Ribonucleic Acid Transcriptase Activity in Purified Wound Tumor Virus¹

D. R. BLACK AND C. A. KNIGHT

Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720

Received for publication 26 May 1970

Purified wound tumor virus was found to possess an associated ribonucleic acid (RNA) transcriptase. The product of transcriptase synthesis was shown to be single-stranded RNA which annealed specifically to wound tumor viral RNA.

Reovirus has an associated ribonucleic acid (RNA) transcriptase (3, 8) which has recently been shown to synthesize reovirus messenger RNAs (9). Cytoplasmic polyhedrosis virus (CPV) has also been shown to contain an associated RNA transcriptase (5); the present report will establish that wound tumor virus (WTV), a virus which multiplies in both insect and plant hosts, contains a similar RNA transcriptase.

MATERIALS AND METHODS

Chemicals. ³H-uridine 5'-triphosphate (22.5 Ci/mmole) and ³H-guanosine 5'-triphosphate (6.05 Ci/mmole) were obtained from New England Nuclear Corp., Boston, Mass.; ribonuclease-free sucrose was obtained from Mann Research Laboratories, New York, N.Y.; pancreatic ribonuclease came from Worthington Biochemical Corp., Freehold, N.J.; pyruvic kinase and 2-phosphoenolpyruvic acid were from Calbiochem, Los Angeles, Calif.; nucleoside triphosphates came from Pabst Laboratories, Milwaukee, Wis.; and actinomycin D was obtained from Merck Sharp and Dohme Research Laboratories, Rahway, N.J. All other chemicals were of reagent grade.

Purification of WTV. WTV was isolated from infected sweet clover root tumors. The freshly harvested tumors were stored at 0°C; all the following operations were performed at 4 to 6°C. After macerating the tumors in the presence of 0.3 M glycine, 0.03 M MgCl₂, 0.03 M tris(hydroxymethyl)aminomethane (Tris), pH 7.5 (GMT buffer), the mixture was briefly treated with carbon tetrachloride, and the bulk of cellular debris was removed by centrifugation at 1,100 × g for 5 min. The supernatant was briefly treated with asymmetric-trichlorotrifluoroethane (Genesolv-D, electronic grade, Allied Chemical Corp.) followed by centrifugation at 1,100 × g for 5 min. The supernatant was centrifuged at 35,000 × g for 1 hr, and the pellet was resuspended in GMT buffer. The resuspended pellet was layered on a linear 30 to 60% sucrose gradient and centrifuged at 65,000 × g

in an SW 25.2 rotor for 3.5 hr. The centrifuge tube was punctured and fractions were collected. The viral fractions were pooled and diluted with GMT buffer, and the virus was pelleted by centrifuging at 35,000 × g for 2 hr. The viral pellet was suspended in GMT buffer (one-third concentration) and stored at 0°C. Except for minor modifications, this was essentially a purification procedure devised by I. Kimura, Department of Botany, University of Illinois, Urbana, Ill., who kindly supplied the information.

Assay of polymerase activity. The standard assay mixture (3, 5, 8) contained in a volume of 0.25 ml: uridine-5'-triphosphate (UTP) and cytidine 5'-triphosphate (CTP), 0.1 μmole; guanosine 5' triphosphate (GTP), 0.2 μmole; adenosine 5' triphosphate (ATP), 0.5 μmole; Tris (pH 8.0), 25 μmoles; MgCl₂, 3 μmoles; 2-mercaptoethanol, 3 μmoles; actinomycin D, 4 μg; ³H-UTP, 1 to 2.5 μCi; and purified WTV equivalent to 40 to 60 μg of protein (except where noted). After incubation for 2 hr at 27 to 28°C, the reaction was terminated by the addition of 2.0 ml of 7.5% trichloroacetic acid and 0.02 M sodium pyrophosphate (NaPP). The reaction mixture was filtered onto Schleicher and Schuell B-6 membrane filters and washed seven times with 2.0 ml of trichloroacetic acid-NaPP solution and once with 2 ml of 95% ethanol. After drying, membrane filters were placed in 5 ml of Omniflor toluene, and radioactivity was determined by liquid scintillation spectrometry. With each preparation of WTV assayed for enzymatic activity, the protein content was determined by the method of Lowry, using prior trichloroacetic acid precipitation (6).

Isolation of viral RNAs. Purified WTV or tobacco mosaic virus (TMV) was treated with Tris (pH 8.0)-buffered phenol, and the resulting aqueous phase was extracted twice more with buffered phenol. The aqueous phase was then extracted three times with ether and finally treated with 2 volumes of 95% ethanol saturated with NaCl. The RNA precipitate obtained by this treatment was washed once with 70% ethanol and then redissolved in 0.01 M Tris (pH 7.2). CPV RNA was isolated as previously described (5).

Preparation of ³H-labeled polymerase product. Approximately 0.3 mg of purified WTV in a volume of 0.8 ml was incubated at 28°C for 2 hr, the reagent concentrations being those of the standard assay.

¹ Preliminary report of this work was presented at the First International Conference on Comparative Virology, 2-4 September 1969.

The reaction mixture was cooled to 0°C, after which 0.1 g of triisopropylphenyl sulfonate was added. The mixture was extracted twice with 1.0 ml of phenol buffered with 0.1 M Tris at pH 7.2. After ether extraction, the reaction mixture was made 0.05 M with sodium phosphate buffer (equimolar concentrations of Na_2HPO_4 and NaH_2PO_4) and placed on a 0.3-g hydroxyapatite column. The column was extensively washed with 0.05 M sodium phosphate at 25°C to remove nucleosides, nucleotides, and small oligonucleotides (1). The column temperature was raised to 60°C and eluted with 0.14 M sodium phosphate which removes synthesized single-stranded RNA product but not viral double-stranded RNA (1). The ^3H -RNA product was ethanol-precipitated and dialyzed versus 0.01 M Tris (pH 7.2).

Annealing of ^3H -labeled polymerase product with viral RNAs. Viral RNAs and dialyzed ^3H -labeled product were mixed in 0.01 M Tris (pH 7.2) in assay tubes at the concentrations shown in Fig. 5. The assay tubes were heated to 95°C for 4 min and then rapidly cooled to 0°C. The assay mixtures were then heated to 65°C, made 0.3 M with NaCl, and annealed for 3 hr. As indicated in Fig. 5, the assay mixtures were treated with ribonuclease at an assay concentration of 2 $\mu\text{g}/\text{ml}$ for 30 min at 37°C. The samples were chilled at 0°C; 97 μg of TMV-RNA was added to each and precipitated with 7.5% trichloroacetic acid. The precipitates were filtered, washed, and assayed for radioactivity.

RESULTS

Cosedimentation of WTV and ability to incorporate ^3H -UTP. Sucrose density gradient cen-

trifugation separates WTV from viral shells produced mainly by the action of the organic solvents used during isolation. Electron microscopy shows that the faster sedimenting component consists of virus particles and the slower sedimenting component consists of viral shells (I. Kimura, *personal communication*). When these two components were isolated from preparative sucrose gradients, treated with 1% sodium dodecyl sulfate (SDS), and sedimented in parallel SDS sucrose density gradients, only the faster sedimenting component yielded RNA as revealed by ultraviolet spectrophotometry. Finally, analytical boundary sedimentation showed the faster sedimenting component had a sedimentation coefficient of 514S, whereas the slower sedimenting component had a sedimentation coefficient of 327S as would be expected for a virus and its viral shell devoid of RNA, respectively (4).

The optical density and trichloroacetic acid precipitable protein values (Fig. 1) show that fractions 12 to 17 contain WTV, whereas fractions 18 to 25 contain the viral shells. Only WTV incorporates ^3H -UTP into trichloroacetic acid-precipitable material. The viral shells do not possess any ability to incorporate ^3H -UTP, an observation strengthening the hypothesis that the assayed level of trichloroacetic acid-precipitable radioactivity is due to enzymatic polymerization rather than adsorption of nucleoside triphosphates.

FIG. 1. Cosedimentation of WTV and the ability to incorporate ^3H -UTP. A 7.5-ml amount of WTV and WTV shells in GMT buffer purified with one sucrose gradient was layered on a 15-ml linear 30 to 60% sucrose gradient. All solutions used were buffered with 0.1 M glycine, 0.01 M MgCl_2 , 0.01 M Tris (pH 7.5). Centrifugation was at $51,000 \times g$ for 4 hr at 4°C with a Spinco SW 25.1 rotor and ZU ultracentrifuge. Fractions were collected by puncturing the bottom of the centrifuge tube. Samples were removed for optical density and trichloroacetic acid-precipitable Lowry protein determinations. The remainder of each fraction was diluted with buffer and pelleted by using Spinco 2-ml nitrocellulose tubes and a 40 rotor. Centrifugation was at $40,000 \times g$ for 1 hr. The pellets were resuspended in the standard assay mixture and incubated for 4 hr. The optical density and protein determinations correspond to the quantity of virus used in the incorporation assay.

Enzymatic characterization of WTV polymerase. The ability of WTV to incorporate ^3H -UTP was further characterized as shown in Table 1. This activity is dependent on all four ribonucleoside triphosphates, and uridine will not substitute for UTP. Both of these observations argue against microbial contamination being responsible for the enzymatic activity. The addition of pyruvic kinase and phosphoenol pyruvic acid resulted in only a small increase in activity. The presence of actinomycin D slightly enhanced the level of activity. All the foregoing observations show that purified WTV contains an RNA-dependent RNA polymerase activity.

The effect of pH on the level of WTV polymerase activity can be seen in Fig. 2. The results are very similar to those for reovirus polymerase (8) and CPV polymerase (5). The temperature optimum, as might be expected for a plant insect virus, is about 28 to 30 C (Fig. 3). These results are in interesting contrast to those reported for a cucumber mosaic virus-induced RNA polymerase activity which is maximal at 37 C (7). They are, however, in agreement with the observed temperature optimum of CPV polymerase (5). The assay is time-dependent as can be seen in Fig. 4.

In the foregoing experiments, different preparations of WTV were used in each figure and table, with the exception of Fig. 3 and Table 1. Consequently, there was a considerable range in the

FIG. 2. Dependence of polymerase activity on pH. Same conditions of standard assay, except 25 μmoles of Tris-maleate of noted pH added in place of Tris-hydrochloride.

TABLE 1. Enzymatic characterization of wound tumor virus

Assay mixture	^3H -UMP incorporated (nmoles/mg of protein)
Standard ^a	24.62
—Virus.....	0.25
—GTP.....	0.59
—ATP.....	0.58
—CTP.....	0.49
—UTP + 2.5 μCi of ^3H -GTP.....	0.84
+UTP + 2.5 μCi of ^3H -GTP.....	30.91
—2-Mercaptoethanol.....	27.68
+Pyruvic kinase +PEP.....	29.93
— ^3H -UTP + 2.5 μCi /0.1 μmole of ^3H -uridine.....	0.26
—Act-D.....	21.84
—Act-D —GTP.....	0.65
Standard at 0 C.....	0.66

^a Standard assay mixture, in a volume of 0.25 ml, consisted of UTP and CTP, 0.1 μmole ; GTP, 0.2 μmole ; ATP, 0.5 μmole ; Tris (pH 8.0), 25 μmoles ; MgCl_2 , 3 μmoles ; 2-mercaptoethanol, 3 μmoles ; actinomycin D, 4 μg ; ^3H -UTP, 2.5 μCi ; and purified WTV equivalent to 60 μg of protein.

specific polymerase activity of the different virus preparations (0.3 to 28 nmoles of ^3H -UTP incorporated/mg of protein). The higher levels of polymerase activity were observed for WTV isolated rapidly from recently harvested plant tumors. The acidic pH of the plant tumors and plant nucleases is probably responsible for this decrease in activity for WTV isolated from older plant tumors. Purified WTV stored at 0 C underwent a fourfold decrease in polymerase activity in a 3-week period.

Characterization of the ^3H -labeled product synthesized by WTV. On the basis of the above observations, WTV contains an RNA polymerase. Moreover, it is highly probable that such an enzyme would operate as a transcriptase. To support this hypothesis, ^3H -labeled WTV polymerase product was annealed with viral RNAs (Fig. 5). The specificity of annealing is clear: only WTV-RNA forms ribonuclease-stable helices. TMV and CPV RNAs give no increase in trichloroacetic acid-precipitable radioactivity relative to annealing of ^3H -RNA alone. Thus, the present annealing studies involving CPV and WTV RNAs revealed no common relationship.

FIG. 3. Dependence of polymerase activity on incubation temperature. Same conditions of standard assay, except incubation temperature.

However, the *in vitro* transcription of the WTV genome may have been incomplete and hence does not rule out the possibility of some homology between CPV and WTV RNAs.

³H-RNA annealed with WTV-RNA, followed by heat denaturation and ribonuclease digestion, also produced a background level of radioactivity. Such a melting-out control shows that the ribonuclease-resistant radioactivity is not due to a protein contaminant in the WTV-RNA preparation. Only 53% of the trichloroacetic acid-precipitable product is converted to a ribonuclease-resistant form after annealing; it is likely that some of the product is large enough to be retained by membrane filtration but too small for helix formation at 65 C.

DISCUSSION

As has been shown with reovirus (3, 8, 9) and CPV (5), WTV has an associated enzyme activity capable of transcribing single-stranded RNA from the double-stranded RNA genome. Still in question is the origin of such an enzyme. Given the diverse host range of WTV (2) and the lack of known cellular RNA transcriptase activities, it is likely that the transcriptase is coded for by the viral genome. Isolation of WTV possessing

FIG. 4. Dependence of polymerase activity on length of incubation. Same conditions of standard assay, except length of incubation.

FIG. 5. Annealing of ³H-labeled polymerase product with viral RNAs. Symbols: ●, WTV-RNA without ribonuclease digestion; ○, WTV-RNA; ▼, TMV-RNA; Δ, CPV-RNA (kindly provided by J. Kalmakoff and L. J. Lewandowski); ■, WTV-RNA (after annealing, heat denatured at 100 C for 4 min before ribonuclease digestion). Each annealing assay initially contained 1,950 counts/min of dialyzed ³H-labeled polymerase product. Except as noted above, each sample after annealing was treated with ribonuclease at an assay concentration of 2 μg/ml for 30 min at 37 C. See text for further details.

transcriptase from infected *Agallia constricta* (AC) cells would support this hypothesis. The present study suggests that the mechanism of WTV viral synthesis is also similar to that hypothesized for reovirus (9): after virus penetration into the host cell, the WTV transcriptase produces viral messenger RNAs which are subsequently

translated into viral proteins. Such messenger RNAs could be translatable by both plant and insect hosts, or certain of these messenger RNAs might be translatable only by one cell system or the other. The close similarity in RNA subunits isolated from WTV prepared from plant and insect cells indicates that the replication of viral double-stranded RNA must have a comparable degree of similarity in the two hosts (4). However, WTV has no observable effect on insect hosts while its tumorigenic effect on plant hosts is widespread. This great difference in insect and plant host response to WTV infection remains a most intriguing question about this virus.

ACKNOWLEDGMENTS

The authors are especially grateful to I. Kimura for virus purification procedures and to L. M. Black for WTV infected plant tumors. We are also grateful to J. Kalmakoff, L. J. Lewandowski, and M. E. Reichmann for helpful experimental suggestions and discussions.

This investigation was supported by Public Health Service research grant AI 00634 from the National Institute of Allergy and Infectious Diseases and by training grant GM 01389 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Bernardi, G. 1969. Chromatography of nucleic acids on hydroxyapatite. III. Chromatography of RNA and polyribonucleotides. *Biochim. Biophys. Acta* 174:449-457.
2. Black, L. M. 1965. Physiology of virus-induced tumors in plants, p. 236-266. *In* Encyclopedia of plant physiology. Springer, Berlin.
3. Borsa, J., and A. F. Graham. 1968. RNA polymerase activity in purified virions. *Biochem. Biophys. Res. Commun.* 33: 895-901.
4. Kalmakoff, J., L. J. Lewandowski, and D. R. Black. 1969. Comparison of the ribonucleic acid subunits of reovirus, cytoplasmic polyhedrosis virus, and wound tumor virus. *J. Virol.* 4:851-856.
5. Lewandowski, L. J., J. Kalmakoff, and Y. Tanada. 1969. Characterization of a ribonucleic acid polymerase activity associated with purified cytoplasmic polyhedrosis virus of the silkworm *Bombyx mori*. *J. Virol.* 4:857-865.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
7. May, J. T., J. M. Gilliland, and R. H. Symons. 1969. Plant virus induced RNA polymerase: properties of the enzyme partly purified from cucumber cotyledons infected with cucumber mosaic virus. *Virology* 39:54-65.
8. Shatkin, A. J., and J. D. Sipe. 1969. RNA polymerase activity in purified reoviruses. *Proc. Nat. Acad. Sci. U.S.A.* 61: 1462-1469.
9. Skehel, J. J., and W. K. Joklik. 1969. Studies in the *in vitro* transcription of reovirus RNA catalyzed by reovirus cores. *Virology* 39:822-831.