Reovirus Nonstructural Protein µNS Binds to Core Particles but Does Not Inhibit Their Transcription and Capping Activities

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Previous studies provided evidence that nonstructural protein µNS of mammalian reoviruses is present in particle assembly intermediates isolated from infected cells. Morgan and Zweerink (Virology 68:455–466, 1975) showed that a subset of these intermediates, which can synthesize the viral plus strand RNA transcripts in vitro, comprise core-like particles plus large amounts of µNS. Given the possible role of µNS in particle assembly and/or transcription implied by those findings, we tested whether recombinant µNS can bind to cores in vitro. The µNS protein bound to cores, but not to two particle forms, virions and intermediate subvirus particles, that contain additional outer-capsid proteins. Incubating cores with increasing amounts of µNS resulted in particle complexes of progressively decreasing buoyant density, approaching the density of protein alone when very large amounts of µNS were bound. Thus, the µNS-core interaction did not exhibit saturation or a defined stoichiometry. Negative-stain electron microscopy of the µNS-bound cores revealed that the cores were intact and linked together in large complexes by an amorphous density, which we ascribe to µNS. The µNS-core complexes retained the capacity to synthesize the viral plus strand transcripts as well as the capacity to add methylated caps to the 5′ ends of the transcripts. In vitro competition assays showed that mixing µNS with cores greatly reduced the formation of recoated cores by stoichiometric binding of outer-capsid proteins µ1 and µ3. These findings are consistent with the presence of µNS in transcriptase particles as described previously and suggest that, by binding to cores in the infected cell, µNS may block or delay outer-capsid assembly and allow continued transcription by these particles.

The infectious virion of mammalian orthoreoviruses (reoviruses), prototype members of the Reoviridae family, has a genome composed of 10 double-stranded RNA (dsRNA) segments surrounded by two concentric protein capsids. The virion can be proteolytically cleaved in vitro to generate the intermediate subvirus particle (ISVP), which lacks outer-capsid protein µ3 and contains fragments of outer-capsid protein µ1. Further proteolysis removes the µ1 fragments and releases outer-capsid protein µ1, yielding the core particle (reviewed in reference 34). When provided with substrates, the core is transcriptionally active in vitro, using the dsRNA genome segments as templates for synthesis of the 10 full-length plus strand transcripts (3, 24, 39). In addition, these transcripts are modified to have a cap 1 structure (m7NGpppGm2ΨG) at their 5′ ends by viral enzymes within the core (15, 37). The resulting capped transcripts, which are released from the core as they are synthesized (4), are competent for translation into the reovirus proteins (38). Three of these proteins, µNS, σNS, and σ1s, are synthesized in infected cells but are not found in purified virions (36, 49). The functions of these “nonstructural” proteins are not well understood, but all members of the Reoviridae family encode such proteins suggesting that they play important roles during infections by these viruses (13).

µNS (called µ0 in older papers), an 80,000-Mr (80K) nonstructural protein, is encoded by the reovirus M3 genome segment (27, 32). M3 encodes another protein, µNSC, whose Mr is approximately 5,000 smaller than that of µNS and that is recognized by µNS-specific monoclonal antibodies (23). µNS is thought to be generated by translation initiation at a downstream start codon in the open reading frame that encodes µNS, such that µNSC lacks approximately 5,000 Da of sequences that are present at the amino (N) terminus of µNS (28, 44). Both µNS and µNSC are present in cells infected with the prototype isolates of all three reovirus serotypes (23), are expressed to moderate levels throughout infection (17), and are present in the infected cell at a µNS:µNSC ratio of 1:1 to 4:1 (44). Whether the two proteins have functional differences has not yet been addressed. Because the activities of µNSC have not been differentiated from those of µNS, the two proteins are generally referred to as µNS in this report.

Although the roles of µNS in the reovirus life cycle remain poorly understood, previous observations suggest several possibilities. In one study, antibodies to µNS coimmunoprecipitated the viral plus strand RNA transcripts soon after the transcripts were synthesized in infected cells (1). Through this RNA-protein interaction, µNS may be involved in translation of the viral transcripts, packaging of the RNA segments into new reovirus particles, synthesis of the minus strand RNA, or recognition and sorting of the 10 distinct RNA segments prior to packaging (1). In other studies, µNS was isolated from infected cells in association with different types of viral particles (30, 31, 50). These particles were believed to be assembly intermediates because they chased into virions later in infection (50). In one of these studies, newly assembled “transcriptase particles,” capable of synthesizing the viral plus strand transcripts in vitro, were isolated from cells and shown to comprise core-like particles plus large amounts of µNS (30).
The precise function of μNS in these particles was unclear because cores are transcriptionally active in vitro in the absence of μNS (3, 24, 39) and because transcriptionally active particles isolated from cells in other studies contained less or no μNS (31, 40). Nevertheless, the findings of Morgan and Zweerink (30) suggest that μNS may play a role in the regulation of reovirus transcription or particle assembly. Other observations concerning μNS include its association with the cytoskeletal fraction from infected cells (29), possession of predicted α-helical-coil-coil motifs in the carboxyl (C)-terminus of the μNS sequence (28), and common promoter and at transcription by 2.5 μg of NS per ml of lysate as estimated from Coomassie blue-stained gels with bovine serum albumin standards. The μNS protein used in the experiments described in this paper was T3D μNS unless otherwise specified. Production of μNS polycationic antibodies. To direct expression of μNS with a N-terminal histidine tag, the T3D μNS gene was removed from pGEM4Z-M3(T3D) at the BamHI and KpnI sites and ligated to pRSETB (Invitrogen) that had been cut with the same enzymes. The plasmid was transformed into BL2-DH5α (Invitrogen, Madison, Wis.), and the histidine-omega protein was expressed and purified following the protocol in the PET system manual (Novagen). In brief, expression was induced with isopropyl-β-D-thiogalactopyranoside (0.19 mg/ml), and the cells were grown at 37°C for 3 h. Intact cells were pelleted, resuspended, and induced by sonication. The insoluble fraction containing μNS was spun down and solubilized in 8 M urea. The histidine-tagged μNS was purified with His-bind resin (Novagen) in column format. The eluent was dialyzed into phosphate-buffered saline and concentrated with polyethylene glycol. The anti-serum was generated in a rabbit by the polyclonal antibody service in the animal care unit of the University of Wisconsin Medical School (Madison, Wis.).

Growth of reovirus and purification. Infections and purification of reovirus T3D and T3D viruses were performed as described previously (14). ISVPs were prepared by digestion of particles with chymotrypsin as described previously (33). Reovirus cores were prepared by digestion as described for reovirus T3D (25) and T3D (12). Cores were alternatively prepared using an expedited protocol as described previously (9). To obtain particles labeled with 35S-methionine and 35S-lysine, 5 ml of Easy Tag protein expression labeling mixture (Dupont, Wilmington, Del.) was added per 4 × 10^8 cells in spinner culture. All particles were purified using equilibrium centrifugation in CsCl density gradients, followed by dialysis into viroin buffer (VB) (10 mM Tris [pH 7.5], 10 mM MgCl2, 150 mM NaCl, and storage at 4°C at concentrations of 6 to 10 mg/ml). To determine 1.0 A260 = 2.1 × 10^3 viroin/ml (42) and 1.0 A230 = 4.2 × 10^2 cores/ml (10).

SDS-PAGE and immunoblot analysis. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels as described previously (18). Proteins were visualized by staining with Coomassie brilliant blue R-250 (Coomassie blue). Gels with radiolabeled proteins were dried and stored for use in Western blots. For immunoblots, protein samples were subjected to SDS-PAGE and transferred to nitrocellulose (Bio-Rad, Hercules, Calif.) at 4°C for 1 h at 100 V in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3). Binding of the primary antibody was detected with alkaline phosphatase-coupled goat anti-rabbit or goat anti-mouse immunoglobulin (Bio-Rad) and colorimetric reagents p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (Bio-Rad).

Incubation of μNS lysate with reovirus particles and gradient fractionation. The buffer conditions for binding were 10 mM Tris, 100 to 120 mM NaCl, 3 to 6 mM MgCl2, and 0.3 to 0.5% Triton X-100. Fifty microliters of insect cell lysate containing μNS was mixed with 2 × 10^11 particles (cores, ISVPs, or virions) in VB. For the lysate-alone gradients, 50 μl of insect cell lysate containing μNS was mixed with VB. For the particle-alone gradients, 2 × 10^12 particles in VB were mixed with ysis buffer. Samples were incubated for 1 h at 37°C, except for ISVPs, which were incubated at room temperature due to instability at 37°C. The samples were layered onto preformed 3.5-ml CsCl density gradients (1.26 to 1.47 g/ml). Samples (1 ml) were loaded on IN/2 ml of ISVPs and virus samples were spun in a Beckman ultracentrifuge for 2 h at 50,000 rpm at 5°C. The gradients were fractionated with a peristaltic pump into 200-μl fractions. The optical density at 280 nm (OD280) and refractive index of each fraction were determined. The OD280 of each fraction was plotted against its refractive index. For ISVPs, the density of each fraction was determined based on its refractive index.

Identification of proteins bound to reovirus particles. Cores, virions, or ISVPs (10^12) were mixed with 140 μl of insect cell lysate containing μNS as described
above except that all samples were incubated at room temperature. After centrifugation, the particle bands were visualized by light scattering and were harvested by puncturing the bottom of the tube, except for μNS cores, which were harvested with a Pasteur pipette from the top of the gradient. Samples were dialyzed into VB prior to analysis by SDS-PAGE and immunoblotting.

NS-expressing recombinant baculovirus and protein expression. The recombinant baculovirus expressing the TIL NS protein was previously described (18). T3D cells were infected with recombinant baculovirus as described previously (18). Cell lysate was prepared as described above for μNS.

RNase A treatment of μNS cores and μNS lysate. μNS cores (approximately 2 × 10^10) purified on a CsCl gradient and dialyzed into VB were treated with 20 μg of RNase A for 2 h at 37°C and then purified on another CsCl gradient. Untreated μNS cores were analyzed in parallel as a control. μNS lysate (50 μl) was treated with 4 μg of RNase A for 30 min at 25°C and then incubated with 2 × 10^10 cores for 1 h at 25°C. Untreated μNS lysate was also analyzed in parallel as a control. The samples were layered on CsCl gradients and spun to equilibrium. Particles were removed from the gradients with a Pasteur pipette and analyzed by SDS-PAGE.

Gradients with different amounts of μNS. Cores (10^12) in 20 μl of VB were mixed with twofold-increasing amounts of insect cell lysate containing μNS (4.3, 8.5, 17, 34, 68, and 140 μg) and 1% mercaptoethanol with lysate buffer. Cores (10^12) in 20 μl of VB were mixed with 150 μl of lysis buffer or 140 μl of lysate from insect cells infected with wild-type baculovirus and 10 μl of lysis buffer. For the lysate-alone sample, 140 μl of insect cell lysate containing μNS was mixed with 10 μl of lysis buffer. The samples were incubated at room temperature for 1 h and then layered onto preformed 3.5-ml CsCl gradients (1.31 to 1.50 g/cm^3) in an SW60 tube. The samples were spun at 50,000 rpm for 2 h at 5°C and visualized with a high-intensity lamp.

Negative stain electron microscopy (EM). Samples were negatively stained with 1% uranyl acetate and viewed with a Phillips 120 transmission electron microscope at 100 kV as described previously (8).

Sucrose gradient velocity sedimentation of lysate containing μNS. Insect cell lysate containing μNS (150 μl) was layered on a preformed 11-mL sucrose gradient (5 to 20%) in an SW41 tube and spun in a Beckman ultracentrifuge at 37°C, and then 20 μl of μNS lysate was added, followed by incubation for 1 h at 37°C. The amount of μNS lysate was determined by OD_260. Samples from the gradient of μNS lysate were analyzed by immunoblotting.

Transcription and methylation assay. T3D cores were mixed with various amounts of μNS lysate, incubated for 1 h at room temperature, purified on CsCl gradients, and dialyzed into VB. Cores were also incubated with lysate from wild-type baculovirus-infected cells (the same amount of lysate used to make μNS cores with 980 molecules of μNS per core) and purified on CsCl gradients. μNS lysate was treated with 20 μg of RNase to remove any RNA. The samples were incubated at 37°C for 1 h and then layered onto preformed 3.5-mL CsCl gradients (1.31 to 1.50 g/cm^3) in an SW60 tube. The samples were spun at 50,000 rpm for 2 h at 5°C and visualized with a high-intensity lamp.

Images for the figures were scaled uniformly and adjusted for optimal brightness and contrast in Photoshop 4.0 (Adobe System, San Jose, Calif.). All figures were produced in Illustrator 7.0 (Adobe).

RESULTS

Reovirus μNS protein is expressed to high levels in insect cells. To obtain large amounts of μNS for study, we generated recombinant baculovirus M3(T3D)-bac containing the entire coding region of the T3D reovirus M3 genome segment under transcriptional control of the baculovirus polyhedrin promoter. Insect cells infected with this virus produced a prominent protein doublet with a size of approximately 80 kDa in SDS-polyacrylamide gels, similar to those of the μNS and μNSC proteins from reovirus-infected L cells (Fig. 1A). The doublet is found mostly in the cytoplasmic fraction of lysed insect cells (data not shown). Both the 80 kDa doublet from M3(T3D)-bac-infected cells and μNS and μNSC from reovirus-infected cells were recognized in immunoblots by a polyclonal antiserum specific to μNS from infected L cells (Fig. 1B). These proteins were also recognized in immunoblots by a monoclonal antibody specific to μNS expressed in Escherichia coli (Fig. 1B). These proteins were also recognized in immunoblots by a monoclonal antibody specific to μNS (data not shown). The ratio of the two proteins in the doublet varied with each preparation (data not shown). Experiments are in progress to determine whether the lower protein in this doublet has the same origin as μNSC from reovirus-infected cells (44) or may instead represent a breakdown product of μNS. In the subsequent text, we refer to both proteins in the 80 kDa doublet from M3(T3D)-bac-infected cells as μNS. A recombinant baculovirus M3(T3D)-bac containing the T1L M3 gene was also constructed and directed expression of an 80 kDa doublet, recognized by the polyclonal μNS antiserum, to levels as high as those for the T3D protein (data not shown).
Density of cores, but not virions and ISVPs, is decreased after incubation with insect cell lysate containing μNS. Based on the observation from Morgan and Zweerink (30) that transcriptase particles from reovirus-infected cells represent core-like particles plus μNS protein, we tested the capacity of μNS from M3(T3D)-bac-infected insect cells to bind reovirus cores as well as other particle types. In initial experiments, lysate from insect cells expressing μNS (μNS lysate) was mixed with purified T3D cores, T1L ISVPs, or T3D virions. T3D ISVPs were not tested because of their instability at high concentrations. As controls, cores, ISVPs, and virions without insect cell lysate and μNS lysate without cores were analyzed separately. After a period of incubation, a floccular precipitate became visible in the cores-plus-μNS sample, but not in the other samples (data not shown). To separate the reovirus particles and particle-bound proteins from nonbound proteins, the samples were subjected to equilibrium centrifugation in CsCl density gradients. Following centrifugation, virus particles and/or other abundant proteins formed visible bands in the gradients (data not shown). The virions-plus-μNS and ISVPs-plus-μNS gradients contained well-defined bands near the positions of virions and ISVPs observed in the gradients with each of those particles alone (ρ ~ 1.36 and 1.38 g/cm³, respectively). In the cores-plus-μNS gradient, however, the core band was absent from its expected position, and instead a flocculent white band was seen at a higher position (lower density) than that of cores in the gradient with cores alone (ρ ~ 1.43 g/cm³). A second prominent band was observed at the top of the cores-plus-μNS, ISVPs-plus-μNS, and virions-plus-μNS gradients, at the same position as the band in the gradient with μNS lysate alone (ρ ~ 1.30 g/cm³). The loss of the core band and the appearance of a new band at lower density suggested that the RNA-to-protein ratio of the particles had been lowered by the binding of a lysate protein(s) to the cores.

To provide better documentation of the preceding results, the gradients were fractionated and the fractions were analyzed for absorbance at 280 nm and buoyant density. The strong absorbance seen at the top of all gradients was attributable to Triton X-100 in the lysis buffer (data not shown). The gradient profile of μNS lysate alone showed a peak of absorbance near the expected density of protein alone, 1.30 g/cm³, and that of cores alone showed a peak of strong absorbance near the expected density of cores, 1.43 g/cm³ (Fig. 2A). In contrast, the gradient profile of cores plus μNS lacked an absorbance peak at the expected density for cores but exhibited a strong absorbance peak at a lower density, near 1.39 g/cm³ (Fig. 2A), in agreement with the qualitative results described above. Cores were found to migrate near the expected density of 1.43 g/cm³ after incubation with insect cell lysate that lacked μNS, either from uninfected cells or from wild-type baculovirus-infected cells (data not shown). In contrast to what was found for cores, strong absorbance peaks near the densities expected for virions and ISVPs, 1.36 and 1.38 g/cm³, respectively, were noted for these particles in either the presence or absence of μNS lysate (Fig. 2B and C). Taken together, these data indicate that μNS-containing lysate altered the density of reovirus cores, but not those of virions and ISVPs, and that lysate which lacked μNS did not alter the density of cores.

μNS binds cores but not virions or ISVPs. To determine if a specific lysate protein was interacting with cores, experiments similar to those described above were performed with an increased amount of μNS lysate to allow for detection of particle-bound protein by Coomassie blue staining. The prominent bands in the CsCl gradients were collected and analyzed by SDS-PAGE. The sample collected from the gradient of cores plus μNS contained both core proteins and an additional protein of approximately 80K (Fig. 3A, lane 2). This protein was not present in the samples collected from gradients of cores alone or of cores plus wild-type baculovirus lysate (Fig. 3A, lanes 1 and 3). Samples isolated from gradients containing virions and ISVPs, whether previously incubated with μNS lysate or not, contained only proteins present in the respective virus particles (Fig. 3A, lanes 4 through 7). Immunoblot analysis of the gradient-isolated material confirmed that μNS was present as an 80K protein doublet comigrating with cores (Fig. 3B, lane 2). μNS and cores comigrated in gradients after incubation at temperatures from 4 to 37°C and with core concentrations between 1.8 × 10¹² and 2.4 × 10¹³ cores/ml (data not shown). At all ratios of cores to μNS lysate tested to date, both proteins in the μNS doublet bound to cores (data not shown). No detectable μNS comigrated with virions or ISVPs (Fig. 3B, lanes 5 and 6). Similar results were obtained for T3D μNS binding to reovirus T1L particles: the μNS doublet bound to cores but not to virions (data not shown). Thus, the capacity to interact with cores was not specific to the strain or serotype of the virus particles. Moreover, the T1L μNS protein doublet was shown to bind to T1L and T3D cores but not to T1L and T3D virions or T1L ISVPs (data not shown), demonstrating that the selectivity of μNS binding to cores was not specific to the strain or serotype of the virus from which μNS was derived.

To determine whether similar results might be obtained with σNS, the other major nonstructural protein of reovirus, cores and insect cell lysate containing σNS were mixed and then
subjects to equilibrium centrifugation in a CsCl density gradient. The appearance and migration of the core band were unchanged in the presence of \(\sigmaNS\). In addition, no \(\sigmaNS\) was detected in the harvested core band by immunoblot analysis with polyclonal antiserum to \(\sigmaNS\) (data not shown). Thus, \(\sigmaNS\) and \(\muNS\) have different capacities to bind to cores.

Because \(\muNS\) may bind RNA (1) and because cores produce RNA transcripts, we performed additional experiments to address the possibility that RNA may serve as a required intermediate for \(\muNS\) binding to cores. Gradient-purified \(\muNS\) cores were treated with RNase A and then purified in another CsCl gradient. This treatment was not sufficient to disrupt the \(\muNS\)-core interaction (data not shown). \(\muNS\) lysis was also treated with RNase A prior to incubation with cores, and this treatment did not prevent \(\muNS\) cores from forming (data not shown). These findings suggest that \(\muNS\) associates with cores via protein-protein interactions and not through a single-stranded RNA intermediate.

Density of \(\muNS\)-core complexes changes with the amount of \(\muNS\). To determine if \(\muNS\) forms a defined layer on the outside of the core similar to the outer-capsid proteins \(\muL\) and \(\sigma3\) in virions (9), we investigated whether \(\muNS\) binding to cores is saturable. Equal numbers of cores were incubated with increasing volumes of \(\muNS\) lysis. The mixtures were then analyzed in CsCl density gradients to determine their buoyant densities. As controls, cores alone, \(\muNS\) lysis alone, and cores mixed with wild-type baculovirus lysis were also analyzed. Following centrifugation to equilibrium, the abundant proteins were visualized by direct observation. \(\muNS\) cores generated with different volumes of \(\muNS\) lysis were found at different positions in the separate gradients and, therefore, exhibited different buoyant densities. As the volume of added \(\muNS\) lysis was increased in gradients 5 through 7 (Fig. 4), the buoyant densities of \(\muNS\) cores continuously decreased. The complexes formed with smaller amounts of \(\muNS\) lysis in gradients 3 and 4 (Fig. 4), appeared to migrate at a slightly higher density than cores alone in this experiment. This may have been due either to a small increase in the density of the complexes or to slight inconsistencies in the way the different gradients were formed.

FIG. 4. CsCl density gradients of cores incubated with increasing amounts of lysis containing \(\muNS\). Cores (10^12) were incubated alone (gradient 1); with 4.3 (gradient 2), 8.5 (gradient 3), 17 (gradient 4), 34 (gradient 5), 68 (gradient 6), or 140 \(\muL\) (gradient 7) of \(\muNS\) lysis; or with 140 \(\muL\) of wild-type baculovirus lysis (gradient 9). \(\muNS\) lysis (140 \(\muL\)) was incubated alone (gradient 8). The samples were subjected to equilibrium centrifugation in CsCl density gradients and visualized with a high-intensity light. Solid arrowheads, bands of cores; open arrowheads, complexes of \(\muNS\) cores; line, position of bands from the lysis alone.

Negative-stain EM of \(\muNS\) cores reveals cores linked together in large complexes. To investigate the morphology of \(\muNS\) cores, the complexes from the gradients in Fig. 4 were examined by negative-stain EM. Micrographs of \(\muNS\) cores from gradients 5 and 6 revealed intact cores embedded within large complexes (Fig. 5A and B). Cores, the appearance of which is well defined (8, 42), are shown for comparison (Fig. 5B, inset). The cores in the \(\muNS\) core samples were linked together by an amorphous density which we attribute to \(\muNS\). The large complexes and amorphous density surrounding the cores were not present in samples containing either cores alone or cores that had been mixed with wild-type baculovirus-infected cell lysates before gradient isolation (Fig. 5B, inset, and data not shown). \(\muNS\) cores from other gradients exhibited similar morphology (data not shown). Notably, the cores in samples with smaller amounts of \(\muNS\) were also linked together in complexes (data not shown). Since nonphysiological cysteine bond formation between \(\muNS\) molecules was one possible explanation for the observations, in a subsequent experiment 1 mM dithiothreitol was added to the lysis buffer, the core-binding reaction mixture, and the CsCl gradient; however, the presence of this reducing agent did not affect the aggregated nature of \(\muNS\) cores (data not shown). In sum, these data provide evidence that (i) \(\muNS\) bound to cores such that a regularly structured outer capsid of protein was not formed and (ii) the intact particles were linked together.
μNS may have been present in the lysate as large complexes before binding to cores. We could not test this by visualization with EM because the lysate contained too many proteins and other contaminants. Instead, we analyzed μNS lysate by velocity sedimentation in sucrose gradients to estimate the size of μNS complexes present in the lysate prior to incubation with cores. μNS sedimented as a single peak near the 7S marker (Fig. 5C). The 19S marker was pelleted under these sedimentation conditions. The predicted S values for a globular 80-kDa protein are 4.7 for a monomer, 7.4 for a dimer, and 9.7 for a trimer (47). Thus, μNS in the lysate appears to be a monomer or small oligomer that forms large complexes only when incubated with cores.

μNS cores retain transcriptional activity. If the μNS-core complexes formed in vitro are similar to transcriptase particles isolated from reovirus-infected cells (30), the complexes should be transcriptionally active. The transcriptional activities of μNS cores containing different amounts of bound μNS were therefore tested and compared to that of cores. Cores mixed with wild-type baculovirus-infected lysate and then gradient purified were included as a control to address whether cellular factors from the lysate could affect the transcriptional activity of cores. Because the aggregated nature of μNS cores made them difficult to aliquot consistently, the transcriptional activity of each sample was standardized to the number of input particles by use of cores labeled with [35S]methionine and [35S]cysteine. Transcriptional activity was expressed as the ratio of [32P] incorporated into acid-precipitable counts to the [35S] counts in each reaction mixture. Results of a representative experiment are shown in Fig. 6A. μNS cores containing approximately 50 or 1,300 μNS molecules per core were slightly less active at transcription than either the original cores or cores mixed with wild-type baculovirus-infected lysate prior to purification. In four different experiments, the transcriptional activities of μNS cores ranged from 46 to 84% (mean, 66% ± 13%) of that of cores. The transcripts from cores and μNS cores were indistinguishable when separated on denaturing gels (data not shown). To verify that μNS remained bound to cores during transcription, transcription reaction mixtures containing μNS cores were layered on CsCl density gradients, spun to equilibrium, and fractionated. μNS continued to comigrate with μNS on the capping of the viral transcripts, μNS cores and cores were quantitatively compared to that of cores. Cores mixed with wild-type baculovirus-infected lysate and then gradient purified were included as a control to address whether cellular factors from the lysate could affect the transcriptional activity of cores. Because the aggregated nature of μNS cores made them difficult to aliquot consistently, the transcriptional activity of each sample was standardized to the number of input particles by use of cores labeled with [35S]methionine and [35S]cysteine. Transcriptional activity was expressed as the ratio of [32P] incorporated into acid-precipitable counts to the [35S] counts in each reaction mixture. Results of a representative experiment are shown in Fig. 6A. μNS cores containing approximately 50 or 1,300 μNS molecules per core were slightly less active at transcription than either the original cores or cores mixed with wild-type baculovirus-infected lysate prior to purification. 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To verify that μNS remained bound to cores during transcription, transcription reaction mixtures containing μNS cores were layered on CsCl density gradients, spun to equilibrium, and fractionated. μNS continued to comigrate with μNS in this experiment (data not shown), suggesting that it remained bound to cores. In sum, the results indicate that the binding of μNS to cores did not inhibit the core transcriptional activity as assembly of outer-capid proteins is believed to do (2, 11, 21, 46; D. L. Farsetta, K. Chandran, and M. L. Nibert, unpublished data).

μNS cores may have elevated transcript 5'-end capping activities. To evaluate the effects of μNS on the capping of the viral transcripts, μNS cores and cores were quantitatively compared for 5' cap methylation. RNA methylation activity was assayed in a standard transcription assay mixture containing [α-32P]GTP and the methyl donor S-adenosyl-L-[methyl-3H]-methionine ([3H]SAM). In addition to assaying cap methylation, this approach indirectly assesses the RNA triphos-
phatase and guanylyltransferase capping activities of particles since these reactions must precede methylation (15). Methylation activity was expressed as a ratio of acid-precipitable $^3$H counts to acid-precipitable $^{32}$P counts to adjust for any differences in transcription activity between samples. The activities of $\mu$NS cores with approximately 60 or 350 molecules of $\mu$NS bound per core and of cores mixed with wild-type baculovirus-infected lysate and then purified were similar to that of cores (Fig. 6B). However, when more $\mu$NS molecules were bound per core (530 to 3,800 molecules of $\mu$NS), the methylation activity was increased to approximately twofold that of cores (Fig. 6B). To test if the increased methylation activity was due to a contaminating methylase or nonspecific trapping of $^3$H|SAM when such large amounts of $\mu$NS were bound to cores, we repeated the assay using $\mu$NS cores containing either 60 or 980 molecules of $\mu$NS under conditions that are not permissive for full-length transcript production: in the presence of GTP only at 45°C, ATP only at 45°C, or all four nucleotides at 4°C. None of these conditions supported detectable methylase activity with either preparation of $\mu$NS cores or cores alone (data not shown). Thus, we conclude that the increased methylation observed with higher levels of $\mu$NS per core was dependent on transcript production, suggesting that it was due to an increase in transcript capping.

$\mu$NS incubation with cores greatly decreases the efficiency of recoated core formation. To test if $\mu$NS can inhibit the binding of the outer-capsid proteins to cores, we mixed $\mu$NS lysate and lysate containing $\mu$1 and $\sigma$3 ($\mu$1-$\sigma$3 lysate) with cores in vitro. Relative amounts of the two lysates were chosen to provide the same amount of $\mu$NS and $\mu$1 for binding to the core surface. Cores were incubated alone, with $\mu$NS lysate, or with $\mu$1-$\sigma$3 lysate to provide controls for the positions of the resulting particles, cores, $\mu$NS cores, and recoated cores, respectively, in CsCl gradients. Cores were incubated with both $\mu$NS lysate and $\mu$1-$\sigma$3 lysate together, with $\mu$NS lysate first then with $\mu$1-$\sigma$3 lysate, or with $\mu$1-$\sigma$3 lysate first then with $\mu$NS lysate to see if the incubation of $\mu$NS with cores during or before the addition of $\mu$1 and $\sigma$3 would affect the formation of recoated cores. The samples were layered on CsCl density gradients, spun to equilibrium, fractionated, and subjected to immunoblot analysis. Cores alone migrated to the bottom of the gradient (Fig. 7A), whereas recoated cores migrated into the lower half of the gradient (Fig. 7B). Note that some core proteins and $\mu$1 remained trapped at the top of the gradient, migrating with protein alone (Fig. 7B). $\mu$NS cores migrated only into the upper half of the gradient (Fig. 7C), near protein alone at the top of the gradient. When cores were incubated first with $\mu$1-$\sigma$3 lysate, allowing the formation of recoated cores before $\mu$NS lysate was added, both the core proteins and $\mu$1 were detected in the lower half of the gradient at the position of recoated cores (Fig. 7D). In addition, no $\mu$NS was detected in the fractions containing core proteins and $\mu$1 at the position of recoated cores, confirming that recoated cores had been formed. This agrees with the result that $\mu$NS does not bind to virions (Fig. 3). When cores were incubated first with $\mu$NS lysate, allowing the formation of $\mu$NS cores before $\mu$1-$\sigma$3 lysate was added, little or no core proteins or $\mu$1 was detected in the lower half of the gradient (Fig. 7E), suggesting that the formation of recoated cores was greatly reduced by prior formation of $\mu$NS cores. When $\mu$1-$\sigma$3 lysate and $\mu$NS lysate were mixed prior to addition of cores, little or no core proteins or $\mu$1 was detected in the lower half of the gradient (Fig. 7F), suggesting that formation of recoated cores was greatly reduced by simultaneous incubation with $\mu$NS. From these results, we conclude that the incubation of $\mu$NS with cores greatly reduces the capacity of $\mu$1 and $\sigma$3 to bind to cores in a manner conducive to recoated core formation.

**DISCUSSION**

$\mu$NS cores are similar to transcriptase particles. The $\mu$NS-coated cores that we formed in vitro from purified cores and recombinant $\mu$NS protein share a number of characteristics with the transcriptase particles previously isolated from reovirus-infected cells (30). (i) They have similar protein compositions, including $\mu$NS in place of outer-capsid proteins. (ii) They have a complete dsRNA genome (30, 31), in contrast to “replicase” particles isolated from infected cells, which contained little or no $\mu$NS and were in the process of converting single-stranded RNA to dsRNA (30, 31). (iii) They are capable of synthesizing the viral plus strand transcripts, although the transcription activity of $\mu$NS cores was slightly lower than the activity of cores in this study (Fig. 6A). The activity of transcriptase particles was not quantitatively compared to that of cores in the previous study (30). The $\mu$NS cores that we generated in vitro also exhibit mRNA capping activities (Fig. 6B), whereas the capping activities of transcriptase particles were not tested in the previous study (30).

Transcriptase particles isolated from infected cells by another group were inactive for capping of transcripts (40). Gel electrophoresis indicated that these particles did not contain $\mu$NS, but they were extracted with Freon prior to electrophoresis, which may have removed $\mu$NS (40). Additionally, late transcripts isolated from infected cells by these investigators were uncapped (41), leading to a hypothesis that the presence of $\mu$NS may inactivate the capping activities of cores (48). We did not find this to be the case with in vitro-assembled $\mu$NS cores;

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**FIG. 7.** Incubation of cores with $\mu$NS lysate and/or $\mu$1 and $\sigma$3 lysate. Cores were incubated alone or with combinations of lysate containing $\mu$NS or $\mu$1 and $\sigma$3. Samples were layered on CsCl density gradients and spun to equilibrium. Gradients were fractionated, and the fractions were subjected to immunoblot analysis. (A) Cores incubated with lysis buffer. (B) Cores incubated with $\mu$1 and $\sigma$3 lysate. (C) Cores incubated with $\mu$NS lysate. (D) Cores incubated with $\mu$1 and $\sigma$3 lysate followed by addition of $\mu$NS lysate and further incubation. (E) Cores incubated with $\mu$NS lysate followed by addition of $\mu$1 and $\sigma$3 lysate and further incubation. (F) $\mu$1 and $\sigma$3 lysate and $\mu$NS lysate mixed, followed by addition of cores and incubation. Antibodies used in the immunoblots are listed to the left, and proteins are labeled on the right. The top and bottom of the gradients are labeled below. The positions of recoated cores and cores are labeled at the top.
instead, cap methylation was enhanced to twofold that of cores when μNS cores had 530 or more molecules of μNS per core (Fig. 6B). While these data do not directly refute the idea that “late” transcripts are uncapped, they suggest that binding of μNS to cores is not sufficient to result in uncapped transcript production.

The addition of cap 1 structures to reovirus transcripts can approach 100% efficiency in vitro under appropriate conditions: high GTP concentration (0.5 mM), addition of SAM, and inclusion of pyrophosphatase (16). Reaction mixtures that approach 100% efficiency in vitro under appropriate conditions are thus required for outer-capsid assembly. Furthermore, the results from mixing cores with μNS, μ1, and σ3 in vitro suggest that μNS prevents outer-capsid assembly (Fig. 7). μNS might also function during the assembly of cores. However, assembly of core-like particles containing σ2, A1, and A2 has been shown to occur in the absence of σ3 (45; J. Kim, S. Noble, and M. L. Nibert, unpublished data), suggesting that μNS is not strictly required for assembling the protein components of cores, although it might be needed to get RNA inside the particle (see next paragraph). Characterization of μNS-bound particles from reovirus-infected cells may provide further evidence for μNS involvement in assembly.

Yet another possibility is that μNS is closely associated with the transcriptase particles in infected cells in order to bind the plus strand RNA transcripts soon after synthesis, as reported previously (1). Through RNA interactions, μNS may assist either in sorting and packaging the 10 different viral transcripts or during minus strand synthesis in the early steps of progeny particle assembly. It may also serve a function in protein translation from these transcripts. For example, μNS may be similar to the rotavirus nonstructural protein NSP3, which interacts with eIF4GI and is believed to enhance translation of the rotavirus transcripts (35). Experiments investigating μNS-RNA interactions should allow these hypotheses to be tested.

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REFERENCES


