JOURNAL OF VIROLOGY, Nov. 1970, p. 702-704

Vol. 6, No. 5
Printed in U.S.A.

Ribonucleic Acid-Dependent Deoxyribonucleic Acid Polymerase in Visna Virus

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Received for publication 1 September 1970

A ribonucleic acid-dependent deoxyribonucleic acid polymerase was found in virions of visna virus. The enzyme product was resistant to ribonuclease and alkaline hydrolysis but susceptible to the digestion of deoxyribonuclease.

Visna virus is a medium-sized, ether-sensitive virus of sheep (8). It is formed by budding of the cytoplasmic membrane of the host cells and contains ribonucleic acid (RNA) as demonstrated by radioactive labeling of purified virus (2; Lin and Thormar,Virology, *in press*), but its multiplication is inhibited by 5-bromodeoxyuridine and actinomycin D, indicating a requirement for synthesis and function of deoxyribonucleic acid (DNA; reference 7). These properties of visna virus are similar to those of RNA tumor viruses (8).

The demonstration of RNA-dependent DNA polymerase in virions of mouse and avian tumor viruses (1, 5) prompted us to investigate whether visna virus also contains this newly discovered enzyme. This report describes the presence of the enzyme in visna virions.

Visna virus was propagated in monolayer cultures of sheep choroid plexus cells (4) and was purified by a method to be published elsewhere. Briefly, the virus was precipitated from the infectious culture fluid by zinc acetate. The viral precipitate was suspended in a saturated solution of disodium ethylenediaminetetraacetate (EDTA). The suspension was dialyzed and centrifuged at 95,500 × *g* for 1 hr. After resuspending in tris(hydroxymethyl)aminomethane-(Tris)-hydrochloride buffer containing 0.1 M NaCl and 0.001 M EDTA (TNE buffer), the virus was banded in a potassium tartrate gradient and collected. The degree of purification up to this point was 3,000-fold, based on the ratio of viral infectivity titer to the radioactivity of ^{3}H-uridine (Lin and Thormar, Virology, *in press*). It was then pelleted by high-speed centrifugation and resuspended in TNE buffer for use as an enzyme source. Protein was determined by the method of Lowry et al. (3) with bovine serum albumin used as a standard.

A 0.1-ml amount of a standard assay solution consisted of 10 μmoles of Tris-hydrochloride (*p*H 8.3); 1.2 μmoles of $MgCl_2$; 12 μmoles of $MgCl_2$; 12 μmoles of NaCl; 4 μmoles of 2-mercaptoethanol; 0.1 μmole each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP) (Sigma Chemical Co., St. Louis, Mo.); 0.001 μmole of ^{3}H-methyl-thymidine triphosphate (TTP; Schwarz Laboratories, Inc., Mount Vernon, N.Y.), and 1% Nonidet P40 (Shell).

By addition of purified visna virus to this mixture, radioactivity from ^{3}H-TTP was incorporated into acid-insoluble product (Table 1). All four deoxyribonucleoside triphosphates were required for maximal incorporation but there was some activity in the absence of dATP and dCTP. No activity was observed in the absence of dGTP. Mg^{2+} and 2-mercaptoethanol were essential for the maximal incorporation of ^{3}H-thymidine monophosphate (TMP). To obtain full activity the virions must be disrupted by Nonidet, a non-ionic detergent.

Figure 1 shows the kinetics of incorporation of radioactivity into acid-insoluble product. The rate of reaction in the first 50 min was linear and then decreased. The reaction continued for up to 5 hr of incubation.

The nature of the acid-insoluble product was determined by the experiment shown in Table 2. The acid-insoluble product was fully resistant to ribonuclease. After alkaline hydrolysis, 80% of the radioactive acid-insoluble material remained in the reaction mixture. On the other hand, treatment of the reaction mixture with 50 μg/ml of deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.; ribonuclease free) reduced the acid-insoluble radioactivity to 4.4%. These results strongly suggest that the product consisted of DNA and, therefore, that the viral preparation contained DNA polymerase.

To determine the association of the enzyme

TABLE 1. *Requirements for enzyme activity*[a]

Reaction system	^{3}H-TMP incorporated (counts per min)
Complete	2,529
Minus dATP	576
Minus dCTP	270
Minus dGTP	0
Minus Mg^{2+}	1,073
Minus mercaptoethanol	1,523
Minus Nonidet	509

[a] To the standard assay solution which contained 1% Nonidet P40, virus containing 10 μg of protein (10^7 $TCID_{50}$) was added. The mixture was incubated at 37 C for 1 hr and chilled; 0.1 ml of yeast RNA (0.1 mg) was added, followed by addition of 0.3 ml of cold 10% trichloroacetic acid. The mixture was filtered through a B-6 membrane filter (Schleicher & Schuell Co., Keene, N.H.) and washed three times with 5 ml of 5% trichloroacetic acid. The acid-insoluble count was measured in a Picker Nuclear Liquimat 330 scintillation counter. The radioactivity of ^{3}H-TTP was 12 Ci/mmole (802 counts per min per pmole). The count (698 counts/min) of a control, in which buffer was substituted for viral preparation, was subtracted from that of the sample.

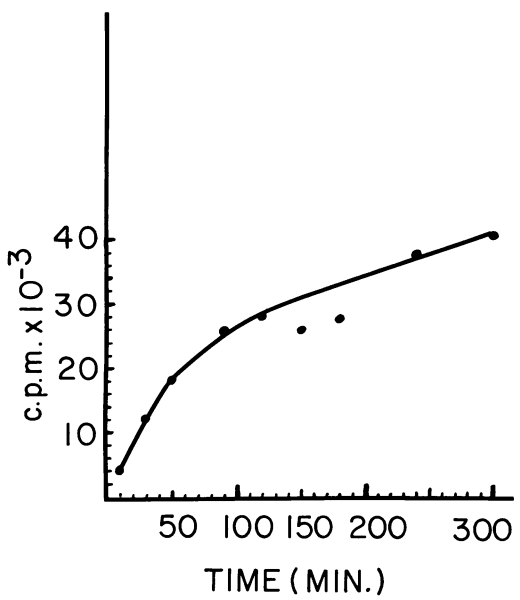

FIG. 1. *Kinetics of incorporation of ^{3}H-TTP by visna virus polymerase. To 1 ml of the standard assay solution, 0.5 ml of viral suspension (17 μg per 0.1 ml of viral protein) was added. Mixture was incubated at 37 C, and 0.1-ml samples were withdrawn at time intervals as indicated for measurement of acid-precipitable product. The specific activity of ^{3}H-TPP was 1,488 counts per min per pmole.*

TABLE 2. *Property of enzyme product*[a]

Treatment	Acid-insoluble radioactivity (counts per min)	Untreated product (%)
Untreated	37,212	100
Ribonuclease (50 μg/ml)	38,082	102
Deoxyribonuclease (50 μg/ml)	1627	4.4
KOH	30,093	80

[a] A mixture of the standard assay solution and viral preparation was incubated at 37 C for 90 min. Portions of the product were withdrawn and treated as indicated. Mixtures were incubated at 37 C for 30 min for the treatment with the nucleases, and for 20 hr at 37 C for treatment with 0.3 M KOH. The acid-insoluble radioactivity was measured as described in Table 1. The radioactivity of ^{3}H-TTP was 1,678 counts per min per pmole.

with the virus, the viral preparation was centrifuged to equilibrium in a 10 to 50% potassium tartrate gradient. Fractions of the gradient were assayed for DNA polymerase activity. Maximal radioactivity of acid-insoluble product was detected at a density of 1.166 g/ml (Fig. 2B) where infectious visna virus was banded (Fig. 2A). There were no significant viral and enzyme activities on the top of the gradient, although the absorbance at 280 nm was very high. The results of these experiments demonstrate that DNA polymerase was localized in the virions of visna virus.

To study a possible template dependence, the virions were disrupted by Nonidet and incubated with ribonuclease (Calbiochem, Los Angeles, Calif.) before the addition of the standard assay solution. Table 3 demonstrates that preincubation of the disrupted viruses with 50 μg of ribonuclease per ml at 25 C reduced the incorporation of ^{3}H-TMP to 42%. When the amount of ribonuclease was increased to 500 μg/ml, the incorporation of radioactivity from ^{3}H-TTP by the reaction was completely prevented, indicating that the activity of the enzyme was dependent on viral RNA. Since the ribonuclease solution was boiled for 10 min before use, it seems unlikely that the solution was contaminated with deoxyribonuclease.

The data obtained indicate that visna virus contains an RNA-dependent DNA polymerase and suggest that this enzyme exists in RNA viruses that require DNA synthesis for their replication.

Visna virus causes proliferation of reticuloendothelial elements in the lungs and the central

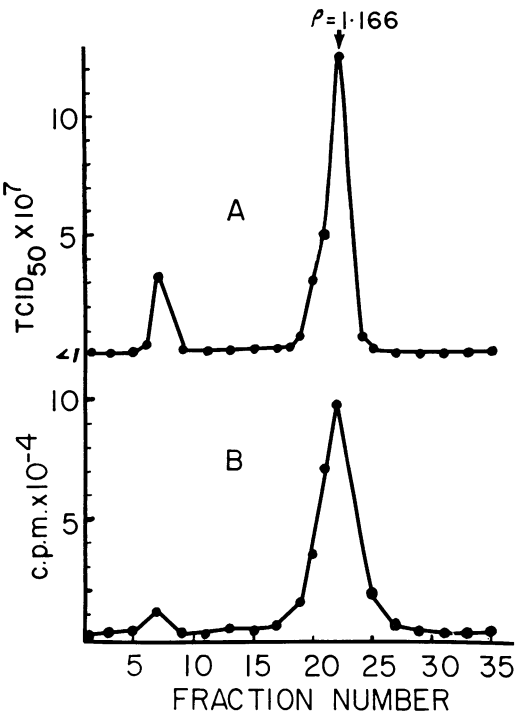

FIG. 2. *Viral and DNA polymerase activities in fractions of potassium tartrate gradient. Five milliliters of a partially purified viral suspension ($10^{9.4}$ $TCID_{50}$) was layered over a 33-ml gradient of potassium tartrate in TNE buffer and centrifuged for 3 hr at 27,000 rev/min in a Beckman SW27 rotor. One-milliliter fractions were collected from the bottom of the centrifuge tube. A, Viral infectivity (6), after measuring the absorbance at 260 and 280 nm; 0.1-ml samples were withdrawn from each fraction for viral infectivity assay. B, Enzyme activity, 0.1-ml portions of each fraction were transferred to IEC type A 321 centrifuge tubes, diluted with 1 ml of TNE buffer, and centrifuged at 35,000 rev/min for 1 hr. The supernatant was discarded, and the pellet was suspended in 0.1 ml of TNE buffer by sonic treatment (with a Bronwill Needle Probe at an intensity of 30 for 30 sec). A 0.1-ml amount o the standard assay solution was added to the suspension. The mixture was incubated at 37 C for 90 min, and the acid-insoluble radioactivity was measured as described in Table 1, except that no background correction was made.*

nervous system of sheep, but has not been associated with tumorogenicity in the animal host (8). It causes cytopathic effect in tissue cultures, leading to cell degeneration and death. Transformation of tissue culture cells by visna virus has not been observed, but experiments are being initiated to study whether the virus is capable of cell transformation under certain conditions.

TABLE 3. *Template requirement for enzyme activity*[a]

Conditions	^{3}H-TMP incorporated (counts per min)
Preincubated with water	2,776
Preincubated with 50 μg of ribonuclease per ml	1,173
Preincubated with 500 μg of ribonuclease per ml	30

[a] Ribonuclease was dissolved in deionized water, boiled for 10 min, and chilled. The viral suspension was incubated with 0.5% Nonidet at 0 C for 30 min. One-tenth milliliter of a mixture containing the disrupted virions (10 μg of viral protein) and the indicated amount of ribonuclease was preincubated at 25 C for 30 min. To the treated mixture, 0.1 ml of standard assay solution with the omission of Nonidet was added, and the mixture was incubated at 37 C for 1 hr. Acid-insoluble radioactivity was measured as described in Table 1. The specific activity of ^{3}H-TTP was 1,237 counts per min per pmole.

We thank Marilyn Genovese, Hannah Brown, and Joann Ingulli for excellent technical assistance.

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