Analysis of the In Vitro Product of an RNA-Dependent RNA Polymerase Isolated from Influenza Virus-Infected Cells

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The products synthesized in vitro by an RNA-dependent RNA polymerase isolated from influenza virus-infected BHK21-F cells were analyzed by velocity sedimentation, annealing techniques, and acrylamide-agarose gel electrophoresis. Approximately 50% of the RNA synthesized in vitro remains associated with the 50 to 70S ribonucleoprotein complex containing polymerase activity; the remainder of the RNA polymerase product sediments heterogeneously with a peak at 13S. At least 90% of the in vitro product hybridizes with virion RNA. If polypeptides are labeled early in the growth cycle, both the P and NP polypeptides are detected in the ribonucleoprotein complex by acrylamide gel electrophoresis. The results suggest that the polypeptide composition and the products of the cell-associated RNA polymerase are similar to those of the RNA transcriptase associated with influenza virus particles.

We have recently described the purification of an RNA-dependent RNA polymerase from cytoplasmic extracts of influenza virus-infected cells (5). This enzyme activity is associated with a ribonucleoprotein (RNP) complex that sediments heterogeneously in sucrose gradients with a peak in the 50 to 70S region. Analysis of the RNP complex revealed that the morphology and polypeptide composition of the complexes are similar to those of the internal ribonucleoproteins of the virion. Several reports have described an RNA-dependent RNA polymerase activity associated with influenza virions (4, 11, 18). The virion-associated enzyme activity synthesizes only complementary RNA in vitro and it has been designated an RNA transcriptase (1). Disruption of the virion revealed that the RNA transcriptase is associated with the internal RNP of the virus (2).

To study the function of the RNA polymerase isolated from infected cells and its relationship to the virion-associated transcriptase, the in vitro products of the viral RNA polymerase isolated from infected cells were analyzed. The present results suggest that ~90% of the RNA synthesized in vitro is complementary to virion RNA, and that ~15% of the RNA template is involved in the process of RNA synthesis. All size classes of RNA appear to be represented by the in vitro RNA products. Further analysis of the polypeptide composition of the cell-associated enzyme complex is also reported.

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MATERIALS AND METHODS

Virus and cells. Growth of BHK21-F cells and the propagation of the A₁/WSN strain of influenza virus in MDBK cells were carried out as described previously (3, 8). Radioactively labeled virus was grown as described (6) and purified by polyethylene glycol precipitation, followed by isopycnic centrifugation in potassium tartrate gradients (10).

Isolation of viral RNA polymerase. Fractionation of cytoplasmic extracts by equilibrium sedimentation in discontinuous sucrose gradients, isolation of fraction 4, and further purification of the RNP complex containing RNA-dependent RNA polymerase by means of velocity sedimentation have been described (5).

RNA polymerase assay. The conditions of the in vitro reaction were described previously (5).

Chemicals and isotopes. [5-³H]uridine-5' triphosphate, [14C]uridine, and ultra-pure sucrose were obtained from Schwarz-Mann, Orangeburg, N.Y. [32P]uridine-5' triphosphate-α was obtained from New England Nuclear Corp., Boston, Mass. Unlabeled nucleoside triphosphates were obtained from P.L. Biochemicals, Inc., Milwaukee, Wis. Tris buffer was obtained from Sigma Chemical Company, St. Louis, Mo. Components for polyacrylamide gel electrophoresis were obtained from Canal Industrial Corporation, Rockville, Md. Agarose was obtained from Seakem through Bausch & Lomb, Rochester, N.Y. SSE is 0.15 M sodium chloride, 0.015 M sodium citrate, and 0.01 M EDTA. RSB is 0.01 M Tris (pH 7.4), 0.01 M potassium chloride, and 0.0015 M magnesium chloride.

RNA extraction and polyacrylamide gel electrophoresis. The RNA polymerase reaction mixtures and purified influenza virus were made 0.1 M in EDTA and 1% in SDS, and the samples were

dialyzed against 0.5% SDS in SSE overnight. RNA was extracted with phenol saturated with SSE for 10 min at room temperature. The aqueous and phenol phases were separated by centrifugation at $10,000 \times g$ in a Sorvall RC2B centrifuge. The extraction procedure was repeated twice and the samples were precipitated in 70% ethanol at -20°C overnight. Electrophoresis of the extracted RNA was performed in 2.4% acrylamide gels containing 0.5% agarose for 3.5 h at 5 mA per tube. The buffers and conditions for electrophoresis were the same as those described by Pons and Hirst (13).

RNA hybridization. RNA extracted from radioactively labeled samples or unlabeled purified influenza virus were precipitated in 70% ethanol and resuspended in distilled water. Samples containing from 500 to 1,000 counts/min in a volume of 10 μl were mixed with 10 μl of 4 M sodium chloride, 1 mM EDTA. Varying concentrations of unlabeled RNA extracted from purified virions were added to some samples (final concentration from 0 to 65 $\mu\text{g}/\text{ml}$). Distilled water was added in sufficient quantity to bring the total volume to 100 μl . Total acid-precipitable radioactivity in the unheated samples was determined after addition of bovine serum albumin (BSA) (200 μg). The samples to be annealed were sealed in capillary tubes and placed at 100°C for 3 min, 85°C for 2 h, and 68°C overnight, and cooled slowly to room temperature. To determine the total radioactivity remaining after annealing, BSA was added and acid-precipitable radioactivity was collected on Whatman GF/C glass fiber filters and washed extensively with 5% trichloroacetic acid. The extent of hybridization was determined by treating each sample with 100 μg of ribonuclease A per ml and 5 μg of T-1 ribonuclease per ml in $2 \times \text{SSE}$, at 37°C for 30 min. BSA (200 μg) was added and acid-precipitable radioactivity was determined. Duplicate samples were analyzed for radioactivity by liquid scintillation counting.

RESULTS

Polypeptides in ribonucleoprotein complexes with polymerase activity. We have previously shown that RNP complexes labeled with amino acids from 3 to 4 h postinfection contain only the NP polypeptide in detectable amounts (5). This labeling period had been selected because it corresponded to the time in the growth cycle when a significant increase in polymerase activity was first detected. To examine the possibility that a polypeptide might be synthesized earlier and incorporated into ribonucleoproteins, cells were labeled from 2 to 4 h and the RNP complexes were isolated. As shown in Fig. 1, under these labeling conditions, both the P and NP polypeptides are detected. The relative amounts of the two polypeptides are similar to their proportions in influenza virions. Therefore, the P polypeptide appears to be present in RNP complexes, but labeled preferentially during very early times in the growth

FIG. 1. Electropherogram of polypeptides of the RNP complex labeled early in the growth cycle. Influenza virus-infected BHK21-F cells were labeled with [^3H]leucine (5 $\mu\text{Ci}/\text{ml}$) from 2 to 4 h. Cytoplasmic extracts were prepared and fractionated as described in Materials and Methods. After lysis of fraction 4 with 1% NP40, the RNP complex was isolated by sedimentation in 15 to 30% (wt/vol) sucrose (RSB) gradients in an SW50 rotor at $165,000 \times g$ (R_{av}) for 90 min at 4°C . The radioactivity of a portion (50 λ) of each fraction was determined, and the peak fractions in the 50 to 70S region of the gradient were pooled. Influenza virions, labeled with ^{14}C -amino acid mixture and purified as described in Materials and Methods, were included as a reference marker. Polypeptides were separated by electrophoresis in a 10% acrylamide gel as described previously (6). The origin in this and subsequent figures is to the left.

cycle, which accounts for its lack of detection in our previous report (5).

Hybridization of the in vitro products with influenza virus RNA. To determine the extent to which the products synthesized in vitro are complementary to viral RNA, annealing experiments were performed with RNA synthesized at different times after incubation in vitro. The radioactive products were heat-denatured and annealed with either no additional unlabeled RNA or with varying concentrations of RNA extracted from unlabeled purified WSN virus. The results in Table 1 indicate a degradation of up to 14% of the total labeled RNA which occurs after incubation at 65°C overnight, suggesting that low levels of ribonuclease may be present in the preparations. The amount of RNA used to calculate the proportion annealed to WSN RNA was that amount of labeled RNA which remained acid precipitable after annealing. Since maximum hybridization occurred at concentrations greater than 20 $\mu\text{g}/\text{ml}$ (R. W. Compans and L. A. Caligiuri, manuscript in preparation), sufficient unlabeled WSN RNA was added to provide a final concentration of 65 $\mu\text{g}/\text{ml}$. Results indicate that 70 to 90% of the newly synthesized product contains base sequences complementary to WSN RNA. Compe-

TABLE 1. Hybridization of RNA synthesized *in vitro* and influenza virus RNA

Incubation time (min)	Counts per minute				Annealed to WSN RNA (%)
	Total before annealing	Total after annealing	Self annealed	WSN RNA 65 µg/ml	
20	175	174	21	124	71
60	298	282	40	234	82
120	528	464	65	354	76
360	468	400	57	360	90

tition experiments using either ribosomal RNA from S3 HeLa cells or poliovirus RNA did not alter the extent of hybridization. The extent of hybridization is similar to that described previously for products of RNA polymerase preparations isolated from influenza virus-infected cells (15) and for products of virion-associated RNA-dependent RNA polymerase (1). Since the intracellular RNA polymerase reported here synthesizes predominantly complementary RNA, it may be termed an RNA transcriptase.

Polyacrylamide gel electrophoresis of the products synthesized *in vitro*. To determine the size of the RNA synthesized *in vitro*, analysis by acrylamide-agarose gels was performed. ^{14}C -labeled viral RNA was used as a reference marker, and the RNA synthesized *in vitro* was labeled with ^3H UTP. Electrophoresis of the phenol-extracted RNA product shows a bimodal distribution with a large peak of RNA migrating slower than, and a smaller peak migrating faster than, viral RNA (Fig. 2A). After heating and rapid cooling, the electrophoretic mobility of the *in vitro* product increases and the bulk of RNA migrates faster than viral RNA (Fig. 2B). Ribonuclease resistance of the native product before heat-denaturation in this experiment was 70%, and after denaturation and fast cooling ribonuclease resistance fell to 1%. These results indicate that most of the RNA synthesized *in vitro* is double stranded after phenol extraction. Furthermore, after denaturation most of the RNA synthesized *in vitro* in this experiment migrated more rapidly than the smallest virion RNA segment.

To determine whether a change in the size of the RNA product was observed at different times after incubation, the products were analyzed by acrylamide-agarose gel electrophoresis. RNP complexes were labeled with ^3H uridine in infected cells and incubated with an RNA polymerase assay mixture containing ^{32}P UTP, and the RNA was extracted. The results were normalized to yield a uniform amount of ^3H RNA. The pattern of migration for the RNA species labeled in infected cells is similar to that described for RNA extracted from purified virions, as shown in Fig. 2, and reported by

FIG. 2. Electropherogram of *in vitro* product before and after denaturation. After a 4-h incubation *in vitro* of the fraction 4 pellet with a reaction mixture containing ^3H UTP, the RNA was extracted as described in Materials and Methods. One half of the extracted RNA was denatured at 100 C for 3 min and quick-frozen in an alcohol-dry-ice bath. The samples were mixed with ^{14}C -labeled RNA extracted from purified virions before electrophoresis on acrylamide-agarose gels. A, Before denaturation; B, after denaturation.

others (2). There is an increase in the amount of product synthesized *in vitro* from 40 min (Fig. 3A) to 320 min (Fig. 3D), as indicated by the change in scale of ^{32}P -radioactivity. The first three samples taken at 40, 60, and 120 min, respectively, showed a distinct bimodal distribution of the ^{32}P RNA product (Fig. 3A-C). By 320 min after incubation, there was an increase in the amount of ^{32}P RNA in the intermediate range between the fast and slow migrating RNA product, and many of the peaks of ^{32}P RNA

FIG. 3. Electropherograms of prelabeled RNA and product RNA synthesized after different periods of incubation *in vitro*. Infected cells were labeled with [^3H]uridine (10 $\mu\text{Ci/ml}$) from 4 h to 5 h postinfection and harvested at 5 h. Cytoplasmic extracts were prepared and fractionated as described in Materials and Methods. The pellet of fraction 4 was incubated *in vitro* with a reaction mixture containing [^{32}P] α -UTP for different periods. RNA was extracted and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. A, 40-min incubation; B, 60-min incubation; C, 120-min incubation; D, 320-min incubation. Symbols: ●, [^3H]RNA labeled *in vivo*; ○, [^{32}P]RNA labeled *in vitro*.

coincide with the [^3H]RNA peaks (Fig. 3D). Furthermore, there was a constant increase in the amount of [^{32}P]RNA in the slower migrating peaks (Fig. 3A to D), whereas the RNA in the faster migrating peaks appeared to be labeled preferentially after short periods of incubation (Fig. 3A and B). Although this may suggest a difference in the time course of appearance of these products, further investigation will be necessary to establish the significance of this finding. The [^{32}P]RNA synthesized *in vitro* was 70 to 80% ribonuclease resistant after phenol extraction, which suggests that most of the product is double stranded and is consistent with the results of acrylamide gel electrophoresis.

To determine the proportion of prelabeled RNA involved in RNA synthesis *in vitro*, the amount of prelabeled RNA that became ribonuclease resistant after incubation at 33.5 C was measured. The amount of radioactivity that was ribonuclease resistant increased from 118 counts/min at 0 time to about 300 counts/min after 4 h of incubation; the rate of increase was fastest during the first 60 min (Table 2). There

TABLE 2. Ribonuclease-resistant prelabeled RNA after incubation *in vitro*^a

Incubation time (min)	Total counts/min	RNase-resistant counts/min	RNase-resistant (%)
0	2,003	118	5
20	1,975	152	8
60	1,526	192	13
120	1,679	218	13
240	1,915	294	15

^a Infected cells were labeled with [^3H]uridine (10 $\mu\text{Ci/ml}$) from 4 to 5 h postinfection and harvested at 5 h. Fraction 4 was isolated as described in Materials and Methods and incubated *in vitro* for different periods. RNA was extracted from the reaction mixtures, and resistance to ribonuclease A and T-1 ribonuclease was determined as described in Materials and Methods.

was a corresponding increase in the proportion of double-stranded RNA from 5 to 15%. These results suggest that as much as 15% of the prelabeled RNA may be involved in RNA synthesis *in vitro*.

Association of the RNA polymerase prod-

ucts with ribonucleoprotein complexes. Previous results have shown that the RNP complex isolated from infected cells sediments heterogeneously with a broad peak of 50 to 70S, which coincides with the distribution of RNA polymerase activity (5). To determine the distribution of RNA after *in vitro* incubation, the RNA polymerase reaction mixture was analyzed by velocity sedimentation. Infected cells were labeled with [^3H]uridine for 15 min, and fraction 4 was isolated and incubated in an RNA polymerase reaction mixture containing [^{32}P]UTP. At the end of the incubation period, the entire reaction mixture was treated with 0.1 volume of 10% Nonidet P-40 and analyzed in a 15 to 30% sucrose gradient (Fig. 4). RNA labeled with [^3H]uridine in infected cells shows a broad distribution throughout the middle of the gradient, and appears to reflect several size classes of RNP structures. This sedimentation pattern is similar to that of RNPs from virions (7, 12) and it is therefore apparent that the RNP-complex

is not dissociated *in vitro*. In contrast, the ^{32}P -polymerase product sediments in a bimodal pattern. There is a sizeable peak of [^{32}P]RNA near the top of the gradient which sediments heterogeneously with a peak at approximately 13S. Only a small amount of the [^3H]RNA is detected in the 13S region. The [^{32}P]RNA at 13S represents RNA synthesized *in vitro* which is released from the RNP complex and constitutes between 40 to 50% of the total product made. Although the remainder of the product sediments as a broad peak in the region of the RNP complex, the major peak of ^{32}P -product is skewed toward the more slowly sedimenting ^3H -RNP from infected cells. These results suggest that approximately 50% of the product synthesized *in vitro* remains associated with the RNP structures isolated from infected cells, and the remainder is released 13S RNA. Hybridization with viral RNA showed that 80 to 90% of the released or complex-bound product contained base sequences that are complementary to WSN RNA.

FIG. 4. Velocity sedimentation of prelabeled ribonucleoprotein structures with RNA synthesized *in vitro*. Conditions for labeling infected cells and isolation of fraction 4 were the same as described in Fig. 3. *In vitro* incubation was for 3.5 h with a reaction mixture containing [^{32}P]α-UTP. Sedimentation was in 15 to 30% (wt/vol) sucrose (0.5× SSE) gradient in an SW41 rotor at $140,000 \times g$ (R_{av}) for 16 h at 4°C. Symbols: ●, [^3H]RNA labeled *in vivo*; ○, [^{32}P]RNA labeled *in vitro*.

It was important to determine the nature of the released and complex-associated RNA synthesized *in vitro*. The degree of double strandedness of the released and complex-bound RNA was estimated by ribonuclease digestion. In this experiment, only the RNA synthesized *in vitro* was labeled and the reaction mixture was sedimented in sucrose gradients after detergent treatment. The acid-precipitable radioactivity of alternate fractions was determined with and without ribonuclease digestion. Figure 5 shows a bimodal distribution of the RNA product similar to that seen above. There is a significant difference in the proportion of ribonuclease resistance of the released and complex-bound RNAs. Before phenol extraction, less than 30% of the RNA associated with the RNP complex is ribonuclease resistant. In contrast, the ribonuclease resistance of the released 13S product is variable, from 30 to 100% resistant. This variability suggests that the 13S peak may be heterogeneous, containing a mixture of single-stranded and double-stranded RNAs. Furthermore, these results suggest that the RNA product synthesized *in vitro* is either partially double stranded before phenol extraction, or protected by protein. Results of experiments in which viral proteins were labeled showed that less than 3% of the viral proteins associated with the RNP complex were released during RNA synthesis *in vitro*.

DISCUSSION

Previous studies have indicated that RNA-dependent RNA polymerase activity in the

FIG. 5. Ribonuclease-sensitivity of *in vitro* product before phenol extraction. Fraction 4, isolated 5 h postinfection as described in Materials and Methods, was incubated *in vitro* for 4 h with a reaction mixture containing [3 H]UTP. Sedimentation conditions were the same as described in Fig. 4. A portion (25 λ) of each fraction was treated with RNase (100 μ g/ml in $2\times$ SSE) for 30 min at 37 C. Symbols: ●, before RNase treatment; ○, after RNase treatment.

cytoplasm of influenza virus-infected cells is associated with RNP complexes similar in morphology to RNPs isolated from influenza virions (5). Under the labeling conditions described here, the polypeptide composition of the cell-associated RNP complexes is similar to that described for the virion RNPs containing RNA polymerase activity, which contain the P and NP polypeptides (2). Preferential labeling of P in whole infected cells has also been observed by Skehel (19) at early times during the growth cycle. However, the lack of detectable P in RNP complexes labeled from 3 to 4 h as reported previously (5) is not due to lack of synthesis of

this polypeptide, since crude cell fractions labeled under these conditions contained significant amounts of this polypeptide. It appears, therefore, that P is incorporated slowly into RNP structures.

The present results also indicate that the *in vitro* product of this RNA polymerase is similar to that of the RNA polymerase contained within influenza virions (1, 18). Others have obtained similar results with viral RNA polymerase present in microsomal fractions (16, 20), although one report has described an RNA polymerase which synthesized RNA similar to virion RNA in base composition (14). These workers used a different strain of virus and prepared cell extracts later in the growth cycle. Our results suggest that the cytoplasmic RNP complexes with polymerase activity are viral RNPs which have not been incorporated into virions.

From 50 to 60% of the RNA synthesized *in vitro* remained associated with the RNP complexes after prolonged incubation. This high level suggests a limited degree of completion of RNA transcripts. Either the process of initiation or completion of RNA chains may require factors which do not function in the *in vitro* system, and this may account for the relatively low levels of RNA synthesis observed with influenza viral RNA polymerase preparations. The lack of coincidence in the sedimentation profiles of template and product RNA species associated with RNP complexes has also been observed with virion RNP complexes (2). This observation may reflect the fact that slower-sedimenting RNP species contain smaller RNA species (7, 12), whereas the amount of RNA synthesized *in vitro* by each size class of RNP complex may depend on factors other than the size of the template.

The finding of a high level of RNase resistance of the *in vitro* product after phenol extraction indicates that it is predominantly a double-stranded structure. However, this may be in part due to the extraction procedure, since before phenol extraction less than 30% of the product RNA bound to the RNP complex was resistant to RNase. The higher level of RNase resistance in the released 13S product species may result from the presence of low levels of RNase in the cytoplasmic fractions, which may preferentially degrade released single-stranded product species. The mechanism by which RNase-resistant structures are released from RNP complexes is unclear.

Analysis of the RNA product by gel electrophoresis also suggests that it is predominantly double stranded after phenol extraction, migrating more slowly than single-stranded viral RNA. After heating and rapid cooling, this

material was converted to a form which migrated more rapidly than viral RNA, probably representing incomplete single-stranded transcripts. With prolonged incubation, there was a constant increase in the amount of RNA migrating slower than viral RNA in acrylamide gels.

The RNA product of polymerase preparations we have obtained, as well as those obtained by others (16, 20), is complementary to virion RNA. Most of the virus-specific RNA synthesized in infected cells is virion-type RNA (17). Furthermore, the synthesis of complementary RNA appears to cease after 4 h postinfection (9). Thus the RNP complexes we have isolated may be inactive *in vivo*, and a different structure which remains to be identified may be involved in the synthesis of virion RNA. Alternatively, the activity of the RNA polymerase may be altered *in vitro*, and the RNP complexes may contain structures which are involved in the synthesis of virion RNA, as well as complementary RNA in infected cells.

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