In Vitro Replication of Sendai Virus Wild-Type and Defective Interfering Particle Genome RNAs

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A system for studying the in vitro replication of the genome RNAs of Sendai virus and its defective interfering particle DI-H has been developed. Cytoplasmic extracts of baby hamster kidney cells infected with wild-type Sendai virus or coinfected with wild-type Sendai virus plus DI-H were prepared after lysolecithin treatment at 12 h postinfection. The extracts supported the transcription of six viral mRNAs as well as the replication of the Sendai virus 50S (wild-type) and 14S DI-H genome RNAs and their encapsidation into nucleocapsids in the absence of de novo protein synthesis. RNA replication in vitro represented more than 50% of total RNA synthesis, a relative level higher than that found in the infected cell. The proteins required for Sendai virus RNA replication were present in a soluble protein pool at the time of extract preparation. Depletion of the protein pool by prior treatment of infected cells with cycloheximide inhibited subsequent in vitro genome replication without affecting transcription. The cytoplasmic extract may be separated by high-speed centrifugation into two components: the Sendai virus wild-type and DI-H nucleocapsid templates containing the RNA and associated NP, L, and P proteins and the soluble protein fraction containing primarily the P, NP, and M viral proteins with trace amounts of the L, HN, Fo, and nonstructural C proteins. The isolated intracellular DI-H nucleocapsid template alone cannot replicate its RNA, but when recombined with the Sendai virus soluble protein fraction it catalyzes the replication and encapsidation of viral RNAs. The initiation of RNA replication in vitro can be demonstrated because detergent-disrupted purified DI-H virions replicate both positive- and negative-strand RNAs in the presence, but not in the absence, of the soluble protein fraction from an extract of infected cells.

Sendai virus, a member of the parainfluenza family, contains a negative-strand [(−)-strand] RNA genome with a molecular weight of 5×10^6 (9, 10, 13). The Sendai virus genome RNA is always found associated with virus-specific proteins in the form of a helical nucleocapsid both in the virion and in the infected cell. The major nucleocapsid protein, NP, of 60 kilodaltons (60 kDa), is tightly associated with the RNA genome and renders it ribonuclease resistant. Two other viral proteins, the P (79 kDa) and L (200 kDa) proteins, are less tightly associated with the nucleocapsid and are thought to catalyze the enzymatic functions required for the transcription and replication of the Sendai virus RNA (17, 20, 27). There are three additional virion structural proteins, HN (72 kDa), Fo (65 kDa), and M (34 kDa), which are associated with the viral envelope (9, 20).

In infected cells the (−)-strand RNA nucleocapsid serves as the template for the transcription of at least six mRNAs and for the synthesis of a full-length antigenome (+ sense) RNA which is also always found encapsidated in a nucleocapsid (9). The antigenome RNA then serves as a template for the synthesis of the progeny (−)-strand nucleocapsid-associated RNA. Viral protein synthesis is thought to be required for RNA replication, since the addition of cycloheximide to an infected cell inhibits RNA replication, although there is a 2- to 3-h lag in the inhibition which is thought to be due to the utilization of preformed proteins for continued replication (22). During the process of maturation by budding through the plasma membrane of the host cell, Sendai virus nucleocapsids containing RNAs of both (+)- and (−)-strand sense are packaged into virions (14).

To facilitate the study of Sendai virus genome RNA replication, the Sendai virus defective interfering particle DI-H was used extensively for the development and characterization of an in vitro replication system. The DI-H virion contains an RNA genome of 0.5×10^6 Da (14S) which retains only 11% of the wild-type (WT) Sendai virus genome RNA (12). The DI-H genome consists only of the 5' terminus of the parental virus, representing a portion of the L cistron, and contains copyback termini with a 3' terminus complementary to the 5' terminus (21). The DI-H particle is thus defective in transcription and requires the presence of WT Sendai virus as helper to provide the mRNAs and proteins necessary for its reproduction. Defective interfering particles, however, do effectively interfere with the replication but not the transcription of the helper WT Sendai virus in a mixed infection (11). The use of intracellular DI-H nucleocapsids for in vitro RNA replication should therefore provide small templates with 14S RNAs of both the (+)- and (−)-strand sense that are involved specifically in the process of replication.

MATERIALS AND METHODS

Growth and purification of virus. Sendai virus (Harris strain) obtained from R. Lamb (Northwestern University, Evanston, Ill.) was propagated in the allantoic fluid of 9-day embryonated chicken eggs (SPAFAS, Roanoke, Ill.). The eggs were inoculated with 10^4 PFU of Sendai virus per egg and incubated at 33°C for 3 days. The allantoic fluid was harvested and clarified by centrifugation at $1,000 \times g$ for 10 min at 4°C. The titer of Sendai virus in the supernatant fluid was determined by plaque assay on Maden-Darby bovine kidney cells as described by Scheid and Choppin (26). WT Sendai virus at 10 PFU per cell was used to inoculate

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subconfluent monolayers of baby hamster kidney (BHK) cells which were used for all experiments.

The Sendai virus defective interfering particle, DI-H, was obtained from D. Kingsbury (St. Jude Children's Research Hospital, Memphis, Tenn.). Stocks were prepared by inoculating 1 hemagglutination unit per egg as described above. The amount of the DI-H stock to be used in coinfection of BHK cells with Sendai virus was determined empirically. A mixture of WT and defective Sendai viruses was purified from the allantoic fluid by pelleting the virus through 25% (vol/vol) glycerol in TNE buffer (20 mM Tris-hydrochloride [pH 7.4]–100 mM NaCl–1 mM EDTA). The virus pellet was resuspended in ET buffer (1 mM EDTA–10 mM Tris-hydrochloride [pH 7.4]) and purified by banding on gradients of 30% (wt/wt) glycerol–50% (wt/wt) potassium tartrate in ET buffer (14).

Preparation of cell extracts and in vitro RNA synthesis. Subconfluent monolayers of BHK cells (10^7 cells in a 100-mm dish) were infected with Sendai virus at 10 PFU/cell in the absence or presence of the DI-H stock (10 μl per 10^7 cells), an amount which gives maximum DI-H replication and 90% inhibition of WT Sendai virus genome RNA synthesis in vivo. Actinomycin D (2 μg/ml) was added at 1.0 h after infection, and cell extracts were prepared at 12 h postinfection as described by Peluso and Moyer (19). Briefly, washed infected cells were treated with lysolecithin (L-α-lysophosphatidylcholine, palmitoyl; Sigma Chemical Co., St. Louis, Mo.) at 250 μg/ml for 1 min at 4°C. The treated cells were scraped into 300 to 500 μl of a reaction mixture containing 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) adjusted to pH 8.5 with KOH, 0.15 M NH₄Cl, 7 mM KCl, 4.5 mM magnesium acetate, 1 mM dithiothreitol (DTT), 1 mM ATP, 1 mM CTP, 1 mM GTP, 1 mM spermidine, creatine phosphokinase at 40 U/ml, 10 mM creatine phosphate, and actinomycin D at 2 μg/ml. The cells were disrupted by pipetting 15 times with a Pasteur pipette and then centrifuged at 800 × g for 5 min to remove nuclei and cell debris. The resulting cell supernatant fluid was used for in vitro RNA synthesis after the addition of [³H]UTP (100 μCi, 35 to 40 Ci/mmol; ICN) to each reaction in a final volume of 0.5 ml. Samples were usually incubated at 30°C for 2 h or for various periods as described in Fig. 1. Purified DI-H particles were disrupted with 0.1% Triton X-100 for 5 min at 4°C before being added to in vitro reaction mixtures such that the final Triton concentration was <0.006%.

Alternatively, viral nucleocapsids were pelleted from cytoplasmic extracts of infected cells by centrifugation in an SW65 rotor at 50,000 rpm for 90 min at 4°C in 0.7-ml tubes that contained 100 μl of 30% (vol/vol) glycerol in HD buffer (10 mM HEPES [pH 7.4], 1 mM DTT) on a 25-μl cushion of 96% (vol/vol) glycerol in HD buffer as described by Abraham and Banerjee (1). The viral nucleocapsid fraction was collected from the top of the 96% glycerol cushion; the remaining supernatant fluid constituted the Sendai soluble protein fraction, and these two fractions were combined for the reconstitution experiments.

RNA analysis. After the in vitro reaction, for some experiments the products were separated directly by the method of Kolakofsky (12) on 20 to 40% (wt/wt) CsCl gradients at 36,000 rpm for 17 h at 4°C in an SW41 rotor. Under these conditions, the Sendai virus nucleocapsid-associated RNA bands at a density of 1.33 g/ml and the viral mRNA pellets. Alternatively, the products in the reaction mixture were diluted twofold with water, the pH was adjusted to 7.4 with HCl, and then in most cases the products were digested with

micrococcal nuclease (10 μg/ml) in the presence of 1 mM CaCl₂ for 30 min at 30°C, conditions which degrade mRNA but leave nucleocapsid-associated RNA intact. Ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid was then added to 7.8 mM to inhibit the nuclease, and the proteins were digested by treatment with proteinase K (500 μg/ml) for 30 min at 37°C. The RNA was extracted with phenol-CCl₃ and precipitated by the addition of 2.5 volumes of ethanol. The RNA was analyzed by electrophoresis on acid–urea–1.5% agarose gels (23) after denaturation at 90°C for 5 min. The gels were processed for fluorography (3) and exposed to X-ray film at −70°C.

Protein analysis. Cell extracts were prepared as described above at 12 h postinfection from Sendai virus-infected BHK cells labeled with [³H]leucine (100 μCi/ml, 40 to 60 Ci/mmol; ICN) from 10 to 12 h postinfection. The Sendai virus soluble protein fraction was isolated by high-speed centrifugation as described above. To prepare radiolabeled virus marker, Sendai virus-infected cells were labeled with [³H]leucine (30 μCi/ml) at 6 h postinfection. The supernatant fluid was harvested at 48 h postinfection, and the virus was purified by banding on 30% (wt/wt) glycerol–50% (wt/wt) potassium tartrate gradients (14).

Antiserum to the Sendai virus proteins was prepared in rabbits by the method of Roux and Holland (24). Purified virus (2 mg [1.5 ml]) was inactivated by UV light (15 min under a UVS 11 Mineralite lamp at a distance of 30 cm). The virus was emulsified with 1.5 ml of complete Freund adjuvant (GIBCO Laboratories, Grand Island, N.Y.). One-half of the emulsion was injected intramuscularly into a rabbit; the remainder was injected 1 week later. Blood was taken 15 days later, and the serum was isolated and stored at −70°C. Immunoprecipitation of viral proteins was perfomed by the method of Kessler (8). The Sendai virus soluble protein fraction was incubated with the anti-Sendai virus serum for 16 h at 4°C. The antigen-antibody complexes were collected by binding to Staphylococcus aureus (Cowan strain) and eluted by boiling for 2 min in lysis buffer (4% sodium dodecyl sulfate, 3% DTT, 40% glycerol, 62.5 mM Tris [pH 6.8]). The bacteria were removed by centrifugation and the proteins were analyzed by 10% polyacrylamide-sodium dodecyl sulfate gel electrophoresis (15). The gel was processed for fluorography and exposed to X-ray film at −70°C.

RESULTS

Analysis of RNA synthesis in Sendai virus-infected BHK cells. To develop an in vitro RNA replication system from Sendai virus-infected cells, it was first necessary to establish the kinetics of viral RNA synthesis in infected BHK cells. Extracts would then be prepared from these cells at the time optimal for in vivo RNA replication. BHK cells were infected with Sendai virus and labeled with [³H]uridine for 2-h intervals at various times after infection. At the end of each labeling period, cytoplasmic cell extracts were prepared after lysolecithin treatment, and the products were analyzed by CsCl gradient centrifugation as described above. Replication of the Sendai virus genome RNA and transcription were quantitated as the material banding in the nucleocapsid peak and in the mRNA pellet, respectively (Table 1). Nucleocapsid-associated RNA synthesis was maximal between 8 and 14 h after infection and represented over 40% of the total RNA being synthesized. By 14 h postinfection, however, cells were detaching from the dish and mRNA synthesis was considerably reduced. Analysis of the time course of RNA synthesis in a coinfection with WT Sendai virus plus DI-H gave identical kinetics with maximal WT Sendai virus and

TABLE 1. Time course of Sendai virus RNA synthesis in vivo[a]

Time postinfection (h)	Nucleocapsid RNA synthesis		mRNA synthesis	
	cpm	%	cpm	%
2–4	5,799	24.5	17,910	75.5
4–6	35,276	29.1	85,816	70.9
6–8	64,582	40.0	96,918	60.0
8–10	58,644	43.3	76,907	56.7
10–12	63,478	43.1	83,941	56.9
12–14	56,565	66.9	28,008	33.1

[a] The RNA synthesized in WT Sendai virus-infected BHK cells was labeled with [³H]uridine (70 μCi/ml) in the presence of actinomycin D (2 μg/ml) for the times indicated. A cytoplasmic cell extract was prepared and analyzed by CsCl gradient centrifugation as described in the text. RNA synthesis was quantitated by determining the acid-precipitable radioactivity in the gradient fractions containing the nucleocapsid-associated RNA (1.33 g/ml) and in the pelleted mRNA, and each is expressed as a percentage of the sum of the two RNA species at each time point.

DI-H nucleocapsid RNA synthesis occurring from 8 to 14 h postinfection (data not shown).

We have also used similar methodology to test other methods for the preparation of extracts from [³H]uridine-labeled Sendai virus-infected cells at 12 h postinfection. Methods such as Dounce homogenization or the use of nonionic detergents to rupture cells gave significantly reduced yields of all viral RNA products in the extract (data not shown). In addition, we tested the kinetics of in vivo Sendai virus RNA replication in two other cell lines, Maden-Darby bovine kidney and LLCMK2, commonly used for Sendai virus infections. In each case the amount of intracellular Sendai virus nucleocapsid RNA was less than 20% of that observed in infected BHK cells up to 14 h postinfec-

tion (data not shown). Furthermore, extracts prepared from Sendai virus-infected Maden-Darby bovine kidney or LLCMK2 cells synthesized proportionately less Sendai virus nucleocapsid RNA in the in vitro reaction.

A final feature of the infection protocol used here was that actinomycin D (2 μg/ml) was present in the infection medium from 1 to 12 h postinfection. This concentration of actinomycin D effectively inhibits cellular RNA synthesis and actually shortens the latent period of the Sendai virus infection by about 6 h, as was reported previously (22). This treatment thus facilitated the rapid accumulation of product intracellular Sendai virus nucleocapsids and proteins. The actinomycin D did cause severe cell damage by late times after infection (14 to 16 h postinfection), but at 12 h, >90% of the cells remained attached to the dish and were permeabilized by lysolecithin treatment. The cell extracts for all subsequent experiments were therefore prepared from BHK cells infected with WT Sendai virus or coinfected with WT Sendai virus plus DI-H in the presence of actinomycin D at 12 h postinfection after lysolecithin treatment; these conditions gave the optimal yield of intracellular Sendai virus nucleocapsids and proteins.

In vitro synthesis of WT Sendai virus and DI-H RNAs. To characterize the RNA products synthesized in vitro, cytoplasmic extracts of Sendai virus-infected BHK cells were prepared at 12 h postinfection and incubated with [³H]UTP for various periods. The replication and transcription products were quantitated during the in vitro incubation by analysis on CsCl gradients (12). This method of analysis is shown, for example, after a 2-h in vitro incubation (Fig. 1A). The nucleocapsid-associated RNA, the product of the replication reaction, banded at a density of 1.33 g/ml, identical with that of intracellular Sendai virus nucleocapsids (not shown), whereas the mRNAs pelleted to the bottom of the
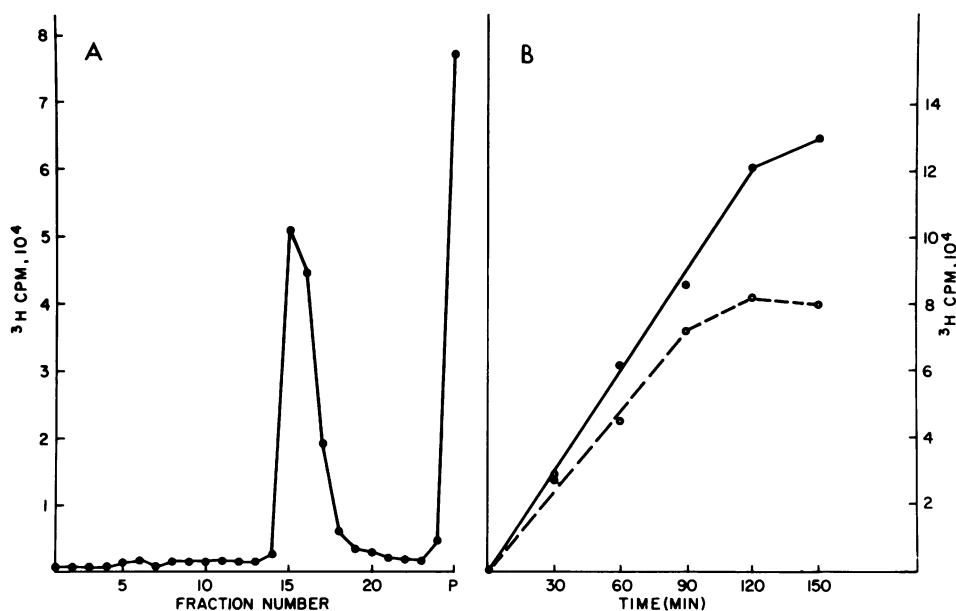

FIG. 1. Analysis of the RNA products synthesized in vitro in extracts of Sendai virus-infected cells. (A) A cytoplasmic extract of 12-h Sendai virus-infected BHK cells was incubated with [³H]UTP (200 μCi/ml) for 2 h at 30°C. The products were directly analyzed by centrifugation on 20 to 40% (wt/wt) CsCl gradients as described in the text. The acid-precipitable radioactivity in each fraction was determined (sedimentation is from left to right). P indicates radioactivity in the mRNA pellet. (B) Equal samples of a cytoplasmic extract of 12-h Sendai virus-infected BHK cells were incubated in vitro with [³H]UTP for various times, and the products were analyzed by centrifugation on CsCl gradients as in (A). Nucleocapsid formation (●) was determined by quantitation of the product banding at a density of 1.33 g/ml, and mRNA synthesis (○) was determined by quantitation of pelleted RNA.

tube. Using this assay for RNA replication, we determined the optimal in vitro reaction conditions by varying the pH and the NH₄Cl, DTT, and magnesium acetate concentrations. Quantitation of the levels of RNA synthesis with time showed that under the optimal reaction conditions described above, nucleocapsid RNA synthesis was linear for approximately 2 h, whereas mRNA synthesis was linear for only 90 min (Fig. 1B). The relative level of nucleocapsid RNA synthesis was actually greater in vitro (50 to 60% of total RNA synthesis) than in vivo (40 to 45% of total RNA synthesis). Similar yields and kinetics for nucleocapsid-associated RNA synthesis were observed in extracts of cells infected with WT Sendai virus plus DI-H (data not shown). Under the reaction conditions used here at pH 8.5 in the absence of added amino acids, there was no detectable protein synthesis in vitro, and even with the addition of amino acids, protein synthesis was negligible (data not shown).

To determine the sizes of the products synthesized in vitro, the RNA was isolated and analyzed by acid-urea-agarose gel electrophoresis and compared with Sendai virus RNAs synthesized in the infected cell (Fig. 2). The six mRNAs and some 50S Sendai virus genome RNA were synthesized in vitro and were identical in size to the Sendai virus RNAs found in infected cells (Fig. 2 IA, IIA). Treatment of the reaction products with micrococcal nuclease degrades the mRNAs; however, the 50S genome RNA product was resistant to nuclease, confirming its encapsidation in vitro, like in vivo, into a nucleocapsid structure (Fig. 2 IB, IIB). Several nuclease-resistant RNAs migrating more slowly than the full-length 50S genome RNA were also synthesized in vitro. Similar RNA products were also seen in vivo, although at much lower levels (Fig. 2 IB).

In similar experiments, the total products synthesized in extracts of cells coinfected with WT Sendai virus plus DI-H at low levels of interference were identical to the RNA products synthesized in vivo (Fig. 2 IC, IIC). In addition to the six mRNAs and the 50S genome RNA, there was the in vitro synthesis of the 14S DI-H genome RNAs. The acid-urea-agarose gel system is not fully denaturing and will separate the (+)- and (−)-sense RNA strands of the DI particle of another (−)-strand RNA virus, vesicular stomatitis virus (7). The two 14S bands observed here probably also represent the (+)- and (−)-sense strands of the DI-H RNAs, although the strand sense of each band has not been identified. The newly replicated DI-H genome RNAs were encapsidated in vitro, like in vivo, since they are nuclease resistant (Fig. 2 ID, IID). Furthermore, after quantitation, these data as well as results of other experiments show that in vitro 14S DI-H RNA synthesis was nearly the same (within 10%) in both the subsequently untreated and micrococcal nuclease-treated samples. This shows that virtually all the 14S RNA synthesized was also encapsidated in these reactions. RNA replication will therefore be defined as RNA synthesis and encapsidation and will subsequently be measured mostly by the quantitation of micrococcal nuclease-resistant RNA products.

Although we did observe some synthesis of the 50S genome RNA in vitro, the amounts were often variable, with many incomplete products. The following experiments therefore involved the use of the intracellular DI-H nucleocapsids as templates for in vitro replication because of the fidelity and magnitude of this reaction. In summary, cell extracts of infected cells prepared as described here faithfully synthesized mRNAs and replicated and encapsidated Sendai virus WT and defective genome RNAs.
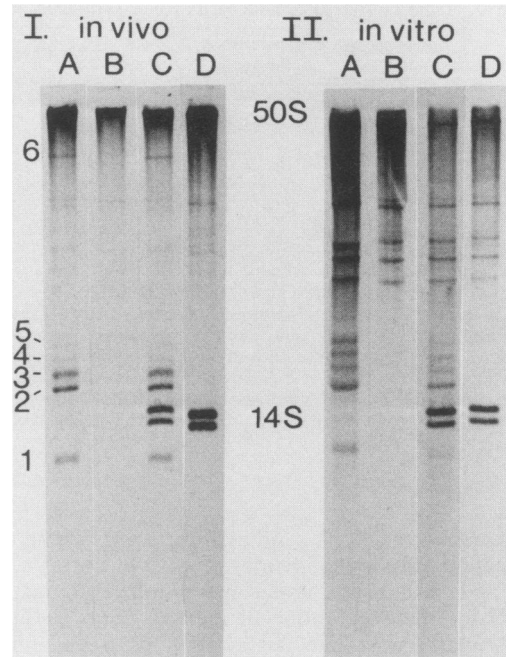

FIG. 2. Agarose gel analysis of viral RNA synthesized in vitro in extracts of cells infected with Sendai virus in the presence or absence of DI-H. (Panel I) BHK cells infected with WT Sendai virus or Sendai virus plus DI-H were labeled with [³H]uridine (50 μCi/ml) from 2 to 12 h postinfection. At 12 h, cytoplasmic cell extracts were prepared and one-half of each was treated with micrococcal nuclease as described in the text. The RNA was then isolated from each sample and analyzed by acid-urea-agarose gel electrophoresis. In vivo RNA products: WT Sendai virus infection untreated (IA) or treated with nuclease (IB); WT Sendai virus plus DI-H coinfection untreated (IC) or treated with nuclease (ID). (Panel II) Cytoplasmic extracts were prepared at 12 h postinfection from BHK cells infected with WT Sendai virus or Sendai virus plus DI-H. The extracts were incubated in the presence of [³H]UTP for 2 h; one-half of each sample was treated with micrococcal nuclease, and the RNA was isolated as described in panel I. In vitro RNA products in extracts of WT Sendai virus untreated (IIA) or treated with nuclease (IIB) or WT Sendai virus plus DI-H untreated (IIC) or treated with nuclease (IID) are shown. Numbers 1 through 6 indicate the positions of the Sendai virus mRNA species, and 50S and 14S indicate the positions of the genome RNAs of WT Sendai virus and the DI-H particle, respectively.

Inhibition of in vitro Sendai virus genome RNA replication by cycloheximide pretreatment. The RNA replication and encapsidation activity in Sendai virus-infected cell extracts appeared to be dependent on a preformed pool of viral proteins, since no de novo protein synthesis occurred under these reaction conditions as discussed above. A previous report on the effect of cycloheximide on in vivo Sendai virus genome replication (22) also suggested that a preformed pool of viral proteins may be involved in the replication and encapsidation processes. To directly test the requirement for preformed proteins in this in vitro replication system, Sendai virus-infected cells were treated with cycloheximide, a potent inhibitor of protein synthesis, for various times before extract preparation. The cytoplasmic extracts were then incubated with [³H]UTP, and the replication (nucleocapsid RNA) and transcription (mRNA) products were quantitated by CsCl gradient centrifugation as described in the legend to Fig. 1. Pretreatment with cycloheximide for 1 to 2 h before extract preparation drastically reduced nucleocapsid RNA

TABLE 2. Effect of cycloheximide pretreatment on in vitro Sendai virus RNA synthesis[a]

Time (h) of cycloheximide pretreatment	Nucleocapsid RNA synthesis		mRNA synthesis	
	cpm	%	cpm	%
0	37,086	100.0	53,475	100.0
1	8,214	22.1	48,753	91.2
2	5,374	14.5	53,795	100.6
3	2,134	5.8	39,200	73.3

[a] RNA was synthesized in vitro for 2 h in the presence of [³H]UTP in cytoplasmic extracts of 12-h Sendai virus-infected BHK cells that were treated with cycloheximide (100 μg/ml) for 1 to 3 h before the preparation of the extract. The RNA products were quantitated after centrifugation on CsCl gradients as described in the text by determining the acid-precipitable radioactivity in the gradient fractions containing the nucleocapsid-associated RNA and in the pelleted mRNA. The data for each RNA species are presented as a percentage of the control sample without cycloheximide treatment (100%).

synthesis by >78% without significantly affecting mRNA synthesis (Table 2). After 3 h of cycloheximide pretreatment, mRNA synthesis was inhibited only by 27%, whereas nucleocapsid synthesis was reduced by 94%. These data showed that a preformed pool of viral proteins which could be depleted by cycloheximide pretreatment was required for the in vitro replication and encapsidation of Sendai virus genome RNAs.

Reconstitution of the in vitro replication reaction. As a first step toward the characterization of the components essential for in vitro Sendai virus RNA replication, extracts of BHK cells infected with WT Sendai virus plus DI-H (at high levels of interference) were fractionated by high-speed centrifugation as described above. The pellet contained the DI-H and a small amount of WT Sendai virus nucleocapsid templates with their associated L and P proteins, and the remaining supernatant fluid was designated the Sendai soluble protein fraction. The in vitro RNA replication activity of the components in various combinations were tested by incubation with [³H]UTP (Fig. 3). There was little or no replication of DI-H nucleocapsids in the absence of added proteins (Fig. 3A), but RNA replication and encapsidation, as demonstrated by the synthesis of the nuclease-resistant 14S DI-H RNAs, was restored by the addition of the Sendai soluble protein fraction (Fig. 3B). These data show that RNA replication and encapsidation may be reconstituted after fractionation of infected cell extracts and was, in fact, dependent on soluble proteins found in the cytoplasm of infected cells.

To examine the components of the Sendai soluble protein fraction, Sendai virus-infected cells were labeled with [³H]leucine. A cytoplasmic cell extract was prepared and fractionated by centrifugation as described above. Analysis by polyacrylamide gel electrophoresis showed that, compared with the virus marker (Fig. 4A), Sendai viral proteins were present in the total soluble protein fraction (Fig. 4B). Immunoprecipitation of the soluble protein fraction with anti-Sendai virus sera showed that the predominate viral proteins in the soluble protein fraction are the NP, P, and M proteins (Fig. 4C). On longer exposure of the gel, the glycoproteins HN and Fo, as well as L protein, were also found in the immunoprecipitated sample but were present in only trace amounts. In other experiments, involving the use of 13% polyacrylamide gels (16), the Sendai virus nonstructural C protein was also found to be present in the soluble protein fraction of infected cells (data not shown). Quantitation of the relative levels of the viral proteins in the

cytoplasmic extract and the soluble protein fraction showed that about 20% of the NP, P, M, and C proteins in the cytoplasmic extract were in a soluble form (data not shown). We are currently investigating which of these soluble viral proteins are essential for the replication and encapsidation of the Sendai virus genome RNAs in the in vitro replication system.

Initiation of DI-H genome RNA replication in vitro. The templates used in the reconstitution reactions described above were the nucleocapsid RNAs pelleted from cells coinfected with WT Sendai virus plus DI-H for 12 h. These intracellular nucleocapsids would probably contain nascent RNA chains already in the process of replication, and so this reaction might represent only the in vitro elongation of the preinitiated RNAs. To directly test whether the initiation of genome replication could also occur in vitro, detergent-disrupted purified DI-H virions which should not contain initiated RNA chains were used as templates in the in vitro replication reaction. The virus preparation used here was in fact a mixture of WT Sendai and DI-H virions purified from the defective stock which is greatly enriched for DI-H particles. Both the (+)- and (−)-strand DI-H RNAs are
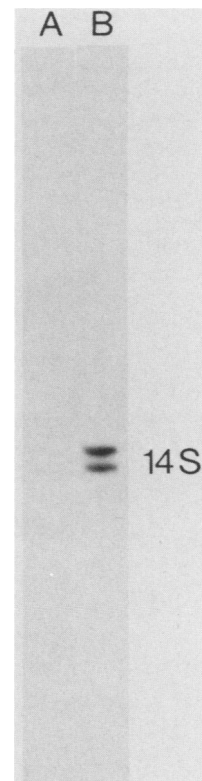

FIG. 3. Agarose gel analysis of DI-H nucleocapsid RNA synthesized in vitro in reconstitution experiments. At 12 h postinfection, cytoplasmic extracts were prepared from BHK cells coinfected with WT Sendai virus plus DI-H. Extracts were separated by centrifugation into the nucleocapsid pellet and soluble protein fractions as described in the text. Selected fractions were recombined and incubated in the presence of [³H]UTP. The samples were then treated with micrococcal nuclease, and the remaining RNA was isolated and analyzed by acid-urea-agarose gel electrophoresis. Nucleocapsid RNA synthesized in vitro from the nucleocapsid template alone (A) or in the presence of the Sendai soluble protein fraction (B) from 10⁷ infected cells is shown. The positions of the 14S DI-H genome RNAs are indicated.

packaged in virions (14). The detergent-disrupted DI-H virions gave no RNA replication and encapsidation in the absence of added proteins (Fig. 5A), as expected; however, the addition of the Sendai soluble protein fraction did support the initiation and complete synthesis of nucleocapsid-associated (+)- and (−)-strand DI-H RNAs (Fig. 5B). Thus, the proteins required for the initiation of Sendai virus genome replication from both RNA strands were present in the Sendai soluble protein fraction.

DISCUSSION

We describe here an efficient cell-free system for the in vitro replication and encapsidation of the genome RNAs of Sendai virus, a prototype of the parainfluenza group, and its defective interfering particle DI-H. In developing this system, we took advantage of the methodology described previously (19) for an in vitro RNA replication system for vesicular stomatitis virus (VSV), a (−)-strand RNA virus of the rhabdovirus group. A key feature for successful in vitro VSV genome RNA synthesis was the treatment of infected cells with lysolecithin before extract preparation, followed by simply pipetting the cells and removing nuclei by low-
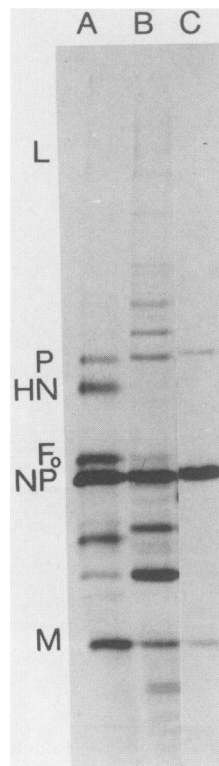

FIG. 5. Agarose gel analysis of DI-H RNA synthesized in vitro from purified DI-H virions. A mixture of purified WT Sendai and DI-H virions (65 μg per reaction) were disrupted with Triton X-100 and added to the soluble protein fraction prepared from 10^7 WT Sendai virus-infected BHK cells. The samples were incubated in the presence of [^{3}H]UTP for 2 h and then treated with micrococcal nuclease, and the remaining nucleocapsid RNA was isolated and analyzed by acid-urea-agarose gel electrophoresis. Nucleocapsid RNA synthesized in vitro from DI-H virions alone (A) or in the presence of the Sendai soluble protein fraction (B) is shown. The positions of the 14S DI-H genome RNAs are indicated.

FIG. 4. Polyacrylamide gel electrophoresis of the Sendai soluble protein fraction. WT Sendai virus-infected BHK cells were labeled with [^{3}H] leucine (50 μCi/ml) from 2 to 12 h postinfection in the presence of actinomycin D (2 μg/ml). The Sendai soluble protein fraction was prepared from the extract by high-speed centrifugation and analyzed by polyacrylamide-sodium dodecyl sulfate gel electrophoresis as described in the text. [^{3}H]leucine-labeled Sendai virus (A), total proteins in the Sendai soluble protein fraction (B), and proteins immunoprecipitated by rabbit antisera to Sendai virions from the Sendai soluble protein fraction (C) are shown. The letters to the left identify the Sendai virus proteins.
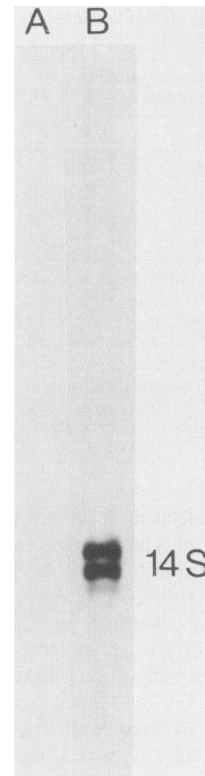
speed centrifugation. This methodology developed for VSV also proved the best for studying Sendai virus RNA replication. Both systems utilize cytoplasmic extracts prepared from lysolecithin-permeabilized virus-infected BHK cells to catalyze the initiation and replication of genome RNAs. Further characterization of the individual components required for the in vitro replication of these two (−)-strand RNA viruses may then allow us to compare and contrast the mechanism(s) of RNA replication for the rhabdo- and parainfluenza virus groups.

Extracts prepared from Sendai virus-infected cells at 12 h postinfection, which is the time of maximal in vivo RNA replication, catalyze the replication and encapsidation of Sendai virus genome RNA, as well as the synthesis of the viral mRNAs in the absence of de novo protein synthesis (Fig. 1B and 2). Six viral mRNAs have been tentatively identified by their ribonuclease sensitivity and labeled 1 through 6 by increasing size. Based on the sizes of the proteins and RNAs, these mRNAs probably code for the M (34 kDa), NP (60 kDa), Fo (65 kDa), HN (74 kDa), P + C (79 kDa + 22 kDa), and L (200 kDa) proteins, respectively. Previous studies report only five size classes of Sendai virus mRNAs, with two of the mRNAs unresolved (2). In vitro translation of individual mRNAs has not yet been done to confirm the polypeptide assignments.

The total amount of RNA synthesis in extracts of Sendai virus-infected cells was two- to threefold greater than that for an equivalent extract of VSV-infected cells. This could reflect either a greater intrinsic replication activity of Sendai virus or perhaps a difference in the amount of intracellular nucleocapsid templates in the two systems. This result was somewhat surprising, since it had been previously shown that in vitro transcription from purified parainfluenza viruses was much poorer than that from purified VSV (6). Sendai virus genome replication, moreover, occurred at a higher relative level in vitro (50 to 60% of total RNA synthesis) than in the infected cell (40 to 50% of total RNA synthesis). This may be a reflection either of the preferential synthesis of the RNA replication product in vitro or of the greater stability of the nucleocapsid RNA versus mRNA in the reaction.

The 50S WT Sendai virus RNA synthesized and encapsidated in the in vitro replication reaction appeared as a ladder of partially completed, micrococcal nuclease-resistant RNAs up to the size of genome RNA (Fig. 2 IIB). The majority of the RNA replication products from WT Sendai virus nucleocapsids were in fact smaller than 50S. Similar results were also observed in a different Sendai virus in vitro replication system (20). It appears that these incomplete, yet distinct, RNAs may represent specific pause sites in the replication process in vitro, and this was also observed to a lesser extent in vivo. In contrast, only full-length, nuclease-resistant RNAs were synthesized in vitro, with intracellular DI-H nucleocapsids as templates (Fig. 2 IID). Perhaps this difference compared with that in the WT Sendai virus replication reaction is due to the small size of the DI-H RNA or to the nature of the sequences contained in the DI-H genome RNA.

An important feature of this Sendai virus in vitro RNA replication system is that the essential components in the cytoplasmic extract can be separated and then recombined and will still retain activity. The cytoplasmic extract could be fractionated by centrifugation into two components: the nucleocapsid template and the soluble protein fractions, both of which were required for RNA replication and encapsidation (Fig. 3). This successful reconstitution experiment showed that in vitro Sendai virus RNA replication did not require ongoing protein synthesis but instead relied on the use of soluble proteins in the cytoplasm of the infected cells, as had been also found for in vitro VSV RNA replication (19, 25). Robinson (22) first showed that significant Sendai virus genome replication could occur in infected cells utilizing a pool of virus proteins, in which de novo protein synthesis was inhibited by cycloheximide. Similarly, we showed that infected cells were depleted of viral proteins by the addition of cycloheximide for 2 to 3 h, and an extract prepared from these cells gave a significant reduction of in vitro RNA replication (>85%) without affecting viral transcription (Table 2).

The Sendai soluble protein fraction contains primarily the P, NP, and M proteins, with trace amounts of HN, Fo, L, and C proteins as well (Fig. 4). This protein fraction not only was required for genome RNA synthesis from intracellular nucleocapsid templates, a reaction which probably involves primarily the elongation of RNA chains initiated in the cell, but also was necessary and sufficient for the initiation of RNA synthesis from detergent-disrupted DI-H virion RNAs (Fig. 5).

The replication reconstitution assay we describe here may now be used to identify the specific viral and possibly host proteins required for Sendai virus RNA replication. In contrast to the system previously reported for Sendai virus

genome replication (20), this in vitro replication system demonstrates the initiation, elongation, and encapsidation of replicating RNA by the soluble protein fraction of Sendai virus-infected cells. The purification of the viral proteins from the soluble protein fraction may allow the elucidation of the minimal protein requirements for Sendai virus RNA replication. By comparison with the data obtained from in vitro VSV replication systems (4, 18, 19), it will be very interesting to determine whether there are similar or divergent mechanisms for RNA transcription and replication for these different groups of (−)-strand RNA viruses.

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