JOURNAL OF VIROLOGY, Dec. 1969, p. 857-865
Vol. 4, No. 6

Characterization of a Ribonucleic Acid Polymerase Activity Associated with Purified Cytoplasmic Polyhedrosis Virus of the Silkworm *Bombyx mori*[1]

L. J. LEWANDOWSKI,[2] J. KALMAKOFF, AND Y. TANADA

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

Received for publication 20 August 1969

Purified cytoplasmic-polyhedrosis virus has been found to have associated with it a polymerase activity capable of catalyzing the synthesis of virus-specific, single-stranded ribonucleic acid (RNA) from the double-stranded RNA genome.

The cytoplasmic polyhedrosis virus (CPV) of the silkworm is known to contain double-stranded ribonucleic acid (RNA) as its genetic material (10). In an accompanying report (5), the number and molecular weight of the double-stranded RNA subunits which comprise the CPV genome are described. The substructuring of the RNA genome into distinct subunits, also a characteristic for the genomes of reovirus and wound tumor virus (5, 16, 18), might well require a mechanism of replication peculiar to double-stranded RNA viruses.

The early steps in the replicative cycle of double-stranded RNA viruses have been extensively studied only with reovirus (2*a*, 14). In this system, double-stranded RNA itself is unable to function as messenger RNA (3, 13), and during infection a strand separation of the genome into single-stranded message does not seem to occur (17). Attempts to demonstrate cellular polymerases capable of transcribing single-stranded RNA from viral double-stranded RNA have been unsuccessful (13). Reovirus type 3 has now been shown to contain an enzyme capable of catalyzing the synthesis of virus-specific single-stranded RNA in vitro (1, 15).

Little is known about the process of replication of CPV. Except for the work of Kawase (8), showing that de novo synthesized double-stranded RNA of CPV first becomes measurable between 33 and 57 hr after oral infection of silkworm larvae, early events in transcription and replication have not been reported.

The present study shows that purified particles of CPV also contain an in vitro RNA polymerase activity which in many respects is analogous to that found in reovirus. The characteristics of this system are described and a suggestion as to how the initial transcription of the CPV genome might occur in the host cell is discussed.

MATERIALS AND METHODS

Chemicals. ^{3}H-uridine 5'-triphosphate (22.5 c/mmole) and ^{3}H-guanosine 5'-triphosphate (6.05 c/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Carrier-free ^{32}P phosphoric acid (10 mc/ml) came from Mallinckrodt Nuclear, St. Louis, Mo. Ribonuclease-free sucrose was obtained from Mann Research Laboratories, New York, N.Y. Pancreatic ribonuclease, trypsin, α-chymotrypsin and Pronase were purchased from Worthington Biochemical Corp., Freehold, N.J. Pyruvate kinase, 2-phosphoenolpyruvic acid and the nucleoside triphosphates were products of Calbiochem, Los Angeles, Calif. Genesolv-D came from Allied Chemical, Morristown, N.J. Actinomycin D was a gift from Merck, Sharp, and Dohme Research Labs, Rahway, N.J., and rifampin was a gift from Ciba Pharmaceuticals, Summit, N.J. All other chemicals were the commonly available reagent grade preparations.

Preparation of cytoplasmic polyhedrosis virus from Bombyx mori. Fifth-instar larvae of the silkworm (*Bombyx mori*, strains C-124, Daizo, or Hosho x Shungetsu from Kanegafuchi Co., Tokyo, Japan) were infected by feeding them mulberry leaves which had been painted with a suspension of polyhedra. After 8 days at 24 C, just prior to pupation, infected guts were removed, freed from the bulk of their food

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

[2] Present address: Department of Microbiology, University of Otago, Dunedin, New Zealand.

contents, and suspended in several volumes of ice-cold 0.01 M tris (hydroxymethyl)aminomethane-(Tris)-hydrochloride, *p*H 7.6. The suspension was homogenized at full speed in a Waring Blendor for 30 sec and filtered first through cheesecloth and then through a layer of organdy. This treatment effectively removed large cell debris and most of the remaining food particles. All subsequent operations were performed at 4 C unless otherwise stated.

To obtain semipurified polyhedra, 10 ml of the above suspension was layered on a step-gradient composed of 6 ml each of 60, 50, and 40% sucrose (w/v) in Tris buffer and sedimented in an SW 25.1 rotor for 10 min at 10,000 × *g*. The resulting pellet consisted mainly of polyhedra. For a greater degree of purity, this pellet was resuspended, and the above step-gradient centrifugation was repeated.

Polyhedra were treated with weak alkali to release the virus particles by a modification of the method of Hosaka and Aizawa (4). Polyhedra were suspended and dissolved at room temperature in a solution which consisted of 0.1 M NaCl and 0.05 M Na_2CO_3, *p*H 10.6. After 1 hr the solution was brought to *p*H 7.2 to 7.4 with 0.2 M HCl and centrifuged for 10 min at 12,000 × *g* in a refrigerated Sorvall centrifuge. The supernatant fluid was removed and centrifuged for 90 min at 75,000 × *g* in a Spinco 30 rotor. The pellets were allowed to resuspend in buffer overnight and were then extracted with 1 volume of Genesolv-D: 2 volumes of virus for 5 min. After centrifugation, the top buffer layer was carefully removed from the interphase debris and immediately layered on a linear 10 to 40% sucrose gradient in Tris buffer, and sedimented for 90 min in an SW 25.1 Spinco rotor at 51,000 × *g* at 6 C. The gradient was fractionated by piercing the tube and collecting 1-ml samples through sterile tubing into sterile tubes.

For ^{32}P-labeled virus, the above procedure was used with the following modification. Three days after infection the larvae were starved for a period of 12 hr and were then fed leaf discs on which about 0.35 mc of ^{32}P-phosphoric acid was dried. The larvae were starved for an additional 12-hr period, after which time they were fed in the usual manner. Assays for the infectivity of purified virus, purified double-stranded RNA, or total RNA from infected gut tissue were performed in fourth-instar larvae of the hybrid Hosho x Shungetsu via intrahemocoelic inoculation through the insect proleg.

Preparation of RNA. Preparation of double-stranded RNA from purified virus was accomplished by incubating the virus with 1% sodium dodecyl sulfate (SDS) at 45 C for 10 min, followed by three phenol treatments at room temperature. After this extraction, the RNA was precipitated by the addition of 3 volumes of NaCl-saturated 95% ethyl alcohol at −20 C. The precipitate was washed with several volumes of 70% ethyl alcohol and stored at −20 C. This same procedure was also used to extract total RNA directly from infected midgut tissues. Heat-denatured ^{32}P-labeled CPV-RNA was obtained by boiling double-stranded CPV-RNA for 8 min in 0.01 M Tris-hydrochloride (*p*H 7.4), followed by quick chilling in an ice bath.

The conditions for the separation of single-stranded RNA from viral double-stranded RNA by hydroxyapatite chromatography were as follows. The sample was applied to a column (3.0 by 0.9 cm) of hydroxyapatite, previously equilibrated with 0.1 M phosphate (*p*H 6.7) at room temperature. When this column was heated to 90 C by water flowing from a Haake thermostatically controlled bath, single-stranded RNA was eluted. Double-stranded RNA was released from the column with 0.3 M phosphate (*p*H 6.7) at 90 C.

The conditions for polyacrylamide gel electrophoresis and sucrose gradient analysis of RNA have been described in the accompanying report (5) and are noted in the figure legends.

Assay for RNA synthesis in vitro. The standard reaction mixture for RNA synthesis in vitro contained each of the following constituents: 0.1 μmole of cytidine 5′-triphosphate (CTP), guanosine 5′-triphosphate (GTP), and uridine 5′-triphosphate (UTP); 0.5 μmole of adenosine 5′-triphosphate (ATP); 1 μc of ^3H-UTP; 25 μmoles of Tris-hydrochloride (pH 8.0); 1.5 μmoles of $MgCl_2$; 0.7 μmole of phosphoenolpyruvate (PEP); 25 μg (6 units) of pyruvate kinase; and, except where noted, purified CPV equivalent to approximately 27.5 μg of protein and 8.2 μg of RNA. The total volume of the reaction mixture was 0.25 ml. This mixture was incubated for 120 min at 27 C, and the reaction was terminated by the addition of 3 ml of ice-cold 7.5% trichloroacetic acid containing 0.02 M Na pyrophosphate (NaPP). The precipitate was filtered through Schleicher and Schuell membrane filters (0.45 μm pore-size) and washed with 20 ml of 7.5% trichloroacetic acid containing 0.02 M NaPP, and then with 95% ethyl alcohol. The filters were placed in scintillation vials, dried, and the radioactivity was determined by liquid scintillation spectrometry.

Ribonuclease sensitivity of single-stranded RNA. Isotopically labeled RNA samples made 0.3 M in NaCl were treated with 2 μg/ml of pancreatic ribonuclease at 37 C for 1 hr. Single-strandedness was measured by the amount of radioactivity rendered acid-soluble under the above conditions.

RNA-RNA annealing studies. The ^3H-labeled product to be tested (whether purified by hydroxyapatite or by phenol extraction of the assay mixture) was combined with 40 μg of double-stranded CPV-RNA in the presence of 0.3 M NaCl; this mixture was boiled for 8 min, chilled quickly on ice, and divided into three portions. One sample was allowed to reanneal at 65 C for 1 hr. This reannealed sample and a second portion of the boiled mixture were each incubated with 2 μg/ml of pancreatic ribonuclease at 37 C for 30 min. These two samples, along with a third untreated sample, were precipitated with 7.5% trichloroacetic acid at 4 C. The precipitates were filtered, washed, and assayed for radioactivity.

All other experimental details can be found in the figure legends.

RESULTS

Cosedimentation of CPV and the capacity to polymerize ribonucleoside triphosphates in vitro.

Figure 1 shows that in vitro polymerase activity cosediments in a sucrose gradient with the virion of cytoplasmic polyhedrosis virus. Maximal activity of the enzyme (or enzymes) involved coincides with the major peak of ultraviolet (UV)-absorbing material present in fraction no. 8.

To establish that this peak represented intact purified virus, a portion of this peak material was dialyzed against 0.01 M Tris buffer and subjected to velocity sedimentation in a Spinco model E ultracentrifuge. A single component having an S value of 440 ± 20 was observed (5). When examined under the electron microscope this material had a diameter of 65 to 70 nm, in agreement with the value reported for intact virus (4). Analysis on polyacrylamide gel columns of the total RNA content of this peak showed only double-stranded subunits as previously observed (5). Furthermore, a qualitative test for infectivity was performed by inoculating 2 μliters of this 440S material into each of 20 fourth-instar silkworm larvae. Gross histopathological assay revealed that within 11 days after infection 18 of 20 larvae developed thorough cytoplasmic polyhedrosis of the midgut.

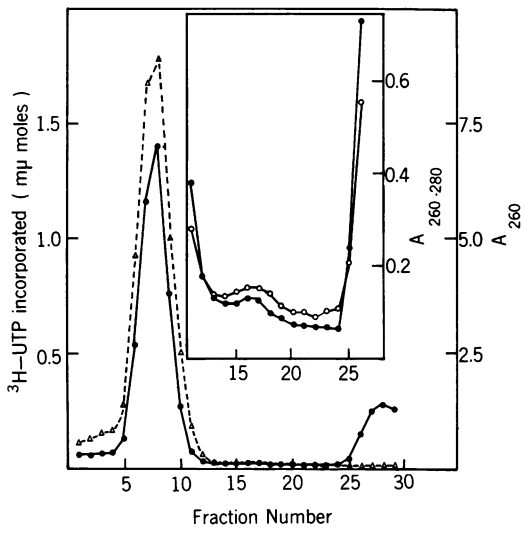

FIG. 1. *Cosedimentation of CPV and in vitro polymerase activity. Virus was extracted as noted in Materials and Methods, layered on a 10 to 40% linear sucrose gradient in Tris buffer (pH 7.6) and sedimented at 23,000 rev/min at 6 C for 90 min by using an SW 25.1 rotor in a Spinco L-2 centrifuge. Fractions (1 ml) were collected, and after determining optical density at 260 and 280 nm, 25-μliter samples from each fraction were added to tubes containing the standard assay mix without virus. The mixtures were tested for their ability to synthesize RNA in vitro over a 120-min incubation period at 27 C. The insert represents an enlargement of the absorbance profiles from fractions 11 to 26. Symbols: ●, A_{260}; ○, A_{280}; △, ^{3}H-UTP incorporated per reaction mixture.*

Fractions 6–9 of the viral peak (Fig. 1) were pooled and, unless otherwise stated, were used without dialysis for all subsequent studies examined in this paper. The convenient method of storing the virus pool in sucrose was made possible by the fact that the presence of 5% (or less) sucrose does not affect the levels of polymerase activity (Table 1).

In addition to the viral component having a spectral 260 to 280 ratio of 1.48 (uncorrected for light scattering), there is present in fractions 16 and 17 of the gradient (insert) a minor protein-rich component with a 260 to 280 ratio of approximately 0.83. This minor component, which sediments in sucrose at approximately 260S, is unstable in comparison to the intact virion, since further attempts to concentrate and repurify it produced material having a lower S value. Concentrates of this component contained no demonstrable viral RNA when analyzed on polyacrylamide gels, and, probably as reflection of this lack of template, the material was not able to stimulate RNA synthesis under standard assay conditions.

Characteristics of CPV-associated RNA polymerase activity. The requirements for in vitro uptake of labeled UTP into acid-insoluble product by purified CPV are examined in Table 1. The reaction is strictly dependent upon the presence of the ribonucleoside triphosphates and of virus. Deletion of the energy-generating system appreciably reduces the amount of label incorporated. Divalent cation is essential, and although Mg^{2+} is optimal, a low level of incorporation can occur when the Mg^{2+} is replaced by the same amount of Mn^{2+}. The extent of incorporation is somewhat reduced by the presence of 2-mercaptoethanol, but not by the presence of either actinomycin D or sucrose up to 5%. The presence of added primer, whether in the double-stranded or heat-denatured form, neither stimulates nor inhibits the reaction to any appreciable degree at those concentrations tested.

In each series of experiments a complete reaction mixture was examined in the light microscope for the presence of visible contamination. On those occasions when microscopically detectable contaminants were present, their presence was reflected in high background levels of incorporation in the absence of GTP. It therefore became standard operating procedure to include a "minus GTP" assay tube in all experiments as a reliable measure of the presence

TABLE 1. *Characteristics of the in vitro synthesis of RNA by purified CPV*

Reaction mixture[a]	nmole ^{3}H-UTP incorporated[b]
Complete	1.311
−ATP	0.029
−CTP	0.019
−GTP	0.017
−Virus	0.005
−PEP, Pep-kinase	0.233
$-Mg^{2+}$	0.011
$-Mg^{2+}$, $+Mn^{2+}$	0.219
−Actinomycin D	1.300
−Sucrose	1.241
+2-Mercaptoethanol (3 μmoles)	0.992
+CPV heat-denatured RNA (4 μg)	1.307
+CPV double-stranded RNA (4 μg)	1.278
+CPV double-stranded RNA (10 μg)	1.178
+Uridine (0.1 μmole)	1.005
−^{3}H-UTP + ^{3}H-uridine (1 μc/0.1 μmole)	0.011
+Rifampin (40 μg/ml)	1.252

[a] Standard assay conditions present as described in Materials and Methods, except where noted.

[b] The studies reported in this present paper were all done with a single virus preparation. With different preparations, however, the properties of the enzyme remained reproducible, except for the level of enzyme activity per unit of virus which sometimes varied as much as threefold from preparation to preparation.

of gross levels of contamination. Furthermore, to eliminate the possibility that the observed levels of incorporation might be due to the presence of contaminating microorganisms sufficiently dilute to escape microscopic detection, the following controls were performed. Since in general, microorganisms must first dephosphorylate UTP to take up and utilize the uridine moiety, a large excess of unlabeled uridine was used in the reaction mixture to dilute any radioactive uridine which might result from dephosphorylation of the ^{3}H-labeled UTP. In this way, incorporation levels should be reduced to background, provided of course that incorporation was indeed due to the presence of a contaminant. Table 1 shows only slight reduction of incorporation of labeled UTP. When ^{3}H-UTP was replaced by ^{3}H-uridine (1 μc/0.1 μmole), incorporation remained at background. Finally, addition to the assay system of rifampin, a drug known to be a potent inhibitor of both in vivo and in vitro bacterial deoxyribonucleic acid (DNA)-dependent RNA polymerase activity (19), had little, if any, effect on the level of isotope incorporation. It seems clear, therefore, that the RNA polymerase activity having the characteristics shown in Table 1 is not due to the presence of contaminating microorganisms.

The *p*H and temperature requirements for the polymerase reaction are shown in Fig. 2a and b. Although incorporation occurs within the broad range of *p*H 7.5 to 9.0, and from 18 C to 37 C, optima are clearly reached at *p*H 8.0 (Fig. 2a) and approximately 27 C (Fig. 2b).

The kinetics of in vitro RNA synthesis by purified CPV are examined in Fig. 3a, and show that the specific activity of the enzyme is directly proportional to the concentration of virus present

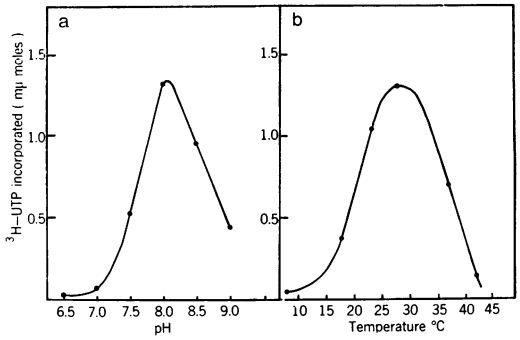

FIG. 2. *Polymerase activity. (a) Dependence of polymerase activity on pH. Virus was incubated at 27 C in the standard assay mixture buffered with Tris-maleate to the above pH values and titered for polymerase activity. (b) Dependence of polymerase activity on temperature. Virus was incubated at various temperatures in the standard assay mix at pH 8.0 and titered for polymerase activity.*

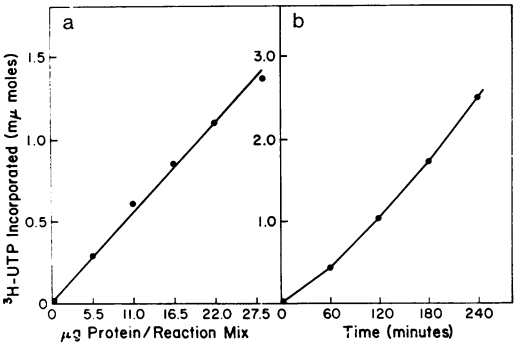

FIG. 3. *RNA synthesis. (a) Dependence of RNA synthesis on virus concentration. Virus at the concentration noted was incubated in the standard assay. (b) Time course of RNA synthesis. Virus was incubated for the indicated intervals in the standard assay.*

in the assay tube. Furthermore, analysis of the time course of the reaction (Fig. 3b) illustrates that there is an increase in the rate of incorporation as a function of time of incubation for at least 4 hr, at which time this particular experiment was terminated. This lag, however, was not detectable when virus was used which had been extracted from polyhedra contained in gut tissue that had been under refrigeration for about 6 years. Although such virus possessed the same general requirements for in vitro incorporation as virus from freshly prepared polyhedra, it exhibited only about 20% of total enzyme activity on a "per A_{260}" basis. This suggests that enzyme activity still present in the older preparation may be more quickly accessible to the substrates than enzymes in the fresh virus stock.

The absolute requirement of the polymerase reaction for divalent cations has already been noted in Table 1. The upper curve in Fig. 4 shows that maximal incorporation occurs in the presence of from 1 to 2 μmoles of Mg^{2+}. The two lower curves show that although Mn^{2+} can substitute somewhat for Mg^{2+} in this reaction, enzyme activity is negligible when Mg^{2+} is replaced by Ca^{2+}.

The extent of polymerization of ^{3}H-UTP or ^{3}H-GTP into a trichloroacetic acid-precipitable product was measured as a function of the concentration of one of the ribonucleoside triphosphates while the three others were kept constant at the concentrations used in the standard assay. Little increase in RNA synthesis was observed with the pyrimidine triphosphates at concentrations above 0.1 μmole. With the purine triphosphates as variables, however, maximal incorporation of ^{3}H-UTP was obtained with 1.0 μmole of GTP and 2.0 μmoles of ATP. Furthermore, at higher levels of any triphosphate, there was a marked inhibition of polymerizations of labeled triphosphate. Purine-dependent stimulation of in vitro RNA synthesis has previously been observed with the vaccinia-virus associated polymerase system (12).

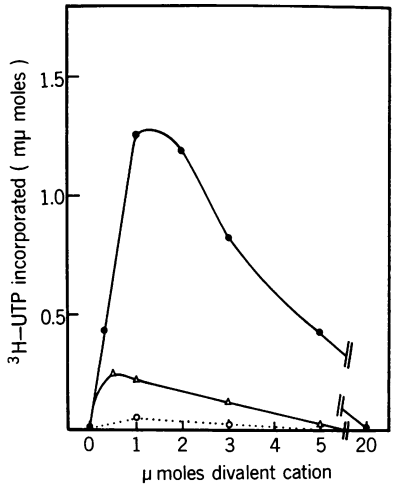

FIG. 4. *Dependence of RNA synthesis on divalent cations. Virus was incubated in the standard assay modified to contain the concentration of divalent cation as noted. Symbols:* ●, Mg^{2+}; △, Mn^{2+}; ○, Ca^{2+}.

Attempts to increase RNA polymerase activity of purified CPV. Both reovirus type 3 and the poxviruses have been shown to contain RNA polymerase activities which are demonstrable only after the virion has been structurally altered (1, 6, 15). This is not the case with purified CPV, since it clearly exhibits a polymerase activity without further "activation." Since, however, the CPV-associated enzyme might represent only a fraction of that potentially available, tests were made for conditions which might allow further expression of activity. Virus was preincubated under various conditions, and then assayed under the standard procedure. The observation that virus extracted from polyhedra by means of a Na_2CO_3 treatment at *p*H 10.6 is already capable of expressing polymerase activity suggested that further controlled exposure of the purified virus to these same conditions might cause a further increase in the level of activation. This was not the case; not only was there a lack of further stimulation, but enzyme activity dropped slightly for the first 60 min and then fell off precipitously. Therefore, if conditions of high *p*H do serve to activate the virus, such activation is restricted to the initial exposure during virus purification.

The conditions and results of the next series of preincubations are outlined in Table 2. In brief, the most significant increase (twofold) was observed when the virus stock was preincubated for 1 hr at 37 C in distilled water or 0.01 M Mg^{2+}. The treatments used to activate reovirus, type 3, i.e., enzymatic digestion of the outer virus shell protein (15) and controlled heat-shocking of the virion (1), were unsuccessful in causing any appreciable increase in the level of polymerase activity. In fact, heat-shocking of the virion for 30 sec at 50 or 60 C prior to assay significantly reduced the levels of incorporation. Though not shown in Table 2, virus exposed to ultrasonic sound before assay showed no appreciable increase in enzyme activity.

It seems, therefore, that under the conditions tested, the virus can undergo no more than a twofold stimulation in the level of its polymerase activity.

Properties of the RNA product of in vitro polym-

TABLE 2. *Effects of various treatments on RNA polymerase activity of purified CPV*

Time and temperature	Preincubation conditions (additions)	^{3}H-UTP incorporated (nmoles)[a]
No preincubation	Control	1.287
60 min, 4 C	0.15 M Tris-hydrochloride (*p*H 8.0) + 0.01 M Mg^{2+}	1.285
60 min, 37 C	same as above	1.445
60 min, 37 C	same as above + chymotrypsin (200[b] μg/ml)	1.780
	(2,000 μg/ml)	1.028
60 min, 37 C	same as above + Pronase (200 μg/ml)	0.951
	(2,000 μg/ml)	0.321
60 min, 37 C	0.15 M Tris-hydrochloride (*p*H 8.0) with no Mg^{2+}	1.285
60 min, 37 C	0.01 M Mg^{2+} with no buffer	2.585
60 min, 37 C	Distilled H_2O	2.198
60 min, 37 C	Distilled H_2O + chymotrypsin (200 μg/ml)	1.971
	+ trypsin (200 μg/ml)	2.036
	+ Pronase (200 μg/ml)	0.850
30 sec, 50 C	Distilled H_2O	0.855
30 sec, 60 C	Distilled H_2O	0.091

[a] Standard assay conditions present as described in Materials and Methods, except where noted.
[b] Chymotrypsin (20 μg/ml) showed no significant increase in polymerase activity over 200 μg/ml.

erase activity. To determine whether the RNA product of the in vitro polymerase reaction was associated with the virion or was free in solution, an assay was performed and the entire contents of the assay tube were combined with a sample of heat-denatured ^{32}P CPV-RNA and unlabeled virus and immediately sedimented through a 10 to 40% sucrose gradient (Fig. 5). The ^{32}P label was added to measure any fortuitous attachment of single-stranded RNA to whole virus particles. After centrifugation, the bulk of the ^{3}H-labeled, trichloroacetic-precipitable product remained at the top of the gradient along with the ^{32}P marker. A minor amount of labeled product, however, remained associated with the virus band. Thus if the intact virion is the site of in vitro synthesis of product, most of this product becomes virus-free after a 2-hr period of synthesis.

The size distribution of the product of in vitro RNA polymerase activity was analyzed by two complementary techniques. Upon completion of a standard assay the reaction mixture was treated with SDS-phenol and RNA was precipitated from the aqueous phase. A sample of this RNA was mixed with double-stranded ^{32}P-labeled CPV-RNA and analyzed by polyacrylamide gel electrophoresis (Fig. 6). Heat-denatured ^{32}P-labeled RNA was analyzed on a companion gel, and the results were superimposed. Although the bulk of the in vitro product advanced as a sharp band just ahead of the dye front, a small portion of the total migrated as a heterogeneous population approximately in the region of the superimposed position of heat-denatured CPV-RNA.

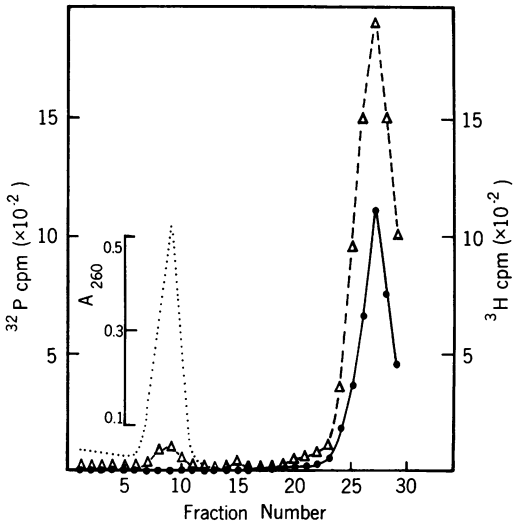

FIG. 5. *Sedimentation analysis of trichloroacetic acid-precipitable product RNA. ^{32}P-labeled heat-denatured CPV-RNA and unlabeled virus were added as markers to an assay tube upon completion of incubation and this mixture was layered on a 10 to 40% linear sucrose gradient; the sedimentation conditions were those used in Fig. 1. Fractions (1 ml) were collected and optical density at 260 nm was determined. The samples were combined with 1 ml of ice-cold 15% trichloroacetic acid and acid-precipitable counts were determined as noted in Materials and Methods. Symbols: △, ^{3}H counts/min present in in vitro synthesized RNA; ●, heat-denatured, ^{32}P-labeled RNA; (dotted line) A_{260} intact CPV.*

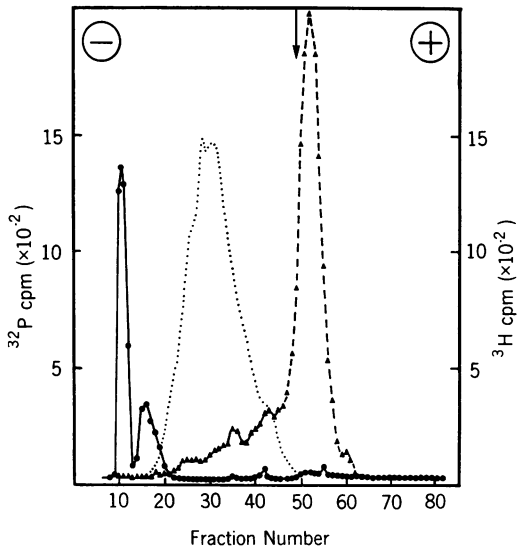

FIG. 6. *Comparison of the sizes of in vitro reaction product and virus RNA by polyacrylamide gel electrophoresis. Electrophoresis in an 8-cm, 2.5% acrylamide, 1% agarose gel was performed at 23 C and 5 ma/column for 2 hr by using 0.05 M Tris buffer (pH 8.4), 10^{-4} M ethylenediaminetetraacetic acid as running buffer. The gel was sliced, and the 1-mm slices were dried on glass fiber filters and counted by liquid scintillation. The arrow marks the forward edge of the bromophenol tracking dye. Symbols: △, ^{3}H-labeled product RNA; ●, ^{32}P-labeled CPV double-stranded RNA; (dotted line) ^{32}P heat-denatured CPV-RNA superimposed from a companion gel.*

For a more accurate determination of the size of the in vitro product, a second sample of labeled product was analyzed by sucrose density gradient centrifugation (Fig. 7). The position of the 15.5*S* and 12.5*S* double-stranded RNA markers allowed an approximation of the sedimentation coefficients of the mixed population of acid-precipitable ^{3}H label. Although the bulk of the RNA sedimented in several distinct bands between 7*S* and 11*S*, a small amount of the product was larger and sedimented along with the marker RNA. As shown by two independent techniques, therefore, most of the in vitro product, at least when it is extracted after a 2-hr period of incubation in the assay tube, is relatively small when compared to what might be expected if the polymerase were copying entire sequences of the genome.

To examine whether the product of the polymerase reaction was double- or single-stranded in nature, the standard reaction was allowed to occur, and the entire contents of the assay tube were analyzed by hydroxyapatite column chromatography. Under the conditions employed, double- and single-stranded RNA can be clearly separated. Figure 8 shows that the acid-precipitable, ^{3}H-labeled product elutes at 90 C in 0.1 M phosphate (*p*H 6.7), conditions that retain double-stranded RNA on the column. Fractionation of the product into several distinct species occurred and the distribution pattern resembled that obtained from sucrose gradient sedimentation. When the phosphate concentration was raised to 0.3 M, no ^{3}H-labeled RNA appeared at the position (fraction 60) where double-stranded RNA elutes under these conditions. When treated with ribonuclease, no ^{3}H-labeled trichloroacetic acid-precipitable counts remained. By two separate criteria the product behaves like single-stranded RNA.

The "remainder" of fractions 59-38 was pooled and, after the addition of 500 μg of tobacco mosaic virus (TMV)-RNA as carrier, was precipitated from solution by the acetone-potassium acetate method of Kirby (9). The precipitate was dissolved in Tris buffer and reprecipitated with 95% NaCl-saturated ethyl alcohol. This material, which we term sample A, was subsequently tested for its ability to anneal with heat-denatured CPV-RNA.

The reannealing studies consisted of the following. Two reaction mixtures were prepared, one

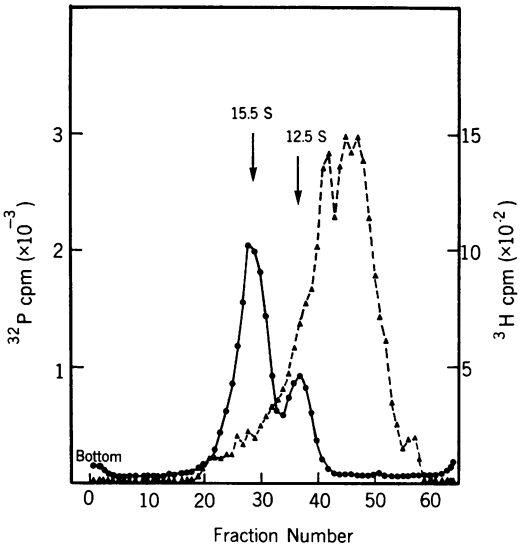

FIG. 7. *Size distribution of RNA synthesized by CPV in vitro as determined by sucrose gradient analysis. Sedimentation was performed through a linear 5 to 20% sucrose gradient containing 0.1 M NaCl, 0.01 M Tris buffer (pH 7.6), 10^{-3} M ethylenediaminetetraacetic acid, and 1% SDS in an SW 41 rotor at 40,000 rev/min at 6 C for 6.5 hr. Symbols: △, ^{3}H-labeled product RNA; ●, ^{32}P-labeled CPV double-stranded RNA.*

utilizing ^{3}H-UTP (sample B) and the other ^{3}H-GTP (sample C). After incubation, the reaction mixtures were extracted by SDS-phenol in the presence of 100 μg of carrier TMV-RNA and the RNA in the aqueous phases was precipitated with ethyl alcohol. The annealing procedure is described in detail above.

Whether the product is labeled in the uridine or the guanidine moiety, and whether it is first purified by column chromatography or directly extracted from the assay mixture, on the average between 83 and 93% of the product is ribonuclease resistant after annealing with heat-denatured unlabeled CPV-RNA (Table 3). The results of this experiment are unambiguous: ^{3}H-labeled RNA synthesized in the in vitro polymerase reaction was copied from the double-stranded viral RNA template.

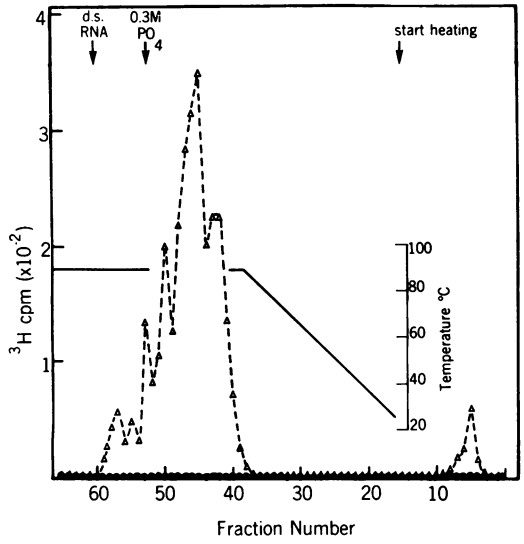

FIG. 8. *Ribonuclease sensitivity of product RNA fractionated by hydroxyapatite column chromatography. A standard assay was performed, and, after a 10-min incubation at 45 C in 1% SDS, the total reaction mixture was loaded onto a jacketed hydroxyapatite column (3.0 by 0.9 cm) equilibrated with 0.1 M phosphate (pH 6.7) at room temperature. Small nucleotides passed through in fractions 1–15. The positions at which heat and 0.3 M phosphate were applied are noted by arrows, as is the reference position at which double-stranded RNA would begin to elute under these conditions. Fractions (2 ml) were collected and a 250-μliter portion of each sample was precipitated with 7.5% trichloroacetic acid. A second sample was made 0.3 M in NaCl and incubated with pancreatic ribonuclease (2 μg/ml) for 1 hr at 37 C prior to precipitation with trichloroacetic acid. Samples were assayed for radioactivity as noted in Materials and Methods. Symbols: △, ^{3}H-labeled product RNA; ●, after ribonuclease treatment.*

TABLE 3. *Annealing of RNA from reaction product with CPV-RNA*

Sample	Treatment	Acid-precipitate (counts/min)	Per cent ribonuclease resistant
A (^{3}H-UTP from column)	None	3,020	
	Ribonuclease	450	15
	Annealed + ribonuclease	2,540	84
B (^{3}H-UTP)	None	3,305	
	Ribonuclease	545	16
	Annealed + ribonuclease	2,755	83
C (^{3}H-GTP)	None	2,032	
	Ribonuclease	373	20
	Annealed + ribonuclease	1,896	93

DISCUSSION

The above results show clearly that CPV purified and assayed in the manner described in this report contains a polymerase capable of transcribing single-stranded RNA in vitro. The observation that this RNA contains both uridine and guanidine moieties and specifically hybridizes with heat-denatured CPV genome would indicate that this product is not simply a homopolymer artifact. Although the requirements of the polymerase and the properties of its product generally resemble those reported for the reovirus-associated enzyme, there seem to be some notable differences between the two systems. As with reovirus, one of the major questions to arise concerns the size of the product RNA. Although the bulk of this product consists of low molecular weight components, about 10% is large enough to represent the product of transciption of at least the smaller subunits of the CPV genome. Whether these small, single-stranded pieces result from the copying of short sequences in the template or from degradation during incubation is not clear. Preliminary studies, however, indicate that the latter situation may be the case.

Although the presence of the polymerase of CPV is firmly established, its exact location and origin are yet unknown. In contrast to purified reovirus whose structure must first be altered to allow expression of polymerase activity, purified CPV is already active. This major difference may reflect the variation in structure between these two viruses (4, 15) or a greater degree of instability on the part of CPV, or both. It is also possible that the alkali treatment during virus purification may have altered the structure of CPV

enough to make the polymerase and the template more accessible to substrates yet not enough to grossly affect the sedimentation coefficient.

Allied to the problem of location is the question of whether the CPV-associated polymerase is genetically coded for by the virus or by the host, *Bombyx mori*. Kawase and Miyajima (7) have reported that the RNA extracted from the midguts of infected larvae is infectious in that it induces polyhedrosis; this implies that the host cells possess an enzyme capable of transcribing single-stranded RNA from the double-stranded viral RNA. Indeed, that this biosynthetic mechanism may not be restricted to RNA viruses can be inferred from the studies of Montagnier (11) and Colby and Duesberg (2) who have isolated double-stranded RNA moieties in several uninfected animal cells and in a DNA-virus-infected cell system, respectively.

Repeated trials in our laboratory, however, would indicate that neither the RNA extracted from purified virus nor that from infected midgut tissue appears to be infectious for silkworm larvae. We raise the possibility, therefore, that the preparations used by Kawase and Miyajima may not have been pure RNA.

Thus, although many questions remain to be answered, our data allow us to make the following tentative proposal. After virus entrance into the host cell the virus-associated polymerase transcribes the first messenger RNA species from the double-stranded CPV template. Translation of these messenger RNA species then provides the virus proteins required for successful multiplication of the virion.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AI 00634 and AI 03930 from the National Institute of Allergy and Infectious Diseases, and training grant CA 05028 from the National Cancer Institute. One of us (J. K.) is a Postdoctoral Fellow from the National Research Council (Canada).

The authors are especially grateful to Joseph Borsa for his many helpful suggestions and to C. A. Knight for encouragement and support during the course of this research.

We also thank T. Hukuhara, visiting Postdoctoral Fellow from the University of Tokyo, Esther M. Omi and Miss Cindy M. Black for their assistance in the infectivity assays and the preparation of virus, and H. Watanabe of the University of Tokyo for providing the silkworm eggs.

LITERATURE CITED

1. Borsa, J., and A. F. Graham. 1968. Reovirus: RNA polymerase activity in purified virions. Biochem. Biophys. Res. Commun. 33:895–901.
2. Colby, C., and P. H. Duesberg. 1969. Double-stranded RNA in vaccinia infected cells. Nature 222:940–944.
2a. Gauntt, C. J., and A. F. Graham. 1969. The reoviruses, p. 259–291. *In* H. B. Levy (ed.), Biochemistry of viruses. Marcel Dekker, New York, N.Y.
3. Gomatos, P. J., R. M. Krug, and T. Tamm. 1964. Enzymatic synthesis of RNA with reovirus RNA as template. 1. Characteristics of the reaction catalyzed by the RNA polymerase from *Escherichia coli*. J. Mol. Biol. 9:193–207.
4. Hosaka, Y., and K. Aizawa. 1964. The fine structure of the cytoplasmic-polyhedrosis virus of the silkworm *Bombyx mori* Linnaeus. J. Insect Pathol. 6:53–77.
5. Kalmakoff, J., L. J. Lewandowski, and D. R. Black. Comparison of the ribonucleic acid subunits of reovirus, cytoplasmic polyhedrosis virus, and wound tumor virus. J. Virol. 4:851–856.
6. Kates, J. R., and B. R. McAuslan. 1967. Poxvirus DNA-dependent RNA polymerase. Proc. Nat. Acad. Sci. U.S.A. 58:134–141.
7. Kawase, S., and S. Miyajima. 1968. Infectious ribonucleic acid of the cytoplasmic-polyhedrosis virus of the silkworm, *Bombyx mori*. J. Invert. Pathol. 11:63–69.
8. Kawase, S., and I. Kawamori. 1968. Chromatographic studies on RNA synthesis in the midgut of the silkworm, *Bombyx mori*, infected with a cytoplasmic-polyhedrosis virus. J. Invert. Pathol. 12:395–404.
9. Kirby, K. S. 1965. Isolation and characterization of ribosomal RNA. Biochem. J. 96:266–269.
10. Miura, K., I. Fujii, T. Sakaki, M. Fuke, and S. Kawase. 1968. Double-stranded ribonucleic acid from cytoplasmic polyhedrosis virus of the silkworm. J. Virol. 2:1211–1222.
11. Montagnier, L. 1968. Presence d'un acide ribonucleique en double chaine dans des cellules animales. C. R. Acad. Sci. 267:1417–1420.
12. Munyon, W., E. Paoletti, and J. T. Grace, Jr. 1967. RNA polymerase activity in purified infectious vaccinia virus. Proc. Nat. Acad. Sci. U.S.A. 58:2280–2287.
13. Shatkin, A. J. 1965. Inactivity of purified reovirus RNA as a template for *E. coli* polymerases *in vitro*. Proc. Nat. Acad. Sci. U.S.A. 54:1721–1728.
14. Shatkin, A. J. 1969. Replication of reovirus. Advan. Virus Res. 14:63–87.
15. Shatkin, A. J., and J. D. Sipe. 1968. RNA polymerase activity in purified reoviruses. Proc. Nat. Acad. Sci. U.S.A. 61:1462–1469.
16. Shatkin, A. J., J. D. Sipe, and P. Loh. 1968. Separation of ten reovirus genome segments by polyacrylamide gel electrophoresis. J. Virol. 2:986–991.
17. Silverstein, S. C., and S. Dales. 1968. The penetration of reovirus RNA and initiation of its genetic function in L-strain fibroblasts. J. Cell Biol. 36:197–230.
18. Watanabe, Y., S. Millward, and A. F. Graham. 1968. Regulation of transcription of the reovirus genome. J. Mol. Biol. 36:107–123.
19. Wilhelm, J. M., N. L. Oleinick, and J. W. Corcoran. 1968. The inhibition of bacterial RNA synthesis by the rifamycin antibiotics. Biochim. Biophys. Acta 166:271–273.