

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

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Reovirus nonstructural protein σ NS 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 σ NS was analyzed for in vitro binding to nucleic acids using gel mobility shift assays. Multiple units of σ NS bound to single-stranded RNA molecules with positive cooperativity and with each unit covering about 25 nucleotides at saturation. The σ NS 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, σ NS 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, σ NS displaced this oligonucleotide from the partial duplex. The strand displacement activity did not require ATP hydrolysis and was inhibited by $MgCl_2$, distinguishing it from a classical ATP-dependent helicase. These properties of σ NS are similar to those of single-stranded DNA binding proteins that are known to participate in genomic DNA replication, suggesting a related role for σ NS in replication of the reovirus RNA genome.

Mammalian orthoreoviruses (reoviruses) encode three nonstructural proteins, μ NS, σ NS, and σ 1s, whose roles during viral infection remain poorly understood. This report concerns σ NS, 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 σ NS-encoding S3 gene segment (9, 27). The σ NS protein comprises 366 amino acids and has a molecular mass of 41 kDa. Interaction of σ NS with the viral plus-strand RNAs in infected cells is well documented (3, 14). Moreover, when σ NS 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 σ NS binds specifically to those regions (34). In addition, σ NS and two other reovirus proteins, μ NS and σ 3, 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 σ NS 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 σ NS in translation of proteins from the reovirus plus-strand RNAs has also been suggested (10, 14).

Evidence for a direct role of σ NS 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 σ NS provides a required function at or before minus-strand synthesis in the replication cycle. Other previous work demon-

strated that σ NS-containing ribonucleoprotein complexes isolated from reovirus-infected cells display a poly(C)-dependent poly(G) polymerase activity (12, 13). However, recombinant σ NS expressed in *Escherichia coli* or insect cells does not display this activity (10, 28), whereas recombinant reovirus protein λ 3 obtained from mammalian or insect cells does (35; D. L. Farsetta and M. L. Nibert, unpublished data). These findings suggest that the σ NS-containing complexes characterized by Gomatos et al. (12, 13) also contained λ 3, which is a known component of the reovirus RNA polymerase (22, 35). Thus, despite not having polymerase activity itself, σ NS might play a role in minus-strand synthesis within these λ 3-containing complexes.

In the present study, we performed additional characterizations of the RNA-binding properties of σ NS to learn more about its roles in reovirus replication. In initial experiments, we analyzed purified, baculovirus-expressed σ NS (10) for its capacity to bind single-stranded RNA (ssRNA) molecules in gel mobility shift assays. The results indicated that multiple units of σ NS bind to single molecules of ssRNA with positive cooperativity and in numbers dependent on RNA length such that each unit of σ NS covered approximately 25 nucleotides at saturation. Competition experiments with various nucleic acids addressed the specificity of σ NS binding. These studies revealed a preference for σ NS to bind single-stranded over double-stranded nucleic acids, with a slight preference for RNA over DNA, but they showed no preference for σ NS to bind reovirus over nonreovirus RNA. We also characterized the capacity of σ NS 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 σ NS is distinct from that of a classical helicase. The observed activities of σ NS are similar to those of several well-characterized single-stranded DNA (ssDNA)

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

Transcript ^a	Plasmid ^b	Primer ^c	
		Upstream	Downstream
S4 full length	pGEM-DS4	2787-GGCTTGACATATTGTCG-2804	1196-GATGAATGAAGCCTGTCC-1179 ^d
5'S4-121	pGEM-DS4	2787-GGCTTGACATATTGTCG-2804	121-TCTTGC GCGCTGTAGATTGATACACG-96 ^d
5'S4-151	pGEM-DS4	2787-GGCTTGACATATTGTCG-2804	151-GGCTGTGCTGAGATTGTTTTGTCC-128 ^d
5'S4-181	pGEM-DS4	2787-GGCTTGACATATTGTCG-2804	181-ACGACGGCCACCATACACC-161 ^d
5'S4-211	pGEM-DS4	2787-GGCTTGACATATTGTCG-2804	211-GATCCAACAACACCTAGACAATGC-188 ^d
5'S4-271	pGEM-DS4	2787-GGCTTGACATATTGTCG-2804	271-TGATGACGGATCTGTTGATTACATCTATGG-242 ^d
3'S4-121	pGEM-3'DS4	2654-GCTGCAAGGCGATTAAGTTGG-2674	1196-GATGAATGAAGCCTGTCC-1179 ^d
pGEM-121	pGEM-4Z	2654-GCTGCAAGGCGATTAAGTTGG-2674	121-AGGAAACAGCTATGACCATGATTACGCC-94
T7-17	pGEM-3'DS4	349-GTCAGTGAGCGAAGCGGAAGAGC-324	54-ATGCAAGCTTGTCTCC-70
SP6-146	pGEM-3'DS4	2654-GCTGCAAGGCGATTAAGTTGG-2674	71-AGGGAGACAAGCTTGCATGC-52

^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*). All sequences are written in 5'-to-3' orientation. Sequence numbers that decrease from left to right represent minus strands between those numbers (SP6 transcripts are defined as plus strands). Upstream and downstream are defined relative to the promoter (SP6 or T7) used in generating each transcript.

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

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

MATERIALS AND METHODS

Overexpression and purification of σ NS. σ NS was overexpressed in insect cells infected with a recombinant baculovirus containing the σ 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 σ NS. As noted in the previous study (10), the σ NS protein obtained by this protocol was $\geq 95\%$ 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 9S in 5 to 20% sucrose gradients in the absence of RNA. Nonetheless, whether these complexes represent one discrete type of σ 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-3'DS4. 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 *XhoI* and ligated to the purified *SpeI-XhoI* fragment of the cloned S4 gene to generate new plasmid pGEM-DS4, with the entire S4 gene positioned immediately 3' to the SP6 RNA polymerase promoter such that the first nucleotide of the SP6 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 new plasmid pGEM-3'DS4. The first 6 nucleotides of the SP6 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) (Table 1) and Deep Vent DNA polymerase (New England Biolabs). For making either full-length or partial (5'S4-121, 5'S4-151, 5'S4-181, 5'S4-211, 5'S4-271, and 3'S4-121) S4 plus-strand transcripts, one primer represented the plus strand of pGEM-DS4 or pGEM-3'DS4 in vector sequences 5' to

the SP6 promoter and the second primer represented the minus strand of pGEM-DS4 or pGEM-3'DS4 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-121), one 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 Qiaex II kit (Qiagen, Valencia, Calif.).

To generate RNA transcripts from each amplified DNA fragment, 1 to 2 μ g of the purified fragment was combined with transcription buffer (50 mM HEPES [pH 7.5], 16 mM MgCl₂, 2 mM spermidine, 40 mM dithiothreitol); 3 mM ATP, CTP, and UTP; 1 mM GTP (Pharmacia Biotech, Piscataway, N.J.); 200 U of SP6 RNA polymerase (Epicentre, Madison, Wis.); 20 U of RNasin (Promega); and 0.1 U of inorganic pyrophosphatase (Sigma, St. Louis, Mo.). To radiolabel the RNA transcripts, 0.13 nmol of [α -³²P]GTP (specific activity, 3,000 Ci/mmol) (NEN, Boston, Mass.) was also added. The reaction mixtures were incubated at 37°C for 90 min prior to the addition of another 130 U of SP6 RNA polymerase, 13 U of RNasin, and 0.067 U of inorganic pyrophosphatase. The reaction mixtures were then incubated for another 90 min, after which they were extracted with phenol-chloroform, and the RNA was precipitated with 1 volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol. The RNAs were gel isolated from a 5% polyacrylamide Tris-glycine native gel (25 mM Tris, 192 mM glycine) and eluted overnight at room temperature in elution buffer (0.1% sodium dodecyl sulfate, 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA; filter sterilized). The eluted RNA was then extracted with phenol-chloroform and precipitated with 0.3 M sodium acetate and 2 volumes of 100% ethanol. The RNA concentration was determined by scintillation counting (11).

Gel mobility shift assays. Various amounts of purified σ NS in phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8.4 mM Na₂HPO₄, 1.6 mM KH₂PO₄) were incubated with various amounts of ³²P-labeled RNA in gel shift buffer (12 mM Tris-HCl [pH 7.5], 0.1 mg of bovine serum albumin/ml, 10 mM β -mercaptoethanol, 0.2% Tween 20, 10 U of RNasin) (32) for 15 min at room temperature in a total volume of 10 μ l. The samples were mixed with gel loading buffer (25 mM Tris, 192 mM glycine, 0.25% bromophenol blue, 0.25% xylene cyanole FF, 30% glycerol) and subjected to electrophoresis through either a 1% agarose-Tris-acetate-EDTA gel (40 mM Tris-acetate, 1 mM EDTA) at 90 V or a 5% polyacrylamide Tris-glycine native gel (described above) at 10 mA. The times of electrophoresis varied depending on the size of the RNA probe used in the assay. The gels were dried onto filter paper under vacuum, and the samples were visualized by phosphorimaging (Molecular Dynamics, Sunnyvale, Calif.). The amount of radioactivity in the samples was quantitated using ImageQuant software (Molecular Dynamics). Concentrations of NaCl from 0 to 300 mM had little effect on the binding of σ NS to RNA. The standard binding reaction mixture used throughout this study contained less than 30 mM NaCl.

Immunoblots. Proteins in the 5% Tris-glycine polyacrylamide gels were blotted onto a nylon membrane (Bio-Rad, Hercules, Calif.) at 30 V overnight at 4°C in transfer buffer (25 mM Tris, 192 mM glycine). The membrane was probed with the anti- σ NS antibody (10) at a dilution of 1/500 using the protocol supplied with the Bio-Rad color development reagent kit. The secondary antibody was visualized with color development reagents 5-bromo-4-chloro-3-indoylphosphate *p*-toluidine salt and *p*-nitroblue tetrazolium chloride (Bio-Rad).

Hill plots. A Hill plot was generated for the 121-, 151-, and 181-nucleotide RNAs in Fig. 3 as well as for three other assays done with a 121-nucleotide RNA and σ NS amounts from 0.24 to 6.0 pmol (1 μ g \approx 24 pmol) (26). The amount of bound σ NS was calculated as follows. First the amount of radiolabeled RNA in

TABLE 2. Oligonucleotides used in forming partial duplexes, RNA-DNA or RNA-RNA

Type of oligonucleotide	Sequence ^a	Region of longer RNA strand to which oligonucleotide was hybridized ^b	T_m^c (°C)
DNA	121-TCTTGC GCGCTGTAGATTG-103	3' end	43.1
DNA	68-CACGACCTGATGACCGTTG-50	Middle	44.1
DNA	19-GGGAAGAGGCCAAAAATAGC-1	5' end	35.5
RNA	70-GGGAGACAAGCTTGCAT-54	3' end ^d	48.8

^a 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).

^b DNA oligonucleotides were individually hybridized to RNA 5'S4-121. The RNA oligonucleotide was hybridized to RNA SP6-146.

^c Melting temperatures calculated for RNA-DNA or RNA-RNA duplexes of the indicated sequences at 25 nM duplex 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.

^d The 5' end of the RNA oligonucleotide was hybridized to the penultimate 3' nucleotide of the longer RNA.

each shifted band was quantitated and expressed as a percentage of total RNA in the reaction mixture. Next the percentage was multiplied by the number of σ NS units in each band (e.g., one σ NS unit in the fastest-migrating band and four in the slowest). Finally, the amounts of σ NS in all four bands were summed and the sum was divided by the total amount of protein added to each sample. The amount of unbound σ NS = 1 - the amount of bound σ NS. The \log_{10} (bound σ NS/unbound σ NS) versus the \log_{10} (σ NS concentration) was plotted, and the best-fit line to the linear portion of the graph was calculated using Excel (Microsoft, Redmond, Wash.). The slope of the line is the Hill coefficient (40) and was calculated for all six assays and averaged.

Competition assays. A radiolabeled RNA probe (0.24 pmol each) was mixed with the unlabeled competitors in various relative amounts, after which 4.8 pmol of σ NS was added, and the samples were incubated for 15 min at room temperature. For one set of samples, σ NS was combined with radiolabeled RNA probe and incubated for 15 min as described above. The competitor was then added, and the samples were incubated for an additional 15 min or 2 h. Equivalent weights of RNA (concentration determined by A_{260} [30]) were used. The radiolabeled RNA probe used for both competition experiments was the 121-nucleotide fragment from the 5' end of S4 in the pGEM-DS4 plasmid. The nucleic acid competitors used for competition between ss- and dsRNA and ss- and dsDNA were the following: ssRNA, full-length in vitro-transcribed S4 gene; dsRNA, reovirus type 3 Dearing genomic RNAs; ssDNA, circular M13 plasmid containing rhinovirus sequences; dsDNA, *Sma*I-linearized pGEM-4Z vector containing the reovirus type 1 Lang S3 gene (10). The unlabeled RNA competitors used for competitions were the following: the 5' 121 nucleotides of S4 (same RNA used as the labeled probe), the 3' 121 nucleotides of S4, and 121 nucleotides from the multiple-cloning region of the pGEM-4Z vector (Promega) that did not contain reovirus sequences. The amount of radiolabeled RNA bound was determined as the amount of RNA in the upper four shifted bands versus the total amount of RNA. All results are expressed relative to the sample containing no competitor.

RNA-DNA and RNA-RNA partial duplexes. Nineteen-nucleotide DNA oligonucleotides (Integrated DNA Technologies) were designed to be complementary to the 3' end, the middle, or the 5' end of the 5'S4-121 RNA (Table 2). The oligonucleotides were 5'-end-labeled with polynucleotide kinase (New England Biolabs) and [γ -³²P]ATP, extracted with phenol-chloroform, and precipitated with ethanol. The oligonucleotides were then hybridized to the 5'S4-121 RNA in hybridization buffer (100 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA) at 90°C for 5 min and cooled to room temperature.

To synthesize linear DNA templates for transcribing the 17-nucleotide RNA oligonucleotide (T7-17) (Table 2) and the 146-nucleotide RNA (SP6-146) to which T7-17 was later hybridized, we performed PCR with different primer pairs (Integrated DNA Technologies) (Table 1) and Deep Vent DNA polymerase (New England Biolabs). For making T7-17, one primer represented the minus strand of pGEM-3'DS4 in vector sequences 5' to the T7 promoter and the second primer represented the plus strand of pGEM-3'DS4 in the appropriate downstream vector sequences. For making SP6-146, one primer represented the plus strand of pGEM-3'DS4 in vector sequences 5' to the SP6 promoter and the second primer represented the minus strand of pGEM-3'DS4 in the appropriate downstream vector sequences. Both RNA transcripts were generated by runoff

transcription in vitro as described above except that T7 RNA polymerase (Epicentre) and 12 mM MgCl₂ were used in making T7-17. [α -³²P]GTP was included in the reaction mixture to provide radiolabeling of T7-17, SP6-146, or both in different experiments. T7-17 was hybridized to the 3' end of SP6-146 as described above for the RNA-DNA hybrids.

ATPase and displacement assays. In the σ NS samples for the ATPase assay, 75 pmol of purified σ NS was incubated with 3 μ Ci of [α -³²P]ATP (NEN) in gel shift buffer with or without EDTA (12.5 mM). In the core samples for the ATPase assay, 6×10^9 reovirus cores were incubated with 3 μ Ci of [α -³²P]ATP (NEN) in nucleoside triphosphatase buffer (50 mM Tris-morpholinoethanesulfonic acid [MES] [pH 8.5], 5 mM MgCl₂) (25) with or without EDTA (12.5 mM). The RNA-DNA hybrid (0.11 pmol) was added to all samples, which were incubated at room temperature or 35°C (cores) for 30 min in a total volume of 5 μ l each. One microliter of each sample was then spotted onto a polyester-backed polyethyleneimine-cellulose thin-layer chromatography plate (Sigma) and developed with ascending solvent (1 M formic acid, 0.5 M LiCl). The reaction products were visualized by phosphorimaging (Molecular Dynamics). To assay for DNA oligonucleotide displacement activity in parallel with ATPase assays, samples were prepared in the same manner except that no [α -³²P]ATP was added. The entire volume of each sample was then analyzed on a 5% polyacrylamide Tris-glycine native gel as described above. For other experiments in which DNA or RNA oligonucleotide displacement activity was measured, various amounts of purified σ NS and 0.24 to 0.26 pmol of RNA-DNA or RNA-RNA were added to gel shift buffer in a total volume of 10 μ l and then the mixtures were incubated at room temperature for 15 min before running the entire sample on a 5% polyacrylamide Tris-glycine native gel.

RESULTS

Gel mobility shift assays for σ NS binding to 1,196- or 151-nucleotide ssRNAs. The RNA-binding activity of purified σ NS was examined using an agarose gel mobility shift assay. In initial experiments, increasing amounts of σ NS 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 σ NS binding using a polyacrylamide gel mobility shift assay (Fig. 1A). For initial experiments with smaller ssRNA molecules, we used runoff transcripts representing the 5' 151 nucleotides of the S4 plus strand (5'S4-151). As the amount of σ NS was increased, the mobility of the 5'S4-151 RNA was progressively retarded and clearly distinguishable, intermediate-size complexes were observed. At the larger amounts of σ NS (>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 σ NS amounts added, we concluded that more than one unit of σ NS 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 σ NS units. Each binding unit of σ NS 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 σ NS, a gel from a mobility shift assay with unlabeled 5'S4-151 RNA was subjected to immunoblotting using a polyclonal antiserum for σ NS (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 σ NS protein did not show this pattern of banding in the absence of ssRNA (Fig. 1B, - RNA), although σ NS 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

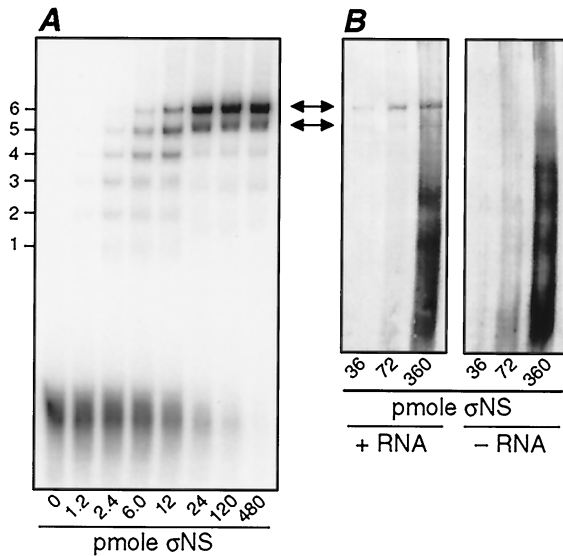


FIG. 1. Gel mobility shift assay with a 151-nucleotide fragment of reovirus S4 plus strand and immunoblot analysis of a similar assay with anti- σ NS serum. (A) Increasing amounts of purified σ NS were incubated with 0.21 pmol of radiolabeled ssRNA comprising the 5' 151 nucleotides of the reovirus S4 plus strand (5'S4-151). The samples were then subjected to electrophoresis in a 5% Tris-glycine polyacrylamide native gel until the free RNA was near the bottom of the gel. The radiolabeled RNA in the dried gel was visualized by phosphorimaging. σ NS-RNA complexes with six distinct mobilities are indicated at left. (B) Increasing amounts of σ NS were incubated with and without unlabeled 5'S4-151 RNA (0.8 pmol) and subjected to electrophoresis as described above. An immunoblot assay was performed to detect the protein. The amounts of RNA and protein in these samples were increased so that the ratios were similar to those in panel A but the protein was more easily detected. Arrows, comparable bands in panels A and B.

of σ NS to self-associate into higher-order multimers in the absence of RNA under these conditions. Their appearance only with excess amounts of σ NS suggests that they hold limited significance for the RNA-binding results. The findings confirm that σ NS was present in the shifted RNA-protein complexes.

Gel mobility shift assay for σ NS 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 σ NS (Fig. 1A). The presence of six shifted bands suggested that if the larger amounts of σ NS were fully saturating the RNA, then a maximum of six units of σ NS were binding to each RNA molecule. Moreover, since the RNA was 151 nucleotides long, each unit of σ NS must be covering about 25 nucleotides if the σ NS 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 σ NS 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 σ NS (Fig. 2A). To compare the mobilities of the different complexes formed with each RNA, all four RNAs were incubated with the same amount of σ NS (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 σ NS (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 σ NS covers a 24- to 27-nucleotide region of RNA and that the total number of σ NS units that bound per RNA molecule is determined by the overall length of the RNA.

Binding cooperativity. Since multiple units of σ NS bound to a single RNA molecule, the binding could have exhibited positive, negative, or no cooperativity. To address these possibil-

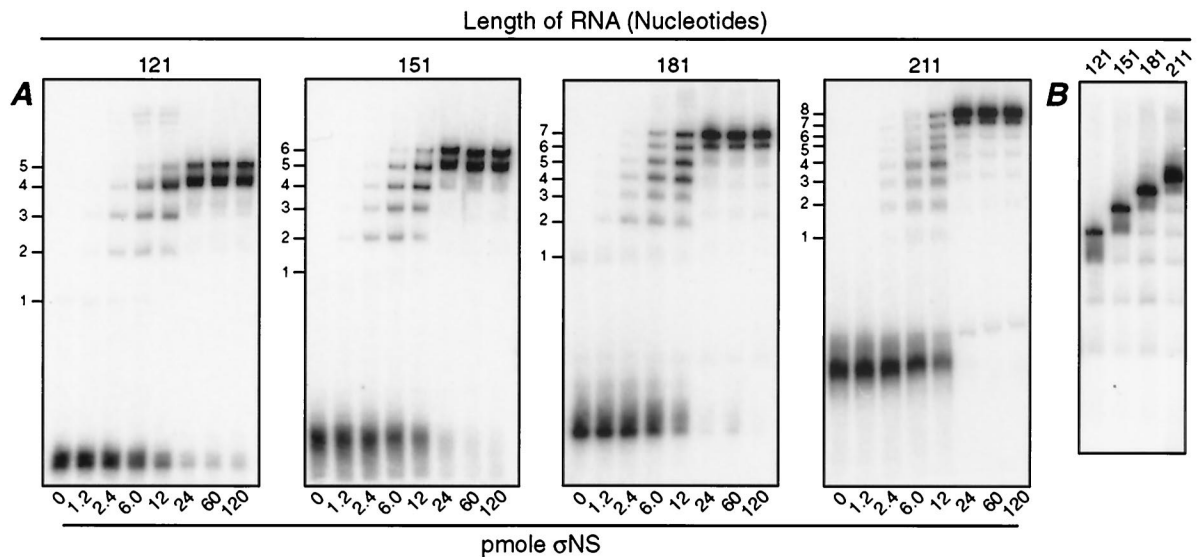


FIG. 2. Gel mobility shift assays with S4 plus-strand RNA fragments ranging in size from 121 to 211 nucleotides in 30-base increments. Radiolabeled ssRNA fragments comprising the following 5' portions of the S4 plus strand were synthesized: 121 nucleotides, 5'S4-121; 151 nucleotides, 5'S4-151; 181 nucleotides, 5'S4-181; and 211 nucleotides, 5'S4-211. (A) Increasing amounts of purified σ NS were separately incubated with 0.21 to 0.22 pmol of each RNA, and the samples were analyzed as described for Fig. 1A. σ NS-RNA complexes with five to eight distinct mobilities are indicated to the left of each gel. (B) Purified σ NS (18 pmol) was incubated with each of the four RNAs as described for panel A (RNA sizes are indicated above the lanes) and subjected to electrophoresis as described for Fig. 1A. To provide better separation of the shifted complexes, the gel was run longer in this experiment such that the free RNA was run off the bottom.

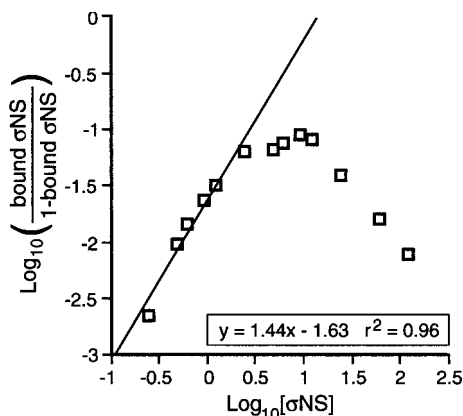


FIG. 3. Hill plot of σ NS binding to ssRNA to determine cooperativity. A gel shift assay with 0.24 pmol of radiolabeled 5'S4-121 RNA was performed as described for Fig. 2A except that the concentrations of purified σ NS ranged from 0.24 to 6 pmol. The amount of σ NS bound at each concentration was calculated as described in Materials and Methods. $\text{Log}_{10}(\text{bound } \sigma\text{NS} / 1 - \text{bound } \sigma\text{NS})$ was plotted relative to $\text{log}_{10}(\sigma\text{NS concentration})$, 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).

ities, the ratio of bound to total σ NS 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 σ NS binds ssRNA with positive cooperativity. When the data from all six experiments were averaged and σ NS was assumed to bind ssRNA as a monomer, the Hill coefficient was calculated at 1.43 ± 0.10 . Since the RNA-binding unit of σ NS is thought to be a small oligomer (10), the calculations were also done for σ NS 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 σ NS 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 σ NS 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 σ NS 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 σ NS. 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 σ NS binding, whereas dsRNA and dsDNA competed much less efficiently (Fig. 4A). For example, when 10-fold more ssRNA or ssDNA competitor was added only $23\% \pm 2\%$ or $37\% \pm 11\%$, respectively, of the radiolabeled 5'S4-121 RNA was bound. In contrast, when 20-fold more dsRNA or dsDNA competitor was added, $82\% \pm 7\%$ or $98\% \pm 4\%$, 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 σ NS than did DNA (Fig. 4A). In summary, σ NS bound efficiently to single-stranded, but not double-stranded, nucleic acids and exhibited a slight preference for RNA over DNA.

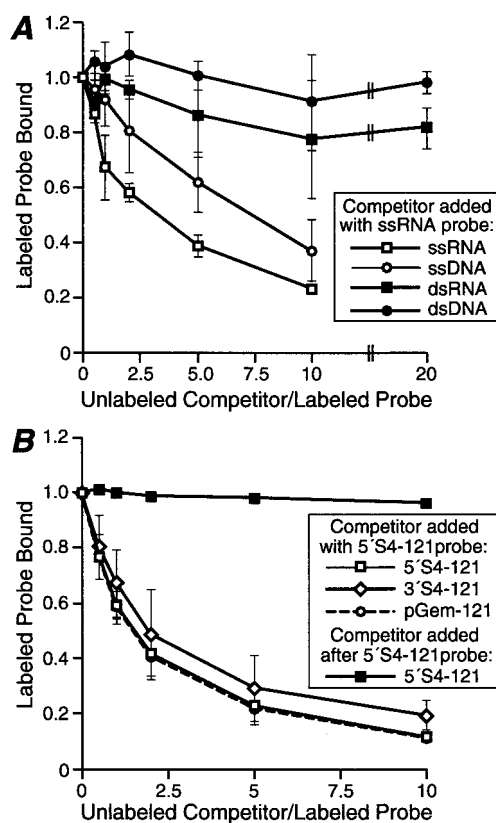


FIG. 4. Competition assays for σ NS binding to ssRNA using ssRNA, dsRNA, ssDNA, or dsDNA as the competitor. Each data point represents the mean from three experiments, and the error bars represent the standard deviation of the mean. (A) Increasing concentrations of unlabeled competitor ssRNA, dsRNA, dsDNA, or ssDNA (see Materials and Methods) were combined with 0.24 pmol of radiolabeled 5'S4-121 ssRNA. Purified σ NS (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 σ NS 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, σ NS was added to the labeled RNA prior to addition of the 5'S4-121 competitor.

Competition assays with reovirus and nonreovirus ssRNAs. Previous work demonstrated σ NS binding to reovirus RNA in infected cells (3, 14) and to nonreovirus RNA in vitro (14, 28). However, no experiments addressed whether σ NS exhibits a preference for reovirus RNA (14, 28). Sequences at the 5' and 3' termini of all 10 reovirus RNAs are conserved (2) and therefore may be involved in distinguishing reovirus from nonreovirus RNA for binding by proteins such as σ NS during steps in reovirus replication. To test this hypothesis, we used a competition assay to compare the affinities of σ NS for RNAs representing the 5' end of the reovirus S4 plus strand, the 3' end of the reovirus S4 plus strand, and nonreovirus sequences derived from pGEM-4Z. Radiolabeled 5'S4-121 RNA was incubated with unlabeled competitor RNAs (5'S4-121, 3'S4-121, or pGEM-121) before addition of σ NS. The RNA-protein complexes were then analyzed using the polyacrylamide gel mobility shift assay (Fig. 4B). In this assay, all three competitor RNAs competed to approximately the same level with radiolabeled 5'S4-121 RNA for binding to σ NS. For example, when fivefold more 5'S4-121 competitor RNA was added, only 13%

$\pm 3\%$ of the radiolabeled 5'S4-121 RNA was bound by σ NS. When fivefold more 3'S4-121 competitor RNA was added, only $22\% \pm 12\%$ of the radiolabeled 5'S4-121 RNA was bound. Last, when fivefold more pGEM-121 competitor RNA was added, only $13\% \pm 9\%$ of the radiolabeled 5'S4-121 RNA was bound. Although these data suggested that σ NS 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 σ NS 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 σ NS. This suggests that σ NS 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 σ NS 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 σ NS, even when the competitor was added in 10-fold excess. The complex of 5'S4-121 RNA and σ NS was stable for at least 2 h after addition of this competitor (data not shown). These results suggest that, once σ NS 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 σ NS binding to partially duplex RNA-DNA hybrids and evidence for a strand displacement activity. We hypothesized that σ NS 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 σ NS 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 σ NS (Fig. 5A). Moreover, the amount of σ NS that shifted the majority of this RNA-DNA hybrid (30 pmol of σ NS 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 σ NS 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 σ NS 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 σ NS 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 σ NS 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 σ NS were incubated with the respective hybrids (data not shown), suggesting that the displacement activity was not direction dependent.

Evidence that σ NS is not an ATP-dependent helicase. Since σ NS displaced DNA oligonucleotides from RNA-DNA hybrids, we recognized that the protein might possess helicase activity. If so, then σ NS 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 de-

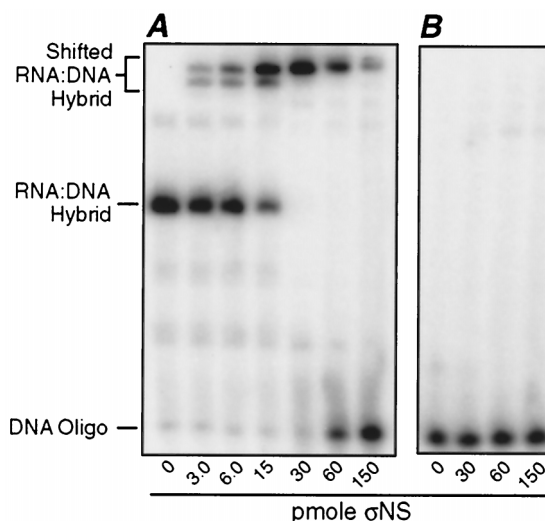


FIG. 5. Gel shift assay with ssRNA having an RNA-DNA duplex region at its 3' end. A 19-nucleotide DNA oligonucleotide that was exactly complementary to the 3' end of the RNA was 32 P-labeled at its 5' end. (A) The oligonucleotide was hybridized to unlabeled ssRNA (5'S4-121) and purified. Differing amounts of purified σ NS were then mixed with 0.26 pmol of hybrid and assayed as described for Fig. 1A. (B) An unhybridized 32 P-labeled oligonucleotide was incubated in the presence of increasing amounts of σ NS and also assayed as described for Fig. 1A. For both panels, the amounts of σ NS added to samples are indicated beneath the lanes, and the positions of the RNA-DNA hybrid and the DNA oligonucleotide are also indicated.

scribed 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 σ NS 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 - 32 P]ATP was added to parallel samples, which were then analyzed by thin-layer chromatography to determine if ATP had been hydrolyzed. The σ NS 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 σ NS does not require hydrolysis of ATP during displacement of the DNA oligonucleotide from the RNA-DNA hybrid.

To assess the effect of $MgCl_2$ on the σ NS-associated strand displacement activity, the displacement assay was performed in the presence of increasing concentrations of $MgCl_2$. The activity was progressively decreased with increasing $MgCl_2$ (data not shown). At 10 mM $MgCl_2$, for example, only 30% of the DNA oligonucleotide was displaced relative to the amount displaced in the absence of $MgCl_2$ (data not shown), suggesting that Mg^{2+} inhibited rather than stimulated the displacement activity. This response to $MgCl_2$ provided additional evidence that the strand displacement activity of σ NS is distinguishable from that of a classical helicase (16).

Test for strand displacement activity of σ NS 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-

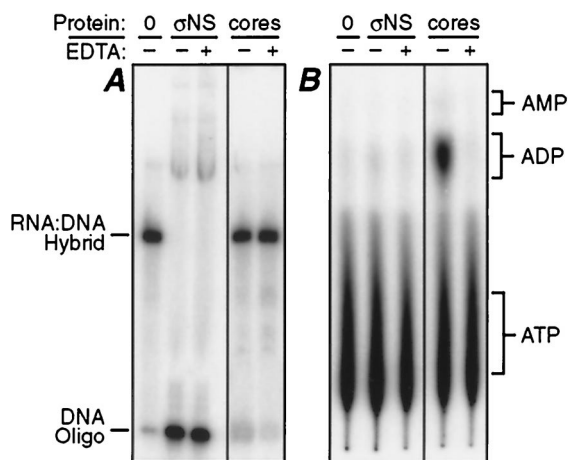


FIG. 6. Analysis of ATP hydrolysis during strand displacement by σ NS. Either 12.5 mM EDTA (+) or H_2O (-) was added to 75 pmol of purified σ NS (σ NS) or 6×10^9 reovirus core particles (cores) in each sample. Cores were included as a positive control for ATP hydrolysis. All samples were analyzed in duplicate. (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 [α - ^{32}P]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.

scripts. The strand displacement activity that we demonstrated for σ NS using RNA-DNA hybrids may therefore reflect an essential activity of this protein at disrupting regions of RNA-RNA duplex during one or more steps in reovirus replication. The poor capacity of σ NS to bind to fully duplex RNA molecules (Fig. 4A) suggested that partial duplexes were more appropriate for further testing. In experiments with one such partially duplex RNA, we found that σ NS could not displace a fully complementary 17-nucleotide RNA oligonucleotide (Table 2) from the 3' end of a longer RNA to which it was hybridized (data not shown). Notably, however, this RNA-RNA duplex had a higher calculated melting temperature than the RNA-DNA duplexes used in the preceding experiments (Table 2), which may have limited the capacity of σ NS to act on it. Further experiments are warranted before concluding that σ NS can or cannot disrupt dsRNA regions in partially duplex molecules.

DISCUSSION

σ NS 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 σ NS can bind to a single ssRNA molecule. Such molecules, bound by multiple units of σ NS, may represent the large nucleoprotein complexes obtained from both reovirus-infected mammalian cells (14) and insect cells infected with a recombinant baculovirus expressing σ NS (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]), σ NS 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 σ NS that bind to an RNA mol-

ecule stretch out like beads on a necklace or self-associate to form a more compact structure.

The positive cooperativity demonstrated for σ NS binding to ssRNA may arise either from changes in RNA conformation upon σ NS binding that promote subsequent σ NS units to bind more readily or from σ NS- σ NS interactions, similar to the case for poliovirus RNA polymerase (4, 26). Certain observations are consistent with each possibility. Data indicating that σ NS 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 σ NS oligomers might self-associate to form higher-order multimers under certain conditions (Fig. 1B) suggests that positive cooperativity may arise from such σ NS- σ NS interactions. Additional analyses of σ NS-RNA and σ NS- σ NS 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 σ NS for each RNA, despite their different sizes (Fig. 2A). Assuming that σ NS 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 σ NS as saturation of the 121-nucleotide RNA. However, since the interval between amounts of added σ NS 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 σ NS when 15 pmol of σ NS was added in this assay, then the 211-nucleotide RNA should have been saturatively bound by eight units of σ NS when 24 pmol of σ NS 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 σ NS were added (Fig. 1A and 2). These complexes showed mobilities distinct from the major ones seen with smaller amounts of σ NS (Fig. 1A and 2A). Moreover, the higher-mobility complexes seen with saturative amounts of σ NS 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 σ NS was also saturatively bound. Enumeration of these complexes in Fig. 2B lends support to the conclusion that each σ NS binding unit covers about 25 nucleotides of RNA.

What are the unit of σ NS that binds to ssRNA and the number of nucleotides of RNA that each unit contacts? The purified σ NS 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 σ NS. As stated earlier, whether these complexes represent one discrete type of σ NS oligomer or a mixture and whether they can self-associate to form higher-order multimers in the absence of RNA remain unknown. We nonetheless hypothesize that one of these oligomers or multimers represents the unit of σ NS that binds to ssRNA in a number of copies dependent on RNA length. The molar ratio of σ NS to RNA at saturation in this study may indicate the number of σ NS subunits in the binding unit, that is, if the concentrations of σ NS are not limiting and if most or all of the added σ NS is competent for RNA binding. In this case, we can calculate that saturative binding was achieved with σ NS in 60- to 120-fold molar excess to each RNA in Fig. 2A

(12 to 24 pmol of σ NS for about 0.2 pmol of RNA). Considering that only five to eight units of σ NS 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 σ NS. If the RNA-free, 7- to 9S σ NS 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 σ NS 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 σ NS oligomer that sediments in the 7- to 9S range in the absence of RNA (10).

The finding that each binding unit of σ NS covers about 25 nucleotides of RNA is consistent with previous work demonstrating that σ NS 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 σ NS from the number of nucleotides that constitutes the minimum or preferred size of binding site for each unit of σ NS. No experiments in this report directly address the latter, but further analyses of σ NS binding to different-size RNAs might be used to establish this number. For example, if a 140-nucleotide RNA binds five σ NS units covering 25 nucleotides each, are the 15 "extra" nucleotides enough to bind a sixth σ NS unit?

No evidence for sequence specificity in σ NS binding to ssRNA. Since reovirus replication is cytoplasmic (reviewed in reference 24), there is no selective advantage for σ NS to have a strong preference for binding ssRNA over ssDNA. Accordingly, we found that σ NS binds to ssRNA with only slightly greater efficiency than to ssDNA. Our evidence that neither dsRNA nor dsDNA is an efficient competitor for σ NS 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 σ NS 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 σ NS 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 σ NS protects many regions along the length of the RNAs, including the 3' ends. We have in fact demonstrated that σ NS binding to ssRNA partially protects it from *in vitro* digestion with RNase A (data not shown). Since our data indicate that σ NS 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 σ NS 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.

σ NS displaces DNA oligonucleotides from RNA-DNA hybrids but is not a classical helicase. Since the strand displacement activity of σ NS bound to RNA-DNA hybrids occurred independently of ATP hydrolysis and was inhibited by MgCl₂ but not by EDTA, the data strongly suggest that σ NS is not a classical, ATP-dependent helicase. Consistent with this conclusion is the fact that σ NS lacks the nucleoside triphosphate-binding motifs characteristic of those enzymes (16). Instead, it appears most likely that σ NS 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 σ NS (Table 2). We favor this explanation for why unwinding activity was not demonstrated by σ NS on the one type of partially duplex RNA-RNA molecule that we examined in this study. Rather than concluding that σ NS is inactive at melting any RNA-RNA duplexes, we propose that the duplex we analyzed was simply too stable to be unwound by σ NS. In future work, we will more systematically test for the potential RNA-melting activity of σ NS 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 σ NS *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. σ NS 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 σ NS is not packaged.

Similarities between σ NS 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 σ NS 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 σ NS: (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 σ NS 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 σ NS but cannot destabilize DNA duplexes (7). Of course, a clear difference between σ NS 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 σ NS plays a role in genome replication similar to that played by the ssDNA binding proteins. Specifically, σ NS 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 σ NS (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 σ NS binding (similarly to gp32 [7]). Studies to address interactions among σ NS, the polymerase, and other proteins should provide further insight into the potential role of σ NS 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 bluetongue virus (orbivirus) nonstructural protein NS2 (41 kDa) binds ssRNA and forms 7S complexes (39). Similarly, the rotavirus nonstructural protein NSP2 (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 σ NS 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 σ NS in this study, but was reported to have nucleoside triphosphatase and autophosphorylation activities (37). The latter have not been demonstrated for σ NS (Fig. 6). It remains to be determined whether reovirus σ NS, 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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REFERENCES

- Acs, G., H. Klett, M. Schonberg, J. Christman, D. H. Levin, and S. C. Silverstein. 1971. Mechanism of reovirus double-stranded ribonucleic acid synthesis in vivo and in vitro. *J. Virol.* **8**:684-689.
- Antczak, J. B., R. Chmelo, D. J. Pickup, and W. K. Joklik. 1982. Sequence at both termini of the 10 genes of reovirus serotype 3 (strain Dearing). *Virology* **121**:307-319.
- Antczak, J. B., and W. K. Joklik. 1992. Reovirus genome segment assortment into progeny genomes studied by the use of monoclonal antibodies directed against reovirus proteins. *Virology* **187**:760-776.
- Beckman, M. T. L., and K. Kirkegaard. 1998. Site size of cooperative single-stranded RNA binding by poliovirus RNA-dependent RNA polymerase. *J. Biol. Chem.* **273**:6724-6739.
- Boehmer, P. E., and I. R. Lehman. 1993. Herpes simplex virus type 1 ICP8: helix-destabilizing properties. *J. Virol.* **67**:711-715.
- Borsa, J., and A. F. Graham. 1968. Reovirus: RNA polymerase activity in purified virions. *Biochem. Biophys. Res. Commun.* **33**:895-901.
- Chase, J. W., and K. R. Williams. 1986. Single-stranded DNA binding proteins required for DNA replication. *Annu. Rev. Biochem.* **55**:103-136.
- Cormack, B. 1991. Mutagenesis by the polymerase chain reaction, p. 8.5.1-8.5.9. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. John Wiley and Sons, Inc., New York, N.Y.
- Cross, R. K., and B. N. Fields. 1972. Temperature-sensitive mutants of reovirus type 3: studies on the synthesis of viral RNA. *Virology* **50**:799-809.
- Gillian, A. L., and M. L. Nibert. 1998. Amino terminus of reovirus nonstructural protein σ NS is important for ssRNA binding and nucleoprotein complex formation. *Virology* **240**:1-11.
- Gillian-Daniel, D. L., N. K. Gray, J. Astrom, A. Barkoff, and M. Wickens. 1998. Modifications of the 5' cap of mRNAs during *Xenopus* oocyte maturation: independence from changes in poly(A) length and impact on translation. *Mol. Cell. Biol.* **18**:6152-6163.
- Gomatos, P. J., O. Prakash, and N. M. Stamatou. 1981. Small reovirus particle composed solely of σ NS with specificity for binding different nucleic acids. *J. Virol.* **39**:115-124.
- Gomatos, P. J., N. M. Stamatou, and N. H. Sarkar. 1980. Small reovirus-specific particle with polycytidylate-dependent RNA polymerase activity. *J. Virol.* **36**:556-565.
- Huismans, H., and W. K. Joklik. 1976. Reovirus-coded polypeptides in infected cells: isolation of two native monomeric polypeptides with affinity for single-stranded and double-stranded RNA, respectively. *Virology* **70**:411-424.
- Ito, Y., and W. K. Joklik. 1972. Temperature-sensitive mutants of reovirus. I. Patterns of gene expression by mutants of groups C, D, and E. *Virology* **50**:189-201.
- Kadare, G., and A.-L. Haenni. 1997. Virus-encoded RNA helicases. *J. Virol.* **71**:2583-2590.
- Kattoura, M. D., X. Chen, and J. T. Patton. 1994. The rotavirus RNA-binding protein NS35 (NSP2) forms 10S multimers and interacts with the viral RNA polymerase. *Virology* **202**:803-813.
- Kattoura, M. D., L. L. Clapp, and J. T. Patton. 1992. The rotavirus nonstructural protein, NS35, possesses RNA-binding activity in vitro and in vivo. *Virology* **191**:698-708.
- Kedl, R., S. Schmechel, and L. Schiff. 1995. Comparative sequence analysis of the reovirus S4 genes from 13 serotype 1 and serotype 3 field isolates. *J. Virol.* **69**:552-559.
- Kornberg, A., and T. A. Baker. 1991. *DNA replication*, 2nd ed. W. H. Freeman & Co., New York, N.Y.
- Monaghan, A., A. Webster, and R. T. Hay. 1994. Adenovirus DNA binding protein: helix destabilizing properties. *Nucleic Acids Res.* **22**:742-748.
- Morozov, S. Y. 1989. A possible relationship of reovirus putative RNA polymerase to polymerases of positive-strand RNA viruses. *Nucleic Acids Res.* **17**:5394.
- Nam, S. C., and C. Kang. 1988. Transcription initiation site selection and abortive initiation cycling of phage SP6 RNA polymerase. *J. Biol. Chem.* **263**:18123-18127.
- Nibert, M. L., L. A. Schiff, and B. N. Fields. 1996. Reoviruses and their replication, p. 1557-1596. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fields virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
- Noble, S., and M. L. Nibert. 1997. Characterization of an ATPase activity in reovirus cores and its genetic association with core shell protein λ 1. *J. Virol.* **71**:2182-2191.
- Pata, J. D., S. C. Schultz, and K. Kirkegaard. 1995. Functional oligomerization of poliovirus RNA-dependent RNA polymerase. *RNA* **1**:466-477.
- Ramig, R. F., T. A. Mustoe, A. H. Sharpe, and B. N. Fields. 1978. A genetic map of reovirus. II. Assignment of the double-stranded RNA-negative mutant groups C, D, and E to genome segments. *Virology* **85**:531-534.
- Richardson, M. A., and Y. Furuichi. 1985. Synthesis in *Escherichia coli* of the reovirus nonstructural protein σ NS. *J. Virol.* **56**:527-533.
- Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Sharf, R. Hiuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487-491.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schonberg, M., S. C. Silverstein, D. H. Levin, and G. Acs. 1971. Asynchronous synthesis of the complementary strands of the reovirus genome. *Proc. Natl. Acad. Sci. USA* **68**:505-508.
- Schoumacher, F., C. Erny, A. Berna, T. Godefroy-Colburn, and C. Stussi-Graud. 1992. Nucleic acid-binding properties of the alfalfa mosaic virus movement protein produced in yeast. *Virology* **188**:896-899.
- Shatkin, A. J., and J. D. Sipe. 1968. RNA polymerase activity in purified reoviruses. *Proc. Natl. Acad. Sci. USA* **61**:1462-1469.
- Stamatou, N. M., and P. J. Gomatos. 1982. Binding to selected regions of reovirus mRNAs by a nonstructural reovirus protein. *Proc. Natl. Acad. Sci. USA* **79**:3457-3461.
- Starnes, M. C., and W. K. Joklik. 1993. Reovirus protein lambda 3 is a poly(C)-dependent poly(G) polymerase. *Virology* **193**:356-366.
- Sugimoto, N., S. Nakano, M. Katoh, A. Matsumura, H. Nakamura, T. Ohmichi, M. Yoneyama, and M. Sasaki. 1995. Thermodynamic parameters to predict stability of RNA/DNA hybrid duplexes. *Biochemistry* **34**:11211-11216.
- Taraporewala, Z., D. Chen, and J. T. Patton. 1999. Multimers formed by the

- rotavirus nonstructural protein NSP2 bind to RNA and have nucleoside triphosphatase activity. *J. Virol.* **73**:9934–9943.
38. **Tsurumi, T., J. Kishore, N. Yokoyama, M. Fujita, T. Daikoku, H. Yamada, Y. Yamashita, and Y. Nishiyama.** 1998. Overexpression, purification and helix-destabilizing properties of Epstein-Barr virus ssDNA-binding protein. *J. Gen. Virol.* **79**:1257–1264.
 39. **Uitenwerde, J. M., J. Theron, M. A. Stoltz, and H. Huismans.** 1995. The multimeric nonstructural NS2 proteins of Bluetongue virus, African horse-sickness virus, and epizootic hemorrhagic disease virus differ in their single-stranded RNA-binding ability. *Virology* **209**:624–632.
 40. **van Holde, K. E.** 1985. *Physical biochemistry*, 2nd ed. Prentice-Hall, Inc., Englewood Cliffs, N.J.
 41. **Xia, T., J. SantaLucia, Jr., M. E. Burkard, R. Kierzek, S. J. Schroeder, X. Jiao, C. Cox, and D. H. Turner.** 1998. Thermodynamic parameters for an expanded nearest-neighbor model for formation of RNA duplexes with Watson-Crick base pairs. *Biochemistry* **37**:14719–14735.
 42. **Zijderveld, D. C., and P. C. van der Vliet.** 1994. Helix-destabilizing properties of the adenovirus DNA-binding protein. *J. Virol.* **68**:1158–1164.