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Studies on Reverse Transcriptase of RNA Tumor Viruses

I. Localization of Thermolabile DNA Polymerase and RNase H Activities on One Polypeptide

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Purified reverse transcriptase from avian myeloblastosis virus or Rous sarcoma virus consists of two subunits of average mol wt of 100,000 and 60,000. The lower-molecular-weight subunit, α, has been isolated from avian myeloblastosis virus, Rous sarcoma virus, and a temperature-sensitive mutant of Rous sarcoma virus, *LA337*. Subunit α manifests both the DNA polymerase and RNase H activities associated with purified reverse transcriptase of avian RNA tumor viruses. The thermal inactivation of these enzymatic activities of α subunit from *LA337* was compared with the isolated α subunit from the wild-type virus. The results show that both DNA polymerase and RNase H activities associated with the α subunit of *LA337* are five to seven times more thermolabile than the corresponding α subunit from the wild-type virus. It is concluded that (i) both the polymerase and nuclease activities reside on the same polypeptide chain, and (ii) at least the lower-molecular-weight subunit α is coded for by the viral RNA.

Avian RNA tumor viruses contain a DNA polymerase which can faithfully transcribe the viral RNA into complementary DNA (11, 12). The DNA polymerase can be solubilized from the virions and purified by column chromatography (3, 6, 8, 13). Purified DNA polymerase from avian myeloblastosis virus (AMV) or Rous sarcoma virus (RSV), upon analysis on sodium dodecyl sulfate-containing polyacrylamide gels, dissociates into equimolar amounts of two polypeptide chains (subunits) with average mol wt of 100,000 and 60,000 (4, 8, 11, 15). The lower-molecular-weight subunit (α) has been isolated from AMV (4) and has been shown to contain the three known enzymatic activities associated with the DNA polymerase from avian RNA tumor viruses: RNA-directed DNA synthesis, DNA-directed DNA synthesis, and RNase H (which selectively degrades the RNA moiety of RNA-DNA hybrids) (11, 12). DNA polymerases isolated from two temperature-sensitive mutants of RSV with defects in a very early event of the virus growth cycle (10) have been shown to be more thermolabile than the DNA polymerase from the wild-type parent (14). Since the purified DNA polymerase studied in those experiments contained both subunits, it could not be conclusively established that the temperature-sensitive lesion resides in the subunit containing the polymerase and nuclease activities. To demonstrate that the polypeptide chain manifesting the synthetic and the degradative activities is thermolabile, the lower-molecular-weight subunit α has been isolated from the temperature-sensitive mutant *LA337*. The heat-inactivation properties of the DNA polymerase and RNase H activities of α subunit from the mutant virus were compared with the α subunit isolated from the wild-type parent. The results indicate that both the DNA polymerase and the RNase H activities of α from *LA337* are five to seven times more thermolabile than those of the α subunit of the wild-type parent.

MATERIALS AND METHODS

Materials. Tritium-labeled and unlabeled deoxyribonucleoside triphosphates were obtained from Schwarz/Mann and New England Nuclear. Polynucleotides were obtained from either Miles Laboratory or P-L Biochemicals. Oligonucleotides were 8 to 18 in length and were obtained from Collaborative Research. AMV in plasma was provided by the Office of Program Resources and Logistics of the Virus Cancer Program of the National Cancer Institute. [^{3}H]poly(A) was synthesized as described previously (2). *Escherichia coli* DNA polymerase was a gift from A. Kornberg to D. Baltimore.

Purification of virion DNA polymerase. Purified virions of *LA337* and wild-type RSV, Prague strain (subgroup C), were obtained as described previously (10). AMV was purified from chicken plasma as described (13). About 4.5 mg of purified virions from *LA337* and 14.0 mg of purified virions from wild-type RSV were lysed with Nonidet P-40 (Shell Chemicals),

and DNA polymerase was purified by column chromatography on DEAE-Sephadex A-25 (13). Enzymatic assays were performed as described previously (13). The peak fractions with enzymatic activities were dialyzed overnight against buffer B containing 50 mM Tris-hydrochloride (pH 7.5), 0.1 mM EDTA, 0.1 M β-mercaptoethanol, 20% glycerol, and 0.15% Nonidet P-40. The dialyzed enzyme was further purified on a phosphocellulose column (13). AMV DNA polymerase was purified as previously described (13).

RNA-directed DNA synthesis was assayed using poly(C)·oligo(dG) as template-primer; DNA-directed DNA synthesis was assayed using poly(dC)·oligo(dG) as template-primer. RNase H activities were assayed as described previously (2, 14). All enzyme assays were carried out for times during which synthesis or degradation was a linear function of time. The details of various reactions are described in the legends to the figures.

RESULTS

Isolation and characterization of α from AMV, wild-type RSV, and LA337. The lower-molecular-weight subunit α was isolated by sequential chromatography on phosphocellulose columns. Figure 1 shows the chromatographic profiles of DNA polymerases isolated from *LA337* and wild-type RSV. The major peak of enzymatic activity obtained from the first phosphocellulose chromatography was dialyzed overnight against buffer B and rechromatographed on a second phosphocellulose column. When assayed with poly(C)·oligo(dG) as template-primer, two peaks of enzymatic activity could be distinguished. The minor peak of enzyme activity, α, eluting at a salt concentration of 0.1 M, represents 3 to 10% of the total enzyme activity. The major peak of enzyme activity, $\alpha\beta$, elutes at a salt concentration of 0.24 M KCl. Similar separation of two enzyme activities can be obtained from AMV. In all subsequent experiments, α and $\alpha\beta$ obtained from the second phosphocellulose column were used.

The molecular size of α and $\alpha\beta$ was analyzed by chromatography on Sephadex G-100 columns. Figure 2 represents a composite of three sets of experiments and was drawn by aligning the *E. coli* DNA polymerase I activity in all three experiments. It can be seen that $\alpha\beta$ from AMV, which has an average mol wt of 150,000 to 170,000, elutes in the void volume considerably ahead of the marker, *E. coli* DNA polymerase I, which has a mol wt of 110,000 (7). This is consistent with the observation that the holoenzyme is composed of two subunits, with a total mol wt of 170,000 (6, 8, 15). Subunit α from both AMV and wild-type RSV elutes from the column after *E. coli* DNA polymerase I. Analysis of α from AMV in sodium dodecyl sulfate-containing agarose slab gels shows one major polypeptide band with an average mol wt of 60,000 (I. M. Verma and W. Gibson, unpublished data). Thus, based upon Sephadex G-100 column chromatography and polyacrylamide gel electrophoresis, α from AMV or RSV wild-type appears to consist of only one polypeptide chain with a mol wt of 60,000 to 70,000. This is in agreement with the results of Grandgenett et al. (4), although their estimate of the molecular weight of α of AMV is somewhat higher. The molecular size of α isolated from *LA337* was not characterized because of insufficient material. It has, however, been shown previously that $\alpha\beta$ obtained either from *LA337* or wild-type RSV has the same molecular size and number of subunits (14).

DNA polymerase and RNase H assay of α subunit of LA337 and wild-type RSV. The three known enzymatic activities associated with the DNA polymerase of avian RNA tumor viruses were compared using poly(C)·oligo(dG) to measure RNA-directed DNA synthesis, poly(dC)·oligo(dG) to assay DNA-directed DNA synthesis and [^{3}H]poly(A)·poly(dT) to assay for RNase H activity (Fig. 3). The average time of inactivation of one-half of the activity of α from *LA337* and wild-type RSV for these three activities has been calculated (Table 1). The α subunit isolated from the virions of *LA337* showed a five- to sevenfold greater thermolability than the α subunit isolated from wild-type RSV for all three activities.

Comparison of α and $\alpha\beta$ from LA337 and wild-type RSV. Both the RNA-directed DNA synthesis and DNA-directed DNA synthesis activities associated with α subunit of *LA337* are slightly more resistant to thermal inactivation than the activities of the corresponding holoenzyme, $\alpha\beta$ (Fig. 4). Subunit α from the wild-type parent is, however, slightly more thermolabile than the $\alpha\beta$ complex. The RNase H activity of α from both *LA337* and wild-type parent are reproducibly more thermolabile than that of the two-subunit complex.

DISCUSSION

DNA polymerase isolated from two conditional temperature-sensitive mutants of RSV (*LA335* and *LA337*) has been shown to be three- to fivefold more thermolabile than the DNA polymerase from the wild-type parent (14). The thermal inactivation profile of the DNA polym-

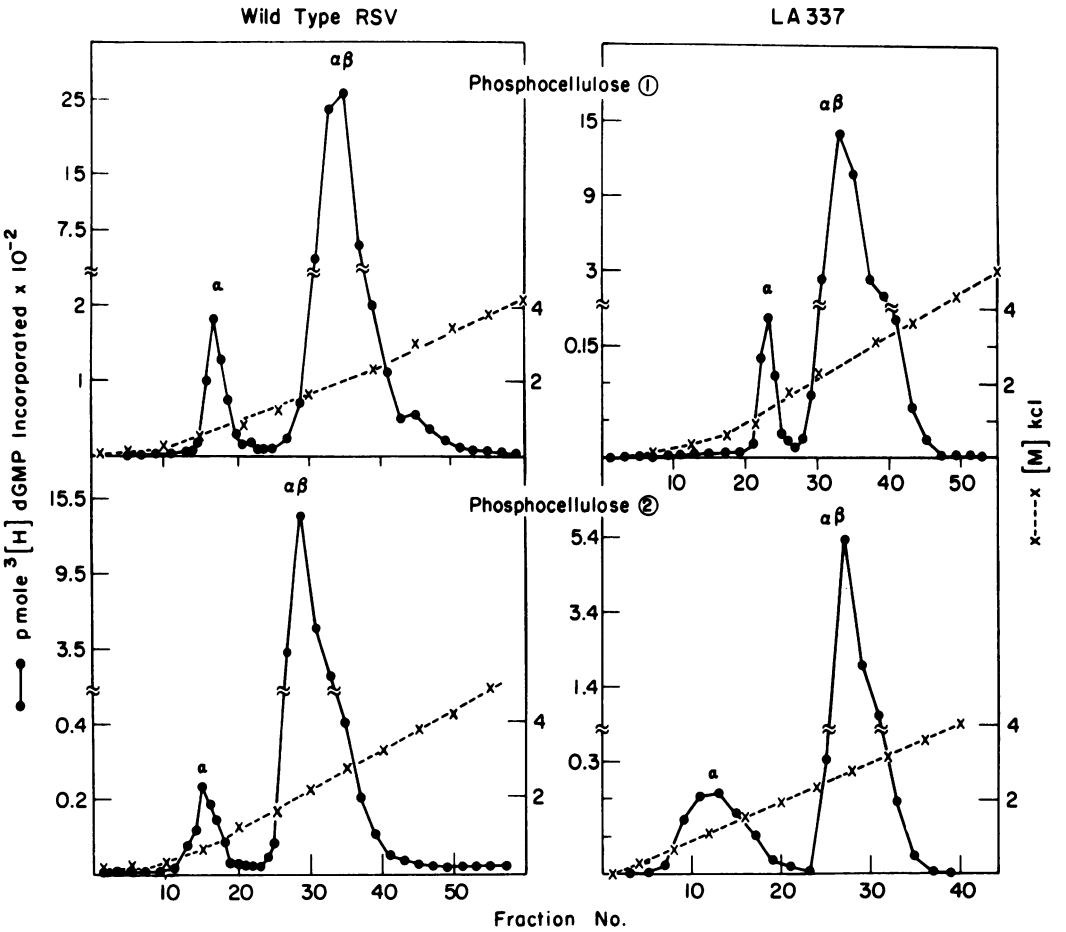

FIG. 1. *Sequential column chromatography of DNA polymerase from wild-type RSV and LA337. The major peak of enzymatic activity from the first phosphocellulose column was pooled, dialyzed overnight against buffer B, and rechromatographed on a second phosphocellulose column. Two peaks of enzymatic activities, α and αβ, could be distinguished. Peak α was stored in 50% glycerol at −20 C, whereas αβ was stored at −70 C. The recovery of the enzymatic activity from the second phosphocellulose column was over 50%. Enzyme assays were performed utilizing poly(C)·oligo(dG) as template-primer and ³[H]dGTP (100 counts per min/pmol) as substrate. Peaks α and αβ from LA337 virions were isolated in an identical fashion, except the dialysis was carried out for 5 to 8 h instead of overnight to minimize thermal inactivation of the enzyme during the purification procedure. The recovery of total enzyme activity after first phosphocellulose column was over 20%. On the second phosphocellulose column over 75% of the input enzymatic activity was recovered. Peak α constituted about 10% of the total enzyme activity. Both α and αβ obtained from LA337 virions were stored at −70 C.*

erase activity from the wild-type revertants (wt *LA335* and wt *LA337*) is indistinguishable from that of wild-type virus (14). Mason et al. (Virology, in press) have recently characterized the biological and biochemical properties of these mutants, their revertants, and several recombinants with leukosis viruses and have shown that the lesion in these mutants is the increased thermolability of their DNA polymerase activity. Varmus et al. (Cold Spring Harbor Symp. Quant. Biol., in press) have reported that less viral DNA is produced in *LA335* and *LA337* at the nonpermissive temperature than is produced by the wild-type virus.

Since all three known enzyme activities associated with the RNA tumor virus DNA polymerase were thermolabile, it was suggested that all three activities were located on the same polypeptide chain (14). To establish that the polypeptide chain manifesting all three enzyme

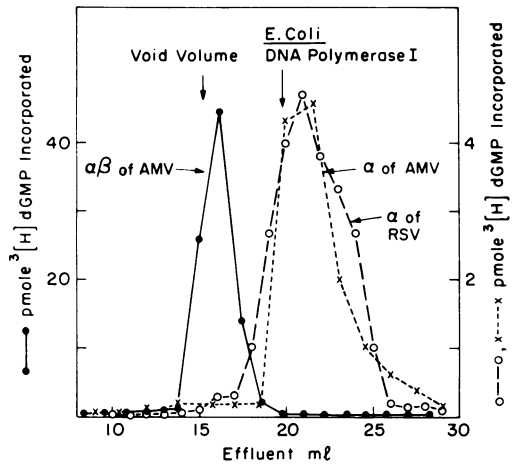

FIG. 2. *Size determination of α and αβ from second phosphocellulose column. Peaks α and αβ from AMV were obtained as described in the legend to Fig. 1. Peak α from wild-type RSV and α and αβ from AMV were separately chromatographed on a Sephadex G-100 column (1 by 70 cm). The elution buffer contained 50 mM Tris-hydrochloride (pH 7.9), 0.2 M KCl, 0.1 mM EDTA, 10 mM mercaptoethanol, and 0.1% Nonidet P-40. In each case, E. coli DNA polymerase I was added as a standard marker. Dextran blue was used to determine the void volume. Peaks α and αβ were assayed for enzyme activity using poly(C)·oligo(dG) as template-primer (15). The E. coli DNA polymerase was assayed utilizing poly(dA)·oligo(dT) as template-primer and ³[H]TTP as substrate (15). The figure was drawn by aligning the E. coli DNA polymerase activities of the three separate experiments. The recovery of enzyme activity of αβ from the column was over 90%, whereas α and E. coli DNA polymerase showed a recovery of over 30% of the input enzyme activity.*

activities is thermolabile and is encoded by the viral RNA, I isolated α subunit from *LA337* and compared its thermal inactivation properties with isolated α subunit from wild-type RSV. The α subunit was isolated from purified DNA polymerase which had both subunits in equimolar ratio. This approach assures that the isolated lower-molecular-weight subunit α is part of the viral DNA polymerase. The mechanism of separation of α from αβ (two subunit complex) is not clear. A comparison of the tryptic peptides of isolated α and β (large subunit) from either AMV or wild-type RSV reveals that the two subunits share at least a portion of their amino acid sequences (Gibson and Verma, unpublished data). Thus, based upon the structural relatedness of the two subunits and polyacrylamide gel electrophoretic profile of aged reverse transcriptase from AMV (K. Mölling, Cold Spring Harbor Symp. Quant. Biol., in

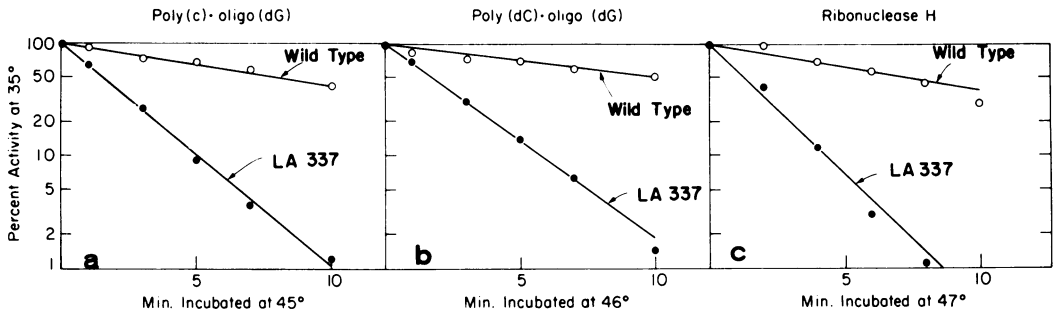

FIG. 3. *Thermal inactivation profiles of DNA polymerase and RNase H activities of isolated α subunit from LA337 and wild-type RSV. Assays of heat lability were performed as described (15). Briefly, the enzyme was preincubated in reaction mixture 1, which contained 100 mM Tris-hydrochloride (pH 8.3), 20 mM dithiothreitol, 12 mM Mg acetate, and 120 mM NaCl. The reaction mixture was incubated at the high temperature shown in the figure, and at various intervals portions (0.05 ml) were withdrawn and added to 0.05 ml of reaction mixture 2, which contained template-primer and the substrates. This mixture was kept on ice for a maximum of 10 to 15 min, then further incubated at 35 C for 30 min. Acid precipitable radioactivity was determined as described previously (1). (a) Approximately 0.005 to 0.05 units of purified α subunit was used per assay. (A unit of enzyme activity is defined as the amount of enzyme required to incorporate 100 pmol of ³[H]dGMP at 37 C in 15 min.) One microgram of poly(C) and 0.5 μg of oligo(dG) were used as template-primer, and 10 nmol of ³[H]dGTP (1,000 counts per min/pmol) was used as substrate. The enzyme was preincubated at 45 C. (b) The same reaction conditions as described above were employed, except 1.0 μg of poly(dC) was substituted for poly(C), and the preincubation was carried out at 46 C. (c) RNase H assays were performed as previously described [Verma et al., Nature (London), in press]. About 1 to 2 units of enzyme activity was used, and the preincubations were carried out at 47 C.*

TABLE 1. *Average one-half time for inactivation of DNA polymerase and RNase H activity of isolated α from LA337 and wild-type RSV*

Source	$t_{½}$ (min)[a]		
	RNA-directed DNA synthesis poly(C)·oligo(dG)	DNA-directed DNA synthesis poly(dC)·oligo(dG)	RNase H [3H] poly(A)·poly(dT)
LA337	1.8	2.1	1.5
Wild-type RSV	8.8	9.5	8.0

[a] $t_{½}$ is the time required for inactivation of 50% of activity at the temperatures indicated in Fig. 3.

press), it appears that α is derived from β. Experiments performed on the mechanism of RNase H (5) and heat stability of isolated α subunit in the presence or absence of template (A. Panet, I. M. Verma, and D. Baltimore, Cold Spring Harbor Symp. Quant. Biol., in press) suggest that β subunit enhances the affinity of α for binding to the template. We are now investigating whether β is enzymatically active and how α is generated from β.

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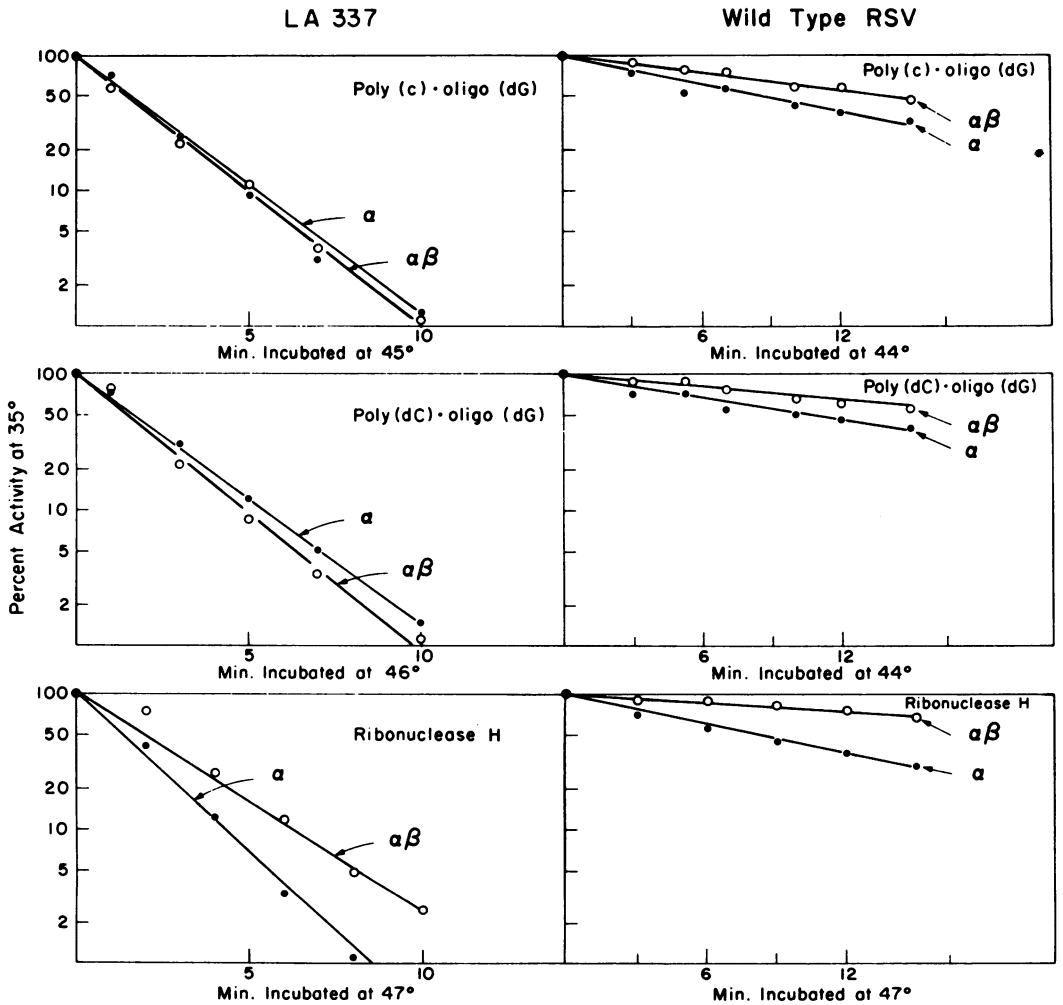

FIG. 4. *Comparison of thermal inactivation profiles of DNA polymerase and RNase H activities of α and αβ from LA337 and wild-type RSV. Assays for heat lability were performed as described. About 0.5 to 1.0 units of αβ was used per assay. Preincubations were carried out at temperatures shown in the figure.*

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