In Vitro Product of a Ribonucleic Acid Polymerase Induced by Influenza Virus

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The ribonucleic acid (RNA)-dependent RNA polymerase induced in the microsomal fraction of cells infected with influenza virus synthesized a mixture of single- and double-stranded RNA *in vitro*. The single-stranded RNA sedimented mainly in the 8S region on sucrose density gradients, with a smaller proportion of the RNA sedimenting at 18S. This sedimentation pattern corresponds closely to that of incomplete influenza virus RNA. The double-stranded RNA formed *in vitro* sedimented at 11S, but molecules which may be replicative intermediate, sedimenting at 14 to 20S, were also detected in the *in vitro* reaction product. Similar species of RNA were detected *in vivo* by pulse-labeling infected cells at the time of polymerase harvest, but the proportion of each RNA species was different, most of the RNA being single-stranded and sedimenting in the 18S region. An 11S double-stranded RNA was also synthesized *in vivo*. Pulse chase analysis of the double-stranded RNA synthesized *in vitro* showed that most is stable, and only a small proportion turns over during the reaction. A proportion of the RNA formed *in vitro* could be annealed to RNA formed in infected cells and to RNA extracted from purified virus.

The replication of most single-stranded ribonucleic acid (RNA) viruses is accompanied by the induction of RNA-dependent RNA polymerase activity in the cytoplasm of the host cell (27, 48). The multiplication of these viruses does not appear to involve functional host cell deoxyribonucleic acid (DNA) and proceeds normally in cells which have been treated with inhibitors of DNA-dependent RNA synthesis, such as actinomycin D (40). Influenza virus represents an anomaly in that host cell DNA does appear to play an essential role in replication, at least during the first 2 hr after infection (5, 8, 10, 41, 49). Subsequently, however, RNA-dependent RNA polymerase activity which is insensitive to actinomycin D *in vitro* can be detected in the infected cells (21, 42-45); the enzyme is not induced if virus replication is inhibited by actinomycin D (21, 25, 44). RNA polymerase activity increases significantly in the nuclear cell fraction but reaches much higher levels in the microsomal fraction (44, 45).

If the microsomal RNA polymerase is involved in the replication of influenza virus RNA, the product of enzyme activity *in vitro* should resemble RNA synthesized in infected cells *in vivo*. In this study, we have compared RNA synthesized *in vitro* by a microsomal preparation from cells infected with avian influenza virus with

RNA extracted either from infected cells or from purified virus. A considerable proportion of the polymerase product RNA was found to be partially resistant to ribonuclease. A preliminary account of some of these results has already been published (26).

MATERIALS AND METHODS

Cells and virus. Monolayer cultures of chick embryo fibroblast cells, prepared as previously described (10), were grown on the inside surface of 2.5-liter glass bottles maintained at 37°C on a roller apparatus providing 0.7 rev/min. Confluent cell sheets comprising about 10^8 cells per bottle were formed by 24 to 48 hr.

Influenza virus (fowl plague virus, Rostock strain) was used as previously described (10). For infection, the growth medium was discarded and the cells were washed with phosphate-buffered saline (PBS) and then exposed to influenza virus [5 to 10 plaque-forming units (PFU) per cell] in 20 ml of PBS containing 0.1% gelatin. After 30 min at 37°C, the virus suspension was discarded, and 50 ml of medium (199 containing 5% calf serum) was added to each bottle. Zero time corresponded to the time of addition of fresh growth medium after infection.

Enzyme preparation. Unless otherwise stated, the microsomal fraction from cells which had been infected with influenza virus for 6 hr was used as the enzyme source. After removing the medium, cell sheets were washed briefly in ice-cold 0.9% NaCl (saline) and then removed from the glass surface in a

further 20-ml amount of saline per bottle by gentle agitation with glass beads. All subsequent procedures were carried out at 0 to 2 C.

The cells were collected by centrifugation at $400 \times g$ for 10 min and resuspended in B4 buffer [10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4; 10 mM NaCl; 1.6 mM $MgCl_2$; 1 mM $CaCl_2$; and 1 mM triethanolamine]. After 10 min, the cells were disrupted with a Dounce homogenizer, and the nuclei were separated by centrifugation at $600 \times g$ for 15 min. The supernatant was centrifuged at $12,000 \times g$ for 10 min to remove mitochondria and cell debris, and finally centrifuged at $105,000 \times g$ for 1 hr to sediment the microsomal fraction. The surface of the microsomal pellet was rinsed in B2 buffer (10 mM Tris-hydrochloride, pH 7.4; 10 mM NaCl; and 1.6 mM $MgCl_2$), and the pellet was either stored for up to 1 week at $-70^\circ C$ or used immediately. Storage in this manner did not significantly affect enzyme activity. Before addition to the polymerase reaction mixture, the microsomes were resuspended in an appropriate volume of B2 buffer to a protein concentration of 2 to 6 mg/ml.

Polymerase assay. The standard reaction mixture for RNA polymerase assay contained, per ml: Tris-hydrochloride (pH 8.0), 250 μ moles; $MgCl_2$, 2.5 μ moles; 2-mercaptoethanol, 25 μ moles; the 5'-triphosphates of adenosine and cytidine (ATP, CTP), 0.3 μ moles of each; and either uridine-5'-triphosphate (UTP), 0.3 μ moles, and radioactive guanosine-5'-triphosphate (3H -GTP, specific activity 1.3 Ci/mmole or ^{14}C -GTP, specific activity 36.6 Ci/mole), 5 nmoles, or GTP, 0.3 μ moles, and radioactive UTP (3H -UTP, specific activity 0.82 Ci/mole, or ^{14}C -UTP, specific activity 56 Ci/mole), 5 nmoles. For routine RNA polymerase assay, 0.3 ml of this reaction mixture was mixed with 0.1 ml of enzyme preparation, but, when the product of the reaction was to be studied, these quantities were increased 10-fold. The mixtures were incubated at 28 C for 30 min unless otherwise stated. All assays were made in duplicate.

Determination of acid-insoluble radioactivity. Samples were mixed with 1 ml of 0.125 M $Na_4P_2O_7$ containing bovine serum albumin (0.2%) as carrier, and acid-insoluble radioactivity was precipitated by addition of 2.5 ml of 1 N perchloric acid-1% hyflosupercel (34, 35). After standing at 0 C for 30 min, the precipitate was collected by centrifugation and washed twice with 4 ml of 0.5 N perchloric acid and once with 4 ml of 0.5 N trichloroacetic acid. Finally, the precipitate was resuspended in 0.2 ml of 0.5 N trichloroacetic acid, heated to 70 to 90 C for 30 min, cooled and then resuspended in 10 ml of Bray's scintillation fluid (11).

Assay for protein. Protein concentration was determined by the method of Lowry et al. (24) with bovine serum albumin as standard.

Preparation and extraction of ^{32}P -labeled influenza virus RNA. Chick embryo fibroblast cell monolayers were grown in 2.5-liter roller bottles as described above; at 24 hr, the growth medium was replaced with phosphate-free 199 medium, supplemented with 0.5% glucose and 0.5% lactalbumen hydrolysate and containing 50 μ Ci of carrier-free ^{32}P -orthophosphate per ml. At 48 hr, the cells were infected with influenza

virus at a multiplicity of infection of 10^{-1} to 10^{-2} PFU/cell. At 30 hr after infection, the medium was collected and centrifuged at $2,000 \times g$ for 10 min to remove cell debris; the virus was recovered by addition of formalinized fowl erythrocytes to a final concentration of 0.5%. After 30 min at 4 C, the erythrocytes were collected by centrifugation at $2,000 \times g$ and were washed twice with ice-cold 0.9% saline. The virus was eluted by stirring the erythrocytes in 8 ml of 0.9% saline at 37 C for 2 hr and was further purified on a 15 to 60% sucrose gradient (6). Peak tubes of virus, as assayed by hemagglutination, were pooled, and the virus was deposited by centrifugation at $90,000 \times g$ for 60 min at 4 C.

The virus was resuspended in 2 ml of Tris-ethylene-diaminetetraacetic acid (EDTA) buffer [0.01 M Tris-hydrochloride, 0.001 M EDTA, 0.1 M NaCl (pH 7.4)]; sodium dodecyl sulfate (SDS) was added to a final concentration of 2%, and the suspension was shaken for 5 min at 20 C. An equal volume of aqueous phenol was added, and the mixture was shaken at 4 C for 15 min; the phases were separated by centrifugation at $2,000 \times g$ for 10 min at 4 C. The aqueous phase was re-extracted once with an equal volume of aqueous phenol, and the RNA was precipitated in the presence of carrier yeast RNA (250 μ g) by the addition of two volumes of ethanol and 0.05 volumes of 3 M sodium acetate and storage at $-20^\circ C$ overnight.

For preparation of unlabeled influenza virus RNA, 11-day fertile eggs were infected with fowl plague virus and the allantoic fluid was harvested after 30 hr. Purification of the virus and extraction of viral RNA were carried out as described above.

Extraction of RNA from polymerase reaction mixture. Two methods were employed for purification of the RNA product of the polymerase reaction, phenol-cresol or phenol-SDS. RNA prepared by either method gave similar results.

For phenol-cresol extractions, the reaction mixture (4 ml) was cooled to 0 C and immediately supplemented with 0.2 ml of 1 M sodium acetate, 1.2 ml of bentonite (1%), 0.3 g of 4-aminosalicylic acid, and 7 ml of phenol-metacresol mixture (22). After stirring at 0 C for 30 min and centrifuging at $2,000 \times g$ for 10 min, the aqueous phase was extracted with an equal volume of 80% (v/v) aqueous phenol by shaking 30 min at 0 C. The resulting emulsion was centrifuged at $2,000 \times g$ for 10 min; the aqueous layer was removed and re-extracted with aqueous phenol as before. Two volumes of ethanol and 0.05 volumes of 3 M sodium acetate were added to the final aqueous extract, and the mixtures were stored at $-20^\circ C$ overnight. The precipitated material was washed twice in ice-cold ethanol before further analysis.

For phenol-SDS extraction, the reaction mixture (4 ml) was cooled to 0 C and immediately supplemented with 0.4 ml of 1 M sodium acetate, 0.5 ml of bentonite (1%), and 10 ml of ethanol. The precipitate, after standing at 0 C for 1 hr, was washed in ethanol, dissolved in 5 ml of 0.1 M sodium acetate, and mixed with 1 ml of SDS (4%) and 5 ml of 80% (v/v) aqueous phenol. The mixture was shaken for 1 hr at 0 C, and, after centrifugation at $2,000 \times g$ for 10 min, the aqueous phase was re-extracted with an equal

volume of aqueous phenol. RNA was precipitated from the final aqueous extract as for phenol-cresol extraction.

Extraction of RNA from infected cells. RNA was prepared from infected cell microsomes after labeling with ^3H -uridine as described in the appropriate experiments. The microsomal pellet was resuspended in 1 ml of B2 buffer, and the RNA was extracted by the phenol-cresol method described above.

Ribonuclease resistance. Samples of 25 to 100 μliters were diluted to 1 ml with twice concentrated standard saline citrate buffer ($2\times \text{SSC}$; $\text{SSC} = 0.15 \text{ M}$ sodium chloride, 0.015 M sodium citrate, $\text{pH } 7$) containing ribonuclease A to a final concentration of $1 \mu\text{g/ml}$. The mixtures were incubated at 28°C for 30 min, unless otherwise stated, before determination of acid-insoluble radioactivity.

Thermal denaturation. Aliquots of the RNA sample in 25 μliters of $0.1\times \text{SSC}$ or $2\times \text{SSC}$ buffer were heated in sealed glass ampoules for 10 min at the indicated temperatures and then rapidly cooled in a dry ice-ethanol bath. The samples were then diluted to 1 ml with $2\times \text{SSC}$ buffer for determination of ribonuclease-resistant acid-insoluble radioactivity.

Sucrose-gradient analysis of RNA. Linear 5 to 20% gradients of sucrose in 5 ml of Tris-EDTA buffer were prepared by the method of Britten and Roberts (12). The RNA sample dissolved in 0.1 ml of Tris-EDTA buffer was layered onto the gradient before centrifugation as described in the appropriate experiments. Ten-drop fractions (0.2 ml) were collected from the bottom of the tube, and, after measuring the optical density at 260 nm, one-half of each fraction was used to determine total and ribonuclease-resistant acid-insoluble radioactivity, respectively.

Annealing assay. Samples of RNA for annealing assay were mixed in sealed glass ampoules in a total volume of 50 μliters of $2\times \text{SSC}$ buffer. The ampoules were heated for 1 hr to 90°C and then slowly cooled to room temperature. The contents of the ampoules were quantitatively transferred to conical centrifuge tubes with 1 ml of $2\times \text{SSC}$ buffer and ribonuclease-resistance was assayed as described above. Controls were as detailed in the appropriate experiments, and all assays were carried out in duplicate.

Radioactivity counting. Samples were counted in 10 ml of Bray's scintillation fluid (11) with a Packard Tri-Carb liquid scintillation spectrometer, series 4000. The counting efficiencies in the presence of 0.2 ml of 0.5 N trichloroacetic acid were as follows. Channel 1 excluded ^3H and ^{14}C and counted ^{32}P at 71% efficiency. Channel 2 excluded ^3H and counted ^{14}C and ^{32}P at efficiencies of 25 and 13%, respectively. Channel 3 counted ^3H , ^{14}C , and ^{32}P at efficiencies of 14, 52, and 5%, respectively. Results for double- and triple-labeled samples were calculated from these efficiencies by computer.

Reagents. Actinomycin D was a gift from Merck and Co., Inc., Rahway, N.J. Ribonuclease A was obtained from Worthington Biochemical Corp., Freehold, N.J. ATP, 2-mercaptoethanol, SDS, and sodium deoxycholate were obtained from Sigma (London) Chemical Co. Ltd., and CTP, GTP, and UTP were from Calbiochem, Los Angeles, Calif. Bentonite

(British Drug Houses Ltd.) was purified by the method of Fraenkel-Conrat et al. (17). ^3H -uridine (27.6 Ci/mmol), ^3H -UTP (0.82 Ci/mmol), ^{14}C -GTP (36.6 Ci/mmol), ^{14}C -UTP (56 Ci/mmol), and sodium phosphate, ^{32}P (PBS-I), were obtained from the Radiochemical Centre, Amersham, Bucks. ^3H -GTP (1.3 Ci/mmol) was obtained from Schwarz Bio-Research Inc., Orangeburg, N.Y.

RESULTS

Appearance and properties of microsomal RNA polymerase activity. The localization of an RNA-dependent RNA polymerase in the microsomal fraction from influenza-infected cells has been described (21, 25, 44, 45). Table 1 shows some properties of the enzyme obtained from cells at 5 hr after infection. Incorporation of ^3H -GTP into acid-insoluble material by the microsomal fraction of infected cells was more than 10-fold higher than by a comparable fraction from uninfected cells. Enzyme activity was dependent on the presence of four nucleoside triphosphates in the reaction mixture. There was an absolute requirement for Mg^{2+} , and no significant activity was obtained in the presence of Mn^{2+} at similar concentration. The results of other studies demonstrated that 0.75 μmoles of Mg^{2+} per assay gave maximal activity and that the pH optimum was 8.0. In the presence of 20 μg of ribonuclease (50 μg per ml), net incorporation of ^3H -GTP was inhibited by 90% (Table 1).

RNA polymerase activity in the microsomal fraction of infected cells increased between 2 and 4 hr postinfection and reached a maximum at 6 hr (Fig. 1). In the late stages of infection, the activity declined, but even after 12 hr was still fourfold higher than in uninfected cells. Addition of 10 μg of actinomycin D to the reaction mixture was

TABLE 1. Requirements of the microsomal RNA polymerase

Reaction mixture	Amt of ^3H -GTP incorporated per mg of protein (pmoles)	
	Uninfected cells	Infected cells ^a
Complete ^b	1.4	20.1
Minus ATP, CTP, UTP.....	1.3	2.1
Minus Mg^{2+}	1.2	1.7
Minus Mg^{2+} , plus Mn^{2+} (0.75 μmole).....	1.3	2.3
Plus ribonuclease (20 μg).....	1.3	1.8

^a Microsomal fraction was prepared from cells uninfected or infected with influenza virus for 5 hr.

^b Complete reaction mixture was as described in Materials and Methods.

without effect on RNA polymerase activity (Fig. 1). In view of these results, all further experiments were carried out with the microsomal fraction from cells at 6 hr after infection as the source of influenza virus-induced RNA polymerase.

The rate of incorporation of ^3H -UTP into RNA by influenza virus-induced RNA polymerase *in vitro* is shown in Fig. 2. The reaction proceeded linearly for 60 min, and then the rate of RNA synthesis declined. The time at which the rate of incorporation decreased varied slightly with different microsomal preparations and appeared to depend on the stability of the enzyme *in vitro* rather than availability of substrate. The enzyme is less stable at 37 C, and reactions carried out at 37 C terminated more rapidly and reached completion within 20 to 30 min; for this reason, all studies on RNA synthesis by the en-

zyme *in vitro* were made at 28 C and during the linear period of the reaction.

Ribonuclease resistance of polymerase product.

Although the presence of ribonuclease in the reaction mixture inhibited enzyme activity completely (Table 1), when samples of the polymerase reaction mixture were removed at any time up to 45 min of incubation and treated with ribonuclease, only about 50% of the incorporated radioactivity was rendered acid-soluble (Fig. 2). Similar results were obtained when samples were treated with sodium deoxycholate to release any membrane-bound acid-insoluble radioactivity before ribonuclease treatment (36).

To confirm the ribonuclease-resistant nature of the polymerase product, RNA was extracted from the reaction mixture after 30 min and treated with various concentrations of ribonuclease. Samples of RNA extracted from an identical preparation of infected cell microsomes labeled from 3.5 to 6 hr with ^3H -uridine in the presence of actino-

FIG. 1. RNA polymerase activity in the microsomal fraction of influenza virus-infected cells. Chick embryo fibroblast cells, either uninfected or infected with influenza virus, were fractionated at various times after infection, and RNA polymerase activity of the microsomal fraction was determined as in Materials and Methods. Where shown, 10 μg of actinomycin D was added to the reaction mixture in a volume of 0.04 ml. Symbols: released hemagglutinin assayed in the medium at intervals ($\blacktriangle$); uninfected cells ($\bullet$, solid line); uninfected cells + actinomycin D ($\bullet$, broken line); infected cells ($\circ$, solid line); infected cells + actinomycin D ($\circ$, broken line).

FIG. 2. Time course of RNA-dependent RNA polymerase reaction, pulse chase analysis of double-stranded RNA *in vitro*. Duplicate reaction mixtures, each containing 1 ml of enzyme preparation and 3 ml of reaction mixture as described in Materials and Methods, were incubated at 28 C, and, at intervals, four 0.1-ml samples were removed from each and frozen in dry ice-ethanol mixture. To one of the reaction mixtures, 0.1 ml of cold UTP (1.2 $\mu\text{moles/ml}$) was added at 18 min. Duplicate samples were then analyzed for incorporation of ^3H -UTP into total or ribonuclease-resistant acid-insoluble material as in Materials and Methods. Symbols: ($\bullet$) continuous reaction, total incorporation; ($\circ$) ribonuclease-resistant incorporation; ($\blacktriangle$) reaction stopped by UTP, total incorporation; ($\triangle$) reaction stopped by UTP, ribonuclease-resistant incorporation. Each point is the average of duplicate samples.

mycin D and from ^{32}P -labeled purified influenza virus were similarly treated (Fig. 3). The polymerase product RNA was 40 to 50% resistant to ribonuclease, even at a concentration of 500 $\mu\text{g}/\text{ml}$, which confirms previous results obtained without deproteinization (Fig. 2). By comparison, labeled microsomal RNA from infected cells was much less resistant to ribonuclease, but 5 to 10% remained acid-insoluble after treatment. Very similar amounts of ribonuclease-resistant RNA have been found in chick fibroblast cells infected with PR8 or equine strain influenza virus by labeling infected cell RNA in the presence of actinomycin D (15, 30). Purified virus RNA was rendered completely acid-soluble by treatment with ribonuclease at a concentration of 10 $\mu\text{g}/\text{ml}$ or greater.

Thermal denaturation of polymerase product. The above results suggest that the polymerase product RNA consists of a mixture of single-

FIG. 3. Ribonuclease resistance of polymerase product RNA, influenza virus RNA, and virus-specific RNA from infected cells. Polymerase product ^3H -RNA was extracted from the reaction mixture by using phenol-cresol and ^{32}P -labeled influenza virus RNA was extracted from purified virus by using phenol-SDS, as in Materials and Methods. For preparation of virus-specific ^3H -RNA, chick fibroblast cells were infected with influenza virus, and actinomycin D (2 $\mu\text{g}/\text{ml}$) was added to the medium at 3 hr, followed by ^3H -uridine (27.6 Ci/mole, 1 $\mu\text{Ci}/\text{ml}$) at 3.5 hr. RNA was extracted from the cells by using phenol-cresol at 6 hr. Samples of the RNA preparations were treated with various concentrations of ribonuclease A for 10 min at 28°C in 1 ml of 2X SSC. Acid-insoluble radioactivity was then determined and results were expressed as a percentage of the radioactivity in samples not treated with ribonuclease.

stranded and double-stranded (ribonuclease-resistant) RNA. This was confirmed by determining the thermal transition profile of the RNA (18). The ribonuclease-resistant RNA from the polymerase product displayed a sharp thermal transition profile characteristic of double-stranded RNA. Furthermore, an increase in thermal transition temperature was observed when the RNA was melted in higher salt concentration (Fig. 4), which is further evidence that the RNA is double-stranded (9).

Pulse chase analysis of double-stranded RNA formed in vitro. It has been reported that influenza virus-specific replicative intermediate RNA formed in cells in the presence of actinomycin D behaves as a precursor of 18S virus-specific RNA. ^3H -uridine incorporated into double-stranded RNA during labeling for 10 min could be chased into single-stranded RNA following addition of excess unlabeled uridine to the cells (31). An experiment similar to this was carried out to determine whether the double-stranded RNA formed by influenza virus-induced microsomal RNA polymerase in vitro is an intermediate in viral RNA replication. At 18 min from the com-

FIG. 4. Thermal denaturation of ribonuclease-resistant polymerase product RNA at different salt concentrations.

mencement of a polymerase reaction, excess unlabeled UTP was added to half the reaction mixture, and subsequent labeling of both single- and double-stranded RNA was determined (Fig. 2). Addition of unlabeled UTP completely inhibited further incorporation of ^3H -UTP into RNA in each case. Ten to twenty per cent of the tritium label was lost from double-stranded RNA over a period of 15 to 20 min from the time of addition of unlabeled UTP, indicating that only a small proportion of the double-stranded RNA formed in vitro under these conditions is converted into single-stranded RNA during the reaction. When this experiment was repeated, adding unlabeled UTP earlier, at 9 min from the start of the reaction, essentially the same result was obtained.

Sucrose-gradient analysis of the RNA polymerase product. The sedimentation characteristics of polymerase product RNA on sucrose gradients are shown in Fig. 5. The bulk of the RNA formed

in vitro sedimented in the 8S region of the gradient, and a minor component was found at 18 to 20S. This sedimentation profile of polymerase product RNA was consistently observed whether the RNA was extracted by the phenol-cresol or the phenol-SDS method.

When sucrose gradient fractions were treated with ribonuclease and the remaining acid-insoluble radioactivity was determined (Fig. 5), the bulk of the ribonuclease-resistant RNA was seen to sediment at 12S, with a rather broad spread in the 14 to 20S region. This spread of ribonuclease-resistant material was not seen if the polymerase product RNA was treated with ribonuclease before centrifugation; in these conditions, all of the ribonuclease-resistant material banded sharply at 11 to 12S (Fig. 5).

Sucrose-gradient analysis of RNA formed in infected cells. The above results suggest that the microsomal RNA polymerase from influenza virus-infected cells synthesizes in vitro a mixture of single- and double-stranded RNA, the components of which correspond in sedimentation characteristics to RNA species which have been reported to occur in cells infected with other influenza virus strains. To provide a more direct comparison between the actions of the polymerase in vivo and in vitro, infected cells were exposed to actinomycin D at 5.5 hr and then pulse labeled with ^3H -uridine for 15 min at 6 hr after infection, when RNA polymerase was normally harvested from the cells. After cell fractionation, RNA was extracted from the microsomes and analyzed on a 5 to 20% sucrose gradient (Fig. 6). The bulk of the microsomal RNA labeled in vivo under these conditions sedimented in a rather broad peak in the 18S region. In addition, a peak of ribonuclease-resistant RNA occurred at 11S and comprised not more than 20% of the total radioactivity.

Characterization of polymerase product RNA by annealing experiments. If the product of influenza virus-induced RNA polymerase is virus-specific, it should contain base sequences complementary to virus RNA on one of the two RNA strands synthesized in vitro. Preliminary experiments in which ^3H -labeled polymerase product RNA was annealed to ^{32}P -labeled influenza virus RNA indicated that unlabeled RNA species present in the mixture were competing in the hybridization reaction (25). These species presumably include unlabeled viral plus and minus strands present in the microsomal enzyme preparation at the time of harvest. To obviate this difficulty, virus-specific RNA synthesized from 3.5 to 6 hr after infection was labeled with ^3H -uridine in the presence of actinomycin D. The microsomal fraction was harvested in the usual way, and one-half of the preparation was used to synthesize a

FIG. 5. Sucrose gradient characterization of polymerase product RNA. Polymerase product ^3H -RNA was extracted from the reaction mixture by using phenol-SDS as in Materials and Methods. A sample of the RNA was sedimented in a 5 to 20% linear gradient of sucrose in Tris-EDTA buffer for 150 min at 50,000 rev/min in a Spinco SW 50 rotor, and fractions were analyzed for total (●) and ribonuclease-resistant (●) acid-insoluble radioactivity as in Materials and Methods. Another sample was treated with ribonuclease (1 $\mu\text{g}/\text{ml}$, 28 C, for 10 min in SSC buffer) before centrifugation and analysis for total acid-insoluble radioactivity as above (○). The S values were calculated from the optical density peaks of 28S and 18S microsomal RNA. The direction of sedimentation is from right to left.

FIG. 6. Sucrose gradient characterization of RNA formed in infected cell microsomes. Chick fibroblast cells were infected with influenza virus (10 PFU per cell), and, at 5.5 hr after infection, actinomycin D (2 μ g/ml) was added to the medium. At 6 hr, 3 H-uridine (27.6 Ci/mmol, 10 μ Ci/ml) was added, and after 15 min the cells were harvested and fractionated as in Materials and Methods. RNA was extracted from the microsomal fraction by using phenol-cresol and analyzed on a linear 5 to 20% sucrose gradient as in the legend to Fig. 5. The direction of sedimentation is from right to left. Symbols: total acid-insoluble radioactivity ($\circ$); ribonuclease-resistant acid-insoluble radioactivity ($\bullet$).

14 C-labeled RNA product in vitro. Virus-specific RNA synthesis in the cytoplasm of influenza-infected cells becomes insensitive to actinomycin D from 3 hr, (15) and in this way more than 90% of the virus-specific RNA present at the time of harvest was 3 H-labeled, and all RNA synthesized by the polymerase was 14 C-labeled. The microsomal preparation not used as enzyme served as a control from which RNA was extracted in parallel with the extraction of polymerase product RNA. 32 P-labeled influenza virus RNA was then used for annealing studies (Table 2).

Heating 32 P-influenza virus RNA followed by slow cooling did not result in significant self-annealing. However, when mixed with virus-specific 3 H-RNA, there was a significant increase in ribonuclease-resistance of both RNA forms (Table 2), confirming that some minus strands are present in infected cell microsomes, although the predominant RNA species synthesized in vivo consists of plus strands.

Sixty per cent of the polymerase product RNA

was ribonuclease-resistant, and this material also contained virus-specific 3 H-RNA which was 22% ribonuclease-resistant. Since the percentage of ribonuclease-resistant 3 H-RNA was identical to that found in microsomes not used as an enzyme source (Table 2), it is likely that 14 C-RNA synthesized in vitro is genuinely double-stranded and not annealed to RNA already formed in the infected cell. However, if the microsomal polymerase product RNA was heated so as to cause strand separation and then reannealed, there was an increase in ribonuclease-resistance of 3 H-RNA from 22 to 83% and of 14 C-RNA from 60 to 75%. The conclusion from this experiment is that much of the RNA synthesized in vitro is complementary to RNA already formed in the infected cells. Since infected cell RNA labeled under the conditions of this experiment is mainly plus strand (15, 30), the predominant RNA species synthesized in vitro must be minus strand. Further evidence for this conclusion was obtained by annealing experiments in which influenza virus 32 P-RNA was included in the mixture before heating and slow cooling (Table 2). This caused a

TABLE 2. Annealing between polymerase product RNA, virus-specific RNA, and influenza virus RNA^a

RNA source	Treatment	Ribonuclease-resistant counts/min (%)		
		14 C	3 H	32 P
Influenza virus	None			0.6
Influenza virus	Annealed			3
Infected cell microsomes	None		22	
Influenza virus + infected cell microsomes	Annealed		36	37
Microsomal polymerase product	None	60	22	
Microsomal polymerase product	Annealed	75	83	
Influenza virus + microsomal polymerase product	Annealed	68	68	40

^a Chick fibroblast cells were infected with influenza virus, and virus-specific RNA which formed from 3.5 to 6 hr post-infection was labeled with 3 H-uridine in the presence of actinomycin D as in the legend to Fig. 3. At 6 hr, the microsomal fraction was prepared as in Materials and Methods and divided into two equal portions; one was used for extraction of RNA directly, and the other portion first reacted in vitro for 30 min with a polymerase reaction mixture as in Materials and Methods, with 14 C-UTP as the labeled nucleoside triphosphate. RNA was then extracted from both portions and from purified 32 P-labeled influenza virus by phenol-SDS as in Materials and Methods. Annealing was carried out as in Materials and Methods, and the results were analyzed by computer by using efficiencies of counting for each isotope obtained under experimental conditions of quenching. Each result is the average of duplicate determinations and is expressed as a percentage of radioactivity before ribonuclease-treatment.

decrease in the proportion of ribonuclease-resistant ^3H -RNA from 83 to 68% and of ^{14}C -RNA from 75 to 68%. Thus viral ^{32}P -plus strands compete in the annealing reaction for ^3H - and ^{14}C -minus strands, thereby rendering ribonuclease-sensitive a proportion of both ^3H - and ^{14}C -plus strands.

The total proportion of in vitro product RNA complementary to viral RNA was quantitatively determined by annealing in the presence of saturating concentrations of unlabeled viral plus strands (Fig. 7). As much as 55% of ^3H -labeled product RNA could be annealed to viral RNA under these conditions, showing that not more than 45% of the in vitro product is viral plus strand.

DISCUSSION

The experiments described here are concerned with the nature of the RNA polymerase which is induced in the microsomal fraction of cells infected with influenza virus (21, 42-45). Conditions of enzyme reaction in vitro were chosen such that RNA synthesis proceeded linearly for at least 60 min, and all studies were made with enzyme prepared 6 hr after infection when polymerase activity was found to be maximal.

There have been conflicting reports concerning the nature of the RNA synthesized by this enzyme in vitro. Scholtissek (43) reported that 85 to 100% of the product was single-stranded RNA of the minus type, i.e., with a base sequence complementary to parental virus RNA. Using a dif-

ferent cell system, Ruck et al. (42) found that influenza A2-induced RNA polymerase synthesized mainly viral plus strand RNA, as well as some RNA which was resistant to ribonuclease.

Our data clearly show that influenza virus-induced RNA polymerase synthesizes both plus and minus strand RNA in vitro, and up to 60% of the RNA product is in the form of ribonuclease-resistant double-stranded RNA. In this respect, the enzyme induced by influenza virus functions in vitro in a similar manner to the RNA polymerases induced by foot-and-mouth disease virus (1), mengovirus (36), poliovirus (47), Sindbis virus (46), and the bacteriophage Q β (33), all of which synthesize a mixture of single- and double-stranded RNA. Prelabeling the RNA present in the microsomal fraction used as enzyme source shows that RNA synthesized during the in vitro reaction is genuinely double-stranded and not annealed to RNA synthesized in the cell before harvest. However, intracellular virus-specific RNA can be annealed to polymerase product RNA by heating to 90 C and slow cooling in vitro, and, since the predominant RNA species synthesized in vivo is plus strand (15, 30), we conclude that, among the single-stranded RNA synthesized in vitro, complementary RNA species are present in excess. Even so, of the total RNA synthesized in vitro, as much as 40% appears to be of the parental plus type.

Sedimentation of the polymerase product RNA on sucrose density gradients shows that it consists of heterogeneous RNA species ranging from 8S to 21S, in agreement with previous reports (42, 45). The RNA extracted from purified preparations of influenza virus is also heterogeneous (7, 15). It is currently believed that influenza virus RNA exists in the mature virus particle as sub-genomic fragments (13, 20) which can be partially resolved into three or four components by sucrose gradient analysis (6, 15, 29) or five by polyacrylamide gel electrophoresis (7, 14, 38). These components range in sedimentation value from 7 to 8S for the smallest RNA species to 21S for the largest, and in standard virus preparations the major RNA species sediments at 18S (6, 15, 29). However, the relative proportion of the various RNA species is different in noninfectious von Magnus-incomplete virus grown from high input multiplicity (28, 39) and in "late yield" influenza virus, i.e., virus harvested after 48 hr of growth from low input multiplicity in tissue culture (7). In both of these cases, the small-molecular-weight RNA species (8S) are present in a greater proportion relative to the large RNA molecules (18 to 21S), and we found a very similar RNA profile for the microsomal polymerase product. Production of small molecular weight

FIG. 7. Annealing between polymerase product RNA and influenza virus RNA. Samples (25 μ liters) of ^3H -labeled polymerase product RNA in $2\times$ SSC were mixed with 25 μ liters of $2\times$ SSC containing amounts of unlabeled influenza virus RNA to give final concentrations as indicated. Annealing was carried out as in Materials and Methods, and the results are expressed as a percentage of the total acid-insoluble radioactivity of polymerase product RNA added to each ampoule (928 counts/min). Each point is the mean of duplicate determinations.

RNA species in vitro does not seem to be the result of conditions of infection in vivo, since RNA extracted from infected cell microsomes at the time of enzyme harvest contained predominantly high-molecular-weight RNA species.

The ribonuclease-resistant RNA was found in fractions sedimenting at 10 to 20S, and probably includes structures containing both double- and single-stranded RNA. Such replicative intermediate RNA, i.e., double-stranded RNA with attached nascent single strands (3), has also been found in influenza-infected cells (31). When polymerase product RNA was digested with ribonuclease before centrifugation, to remove all single-stranded regions, it then banded sharply at 11S. A similar species of RNA was present in the microsomal RNA labeled in vivo and has also been found in cells infected with other influenza viruses (30, 37). The melting temperature of the double-stranded RNA from the polymerase product was 84°C in $0.1 \times$ SSC, which agrees well with that of ribonuclease-resistant 12S RNA obtained by self-annealing of influenza virus-specific RNA, which was 83°C at the same salt concentration (32).

Pulse chase analysis of the ribonuclease-resistant RNA formed in vitro indicated that only a small proportion of double-stranded RNA turned over during the reaction. It has been reported that mengovirus-induced RNA polymerase synthesizes two types of double-stranded RNA in vitro, one of which can be converted into a ribonuclease-sensitive form by mild heat treatment: only the latter RNA species is converted into single-stranded RNA during the in vitro reaction (36). Similar experiments with influenza virus-induced RNA polymerase gave no evidence that such a species of low-melting RNA was present in the enzyme product.

Double-stranded RNA appears to be synthesized in greater proportion in vitro compared to in vivo. There are several possible explanations for this. It has been suggested, for the Q β virus-induced RNA polymerase, that under in vitro conditions the enzyme may bind growing plus strands in close proximity, but not hydrogen-bonded, to the minus strands of the templates. Removal of the protein by phenol-SDS could give rise to the formation of a hydrogen-bonded duplex (16). However, such a mechanism would not explain the high proportion of ribonuclease-resistant RNA which we observed without deproteinization.

Double-stranded RNA might alternatively result from some side reaction in vitro that is not actively involved in the synthesis of viral RNA (36). An increase in ribonuclease-resistant RNA has been observed in poliovirus-infected cells

towards the end of infection (4), and it has been suggested that this represents the accumulation of nonfunctional template molecules (19).

Finally, two enzymes may be present in infected cells, one of which makes single-stranded and the other double-stranded RNA (23). The latter enzyme might be present in excess in the polymerase preparation used for these experiments. It is of interest that two polymerase-RNA complexes, one synthesizing single-stranded viral RNA and the other double-stranded RNA, have recently been separated in extracts from cells infected with foot-and-mouth disease virus (2).

In addition to the microsomal RNA-dependent RNA polymerase discussed here, increases in both DNA-dependent and RNA-dependent RNA polymerase activities are found in the nuclear fraction from cells infected with influenza virus (10, 25, 44, 45). Further studies are in progress to elucidate the role of the various polymerase activities in the replication of influenza virus.

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