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Ribonucleic Acid Transcriptases in Sendai Virions and Infected Cells

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Sendai virions contain an enzyme which catalyzes the incorporation of ribonucleotides into ribonucleic acid (RNA). Enzyme activity was optimal at *p*H 8.0 and 28 C; otherwise conditions were similar to those reported for Newcastle disease virion (NDV) RNA polymerase. The initial rate of RNA synthesis by the Sendai virion enzyme was about 10 pmoles per mg of protein per hr, but after 3 hr of incubation the rate increased about fivefold. The virion enzyme was compared with an RNA polymerase in the microsomal fraction of infected cells. Both enzymes made predominantly single-stranded RNA which was complementary in base sequences to 50*S* virion RNA. Most of the RNA synthesized by the virion polymerase sedimented at 16*S*, but the product of the microsomal enzyme sedimented at about 8*S*.

There is evidence that paramyxovirus genomes cannot function as messenger ribonucleic acid (RNA) species (7) and that smaller RNA species complementary in base sequences to viral genomes are the templates for viral proteins (2, 3). If this is correct, an enzyme capable of transcribing virion RNA must pre-exist in the cell or must reside in the virion. Huang, Baltimore, and Bratt (6) have recently shown that virions of an avian paramyxovirus, Newcastle disease virus (NDV), contain an enzyme which synthesizes RNA complementary in base sequences to the virion RNA, indicating that the latter possibility is correct.

In this paper, we report that the mammalian paramyxovirus, Sendai virus, also contains an RNA polymerase, and we compare the virion enzyme with the RNA polymerase previously described in the microsomal fraction of infected cells (11).

MATERIALS AND METHODS

Virus. Virus used in these experiments was plaque-purified from the Enders strain of Sendai virus and was free from incomplete virions. Methods for preparing chick embryo lung (CEL) cell cultures and for growing Sendai virus in these cells have been described (9). The only modification we made in the present work was to incubate cells infected with 0.1 plaque-forming unit (PFU)/cell at 30 C, since we found that by 3 to 4 days after infection about fivefold more of this virus clone was made at this temperature than at 37 C.

Culture medium containing released virus was centrifuged for 10 min at 3,600 × *g* to remove cells and debris. Virus was pelleted by centrifugation at 80,000 × *g* for 45 min; was resuspended in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.03 M NaCl (*p*H 7.3); and was centrifuged at 12,000 × *g* for 10 min to remove virus clumps and remaining debris. Such virus preparations contained 1 to 2 mg per ml of protein and about 10^4 hemagglutinating units/ml. Protein was determined by the method of Lowry et al. (10) with bovine serum albumin as standard.

RNA extraction, rate zonal centrifugation, and hybridization. These methods have all been described previously as has the method for determining radioactivity (8, 9).

Isolation of RNA polymerase from infected cells. RNA polymerase was prepared from CEL cells incubated at 30 C for 72 to 96 hr after infection with 0.1 PFU/cell. The procedure of Mahy et al. (11) was used, except that cells were swollen in RSB (12) for 30 min before homogenization.

Materials. Unlabeled nucleoside triphosphates were obtained from P-L Biochemicals. ^{3}H-guanosine triphosphate (GTP; 1.3 Ci/mmole) was purchased from Schwarz BioResearch. Actinomycin D was a gift from Merck, Sharp and Dohme. Triton N-101 was purchased from Sigma Chemical Co. Bovine pancreatic deoxyribonuclease I, electrophoretically separated from contaminating ribonuclease activity (DPFF), and bovine pancreatic ribonuclease A (RAF) were products of Worthington Biochemical Corp.

RESULTS

Requirement for virion polymerase activity. When purified Sendai virions were added to the appropriate reaction mixture, ^{3}H-guanosine

monophosphate (GMP) was incorporated into an acid-insoluble material (Table 1). Except for the temperature optimum, which was 28 C (Fig. 1A), and the *p*H optimum, which was *p*H 8.0 (Fig. 1B), the reaction conditions were similar to those reported for the NDV and vesicular stomatitis virus (VSV) virion polymerases (1, 6). The reaction was totally dependent upon virions and Mg^{2+}. Omission of cytidine triphosphate (CTP) or the nonionic detergent Triton N-101 virtually-eliminated the activity (Table 1). Full activity was obtained in the absence of uridine triphosphate (UTP), and there was some activity without adenosine triphosphate (ATP; Table 1). Since the amount of acid-insoluble product was about 10^{-5} the amount of triphosphates present, these findings may have resulted from contamination of CTP or GTP with other triphosphates. Other possibilities, such as the existence of another ribonucleotide-polymerizing enzyme in virions, have not been ruled out.

Ribonuclease added to the reaction mixture at zero time completely inhibited the reaction (Table 2). Actinomycin D or deoxyribonuclease did not inhibit incorporation, indicating that DNA was not involved. Pronase reduced the incorporation, indicating the involvement of protein in the activity.

Association of polymerase activity with Sendai virions. The association of the polymerase activity with Sendai virions was established by rate zonal centrifugation in sucrose gradients. The virus was centrifuged and its distribution in the gradient was determined by ultraviolet absorption and by assaying each fraction for hemagglutinating activity. Each fraction was then assayed for RNA polymerase. We have shown before that Sendai virions are pleomorphic and are found at more than one position in rate zonal gradients (9). As shown in Fig. 2, peaks of HA were found in the gradient at fractions 18 to 20 (1,000*S*) and on the sucrose cushion at fractions 30 to 33. Over 80% of the enzyme activity in the gradient was located in these two peaks. There was no evidence of polymerase activity in fractions which lacked virus, indicating that no soluble enzyme exists (fractions 1 to 15). The specific activity of the enzyme was not identical throughout the gradient, and it is not ruled out that there is enzyme associated with structures other than virus.

Time course of ^{3}H-GMP incorporation. The rate of ^{3}H-GMP incorporation into an acid-insoluble product increased with time of incubation at 28 C. Amounts incorporated per milligram of protein during the first, second and third hours of incubation were 11, 20, and 49 pmoles in a typical experiment (Fig. 3A). During the first hour, slightly over 50% of the incorporated radioactivity was in a ribonuclease-resistant form; thereafter, little increase of ribonuclease-resistant counts was observed, but the synthesis of ribonuclease-sensitive material increased (Fig. 3A). All of the product was RNA, since all of it was digested by ribonuclease when the enzyme treatment was performed in the absence of salt.

TABLE 1. *Requirements for Sendai virion RNA polymerase activity*[a]

Reaction mixture	Counts per min per mg of protein
Complete	11,000
Minus virions	<28
Minus Mg	<28
Minus ATP	2,252
Minus CTP	630
Minus UTP	15,828
Minus Triton N-101	257
Minus NaCl	2,350
Minus dithiothreitol	6,160

[a] Complete reaction mixture consisted of the following components, in a total volume of 0.1 ml: 5 μmoles of Tris-hydrochloride, *p*H 8.0; 10 μmoles of NaCl; 0.4 μmole of magnesium acetate; 0.3 μmole of dithiothreitol; 0.07 μmole each of ATP, CTP, UTP; 0.001 μmole of ^{3}H-GTP (3,040 disintegrations per min per pmole); 80 μg of Triton N-101; and 35 μg of viral protein. The reaction mixtures were incubated in duplicate at 28 C for 90 min. The reaction was terminated by chilling in an ice bath and 0.2 ml of 0.08 M sodium pyrophosphate and 0.5 ml of bovine serum albumin (1 mg/ml) were added. The sample was precipitated with 3 ml of 5% cold trichloroacetic acid and was processed and counted as described previously (9). The counting efficiency of ^{3}H under our conditions was 36%. A complete sample with virions which had been incubated at 4 C for 90 min contained 44 counts/min and this value was subtracted from all of the experimental values.

^{3}H-GMP incorporation mediated by an enzyme from infected cells. Mahy, Hutchinson, and Barry (11) described an RNA-dependent RNA polymerase in the microsomal fraction of primary chick embryo fibroblasts infected with Sendai virus. The optimal conditions reported for this enzyme were similar to those we found for the virion polymerase (Table 1). We examined infected CEL cells for such an enzyme by using the same reaction mixture which we employed for the virion polymerase (Table 1), except that the detergent Triton N-101 was omitted. The enzyme from cells had a higher specific activity than the enzyme from virions, but the kinetics of the reaction were similar. In the first, second, and third hours of incubation, 57, 131, and 260 pmoles of GMP, respec-

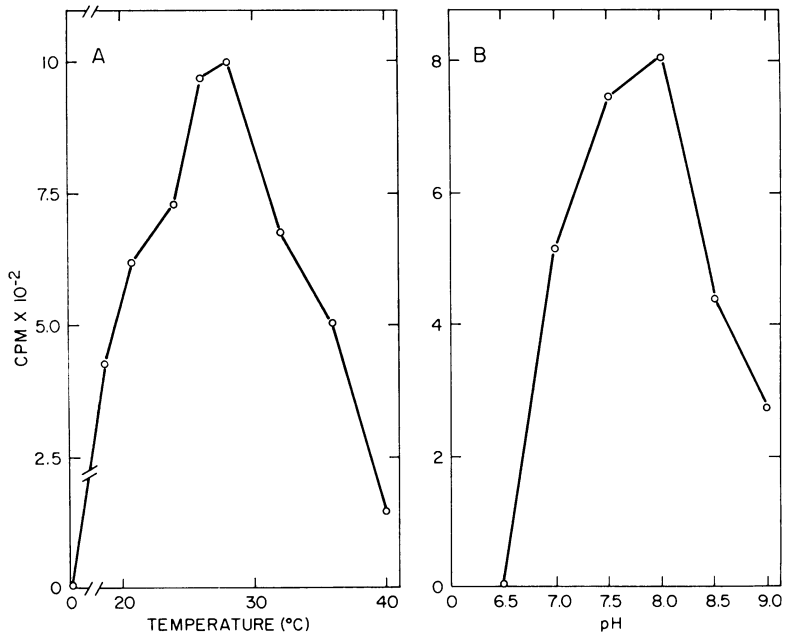

FIG. 1. *Optimum temperature and pH for Sendai virion RNA polymerase. (A) Standard 0.1-ml reaction mixtures (as described in Table 1) containing 40 μg of virus protein were incubated in duplicate for 90 min at the indicated temperatures. (B) Standard 0.1-ml reaction mixtures were used except that the pH 8.0 Tris-hydrochloride buffer was replaced by 5 μmoles of Tris-hydrochloride at the indicated pH values, measured at 28 C. Reaction mixtures containing 35 μg of virus protein were incubated in duplicate at 28 C for 90 min. Complete reaction mixtures at each pH containing the same amount of viral protein were run at 4 C for 90 min, giving background values of 50 to 80 counts per min, which were subtracted from the experimental values.*

TABLE 2. *Effect of enzymes and inhibitors on Sendai virion polymerase*[a]

Reaction mixture	Counts per min per mg of protein
Complete	34,000
Plus ribonuclease (50 μg/ml)	<44
Plus actinomycin D (20 μg/ml)	31,300
Plus deoxyribonuclease (100 μg/ml)	34,400
Plus Pronase (50 μg/ml)	12,300

[a] Duplicate 0.1-ml reaction mixtures, as in Table 1, containing 22.5 μg of Sendai protein and each of the inhibitors were incubated at 28 C for 2.5 hr. The reaction was terminated, processed, and counted as described in Table 1. Complete mixtures with virions which had been incubated at 4 C for 2.5 hr contained 56 counts/min and this value was subtracted from all of the experimental values.

tively, were incorporated per mg protein (Fig. 3B). Relative incorporation into ribonuclease-resistant and ribonuclease-sensitive materials was almost identical to that observed with the virion polymerase (Fig. 3A).

Products of both enzymes were virus-specific. To determine if the products of the polymerases were complementary to viral genomes, annealing experiments were performed. About 20% of the labeled RNA obtained from 5 hr of incubation of the virion polymerase was resistant to the action of ribonuclease without annealing (Table 3). When this RNA was completely denatured and self-annealed at high concentration, about 60% of the product became ribonuclease-resistant, presumably having hybridized with virion template RNA present in the reaction mixture; self-annealing at low concentration gave 17% ribonuclease resistance (Table 3). When the enzyme product was completely denatured and then annealed with unlabeled Sendai virion 50*S* RNA, it became almost entirely resistant to ribonuclease (Table 3).

The ribonuclease resistance of the RNA synthesized by the microsomal polymerase was 8% without annealing, 48% when self-annealed at high concentration, and 73 to 84% when annealed with added 50*S* virion RNA (Table 3). Thus, the products of both enzymes were predominantly complementary in base sequences to Sendai virion RNA.

Sedimentation properties of the enzyme products.

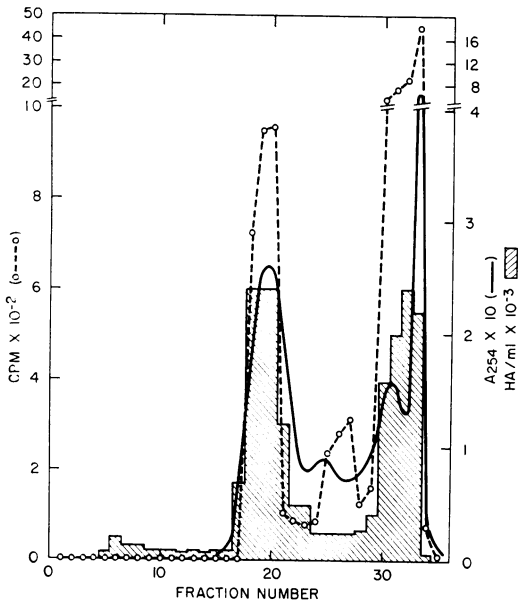

FIG. 2. *Association of polymerase activity with Sendai virions. A 3-mg amount of Sendai virus in 2 ml of 0.01 M Tris-hydrochloride, 0.03 M NaCl (pH 7.3), prepared as described in Materials and Methods, was layered on a 30-ml linear 15 to 30% (w/w) sucrose gradient in 0.005 M Tris-hydrochloride, 0.001 M ethylenediaminetetraacetic acid (EDTA; pH 7.4) which had been formed on a 4-ml 60% (w/w) sucrose cushion in the same buffer. Centrifugation was for 40 min at 20,000 rev/min at 20 C in a Spinco SW 27 swinging-bucket rotor. Absorbance at 254 nm was monitored continuously as the gradient was fractionated. Hemagglutination assays were performed on each fraction, and fractions were then diluted fourfold with 0.05 M Tris-hydrochloride, 0.001 M EDTA (pH 7.4) buffer and centrifuged at 80,000 × g for 45 min. Pellets were resuspended in 0.1-ml enzyme reaction mixtures and incubated for 90 min at 28 C. Peak fractions of hemagglutinin were also assayed for enzyme at 10-fold dilutions. The dilution with the highest specific activity was used to plot the peak values.*

The major product of the Sendai virion polymerase sedimented at about 16*S* relative to 18*S* ribosomal RNA (Fig. 4A, fraction 11). There was an additional peak at about 7*S* (fraction 5) and some heterogeneously sedimenting material in the 30 to 50*S* region (fractions 21–33). None of the reaction products sedimented as sharply as the marker ribosomal RNA species (Fig. 4B).

Ribonuclease treatment of the sucrose gradient fractions revealed that the 16 and 7*S* components were single-stranded RNA, whereas most of the heterogeneous material in the 30 to 50*S* region was ribonuclease-resistant (Fig. 4A). All of the ribonuclease-resistant counts in the unfractionated extract were rendered ribonuclease-sensitive when the RNA was boiled for 2 min and then quickly cooled. When this heat-denatured RNA was centrifuged on a rate zonal gradient, the radioactive material in the 30 to 50*S* region was converted to slower sedimenting components (Fig. 4B). Little change was seen in the 16*S* peak, whereas the 7*S* peak increased. Evidently, single-stranded product RNA which sedimented slower than 16*S* was released from a ribonuclease-resistant structure by boiling.

The product of the microsomal enzyme was examined in the same manner. The major product sedimented at about 8*S*, relative to 18*S* ribosomal RNA (Fig. 5A, fraction 6). This was single-stranded RNA by virtue of its ribonuclease sensitivity, but most of the heterogeneous components in the 30 to 50*S* region were ribonuclease-resistant (Fig. 5A, fractions 22 to 33). When the product was heated and quickly cooled, all the ribonuclease-resistant counts became ribonuclease-sensitive and slower sedimenting (Fig. 5B).

The above results, taken with the kinetic data (Fig. 3) and the hybridization data (Table 3), indicated that the products of both virion and cell enzymes were synthesized on 50*S* RNA templates in a partially double-stranded form and then released as single-stranded RNA species.

DISCUSSION

Optimal conditions for demonstrating the Sendai virion polymerase were different enough from conditions reported for the VSV and NDV virion polymerases (1, 6) to suggest that these various enzymes are different and virus-specific, specified entirely or in part by viral genes. The Sendai virion enzyme may be the same as the enzyme from infected cells; this can only be decided when both enzymes have been purified and extensively compared. Regardless of the origin of the enzyme, the most important question is whether its function is essential for Sendai virus replication. Lack of infectivity of paramyxovirion RNA species (7) and production of large amounts of virus genome transcripts in infection (2, 3, 8) indicate that the genetic information in paramyxovirus genomes is not in a translatable form and that a virion transcriptase is necessary to make early virus messenger RNA species. The virion polymerase products sedimented rapidly enough to be messengers for proteins of respectable size; the 16*S* product sedimented only a little slower than the 18*S* minus strands accumulating in infected cells late in infection. Most of the virion enzyme product was complementary to viral genomes; it will have to be isolated free from template to learn whether it contains any plus strands or self-complementarity (13, 14). It will be espe-

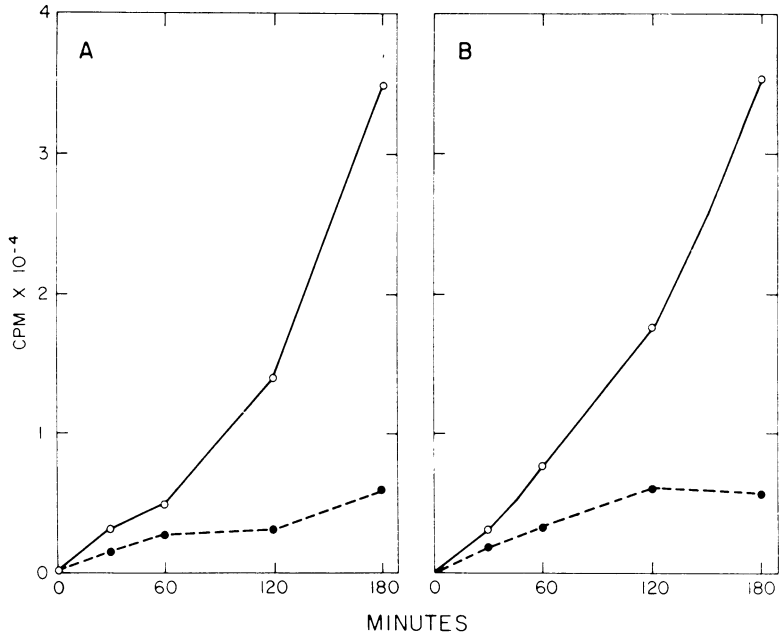

FIG. 3. *Time course of ^{3}H-GMP incorporation by polymerases from virions and infected cells. (A) A 2-ml reaction mixture containing 800 μg of virus protein was incubated for 3 hr at 28 C. At the indicated times, portions were removed and extracted twice with phenol and 0.5% sodium dodecyl sulfate. RNA was recovered by ethanol precipitation and resuspended in 0.005 M Tris-hydrochloride, 0.001 M ethylenediaminetetraacetic acid, 0.1 M NaCl (pH 7.4). One-half of each sample was precipitated with trichloroacetic acid, and the other half was treated with 10 μg of ribonuclease A per ml for 30 min at 24 C before acid precipitation. (B) A 2-ml reaction mixture containing 250 μg of microsomal protein from cells infected with Sendai virus 72 hr previously was incubated for 3 hr at 28 C. At the indicated intervals, portions were removed and processed as described above. Symbols: ○, total counts per minute; (●) ribonuclease-resistant counts per minute.*

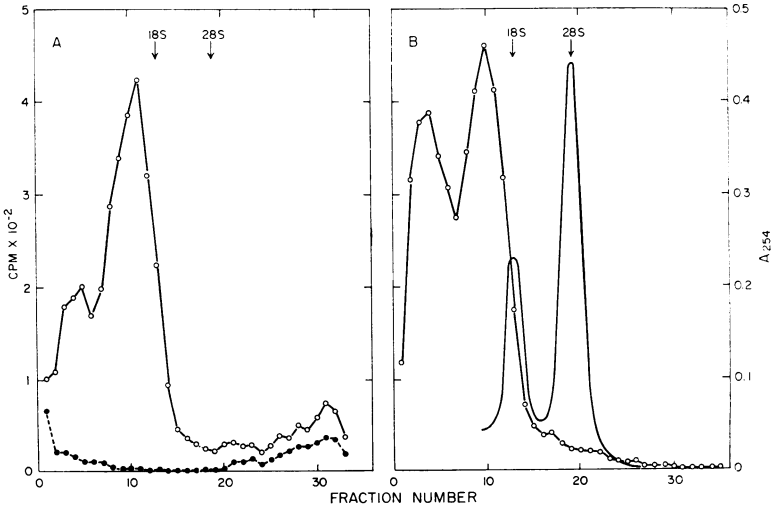

FIG. 4. *Sucrose gradient centrifugation of Sendai virion polymerase product. A reaction mixture containing 350 μg of virus protein per ml was incubated for 5 hr at 28 C. During the last hour of incubation, the specific activity was 69 pmoles of ^{3}H-GMP incorporated per mg of protein. RNA was extracted with phenol in the presence of 0.5% sodium dodecyl sulfate (SDS) and precipitated with ethanol. (A) A portion of the extracted RNA was dissolved in 2 ml of 0.005 M Tris-hydrochloride, 0.001 M ethylenediaminetetraacetic acid (EDTA), 0.1 M NaCl, 0.5% SDS (pH 7.4) and loaded on a 34-ml 15 to 30% sucrose gradient in the same buffer. Centrifugation was at 20,000 rev/min for 16 hr at 20 C in a Spinco SW 27 rotor. Each 1-ml fraction was precipitated by 2 volumes of ethanol with 500 μg of yeast RNA. Pellets were dissolved in gradient buffer lacking SDS. One-half of each sample was acid-precipitated, and the other half was treated with 50 μg of ribonuclease per ml for 30 min at 24 C before acid-precipitation. (B) A portion of the extracted RNA was dissolved in 2 ml of 0.005 M Tris-hydrochloride, 0.001 M EDTA (pH 7.4). The sample was boiled for 2 min and then rapidly cooled in an ice bath. Sodium chloride was added to bring the concentration to 0.1 M, and SDS was added to 0.5%. The sample was centrifuged and processed as described in Fig. 4A. The optical density profile was obtained from RNA extracted from CEL cells and centrifuged in a separate gradient under identical conditions. Symbols: ○, total counts per minute; ●, ribonuclease-resistant counts per minute; —, absorbance at 254 nm.*

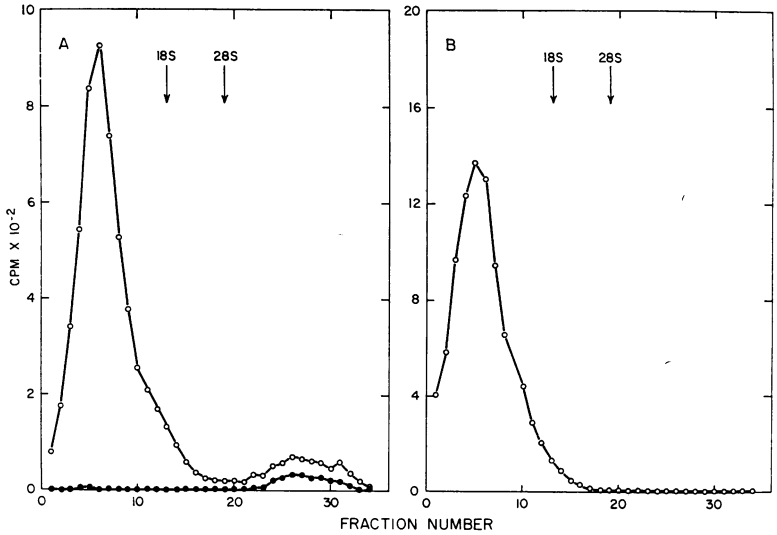

FIG. 5. *Sucrose gradient centrifugation of the microsomal enzyme product. A reaction mixture containing 125 μg of microsomal protein per ml from cells infected 72 hr previously with Sendai virus was incubated for 5 hr at 28 C. During the last hour of incubation, the specific activity was 117 pmoles of ^{3}H-GMP incorporated per mg of protein. RNA was extracted as described in Fig. 4. (A) A portion of the extracted RNA was centrifuged on a sucrose gradient and processed as described in Fig. 4A. (B) A portion of the extracted RNA was dissolved in hypotonic buffer, boiled, centrifuged, and processed as described in Fig. 4B. Symbols: ○, total counts per minute; ●, ribonuclease-resistant counts per minute.*

TABLE 3. *Hybridization of polymerase products with unlabeled Sendai virion 50S RNA*[a]

Labeled RNA (concn annealed)	Treatment	Per cent ribonuclease resistant
Virion enzyme product	Not annealed	21
785,000 counts per min per ml	Self-annealed	61
	Annealed with 100 μg of 50S RNA per ml	91
2,560 counts per min per ml	Self-annealed	17
	Annealed with 4 μg of 50S RNA per ml	100
Microsomal enzyme product	Not annealed	8
820,000 counts per min per ml	Self-annealed	48
	Annealed with 100 μg of 50S RNA per ml	84
2,100 counts per min per ml	Self-annealed	17
	Annealed with 4 μg of 50S RNA per ml	73

[a] Annealing was done at 80 C for 1 hr in 0.3 M NaCl, 0.03 M sodium citrate (*p*H 7.0). A portion of each sample was treated with 10 μg of pancreatic ribonuclease A per ml for 30 min at 24 C before acid precipitation.

cially important to determine how much of the base sequences in the virus genome are represented in the virion polymerase product. The minus strands which accumulate late in infection complement most of the genome (13). Presumably, a transcriptase involved in early messenger production might transcribe a region of the genome not represented by late messages.

Our data are consistent with the idea that both virion and cell enzymes use 50S plus strands as template, although this has not been proven. Ribonuclease-resistant material, probably representing complexes of template and newly synthesized product, sedimented mainly in the range of 30 to 50S. Template-product complexes would be expected to sediment slower than free 50S RNA; to be heterogeneous in size, depending on the number and size of product molecules present; and to be partially ribonuclease-sensitive, reflecting release of the 5′-ends of growing strands from the templates (5).

The enzyme from the microsomal fraction of infected cells would appear to be the transcriptase which functions to make the minus strands found late in infection. It is not ruled out that a viral RNA replicase is present in this relatively crude extract as well, although if present its activity must be considerably less than that of the transcriptase. The specific activity of our extract was 20- to 50-fold higher than that reported by Mahy

et al. (11) for the enzyme obtained from infected chick embryo fibroblasts. It may be that the more productive CEL cells (4) contain more enzyme. Incorporation by the fibroblast enzyme ceased by about 30 min (11), whereas the CEL cell enzyme made product at an accelerating rate for 3 hr in the present work. The CEL cell enzyme product might be larger than the 7S material we observed. The microsomal preparation probably contains nucleases which could have cleaved the product, but it is also possible that the preparation lacks factors needed for faithful transcription.

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