

Encapsidation of Sendai Virus Genome RNAs by Purified NP Protein during In Vitro Replication

SUSAN C. BAKER[†] AND SUE A. MOYER^{‡*}

Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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The ability of the Sendai virus major nucleocapsid protein, NP, to support the in vitro synthesis and encapsidation of viral genome RNA during Sendai virus RNA replication was studied. NP protein was purified from viral nucleocapsids isolated from Sendai virus-infected BHK cells and shown to be a soluble monomer under the reaction conditions used for RNA synthesis. The purified NP protein alone was necessary and sufficient for in vitro genome RNA synthesis and encapsidation from preinitiated intracellular Sendai virus defective interfering particle (DI-H) nucleocapsid templates. The amount of DI-H RNA replication increased linearly with the addition of increasing amounts of NP protein. With purified detergent-disrupted DI-H virions as the template, however, there was no genome RNA synthesis in either the absence or presence of the NP protein. Furthermore, addition of the soluble protein fraction of uninfected cells alone or in the presence of purified NP protein also did not support DI-H genome RNA synthesis from purified DI-H. Another viral component in addition to the NP protein appears to be required for the initiation of encapsidation, since the soluble protein fraction of infected but not uninfected cells did support DI-H genome replication from purified DI-H.

Sendai virus, the prototype virus of the parainfluenza virus family, contains a 15-kilobase negative-strand RNA genome (for a review, see reference 12). The genome RNA is found both in the virion and in the infected cell as an RNase-resistant nucleocapsid by its tight association with the major nucleocapsid protein, NP (60 kilodaltons [kDa]). Two other viral proteins, the P (79 kDa) and L (200 kDa) proteins, are also associated with the nucleocapsid and are thought to function as subunits of the RNA-dependent RNA polymerase. Three additional Sendai virus proteins, the hemagglutinin/neuraminidase (HN, 72 kDa), fusion (F₀, 65 kDa), and matrix (M, 34 kDa) proteins, are associated with the lipid envelope of the virion.

After virion adsorption and penetration by fusion, the reproduction of Sendai virus takes place entirely in the cytoplasm of the infected cell. Both during infection and in detergent-disrupted purified virus the negative-strand nucleocapsid serves as the template for the transcription first of a short leader RNA complementary to the 3' end of the genome RNA (14) and then the sequential synthesis of the viral mRNAs in the order NP, P+C, M, F₀, HN, L (11, 18, 19). In addition to the six structural viral proteins in the infected cell, there is synthesis in the infected cell of two nonstructural proteins of unknown function (13). These proteins, designated C and C' (22 and 21 kDa, respectively), are translated from an alternative open reading frame of the P mRNA (10).

In the infected cell, viral protein synthesis is required for the reproduction of the progeny minus-strand nucleocapsid RNA which occurs via the synthesis of a full-length plus-strand RNA intermediate which is also encapsidated by the NP protein (12). We have developed a cell-free system which

supports the initiation and complete replication and encapsidation of the genome RNAs of both Sendai virus and its defective interfering particle (DI) (6). This in vitro RNA replication system employs isolated intracellular nucleocapsids or purified virus as templates and the soluble protein fraction from Sendai virus-infected cells to supply the proteins required for RNA synthesis and encapsidation. The template used extensively in these experiments was the Sendai virus DI particle DI-H. The DI-H genome RNA (0.5 × 10⁶ Da) is only 11% of that of wild-type (WT) Sendai virus RNA, consists of sequences from the 5' end of the WT RNA, including only a portion of the L cistron, and has copy-back termini, with the 3' terminus complementary to the 5' terminus (17). Like the WT virus, both the plus and minus DI-H RNA-containing nucleocapsids are packaged into virions. The DI-H virus thus provide templates which synthesize two products, a leader RNA and the genome-length RNA, from each RNA strand.

A key question in understanding the RNA replication of negative-strand viruses is the role of the major nucleocapsid protein. It has been proposed that the binding of the nucleocapsid protein to the nascent RNA may act as an attenuator of the mRNA processing steps which occur during transcription, allowing readthrough of the intracistronic regions and, therefore, synthesis and concomitant encapsidation of the progeny genome RNA (1-3). In this study, we have purified the NP protein from WT Sendai virus intracellular nucleocapsids and tested its ability to support Sendai virus DI-H genome replication. We show that the purified NP protein is necessary and sufficient for RNA synthesis and encapsidation from preinitiated templates (that is, for the elongation reaction) but cannot alone initiate Sendai virus RNA replication and encapsidation.

MATERIALS AND METHODS

Cells and virus. Sendai virus (Harris strain) and its defective interfering particle DI-H were propagated in embryonated chicken eggs and purified as described previously (6).

* Corresponding author.

[†] Present address: Department of Microbiology, University of Southern California School of Medicine, Los Angeles, CA 90033.

[‡] Present address: Department of Biochemistry and Molecular Biology, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610.

Subconfluent BHK cells were infected with either Sendai virus alone at a multiplicity of infection of 20 or in a coinfection with Sendai virus plus 10 μ l of DI-H stock, an amount which was empirically determined to give maximum replication of DI-H.

Purification of the NP protein from intracellular Sendai virus nucleocapsids. The Sendai virus NP protein was purified from intracellular nucleocapsids by a modification of the method of Blumberg et al. (3). BHK cells were infected with WT Sendai virus, and a portion were labeled with [3 H] leucine (50 μ Ci/ml, 50 Ci/mmol; ICN) from 10 to 12 h postinfection. At 12 h postinfection, the cells were treated with lysolecithin, and a cytoplasmic cell extract was prepared in buffer containing 0.1 M HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), pH 8.5, 0.15 M NH_4Cl , and 1 mM dithiothreitol (DTT) as previously described (6). We have found that this method of extract preparation gives a higher yield of Sendai virus nucleocapsids (about twofold) than the method with Nonidet P-40 (3). The cytoplasmic extracts were sedimented on 20 to 40% (wt/wt) CsCl gradients in TNE buffer (20 mM Tris hydrochloride [pH 7.4], 100 mM NaCl, 1 mM EDTA) for 2 h at 36,000 rpm at 4°C in the SW41 rotor. The visible nucleocapsid band was collected, diluted threefold in ET buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA), and pelleted onto a 50- μ l pad of 96% (vol/vol) glycerol in buffer containing 0.1 M HEPES, pH 7.4, and 1 mM DTT by centrifugation in an SW65 rotor at 50,000 rpm for 90 min at 4°C. The nucleocapsid pellets were combined, diluted to 1 ml with ET buffer, and sedimented on a single 20 to 40% CsCl gradient in TNE buffer containing 3 M guanidinium chloride in the SW41 rotor for 17 h at 36,000 rpm at 4°C. The dissociated NP protein was located by determination of the trichloroacetic acid (TCA)-precipitable radioactivity of the fractionated gradient. The fractions containing the protein were pooled and dialyzed against buffer containing 0.1 M HEPES, pH 8.5, 1 M NH_4Cl , 7 mM KCl, and 4 mM DTT. Portions were stored at -70°C. The protein concentration was determined as described by Bradford (5), and approximately 0.3 mg of purified NP protein was obtained from 3.6×10^8 Sendai virus-infected BHK cells.

Analysis of the purified NP protein by gradient centrifugation. Purified radiolabeled NP protein (20 to 25 μ g) was adjusted to 1.0 ml at a final concentration of HN buffer (0.1 M HEPES, pH 8.5, 0.15 M NH_4Cl , 7 mM KCl, 4.5 mM magnesium acetate, 1 mM DTT) and analyzed by centrifugation on a 5 to 20% (vol/vol) glycerol gradient in HN buffer in the SW41 rotor for 22 h at 36,000 rpm at 4°C. The position of the NP protein was determined by counting TCA-precipitable material in the fractionated gradient. Bovine serum albumin (350 μ g; Sigma Chemical Co.) or yeast alcohol dehydrogenase (350 μ g; Sigma) in 1 ml of HN buffer was analyzed on parallel gradients, and their sedimentation positions were determined by protein analysis (5).

In vitro Sendai virus DI-H RNA synthesis. Coinfected (Sendai virus plus DI-H) or uninfected BHK cells were treated with lysolecithin, and cytoplasmic extracts were prepared as described previously (6). The extract was further fractionated by centrifugation in the SW55 rotor at 54,000 rpm for 65 min at 4°C in 0.7-ml tubes that contained 100 μ l of 30% (vol/vol) glycerol in HD buffer (10 mM HEPES, pH 7.4, 1 mM DTT) atop a 25- μ l cushion of 96% glycerol in HD buffer. The viral nucleocapsid fraction containing the RNA-NP template with the associated L and P proteins was collected from the top of the 96% glycerol cushion and used as the template for in vitro RNA synthesis

with [3 H]UTP (250 μ Ci/ml, 35 to 40 Ci/mmol; ICN) as described previously (6). The remaining supernatant fluid constituted the soluble protein fraction. Alternatively, purified Sendai DI-H virions (50 μ g/10 μ l) were disrupted with 0.1% Triton X-100 for 10 min at 4°C, and RNA was synthesized in the presence of [3 H]UTP in reaction mix (200 μ l) as described above. The products, as indicated in the text, were either untreated or digested with micrococcal nuclease (10 μ g/ml) in the presence of 1 mM CaCl_2 for 30 min at 30°C to degrade nonencapsidated RNA. The RNA was then isolated after proteinase K digestion by phenol- CCl_3 extraction and ethanol precipitation and analyzed by acid-urea-agarose gel electrophoresis and fluorography as described previously (6).

RESULTS

Purification and analysis of the NP protein. To study the role of the nucleocapsid protein in RNA replication, the NP protein was purified from Sendai virus intracellular nucleocapsids essentially by the method of Blumberg et al. (3) with the modifications described in Materials and Methods. Briefly, intracellular Sendai virus nucleocapsids consisting of the RNA-NP template were isolated on CsCl gradients. The NP protein was then dissociated from the RNA by centrifugation on gradients containing 3 M guanidinium chloride and extensively dialyzed. Analysis by polyacrylamide gel electrophoresis showed that the isolated NP protein was the only protein detected (Fig. 1, lanes B and C), even after prolonged exposure of the gel (not shown).

The NP protein was further analyzed by glycerol gradient centrifugation to determine its native molecular weight. Compared with the protein standards analyzed in parallel gradients, the purified NP protein sedimented predominantly with a molecular weight of 60,000 (Fig. 2), a molecular weight identical to that obtained on denaturing polyacrylamide gels (Fig. 2) (12). Less than 15% of the labeled protein was in an aggregated form pelleting to the bottom of the gradient. The ionic conditions used for the gradient centrifugation were essentially the same as those for in vitro RNA

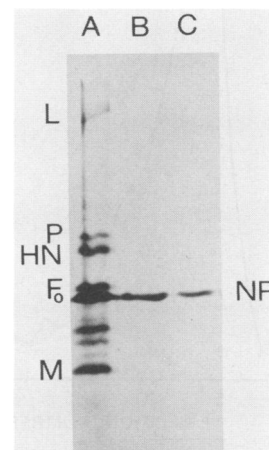


FIG. 1. Polyacrylamide gel analysis of purified Sendai virus NP protein. Sendai virus NP protein was purified from [3 H]leucine-labeled intracellular nucleocapsids as described in Materials and Methods and analyzed by 10% polyacrylamide-sodium dodecyl sulfate gel electrophoresis and fluorography (6). Lanes: Purified [3 H]leucine-labeled Sendai virus marker (A); 1.85 μ g (B) and 0.925 μ g (C) of purified NP protein. The letters designate the marker Sendai virion proteins.

synthesis, suggesting that the purified NP protein remains predominantly as a soluble monomer under the reaction conditions employed in the subsequent experiments.

Effect of NP protein on in vitro DI-H RNA replication. The purified NP protein was then tested for its ability to support in vitro RNA replication. Sendai DI-H nucleocapsids retaining the RNA polymerase were isolated from Sendai virus- and DI-H-coinfected cells at 12 h postinfection and used as the template for reactions in the presence or absence of added protein. The products were treated with micrococcal nuclease to digest any nonencapsidated RNA, and the remaining RNA was isolated and analyzed by agarose-urea gel electrophoresis. As we have shown previously (6), addition of the soluble protein fraction from Sendai virus-infected cells was necessary and sufficient for the synthesis and encapsidation of the two 14S genome RNAs of DI-H (Fig. 3, lane A), since in the absence of added protein little nuclease-resistant RNA was synthesized (Fig. 3, lane B). The addition of increasing amounts of purified NP protein also supported the synthesis and encapsidation of the DI-H genome RNAs (Fig. 3, lanes C to E). Quantitation of the total radioactivity in both the plus and minus 14S RNA products in several similar experiments showed that DI-H RNA replication increased in a linear fashion with the addition of increasing amounts of NP protein (Fig. 4). The DI-H nucleocapsids used here as templates were isolated from infected cells at the time of maximum RNA replication and probably contain nascent replicative RNAs initiated in the cell prior to extract preparation. The RNA replication measured in these experiments, therefore, is primarily the elongation and simultaneous encapsidation of these nascent RNAs, and the data show that purified NP protein alone was necessary and sufficient for this reaction.

We also tested whether the purified NP protein allows the initiation of encapsidation for RNA replication. For this purpose, we used purified detergent-disrupted DI-H virions,

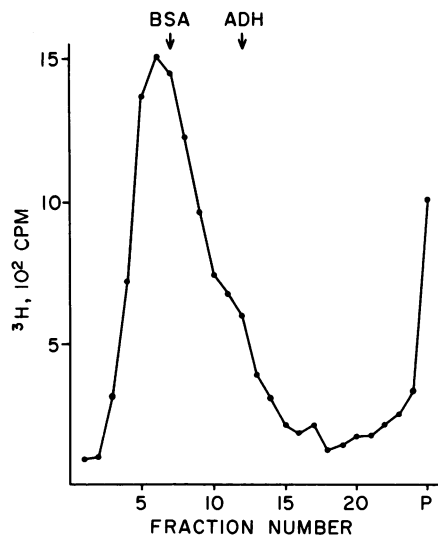


FIG. 2. Glycerol gradient analysis of Sendai virus NP protein. Purified [^3H]leucine-labeled NP protein was analyzed by sedimentation on 5 to 20% glycerol gradients as described in Materials and Methods. The gradient was collected in 0.5-ml fractions, and the TCA-precipitable radioactivity of each fraction was determined. Sedimentation is from left to right. The arrows indicate the sedimentation positions of the bovine serum albumin (BSA, 65 kDa) and alcohol dehydrogenase (ADH, 150 kDa) standards analyzed in parallel gradients.

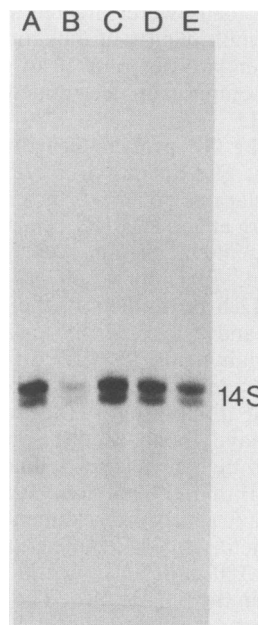


FIG. 3. Agarose gel analysis of the effect of NP protein on DI-H RNA synthesis in vitro. The DI-H nucleocapsid fraction was prepared from Sendai virus- plus DI-H-coinfected BHK cells as described in Materials and Methods. The nucleocapsid samples (from 10^7 infected cells) were incubated in the presence or absence of various protein fractions for 2 h at 30°C in the presence of [^3H]UTP. The products were treated with micrococcal nuclease, and the remaining RNA was isolated and analyzed by acid-urea-agarose gel electrophoresis. Lanes: Nucleocapsid RNA synthesized in the presence (A) or absence (B) of the soluble protein fraction from 10^7 Sendai virus-infected cells, or in the presence of 6.24 μg (C), 4.16 μg (D), or 2.08 μg (E) of purified NP protein. The positions of the two 14S DI-H genome RNA products are indicated.

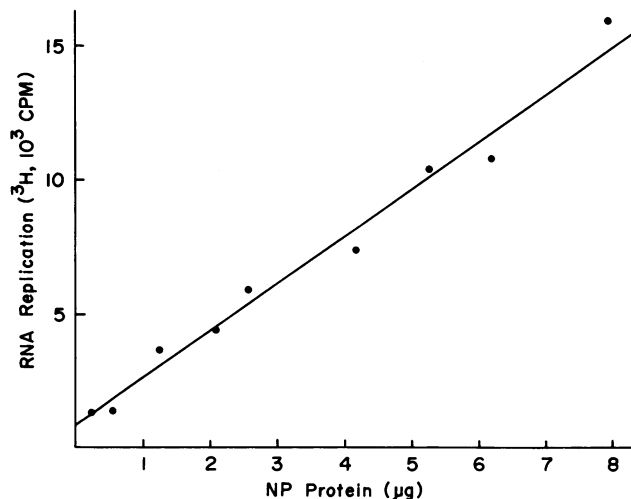


FIG. 4. Effect of NP protein concentration on in vitro DI-H RNA replication. The micrococcal nuclease-resistant RNA products from in vitro reactions with increasing concentrations of purified NP protein alone were analyzed by acid-urea-agarose gel electrophoresis as described in the legend to Fig. 3. The 14S DI-H replication products were identified by fluorography, excised together from the gel, dissolved in 1 ml of 30% H_2O_2 for 17 h at 60°C , and quantitated by liquid scintillation counting in ACS (Amersham). The data are the cumulative results from several different experiments with two different preparations of purified NP protein. The line represents the best fit to the experimental points.

which should have little if any nascent RNA, as the template for *in vitro* RNA replication (6). The purified DI-H particles came from stock grown in eggs at high levels of interference and were not separated from residual WT Sendai virus; however, the amount of WT virus was very low since there was no detectable mRNA synthesis from the Sendai virus in the DI-H preparations. Furthermore, the DI-H stock alone did not support the reproduction of DI-H virions in cell culture (data not shown). As a positive control, addition of the soluble protein fraction from Sendai virus-infected cells to purified DI-H did support the synthesis and encapsidation of the 14S RNA products *in vitro* (Fig. 5, lane A), whereas there was no RNA replication in the absence of added proteins (Fig. 5, lane B), as described previously (6). The addition of purified NP protein to the DI-H particles did not allow RNA replication (Fig. 5, lane C), in contrast to the results obtained when intracellular nucleocapsids were used as templates (Fig. 4). The soluble protein extract from uninfected cells also did not support Sendai virus DI-H genome replication either in the absence (lane D) or presence (lane E) of purified NP protein. These data suggest that in addition to the NP protein, another viral protein, and not a cellular protein, is required for the initiation of encapsidation. This second viral protein, however, is not required for the subsequent elongation events.

DISCUSSION

A major goal of our ongoing studies has been to determine the mechanism of genome replication of negative-strand

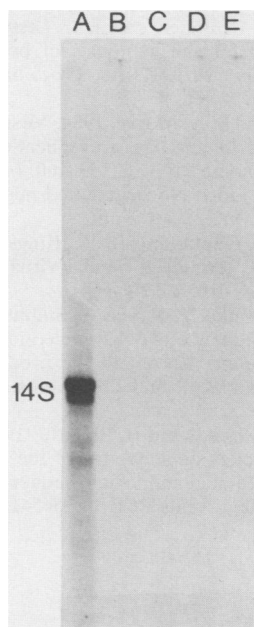


FIG. 5. Agarose gel analysis of *in vitro* RNA replication from purified DI-H virions. Purified Sendai DI-H virions (50 μ g) were disrupted with 0.1% Triton X-100 and incubated in reaction mix (200 μ l) in the presence of [3 H]UTP with or without various protein fractions as described in Materials and Methods. The micrococcal nuclease-resistant RNA was isolated and analyzed by acid-urea-agarose gel electrophoresis. Lanes: Nucleocapsid RNA synthesized in the presence (A) or absence (B) of the soluble protein fraction from 10^7 Sendai virus-infected cells or in the presence of 6.24 μ g of NP protein (C) or the soluble protein fraction from 10^7 uninfected cells alone (D) or with 6.24 μ g of NP protein (E). The positions of the two 14S DI-H genome RNA products are indicated.

RNA viruses. In this discussion, we will compare and contrast the mechanism(s) of RNA replication of the prototypes of the paramyxoviruses (Sendai virus) and the rhabdoviruses (vesicular stomatitis virus [VSV]), similar yet different classes of viruses. Based primarily on studies of VSV RNA replication, a model has been proposed implicating the major nucleocapsid protein of negative-strand viruses as the protein which modulates RNA transcription and replication (for a review, see reference 1). RNA synthesis is initiated at the 3' end of the genome RNA and results in the synthesis of the small leader RNA. In the absence of the viral nucleocapsid protein, the viral mRNAs are subsequently synthesized. However, when it is present, the nucleocapsid protein binds to nascent leader RNA and the encapsidation attenuates the normal transcription signals, allowing full-length genome RNA synthesis and its encapsidation. For the purpose of discussion, therefore, RNA replication can be divided into two stages: (i) the first stage of the initiation of encapsidation in which N protein initially binds to specific sequences in nascent leader RNA, and (ii) an elongation reaction involving the concomitant synthesis and encapsidation, presumably mediated by cooperative binding.

The *in vitro* Sendai virus replication system that we have developed (6) allows separate analysis of the protein requirements for both the initiation and elongation steps of encapsidation during replication. DI-H nucleocapsids (containing the associated RNA polymerase) isolated from infected cells represent a population of molecules in all stages of replication with various lengths of replicative RNA that have already been initiated in the cell. Such templates allow analysis of the elongation step of encapsidation. On the other hand, detergent-disrupted DI-H virions do not contain any nascent product RNAs and can serve as templates to measure the initiation of encapsidation.

We purified the Sendai virus major nucleocapsid protein, NP, from intracellular viral nucleocapsids and tested its ability to support the replication of the genome RNA of the Sendai virus DI particle DI-H. The purified Sendai virus NP protein alone was able to support the synthesis and encapsidation of DI-H genome RNAs from intracellular DI-H nucleocapsid templates (Fig. 3), with the amount of replication dependent on the concentration of NP protein in the reaction (Fig. 4). These data show that the NP protein is the only additional protein required for the elongation reaction of encapsidation. The purified NP protein was shown to be a soluble monomer under the conditions used for the *in vitro* RNA replication reactions. In contrast, the corresponding major nucleocapsid protein (N) of VSV cannot be isolated as a soluble monomer protein, but is found in infected cells as a soluble component only when it is complexed to the VSV NS protein (16), which in addition to this structural role also serves as a subunit of the viral RNA polymerase.

We have shown, however, that the purified NP protein cannot alone initiate encapsidation. Purified detergent-disrupted DI-H virions either alone or in the presence of purified NP protein cannot synthesize the DI-H genome RNAs (Fig. 5). We have recently shown that the addition of either soluble protein from uninfected cells or purified tubulin permits virion-derived nucleocapsids to synthesize DI-H leader RNA but not genome RNA (15; unpublished data). The leader RNA synthesized under these latter conditions is, however, not encapsidated, even when purified NP protein is also added (unpublished data). Another viral protein, in addition to NP protein, is required for the initiation of encapsidation. This is demonstrated by the fact that infected

but not uninfected cell protein supported DI-H genome RNA synthesis from virion-derived nucleocapsids. Our results, therefore, clearly indicate that the protein requirements for the elongation and initiation reactions are different.

There are several possible explanations for the differences in the response observed to added NP protein in the elongation versus initiation steps of encapsidation. First, the NP protein itself, when purified from intracellular nucleocapsids, in contrast to the NP protein present in the soluble protein fraction, may be altered either inherently or by virtue of its isolation with guanidinium chloride, a potent denaturing agent. Alternatively, we favor the hypothesis that the NP protein requires another viral protein for the initial productive binding to nascent leader RNA. By analogy with the VSV N-NS complex, which is required for VSV RNA replication (7, 16), a likely candidate would be the Sendai virus P protein, which is analogous to the VSV NS protein. The P protein may play a role similar to the VSV NS protein in stabilizing or activating the nucleocapsid protein for the initial binding to nascent RNA.

We have used several monoclonal antibodies to the Sendai virus NP and P proteins (8, 9) to test for possible interactions of these two viral proteins in the soluble protein fraction of infected cells. No stable association (i.e., complex) of the NP and P proteins (unpublished observations) was detected under conditions which readily allowed detection of the VSV N-NS complex (16). An NP-P complex could be unstable and thereby escape detection, or the P protein could act as a cofactor necessary for the initiation of encapsidation. Alternatively, the Sendai virus nonstructural proteins C and C', whose functions are to date unknown, may also have a role(s) in the initiation of encapsidation during RNA replication. Further studies with the *in vitro* Sendai virus replication system with nucleocapsid templates supporting different reactions in replication and purified viral components should help to determine the roles of the viral proteins in Sendai virus genome replication.

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