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RNA-Dependent RNA Polymerase Activity Associated with Virions and Subviral Particles of Myxoviruses

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ABSTRACT ***In vitro* RNA synthesis is catalyzed by an enzyme found in purified virions of each of nine influenza strains tested. The extent of [^{3}H]UTP incorporation observed is strain dependent, with an obligate requirement for Mn^{2+} ions and RNA template. At least 30% of purified product RNA shows resistance to degradation by ribonuclease A and T1 before and after annealing with exogenous RNA from influenza virions.**

Infection of permissive animal cells with influenza virus results in the appearance of an RNA-dependent RNA polymerase (RNA nucleotidyltransferase EC 2.7.7.6) associated with microsomal and nuclear cell fractions (1). It is now established that RNA polymerase activity with a similar template dependence is structurally associated with purified particles of reovirus (2) and cytoplasmic polyhedrosis virus (3) containing double-stranded RNA and vesicular stomatitis virus (4) containing single-stranded RNA. Recently, virions of various RNA tumor viruses (5) and a nononcogenic RNA virus (6) were found to possess an RNA-dependent DNA polymerase activity that can be demonstrated *in vitro*. The foregoing observations prompted us to look for an enzyme in the influenza virion that can catalyze the synthesis of virus-specific RNA. This report describes the finding of such an enzyme in several myxovirus strains.

MATERIALS AND METHODS

Materials

ATP, CTP, GTP, and UTP (Boehringer–Mannheim Corp.); [^{3}H]UTP (New England Nuclear Corp.); Nonidet P40 (NP40) (Shell Chemical Co.), and Triton N-101 (Rohm and Haas); α-chymotrypsin (Boehringer–Mannheim Corp.), ribonuclease A and T1, deoxyribonuclease I (Worthington Biochemical Corp.); polyvinyl sulfate (General Biochemicals, Inc.); dextran sulfate (Pharmacia); actinomycin D and rifampicin were kindly supplied by Drs. J. Lampen and T. Kasai, respectively, of this Institute.

Viruses. The myxovirus strains listed in Table 2 were used in this study, as well as vesicular stomatitis virus (Indiana strain). The sources of these viruses and their propagation in embryonated eggs or primary cultures of chick-embryo fibroblasts has been described (7, 8). Influenza A, strain BEL, was provided by Dr. W. G. Laver.

Virus Purification. The method of Pons and Hirst (9) was used with slight modifications. Crude virus pools (250–500 ml), freed of gross cellular debris, were initially clarified by centrifugation at 10,000 $\times$ *g* for 10 min; virus was subsequently pelleted in a Spinco 42 rotor for 30 min at 95,500 $\times$ *g*. All further steps were as described (9). Purified influenza virus stocks in Tris·HCl buffer usually contained 1–2 mg of viral protein per ml [Lowry determination (10)] and a minimum of 10^6 hemagglutinating units per ml as measured by standard methods (7). Vesicular stomatitis virus was purified by a similar procedure (manuscript in preparation). All purified virus preparations were stored at 4°C or in a −70°C freezer.

Preparation of Subviral Particles. Viral "cores"†, stripped of surface glycoproteins, were obtained by the technique of Schulze (11). A purified suspension of WSN influenza virus, containing $10^{5.3}$ hemagglutinating units, was treated with chymotrypsin (1 mg/ml) for 15 hr at 37°C. Fractions of chymotrypsin-resistant particles were collected from glycerol gradients, passed through Sephadex G-50 columns to remove glycerol, and concentrated by sedimentation at 100,000 $\times$ *g* for 1 hr.

RNA Polymerase A ssays. The reaction mixture and conditions used for enzyme assay is described in the legend for Table 1. The method used for assay of RNA polymerase activity found in vesicular stomatitis virus has been described (4).

RESULTS

RNA polymerase activity of influenza virus strain WSN

The experimental conditions necessary for the detection of a nucleotide polymerase in particles of influenza virus evolved from studies with purified stocks of the WSN strain grown in chick-embryo fibroblasts. The following features of the system can be noted:

General Requirements for Enzyme Activity. Incorporation of [^{3}H]UTP into an acid-insoluble product occurs only if the reaction mixture contains virus particles, detergents such as Triton N-101 or Nonidet P40, all four ribonucleoside triphosphates, and Mn^{2+} ions (Table 1). Although not required, both $MgCl_2$ and 2-mercaptoethanol were included in the standard reaction mixture. The template for this reaction is RNA, as suggested by the finding that incorporation is completely blocked by ribonuclease, but not by deoxyribonuclease or inhibitors of DNA-dependent RNA polymerases. Polyanions (e.g., polyvinyl sulfate) strongly inhibit polymerase activity (Table 1).

† The term "core" denotes a chymotrypsin-resistant structure containing viral RNA and three carbohydrate-free proteins and lipids.

TABLE 1. *Requirements for in vitro RNA polymerase activity of influenza virus (WSN) particles*

Assay components	[^{3}H]UTP Incorporation (pmol/mg viral protein per hr)
Complete*	160
Minus Mg^{2+}	158
Minus Mn^{2+}	0
Minus 2-mercaptoethanol	143
Minus CTP and ATP	7
Minus GTP	0
Minus Triton N-101	0
Minus virus particles	0
Plus actinomycin D (2 μg)	147
Plus rifampicin (3 μg)	144
Plus polyvinyl sulfate (20 μg)	0
Plus dextran sulfate (20 μg)	8
Plus deoxyribonuclease I (20 μg)	130
Plus ribonuclease A (10 μg)	0

* The complete assay mixture contained the following components in a final volume of 0.2 ml: 10 μmol Tris·HCl buffer (pH 7.9), 1 μmol $MgCl_2$, 0.38 μmol $MnCl_2$, 0.5 μmol 2-mercaptoethanol, 0.2% Triton N-101, 0.1 μmol each of ATP, CTP, and GTP, 0.01 μmol UTP, [^{3}H]UTP (final specific activity, 200 cpm/pmol), and 50 μg of viral protein (frozen stock of purified WSN virions). The reaction was begun by the addition of virus and incubated in a 37°C water bath. To stop the reaction, 0.1-ml samples were combined with 0.4 ml of a chilled (4°C) carrier solution containing 60 μg each of yeast transfer RNA and bovine serum albumin (fraction V), followed by the immediate addition of 1 ml of cold 30% trichloroacetic acid (equal volumes of 100% Cl_3CCOOH, saturated sodium pyrophosphate, and Na_3PO_4 solutions). After incubation at 4°C for 10 min or longer, precipitates were collected on Bac-T-flex membranes (Schleicher and Schuell, Inc.) and washed with 50 ml of cold 5% Cl_3CCOOH, followed by 20 ml of cold 95% ethanol. Membranes were dried and counted for radioactivity in a Packard 3320 liquid scintillation spectrometer in 10 ml of a toluene cocktail containing 0.4% 2,5-diphenyloxazole and 0.02% dimethyl 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene. All values were corrected for the background radioactivity in unincubated reaction mixtures, which never exceeded 50 cpm.

Effect of Metal Ions. Mn^{2+} ions are absolutely required (Fig. 1*A*), in a concentration from 1.9 to 7.5 mM. Both Mn^{2+} and Mg^{2+} ions, at concentrations of 15 mM or higher, effectively block [^{3}H]UTP incorporation (Fig. 1*A* and *B*). The data presented in Fig. 2 show that optimal conditions for detection of RNA polymerase activity in influenza virions are not suitable for demonstrating similar activity with vesicular stomatitis virus (VSV), which is *inhibited* by Mn^{2+} ions.

Relative Enzyme Activity of Purified Virus before and after Freezing. The polymerase activity of freshly prepared virus was compared with that of stocks taken from frozen storage since variable requirements have been reported for DNA polymerase activity among different RNA tumor viruses with respect to the type of preparation used [Spiegelman *et al.* (5)]. It was found that highest enzyme activity was associated with unfrozen stocks of WSN virus which, if tested fresh, required a higher detergent concentration, presumably to effectively disrupt virions (Fig. 3). Frozen virus tested with 2% detergent

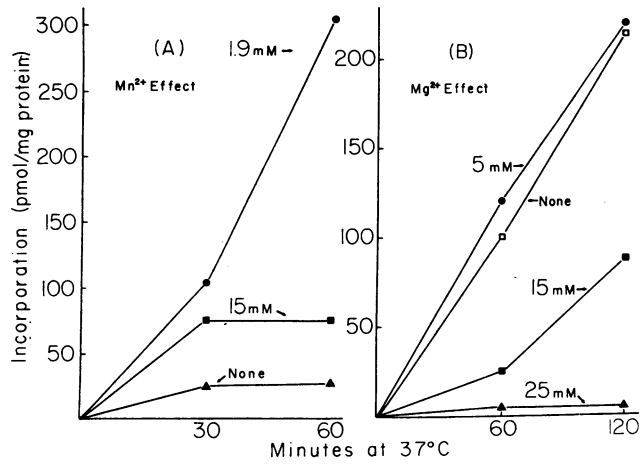

FIG. 1. Effect of divalent cations on RNA polymerase activity of WSN influenza virus. Except for Mn^{2+} and Mg^{2+} ions, the standard reaction mixture (Table 1) was used with 2 μCi of [^{3}H]UTP (100 cpm/pmol) and 50 μg of viral protein. (*A*) Incorporation with 5 mM $MgCl_2$ and 0, 1.9, or 15 mM $MnCl_2$. (*B*) Incorporation in the presence of 1.9 mM $MnCl_2$ and 0, 5, 15, or 25 mM $MgCl_2$.

showed reduced activity. Linear kinetics were observed for the first 2 hr of incubation with each preparation. Both the rate and maximum extent of incorporation obtained with frozen virus stocks were below that of fresh, unfrozen preparations for reasons that are not entirely apparent.

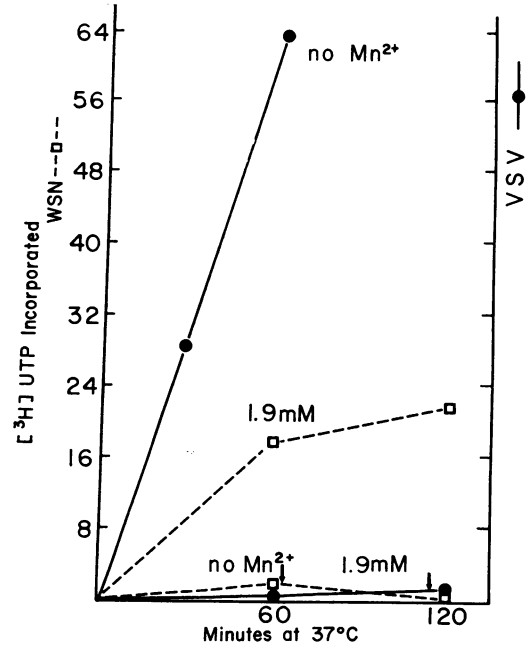

FIG. 2. Differential effect of Mn^{2+} ions on RNA polymerase activity of WSN influenza virus and vesicular stomatitis virus (VSV). The standard assay (Table 1) was used for WSN virus with [^{3}H]UTP (200 cpm/pmol) and 50 μg of viral protein. The VSV reaction mixture (4) contained [^{3}H]UTP (100 cpm/pmol) and 50 μg of viral protein. Each virus was tested for enzyme activity in the presence and absence of 1.9 mM $MnCl_2$. Activity is expressed in pmol/mg of viral protein $\times 10^{-1}$ (WSN) or $\times 10^{-2}$ (VSV).

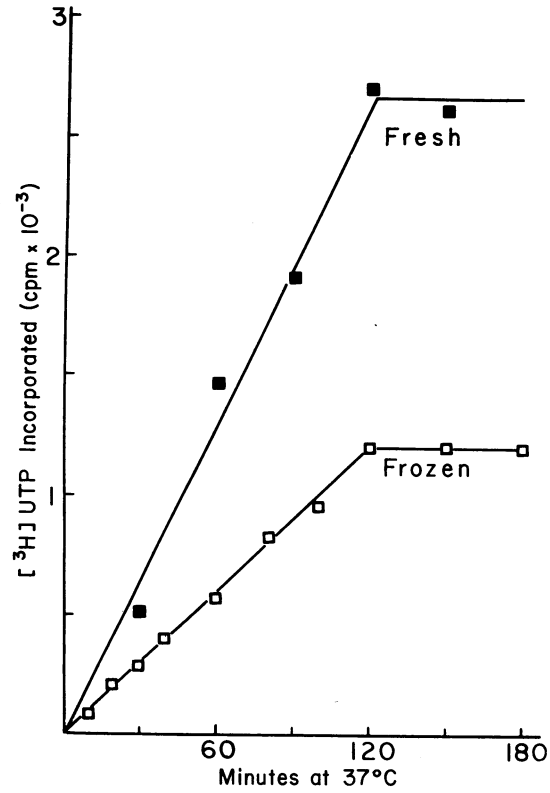

FIG. 3. Effect of freezing on RNA polymerase activity of WSN influenza virus. Purified virus, either freshly prepared or stored at −70°C for 4 days, was tested in the standard assay system (Table 1). The concentrations of detergent used for each of these preparations (2% with fresh, 0.2% with frozen virus) were found to give *maximum* incorporation in comparative tests. Each reaction mixture was adjusted so as to contain [^{3}H]UTP (200 cpm/pmol) and 60 μg of viral protein per 0.2 ml.

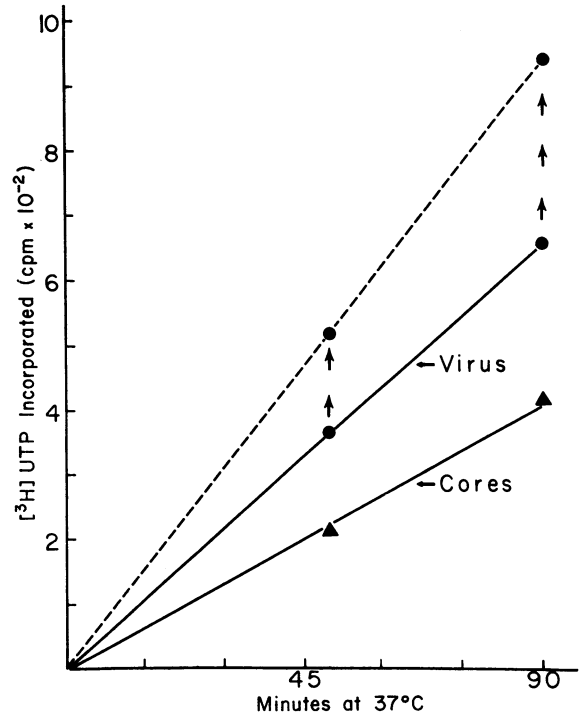

FIG. 4. Relative RNA polymerase activity of intact particles and subviral components of WSN virus. The same virus preparation was tested for enzyme activity before and after removing the virion surface glycoproteins by treatment with chymotrypsin (11) for 15 hr. Untreated intact virus (*circles*) and chymotrypsin-resistant "cores" (*triangles*) were tested under standard conditions (Table 1) with [^{3}H]UTP (200 cpm/pmol) and 35 μg of viral protein. The activity of samples is plotted as a function of time of incubation at 37°C. The upper plot (*broken line*) corresponds to the extent of incorporation that can be calculated for untreated virus if activity is expressed only in terms of the amount of core protein (70%) present in intact virions, as determined in independent experiments with radiolabeled virus.

RNA polymerase activity of different myxovirus strains

It was of considerable interest to examine other influenza viruses for their ability to catalyze *in vitro* RNA synthesis since many distinct serotypes of diverse host origin comprise the myxovirus group. Each of nine influenza strains tested showed such activity, but a paramyxovirus, Newcastle Disease virus, did not (Table 2). In expressing the polymerase activity detected in the different influenza strains relative to that of WSN virus assayed under standardized conditions, we found that amounts of [^{3}H]UTP incorporation ranged from 2-fold below to 10-fold above the WSN virus activity (Table 2). The relatively high incorporation consistently observed in many tests with the WS strain is regarded as a genetic attribute of the virus. There was no apparent correlation between relative enzyme activity and other viral properties.

Location of enzyme activity in the virion

We have confirmed the recent observation of Schulze (11) that extensive treatment of WSN influenza virus with chymotrypsin removes all surface glycoprotein components concomitant with a loss of sialidase activity, hemagglutinating capacity, and viral infectivity (data not shown). When such viral "cores" were tested for RNA polymerase, they were found to possess an activity below that of intact virions (Fig. 4). These data suggest that the enzyme that catalyzes incorporation of [^{3}H]UTP is internally situated in influenza virions.

Preliminary characterization of the enzyme product

Two approaches were used to study the nature of the *in vitro* product.

Resistance to Ribonuclease Digestion. 45 to 52% of the product synthesized during a 2-hr incubation with WSN virus in a standard reaction mixture was not degraded by ribonuclease A (Fig. 5).

Hybridization of Deproteinized RNA Product with Virion RNA. The ability of purified product RNA to anneal with RNA extracted from influenza virus was examined, using the WS strain to synthesize *in vitro* product (Table 3). We found that 30% of the product RNA was resistant to the combined effect of ribonuclease A and T1. It has not been determined whether treatment of crude product (total reaction mixture) shows this lower amount of resistance when treated with both enzymes. This resistance was largely abolished if product

RNA was rapidly cooled after heating, whereas it was restored by annealing with added unlabeled RNA of the WS or WSN strains. The extent of annealing obtained is considered minimal, since the heating conditions used (Table 3) are known to damage influenza RNA (12). Product RNA did not anneal significantly with various heterologous RNAs. We conclude that virion-associated enzyme catalyzes the incorporation of nucleoside triphosphates into RNase-resistant and RNase-sensitive products, including at least 15% of which is RNA complementary to the base sequence of virion RNA.

DISCUSSION

Evidence has been presented that an RNA polymerase physically located in the chymotrypsin-resistant "core" (11) of virus particles is apparently found in most, if not all, influenza strains, including those of human, avian, and porcine hosts (Table 2). It is noteworthy that this enzyme shares several properties with the RNA polymerase found in cells infected with influenza virus, including the requirement for RNA template (1), the dependence on Mn^{2+} ions (Skehel and Burke (1)), the insensitivity to actinomycin D (1), the inhibition by polyanions [Scholtissek and Rott (1)] and the general nature of the *in vitro* product [Mahy and Bromley (1)]. Unlike the enzyme detected in particles of vesicular stomatitis virus (4), the influenza polymerase catalyzes the formation of a large amount of ribonuclease-resistant product (Fig. 5). It is possible that more than one virion-associated enzyme is involved in the synthesis of these products.

Failure to detect RNA polymerase activity in the paramyxovirus, Newcastle disease virus, may simply be due to lack of proper assay conditions or, alternatively, the suggested presence of negative-strand RNA (messenger?) (13) in this virus may preclude the necessity for a virion-specific polymerase. This leads to the important question of whether the functional RNA polymerase detected in influenza virus particles is essential for initiation of a productive infection by carrier virions, or whether it only represents enzyme molecules

TABLE 2. *RNA polymerase activity detected in purified virus particles of various myxovirus strains*

Virus*	[^{3}H]UTP Incorporation (pmol/mg viral protein)†
Influenza A: Strain WS	1590
Influenza A: Strain Japan 305	690
Influenza A: Strain PR8	525
Influenza A: Strain BEL	310
Influenza A: Strain FM_1	279
Influenza A: Strain WSN	150
Influenza A: Strain SW (swine)	154
Influenza A: Strain N (fowl plague)	97
Influenza B: strain Lee	124
Newcastle disease virus (Beaudette)	0

* All test viruses were grown in embryonated eggs and purified as in *Methods*. Standard reaction mixtures were prepared as specified in Table 1 with [^{3}H]UTP (100 cpm/pmol) and 120 μg of viral protein, in a total volume of 0.6 ml.

† Incorporation values are for 60 min at 37°C. The value obtained for the WSN strain is the mean of seven assays (range = 106–290 pmol/mg viral protein).

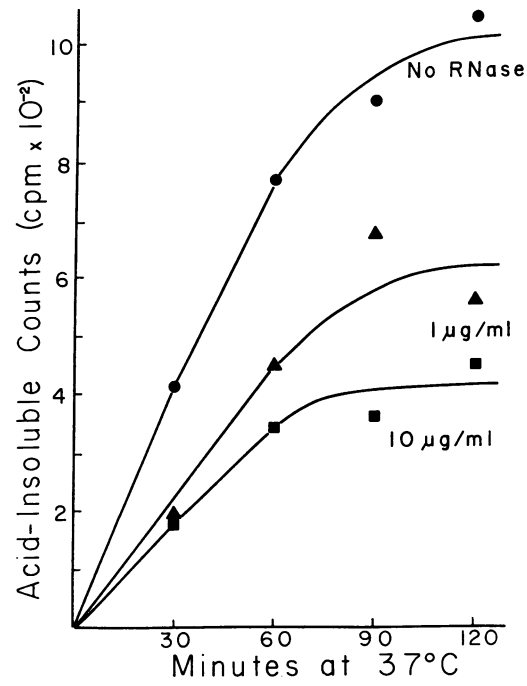

FIG. 5. Ribonuclease resistance of RNA polymerase product of WSN virus. The reaction mixture contained the standard components (Table 1) with [^{3}H]UTP (200 cpm/pmol) and 240 μg of viral protein. At the time intervals indicated, samples were removed from the same reaction mixture and tested for acid-insoluble ^{3}H before and after treatment with 1 or 10 μg/ml of ribonuclease A for 10 min at 37°C.

TABLE 3. *Ribonuclease resistance of polymerase product RNA (WS virus) before and after annealing with influenza virion RNA*

Treatment of product RNA*	Ribonuclease resistance (%)
None	30
Heated and rapidly cooled	2
Heated and self-annealed	15
Heated and annealed with WS RNA (3.4 μg)	24
Heated and annealed with WSN RNA (12.5 μg)	31

* All viruses used were treated with DNase I (5 μg/ml) and RNase A (20 μg/ml) early in the purification procedure (9). Labeled product RNA was derived from a standard reaction mixture (1.6 ml) containing 1.6 mg of purified WS viral protein and 26 μCi of [^{3}H]UTP (62 cpm/pmol) after incubation at 37°C for 150 min. The RNA was extracted with phenol–sodium dodecyl sulfate (9) from purified virus and from the polymerase reaction mixture in the absence and presence of carrier yeast transfer RNA (60 μg), respectively.

Annealing mixtures were prepared by adding 3600 cpm of product RNA in 0.4 ml of buffer (0.1 M Tris·HCl (pH 7.4)–0.1 M NaCl) and 3.4–12.5 μg of virion RNA (WS or WSN strain). After they were heated at 120°C for 2 min, the mixtures were slowly cooled to 68°C and held at this temperature for 15 hr. After they were cooled to room temperature, all samples were diluted to 1 ml in STE buffer (9) and divided into equal fractions, one of which was treated with 10 μg of RNase A and 1 μg of RNase T1 (15 min at 37°C). Acid precipitation and counting of radioactivity were as described in Table 1.

fortuitously "trapped" in association with the viral ribonucleoprotein at the time of viral maturation. Our recent finding (Chow and Simpson, in preparation) that incomplete ("Von Magnus") influenza-virus preparations have both greatly diminished infectivity and *in vitro* polymerase activity would favor the first interpretation. Finally, we can also report that the amount of enzyme activity observed among different strains of influenza virus is a phenotypic expression of the viral genome since genetic recombination occurs for this property (Simpson and Chow, in preparation).

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