JOURNAL OF VIROLOGY, Nov. 1978, p. 584-589
0022-538X/78/0028-0584$02.00/0

Vol. 28, No. 2
Printed in U.S.A.

Ends of the RNA Within Sendai Virus Defective Interfering Nucleocapsids Are Not Free

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Received for publication 16 June 1978

Sendai virus defective interfering nucleocapsids, isolated from infected cell cytoplasm by equilibrium banding in CsCl gradients, contain only the viral N protein. Neither end of the genomic RNA within these nucleocapsids is accessible to RNase digestion.

The genome of the parinfluenza group of viruses is a single-stranded RNA surrounded mainly by a single protein, the nucleocapsid or N protein, in a helical manner similar to that of tobacco mosaic virus (2, 4, 7). This structure, the viral nucleocapsid, is approximately 1 μm long when viewed in the electron microscope (11, 12) and contains a single-stranded RNA of 5.0×10^6 daltons (15,000 nucleotides) or 5 μm in length (15, 16), complexed with approximately 3,000 N proteins (60,000 daltons), 300 P proteins (70,000 daltons), and 40 L proteins (~200 daltons) (17). The functions of the L and P proteins are not as yet known, but it is thought that these proteins are involved in the viral RNA polymerase or transcriptase activity which is present in purified virions and which has been localized in the nucleocapsid core (19, 21, 23). The structural integrity of the nucleocapsid is probably provided by the interaction of the viral RNA and N protein, since nucleocapsids treated with high concentrations of salt (~2 M) lost most, if not all, of their L and P proteins yet remain intact as judged by their appearance under an electron microscope and their buoyant density in CsCl (19; unpublished data).

The viral minus-strand genome is thought to replicate via full-length complementary plus-strand copy, the antigenome, since completely double-stranded forms of the viral RNA which are 5 μm long can be artificially constructed from nucleocapsid RNA (15). Such copies are found in large amounts in the infected cell and to a lesser extent in mature virion. These viral plus strands or antigenomes, like the minus strands, are found only as nucleocapsids; no genome-length viral RNA is ever found free in the infected cell (5, 13, 22). The template for viral replication is, therefore, thought to be a nucleocapsid, and the product of the replicative event is thought to be a complementary nucleocapsid. Presumably, nucleocapsid assembly takes place on the nascent RNA chain of the replicative intermediate and continues during RNA chain elongation so that no free genome-length RNA is ever produced. In the model of genome replication described above, the viral replicase recognizes a structure at the 3′ end of both the genome and antigenome nucleocapsids and initiates synthesis of the complementary RNA strand at the 3′ end of the nucleocapsid template. The site recognized by the viral replicase to initiate replication is therefore thought to be specified at least in part by the 3′ end of the nucleocapsid templates.

To more clearly understand this recognition event, we have investigated the nature of the ends of the viral nucleocapsids. The experiments described in this communication attempt to distinguish between two basic possibilities: (i) the RNA genome template is completely covered with N protein, and the 3′ end of the nucleocapsid RNA is not free, or (ii) a small number of nucleotides at the 3′ end of the nucleocapsids are not complexed with N protein and remain accessible as free RNA for association with the viral replicase. This latter possibility is suggested by the recent finding that in Uukeneimi virus, another minus-strand virus of the Bunyaviridae group, the three nucleocapsid genomes appear circular when viewed in the electron microscope (20), and their RNAs, when extracted, form circular molecules with short panhandles which appear to be held together by complementary sequences at the ends of the three genome RNAs (10). If the circular Bunyavirus nucleocapsids are held together by the same complementary RNA sequences, this would suggest that the ends of these genome RNAs are not completely covered with protein.

If a small number of nucleotides are exposed at the ends of Sendai virus nucleocapsids, these nucleotides should be available to RNase digestion. However, since wild-type Sendai nucleocapsids contain a single RNA chain of approximately 15,000 N, the loss of a small number of

nucleotides would be difficult to detect. More suitable candidates on which to examine the possibility that viral nucleocapsids contain free RNA tails are Sendai virus defective interfering (DI) nucleocapsids. We have recently isolated a 1,200-nucleotide-long Sendai DI nucleocapsid which, when disaggregated with sodium dodecyl sulfate (SDS), liberates its RNA as single-stranded circles held together by a 110-base pair-long stem due to the base complementarity at the ends of the DI RNA (14, 18). We have therefore treated such nucleocapsids with amounts of pancreatic RNase sufficient to completely degrade free RNA and then examined the remaining nucleocapsid RNA. This communication reports that Sendai DI nucleocapsids so treated contain RNA indistinguishable from untreated nucleocapsid RNA.

MATERIALS AND METHODS

Preparation of Sendai virus DI nucleocapsids. Confluent cultures of LLCMK2 cells were infected with a 1:10 dilution of fluid containing a mixture of both Sendai nondefective and H5 DI particles (14). After 45 min, the infecting medium was recovered and replaced with 3.5 ml per 100 mm of tissue culture plate of minimal Eagle medium, containing 1% fetal bovine serum, 1 μg of actinomycin D per ml, and 20 μCi of [^{3}H]uridine per ml (25 Ci/mmol). The infected cells were harvested at 20 h postinfection, suspended in 0.15 M NaCl, 0.05 M Tris-chloride (pH 7.6), and 0.5% NP-40 at a concentration of approximately 25×10^6 cells per ml, and then rapidly disrupted in a Vortex mixer. Nucleic and cell wall fragments were pelleted by centrifugation (5 min at 3,000 × *g*), and the resulting supernatant was made 5 mM in EDTA. Samples (3 ml each) of this cytoplasmic extract were layered onto CsCl-sucrose gradients (6.6 ml of 20 to 40% [wt/wt] preformed CsCl gradient and 2.3 ml of 5% sucrose containing 0.15 M NaCl, 0.02 M Tris-chloride, pH 7.6, and 1 mM EDTA) which were then centrifuged for 16 h at 30,000 rpm in a Spinco SW40 rotor. The visible nucleocapsid band, which was located at the center of the preformed CsCl gradient, was removed through the side of the centrifuge tube with a syringe in a total volume of approximately 0.3 ml and stored at 4°C. It has been our experience that nucleocapsids stored in 30% CsCl are stable for at least 1 month. For RNase digestion studies, the CsCl was removed by chromatographing the nucleocapsids on Sephadex G-50 in TNE (25 mM Tris-chloride, pH 7.4, 50 mM NaCl, and 1 mM EDTA).

Polyacrylamide gel electrophoresis of RNAs. RNAs were subjected to electrophoresis under nondenaturing conditions on 8% acrylamide–0.4% bisacrylamide slab gels (1.5 by 200 by 400 mm) containing 40 mM Tris-chloride, 20 mM sodium acetate, and 1 mM EDTA (pH 7.8). Ethanol precipitates of RNA were dissolved in 20 μl of one-fifth the concentration of the electrophoresis buffer described above, containing 0.1% SDS; 10 μl of 50% glycerol containing 0.1% bromophenol blue was added to each sample, and the samples were subjected to electrophresis for 16 h at 40 V.

RNAs were subjected to electrophoresis under denaturing conditions on 1% agarose (SeaKem, Marine Collids, Inc.) slabs gels (3 by 200 by 400 mm) containing sodium borate buffer as described by Bailey and Davidson (1). Ethanol precipitates of RNA were dissolved in 20 μl of 5 mM sodium borate (pH 8.0), 0.1% SDS, and 10 mM CH_3HgOH; 10 μl of 50% glycerol containing 0.1% bromophenol blue was added to each sample, and the samples were subjected to electrophoresis for 16 h at 60 V. Before being processed for fluorography, the gel was fixed in 10% acetic acid and 10 mM cysteine to complex the CH_3HgOh.

RESULTS

We have previously shown that viral nucleocapsids isolated from LLCMK2 cells infected with a mixture of wild-type and H5 DI particles are composed almost entirely of a single DI nucleocapsid species (14). This DI nucleocapsid has been shown to contain a 1,200-nucleotide-long RNA which sediments at 14S and represents a unique segment of the viral genome (14, 18). When these DI nucleocapsids are disaggregated with SDS and the RNA is isolated by velocity sedimentation on sucrose gradients, the RNA appears in the electron microscope as a circular molecule held together by a short (0.05 μm) double-stranded stem. This double-stranded RNA stem represents approximately 20% of the mass of the 14S DI genome, contains both the original 5′ (pppA) and 3′ (U-OH) ends of the RNA chain and can be quantitatively isolated from the circular single-stranded RNA molecule by virtue of its resistance to RNase digestion in 0.4 M NaCl (18). These Sendai DI nucleocapsids are therefore ideal candidates on which to examine whether the 3′ end of the RNA is covered with N protein or remains exposed.

Sendai H5 DI nucleocapsids were therefore isolated from infected cell cytoplasm by equilibrium banding in CsCl gradients as described above, and the CsCl was removed by gel filtration on Sephadex G-50. Triplicate samples of the nucleocapsids in TNE containing 20 μg of added rRNA per ml were digested with either no (Fig. 1, panel a) 6 μg (Fig. 1, panel b), or 18 μg (Fig. 1, panel c) of pancreatic RNase per ml, and the RNase was then inactivated with diethylpyrocarbonate (8). The nucleocapsids were then disaggregated with SDS, and the released RNAs were sedimented on sucrose gradients. The results (Fig. 1) show that, although a small amount of nucleocapsid RNA is degraded by the RNase digeston, the bulk of the nucleocapsid-RNA continues to sediment at 14S. As a control, panel d of Fig. 1 shows the effect of 6 μg of

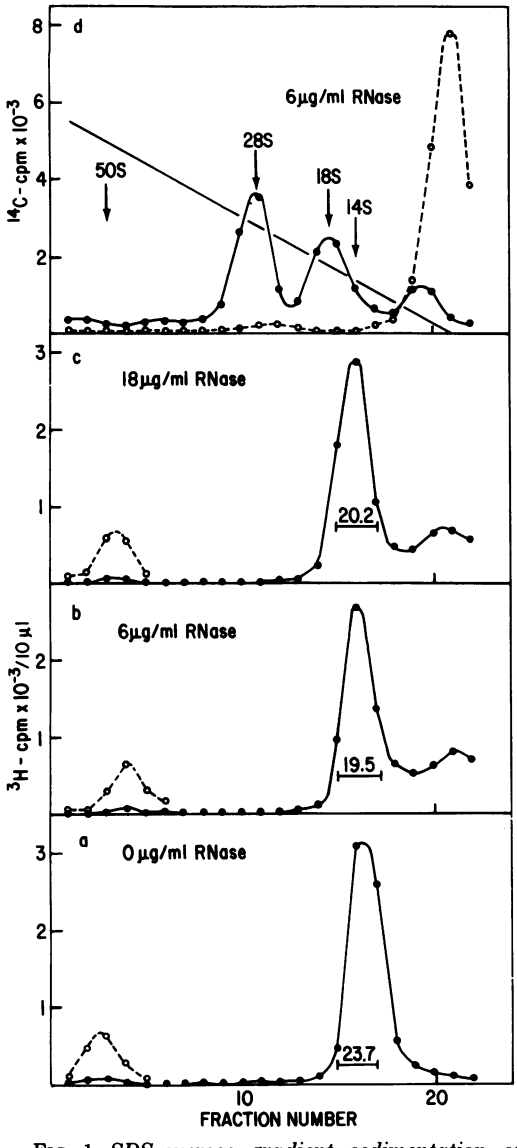

FIG. 1. *SDS-sucrose gradient sedimentation of RNase-treated nucleocapsids. Triplicate 0.2 ml samples of [^{3}H]uridine DI nucleocapsids (see text) in TNE containing 20 μg of added ribosomal RNA per ml were incubated with 0 (a), 6 (b), and 18 (c) μg of pancreatic RNase per ml for 10 min at 25°C. The RNase was inactivated by the addition of 2 μl of diethylpyrocarbonate, and the nucleocapsids were then deproteinized by the addition of 10 μl of 10% SDS. Each sample was then centrifuged on 5 to 23% sucrose gradients (17) for 90 min at 58,000 rpm in the Spinco SW60 rotor, the gradients were fractionated, and 10 μl of each fraction was directly counted by liquid scintillation. The horizontal bars in panels a, b, and c denote those fractions pooled as DI RNAs (cf. Fig. 1, reference 18). The numbers above these horizontal bars represent the percentage of radioactive RNA in the pooled fractions which is resistant to pancreatic RNase per ml on 30 μg of [^{14}C]rRNA per ml under identical conditions. In addition, RNase digestion of the intact nucleocapsids did not appreciably diminish the percentage of the 14S RNA which is further resistant to RNase digestion due to its double-stranded character (see numbers above horizontal bars in Fig. 1). Since this RNase-resistant fraction represents the complementary ends of the RNA chain, these results indicate that very little, if any, of the ends of the DI RNA chain have been removed by RNase digestion of the intact nucleocapsid.*

A more precise way to examine whether any RNA tails have been removed by the RNase digestion is to examine the double-stranded RNA stems isolated from the 14S DI RNA. We have previously shown that H5 DI nucleocapsid RNA yields a 110-base pair-long stem when the single-stranded circular RNA is digested with RNase in a high salt concentration (18). If RNase digestion of intact nucleocapsids has removed a smaller number of nucleotides from either end of the RNA, the length of the double-stranded stem should be reduced accordingly. Three-quarters of each pooled 14S RNA shown in panels a, b, and c of Fig. 1 was therefore treated with RNase in a high salt concentration (see legend to Fig. 2), and the remaining RNA was subjected to electrophoresis as an 8% polyacrylamide slab gel. As shown in Fig. 2, panel a, 14S RNA derived from nucleocapsids which had been digested with RNase yielded a double-stranded RNA stem whose electrophoretic mobility is indistinguishable from the double-stranded RNA stem isolated from 14S RNA derived from untreated nucleocapsids. Since a difference of even 5 base pairs is easily distinguishable on these gels (cf. Fig. 2 of reference 18), these results indicate that less than two nucleotides could have been removed from either end of the RNA chain by RNase digestion of intact nucleocapsids. To demonstrate that the entire RNA chain in nucleocapsids is protected from RNase digestion, 1/10 of each of the pooled 14S RNAs shown in panels a, b, and c of Fig. 1 was recovered by ethanol precipitation, denatured with methyl mercury hydroxide, and subjected to electrophoresis on an agarose slab gel containing methyl mercury hydroxide (1). As

pancreatic RNase digestion (30 μg/ml) in 0.3 M NaCl for 30 min at 25°C. The dashed line over fractions 1 to 5 in panels a, b, and c represents a 10-fold amplification of the radioactivity present in these fractions. Panel d demonstrates the effect of 6 μg of ^{14}C-labeled LLCMK2 cell RNA per ml under identical conditions (open circles); undigested ^{14}C-labeled LLMCMK2 cell RNA is denoted by closed circles.

shown in panel b of Fig. 2, even though less RNA was recovered from RNase-treated nucleocapsids, a single RNA species of identical electrophoretic mobility was detected in each case.

Since the entire RNA chain in our DI nucleocapsids was inaccessible to RNase digestion, it was of interest to determine which viral and/or host proteins remained associated with the DI nucleocapsids throughout our purification procedure. A sample of the nucleocapsid preparation was therefore subjected to electrophoresis on an SDS-polyacrylamide slab gel along with a sample of purified Sendai virus grown in LLCMK2 cells. The gel was stained with Coomassie brilliant blue and then scanned in an Ortec densitometer. Panel b of Fig. 3 demonstrates that only the viral N protein and some of the cellular contaminant actin (protein 7 [17]) remain associated with our DI nucleocapsids. The association of viral N protein with Sendai genome RNA is therefore sufficient to protect this RNA from RNase digestion.

DISCUSSION

The experiments presented in this paper demonstrate that Sendai DI nucleocapsids isolated from infected cell cytoplasm by equilibrium banding in CsCl gradients (i) contain only the viral N protein, and (ii) neither end of the genomic RNA within these nucleocapsids is accessible to RNase digestion.

The possibility that the ends of the RNA in our DI nucleocapsid are not covered with NP protein but are still inaccessible to RNase digestion due to the complementary ends of the RNA having already annealed to form a duplex structure can be discounted for two reasons: (i) when viewed in an electron microscope after negative staining, the vast majority (99%) of the nucleocapsids appear as linear and not circular structures (unpublished data); and (ii) the pancreatic RNase digestion of intact nucleocapsids was carried out under NaCl concentrations (50 mM) in which completely double-stranded MS2 RNA is almost totally degraded (3). Since the viral replicase is thought to recognize the 3′ ends of the genome and antigenome nucleocapsid templates during genome replication, these experiments indicate that the viral replicase recognizes an RNA-N protein complex and not free RNA.

End-to-end aggregation of Sendai nondefective nucleocapsids in virus particles containing multiple nucleocapsids, some reaching 20 times the unit length of 1 μm, was noted as early as 1966 by Hosaka et al. (12). More recently, Thorne and Dermott (24) have found rare forms of what appear to be circular measles DI nucleocapsids. In both of these cases, the presence of

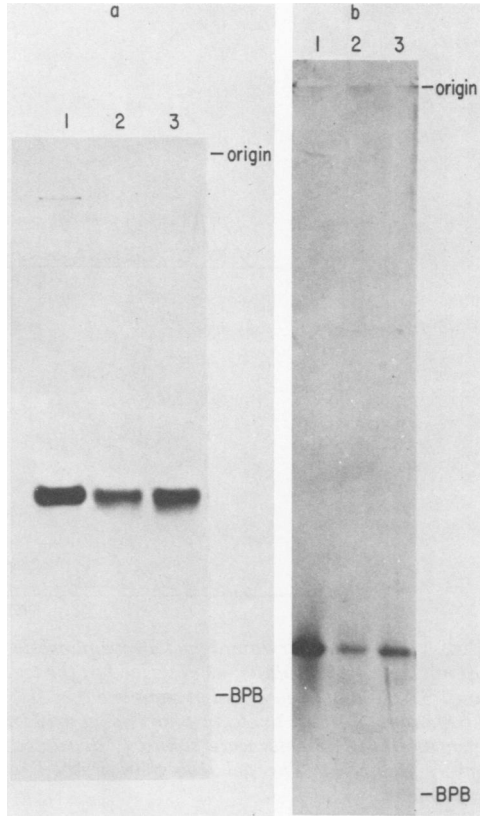

FIG. 2. *(Panel a) Polyacrylamide gel electrophoresis of double-stranded RNA stems from RNase-treated and untreated nucleocapsids. Of each of the pooled DI RNAs denoted by the horizontal bars in panels a, b, and c of Fig. 1, 75% were digested with 20 μg of pancreatic RNase per ml in 0.4 M NaCl followed by Pronase digestion as previously described (18). The remaining RNA was twice precipitated with ethanol and subjected to electrophoresis on a polyacrylamide slab gel as described in the text. The gel was processed for fluorography as described by Bonner and Laskey (6). (Panel b) Methyl mercury hydroxide-agarose gel electrophoresis of RNase-treated and untreated nucleocapsid RNA. Of each of the pooled DI RNAs denoted by the horizontal bars in panels a, b, and c of Fig. 1, 10% were precipitated with ethanol and subjected to electrophoresis on a 1% agarose slab gel containing 10 mM CH_3HgOH as described in the text. The gel was then processed for fluorography as previously described (6), except that methanol rather than dimethyl sulfoxide was used to dehydrate the gel.*

complementarity at the ends of these parainfluenza nucleocapsid RNAs could explain these end-to-end associations if the complementary ends of the RNAs were free to anneal. However, since the results presented in this paper demonstrate that the complementary ends of the RNA in Sendai DI nucleocapsids are not acces-

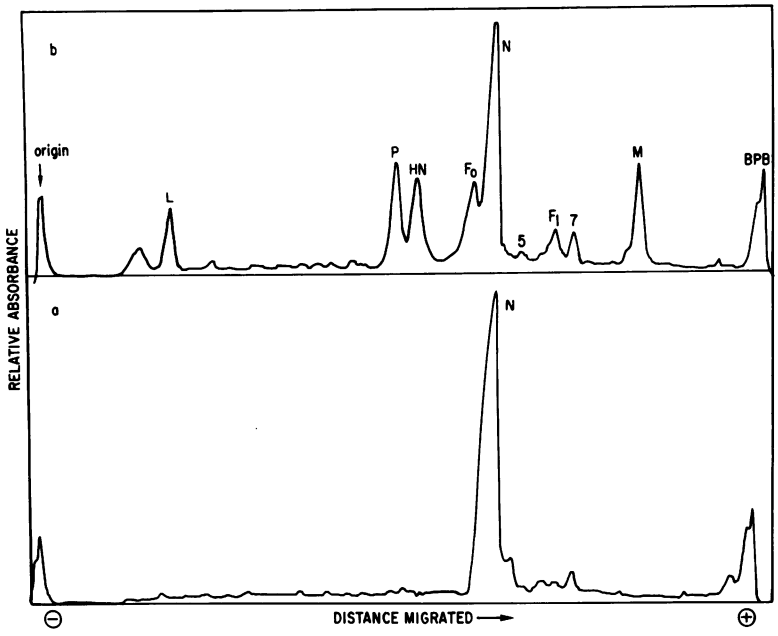

FIG. 3. *SDS-polyacrylamide gel electrophoresis of nucleocapsid and mature virion proteins. Intracellular viral nucleocapsids isolated as described in the text were pelleted by centrifugation (60 min, 50,000 rpm in the Spinco SW50 rotor), disrupted in sample buffer (10% glycine, 5% β-mercaptoethanol, 3% SDS, and 0.06 M Tris, pH 6.8), and subjected to electrophoresis on a 10% polyacrylamide slab gel (panel a). Purified Sendai virions grown in LLCMK2 cells were similarly disrupted and subjected to electrophoresis on the gel as reference markers (panel b). The gel was then stained with Coomassie brilliant blue and scanned in an Ortec densitometer.*

sible to RNase digestion, it seems more likely that the end-to-end aggregation of parainfluenza nucleocapsids noted above is due to the interactions of the nucleocapsid proteins per se. This would not be unusual for proteins which possess the ability to self-assemble into helical nucleocapsids in a manner similar to that of tobacco mosaic virus. End-to-end aggregation of tobacco mosaic virus has also been shown to occur (9).

ACKNOWLEDGMENTS

This work was supported by grant PCM75-21043 from the National Science Foundation and Public Health Service grant AI-12921 from the National Institute of Allergy and Infectious Diseases.

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