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Interactions of Murine Leukemia Virus Core Components: Characterization of Reverse Transcriptase Packaged in the Absence of 70S Genomic RNA

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Virions produced by cells in the presence of actinomycin D (Act D virions) contain reverse transcriptase but are deficient in 70S genomic RNA. To assess the role of genomic RNA in encapsidation of a functional reverse transcriptase and to study the interaction of the enzyme and its template in the cores of intact virions, the reverse transcriptase enzymes of normal and Act D virions were compared. The enzymes were indistinguishable by column chromatography, sedimentation velocity, or template/primer preferences. In addition, these enzymes showed equal sensitivity to inactivation by antibodies directed against Rauscher murine leukemia virus DNA polymerase. The enzymes from Act D and normal virions had similar thermal decay rates and were both protected against heat denaturation by natural and synthetic template/primers. By these criteria, the DNA polymerase molecules synthesized and assembled into virions in the absence of genomic RNA are identical to those packaged under normal conditions. Additional studies designed to measure protection of reverse transcriptase by genomic RNA were carried out by comparing the thermal lability of the enzyme in intact Act D and normal virions. The thermal decay rate of reverse transcriptase in Act D virions was identical to that in control virions. In contrast to the lability of the virion-associated enzyme, however, genomic RNA in control virions was stable to heat treatment.

The role of genomic RNA in RNA tumor virus assembly is a matter of considerable interest, which can be approached experimentally by studying virion production in the presence of actinomycin D (Act D). Previous studies have shown that, when murine leukemia virus (MuLV)-producing cells are treated with Act D, noninfectious virions (Act D virions) are assembled for at least 8 to 12 h (13, 22). These virions have the ultrastructural appearance of normal type C particles and contain reverse transcriptase, but they lack 60 to 70S genomic RNA (13). More recently, in an investigation designed to determine the functional lifetime of viral mRNA, Levin and Rosenak (14) established that all of the virion structural proteins are synthesized in the absence of ongoing viral RNA synthesis and are present in Act D virions in normal amounts. Similar findings have been reported by Jamjoom et al. (12).

Because Act D virions lack 70S genomic RNA, the natural template for reverse transcriptase, the Act D system provides an opportunity to determine whether this RNA has any influence on the processing, packaging, or catalytic activity of the enzyme. Furthermore, comparative studies of Act D and normal virions can be used to explore possible interactions between the enzyme and its RNA template. In the present study, we approached these questions by analyzing the properties of reverse transcriptase from normal and Act D virions. In addition, measurements of the thermal stability of reverse transcriptase in intact Act D and control virions were utilized as a means of assessing the functional relationship of enzyme to template within the virion core.

The experiments reported in this paper indicate that the reverse transcriptase enzymes packaged in Act D and control virions are structurally and catalytically indistinguishable. In addition, the data are consistent with the possibility that, in normal virions, reverse transcriptase may not be functionally associated with 70S genomic RNA. A preliminary account of these findings has appeared (J. G. Levin and B. I. Gerwin, Fed. Proc. 35:1593, 1976).

MATERIALS AND METHODS

Chemicals. Polynucleotides and bovine serum albumin were obtained from Miles Laboratories; oligodeoxythymidylic acid$_{12-18}$ [(dT)$_{12-18}$] and oligodeoxy-

guanidylic acid$_{12-18}$ [(dG)$_{12-18}$] were purchased from Collaborative Research Laboratories. Calf thymus DNA was a product of Worthington Biochemicals Corp. Act D was a gift of Merck Sharp & Dohme. Unlabeled deoxyribonucleoside triphosphates and dithiothreitol were obtained from Calbiochem. Mouse globin mRNA was the generous gift of Stuart Orkin. [*methyl*-^{3}H]dTTP (40,000 cpm/pmol) and [8-^{3}H]-dGTP (10,000 cpm/pmol) were obtained from Schwarz/Mann. [5,6-^{3}H]uridine (specific activity, 34 Ci/mmol) was purchased from New England Nuclear Corp. All other reagents were the best grades commercially available.

Cells and viruses. SC-1 cells (10) and AKR-L1 virus were the generous gifts of Marilyn Lander and Janet Hartley. The cells were infected and maintained as described previously (14). Infectivity was determined by XC plaque assay (24), expertly performed by Elizabeth von Kaenel, Microbiological Associates, Bethesda, Md. Avian myeloblastosis virus (AMV) was obtained from Joseph Beard, Life Sciences, St. Petersburg, Fla.

To obtain enough virus for enzyme purification, the AKR virus-infected cells were grown in plastic roller bottles (Corning Glass Works) at 37°C until 75 to 85% confluent. At zero time, 30 ml of McCoy medium containing 10% fetal bovine serum and 1 μg of Act D per ml, where indicated, were added to each bottle. After incubation for 3 h, fluids from untreated cells were clarified by centrifugation at 1,000 × *g* for 20 min and immediately stored at −70°C; fluids from Act D-treated cells were discarded. Both sets of cells were gently rinsed once with medium, and the incubation then continued for another 3 h with fresh regular or Act D-containing medium, added as described above. At the end of 6 h, the fluids were collected from both treated and untreated cells and were clarified. A portion was removed for determination of infectivity (24). All samples were then frozen at −70°C.

Determination of virus production. The cells were grown on 100-mm plastic petri dishes in Eagle medium containing 10% fetal bovine serum and 1 μg of Act D per ml, where specified. At the indicated times, the extracellular fluids were collected and clarified as described above. Portions were taken from each sample for measurement of infectivity (24). The remaining fluids were concentrated 64-fold by sedimentation in a Beckman SW50.1 rotor at 100,000 × *g* for 75 min. Virus pellets were assayed for DNA polymerase activity in response to polyriboadenylic acid [poly(rA)] · (dT)$_{12-18}$ as described previously (13).

Virus purification. The extracellular fluids were thawed and resedimented at 1,000 × *g* for 20 min. An equal volume of cold, saturated ammonium sulfate (pH 7.4) was added to the clarified fluids, with care taken to avoid foaming. The 50%-saturated solution was allowed to stand at 4°C for 30 min and then centrifuged at 4,000 × *g* for 20 min in a Sorvall GSA rotor. All subsequent steps were performed at 4°C. The supernatant was discarded, and the pellet was suspended in cold NTE buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 1 mM EDTA), at approximately 3 ml of NTE per 140 ml of original culture fluid. The resulting solution was layered over a discontinuous gradient consisting of 9 ml of 20% (wt/wt) sucrose and 6 ml of 60% (wt/wt) sucrose in NTE and centrifuged at 100,000 × *g* for 120 min in a Beckman SW25.2 rotor. Fifteen-drop fractions were collected from the bottom and assayed for DNA polymerase activity in response to poly(rA) · (dT)$_{12-18}$ (7). The virus band was pooled, diluted with NTE, and pelleted at 200,000 × *g* for 60 min.

Enzyme purification. Virus pellets were dissolved in disruption buffer (0.5 M Tris-hydrochloride [pH 7.8], 1 mM dithiothreitol, 0.5 M KCl, 1% [vol/vol] Triton X-100, 20% [vol/vol] glycerol), incubated at 37°C for 15 min, and centrifuged at 100,000 × *g* for 1 h (6). The supernatant fluid was then chromatographed on (dT)$_{12-18}$-cellulose and phosphocellulose at 4°C as detailed previously (6, 7). Sedimentation velocity analysis of enzyme from phosphocellulose columns was performed as described earlier (7).

DNA polymerase assays. DNA polymerase activity in response to poly(rA) · (dT)$_{12-18}$, poly(dA) · (dT)$_{12-18}$, poly(rC) · (dG)$_{12-18}$, and "activated" DNA was assayed as described previously (7). When mouse globin mRNA or AMV 70S RNA was used as a template, 34 or 100 ng, respectively, was added to a 100-μl reaction mixture containing 1.5 μg of (dT)$_{12-18}$, 0.04 M Tris-hydrochloride (pH 8.3), 60 mM Mg(Ac)$_2$, 1 mM Mn(Ac)$_2$, 1 mM dithiothreitol, 0.05 mM TTP, dATP, dCTP, and [8-^{3}H]dGTP (10,000 cpm/pmol). Inhibition of DNA polymerase activity by goat antiserum to Rauscher MuLV polymerase was tested by preincubating enzyme and appropriate immunoglobulin G (IgG) fractions (see below) for 15 min at 4°C, followed by the usual assay with poly(rA) · (dT)$_{12-18}$ (7).

Assay of heat lability of reverse transcriptase. The decay rates of phosphocellulose-purified enzyme in the presence and absence of template were determined at various temperatures by incubating enzyme with or without the indicated template [poly(rA) · (dT)$_{12-18}$ or AMV 70S RNA]. Fifteen-microliter portions [representing 10 μl of enzyme and, where specified, 5 μl of poly(rA) · (dT)$_{12-18}$ (250 ng), AMV 70S RNA (5 ng), or buffer] were removed at the indicated times, added to a 35-μl reaction mixture containing the additional components required for assay of polymerization in response to poly(rA) · (dT)$_{12-18}$ (7), and incubated at 37°C for 1 h.

The thermal lability of the DNA polymerase activity of intact virions was tested by heating clarified tissue culture fluids at 44 or 44.5°C for the specified times. Samples were transferred to 4°C, and viral pellets were prepared by sedimenting the fluids at 100,000 × *g* for 1 h. The pellets were dissolved in the disruption buffer used for enzyme purification (see above), incubated for 15 min at 37°C, and then assayed for DNA polymerase activity in response to poly(rA) · (dT)$_{12-18}$ (7). Samples were diluted in disruption buffer to achieve linearity of [^{3}H]dTMP incorporation with enzyme concentration.

Preparation of immune IgG. Goat immune serum directed against Rauscher MuLV DNA polymerase was obtained by immunization with an enzyme preparation that had been partially purified by (dT)$_{12-18}$-cellulose chromatography (6). This serum was kindly supplied by Huntingdon Research Laboratories through the Office of Program Resources and Logistics, National Cancer Institute. The IgG fraction was obtained as described previously (3). Briefly, se-

rum (ca. 2 ml) was exhaustively dialyzed against 0.01 M potassium phosphate buffer (pH 8) and centrifuged for 10 min at 15,000 × *g*. The supernatant was applied to a DE52 column (0.9 by 15 cm) and eluted with a concave gradient of 10 to 300 mM potassium phosphate buffer (pH 8.0) controlled by an LKB Ultrograd. Appropriate effluent fractions were pooled on the basis of absorbance at 280 nm and concentrated by precipitation from solutions adjusted to 50% of saturation with ammonium sulfate. Pools were redissolved and dialyzed against 10 mM Tris-hydrochloride (pH 8.0), centrifuged at 15,000 × *g* for 10 min, and tested for their ability to inhibit the activity of Rauscher MuLV polymerase (see above). IgG fractions were stored at −70°C.

Isolation and analysis of viral RNA. Cells were labeled by incubating each of 20 petri dishes (100 mm) with 4 ml of Eagle medium containing 10% fetal bovine serum and 125 μCi of [^{3}H]uridine per ml for 3 h at 37°C. The extracellular fluids were clarified as described above and divided into four portions. Samples were heated at 44°C for the indicated times and processed immediately. Virions were purified by sedimentation in a discontinuous sucrose gradient, as described above, except that the gradient contained 1 ml of 60% and 4.6 ml of 20% sucrose, respectively, and an SW27.1 rotor was used. Fractions containing ^{3}H-labeled virus were stored at 4°C overnight. RNA was extracted from pooled peak fractions and analyzed on composite polyacrylamide-agarose gels by procedures outlined previously (13). Since there was some variability in the recovery of viral RNA after alcohol precipitation, the amount of radioactivity added to the gels was not always the same for each sample.

AMV RNA was isolated from AMV, which was received as a frozen pellet. The viral pellet was dispersed in NTE, and the RNA was extracted two times with equal volumes of phenol and chloroform–isoamyl alcohol (24:1, vol/vol) and then one time with chloroform–isoamyl alcohol alone. Two volumes of absolute ethanol were added to the final aqueous phase and the solution was stored at −20°C. The RNA was then pelleted from alcohol, dissolved in NTE containing 0.5% (wt/vol) sodium dodecyl sulfate, and sedimented in a 10 to 30% (wt/wt) sucrose gradient (in NTE) for 2.7 h at 200,000 × *g* in an SW41 rotor. 70S RNA was isolated by alcohol precipitation of appropriate fractions.

RESULTS

Kinetics of noninfectious virion production by Act D-treated cells. To compare the properties of DNA polymerase in Act D virions and control virus particles, it was essential to isolate Act D virions at a time when they represent the major element in the virus population. Virus production was therefore measured at various times after Act D addition (Table 1). In agreement with our earlier findings (14), the data show that fluids collected between 0 and 3 h after Act D treatment contained significant amounts of infectious particles, whereas, in the interval between 3 and 6 h, infectivity dropped by 98%. However, the total number of particles, as measured by DNA polymerase activity, was the same in both harvests (ca. 50% relative to the control). Thus, the time interval from 3 to 6 h after the beginning of Act D treatment was chosen as the period for collection of supernatant fluids to be used for further analysis of virion-associated DNA polymerase activity.

TABLE 1. *Effect of Act D on the kinetics of MuLV production*

Time interval (h)	Percent of control	
	Infectivity[a]	DNA polymerase activity[b]
0–3	32	48
3–6	2.0	48

[a] 100% = 8.7 × 10^6 PFU/ml of culture fluid.
[b] 100% = 115 pmol of [^{3}H]dTMP incorporated in response to poly(rA) · $(dT)_{12-18}$ per μl of concentrated culture fluid.

Physicochemical properties of reverse transcriptase from Act D-treated and control cells. (i) Chromatography on $(dT)_{12-18}$-cellulose. It has previously been shown that $(dT)_{12-18}$-cellulose selectively binds viral RNA-dependent DNA polymerase and provides a useful step in the purification of this enzyme (6). Thus, control and Act D virions were disrupted, dialyzed, and applied in parallel to $(dT)_{12-18}$-cellulose columns (data not shown). Enzyme activity bound to the columns to similar extents (71% for control virions; 82% for Act D virions) and eluted as a sharp peak in both cases. The peak of the elution curve corresponded to a salt concentration of 0.2(±0.05) M KCl for DNA polymerase activity from both Act D and control virions. Elution of activity at this salt concentration is characteristic of DNA polymerases associated with a variety of RNA tumor viruses including murine, feline, and primate type C viruses (7; unpublished data).

(ii) Ion-exchange chromatography on phosphocellulose. Further purification of the enzyme samples eluted from $(dT)_{12-18}$-cellulose was achieved by chromatography on phosphocellulose columns (Fig. 1). Extremely sharp elution profiles were achieved for both control and Act D virion polymerases, and in each case the maximum occurred at a salt concentration of 0.4 M KCl. Enzyme activity was assayed with three different templates and, as may be seen, the relative activities of both enzymes with these templates were similar (see below). The elution profiles of Fig. 1 indicate that parallel purification of the DNA polymerases from Act D and control virions results in enzymes that are chromatographically indistinguishable.

(iii) Sedimentation velocity in glycerol gradients. To determine whether Act D treatment leads to the formation of a virion DNA polymerase that is altered with respect to molecular size, the enzymes eluted from the phosphocellulose columns were sedimented in velocity gradients as described in Materials and Methods. No size difference between the two enzymes was detectable by velocity gradient analysis (Fig. 2). Both the control and Act D virion polymerases migrated with bovine serum albumin, consistent with a molecular weight of approximately 70,000; this value is in the range observed for other murine viral DNA polymerases (5, 18, 19, 28). Thus, analysis of the two enzymes by sedimentation velocity and chromatographic techniques indicates that their physicochemical properties are identical.

Catalytic properties: Template/primer preferences. Initial observations in the course

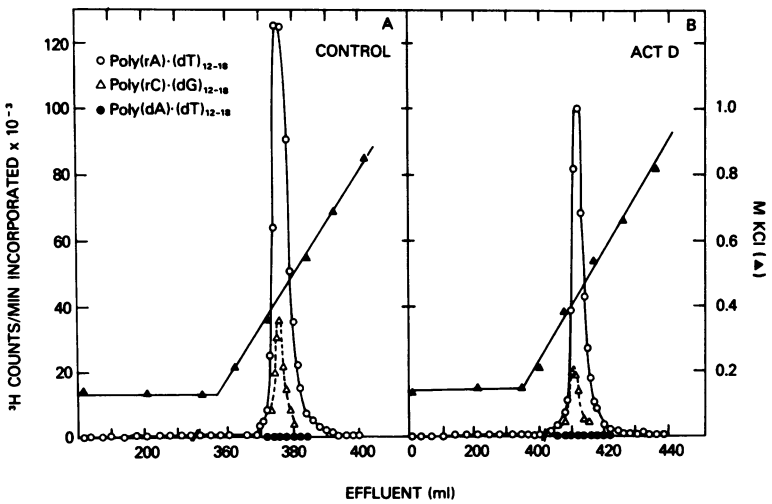

FIG. 1. *Phosphocellulose column chromatography of DNA polymerase from control and Act D virions. Peak fractions from $(dT)_{12-18}$-cellulose chromatograms of control (A) or Act D (B) virion DNA polymerases were pooled, adjusted to a salt concentration (0.16 M KCl) equivalent to that of equilibration buffer, and applied to capillary columns of phosphocellulose as described in Materials and Methods. Portions (10 μl) from each fraction were assayed for homopolymer synthesis with the three synthetic templates shown, using the conditions outlined in Materials and Methods. Salt concentrations were determined conductimetrically.*

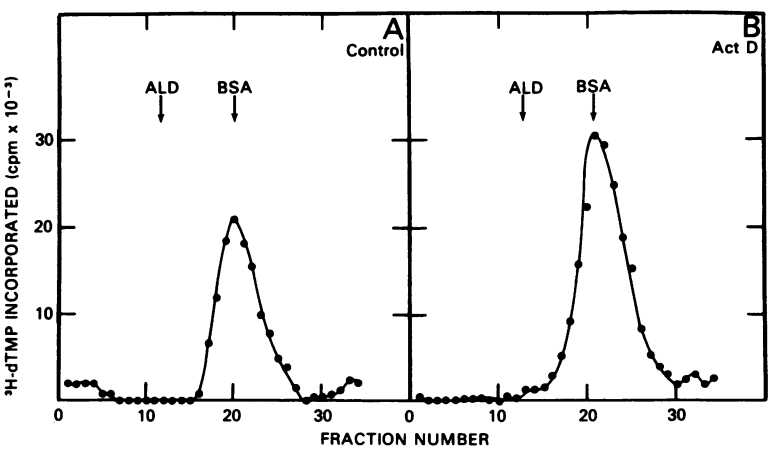

FIG. 2. *Glycerol gradient centrifugation of control and Act D DNA polymerases. Samples (500 μl) from phosphocellulose column-purified control (A) or Act D (B) DNA polymerase preparations were sedimented in 5 to 25% (vol/vol) glycerol gradients as described previously (7). An additional gradient contained aldolase (ALD) and bovine serum albumin (BSA) as external markers. Four-drop fractions were collected from the bottom of the tubes. Portions (10 μl) of each fraction were assayed with poly(rA)·$(dT)_{12-18}$ as described previously (7).*

of purification (Fig. 1) indicated that control and Act D DNA polymerases displayed similar patterns of template/primer utilization. To establish more precisely whether the Act D polymerase might show altered catalytic properties, we tested the relative response of purified enzymes from control and Act D virions to various natural and synthetic template/primers (Table 2). All values were calculated relative to that observed with poly(rA) · $(dT)_{12-18}$.

The data show that poly(rA) · $(dT)_{12-18}$ is the preferred template for both the control and the Act D virion DNA polymerases. Poly(rC) · $(dG)_{12-18}$ is also utilized efficiently as expected for these viral enzymes (2). Polymerization in response to both "activated" DNA and natural templates, AMV 70S RNA and globin mRNA, was also detected. As expected for a viral RNA-dependent DNA polymerase, there was very low activity in response to poly(dA) · $(dT)_{12-18}$. This pattern of template/primer responses is consistent with that observed for other reverse transcriptase enzymes (2, 8).

The metal ion preference and concentration optimum were also determined and found to be identical for both the control and Act D enzymes (data not shown). In agreement with the results of Abrell et al. (1), Mn^{2+} was the preferred metallic cation for all templates including the AMV 70S RNA and globin mRNA, and the optimal concentration ranged from 0.5 to 1.0 mM for both enzymes. Mn^{2+} at a concentration of 0.5 mM was therefore utilized in all reactions.

Sensitivity to anti-polymerase antibody. The data presented thus far indicate that the DNA polymerase associated with Act D virions is indistinguishable from the control viral enzyme with regard to its physicochemical and catalytic properties. To demonstrate possible differences between the two enzymes which would involve their antigenic binding sites or the interaction of these sites with the catalytic portions of the molecules, we tested the sensitivity of both DNA polymerases to inhibition by antibody directed against Rauscher MuLV DNA polymerase.

Our approach in these experiments was to preincubate purified enzyme from Act D or control virions with various amounts of immune IgG and then to measure enzymatic activity in the standard assay. Quantities of DNA polymerase tested were sufficient to incorporate 3 pmol of [^{3}H]dTMP in response to poly(rA) · $(dT)_{12-18}$. It is clear from the results (Fig. 3) that both control and Act D enzymes are essentially identical in the degree to which they are inhibited by comparable amounts of immune IgG. It is of interest that Rauscher DNA polymerase was more sensitive to this antiserum than AKR polymerase; e.g., whereas 10 μg of immune IgG inhibited AKR virus enzymes by only 30%, a similar amount of Rauscher DNA polymerase was inhibited by 95% (data not shown). This suggests, in agreement with observations in the avian system by Panet et al. (21), that reverse transcriptase contains some type-specific determinants.

TABLE 2. *Response of purified reverse transcriptase to several template/primers*

Template/primer	Control enzyme	Act D enzyme
Poly(rA) · $(dT)_{12-18}$	100[a]	100[a]
Poly(rC) · $(dG)_{12-18}$	27	22
"Activated" DNA	4.7	6.2
AMV 70S RNA/$(dT)_{12-18}$	4.5	4.3
Mouse globin mRNA/$(dT)_{12-18}$	3.4	3.2
Poly(dA) · $(dT)_{12-18}$	<1.0	<1.0

[a] 100% represents the incorporation of 5 pmol of ^{3}H-labeled deoxyribonucleoside monophosphate in standard assay. See text.

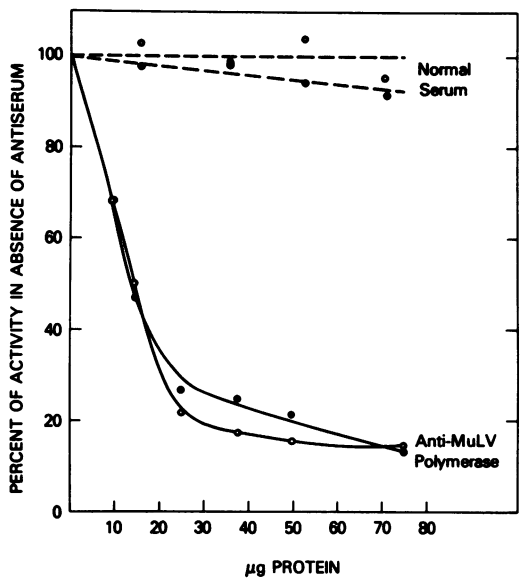

FIG. 3. *Inhibition of control and Act D virion DNA polymerases by antiserum directed against Rauscher MuLV DNA polymerase. Phosphocellulose column-purified enzymes were tested, as described in Materials and Methods, for susceptibility to inhibition by IgG purified from goat anti-Rauscher MuLV DNA polymerase serum or from normal goat serum. For both enzymes, 100% activity represents incorporation of 100,000 cpm of [^{3}H]dTMP. Symbols: ●, control; ○, Act D.*

Thermal inactivation rate of purified viral DNA polymerase. To probe for fine-structural differences undetectable by the preceding methods, the thermal decay rates of DNA polymerase from Act D and normal virions were compared. In addition to measuring the inacti-

vation of enzyme activity, we also examined the degree of protection achieved by heating the enzymes in the presence of template (16, 26, 27, 29).

Data from a typical experiment are shown in Fig. 4. Purified reverse transcriptase from control or Act D virions was incubated at 44°C for specified times in the presence or absence of poly(rA) · $(dT)_{12-18}$ and immediately assayed at 37°C for 1 h. The decay rates calculated from the slopes of these lines were 3.1(±0.5)% loss per min for the unprotected control enzyme and 2.6(±0.5)% loss per min for the unprotected Act D enzyme. These small differences in the decay rates were within the range of experimental variation. Both enzymes were significantly protected by poly(rA) · $(dT)_{12-18}$, and this protection reduced the decay rate by 0.9 to 1.4% activity loss per min at 44°C. As may be seen, Act D and control enzymes were protected to roughly the same extent.

When these experiments were performed at temperatures ranging from 41 to 48°C (data not shown), the decay rates varied from 0.9% loss per min at 41°C to 10% loss per min at 48°C. In every case, thermal inactivation was less extensive when either the control or Act D enzyme was heated with the template/primer poly(rA) · $(dT)_{12-18}$. There was considerable variation within this temperature range in the absolute value of the protective effect, but, in general, protection by poly(rA) · $(dT)_{12-18}$ reduced the rate of decay by 33 to 50% relative to the unprotected rate.

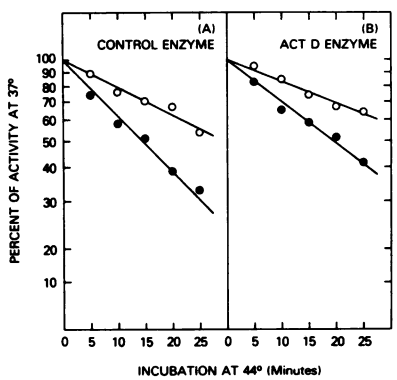

FIG. 4. *Thermal inactivation of purified DNA polymerase from control or Act D virions in the presence and absence of poly(rA) · $(dT)_{12-18}$. Enzymes that had been purified on phosphocellulose columns were incubated at 44°C for the indicated times and immediately transferred to a complete reaction mixture for assay at 37°C as described in Materials and Methods. For both enzymes, 100% activity is equivalent to incorporation of 120,000 cpm of [^{3}H]dTMP. Each point represents 10 μl of enzyme in a 50-μl reaction mixture. Symbols: ●, enzyme alone; ○, enzyme plus poly(rA) · $(dT)_{12-18}$.*

In addition to the experiments with poly(rA) · $(dT)_{12-18}$, the purified enzymes were also tested at 44°C for protection by a natural template, AMV 70S RNA. Table 3 gives a comparison of the extent to which control and Act D enzymes were protected by 70S RNA and poly(rA) · $(dT)_{12-18}$. It is clear that both enzymes showed significant protection from thermal inactivation when preincubated with either template/primer. Thus, in all of these measurements of thermal decay, DNA polymerase purified from Act D virions was indistinguishable from enzyme purified from control virions.

Thermal decay of encapsidated polymerase in control and Act D virions. The experiments presented above indicate that purified DNA polymerases isolated from Act D and control virions are identical with respect to thermal decay and potential for template/primer protection. Although Act D and control virions have the same morphology (13) and structural protein composition (14), they differ in one very fundamental respect: Act D virions lack 70S genomic RNA (13), which serves as the natural template for reverse transcriptase. Thus, a comparison of thermal decay rates of Act D and control DNA polymerases contained within the virion structure provides a unique opportunity to probe the degree of protection afforded the enzyme by association with the template RNA normally found in the virion. Figure 5 illustrates an experiment in which this comparison was made. Clarified supernatant fluids from control and Act D-treated cells were incubated at 44.5°C for the indicated times. Virions were then pelleted and disrupted, and the polymerase activity was tested with poly(rA) · $(dT)_{12-18}$ as described in Materials and Methods. The data show that the thermolability of polymerase in Act D virions is indistinguishable from that in control virions. Furthermore, the decay rate of the virion-associated polymerases (3.5% activity loss per min at 44.5°C) is very similar to that of purified enzymes in the absence of template

TABLE 3. *Protection from thermal decay at 44°C*

DNA polymerase	Template/primer	Protection[a] (%)
Control	AMV 70S RNA	29
Act D	AMV 70S RNA	27
Control	Poly(rA) · $(dT)_{12-18}$	35
Act D	Poly(rA) · $(dT)_{12-18}$	45

[a] Enzyme decay rates were calculated graphically as the slopes of the decay curves in the presence and absence of template/primer. The percent protection is the value obtained by dividing the difference between these slopes by the slope of the unprotected decay curve.

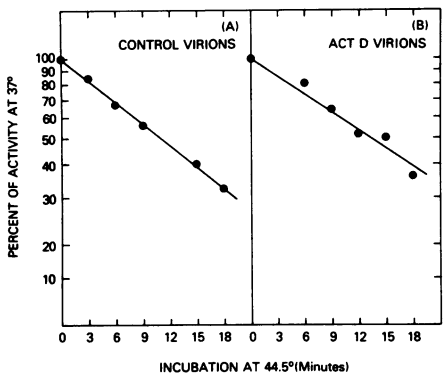

FIG. 5. *Thermal inactivation of DNA polymerase activity in intact control or Act D virions. Clarified tissue culture supernatants containing control or Act D virions were incubated at 44.5°C for the indicated times. After centrifugation at 100,000 × g for 1 h, the virions were disrupted and assayed with poly(rA) · $(dT)_{12-18}$ as described in Materials and Methods. The 100% value represents 150,000 cpm of [^{3}H]dTMP incorporated.*

(2.9% activity loss per min at 44°C and 3.9% activity loss per min at 45°C). These findings suggest a comparable stability for the enzyme in the virion and the purified enzyme in solution.

Thermal lability of activities associated with normal virions. To further explore the finding that thermal decay rates of virion-associated and purified DNA polymerase are similar, we tested the stability of normal virions to heat treatment by measuring the thermal decay of biological and biochemical markers of virion integrity. In the experiment shown in Fig. 6, clarified tissue culture fluids were incubated at 44°C for the specified times and analyzed for infectivity and polymerase activity in response to poly(rA) · $(dT)_{12-18}$, as well as for the percentage of total RNA in the 70S form. As may be seen, the thermal decays of polymerase and infectivity were essentially identical. In both cases, the inactivation occurred very rapidly (3.0% activity loss per min at 44°C), in agreement with the data presented above (Fig. 5); by 60 min, 97% of the infectivity titer and the DNA polymerase activity was lost. In contrast, the relative amount of 70S RNA contained in virions did not diminish significantly and was still 96% of the zero-time value after 60 min of incubation at 44°C.

Polyacrylamide gel analysis of virion RNA. The RNA data in Fig. 6 were obtained by polyacrylamide gel electrophoresis of ^{3}H-labeled RNA samples extracted from virions heated for varying times at 44°C. Figure 7A and C shows the analysis of the 0- and 60-min samples, respectively. As may be seen from a comparison of the gel patterns, very sharp peaks of 70S RNA were obtained in both cases. The percentage of total RNA that migrated as 70S was essentially unchanged even after 60 min at 44°C, and there was no increase in the amount of low-molecular-weight RNA species. As a further measure of the integrity of the 70S RNA, portions were denatured at 100°C for 45 s and analyzed on composite gels of 2% polyacrylamide–0.5% agarose. As shown in Fig. 7B and D, these samples were converted to sharp subunit peaks of approximately 3.0 × 10^6 daltons. These gel analyses indicate that the genomic RNA of normal virions is not degraded by incubation for 60 min at 44°C. This result is in striking contrast to the finding that both infectivity and DNA polymerase activity ultimately drop to 3% of their initial values during the same period of time (Fig. 6).

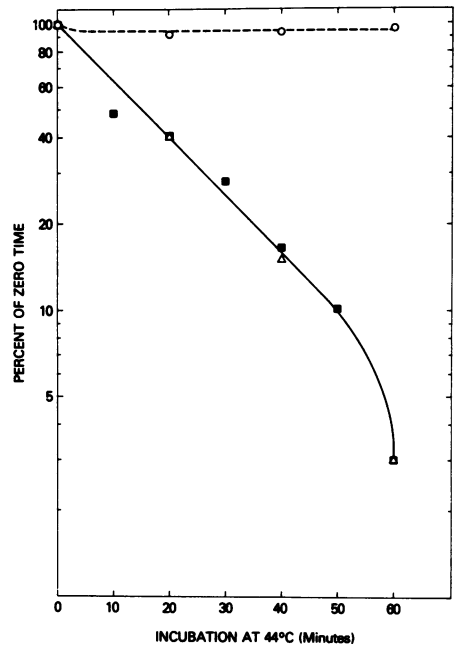

FIG. 6. *Rate of change of infectivity, DNA polymerase activity, and 70S genomic RNA content after heating intact control virions at 44°C. Clarified tissue culture supernatants containing normal virions were incubated at 44°C for the indicated times. Samples were assayed for infectivity (24), for DNA polymerase activity after centrifugation for 1 h at 100,000 × g (13), and for the percentage of total RNA in the form of 70S genomic RNA, as determined by analysis of ^{3}H-labeled virion RNA in composite polyacrylamide-agarose gels (see Materials and Methods). 100% values were: 2.4 × 10^6 PFU/ml; 150,000 cpm of [^{3}H]-dTMP incorporated; and 72% of the total virion RNA as 70S RNA. Symbols: △, infectivity; ■, DNA polymerase activity; ○, 70S RNA.*

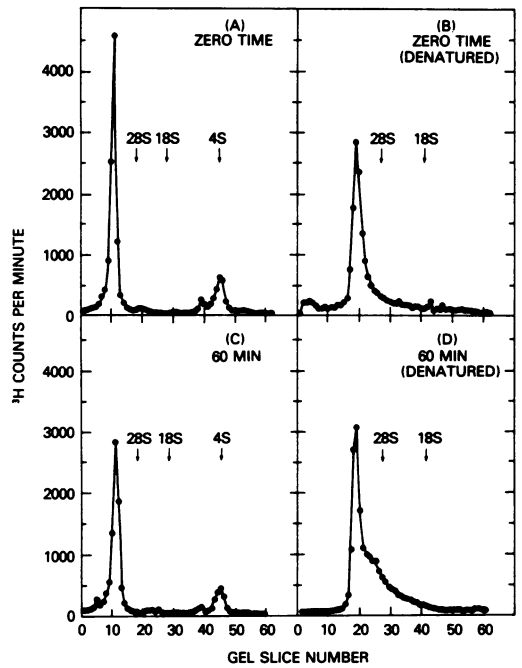

FIG. 7. *Polyacrylamide gel electrophoresis of [³H]-RNA from unheated and heated control virions. RNA was extracted from purified ³H-labeled virions heated at 44°C for the indicated times, as described in Materials and Methods. For detection of 70S RNA, samples were subjected to electrophoresis in composite 1.8% polyacrylamide-0.5% agarose gels for 2.25 h at a constant current of 3 mA/gel (A and C). For detection of subunit RNA, samples were first denatured by heating at 100°C for 45 s, then cooled rapidly in ice, and immediately analyzed on 2% polyacrylamide-0.5% agarose gels run for 2 h, 50 min at a constant current of 4 mA/gel (B and D). ³²P-labeled cell RNA was included in each sample as an internal marker. (A and B) RNA from unheated virions (zero time); (C and D) RNA from virions heated for 60 min at 44°C.*

DISCUSSION

In this study, the role of genomic RNA in the encapsidation of a functional reverse transcriptase was investigated by comparing the properties of the reverse transcriptase enzymes in normal and Act D virions. The results show that virus particles produced by cells after 3 to 6 h of treatment with Act D contain a virion reverse transcriptase that is indistinguishable from the enzyme packaged in virions produced by untreated control cells.

Enzymes from the two classes of particles were purified in parallel and demonstrated identical elution profiles on both $(dT)_{12-18}$-cellulose and phosphocellulose chromatography (Fig. 1). In addition, both enzymes migrated to the same position as bovine serum albumin upon glycerol velocity gradient sedimentation (Fig. 2). Thus, the enzymes from control and noninfectious Act D particles share the physicochemical characteristics measured by these techniques.

When the template preferences of the two partially purified enzymes are compared (Fig. 1; Table 2), it is clear that various template/primer combinations are utilized in the same relative ratios. In addition, the enzymes possess the same antigenic determinants since they were inhibited to the same extent by equivalent quantities of immune IgG directed against Rauscher MuLV DNA polymerase (Fig. 3). These data also indicate that the interaction between the antigenic and catalytic sites of the two classes of enzymes is quantitatively identical.

As a further probe of the molecular identity of reverse transcriptase from virions produced by control or Act D-treated cells, the thermal lability of the two enzymes was tested in the presence and absence of template/primers. In other systems, such studies have made it possible to distinguish between two types of defective reverse transcriptase enzymes. One class consists of enzymes, like those encoded by the avian *ts* mutant LA336 (25, 30) and the mammalian mutant *ts* 29 (26), that are more thermolabile than the wild-type reverse transcriptase but can, nevertheless, be protected from thermal denaturation by template/primer. A second class consists of abnormal reverse transcriptase molecules, like those encoded by the avian *ts* mutants LA335 and LA337 (15, 17, 29), that are more thermolabile than the wild type but cannot be protected by the presence of template/primers. Thus, in addition to evaluating the stability of the native conformation, the quantitative measurement of thermal inactivation can be used to assess the strength of template binding by the enzyme as measured by the enhanced thermal stability of the enzyme-template complex. Such experiments were of particular interest in the Act D system, where any interactions between genomic RNA and reverse transcriptase would be absent. It was thus of great interest to find that reverse transcriptase purified from Act D virions was indistinguishable from the control enzyme with respect to its thermal decay rate (Fig. 4). In addition, the two enzymes were protected to the same extent by heating in the presence of the synthetic template poly(rA) · $(dT)_{12-18}$ or the natural template, AMV 70S RNA (Table 3).

Thus, by several physical and catalytic measurements, the viral DNA polymerase synthesized and packaged into virions in the presence of Act D is indistinguishable with regard both

to structure and function from the enzyme found in normal virions. This finding, for a virus-coded enzyme as distinct from the viral structural proteins, extends the observation of Levin and Rosenak (14) that all of the normal virion proteins are present in Act D particles.

It is of interest, in this regard, to compare Act D virions with noninfectious particles produced by the avian temperature-sensitive mutant LA334 (20, 25) at nonpermissive temperatures. Under these conditions, cleavage of the 76,000-dalton precursor protein pr76 (31) to the core protein p27 occurs incorrectly (11, 23), and several atypical proteins (11, 23), including relatively large amounts of a virus-coded protein p23, are incorporated into the particles (11, 23). As a result, the viral core structure is abnormal (11, 23), and this, in turn, exerts pleiotropic effects on virus assembly. Noninfectious particles formed by LA334 lack the typical morphology of type C virions (4, 11, 23), exhibit aberrant viral budding forms in the electron microscope (4, 11, 23), and are deficient in the normal core components p27 (11, 23), reverse transcriptase (23), and genomic RNA (9). In contrast, the noninfectious particles produced after Act D treatment, although apparently lacking the entire 60 to 70S RNA genome, contain the normal complement of virion structural proteins (14), presumably because viral mRNA functions for at least 8 to 12 h quite independently of viral RNA synthesis (14). Furthermore, Act D virions appear to have normal morphology (13) and, as demonstrated in this report, encapsidate a normal reverse transcriptase enzyme. These findings strengthen the previous conclusion (14) that genomic RNA plays little, if any, role in directing virus assembly and further suggest that it is the proper synthesis and interaction of the viral proteins that are critical.

Although genomic RNA does not appear to play a major role in virus assembly, it was of interest to explore possible functional interactions between genomic RNA and DNA polymerase that might lead to increased stability of these molecules within the virion structure. For this purpose, we compared the thermal lability of purified reverse transcriptase to that of enzyme encapsidated in normal or Act D virions. This approach involves the comparison of intact virions with a reconstructed system containing enzyme in the presence or absence of nucleic acid. Several variables such as ionic conditions, structural effects, and the actual ratio of enzyme to RNA may differ in the two systems, thus limiting the possibilities for rigorous interpretation.

However, the experiments reported here have clearly demonstrated that encapsidated enzyme in both Act D and normal particles decays as rapidly as the purified molecule in the absence of template (Fig. 5). Furthermore, the data of Fig. 6 and 7 show that genomic RNA in normal particles is stable to incubation of virions for 1 h at 44°C. Thus, genomic RNA is much more stable to heating than the virion-associated reverse transcriptase, since only 3% of the original DNA polymerase activity remains after this extended thermal exposure (Fig. 6). In effect, this 1-h treatment provides a simple method for generating a class of defective virions that contain genomic RNA but lack reverse transcriptase activity.

The observation that 70S RNA in normal virions is thermostable, coupled with the finding that the reverse transcriptase enzymes in normal and Act D virions exhibit similar thermal decay rates, suggests that virion-associated DNA polymerase is not stabilized by genomic RNA or other components of the virion. The possibility that the techniques used here mask a slower decay of a minority of enzyme molecules that are functionally associated with and stabilized by genomic RNA cannot be completely excluded. However, in reconstruction experiments (not shown), in which conditions of enzyme excess were achieved by increasing the ratio of purified enzyme to AMV 70S RNA 15-fold over that shown in Table 3, the same level of protection was obtained. In any case, it is clear that the majority of enzyme molecules are packaged in such a way that interactions within the virion do not confer increased thermal stability. Thus, either polymerase and genomic RNA are separated in the virion or their conformations are such that binding sites are masked and the interaction that forms in free solution and provides protection against heat denaturation cannot occur.

In the course of studying the thermolability of the DNA polymerase in intact virions, we measured both infectivity and polymerase activity on separate portions of the same sample. The data of Fig. 6 show a remarkable congruence between the decay rates of these two parameters. This suggests that, in the AKR virions produced in this system, the DNA polymerase or another molecule with identical heat lability is the most thermolabile molecule required for infectivity. In addition, the fact that the decay rates are identical suggests that only one polymerase molecule per virion is essential for infectivity. A model in which a virion contained excess polymerase molecules, any one of which could function in infection, would predict a lag period or "shoulder" before the loss of infectivity would occur at the same rate as the loss of polymerase activity. In contrast, a model in which more

than one polymerase molecule was required for infectivity would result in a more rapid decay of infectivity than of DNA polymerase activity.

In summary, analysis of noninfectious virions produced by cells treated with Act D has enabled us to study, by difference, possible functional relationships of DNA polymerase and genomic RNA within the virion structure. This paper demonstrates that an apparently normal DNA polymerase is synthesized and packaged in the absence of genomic RNA. In addition, the data presented here demonstrate that the genomic RNA of normal virions is not degraded after extended heat treatment. Finally, the data are consistent with the possibility that reverse transcriptase is not associated with genomic RNA as an enzyme-template complex within the virion core.

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