

Ribonucleic Acid Synthesis of Vesicular Stomatitis Virus, II. An RNA Polymerase in the Virion*

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Abstract. The virions of vesicular stomatitis virus contain an enzyme that catalyzes the incorporation of ribonucleotides into RNA. The product of the reaction is mainly RNA complementary in base sequence to that of vesicular stomatitis virus RNA.

Introduction. Two considerations led us to search for an RNA polymerase in the virion of vesicular stomatitis virus, a single-stranded RNA virus. First, the lack of infectivity of vesicular stomatitis virus RNA,¹ which contrasts with the readily demonstrable infectivity of RNA from arboviruses² or picornaviruses,³ could be explained by an obligate requirement for a virion polymerase in order to initiate a vesicular stomatitis virus infection. Second, the recent finding that the messenger RNA's that synthesize vesicular stomatitis virus proteins are complementary to the nucleotide sequence of the virion RNA⁴ led us to wonder how the initial stages of the infection proceed: if the virion RNA is not a messenger, then it must be able to serve as template for messenger RNA synthesis using either a polymerase found in the host cell or one that is a part of the virion.

Two precedents exist for the association of an RNA polymerase with the virion of an animal virus. One is the finding of a DNA-dependent RNA polymerase in vaccinia virions.⁵ The second is the finding of an RNA-dependent RNA polymerase in the virions of reovirus⁶ and other double-stranded RNA viruses.⁷

Materials and Methods. Purified B particles⁸ of vesicular stomatitis virus, the Indiana serotype,⁹ were used throughout these experiments. Details of the growth of high titered viral stocks in Chinese hamster ovary cells and purification of the virions through sucrose gradients were as previously described¹⁰ except for some modifications (manuscript in preparation). A single virus stock was used for all the experiments reported in this paper, although experiments with other stocks have given comparable results. The stock contained 0.5 mg of protein/ml and 10¹⁰ plaque-forming units/ml.

Protein was determined by the method of Lowry *et al.*¹¹

Stocks of purified Newcastle disease virus, type N, and Sindbis virus were kindly provided by Dr. Michael A. Bratt and Dr. Boyce W. Burge, respectively.

Reagents were obtained from the following sources: unlabeled nucleoside triphosphates from P-L Biochemicals; [³H]GTP from New England Nuclear; Triton N-101, deoxyribonuclease and ribonuclease from Sigma. Actinomycin was a gift from Merck, Sharpe and Dohme. Rifampicin was supplied by Dr. Harvey Lodish.

Results. Addition of intact, purified vesicular stomatitis virus to the appropriate reaction mixture led to incorporation of [³H]GMP into an acid-insoluble form (Table 1). The reaction was almost totally dependent on the presence of a detergent, whose role was presumably to strip off the outer lipid-containing en-

velope of the virion, exposing the nucleocapsid. Triton N-101, used in these experiments, can be replaced by other nonionic detergents but not by sodium deoxycholate.

Virtually no incorporation occurred if Mg^{2+} , UTP, or CTP was omitted from the reaction mixture. Mn^{2+} did not replace Mg^{2+} and, in fact, inhibited the reaction if added together with Mg^{2+} . The optimum Mg^{2+} concentration for incorporation was 4–6 mM (Fig. 1). There was a partial dependence for activity on mercaptoethanol and either NaCl or KCl strongly stimulated incorporation.

As expected for the properties of an RNA-dependent synthesis of RNA, the addition of deoxyribonuclease, actinomycin D, or rifampicin to the reaction mixture did not inhibit incorporation. However, the reaction was totally sensitive to ribonuclease (Table 1).

TABLE 1. *Properties of the vesicular stomatitis virus RNA polymerase.*

Reaction system	m μ moles GMP incorporation (mg protein/20 min)
Complete	12.4
Minus Triton N-101	0.38
Minus $MgCl_2$	<0.20
Minus $MgCl_2$, plus 1.6 μ moles $MnCl_2$	<0.20
Minus UTP	<0.20
Minus CTP	0.26
Minus virions	<0.20
Minus mercaptoethanol	7.4
Minus NaCl	1.9
Minus NaCl, plus 30 μ moles KCl	12.0
Plus 10 μ g ribonuclease	0.46
Plus 20 μ g deoxyribonuclease	12.0
Plus 5 μ g actinomycin D	13.0
Plus 5 μ g rifampicin	12.6

The complete reaction mixture consisted of the following components in a total volume of 0.3 ml: 15 μ moles Tris-HCl, pH 7.9; 1.6 μ moles $MgCl_2$; 1 μ mole mercaptoethanol; 30 μ moles NaCl; 0.2 μ mole of ATP, UTP, and CTP; 0.02 μ mole of [3H]GTP (28.7 cpm/ μ mole); 0.25 mg Triton N-101 and 10 μ g of viral protein. The reaction mixture was incubated at 37°C for 20 min. The reaction was terminated by chilling in an ice bath, adding 0.5 ml of 0.08 M Na pyrophosphate, mixing, adding 0.05 ml of 4 mg/ml yeast RNA, and finally precipitating with 0.5 ml of 25% trichloroacetic acid. After at least 10 min at 4°C, the samples were filtered through a Millipore filter, type HA, and the trapped precipitate was assayed for radioactivity in a scintillation spectrometer at 28% efficiency after digestion with 0.4 ml of concentrated NH_4OH for 30 min and addition of 10 ml of cocktail D (5 gm PPO and 100 gm naphthalene in 1 liter of *p*-dioxane). An unincubated sample contained less than 3% of the radioactivity of an incubated sample and this value was subtracted from all of the experimental values.

The kinetics of incorporation are shown in Figure 2. Although the maximal rate was maintained only for 20 min, there was a long period of continued incorporation at a slower rate. The absence of a lag before the maximal rate was reached suggests that the detergent activation was an instantaneous process.

Figure 3 shows the incorporation relative to the amount of added vesicular stomatitis virus, expressed as micrograms of viral proteins. For unknown reasons, the rate of the reaction was not exactly proportional to protein concentration.

In order to determine if the polymerase was located in virions we centrifuged a previously purified preparation of virions through a sucrose gradient and as-

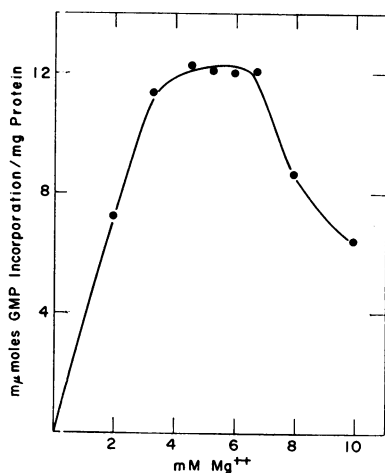


FIG. 1.

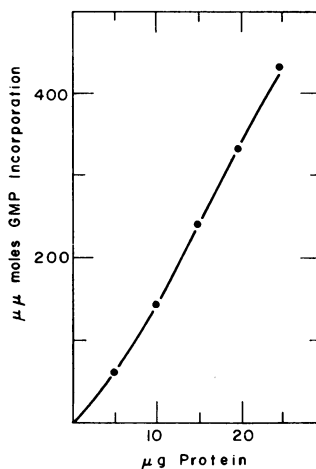


FIG. 3.

FIG. 1.—Vesicular stomatitis virus RNA polymerase activity at different concentrations of Mg^{2+} . Reaction mixtures were incubated under standard conditions (see Table 1) with different concentrations of $MgCl_2$.

FIG. 3.—Incorporation by different amounts of vesicular stomatitis virus virions. Reaction conditions were as described in Table 1.

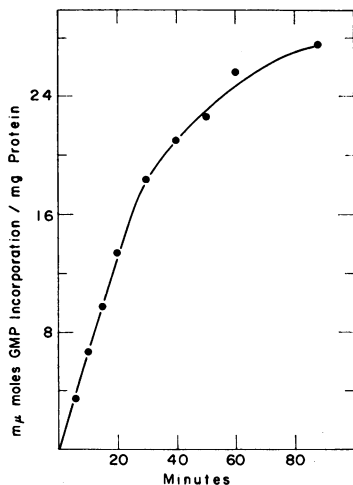


FIG. 2.

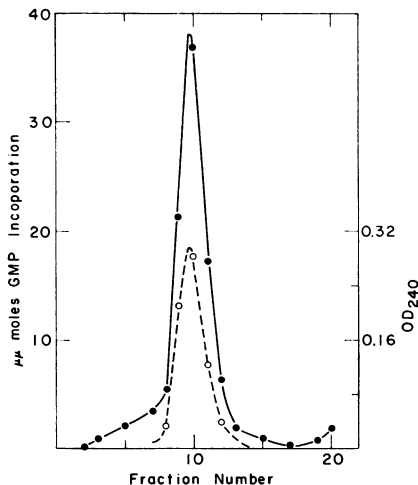


FIG. 4.

FIG. 2.—Incorporation by the vesicular stomatitis virus RNA polymerase after different times of incubation. Reaction mixtures were incubated under standard conditions (see Table 1) for different lengths of time. All the chemical components of the reaction mixture were brought to $37^{\circ}C$ and incorporation was initiated by the addition of virus.

FIG. 4.—Location of vesicular stomatitis virus RNA polymerase activity by sucrose gradient sedimentation of the virions. A purified vesicular stomatitis virus preparation (0.25 ml containing 0.375 mg protein) was layered over a 5–40% sucrose gradient in 0.13 M NaCl, 0.003 M KCl, 0.005 M Na phosphate buffer, pH 7.4, 0.02% EDTA. The 17-ml gradient was centrifuged for 2.25 hr at 18,000 rpm in a small bucket of the SW 27 Spinco rotor. Fractions of 1 ml were collected and 0.2-ml portions were assayed in the standard reaction mixture lacking NaCl (the NaCl in the gradient provided sufficient salt) (●—●). The virions were identified by measuring the absorbance of fractions at 240 $m\mu$ (mainly light scattering) (○—○).

sayed fractions for activity (Fig. 4). The peak of absorbance due to virions coincided with the peak of RNA polymerase activity, suggesting that the enzyme was an integral part of the virion. The template for the incorporation was presumably the viral RNA because no template RNA was added to the reaction mixtures.

The nature of the polymerase product was examined by determining its sensitivity to ribonuclease and its ability to anneal to vesicular stomatitis virus RNA (Table 2). The RNA product obtained from a 30-min reaction mixture was less

TABLE 2. *Annealing of polymerase products with vesicular stomatitis virus RNA.*

Treatment	Ribonuclease-resistance (%)
(1) None	8.8
(2) Boiled and quickly chilled	4.5
(3) Boiled and annealed	20
(4) As (3); annealed for 290 min	20
(5) Boiled and annealed with VSV-RNA	94
(6) Boiled and annealed with poliovirus RNA	21

A standard reaction mixture containing 50 μg of viral protein and [^3H]GTP at 73.2 cpm/ μmole was incubated for 30 min at 37°C. It was then chilled, brought to 0.1% sodium dodecyl sulfate, and precipitated with 2 vol of ethanol and carrier yeast transfer RNA. The labeled RNA was dissolved in H_2O .

Mixtures were made containing about 4000 cpm of the reaction product and either 8 μg of vesicular stomatitis virus RNA, or 8 μg of poliovirus RNA, or no added RNA. They were brought to 0.1% sodium dodecyl sulfate in a final volume of 0.4 ml of H_2O . As indicated in the table, certain samples were boiled for 8 min and then annealed for 120 min at 70°C after addition of 0.1 ml of a solution of 1.5 M NaCl and 0.15 M Na citrate. The samples were chilled and divided into two equal portions, one of which was treated with 50 $\mu\text{g}/\text{ml}$ of ribonuclease A for 30 min at 22°C. All samples were then acid precipitated and assayed as described for Table 1. The results are expressed as percentage of ribonuclease-resistant RNA in each sample.

than 10% resistant to the action of ribonuclease. When the product was allowed to self-anneal at 70°C for 2 hr, ribonuclease resistance reached a level of 20% but did not increase further when annealing was continued for a longer time. When annealed with added unlabeled vesicular stomatitis virus RNA, the product became almost entirely resistant to ribonuclease. Neither poliovirus RNA nor ribosomal RNA from Chinese hamster ovary cells caused any increase in ribonuclease resistance. The product appears, therefore, to be mostly single-stranded RNA complementary in sequence to vesicular stomatitis virus RNA.

Preparations of virions of Newcastle disease virus and Sindbis virus, which had been purified by sucrose gradient centrifugation, were tested for RNA polymerase activity using the standard vesicular stomatitis virus polymerase assay conditions (see Table 1). No activity was demonstrable with these preparations, indicating that they had less than 2% of the specific activity of vesicular stomatitis virus preparations per milligram of protein. The Newcastle disease virus preparation was tested more extensively: neither omitting the NaCl from the reaction mixture nor omitting the detergent revealed any activity and the Newcastle disease virus was not inhibitory to the vesicular stomatitis virus polymerase when the two viral preparations were mixed.

Discussion. The RNA polymerase described here appears to be inside the vesicular stomatitis virion and to use the vesicular stomatitis virus RNA as

template for the synthesis of complementary RNA. This statements follows from the co-sedimentation of virions and polymerase activity, the lack of sensitivity to inhibitors of DNA-dependent RNA synthesis, and the ability of the RNA product to form a ribonuclease-resistant complex with vesicular stomatitis virus RNA.

Experiments to be published suggest that complementary RNA serves as messenger for the translation of viral proteins in vesicular stomatitis virus-infected cells. Since the product of the polymerase found in the virions is also complementary RNA, it is likely that this enzyme is a *transcriptase* rather than a replicase. Whether a second RNA polymerase exists in infected cells is not known. Likewise, the relation of the virion polymerase to the cytoplasmic polymerase that has been demonstrated in vesicular stomatitis virus-infected cells¹² is not clear.

Our inability to anneal more than 20% of the product to the endogenous template (Table 2, lines 3 and 4) suggests that the *in vitro* reaction leads to the synthesis of more than one copy of the region of the vesicular stomatitis virus RNA which is transcribed. We do not know, however, whether all of the vesicular stomatitis virus RNA is being copied or merely a portion of it.

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