Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5537–5541, July 1991
Microbiology

Rescue of a foreign gene by Sendai virus

(paramyxovirus/negative-sense genome/replication/packaging)

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Communicated by Robert M. Chanock, March 29, 1991

ABSTRACT **A simple protocol for the rescue of a synthetic genome into a paramyxovirus has been developed. First, a synthetic Sendai virus-like RNA, containing the antisense coding region of the chloramphenicol acetyltransferase gene replacing the coding region of the Sendai virus genome, was transcribed from a cDNA. When introduced into cells that are infected with Sendai virus, this RNA construct was transcribed, replicated, and packaged into infectious virions. The addition of infected cell extract to the RNA prior to transfection markedly enhanced levels of chloramphenicol acetyltransferase expression and rescue. However, this enhancement is not due to encapsidation of the RNA into nucleocapsids as the RNA remains nuclease-sensitive. Uninfected cell extract also enhances expression and rescue efficiency, implying involvement of a cellular factor(s) with the synthetic viral-like RNA construct that allows for enhanced polymerase recognition. This system should allow for the dissection of the various cis-acting RNA signals within the paramyxovirus genome.**

Sendai virus is a prototype paramyxovirus containing a nonsegmented negative-sense RNA genome of 15.3 kilobases. During transcription, the genome directs the synthesis of a leader RNA and six transcription units (NP, P/C, M, F, HN, and L) (1). After active viral protein synthesis, replication ensues, resulting in the synthesis of full-length positive-strand complementary RNA (cRNA), which is used to create new genomes. The mRNAs are capped and polyadenylylated by the viral polymerase, whereas the leader RNA and the cRNA are unmodified.

In the viral genome, various cis-acting signals have been identified or proposed to function during mRNA synthesis. These include the transcriptase entry signal at or near the 3′ end, transcription termination and restart sequences at the intergenic junctions (2), and the site in the P/C gene at which RNA editing by guanine insertion occurs (3). In contrast, sequences required for replication, encapsidation, and packaging of the genome have not been clearly defined, although they probably reside within the terminal sequences. In this regard, efforts to study such signal(s) have been hampered by the large size of the viral genome and the difficulty in performing reverse genetics, due to the negative-sense polarity of the genome.

Only recently have methods been developed that enable molecular studies of negative-strand RNA viruses *in vivo*. For influenza viruses, Luytjes *et al.* (4) have described a system whereby purified nucleocapsid proteins and synthetic RNA (5) are used to assemble a synthetic gene-like RNA into a biologically active ribonucleoprotein (RNP). Subsequently, this system allowed introduction of mutations into the genome of influenza virus (6) and led to the determination of the minimal subset of viral proteins required for genome replication (7). In the rhabdovirus system, defective interfering (DI) particles of vesicular stomatitis virus (VSV) have been replicated (8) and assembled (9) through the use of viral proteins expressed from recombinant sources. Recombinant infectious virus systems have also been developed for double-stranded RNA viruses such as bacteriophage $\phi 6$ (10) and reovirus (11). Finally, for paramyxoviruses, infectious measles virus has been generated from cloned measles virus cDNA by microinjecting "committed transcription complexes" into helper cells (12). Thus these studies provide examples of alternative and rewarding approaches to manipulate and understand negative-sense RNA virus genomes and their gene products.

In the present study, we describe a simple system similar to that which was developed for influenza virus that allows for efficient expression, replication, and packaging of a foreign gene by Sendai virus. In contrast to the studies described above, we find that expression and rescue of synthetic RNA can be achieved by transfection of naked virus-like RNA followed by helper virus infection. Therefore, no encapsidation or modification of the synthetic RNA is needed prior to transfection. However, the addition of host cell factor(s) can markedly enhance the levels of expression and rescue of the gene. This system should allow the examination of the various cis-acting RNA signals and identification of possible host cell auxiliary proteins and their role during viral gene expression and replication.

MATERIALS AND METHODS

Virus and Cells. Sendai virus (Sendai/52) was grown in embryonated chicken eggs as described (5). Madin–Darby bovine kidney (MDBK) cells were used for transfection experiments and plaque assays. Cytoplasmic extracts were prepared from baby hamster kidney (BHK) cells.

Construction of pSend–CAT. Plasmid pSend–CAT was constructed through a series of three polymerase chain reactions (PCRs) using a total of five primers. Each primer was 60–70 bases long. Primers 1 and 2 contained a chloramphenicol acetyltransferase (CAT) gene sequence fused with a partial sequence of Sendai virus 5′ or 3′ termini. The original PCR was accomplished using primers 1 and 2 on plasmid pCM7 (Pharmacia). This PCR product was then used as template for another PCR using primers 3 and 4. Primer 3 overlaps with the 5′ end of primer 1 and also contained the sequence of the remaining 3′ terminus of the Sendai genome bounded by the engineered *Ear* I site and a *Sac* I restriction site useful for cloning (Fig. 1). Primer 4 overlaps with the 5′ end of primer 1 and contains additional sequence from the 5′ end of Sendai virus but does not complete the 5′ terminal Sendai virus sequence. This PCR product was then used as template for a reaction with primers 3 and 5. Primer 5 overlaps with the 5′ end of primer 4, completing the 5′ terminus of the Sendai virus sequence and adding the T7

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Abbreviations: CAT, chloramphenicol acetyltransferase; cRNA, complementary RNA; RNP, ribonucleoprotein; DI, defective interfering; VSV, vesicular stomatitis virus; MN, micrococcal nuclease; ICE, infected-cell extract; UCE, uninfected-cell extract.

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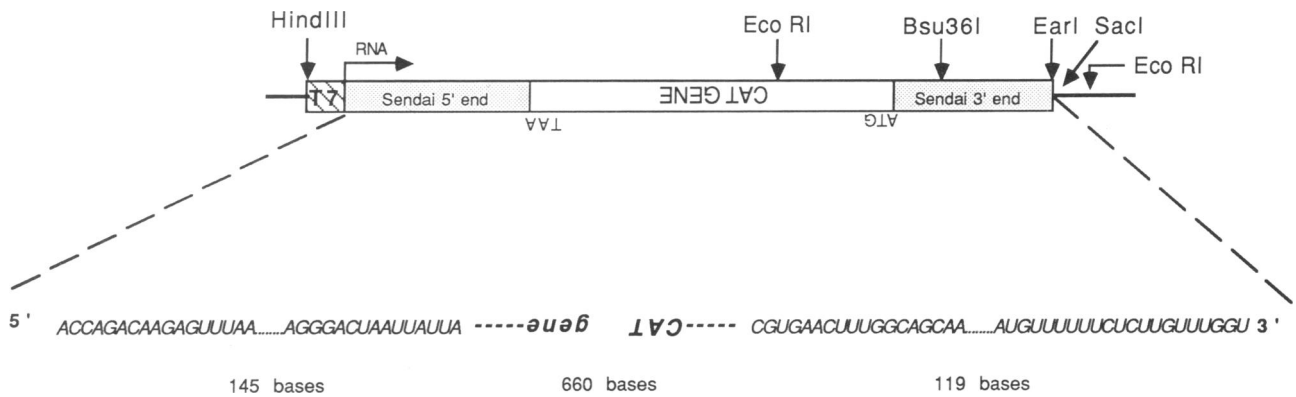

FIG. 1. Schematic representation of the relevant region of plasmid pSend–CAT. The plasmid contains the CAT gene in an antisense orientation (shown upside down and backward) flanked by the 5′ and 3′ terminal sequences of the Sendai virus genome. The Send–CAT RNA, including the partial sequence of the Sendai virus termini and the viral sequences immediately adjacent to the CAT gene, is shown below the clone. Note that *Bsu*36I cuts within the 3′ end of Sendai sequence so that the resulting transcript has an imperfect 3′ terminal sequence but leaves intact the antisense coding region of CAT gene and the complete 5′ terminal sequence.

promoter sequence and a *Hin*dIII site for cloning (Fig. 1). The final PCR product was cloned into the *Hin*dIII/*Sac* I window of pUC19.

Preparation of Cytoplasmic Extracts. Cytoplasmic extracts were prepared essentially as described (13), except that leupeptin (0.3 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and antipain (1 μg/ml) were included in the extraction buffer. For infected cell extracts, subconfluent monolayers of BHK cells in 150-mm dishes were infected with Sendai virus at a multiplicity of infection of ≈10 and incubated for 16 hr at 33°C. Extracts were prepared at a final concentration of 10^8 cells per ml with protein concentrations in the range of 1–2 mg/ml. RNasin (10 units/ml) was added to the final supernatant.

Preparation of RNA and Transfections. Run-off transcription of *Ear* I-digested pSend–CAT (Fig. 1) was performed using standard T7 polymerase reaction procedures (14). The RNA was purified and quantitated as described (5). For synthesis of RNA probes, 100 μCi of [α-^{32}P]CTP was added in the presence of 4 mM ATP, 4 mM UTP, and 4 mM GTP. The full-length product was gel-purified (14). The purified transcript (0.5–1 μg) was used for transfection with or without cytoplasmic extract as described (4).

Micrococcal Nuclease (MN) Digestion. RNA samples were digested with 10 units of MN per 1 μg of RNA in 10 mM Tris·HCl, pH 8.1/1 mM $CaCl_2$ at 37°C for 1 hr. For encapsidation analysis, duplicate reactions were carried out with labeled RNA as described (13) prior to digestion with MN. Resulting RNA samples were either used directly for transfection or extracted with phenol/chloroform, 1:1 (vol/vol), and analyzed on a 1% agarose gel, which was dried and autoradiographed.

RNase Protection and CAT Assays. For RNase protection, cytoplasmic extract was prepared from a 100-mm dish containing 2×10^7 MDBK cells that had been transfected with an appropriate mixture containing 20 μg of Send–CAT transcript as described above. The extract was treated with ≈1000 units of MN to digest mRNA and the resulting nuclease-resistant RNA species were isolated by multiple phenol/chloroform extractions, followed by ethanol precipitation. Hybridization and nuclease treatment with RNase T1 were carried out as described (14). CAT assays were performed 16–18 hr after infection as detailed (15).

Passaging of Virus After Transfection. After MDBK cells were transfected and infected as described, serum-free medium containing 0.2% bovine albumin and trypsin (5 μg/ml) was added. After 24 hr, medium was collected and cleared of cells, and 100 μl of this supernatant was used to infect fresh MDBK cells. For antibody inhibition studies, anti-Sendai virus antiserum or anti-influenza virus (A/Hong Kong/68) antiserum was added to the inoculum prior to infection and incubated for 30 min at 37°C. At 18 hr after infection, cells were harvested and assayed for CAT activity.

RESULTS

Expression of Send–CAT RNA by Sendai Virus. A plasmid, pSend–CAT, was constructed through multiple rounds of PCRs. This plasmid could direct the synthesis of a Sendai virus-like RNA in which the coding region of the genome was replaced by the antisense coding region of the CAT gene. Thus, the CAT gene is flanked by 145 and 119 noncoding nucleotides corresponding to the Sendai virus genome 5′ and 3′ termini, respectively (see Fig. 1). Some known signals present in these sequences include the entire leader RNA, the 5′ noncoding region of the NP gene, and the transcription termination/poly(A) addition signal of the L gene. The construction was such that, after *Ear* I digestion of the plasmid, run-off transcription by T7 RNA polymerase generates a 924-nucleotide RNA molecule sharing the exact 5′ and 3′ ends of the viral genome. When this recombinant CAT RNA was transfected into MDBK cells, no detectable CAT activity was observed (Fig. 2*A*, lane 1). Since this RNA is of negative polarity, CAT activity would be present only if the Send–CAT RNA is transcribed *in vivo*. When the cells were subsequently infected with Sendai virus, significant CAT activity was observed (Fig. 2*A*, lane 2). This expression was specific to Send–CAT RNA since a related RNA, IVA-CAT-1, which contains the antisense RNA of the CAT gene flanked by the 5′- and 3′-terminal sequences derived from the NS gene of influenza A virus (4) was not transcribed (Fig. 2*B*, lane 2). Additionally, RNA derived from transcription of *Bsu*36I-digested pSend–CAT did not result in significant CAT expression (Fig. 2*B*, lane 1). *Bsu*36I cuts within the 3′ Sendai sequence but leaves the CAT sequence intact. Furthermore, if the Send–CAT RNA was preincubated with a cytoplasmic extract prepared from Sendai virus-infected cells, CAT expression was markedly enhanced (Fig. 2*A*, compare lanes 2 and 3). Addition of increasing amounts of the extract resulted in only a minimal increase of CAT activity (Fig. 2*A*, lanes 4–7). However, continued addition of extract at high concentrations eventually resulted in complete inhibition of CAT activity in an extract-dependent manner (see below).

The Send–CAT RNA Is Not Encapsidated *in Vitro*. DI RNA of VSV can be encapsidated *in vitro* through incubation with an infected-cell extract (13). As incubation of Send–CAT RNA with infected-cell extract (ICE) enhances CAT expres-

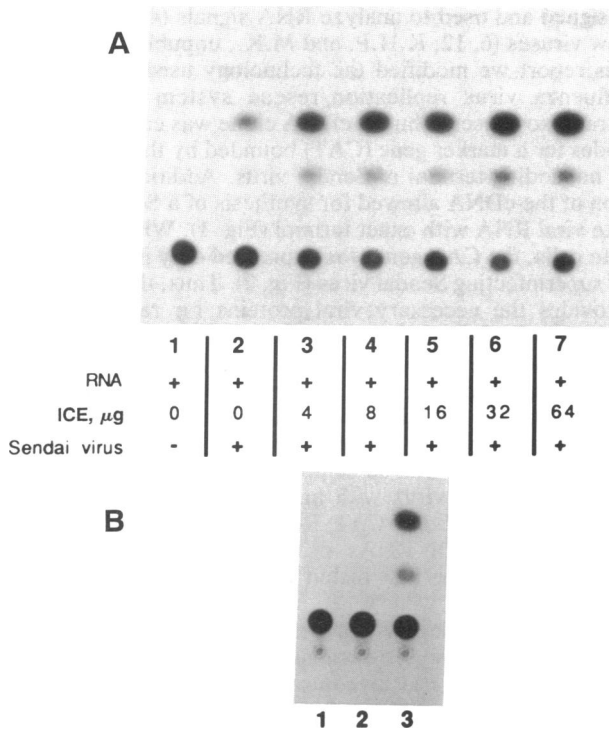

FIG. 2. Specific CAT expression after Send–CAT RNA transfection. (*A*) Conditions used for transfections are indicated below each lane. +, RNA or virus added; −, virus not added. (*B*) CAT activities were measured for the various RNAs used in transfections. The indicated RNA (1 μg) was preincubated with 32 μg of ICE and transfected, followed by helper virus infection. Lanes 1, RNA derived after transcription of *Bsu*36I-digested pSend–CAT; 2, IVA-CAT-1; 3, Send–CAT RNA.

sion, it was postulated that the Send–CAT RNA was assembled into an RNP by the viral proteins present in the extract. Since a characteristic of paramyxovirus nucleocapsids is their resistance to nuclease treatment, labeled Send–CAT RNA was incubated with or without extract and with or without MN, and analyzed by PAGE. As shown in Fig. 3*A*, labeled RNA was completely digested in the absence of added extract (lane 3) and only trace amounts are present when extract is added (lane 4). To determine whether the trace amounts of RNA are sufficient to induce CAT expression, duplicate samples of MN-treated RNA were used to transfect Sendai virus-infected cells. Fig. 3*B*, lane 4, shows that RNA preincubated with ICE and then treated with MN does not induce CAT activity in virus-infected cells. Therefore, the enhancement of expression through incubation with ICE is not due to the assembly of nucleocapsids *in vitro*.

A Cellular Factor(s) Interacts with Send–CAT RNA. Since the biologically active RNA construct was found not to be encapsidated, a cellular factor rather than a viral component may account for the enhancement of CAT expression. To address this, we prepared cytoplasmic extracts in parallel from uninfected cells and infected cells. In the presence of helper virus infection, 5 and 10 μg of uninfected cell extract (UCE) clearly increased Send–CAT expression (Fig. 4*A*, lanes 6 and 7, respectively). Interestingly, 20 and 40 μg of this UCE reproducibly suppressed Send–CAT RNA expression below the level detectable with RNA alone (compare lanes 8 and 9 to lane 1). Inhibition was also observed with ICE, but at much higher protein levels (Fig. 4*A*, lane 5), with complete inhibition occurring with 200 μg of ICE (data not shown).

Send–CAT RNA Is Replicated by Helper Virus. To determine if the Send–CAT RNA is replicated, the presence of complete positive-sense copies of Send–CAT RNA (cRNA) in the transfected cells was assayed by an RNase protection experiment. Cytoplasmic extracts prepared after a typical transfection experiment were first treated with MN. This treatment removes the MN-sensitive mRNAs, but the nucleocapsids remain intact. The resulting MN-resistant RNA species were isolated by extensive phenol/chloroform extractions and used for a standard RNase protection experiment. A radiolabeled Send–CAT RNA probe was used to assay for the presence of full-length positive-sense replicative intermediate cRNA. Fig. 5*A*, lane 1, is the RNase-resistant product after hybridization with a synthetic RNA corresponding to the cRNA of Send–CAT, which was transcribed from a second plasmid construct (M.K., unpublished data). Fig. 5*A*, lane 4, is the negative control whereby probe was hybridized to excess nonspecific RNA. In this case, all probe sequences are digested. However, if nuclease-resistant RNA from helper-virus-infected cells transfected with Send–CAT RNA plus UCE was hybridized to labeled Send–CAT RNA, a full-length product was protected (Fig. 5*A*, lane 2). In addition, the short protected fragments seen in Fig. 5*A* probably represent Sendai virus sequences present in Send–CAT RNA (the 5′ and 3′ ends) that are protected by authentic MN-resistant viral RNAs. Therefore, encapsidated replicative intermediate was present, indicating that the synthetic RNA was indeed replicated.

Send–CAT RNA Is Packaged into Infectious Virus. Since the recombinant Send–CAT RNA can be replicated by the helper Sendai virus, we tested whether progeny viruses released after a transfection experiment contained Send–CAT RNA. This would indicate that the Send–CAT RNA contains all signals necessary for virus packaging. To determine this

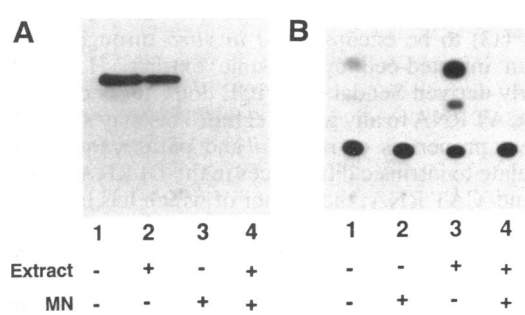

FIG. 3. Analysis of encapsidation reactions. In duplicate samples, labeled Send–CAT RNA was incubated with either ICE or extraction buffer. (*A*) Autoradiography of MN-treated RNAs after electrophoresis. +, Incubation with ICE or MN; −, no addition. (*B*) CAT activities observed after transfection of the samples after encapsidation and MN treatment. All transfections were followed by helper virus infection.

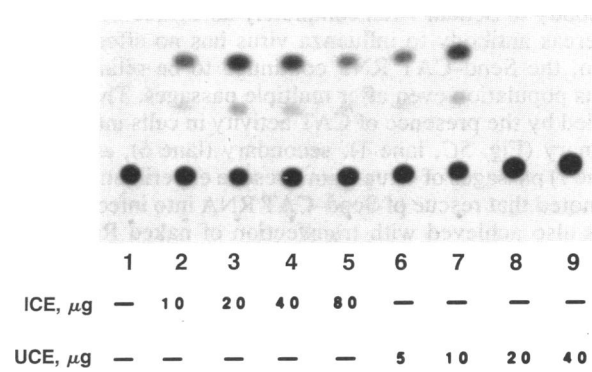

FIG. 4. CAT activities observed after incubation of the Send–CAT RNA with increasing amounts of ICE or UCE. Helper virus infection followed all transfections. Lanes: 1, RNA only; 2–5, increasing amounts of ICE as shown; 6–9, increasing amounts of UCE as shown. −, Extract not added.

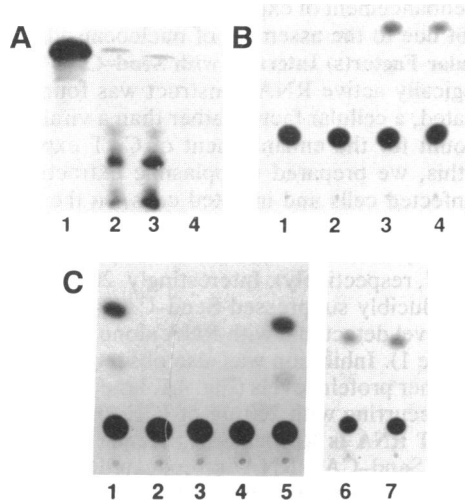

FIG. 5. Analysis of replication and packaging of Send–CAT RNA. (*A*) RNase protection assay was performed using labeled Send–CAT RNA as a probe. The vRNA probe was hybridized to the following samples. Lanes: 1, 10 ng of synthetic full-length cRNA transcript; 2, MN-resistant cytoplasmic RNA isolated after a transfection/infection experiment; 3, MN-resistant cytoplasmic RNA isolated after a secondary infection (18 hours after infection); 4, 10 μg of tRNA. (*B*) Time course of secondary CAT expression. Cells were harvested for CAT assays at various times after infection. Lanes: 1–4, 1, 4, 12, 24 hr after infection, respectively. (*C*) CAT activity induced by infection of fresh MDBK cells by medium from a transfection/infection experiment in the presence of 0 μl (lane 1), 10 μl (lane 2), or 40 μl (lane 3) of anti-Sendai antibody or 10 μl of anti-influenza antibody (lane 5). Lanes 6 and 7 represent CAT activities observed after second and third passages, respectively, of progeny virus obtained from first passage.

possibility, the medium from the transfected (Send–CAT RNA/UCE) virus-infected cells was removed and used for secondary infection of fresh MDBK cells. Cells were harvested at various times after the secondary infection and assayed for CAT activity. Fig. 5*B* shows that the CAT activity was first detected after 4 hr and increased with time, indicating continued CAT gene expression during this secondary passage. In addition, in this secondary passage, replication of the Send–CAT RNP was still occurring, as nuclease-resistant positive-sense RNA was detected in an RNase protection experiment (Fig. 5*A*, lane 3). Also, to rule out the possibility that either Send–CAT RNP or CAT protein was carried along with the infecting medium but not packaged into infectious virions, medium was treated with polyclonal antisera prior to infection. Fig. 5*C* (lanes 2–4) shows that antibody to Sendai virus completely abrogates CAT activity whereas antibody to influenza virus has no effect (lane 5). Also, the Send–CAT RNP continued to be retained in the virus population even after multiple passages. This is exemplified by the presence of CAT activity in cells infected with primary (Fig. 5*C*, lane 1), secondary (lane 6), and tertiary (lane 7) passages of virus from a rescue experiment. It should be noted that rescue of Send–CAT RNA into infectious virus was also achieved with transfection of naked RNA. However, the level of rescued RNA was enhanced upon addition of ICE or UCE. In fact, rescue efficiency of Send–CAT RNA was always proportional to the levels of CAT activity seen in the primary transfected/infected cells (data not shown).

DISCUSSION

The ability to manipulate the genomes of negative-sense RNA viruses will have a profound effect on the study of these virus groups. In recent years, artificial genomes have been designed and used to analyze RNA signals (4, 5) and create new viruses (6, 12, K.H.P. and M.K., unpublished data). In this report we modified the technology used to create the influenza virus replication/rescue system for use with paramyxoviruses. Thus, a cDNA clone was constructed that codes for a marker gene (CAT) bounded by the entire 5′ and 3′ noncoding termini of Sendai virus. Additional manipulation of the cDNA allowed for synthesis of a Sendai genome-like viral RNA with exact termini (Fig. 1). When introduced into cells, the CAT gene was expressed only in the presence of superinfecting Sendai virus (Fig. 2). Thus, the helper virus provides the necessary viral proteins for recognition and transcription of the Send–CAT RNA. That the Send–CAT RNA is also replicated in these cells is shown by the presence of full-length positive-sense transcripts within cells (Fig. 5*A*). This synthetic RNA is also packaged into infectious virions as CAT activity is expressed even after multiple passages in tissue culture and can be completely inhibited by preincubation of progeny virus with antibody against Sendai virus. Therefore, this Send–CAT RNA is analogous to the described fusion DI RNAs of Sendai virus (16, 17). This provides evidence that fusion DI viruses can be transcriptionally active.

A unique finding is that the Send–CAT RNA molecule requires neither *in vitro* encapsidation nor special methods of transfection. This is in contrast to the influenza system, which absolutely requires the addition of purified viral core proteins to reconstitute a biologically active RNP. In addition, the genesis of recombinant measles virus required microinjection of "committed transcription complexes" into helper cells as transfection of genomic RNA by a number of other methods did not yield infectious virus (12). In our experiments, simple DEAE–dextran-mediated transfection of naked Send–CAT RNA transcripts was sufficient for expression and rescue as long as viral proteins were provided by helper virus infection. Perhaps the smaller size of our fusion DI-like RNA (924 nucleotides) compared to the entire measles virus genome (15,894 nucleotides) allows for the transfection, subsequent encapsidation, and recognition by the Sendai polymerase. How can this recognition occur? The basic model for transcription and replication of paramyxovirus is that, during replication, assembly of nucleocapsid by nucleoprotein is concomitant with RNA synthesis from the intact nucleocapsid (18). Therefore, replicative templates are always encapsidated. Since Send–CAT RNA is not encapsidated *in vitro* (Fig. 3), in order for it to be expressed and replicated, it is possible that the helper virus polymerase can recognize and replicate the naked RNA. The subsequent replication products would be biologically active and encapsidated. Alternatively, the full-length naked RNA may be assembled into a nucleocapsid *in vivo* before polymerase recognition. In this regard, naked VSV DI RNA has been shown (13) to be encapsidated *in vitro* through incubation with an infected-cell cytoplasmic extract. However, our similarly derived Sendai virus ICE does not encapsidate the Send–CAT RNA to any great degree. This may simply reflect different properties of rhabdo- and paramyxoviruses or it may relate to intrinsic differences in the DI RNA of VSV and the Send–CAT RNA; the former of which has been selected *in vivo* by its ability to replicate.

The levels of CAT expression can be markedly enhanced by the addition of cytoplasmic extract from either mock-infected or Sendai virus-infected cells. This enhancement is reflected both in the expression of CAT activity and in the replication of the Send–CAT RNA, as the efficiency of rescue into infectious virions mirrors the levels of CAT activity observed during the initial transfection/infection (data not shown). This enhancement is not due to increased transfection efficiencies (data not shown), implying that a specific interaction with a cellular protein(s) is involved. The ability

of host proteins to stimulate transcription of negative-strand viruses is well documented. It has been reported that β-tubulin not only stimulates the transcription of VSV and Sendai virus but also is an absolute requirement for Sendai virus (19). Host factors have also been identified as a requirement for transcription of another paramyxovirus, parainfluenza type IV (20). In addition, a minor microtubule-associated protein has been shown to increase VSV transcription *in vitro* (21). Finally, the replication of other cytoplasmic RNA viruses such as poliovirus and another paramyxovirus, Newcastle disease virus, has also been shown to require host-cell cytoplasmic skeleton factors (22, 23). The stimulatory activity that is detected in our recombinant system may in fact be due to one of the proteins described above or may result from the action of an alternative factor that may act to enhance polymerase recognition or packaging of the Send–CAT RNA *in vivo*. In addition, with increasing concentrations both ICE and UCE completely inhibit replication of Send–CAT RNA. The fact that UCE inhibits at much lower concentrations than ICE may indicate that Sendai virus possesses the ability to modulate the activity of factors inhibitory to its own replication.

Of premier interest, these data show that the synthetic RNA contains all the signals required for efficient expression, replication, and packaging of viral RNA. Thus, the recombinant RNA expression/rescue system described above should allow for simple and quantitative studies of the RNA signals present within the paramyxovirus genome. These include the sequences responsible for RNA editing, the intergenic sequences, and the regulatory sequences present at the termini of the paramyxovirus genome.

We thank Dr. Jerome Schulman for anti-Sendai and anti-influenza antisera and critical reading of the manuscript. We also thank Dr. Peter Palese for Sendai/52 virus. This work was supported by Public Health Service Grant AI-2663 from the National Institutes of Health. M.K. is also an Irma T. Hirschl Scholar. F.F.C. is supported by a JDF postdoctoral fellowship. K.H.P. is partially supported by Medical Scientist Training Grant GM-07280 from the National Institutes of Health.

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