Synthesis of Message and Genome RNAs *In vitro* by Sendai Virus-infected Cell Nucleocapsids

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SUMMARY

Purified Sendai virus nucleocapsids isolated from infected cells were used to programme a transcription system *in vitro* to study virus-specific RNA synthesis. The RNA products were analysed for size by centrifugation before and after denaturation with formamide or glyoxal. The polarity of the products [message (+) or genome (−) strands] was analysed by RNA–RNA hybridization. The non-denatured RNA products sedimented in three groups: 7S to 22S single-stranded RNA transcripts and two partially ribonuclease-resistant complexes. One complex, representing 12% of the total product, sedimented at 26S to 36S. After denaturing the 26S to 36S complex to single-stranded molecules, about half of the RNAs sedimented at 25S to 54S and about half at 6S to 24S. The second complex, representing about 13% of the total RNA product, sedimented at 42S to 52S. After denaturing, about 10% of the single-stranded RNAs sedimented at 38S to 52S and about 90% sedimented at 6S to 19S. In hybridization studies, single-stranded RNAs that sedimented at <19S were predominantly of message sense (+ strand), whereas RNAs that sedimented at 25S to 54S were a mixture of genome and anti-genome type. These results show that transcription and replication activities *in vitro* were associated with Sendai virus nucleocapsids obtained from infected cells and that some of the reaction products approached genome size.

INTRODUCTION

Synthesis of virus RNA in infected cells by RNA-containing negative-strand viruses has been studied to clarify the mechanisms of transcription and replication (Emerson, 1976; Kingsbury, 1977). The use of *in vitro* systems to allow manipulation of environmental conditions and the identification of polypeptides involved in synthetic functions have contributed substantially to our knowledge of the molecular mechanisms involved in negative-strand virus transcription (Choppin & Compans, 1975; Emerson, 1976). For replication, however, success with *in vitro* systems has been limited.

Sendai virus, a paramyxovirus, synthesizes RNA transcripts *in vitro* (Chinchar & Portner, 1981; Stone *et al.*, 1971) that are about the same size as 18S virus messenger RNAs isolated from infected cells (Kingsbury, 1974, 1977). The transcriptase activity found in virions is associated with virus nucleocapsids, a subviral structure isolated from virions or infected cells (Chinchar & Portner, 1981; Marx *et al.*, 1974; Stone *et al.*, 1971). The nucleocapsid, the minimum structural unit required for transcription *in vitro* (Marx *et al.*, 1974), contains the virus genome (a single-stranded 50S RNA molecule, $5 \times 10^6$ mol. wt.) and three virus-specific polypeptides, L, P and NP (Stone *et al.*, 1972; Lamb & Choppin, 1978). These proteins are therefore likely to be involved in the synthesis and modification of virus transcripts.
In addition to the 18S mRNAs synthesized in Sendai virus-infected cells, genome-size (50S) RNA is also synthesized. This RNA accounts for about 20% of the total RNA synthesized and is composed of about 70% 50S negative-strand and 30% positive-strand RNA (Portner, 1977). Although both 'plus' and 'minus' 50S RNA molecules are essential for virus replication, the synthesis of these molecules in vitro has not been demonstrated. The reason for this remains unclear. However, nucleocapsids isolated from infected cells might be a more suitable template for genome synthesis in vitro since some of these structures are actively involved in the replication process (Kingsbury, 1977).

This report provides evidence that Sendai virus nucleocapsids, extracted from infected cells, can synthesize both plus- and minus-strand RNAs, some of which approach genome size. This system therefore provides a means for elucidating the function of the nucleocapsid structural unit in the replication process and may be useful for studying the regulation of transcription and replication.

**METHODS**

**Virus, cells and infection.** Confluent monolayers of chick embryo lung epithelial (CEL) cells (Darlington et al., 1970) grown in 100 mm Falcon plastic Petri dishes were infected with Sendai virus (1 p.f.u./cell) and incubated at 30 °C under Eagle's minimal essential medium (MEM) that contained 3% foetal calf serum. The cultures were allowed to grow for 48 to 72 h under these conditions before the nucleocapsids were isolated. By this time, the average culture had produced about 1.0 × 10^9 p.f.u. (2000 HA units).

**Preparation of enzymically active complex.** Nucleocapsids were purified from infected cells as described previously (Portner, 1977) with some modification of the conditions. To prepare cytoplasmic extracts, we suspended 5 × 10^6 cells/ml in phosphate-buffered saline (PBS), collected them by centrifugation at 500 g for 5 min and then resuspended them in ice-cold RSB (0.01 M-tris-HCl pH 7.4, 0.01 M-KCl, 0.0016 M-MgCl_2) at a concentration of 1 × 10^7 cells/ml. Dithiothreitol was added immediately (final concentration 5 μg/ml). After 15 min at 4 °C, cells were disrupted by 15 strokes of a tight-fitting Dounce homogenizer, and the nuclei and other rapidly sedimenting structures were removed by centrifugation at 12000 g for 10 min. The enzymically active complex (nucleocapsids) in the supernatant was isolated from the interface of a step gradient [6-ml layer of cytoplasmic extract on a 7-ml gradient of 2HEO-glycerol (1.19 g/ml) and 2HEO-Sucrose (1.31 g/ml)], and centrifuged at 40000 rev/min for 90 min at 8 °C in a Spinco SW41 swinging bucket rotor.

**RNA synthesis** in vitro. RNA polymerase reactions in vitro each had a vol. of 1 ml and contained 140 mM-HEPES pH 8, 120 mM-NaCl, 3.2 mM-Mg_2^+ and 3 mM-dithiothreitol, 0.7 mM each of ATP, CTP and GTP (Sigma); 50 μCi [3H]UTP (30 Ci/mmol; New England Nuclear); 10% L-cell extract that contained 17 mg protein/ml and had an absorbance at 260 nm of 36 units/ml (Friedman et al., 1972); 25% glycerol and about 100 μg of nucleocapsids from infected cells. The differences in the reaction mixture from a previous report (Miller & Stone, 1977) were the inclusion of L-cell extract, glycerol and nucleocapsids from infected cells. These mixtures were incubated for 2.5 h at 28 °C and reactions were terminated by the addition of an equal volume of TENS (0.005 M-tris-HCl pH 7.4, 0.001 M-EDTA, 0.1 M-NaCl and 1% SDS); 100 μg yeast RNA was added as carrier.

**Sedimentation analysis of labelled RNA** in vitro. The procedures used for extraction of RNA, rate-zonal centrifugation, ribonuclease treatment, and radioactivity determinations have been detailed previously (Portner & Kingsbury, 1972). Gradients were 34 ml linear 15 to 30% (w/w) sucrose in 0.005 M-tris-HCl pH 7.4, 0.001 M-EDTA, 0.1 M-NaCl (TEN buffer), containing 0.5% SDS. Gradients were centrifuged in a Spinco SW27 swinging-bucket rotor at 52800 g for 16 h at 20 °C. One ml fractions were collected, and RNA was
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recovered from them by precipitation with 2 vol. ethanol. Marker RNAs (18S and 28S ribosomal and 50S virion RNA) were centrifuged in parallel gradients.

Denaturation of extracted RNA. The labelled RNA was concentrated by ethanol precipitation and denatured by formamide (Lehrach et al., 1977) or glyoxal treatment (McMaster & Carmichael, 1977). For formamide (Eastman Chemical Co.) denaturation, RNA samples were dissolved in 99% formamide, and then heated at 56 °C for 10 min, fast-cooled in an ice-bath, and diluted 20-fold in TENS buffer.

Hybridization analysis of RNA products formed in vitro. The RNA products synthesized in vitro were purified from reaction mixtures and analysed by RNA–RNA hybridization. After dissolving the RNA samples in 100 µl 50% (v/v) formamide, 150 mM-tris–HCl pH 7.4, 2 mM-EDTA, 1 mM-NaCl and 0.3% SDS, the RNAs were denatured (95 °C, 90 s) and then annealed to unlabelled 50S Sendai virion RNA (negative-strand) or 18S virus RNA from infected cells (positive-strand) for 2 h at 60 °C. After annealing, RNA samples were diluted 20-fold in 0.15 M-NaCl, 0.015 M-sodium citrate pH 7 (Birnstiel et al., 1972; Guild & Stollar, 1977). Half of each sample was treated with 20 µg/ml pancreatic ribonuclease for 30 min at 24 °C, and acid-insoluble radioactivity was compared with the untreated half.

To establish the polarity of the unlabelled 18S and 50S RNAs, [3H]uridine-labelled 18S and 50S RNAs (prepared in the same manner as unlabelled RNAs) were self-annealed or hybridized to unlabelled RNA of the opposite polarity. Labelled 18S RNAs showed 4% self-annealing and 97% hybridization to unlabelled 50S RNA. Self-annealing of labelled 50S RNA was 18%, indicating that the preparation contained 9% positive strands. To avoid any possible ambiguity caused by the presence of positive strands in our unlabelled 50S RNA preparation, the RNA is self-annealed prior to being used as a reagent in hybridization tests.

RESULTS

Characterization of RNA synthesized in vitro by Sendai virus nucleocapsids isolated from infected cells

Previous reports have demonstrated that paramyxovirions synthesize mRNA species in vitro (Kingsbury, 1974, 1977). However, the genome-length 50S RNA, which is synthesized in addition to mRNA species in infected cells, has not been demonstrated in the RNA product in vitro. Therefore, we examined the RNA products synthesized in vitro by nucleocapsids isolated from infected cells. Accordingly, the sedimentation properties of these labelled products were analysed by centrifugation on sucrose gradients before and after denaturation with glyoxal (Fig. 1) or formamide (Fig. 2). Fig. 1 (a) shows the sedimentation properties of non-denatured UMP-labelled RNA synthesized in vitro by Sendai virus nucleocapsids. The distribution of labelled product is strikingly similar to that in vivo (Portner & Kingsbury, 1972): a major portion sedimented where 18S virus mRNA would be found (line b); a heterogeneous portion sedimented in the position of replicative intermediates (line c); a third portion sedimented between 40S and 56S (line d), where virus genomes and transcriptive intermediates would be found. The RNAs in the c and d portions of the gradient (Fig. 1a) contained RNase-resistant structures and thus are suitable candidates for the RNA-synthesizing intermediates, described for Sendai virus in vivo (Portner & Kingsbury, 1972). The RNase-resistant structures are not represented in Fig. 1 (a), but are demonstrated in a different but identically prepared RNA product (Fig. 2a, lines b and c).

Core size of RNase-resistant structures

The core size of the RNase-resistant structures was analysed to further test the possibility that the products were synthesized on two intermediates, one involved in transcription and the other in the production of genome-size molecules. Before recentrifugation the RNA synthesis
Fig. 1. Sedimentation properties of in vitro synthesized RNA before and after denaturation with glyoxal–dimethyl sulphoxide. The in vitro synthesized RNAs were extracted from the reaction mixture by the SDS–phenol method and then concentrated by ethanol precipitation. (a) Without further treatment the RNAs were centrifuged in 15 to 30% sucrose gradients at 52 800 g for 16 h at 20 °C. Pooled gradient fractions indicated by lines b, c and d in panel (a) were denatured and recentrifuged as before. (b) Fraction b. (c) Fraction c. (d) Fraction d.

intermediates (Fig. 2a; lines b, 26S to 36S and c, 42S to 52S) were treated with a low concentration of ribonuclease (0.1 μg/ml) that digested single-stranded regions without attacking the base-paired 'cores'. Fig. 3 (a, b) shows the sedimentation properties of these structures before and after treatment with ribonuclease. The sedimentation rate of the 26S to 36S structure was changed relatively little (12S to 22S) (Fig. 3a), although 50% of the radioactive RNA was made acid-soluble by RNase treatment. Ribonuclease solubilized about 12% of the radioactivity in structures that sedimented at 42S to 52S. The remaining core structure sedimented at about 5S to 11S (Fig. 3b). From these results, it appears that the double-stranded core of the 24S to 36S structure (Fig. 3a) was appreciably larger than the cores of the 42S to 52S structures (Fig. 3b). The sedimentation rate of the large (12S to 22S) cores (Fig. 3a) was the expected S value of a double-stranded RNA molecule from which some single-stranded RNA would sediment at 50S. Since some complexes contained a 50S
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Fig. 2. Sedimentation properties of in vitro synthesized RNAs before and after formamide denaturation. (a) Sucrose gradient of untreated product. Pooled gradient fractions b and c from (a) were treated with 99% formamide at 56 °C for 10 min, and recentrifuged. Centrifugation conditions were as indicated in Fig. 1. (b) Fraction b and (c) fraction c were then assessed for total acid-insoluble radioactivity (○) and ribonuclease-resistant radioactivity (●). In (b), b' 1 = 9S to 21S, b' 2 = 25S to 37S and b' 3 = 42S to 54S. In (c), c' 1 = 6S to 19S and c' 2 = 38S to 52S.

Fig. 3. Core size of RNA intermediates synthesized in vitro. Isolated RNase-resistant structures shown in Fig. 2(a) fractions b and c were centrifuged after no treatment or treatment with bovine pancreatic ribonuclease (0.1 μg per ml of TEN buffer) for 10 min at 24 °C. (a) Fraction b intermediates; (b) fraction c intermediates. Acid-insoluble radioactivity was determined in ribonuclease-resistant cores (●) and untreated controls (○). Centrifugation was as indicated in Fig. 1.
RNA strand completely base-paired with its complement, these complexes may be involved in the replicative process (Portner & Kingsbury, 1972). Smaller cores (5S to 11S) in the 42S to 52S structures indicated that the template was base-paired to product molecules in the 14S to 20S range and was involved in transcription.

Sedimentation properties of denatured in vitro product

Since a considerable amount of the product was in RNase-resistant structures, denaturing conditions were needed to estimate more precisely the size of the single-stranded RNA components. In this procedure, portions of the gradient were pooled (Fig. 1a, fractions b, c and d), and the isolated RNA was denatured with glyoxal and resedimented in sucrose gradients. Fig. 1(b) shows that isolated RNA, which sedimented at 18S in the first gradient (Fig. 1a, line b), sediments at 18S after glyoxal denaturation. Fig. 1(c) shows a heterogeneous distribution of labelled single-stranded RNAs with most of the RNA sedimenting faster than 18S mRNA, as would be expected if labelled, glyoxal-denatured RNA (from first gradient Fig. 1a, pool c) represented replicative intermediates. Of special note is the labelled product that sedimented at 50S, the position of virus genomes. As shown in Fig. 1 (d) (from Fig. 1a, fraction d), most of the RNAs were smaller than 18S message and probably represented nascent strands from transcriptive intermediates. However, some labelled products sedimented at the position of 50S genomes. This result suggests that the in vitro labelled RNAs were synthesized on two partially RNase-resistant structures, possibly transcriptive and replicative intermediates, and that some of the labelled RNA was genome size.

Formamide denaturation of RNA and separation into size classes for hybridization

Although the previous experiment suggested that replication occurred in vitro, the synthesis of genome-length 50S RNA may have represented aberrant transcription (positive strands), unrelated to the replication process. However, if these products contained negative-strand RNA, a replication process in vitro would be further supported. Therefore, the polarity of the labelled RNAs was analysed by hybridization studies to determine if negative strands were indeed synthesized. Single-stranded products of different size classes were obtained from formamide-treated labelled RNA for hybridization studies; the size distribution of the product after denaturation with formamide was determined by analysis on sucrose gradients (Fig. 2). The distribution of the untreated RNA (Fig. 2a) was very similar to that in the previous experiments (Fig. 1a); a major portion (75%) sedimented between 12S and 18S, and two heterogeneous peaks sedimented between 26S to 34S (12%) and 40S to 56S (13%).

In the preparation of single-stranded molecules of defined size for hybridization, portions of the first gradient (Fig. 2a, fractions a, b and c), were pooled and treated with 99% formamide at 56 °C for 10 min, and the RNA was resedimented. Fig. 2(b, c) (from pools b and c respectively) shows that formamide, like glyoxal treatment, produced a heterogeneous size distribution of labelled product with some RNA sedimenting in the position of 50S genomes (fractions 21 to 24). The distribution of radioactivity was 56% for the 9S to 21S RNAs and 44% for the 25S to 54S (Fig. 2b). The RNAs that sedimented at 6S to 19S and 38S to 52S represented 90% and 10% of the radioactivity respectively (Fig. 2c). In addition, formamide treatment produced RNAs that were completely sensitive to ribonuclease, indicating that single-stranded RNA molecules produced the size distribution shown in Fig. 2(b, c).

Hybridization analysis: in vitro products contain message- and genome-type molecules

In the test of polarity of the labelled, single-stranded product (Fig. 2), pooled RNAs fractions [b' in (b), c' in (c) and a in (a)] were hybridized with 50S RNA from virions.
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Table 1. Hybridization of the in vitro synthesized RNA product after denaturation with formamide

<table>
<thead>
<tr>
<th>RNA from first gradient*</th>
<th>Single-stranded ³H-labelled RNA component†</th>
<th>50S RNA (minus strand)</th>
<th>18S RNA from infected cells (plus strand)</th>
<th>None</th>
</tr>
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<tr>
<td>Fractions a</td>
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<tr>
<td>7–22S</td>
<td>7–18S</td>
<td>83</td>
<td>9</td>
<td>6</td>
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<tr>
<td>Fractions b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9–21S</td>
<td>25–37S</td>
<td>66</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>26–36S</td>
<td>42–54S</td>
<td>27</td>
<td>74</td>
<td>30</td>
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<tr>
<td>Fractions c</td>
<td></td>
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<td></td>
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<tr>
<td>6–19S</td>
<td>42–52S</td>
<td>31</td>
<td>88</td>
<td>61</td>
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<tr>
<td>42–52S</td>
<td></td>
<td>86</td>
<td>21</td>
<td>10</td>
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<td></td>
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<td>41</td>
<td>79</td>
<td>64</td>
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</table>

* ³H-labelled RNA product was extracted from the reaction mixture and centrifuged as described in Fig. 2(a). RNA species from the gradient were selected by pooling the appropriate fractions.

† Selected RNA species from the gradient shown in Fig. 2(a) were denatured with 99% formamide for 10 min at 56 °C and then centrifuged (see also Fig. 2b, c). RNAs from the indicated fractions were pooled for hybridization.

‡ Unlabelled 50S Sendai virion RNA (5 µg/ml) or 18S virus RNA from infected cells (about 5 µg/ml) were added to the indicated sample. Values given were derived from a single experiment. Similar results were obtained in two independent repetitions in which slightly different pools were taken.

(positive-strand probe) or 18S RNA from infected cells (negative-strand probe). The results, shown in Table 1, demonstrate that both message- (+) and genome-sense (−) strands were synthesized in vitro and that some of these RNAs were genome size. As an illustration of these points, the single-stranded RNA components obtained after denaturation of the RNase-resistant structures (Table 1, fractions b and c), contained RNA products of both message and genome sense when titrated with the appropriate reagent. Although positive strands were found in all size classes, they predominated in RNA species that sedimented more slowly than 21S, whereas the proportion of RNA with genome polarity (negative strands) increased with increasing size of the product. Some of these numbers do not add up to 100% because only one set of data was obtained after titration with 50S RNA, the positive-strand probe, under saturating conditions. The high self-annealing values of 61% and 64%, which were due to the presence of input template from the reaction mixture, also indicate that saturation was not reached with the negative-strand probe. However, the data still support the conclusion that strands of both plus and minus polarity were synthesized.

DISCUSSION

Within Sendai virus-infected cells, the negative-strand genome serves as a template for the synthesis of seven mRNA species that are translated into virion proteins and for the synthesis of genome-length plus-strand copies (Kingsbury, 1974, 1977). The latter RNA then provides a template for the synthesis of negative-strand virion genome RNA (Kolakofsky & Bruschi, 1975). The structure responsible for RNA synthesis is the virus nucleocapsid which contains two minor polypeptides, L and P, associated with the NP protein–RNA complex (Chinchar & Portner, 1981; Marx et al., 1974; Stone et al., 1971). Probably both mRNA synthesis and genome replication are carried out by the L and P polypeptides.

Previous reports of RNA synthesis by paramyxovirions or virion nucleocapsids in vitro have not demonstrated the synthesis of full-length genome RNA. In this study, the Sendai virus nucleocapsids extracted from infected cells synthesized a species of RNA in vitro that has properties associated with full-length genome-size 50S RNA synthesized in vivo. The
genome size was established by sedimentation of the in vitro product after treatment with glyoxal or formamide. Furthermore, hybridization results suggest that the genome-length molecules represent replication rather than aberrant transcription since synthesized 50S RNA was of anti-genome sense as well as genome sense. An explanation for this finding came from our observation that nucleocapsids isolated from infected cells contained appreciable (30 to 40%) amounts of anti-genome strand RNA which apparently can serve as a template for synthesis of genome-length molecules in vitro.

Similarly, virions were also tested for genome synthesis under identical reaction conditions to those used with cell nucleocapsids, but 50S RNA molecules were not detected (data not shown). It is not known whether some nucleocapsids from infected cells have been modified in some way to permit genome synthesis in vitro, or whether elongation of nascent RNA strands associated with cell nucleocapsids was responsible for the synthesis of genome-size molecules. Studies of RNA synthesis of vesicular stomatitis virus (VSV) in vitro provide support for the modification concept (Batt-Humphries et al., 1979; Breindl & Holland, 1976). These studies suggest that factor(s) in uninfected or infected cells may modify VSV ribonucleoprotein cores so that they can synthesize 42S anti-genome RNA in vitro. More recently, Testa et al. (1980) reported that in the absence of cell extracts, the ribonucleoprotein core of VSV can be modified by the ATP analogue AMP-PNP to permit the synthesis of genome-length plus strands in vitro. These authors suggested that the phosphorylated state of a putative regulatory protein(s) may determine whether transcription or replication will occur.

Another mechanism suggested for genome replication of Sendai virus involves the binding of NP or P polypeptides to nascent RNA strands to allow polymerization of genome-size molecules rather than mRNA synthesis (Kingsbury, 1974). Recent findings with VSV are consistent with this concept (Blumberg et al., 1981) and suggest that binding of VSV polypeptide N to nascent RNA strands at the leader or leader–N message junction may be responsible for extending leader to full-size genome molecules.

The mechanism of genome replication that applies to this work remains to be determined. However, the in vitro system reported here provides a way to sort out some of the possibilities that may be involved in the transcription and replication process.

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REFERENCES


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