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Ribonucleic Acid Polymerase in Virions of Newcastle Disease Virus: Comparison with the Vesicular Stomatitis Virus Polymerase

ALICE S. HUANG,[1] DAVID BALTIMORE, AND MICHAEL A. BRATT

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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The virions of Newcastle disease virus (NDV) contained an enzyme that catalyzed the incorporation of ribonucleotides into ribonucleic acid (RNA). Optimal conditions for this polymerase activity were identical to the conditions for the vesicular stomatitis virus (VSV) polymerase, and both enzymes were active for longer times at 32 C than at 37 C. However, the specific activity of the NDV polymerase was less than 3% that of the VSV polymerase. Product RNA species from the NDV and VSV polymerase reactions annealed specifically to the homologous virion RNA species. Transcriptive intermediates containing product RNA attached to the respective virion RNA could be identified in both systems.

Ribonucleic acid (RNA) extracted from virions of Newcastle disease virus (NDV) lacks infectivity (14), and during NDV replication there is extensive synthesis of RNA complementary in base sequence to the major species of virion RNA (6, 15). This complementary RNA appears to be attached to polyribosomes and may serve as messenger RNA (6). Similar findings with vesicular stomatitis virus (VSV) led to the characterization of an RNA polymerase in virions of VSV (1). Therefore, NDV is also likely to have such an enzyme.

A previous attempt which failed to detect polymerase activity in purified preparations of NDV led to the conclusion that if the NDV polymerase exists it must have less than 2% of the specific activity of VSV preparations (1). By obtaining a more concentrated preparation of virions and by increasing the sensitivity of the assay, an RNA-dependent RNA polymerase has now been detected in virions of NDV.

MATERIALS AND METHODS

Viruses. NDV strains used were NDV-HP (Israel, HP, 1935), NDV-IM (Milano, Italy, 1945), NDV-N (NJ, La Sota, 1946), and NDV-RO (California RO, 1944). Each strain was cloned, grown, and concentrated as previously described (5, 7) and then partially purified by sedimentation through 20% sucrose (Clavell and Bratt, *in preparation*). The pellet of virus was resuspended and further purified through an isopycnic sucrose-D_2O gradient as described in Fig. 3. Preparations of NDV-HP, containing 1.2 to 2.0 mg of NDV protein (17) per ml and 5×10^{10} to 10×10^{10} plaque-forming units per ml, were used in all experiments unless otherwise indicated.

A preparation of the Indiana serotype of VSV, similar to the stocks previously described (1, 24) and containing 200 μg of VSV protein per ml, was used throughout these experiments.

Procedures and reagents. All of the reagents and procedures were as previously described (1), except for modifications noted in the figure legends.

Virion RNA. For the annealing experiment, virion RNA was extracted and purified as previously described for NDV (10) and for VSV (23). A pool of virion RNA from several NDV strains was used. This is justified by the high degree of cross-annealing between virion RNA and complementary RNA from cells infected with different NDV strains (14; Bratt, *unpublished data*).

RESULTS

General properties of the NDV RNA polymerase. The polymerase activity found in virions of NDV had properties very similar to those of the VSV polymerase (Tables 1 and 2, Fig. 1 and 2), except that the specific activity was 1 to 3% of the specific activity of VSV preparations (Fig. 1 and 2). The amount of activity in NDV preparations was approximately proportional to the concentration of viral protein in the reaction mixture. The activity was dependent on activation by a nonionic detergent, Triton N-101 or Nonidet P-40, and on the presence of a sulfhydryl reagent, dithiothreitol. The omission of uridine triphos-

[1] Present address: Channing Laboratory, 774 Albany St., Boston, Mass. 02118.

TABLE 1. *General properties of the Newcastle disease virus polymerase*[a]

Reaction mixture	Amt (pmoles) of GMP incorporated per mg of protein
Complete	215
Minus dithiothreitol	<20
Minus UTP	28
Minus CTP	<20
Minus CTP, plus dCTP	22
Minus Triton N-101	<20
Minus Triton, plus Nonidet P-40	224

[a] The complete reaction mixture consisted of the following components, in a total volume of 0.1 ml: 5 μmoles of tris(hydroxymethyl)aminomethane-hydrochloride, *p*H 7.3; 0.4 μmole of magnesium acetate; 0.3 μmole of dithiothreitol; 10 μmoles of NaCl; 0.07 μmole each of adenosine triphosphate, uridine triphosphate (UTP), and cytidinetriphosphate (CTP); 0.001 μmole of ^{3}H-guanosine triphosphate (1,160 counts per min per pmole); 80 μg of Triton N-101; and 10 μg of viral protein. The reaction mixture was incubated at 32 C for 1 hr. The reaction was terminated and each sample was assayed for acid-precipitable ^{3}H-guanosine monophosphate (GMP) as previously described (1). An incubated sample without virions contained 16% of the radioactivity of the complete system, and this value was subtracted from all of the experimental values. dCTP, deoxycytidine triphosphate.

TABLE 2. *Susceptibility of the Newcastle disease virus (NDV) polymerase reaction to inhibitors*[a]

Reaction mixture	Amt (pmoles) of ^{3}H-GMP incorporated per mg of protein
Complete	198
Plus ribonuclease (50 μg/ml)	<10
Plus trypsin (5 μg/ml)	20
Plus deoxycholate (1 mg/ml)	<10
Plus actinomycin D (20 μg/ml)	188
Plus rifampin (20 μg/ml)	190
Plus deoxyribonuclease (100 μg/ml)	186

[a] Standard 0.1-ml reaction mixtures, as in Table 1, containing 20 μg of NDV protein and each of the inhibitors, were incubated at 32 C for 1 hr. Mixtures lacking virions but incubated at 32 C, or containing virions but unincubated, contained approximately 10% of the radioactivity of the complete system. This value was subtracted from all of the experimental values. GMP, guanosine monophosphate.

phate or cytidine triphosphate or the substitution of deoxycytidine triphosphate for cytidine triphosphate in the reaction mixture virtually eliminated the activity (Table 1). The enzymatic activity was sensitive to trypsin, ribonuclease, and deoxycholate and insensitive to inhibitors of deoxyribonucleic acid (DNA)-dependent RNA synthesis (Table 2). The magnesium ion concentration required for optimal activity was 4 to 6 mM, and substitution with manganese at concentrations from 2 to 26 mM did not result in any detectable activity. A salt concentration of 0.05 to 0.1 M was necessary for the detection of optimal activity.

Effects of temperature and pH. For both NDV and VSV polymerases, the optimal activity was observed at *p*H 7.3 (Fig. 1). Also, for both viruses incorporation of guanosine monophosphate (GMP) continued for longer times at 32 C than at 37 C (Fig. 2). The kinetics of synthesis by NDV were complicated and require further study. At temperatures higher than 37 C, polymerase activity for NDV and VSV was markedly reduced.

Coincidence of polymerase activity in an isopycnic gradient with NDV infectivity and hemagglutinin. To investigate whether the polymerase activity was part of the virion, a partially purified preparation of NDV was centrifuged to equilibrium in a sucrose density gradient. Polymerase activity coincided with both hemagglutinating activity and infectivity at a buoyant density of 1.19 g/ml (Fig. 3).

Polymerase activity in other NDV strains. To test whether strains of NDV other than NDV-HP

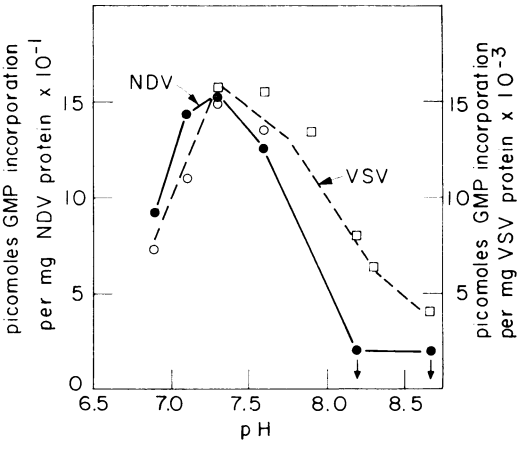

FIG. 1. *Dependence on pH of the Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) polymerase activities. Standard reaction mixtures of 0.1 ml for NDV with 5 μmoles of tris(hydroxymethyl)-aminomethane (Tris) buffer at the different pH values contained 12 μg of viral protein and were incubated at 37 C for 30 min. Standard reaction mixtures for VSV (I) in 0.3 ml with 15 μmoles of Tris buffer at the different pH values contained 13 μg of viral protein and were incubated at 37 C for 20 min. GMP, guanosine monophosphate.*

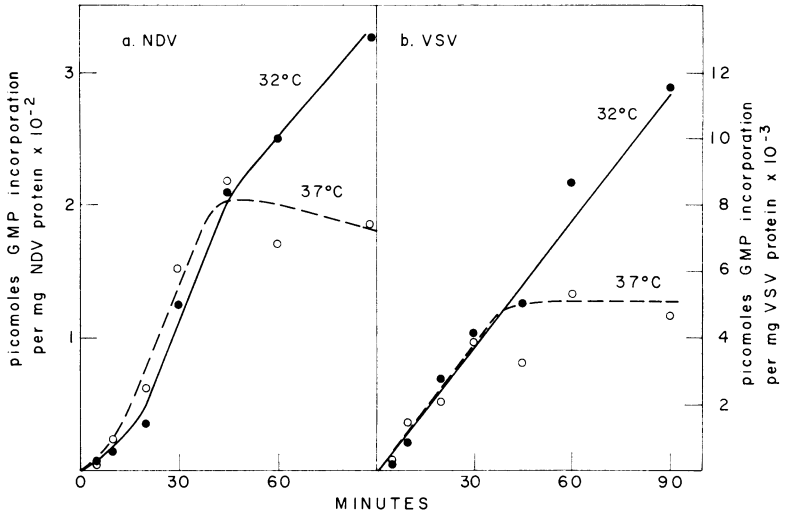

FIG. 2. *Time course of guanosine monophosphate (GMP) incorporation at 32 and 37 C for Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) polymerase reactions. Standard 0.1-ml reaction mixtures were used containing either 16 μg of NDV protein or 2 μg of VSV protein and incubated for the indicated times at 37 C or 32 C.*

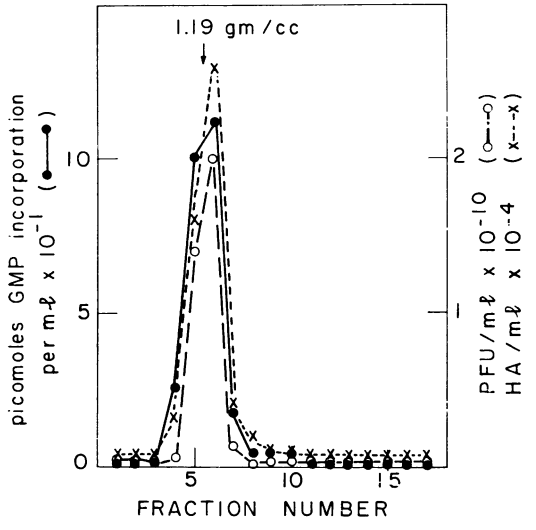

FIG. 3. *Location of the Newcastle disease virus (NDV) polymerase activity by isopycnic sedimentation in a sucrose gradient. Partially purified NDV was layered onto a linear gradient made from 15% sucrose in water and 65% sucrose in D_2O. The buffer was 0.01 M tris(hydroxymethyl)aminomethane (pH 7.4) with 0.03 M NaCl. Centrifugation was in a Beckman SW41 rotor at ~170,000 × g for 3 hr at 4 C. Fractions of 0.7 ml were collected from the bottom. Plaque assay (5) and hemagglutination (HA) titration of NDV (7) were done as previously described, except for some modifications of the hemagglutination assay (Clavell and Bratt, in preparation). GMP, guanosine monophosphate; PFU, plaque-forming unit.*

also contained virion-associated RNA polymerase activity, strains NDV-IM, NDV-RO, and NDV-N were tested for enzymatic activity. All three strains contained polymerase activity but at a slightly lower specific activity (~50 pmoles per mg of protein) than NDV-HP (~200 pmoles per mg of protein) when incubated for 1 hr at 32 C. The presence of an RNA polymerase for four independently purified strains of NDV indicates that the enzymatic activity is not due to contamination of NDV-HP with another virus.

Annealing of the products to virion RNA. To demonstrate that the product made by the NDV polymerase is a transcript of NDV RNA, polymerase products of NDV and VSV were tested for annealing to either NDV or VSV RNA. Table 3 shows that the NDV product annealed to only NDV RNA and not to VSV RNA, whereas the VSV product annealed only to its homologous RNA. The high degree of self-annealing in the absence of added virion RNA probably resulted from the large amount of virion RNA templates already present in the reaction mixture. However, until further experiments are done, we cannot rule out the possibility of self-annealing of the product, especially because there are reports of self-annealing of RNA from virions of NDV (19, 20).

Initial transcriptive intermediates. If virion RNA serves as a template for the NDV and VSV polymerases, labeled products made during the polymerase assay should be initially associated with virion RNA in a ribonuclease-resistant form and

TABLE 3. *Specificity of Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) polymerase products by cross-annealing to NDV and VSV ribonucleic acid (RNA)*[a]

Polymerase product	Annealed with added RNA	Per cent ribonuclease-resistance
NDV	None	39.5
NDV	VSV	42.2
NDV	NDV	107.0
VSV	None	5.3
VSV	VSV	101.2
VSV	NDV	6.2

[a] Standard polymerase reaction mixtures containing virions of NDV or VSV were terminated after 1 hr at 32 C by the addition of 0.1% sodium dodecyl sulfate. Details of annealing were as previously described (12), except that product at 5,000 counts per min per tube and NDV or VSV RNA at 4 μg per tube were used, and all the samples were boiled and then annealed.

should sediment at approximately the same rate as virion RNA. Such a complex will be called a transcriptive intermediate. As more product is synthesized, the transcriptive intermediate should sediment more slowly than virion RNA alone and product RNA should be released from the complex. Because virion RNA species of NDV and VSV sediment at approximately 50 to 57*S* (10, 14, 22; W. R. Adams, Fed. Proc. **24:** 159, 1965) and 40 to 45*S* (13, 23), respectively, examination of the initially labeled products in sucrose gradients should show a difference in size between the two viral systems.

When NDV or VSV products which were made during the first 10 min of the polymerase reaction were deproteinized by sodium dodecyl sulfate and then separated in sucrose density gradients, a peak of radioactivity was observed at 48*S* for NDV and at 38*S* for VSV, which coincided with the optical density at 260 nm of the virion RNA species and contained partially ribonuclease-resistant products (Fig. 4). The different rates of sedimentation for the initial transcriptive intermediates of NDV and VSV indicate the individual specificity of the two polymerase reactions. These results also indicate that the template is not degraded during transcription for 10 min. The data are consistent with the model of a large-template RNA associated by base pairing with small amounts of labeled product. Bishop and Roy (2), with polyacrylamide gel electrophoresis, have also observed that the initial VSV polymerase products are associated with the viral RNA.

With increased time of reaction, the transcrip-

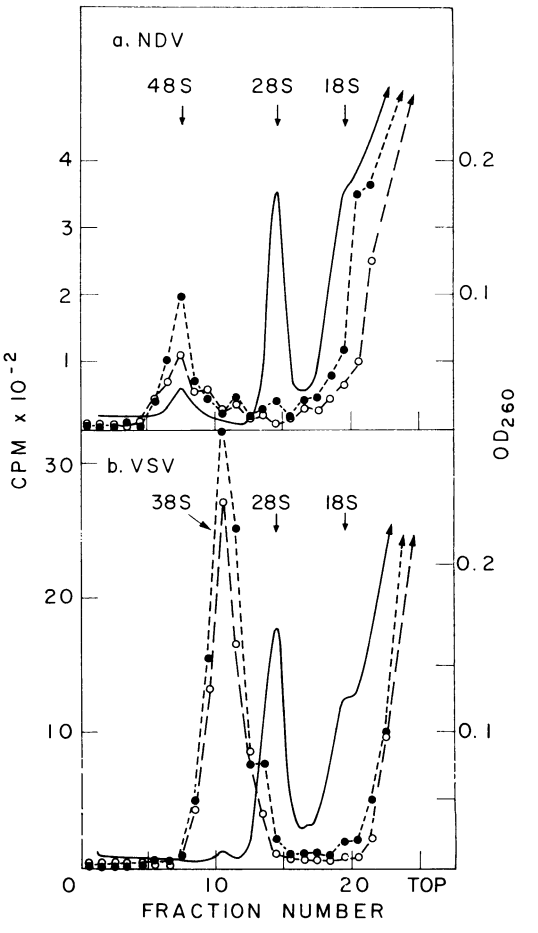

FIG. 4. *Sucrose gradient analyses of Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) polymerase products labeled for 10 min at 32 C. Standard reaction mixtures were made in a volume of 1 ml, containing a total of either 460 μg of NDV protein or 30 μg of VSV protein. After incubation at 32 C for 10 min, sodium dodecyl sulfate was added to a final concentration of 1%, and marker, HeLa cell ribosomal ribonucleic acid was also added. Each reaction mixture was layered onto a linear 15 to 30% sucrose gradient in 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 7.4), 0.1 M NaCl, 0.001 M ethylenediaminetetraacetic acid, and 0.5% sodium dodecyl sulfate. Centrifugation in a Beckman SW27 rotor with large buckets was at ~ 42,000 × g for 18 hr at 22 C. Collection of the gradient into individual fractions, measurement of ultraviolet-absorbing material, and assay of the acid-insoluble radioactivity in each fraction have been previously described (11). Prior to acid precipitation, half of each fraction was ribonuclease-digested as previously described (12). Symbols: solid line, total absorbancy at 260 nm; (●) acid-precipitable radioactivity in half of each fraction; (○) acid-precipitable radioactivity in half of each fraction after digestion by ribonuclease. OD_{260}, optical density at 260 nm.*

tive intermediates for both NDV and VSV sediment even slower and more heterogeneously (*unpublished data*). The top fractions of the sucrose gradient (Fig. 4) represent not only some released single-stranded product but also a large amount of viral protein, unincorporated triphosphates, and detergents. Because of the large amount of material, little can be concluded about the released products until better separation has been achieved. However, for VSV, released RNA products have been identified by polyacrylamide gel electrophoresis (2).

DISCUSSION

These results demonstrate the presence of an RNA polymerase activity in virions of a paramyxovirus, NDV. This activity incorporates ^{3}H-GMP into acid-insoluble material under conditions very similar to the polymerase activity found in VSV (1) but has a much lower specific activity than that of the VSV polymerase. Moreover, the initial labeled products are associated with template RNA of the size expected for NDV RNA, and the products contain base sequences complementary to RNA extracted from virions of NDV. Therefore, the enzyme appears to be an RNA-dependent transcriptase analagous to the enzyme found in virions of VSV (1). The relation of the virion-associated NDV polymerase to the polymerase that has been demonstrated in the microsomal fraction of cells infected by another paramyxovirus, Sendai virus (18), is not clear, but the two enzymatic systems have very similar properties.

The polymerase activity for both NDV and VSV is temperature-sensitive and for VSV at 31 C will proceed linearly for serveral hours given the proper reaction mixture and volume (Baltimore, *unpublished data*). At 37 C, the incorporation of GMP declines after the first 30 min for unknown reasons. Attempts to further activate the NDV and VSV polymerases by deoxycholate or trypsin resulted in marked inhibition of the polymerase activity (reference 1 and Table 2).

Besides the similarity in virion-associated polymerases between a paramyxovirus and VSV, several other striking similarities between the two types of viruses have been noted. First, both viral systems are characterized by the synthesis of large amounts of RNA complementary in base sequence to virion RNA (3, 6, 15, 21). Second, these complementary RNA species are smaller than virion RNA and appear to function as polyribosome-associated messenger RNA (6, 12). Third, nucleocapsids found in the virions of these viruses have an approximate density in CsCl of 1.31 g/ml and are resistant to digestion by ribonuclease (4, 9, 12, 16, 25). Finally, the formation of phenotypically mixed particles after infection with the paramyxovirus SV5 and VSV (8) is an indication of the similarity between the two viral systems. Such evidence supports the recognition of a special group of RNA viruses, including VSV and paramyxoviruses, based on the existence of a unique system of RNA transcription and replication.

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