNA or RNA-RNA duplexes of the indicated sequences at 25 mM dGMP and 25 mM salt using the algorithms of Sugimoto et al. (36) for RNA-DNA and Xia et al. (41) for RNA-RNA as implemented at http://bioweb.pasteur.fr/seqanal/interfaces/melting.html at the Pasteur Institute.

<sup>d</sup> The 5′ end of the RNA oligonucleotide was hybridized to the penultimate 3′ nucleotide of the longer RNA.

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**RESULTS**

**Gel mobility shift assays for sNS binding to 1,196- or 151-nucleotide ssRNAs.** The RNA-binding activity of purified sNS was examined using an agarose gel mobility shift assay. In initial experiments, increasing amounts of sNS were incubated with runoff transcripts representing full-length ssRNA plus strands of the reovirus type 3 Dearing S4 gene (data not shown). More than one band was seen in the mobility shift assay with this long RNA (1,196 nucleotides); however, most of the intermediate-size complexes migrated as a smear, making it difficult to distinguish the number of different complexes. To improve resolution of the intermediate-size complexes, we decreased the length of the ssRNA and assayed for sNS binding using a polycrylamide gel mobility shift assay (Fig. 1A). For initial experiments with smaller ssRNA molecules, we used runoff transcripts representing the 5′-1,196 nucleotides of the S4 plus strand (5′-S4-151). As the amount of sNS was increased, the mobility of 5′-S4-151 RNA was progressively retarded and clearly distinguishable, intermediate-size complexes were observed. At the larger amounts of sNS (>12 pmol), a lowest-mobility complex was formed, accumulated, and was not further retarded with the addition of more protein. Based on the detection of more than one shifted RNA band over the range of sNS amounts added, we concluded that more than one unit of sNS can bind per molecule of 5′-S4-151 plus strand RNA. In addition, we concluded that the lowest-mobility complex represents RNA molecules that have each been saturatively bound by the same maximum number of sNS units. Each binding unit of sNS is hypothesized to be a small oligomer of that protein based on the sedimentation properties of the purified protein (10).

To confirm that the shifted bands contain sNS, a gel from a mobility shift assay with unlabeled 5′-S4-151 RNA was subjected to immunoblotting using the antiserum to sNS (10). The antiserum detected protein migrating in distinct bands (Fig. 1B, + RNA) with the same mobilities as the radiolabeled, protein-shifted RNA bands in Fig. 1A. The sNS protein did not show this pattern of banding in the absence of ssRNA (Fig. 1B, − RNA), although sNS did appear to concentrate in several distinct bands when excess amounts of the protein were present. Bands of similar mobility were visible in the presence of RNA as well (Fig. 1B, + RNA). The nature of these protein-specific bands is not known, but they may reflect a capacity...
of σNS to self-associate into higher-order multimers in the absence of RNA under these conditions. Their appearance only with excess amounts of σNS suggests that they hold limited significance for the RNA-binding results. The findings confirm that σNS was present in the shifted RNA-protein complexes.

Gel mobility shift assay for σNS binding to ssRNAs of other lengths. The 5’S4-151 RNA appeared to be shifted into six distinct bands over the range of amounts of σNS (Fig. 1A). The presence of six shifted bands suggested that if the larger amounts of σNS were fully saturating the RNA, then a maximum of six units of σNS were binding to each RNA molecule. Moreover, since the RNA was 151 nucleotides long, each unit of σNS must be covering about 25 nucleotides if the σNS units are evenly distributed. To test this hypothesis, three additional plus-strand RNAs, differing in length by 30 nucleotides (5’S4-121, 5’S4-181, and 5’S4-211), were generated as runoff transcripts from the same S4 clone used to generate the 5’S4-151 RNA. The new RNAs were incubated with increasing amounts of σNS and were analyzed in the polyacrylamide gel mobility shift assay. As predicted from initial findings with 5’S4-151 (Fig. 1A), 5’S4-121 was shifted into five distinguishable complexes, 5’S4-151 was shifted into six distinguishable complexes, 5’S4-181 was shifted into seven distinguishable complexes, and 5’S4-211 was shifted into eight distinguishable complexes with increasing amounts of σNS (Fig. 2A). To compare the mobilities of the different complexes formed with each RNA, all four RNAs were incubated with the same amount of σNS (18 pmol) and subjected to electrophoresis in adjacent lanes of the same gel (Fig. 2B). This analysis demonstrated that with each 30-nucleotide increase in RNA length over this range, one new lower-mobility complex was seen. In later experiments, 5’S4-271 RNA was also tested and found to be shifted into 11 distinguishable complexes with increasing amounts of σNS (data not shown). Considering the size of each RNA and the number of distinguishable complexes formed with each, we concluded that the binding unit of σNS covers a 24- to 27-nucleotide region of RNA and that the total number of σNS units that bound per RNA molecule is determined by the overall length of the RNA.

Binding cooperativity. Since multiple units of σNS bound to a single RNA molecule, the binding could have exhibited positive, negative, or no cooperativity. To address these possibil-
milities, the ratio of bound to total σNS in each sample was calculated for the 121-, 151-, and 181-nucleotide RNAs from Fig. 2A. In addition, three other experiments using the 121-nucleotide RNA and smaller protein amounts were performed. Hill plots were generated for all six data sets (40). A representative plot for the 121-nucleotide RNA is shown (Fig. 3). The linear region of this curve has a slope >1 indicating that σNS binds ssRNA with positive cooperativity. When the data from all six experiments were averaged and σNS was assumed to bind ssRNA as a monomer, the Hill coefficient was calculated at 1.43 ± 0.10. Since the RNA-binding unit of σNS is thought to be a small oligomer (10), the calculations were also done for σNS binding as a dimer, trimer, and tetramer. The Hill coefficient increased by 0.02 to 0.03 unit with each step in oligomer order (data not shown), indicating that σNS binding to ssRNA exhibited positive cooperativity regardless of the oligomeric nature of the binding unit.

**Competition assays with single- and double-stranded nucleic acids.** Previous work demonstrated that σNS does not bind detectably to dsRNA or dsDNA in filter-binding or sedimentation assays (14, 28) but does bind to ssDNA in the filter-binding assay (28). To compare the affinities of σNS for ssRNA and other nucleic acids in a more quantitative fashion, we performed competition assays. Radiolabeled 5′S4-121 RNA was combined with unlabeled competitor nucleic acids (reovirus ssRNA, reovirus dsRNA, ssDNA, or dsDNA) before the addition of σNS. The RNA-protein complexes were then analyzed using the polyacrylamide gel mobility shift assay (Fig. 4A). Both ssRNA and ssDNA competed efficiently with the labeled ssRNA probe for σNS binding, whereas dsRNA and dsDNA competed much less efficiently (Fig. 4A). For example, when 10-fold more ssRNA or ssDNA competitor was added only 23% ± 2% or 37% ± 11%, respectively, of the radiolabeled 5′S4-121 RNA was bound. In contrast, when 20-fold more dsRNA or dsDNA competitor was added, 82% ± 7% or 98% ± 4%, respectively, of the radiolabeled 5′S4-121 RNA was bound. In addition, with either single- or double-stranded competitors, RNA competed with only a slightly greater efficiency for binding to σNS than did DNA (Fig. 4A). In summary, σNS bound efficiently to single-stranded, but not double-stranded, nucleic acids and exhibited a slight preference for RNA over DNA.

![FIG. 3. Hill plot of σNS binding to ssRNA to determine cooperativity. A gel shift assay with 0.24 pmol of radiolabeled 5′S4-121 RNA was performed as described for Fig. 2A except that the concentrations of purified σNS ranged from 0.24 to 6 pmol. The amount of σNS bound at each concentration was calculated as described in Materials and Methods. Log_{10} (bound σNS/1 - bound σNS) was plotted relative to log_{10} (σNS concentration), and a best-fit line was calculated for the linear portion of the graph (the equation and coefficient of determination for the line are shown in the box).](http://jvi.asm.org/)

![FIG. 4. Competition assays for σNS binding to ssRNA using ssRNA, dsRNA, ssDNA, or dsDNA as the competitor. Each data point represents the mean from three experiments, and the error bars represent the standard deviation of the mean. (A) Increasing concentrations of unlabeled competitor ssRNA, dsRNA, ssDNA, or dsDNA (see Materials and Methods) were combined with 0.24 pmol of radiolabeled 5′S4-121 ssRNA. Purified σNS (4.8 pmol) was added to each sample, and the sample was incubated. The samples were subjected to electrophoresis and visualized as described for Fig. 1A. The upper four shifted RNA bands were included in the quantitation of bound RNA. The ratios of competitor to probe RNA were calculated from weights rather than molar amounts of nucleic acid to reflect numbers of potential σNS binding sites rather than numbers of nucleic acid molecules. (B) The assay was performed as described for panel A except that the unlabeled competitor ssRNAs were 121 nucleotides from the 5′ end of the S4 plus strand (5′S4-121), the 3′ end of the S4 plus strand (3′S4-121), or the vector sequence (pGEM-121). For one set of samples, σNS was added to the labeled RNA prior to addition of the 5′S4-121 competitor.](http://jvi.asm.org/)
± 3% of the radiolabeled 5’S4-121 RNA was bound by σNS. When fivefold more 3’S4-121 competitor RNA was added, only 22% ± 12% of the radiolabeled 5’S4-121 RNA was bound. Last, when fivefold more pGEM-121 competitor RNA was added, only 13% ± 9% of the radiolabeled 5’S4-121 RNA was bound. Although these data suggested that σNS does not preferentially bind reovirus sequences, it was possible that both ends of the RNA together might be required to confer specificity. However, the amount of 5’S4-121 RNA bound to σNS in the presence of the full-length ssRNA competitor in Fig. 4A was similar to the amount bound in the presence of each of the competitor RNAs in Fig. 4B. Thus, all ssRNAs appeared to compete to about the same level with radiolabeled 5’S4-121 RNA for binding to σNS. This suggests that σNS does not distinguish between RNA sequences corresponding to the 5’ and 3’ ends of the S4 gene and does not exhibit preference for binding to reovirus sequences.

To investigate the stability of preformed RNA-protein complexes, radiolabeled 5’S4-121 RNA was incubated with σNS prior to addition of competitor (Fig. 4B). Unlabeled 5’S4-121 RNA did not displace radiolabeled 5’S4-121 RNA that was already bound to σNS, even when the competitor was added in 10-fold excess. The complex of 5’S4-121 RNA and σNS was stable for at least 2 h after addition of this competitor (data not shown). These results suggest that, once σNS bound to RNA, it formed a stable complex that could not be easily disrupted by subsequent addition of other RNA.

**Gel mobility shift assays for σNS binding to partially duplex RNA-DNA hybrids and evidence for a strand displacement activity.** We hypothesized that σNS may require a single-stranded 5’ or 3’ end of RNA to initiate binding. To test this hypothesis, an end-radiolabeled DNA oligonucleotide complementary to 19 nucleotides at the 3’ end of the 5’S4-121 RNA (Table 2) was hybridized to that RNA. The RNA-DNA hybrid was then incubated with increasing amounts of σNS and analyzed using the polyacrylamide gel mobility shift assay (Fig. 5). The mobility of this RNA-DNA hybrid with a duplex region at the RNA 3’ end was retarded by the smaller amounts of σNS (Fig. 5A). Moreover, the amount of σNS that shifted the majority of this RNA-DNA hybrid (30 pmol of σNS to shift 0.26 pmol of hybrid in Fig. 5A) was comparable to the amount that shifted the majority of the 121-nucleotide RNA (24 pmol of σNS to shift 0.22 pmol of RNA in Fig. 2A). Similar results were observed when other end-radiolabeled DNA oligonucleotides (Table 2) were hybridized to either the middle or the 5’ end of the 5’S4-121 RNA (data not shown). We therefore concluded that σNS does not require a large single-stranded region at either the 5’ or the 3’ end of RNA to initiate binding.

Interestingly, when larger amounts of σNS were incubated with the RNA-DNA hybrid having a duplex region at the 3’ end, the shifted hybrids disappeared and increasing amounts of free oligonucleotide were observed (Fig. 5A). This suggests that, as the final unit of σNS was binding to each molecule of the hybrid, it destabilized the duplex region and caused the DNA oligonucleotide to be released. The DNA oligonucleotides bound to the middle or the 5’ end of the RNA were also released when saturating amounts of σNS were incubated with the respective hybrids (data not shown), suggesting that the displacement activity was not direction dependent.

**Evidence that σNS is not an ATP-dependent helicase.** Since σNS displaced DNA oligonucleotides from RNA-DNA hybrids, we recognized that the protein might possess helicase activity. If so, then σNS like other RNA or DNA helicases, should require hydrolysis of ATP as a source of energy for the displacement activity (reviewed in reference 16). Although ATP was not added to the displacement assay mixtures described in the previous section, it is possible that there was contaminating ATP from the reaction mixture used to radiolabel the DNA oligonucleotides (see Materials and Methods). To investigate whether ATP hydrolysis was required for σNS to displace the DNA oligonucleotides, the displacement assay was repeated in the absence or presence of EDTA, a known inhibitor of ATPase and RNA helicase functions through its chelation of divalent cations (16, 25). In addition, [α-32P]ATP was added to parallel samples, which were then analyzed by thin-layer chromatography to determine if ATP had been hydrolyzed. The σNS protein displaced DNA from the RNA-DNA hybrid in the absence or presence of EDTA (Fig. 6A) but did not detectably hydrolyze ATP in either case (Fig. 6B). Reovirus cores, which were analyzed as a positive control for EDTA-sensitive ATPase activity (25), hydrolyzed ATP in the absence, but not in the presence, of EDTA (Fig. 6B). These data suggest that σNS does not require hydrolysis of ATP during displacement of the DNA oligonucleotide from the RNA-DNA hybrid.

To assess the effect of MgCl2 on the σNS-associated strand displacement activity, the displacement assay was performed in the presence of increasing concentrations of MgCl2. The activity was progressively decreased with increasing MgCl2 (data not shown). At 10 mM MgCl2, for example, only 30% of the DNA oligonucleotide was displaced relative to the amount displaced in the absence of MgCl2 (data not shown), suggesting that Mg2+ inhibited rather than stimulated the displacement activity. This response to MgCl2 provided additional evidence that the strand displacement activity of σNS is distinguishable from that of a classical helicase (16).

**Test for strand displacement activity of σNS using a partially duplex RNA molecule.** Regions of RNA-RNA duplex are generated during the normal course of reovirus replication, certainly in the genomic dsRNA segments and probably also in regions of secondary structure within the free plus-strand tran-
**REOVIRUS RNA-BINDING PROTEIN σNS**

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**DISCUSSION**

σNS binds in multiple copies to single ssRNA molecules, but there are unresolved questions about the nature of these complexes. Results from the gel mobility shift assays indicate that multiple units of σNS can bind to a single ssRNA molecule. Such molecules, bound by multiple units of σNS, may represent the large nucleoprotein complexes obtained from both reovirus-infected mammalian cells (14) and insect cells infected with a recombinant baculovirus expressing σNS (10). By binding in multiple units to the viral plus-strand RNAs (minus-strand RNAs are not released from template plus strands during reovirus replication [1, 31]), σNS may provide multiple sites for interactions with other viral or cellular proteins that may play roles in translation, packaging, or minus-strand synthesis. Questions remaining to be answered include whether the multiple units of σNS that bind to an RNA molecule stretch out like beads on a necklace or self-associate to form a more compact structure.

The positive cooperativity demonstrated for σNS binding to ssRNA may arise either from changes in RNA conformation upon σNS binding that promote subsequent σNS units to bind more readily or from σNS-σNS interactions, similar to the case for poliovirus RNA polymerase (4, 26). Certain observations are consistent with each possibility. Data indicating that σNS destabilizes short regions of a nucleic acid duplex and thereby alters the RNA conformation are consistent with an RNA-based explanation for cooperativity. On the other hand, evidence that σNS oligomers might self-associate to form higher-order multimers under certain conditions (Fig. 1B) suggests that positive cooperativity may arise from such σNS-σNS interactions. Additional analyses of σNS-ssRNA and σNS-σNS interactions are needed to establish the basis of the observed cooperativity.

One curious feature of the results is that saturative binding occurred at between 12 and 24 pmol of σNS for each RNA, despite their different sizes (Fig. 2A). Assuming that σNS was present in limiting amounts but not limiting concentrations in these experiments, we would expect saturation of the 211-nucleotide RNA to have required approximately twice the amount of σNS as saturation of the 121-nucleotide RNA. However, since the interval between amounts of added σNS in the saturation range was large (twofold), the experimental findings are reasonable. For example, if the 121-nucleotide RNA was saturatively bound by five units of σNS when 15 pmol of σNS was added in this assay, then the 211-nucleotide RNA should have been saturatively bound by eight units of σNS when 24 pmol of σNS was added. This is consistent with the data in Fig. 2A.

Another interesting feature of the results is that a faint ladder of higher-mobility complexes was seen even when saturative amounts of σNS were added (Fig. 1A and 2). These complexes showed mobilities distinct from the major ones seen with smaller amounts of σNS (Fig. 1A and 2A). Moreover, the higher-mobility complexes seen with saturative amounts of σNS showed the same mobilities regardless of the size of added RNA and also comigrated with the saturated complexes of each smaller added RNA over the range at which those were analyzed (Fig. 2B). There are several possible explanations for these observations, but by far the most likely would seem to be that these higher-mobility complexes represent small amounts of partially degraded RNA molecules that were present in each sample and to which σNS was also saturatively bound. Enumeration of these complexes in Fig. 2B lends support to the conclusion that each σNS binding unit covers about 25 nucleotides of RNA.

What are the unit of σNS that binds to ssRNA and the number of nucleotides of RNA that each unit contacts? The purified σNS protein migrates as 7- to 9S complexes in 5 to 20% sucrose gradients (10), consistent with oligomers containing three to six 41-kDa monomers of σNS. As stated earlier, whether these complexes represent one discrete type of σNS oligomer or a mixture and whether they can self-associate to form higher-order multimers in the absence of RNA remains unknown. We nonetheless hypothesize that one of these oligomers or multimers represents the unit of σNS that binds to ssRNA in a number of copies dependent on RNA length. The molar ratio of σNS to RNA at saturation in this study may indicate the number of σNS subunits in the binding unit, that is, if the concentrations of σNS are not limiting and if most or all of the added σNS is competent for RNA binding. In this case, we can calculate that saturative binding was achieved with σNS in 60- to 120-fold molar excess to each RNA in Fig. 2A.

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**FIG. 6.** Analysis of ATP hydrolysis during strand displacement by σNS. Either 12.5 mM EDTA (+) or H2O (−) was added to 75 pmol of purified σNS (σNS) or 6 × 10^6 reovirus core particles (cores) in each sample. Cores were included as a positive control for ATP hydrolysis. All samples were analyzed in triplicate. (A) The RNA-DNA hybrid described for Fig. 5 (0.11 pmol) was added to each sample in one set. These samples were then analyzed as described for Fig. 1A. The positions of the RNA-DNA hybrid and the DNA oligonucleotide are indicated. (B) Three microcuries of [γ-32P]ATP and 0.11 pmol of the RNA-DNA hybrid were added to each sample in the other set. Products of the reaction with ATP were resolved by thin-layer chromatography and analyzed by phosphorimaging. The positions of unlabeled ATP, ADP, and AMP markers, as determined by UV absorption, are indicated.
(12 to 24 pmol of σNS for about 0.2 pmol of RNA). Considering that only five to eight units of σNS were bound to each RNA in the 121- to 211-nucleotide range at saturation, we can further calculate that each binding unit contained 8 to 24 subunits of σNS. If the RNA-free, 7- to 9S σNS oligomer is a tetramer, for example, this suggests that a multimer comprising two to six tetramers constitutes each binding unit. Given that some of the purified σNS in our preparations might not have been competent for RNA binding, we recognize that these numbers indicate an upper limit for the size of the binding unit. It remains possible that each binding unit comprises one copy of a discrete type of σNS oligomer that sediments in the 7- to 9S range in the absence of RNA (10).

The finding that each binding unit of σNS covers about 25 nucleotides of RNA is consistent with previous work demonstrating that σNS protects 20 to 40 nucleotides of reovirus ssRNAs from RNase T1 degradation (34). However, it is important to distinguish this 25-nucleotide region of ssRNA covered by σNS from the number of nucleotides that constitutes the minimum or preferred size of binding site for each unit of σNS. No experiments in this report directly address the latter, but further analyses of σNS binding to different-size RNAs might be used to establish this number. For example, if a 140-nucleotide RNA binds five σNS units covering 25 nucleotides each, are the 15 “extra” nucleotides enough to bind a sixth σNS unit? No evidence for sequence specificity in σNS binding to ssRNA. Since reovirus replication is cytoplasmic (reviewed in reference 24), there is no selective advantage for σNS to have a strong preference for binding ssRNA over ssDNA. Accordingly, we found that σNS binds to ssRNA with only slightly greater efficiency than to ssDNA. Our evidence that neither dsRNA nor dsDNA is an efficient competitor for σNS binding to ssRNA is consistent with previous findings (28), but our further evidence for strand displacement from short duplex regions is novel (see below).

Although previous work suggested that σNS can bind nonreovirus RNA (14, 28), this study provides the first evidence that nonreovirus RNA efficiently competes with reovirus RNA sequences for binding. Previous authors suggested that σNS binds specifically to the 3′ end of the reovirus plus-strand RNAs (34). Our findings suggest that the reason reovirus 3′ RNA ends were protected from nuclease degradation in the previous study is that σNS protects many regions along the length of the RNAs, including the 3′ ends. We have in fact demonstrated that σNS binding to ssRNA partially protects it from in vitro digestion with RNase A (data not shown). Since our data indicate that σNS does not bind specifically to the short conserved sequences at the 5′ and 3′ ends of all reovirus plus-strand RNAs (2), we hypothesize that σNS is not involved in recognizing these sequences during packaging or minus-strand synthesis. Thus, these sequences are likely to be recognized by other viral or cellular proteins. σNS displaces DNA oligonucleotides from RNA-DNA hybrids but is not a classical helicase. Since the strand displacement activity of σNS bound to RNA-DNA hybrids occurred independently of ATP hydrolysis and was inhibited by MgCl₂ but not by EDTA, the data strongly suggest that σNS is not a classical, ATP-dependent helicase. Consistent with this conclusion is the fact that σNS lacks the nuclease triphosphate-binding motifs characteristic of those enzymes (16). Instead, it appears most likely that σNS melts duplex regions as it completes its cooperative and saturative binding to a primarily ssRNA molecule, with the energy for melting provided by the binding event(s).

From the data in this study, we hypothesize that only short duplex regions, with low thermodynamic stability, are subject to melting by σNS (Table 2). We favor this explanation for why unwinding activity was not demonstrated by σNS on the one type of partially duplex RNA-RNA molecule that we examined in this study. Rather than concluding that σNS is inactive at melting any RNA-RNA duplexes, we propose that the duplex we analyzed was simply too stable to be unwound by σNS. In future work, we will more systematically test for the potential RNA-melting activity of σNS by using molecules containing shorter duplex regions with lower predicted melting temperatures. Such duplexes may be more relevant to reovirus replication in any case since regions of continuous duplex shorter than 17 bp are much more likely to form within the reovirus plus strands according to RNA-folding predictions (J.-Y. Sgro and M. L. Nibert, unpublished data). We will also examine molecules having shorter duplex regions at the 5′ end, the middle, or the 3′ end of the longer RNA strand in order to increase our chances of seeing a specific type of strand displacement activity. If RNA-RNA melting activity can be shown for σNS in vitro, then its role in reovirus-infected cells may be in melting short regions of intra- or intermolecular secondary structure within the viral plus-strand RNAs, regions which might otherwise interfere with the use of the RNAs in packaging and/or minus-strand synthesis during formation of progeny virions. σNS almost certainly does not destabilize the reovirus dsRNA genome for transcription, since this process occurs within the inner capsid of assembled reovirus particles (6, 33), where σNS is not packaged.

Similarities between σNS and ssDNA binding proteins involved in dsDNA replication. The adenovirus DNA binding protein Ad-DBP (21, 42), the herpes simplex virus type 1 protein ICP8 (5), and the Epstein-Barr virus ssDNA-binding protein BALF-2 (38) are all similar to σNS in that they can destabilize regions of a nucleic acid duplex independently of ATP hydrolysis and in a manner that is inhibited by MgCl₂ (5, 21, 38, 42). In addition to ATP-independent strand displacement activity, Ad-DBP and ICP8 share the following properties with σNS: (i) binding in multiple units to a nucleic acid molecule in a sequence-independent manner; (ii) protection of the single-stranded nucleic acid from nuclease degradation (data for σNS are not shown in this paper); (iii) strong preference for single-stranded over double-stranded nucleic acids; and (iv) positive cooperativity for nucleic acid binding (reviewed in references 7 and 20). These and other ssDNA binding proteins have been shown to be involved in dsDNA replication. For example, both in vitro and in vivo studies have shown that Ad-DBP is required for elongation of ssDNA by the adenovirus polymerase (7, 20), possibly involving the ATP-independent helix-destabilizing properties of Ad-DBP (21, 42). Two prokaryotic ssDNA binding proteins, E. coli SSB and bacteriophage T4 gene 32 protein (gp32), are also required for dsDNA replication and share nucleic acid-binding properties with the noted DNA animal virus proteins and σNS but cannot destabilize DNA duplexes (7). Of course, a clear difference between σNS and ssDNA binding proteins is the nucleic acid substrate for genome replication (RNA versus DNA). Nonetheless, considering the large number of similarities, we propose that σNS plays a role in genome replication similar to that played by the ssDNA binding proteins. Specifically, σNS may bind to the reovirus plus-strand RNAs and stabilize them in a conformation that allows the reovirus RNA polymerase and other potential cofactors to mediate minus-strand synthesis to produce the dsRNA gene segments. Some secondary structures in the plus-strand RNAs may be unwound directly by σNS (similarly to Ad-DBP [21, 42]), whereas others may need to be unwound by another viral or cellular protein and then
stabilized in the single-stranded state by eNS binding (similarly to gp32 [7]). Studies to address interactions among eNS, the polymerase, and other proteins should provide further insight into the potential role of eNS in reovirus genome replication.

Similarities to the nonstructural proteins of other Reoviridae members. Other members of the virus family Reoviridae, all of which have dsRNA genomes, also encode nonstructural proteins with ssRNA-binding activity. For instance, the blue-tongue virus (orbivirus) nonstructural protein NS2 (41 kDa) binds ssRNA and forms 7S complexes [39]. Similarly, the reovirus nonstructural protein 7S (35 kDa) has sequence-independent ssRNA-binding activity (18), forms 10S multimers (17), and interacts with the rotavirus RNA polymerase VP1 (17). Recent data indicate that the binding activities of NSP2 are in fact very similar to those of eNS shown in this study, including binding to single ssRNA molecules in multiple copies, with positive cooperativity, with little or no specificity for rotavirus sequences, and with substantially greater affinity for ssRNA than dsRNA (37). NSP2 has not yet been reported to destabilize nucleic acid duplexes, as was eNS in this study, but was reported to have nuclease triphosphatase and autophosphorylation activities (37). The latter have not been demonstrated for eNS (Fig. 6). It remains to be determined whether reovirus eNS, rotavirus NSP2, bluetongue virus NS2, and similar nonstructural proteins from other Reoviridae members play strictly analogous roles in the replication cycles of their respective viruses.

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