Deoxyribonucleic Acid Polymerase Associated with Rous Sarcoma Virus and Avian Myeloblastosis Virus: Properties of the Enzyme and Its Product

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Deoxyribonucleic acid (DNA) polymerase activity can be elicited in purified preparations of avian myeloblastosis virus and Rous sarcoma virus (Schmidt-Ruppin strain) by treatment with nonionic detergent. The enzyme(s) and its synthetic products appear to be virion-associated. Enzymatic activity can be inhibited by pretreatment with either ribonuclease (8- to 10-fold inhibition) or actinomycin D (twofold inhibition). By contrast, rifampin has little, if any effect. The enzyme(s) synthesizes two primary products, a ribonucleic acid (RNA):DNA hybrid and DNA which is free of RNA. The results of both zonal and equilibrium centrifugation indicate that nascent chains of DNA are associated with the 70S viral RNA. It is concluded that at least two enzymatic activities are under study: transcription of DNA from viral RNA, and subsequent, additional synthesis of DNA, utilizing product of the initial reaction as template.

Transformation by the ribonucleic acid (RNA)-containing tumor viruses requires the synthesis of deoxyribonucleic acid (DNA) in the host cell immediately after infection (1, 12). The hypothesis has been advanced that such DNA synthesis is virus-directed, and that it represents transcription of the viral RNA genome into a DNA copy which can then be integrated into the host cell chromosome (13). Substantial support for this hypothesis has been provided by the discovery of a novel DNA polymerase which is associated with the virions of RNA tumor viruses, and which apparently transcribes DNA from an RNA template (3, 14). We have identified such an enzyme in purified preparations of avian myeloblastosis virus (AMV) and Rous sarcoma virus (RSV, Schmidt-Ruppin strain), and we report here some properties of the enzyme and its product. Our data suggest that the enzyme does utilize an RNA template to synthesize DNA, that this synthesis proceeds by way of an RNA:DNA hybrid intermediate, and that a second virus-associated enzymatic reaction synthesizes DNA from either a hybrid or DNA template.

MATERIALS AND METHODS

Reagents. Reagents used were: ³H-thymidine triphosphate (TTP), 5 to 13 Ci/mmole, New England Nuclear Corp.; pancreatic ribonuclease A, Worth-

ington Biochemicals Inc.; deoxyribonuclease, electrophoretically purified and free of ribonuclease activity, Worthington Biochemicals, Inc.; Pronase, B grade, Calbiochem; deoxyribonucleoside triphosphates, deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), and deoxycytosine triphosphate (dCTP), Calbiochem; phenol, reagent grade, Mallinckrodt Chemical Works; Nonidet-P 40 (NP-40), Shell Chemical Company; and Cs₂SO₄, optical grade, Gallard-Schlesinger.

Preparation of virus. AMV and the Schmidt-Ruppin strain of RSV were propagated and purified as described previously (8; J. M. Bishop, W. E. Levinson, N. Quintrell, L. Fanshier, and J. Jackson, *Virology*, *in press*). Concentrated crude virus suspensions were prepared by sedimenting the virus at 100,000 × g for 2 hr and suspending the pellets in 0.15 M NaCl-0.001 M MgCl₂-0.02 M tris(hydroxymethyl)amino-methane (Tris)-hydrochloride (pH 7.4). The protein content of these latter suspensions ranged between 10 and 30 mg/ml, that of the purified virus preparations, 0.5 to 2.0 mg/ml. Protein was measured with the procedure of Lowry et al. (9).

Enzyme assay. In early experiments, enzyme activity was elicited by treating virus suspensions with 1% NP-40 for 15 min at 4 C, followed by the addition of β-mercaptoethanol (2%, v/v). Later, we adopted the procedure of including the appropriate concentration of detergent in the reaction mixture proper and initiating the enzyme reaction immediately upon addition of virus. Polymerase activity was assayed with the following standard reaction mixture: 0.1 M Tris-

hydrochloride, pH 8.1; 0.01 M MgCl_2 ; 8×10^{-6} M dATP, dGTP, and dCTP; 2% β -mercaptoethanol (v/v); and 0.5×10^{-6} to 3.0×10^{-6} M ^3H -TTP (1 to 12 Ci/mmmole). Stability of the reaction over prolonged periods could be improved by the addition of phosphoenol pyruvate (0.01 M) and pyruvate kinase (40 $\mu\text{g}/\text{ml}$). Samples were precipitated for measurement of radioactivity by the sequential addition of 0.01 M sodium pyrophosphate and 3.5% perchloric acid containing 0.01 M sodium pyrophosphate (2). Precipitates were trapped in glass-fiber filters, washed six times with 0.1 N hydrochloride-0.01 M sodium pyrophosphate, and washed once with 95% ethanol. The filters were dried and counted in liquifluor-toluene (Pilot Chemicals Co.) scintillation fluid.

Rate-zonal and equilibrium centrifugation of nucleic acids and virus. Zonal centrifugation was carried out by using gradients of 15 to 30% sucrose in 0.1 M NaCl-0.001 M ethylenediaminetetraacetic acid (EDTA)-0.02 M Tris-hydrochloride (pH 7.4), and formed on top of a cushion of either 60% (w/v) sucrose or 60% (w/w) Cs_2SO_4 (density: 1.9 g/ml). Equilibrium centrifugation in Cs_2SO_4 was performed as described previously (4). A maximum of 5 μg of purified nucleic acid was added to each centrifuge tube in 2.5 to 3.5 ml of Cs_2SO_4 (density: 1.51 g/ml), buffered with 0.01 M EDTA-0.02 M Tris-hydrochloride (pH 7.4).

Extraction of nucleic acids from enzyme reaction mixtures. Nucleic acids were extracted at room temperature with phenol in the presence of 0.5% sodium dodecyl sulfate (SDS). On occasion, the reaction mixtures were adjusted to 0.01 M EDTA, and treated with 0.5% SDS and 500 μg of Pronase per ml at 37 C for 45 min, followed by phenol extraction or direct analysis by zonal centrifugation. Recovery of nucleic acid and the physical properties of the product did not vary significantly from one of these extraction procedures to another.

RESULTS

Requirement for detergent treatment of virions. RSV and AMV must be treated with a nonionic detergent to elicit DNA polymerase activity (Table 1). We have never observed appreciable activity in untreated preparations of virus, even in those stored for as long as 6 months at -70 C. There is a relatively sharp optimal concentration of detergent which is an apparent function of the protein concentration of the virus suspension (Fig. 1). Highly concentrated virus, prepared by differential ultracentrifugation and containing concentrations of protein which are 10-fold greater than those of virus purified by isopycnic centrifugation, requires 10-fold higher concentrations of detergent for maximum polymerase activity. These results resemble those reported from other laboratories (14; M. Green et al., Proc. Nat. Acad. Sci. U.S.A., *in press*; E. M. Scolnick, S. A. Aaronson, and G. Todaro, Proc. Nat. Acad. Sci. U.S.A., *in press*), although the apparent stoichiometric relationship between optimal

TABLE 1. Effect of detergents on avian myeloblastosis virus (AMV)-associated DNA polymerase activity^a

Determination	³ H-Thymidine triphosphate incorporated (pmoles)
No detergent.....	0.09
With Nonidet-P 40.....	2.00
With deoxycholate.....	0.05

^a Standard reaction mixtures containing 15 μg of viral protein (AMV) were incubated at 37 C for 30 min and then acid precipitated. Nonidet-P 40 was omitted from one set of samples, and deoxycholate (1%, w/v) was added to another. The results are expressed as means, computed from duplicate reactions. Identical results have been obtained with Rous sarcoma virus.

FIG. 1. Detergent requirement for RSV-associated DNA polymerase activity. Samples of purified RSV (35 μg of protein per reaction) and of crude, concentrated RSV (300 μg of protein per reaction) were added to equal volumes of the standard reaction mixture, containing the indicated amounts of NP-40, and incubated at 37 C for 2 hr. Acid-precipitable radioactivity was then determined as described in Materials and Methods. The results for different concentrations of detergent are expressed as percentages of the maximal incorporation observed for each type of virus preparation. Symbols: Δ , purified virus; $\circ$, virus prepared by differential ultracentrifugation and containing a 10-fold higher concentration of protein than the purified virus.

FIG. 2. Kinetics of DNA synthesis by RSV-associated DNA polymerase. Purified RSV (Δ ; 55 μ g of protein) was added to 0.4 ml of the standard reaction mixture at pH 8.1 and incubated at 37 C. Samples were withdrawn at the indicated time points for determination of acid-precipitable radioactivity. Similar experiments were carried out with crude suspensions of concentrated virus ($\circ$) and purified virus ($\bullet$), but with reaction mixtures at pH 7.3 and containing phosphoenol pyruvate and pyruvate kinase. The results of incubations in the absence of any of the unlabeled deoxynucleoside triphosphate precursors, or with ribonucleoside triphosphates (including ^3H -GTP) in place of the deoxyprecursors ($\blacktriangle$ for both cases), are also illustrated. Results identical to all of the above were also obtained with AMV. One picomole of precursor incorporated = 2,800 counts/min.

detergent concentration and protein content of virus preparations has not been noted previously.

In contrast to nonionic detergents, deoxycholate cannot elicit polymerase activity (Table 1) and, in fact, promptly terminates the reaction if added to an ongoing incubation (*unpublished data*). These results are not unexpected in view of the fact that deoxycholate, in concentrations much lower than those used here, completely disrupts the virion structure of RSV (P. Duesberg, *personal communication*; *unpublished data* of the authors).

Kinetics of the reaction. Detergent-treated RSV catalyzes the synthesis of DNA for at least 6 hr (Fig. 2). At a pH somewhat below the optimum (see below, Fig. 3b) and in the presence of phosphoenol pyruvate and pyruvate kinase, the reaction is stable for a minimum period of 12 hr, albeit at a reduced rate of DNA synthesis. The enzymatic activities of crude virus suspensions and of purified virus are equally stable, but the purified virus has a higher specific activity (note the two scales in Fig. 2).

As previously reported (3; M. Green et al.,

Proc. Nat. Acad. Sci. U.S.A., *in press*), omission of any of the deoxynucleoside triphosphate precursors completely inhibits the reaction (Fig. 2). In addition, there is no demonstrable polymerization of ribonucleosides (3; M. Green et al., Proc. Nat. Acad. Sci. U.S.A., *in press*; Fig. 2).

Properties of the enzyme reaction. When assayed over a comparatively short period of time (1 hr), the RSV- and AMV-associated DNA polymerases have a temperature optimum of approximately 45 C (Fig. 3a). This is consistent with the ability of both viruses to grow well at relatively high temperatures. (RSV is routinely propagated at 42 C in our laboratory.) However, the temperature optimum drops appreciably if the reaction is carried out for longer periods of time (Fig. 3a), a fact which presumably reflects greater stability of the enzyme aggregate at lower temperatures.

The optimal conditions of pH and magnesium concentration for the AMV and RSV enzymes are essentially as previously reported for both avian and murine RNA tumor viruses (14; Green et al., Proc. Nat. Acad. Sci. U.S.A., *in press*; E. M. Scolnick et al., Proc. Nat. Acad. Sci. U.S.A., *in press*; Fig. 3b, 3d). As in the case of temperature, the reaction is more stable over prolonged periods of time (5 to 10 hr) at a pH below the optimum for brief reactions (*unpublished data*).

The polymerase has a stringent requirement for mercaptan (Fig. 3c). In our experience, dithiothreitol offers no advantage over β -mercaptoethanol.

Inhibition of the reaction by ribonuclease. Pretreatment of the enzyme complex with ribonuclease causes an 8- to 10-fold reduction of enzyme activity (Table 2). The exceptionally high concentrations of ribonuclease required to demonstrate this effect (3, 14; Table 2) presumably reflect difficulty of access to the enzyme-template complex. As reported elsewhere (Scolnick et al., Proc. Nat. Acad. Sci. U.S.A., *in press*) and substantiated below, the enzyme and its product are contained within an aggregate which may represent virtually intact virions. Ribonuclease has no effect when added to an ongoing reaction (at 30 min, Table 2).

Effects of actinomycin and rifampin. High concentrations (20 to 50 μ g/ml) of actinomycin D inhibit the polymerase reaction two- to threefold (Fig. 4a). The inhibition is more pronounced in the later phases of the reaction, i.e., after 1 hr. These concentrations of actinomycin also strongly inhibit the synthesis of DNA by nuclei isolated from HeLa cells (Fig. 4a). We assume, but cannot prove, that this represents a direct effect of actinomycin on the mammalian DNA polymerase.

FIG. 3. Properties of RSV-associated DNA polymerase. (a) Activity at different temperatures. Standard reaction mixtures, containing 20 μ g of viral protein, were incubated at various temperatures for 1 (●) and 3 (○) hr. (b) Activity as a function of pH. Reactions were carried out at 37 C as in (a), except that the pH was varied as indicated. (c) Requirement for mercaptan. Reactions were carried out at 37 C as in (a), except that the concentrations of β -mercaptoethanol in one series (●) and dithiothreitol in another (○) were varied as indicated. (d) Requirement for divalent cation. Reactions were carried out at 37 C as in (a), except that the concentration of $MgCl_2$ was varied as indicated.

reaction, mediated by the binding of actinomycin to template DNA.

Rifampin, by contrast, has only a slight inhibitory effect on the activity of RSV-associated DNA polymerase (Fig. 4b). The concentrations of the antibiotic used in these experiments are far in excess of those required to obtain inhibition of *Escherichia coli* DNA-dependent RNA polymerase in vitro (16) and are of the same order of magnitude as those reported by Diggelman and Weissmann to inhibit cellular transformation in vivo by RSV(6).

Is the polymerase located within virions? We

have made repeated efforts to demonstrate physical association of DNA polymerase with virus particles, utilizing isopycnic centrifugation in density gradients of sucrose. The results have been variable (Fig. 5), possibly because of the relatively poor resolution offered by this technique as opposed to equilibrium centrifugation in solutions of cesium salts. An interesting and possibly significant observation is the fact that biological activity (RSV focus-forming units) and enzyme activity are generally not completely congruent (Fig. 5a, b, c). In fact, the results of some analyses suggest the existence of two populations of en-

zyme activity, one corresponding to focus-forming activity (the broad, leading shoulder of enzyme activity in Fig. 5a), the other, more sharply defined, located at a somewhat lower buoyant density (the sharp, main peak in Fig. 5a).

TABLE 2. *Effect of ribonuclease on the polymerase reaction^a*

Expt	Determination	³ H-Thymidine triphosphate incorporated (pmoles)
1	Control	6.6
	Pretreated with ribonuclease	0.85
2	Control	4.0
	Ribonuclease added at 30 min	4.2

^a Samples (95 μ g of protein) of purified avian myeloblastosis virus were pretreated with Nonidet-P 40 and β -mercaptoethanol as described in Materials and Methods, added to standard reaction mixtures, and incubated at 37 C for 1 hr, followed by acid precipitation. In experiment one, ribonuclease (1 mg/ml) was added to the virus at the same time as the detergent and was, therefore, present throughout the 30-min incubation at 4 C which preceded initiation of the enzyme reaction. In experiment two, ribonuclease (1 mg/ml) was added to an ongoing reaction at 30 min, and incubation was then continued at 37 C for another 30 min.

In general, the relationship between biological activity and radiolabel in virions conforms to previous reports, i.e., the distribution of focus-forming units is slightly skewed towards a heavier density (Fig. 5a). In the absence of bioassay data for AMV, we note that polymerase activity is similarly skewed with respect to the radioactivity in virus particles (Fig. 5d).

After detergent treatment, the enzyme and its product (Fig. 6a, b) are still intimately associated with structures which sediment in a manner similar to that of virus particles during rate zonal analysis. The fact that the product of at least the early reaction is not released from the enzyme-virus aggregate will be further considered below (Fig. 7).

Accessibility of the product to deoxyribonuclease. It has been reported previously that the native, early product of the enzyme reaction is relatively shielded from degradative enzymes unless first diluted into low concentrations of electrolytes (3). We have made a similar observation although, in our hands, dilution of the reaction mixture does not effectively release product into a sensitive state (Fig. 7). However, boiling for 10 min renders the product deoxyribonuclease-sensitive. As the reaction progresses, the product becomes increasingly susceptible to digestion by deoxyribonuclease (Fig. 7). We assume that DNA is now dissociating from the enzyme-virus complex, or the envelope of the complex is de-

FIG. 4. *Effect of actinomycin and rifampin.* (a) Actinomycin. Purified AMV (90 μ g of viral protein) was treated sequentially with NP-40 (15 min) and β -mercaptoethanol (15 min) as described in Materials and Methods. Actinomycin (20 μ g/ml) was added at the same time as the detergent. The standard reaction mixture, added at the conclusion of the detergent treatment to initiate the enzyme reaction, also contained actinomycin (20 μ g/ml). Control reactions contained appropriate amounts of the solvent (50% ethanol) used to dissolve the antibiotic. Parallel reactions were carried out with nuclei isolated from HeLa cells, except that detergent treatment was omitted. Symbols: $\bullet$, control reaction with virus; $\circ$, reaction with actinomycin-treated virus; $\blacktriangle$, control reaction with nuclei; $\triangle$, reaction with actinomycin-treated nuclei. (b) Rifampin. The reactions were carried out exactly as described for (a), except that rifampin was substituted for actinomycin. Symbols: $\bullet$, control; $\blacktriangle$, rifampin, 20 μ g/ml; $\triangle$, rifampin, 50 μ g/ml.

FIG. 5. Association of DNA polymerase with virions. Various preparations of RSV and AMV were subjected to isopycnic centrifugation in preformed gradients of 25 to 55% sucrose containing 0.1 M NaCl-0.001 M EDTA-0.02 M Tris·hydrochloride (pH 7.4). In each instance, centrifugation was carried out at 4 C for 12 to 14 hr. The gradients were fractionated in the usual manner, and samples were taken from each fraction for determination of focus-forming activity, radioactivity, optical density, and DNA polymerase activity. (a) ^{32}P -labeled RSV, centrifuged in a Spinco SW-25.3 rotor at 24,000 rev/min. Symbols: ●, focus-forming units; Δ , ^{32}P counts/min; ○, DNA polymerase activity; $\blacktriangle$, density, determined from refractive index. (b) Unlabeled RSV, centrifuged in a SW-25.3 rotor. Symbols: Δ , absorbance at 260 nm; ●, focus-forming units; ○, DNA polymerase activity. (c) ^{32}P -labeled RSV, centrifuged in a Spinco SW-41 rotor at 35,000 rev/min. Symbols: ●, focus-forming units; Δ , ^{32}P counts/min; ○, DNA polymerase activity. (d) ^{32}P -labeled AMV, centrifuged in a Spinco SW-65 rotor at 33,000 rev/min. Symbols: Δ , ^{32}P counts/min; ○, DNA polymerase activity.

teriorating and allowing external macromolecules more ready access to the site of synthesis. This is substantiated by our unpublished data that, at all time points, most of the product (70 to 80%) is sensitive to hydrolysis by deoxyribonuclease if it is first extracted with SDS-phenol. In addition, it is resistant to ribonuclease (100 $\mu\text{g}/\text{ml}$, 0.01 M MgCl_2 , 37 C, 1 hr) and alkali (0.3 N KOH, 37 C, 1 hr).

Sedimentation analysis of early product. The product of 30 min of enzymatic synthesis consists primarily of DNA which cosediments with 70S viral RNA at neutral pH (Fig. 8a). Treatment of the product with ribonuclease (in high concentration of NaCl) prior to centrifugation eliminates the rapidly sedimenting form of DNA and results in a concomitant increase in radioactivity in the slowly sedimenting population of DNA (Fig. 8b). Thus, the rapidly sedimenting DNA must be

associated with RNA, a substantial portion of which is single-stranded and readily accessible to hydrolysis by ribonuclease. We propose that the DNA in question consists of short, nascent chains, complexed to molecules of their putative template, the 70S viral RNA. The portion of each viral RNA molecule which is not complexed to DNA is sufficiently large so that the entire structure still sediments in a manner approximating that of single-stranded RNA. Such a structure would be expected to have a buoyant density identical to that of single-stranded RNA, and the results of equilibrium centrifugation in Cs_2SO_4 have confirmed this expectation (Fig. 9a). If the 70S complex is treated with ribonuclease prior to equilibrium centrifugation, the DNA is now found in a heterogeneous population of molecules which have buoyant densities corresponding to those expected for hybrids of DNA and RNA

FIG. 6. Zonal centrifugation of AMV-DNA polymerase aggregate and its product. (a) Sedimentation of detergent-treated virus. ^{32}P -labeled AMV was treated sequentially with NP-40 and β -mercaptoethanol and then centrifuged through a gradient of 15 to 30% sucrose, formed on top of a 60% sucrose cushion. Centrifugation was carried out in a Spinco SW-65 rotor for 40 min at 40,000 rev/min, 4 C. After fractionation of the gradient, samples were taken for measurement of ^{32}P radioactivity ($\circ$) and DNA polymerase activity ($\blacktriangle$), the latter being measured in the absence of additional detergent. (b) Association of the early product with the virion-enzyme aggregate. Detergent-treated AMV (labeled with ^{32}P) was incubated in the standard reaction mixture for 30 min and then centrifuged as in (a). Fractions of the gradient were assayed for acid-precipitable ^{32}P ($\circ$) and ^3H ($\blacktriangle$). The tritium radioactivity represents product of the enzyme reaction which was carried out prior to centrifugation.

(Fig. 9b). In addition, there may be a small amount of free DNA, represented by the secondary peak at the top of the gradient in Fig. 9b. These data suggest that most or all of the DNA in the 70S complex is hydrogen-bonded to RNA in a hybrid state. The portion of the viral RNA contained in such a hybrid would be relatively resistant to hydrolysis by ribonuclease under the conditions employed here (8, 15). Results of experiments utilizing denaturation with formamide (11) and dimethyl sulfoxide (*in preparation*) substantiate this conception.

Sedimentation analysis of late product. After

FIG. 7. Susceptibility of the native product to hydrolysis by deoxyribonuclease. A standard reaction mixture (0.8 ml containing 200 μg of viral protein) was incubated at 37 C. At the indicated time points, multiple samples were withdrawn and treated as follows. (a) A portion was acid precipitated directly. This served as a control. (b) Deoxyribonuclease (50 $\mu\text{g}/\text{ml}$) was added directly to another portion. This was incubated at 37 C for 1 hr and then acid precipitated. (c) A third portion was diluted fourfold into 0.01 M MgCl_2 -0.02 M Tris-hydrochloride (pH 7.4) containing deoxyribonuclease (10 $\mu\text{g}/\text{ml}$), incubated at 37 C for 1 hr, and acid precipitated. (d) A final portion was diluted fourfold into 3 mM EDTA and heated at 100 C for 10 min. MgCl_2 (0.01 M) and deoxyribonuclease (10 $\mu\text{g}/\text{ml}$) were then added, and the mixture was incubated at 37 C for 1 hr prior to acid precipitation. The results are expressed as the per cent of the control radioactivity which remained acid-precipitable after the various treatments. Symbols: Δ , deoxyribonuclease added directly to the reaction mixture; $\bullet$, sample diluted into buffered MgCl_2 containing deoxyribonuclease; $\blacktriangle$, sample heated at 100 C prior to deoxyribonuclease treatment.

either 1 or 4 hr of enzymatic synthesis, the 70S complex described above is still detectable by zonal centrifugation (Fig. 10a, b). However, the relative amount of this material is diminished, and a major portion of the product now has a low sedimentation velocity (ca. 4 to 10S). In a subsequent communication, we will report data which indicate that this slowly sedimenting population contains both DNA:RNA hybrids and double-stranded DNA.

When centrifuged to equilibrium in Cs_2SO_4 , the 4 hr product bands mainly at the same density (1.45 g/ml) as the linear, double-stranded DNA of phage lambda (Fig. 11a). This density is the same as that reported for late product by Spiegel-

FIG. 8. Analysis of early product by rate-zonal centrifugation. Standard reaction mixtures (2.5 ml, containing 750 μg of viral protein) were extracted with SDS-phenol after 30 min of incubation at 37 C. The extracted nucleic acids were precipitated with ethanol, by using unlabeled HeLa cell RNA as carrier, and then dissolved in a small volume of 0.01 M EDTA-0.02 M Tris-hydrochloride (pH 7.4). Density gradients of 15 to 30% sucrose were formed on top of a cushion of Cs_2SO_4 (density 1.9 g/ml). Centrifugation was carried out in an SW-65 rotor at 60,000 rev/min for 65 min at 4 C. Identical results were obtained with nucleic acids extracted after incubation with SDS-Pronase (see Materials and Methods), and if centrifugation was performed in the absence of viral RNA marker. (a) Control. ^{32}P -labeled 70S viral RNA was included as a sedimentation marker, and its position is indicated by the arrow. (b) Pretreated with ribonuclease. The extracted nucleic acids were treated with ribonuclease (previously heated at 100 C for 10 min), 10 $\mu\text{g}/\text{ml}$, 15 min, room temperature, in 0.3 M NaCl, and then analyzed simultaneously with the sample illustrated in panel a, by using a separate rotor bucket.

man et al. (11). These data do not provide reliable information regarding the secondary structure of the DNA in question, but the results of chromatography on hydroxyapatite indicate that virtually all of the DNA, other than that which is contained in hybrid structures, is double-stranded (*in preparation*).

An appreciable portion (ca. 25%) of the 4-hr product is spread through the equilibrium gradi-

ent, ranging from the density of a double-stranded RNA:DNA hybrid (ca. 1.50 g/ml, reference 5) to nearly that of single-stranded RNA (ca. 1.66 g/ml, reference 4). Given the sharp band of lambda DNA and the absence of ^{32}P from the rest of the gradient, we assume that the heterogeneous distribution of a fraction of the product DNA population is not due to technical artifact and this material represents DNA:RNA hybrids.

By 8 hr, the product bands almost entirely with the marker DNA (Fig. 11b). There is no appreciable radioactivity in the region of DNA:RNA hybrids. This observation conforms to the fact that little, if any, 70S complex is detectable in 8-hr product (*unpublished data*).

DISCUSSION

The foregoing results confirm preliminary reports from other laboratories (3, 11, 14; Green et al., Proc. Nat. Acad. Sci. U.S.A., *in press*; Scolnick et al., Proc. Nat. Acad. Sci. U.S.A., *in press*) that a DNA polymerase is associated with, and possibly contained within, the virions of RNA tumor viruses. Indirect data, such as the ribonuclease-sensitivity of the reaction and the virtual absence of DNA from purified virus (10; Green et al., Proc. Nat. Acad. Sci. U.S.A., *in press*; W. Levinson et al., Nature (London), *in press*), support the provisional conclusion that the enzyme utilizes single-stranded RNA (presumably, the viral genome) as template. The finding of a complex between nascent DNA and 70S viral RNA further substantiates this view. In addition, there is some evidence for the existence of a second virus-associated enzymatic activity, viz., the synthesis of double-stranded DNA from an RNA:DNA hybrid template and, possibly, from product double-stranded DNA. The evidence for this is as follows. (i) The major long term product of synthesis is double-stranded DNA (*in preparation*), despite the fact that the reaction can be inhibited by pretreatment with ribonuclease; the initial product of transcription from an RNA template should be an RNA:DNA hybrid. (ii) A portion of the enzymatic activity is sensitive to actinomycin D. We would predict that transcription from single-stranded RNA would not be affected by this antibiotic in view of its inability to bind to either single- or double-stranded RNA. It therefore seems more likely that the actinomycin-sensitive reaction utilizes a DNA template. This hypothesis has been substantiated by experiments which demonstrate that the actinomycin-inhibited reaction synthesizes only DNA:RNA hybrid and that the virus-associated enzyme(s) is capable of utilizing exogenous DNA as template (*submitted for publication*).

FIG. 9. Analysis of 70S complex by equilibrium centrifugation. (a) Control. The 70S DNA-RNA complex produced by 30 min of synthesis was isolated by zonal centrifugation as described for Fig. 8. After recovery from the sucrose gradient, it was dialyzed against 0.1 M NaCl-0.002 M EDTA-0.02 M Tris·hydrochloride (pH 7.4) and centrifuged to equilibrium in Cs_2SO_4 (starting density, 1.60 g/ml), SW-50 rotor, 33,000 rev/min, 60 hr at 4 C. ^{32}P -labeled 70S single-stranded viral RNA (less than 1 μg) was included as a density marker, and its position is indicated by the arrow. Identical results were obtained if the viral RNA marker was omitted. (b) Pretreatment with ribonuclease. The 70S complex was isolated as described above and treated with ribonuclease (0.1 $\mu\text{g}/\text{ml}$) for 30 min at room temperature in 0.3 M NaCl. The ribonuclease was removed by phenol extraction before centrifugation of the nucleic acids in Cs_2SO_4 (starting density, 1.55 g/ml). The arrows indicate the approximate densities of single-stranded RNA (1.66 g/ml) and DNA:RNA hybrid (1.54 g/ml in this laboratory, unpublished data). Symbols: $\circ$, ^3H counts/min; $\square$, density, g/ml.

FIG. 10. Analysis of late product by rate-zonal centrifugation. Reaction mixtures similar to those described for Fig. 8 were extracted with SDS-phenol after 1 and 4 hr of incubation at 37 C. The extracted nucleic acids were centrifuged through sucrose gradients as in Fig. 8a. ^{32}P -labeled 70S viral RNA was included as a sedimentation marker. (a) One-hour product. (b) Four-hour product. Symbols: Δ , ^{32}P counts/min; $\bullet$, ^3H counts/min.

We have suggested that the DNA which sediments rapidly in zonal analyses, which loses its relatively high sedimentation velocity upon pretreatment with ribonuclease, and which has a buoyant density identical to that of single-stranded RNA represents short lengths of newly synthesized DNA associated with a long, single chain of RNA template. This material probably represents an intermediate in the synthesis of virus-specific DNA, and it is the initial product of the enzymatic reaction (*in preparation*). In fact, it is the predominant product of the reaction until at least 30 min after initiation of synthesis (Fig. 8a). This observation suggests that the rate

of chain elongation is quite slow in the reaction utilized for the present experiments, and probably reflects the fact that the concentration of radioactive precursor (^3H -TTP) was far below that required to saturate the reaction (ca. 5×10^{-4} M; unpublished data).

Finally, it should be noted that none of the data reported to date provides any sound evidence as to the biological significance of this enzyme (or enzymes) and its product. The hypothesis that the DNA product serves as an intermediate in viral RNA replication and that it is integrated into host DNA to provide for stable transformation (3, 13, 14) is logically persuasive

FIG. 11. Analysis of late product by equilibrium centrifugation. Product was extracted after 4 and 8 hr of synthesis from reaction mixtures similar to those described for Fig. 8. Each reaction yielded approximately 5 μ g of nucleic acids, which were then dialyzed and centrifuged in Cs_2SO_4 as in Fig. 9. The double-stranded DNA of lambda (virulent) phage, labeled with ^{32}P , was used as a density marker. (a) Four-hour product. (b) Eight-hour product. Symbols: $\bullet$, ^3H counts/min; $\circ$, ^{32}P counts/min; $\blacktriangle$, density, g/ml.

but unproven. It can be argued that even the demonstration of sequence complementarity between viral RNA and DNA product (11; P. Duesberg, *personal communication*) does not prove the point. In any event, the reaction under study almost certainly represents a novel biochemical phenomenon—transcription of RNA into DNA. Data to be reported subsequently indicate that a virus-associated enzyme utilizes the initial product of this transcription to synthesize double-stranded DNA.

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