PREPARATION OF RNA-DIRECTED DNA POLYMERASE FROM SPLEENS OF Balb/c MICE INFECTED WITH RAUSCHER LEUKEMIA VIRUS*

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SUMMARY — A high level of RNA-directed DNA polymerase activity is found in spleens of Balb/c mice infected with Rauscher murine leukemia virus. The enzyme is associated with sedimentable subcellular fractions, can be separated from other cellular DNA polymerases, and is present in useful quantity for purification purposes. Complete solubilization of the enzyme, achieved and maintained by using a combination of detergent and high salt solution, is required for a satisfactory purification both in the initial extraction procedure and in subsequent column chromatography.

The importance of RNA-directed DNA polymerase, the enzyme independently discovered by Temin and Mizutani (1) and Baltimore (2) in RNA tumor viruses, has been substantiated by recently accumulated biological evidence (see review by Gallo, ref. 3). Detailed biochemical and biophysical studies of the enzyme have been limited, however, due to difficulty in obtaining sufficient quantities of material. Two problems usually complicate the purification of RNA-directed DNA polymerase — availability of virus and recovery of purified enzyme. Only with the avian myeloblastosis virus, which is available in unusually high quantity, has purification of the polymerase been achieved for biochemical studies (4). In mammalian systems, where isolated virus is merely enough for enzyme detection, attempts to purify the enzyme from isolated virus are impractical, and recovery of the polymerase from chromatographic columns is poor (5). For this reason, we investigated the possibility of purifying RNA-directed DNA polymerase from leukemia virus-infected mouse spleens, which are readily obtainable in large quantity and which were also used as the infectious source in original studies of murine oncogenic RNA viruses (6, 7). This

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communication describes briefly certain preparative procedures that we have found to be useful for a satisfactory purification of this polymerase.

MATERIALS AND METHODS

The original source of Rauscher murine leukemia virus was a 10-fold virus concentrate from mouse plasma (lot #RPV-HL-68-4, Hazleton Laboratory). Specific-pathogen-free Balb/c strain mice of both sexes, 6–10 weeks old, were either supplied by the Mammalian Isolation Program of the Oak Ridge National Laboratory or purchased from the Cumberland View Farm, Clinton, Tennessee. Each mouse was given intraperitoneally either $10^{3.48}$ 50%-splenomegaly units (6) of plasma virus or 0.1 ml of a 10% infectious spleen homogenate. The homogenate was prepared from 21-day postinfection spleens in 0.14 M NaCl and 0.01 M phosphate buffer at pH 7.2, stored at -70°C in 5-ml vials, and used as infectious stock. The animals were killed 3–4 weeks after infection, when the spleens weighed approximately 2 grams.

Spleens were minced and then homogenized in a Kontes glass homogenizer in 4 volumes (v/w) of medium containing 0.4 M sucrose, 50 mM Tris–Cl at pH 8.0, 50 mM KCl, 4 mM $Mg(OAc)_2$, and 2 mM dithiothreitol. Nuclear, mitochondrial, and microsomal fractions were sequentially sedimented by differential centrifugation at 1,000 X g for 10 min, 8,500 X g for 20 min, and 164,000 X g for 90 min. Sediments were washed twice with homogenizing medium by manual dispersion in a glass homogenizer and recentrifugation. The nuclear fraction was further separated into pellet and membranous components by suspension in a glycerol medium (15% glycerol, 50 mM Tris–Cl at pH 8.0, 4 mM $Mg(OAc)_2$, and 2 mM dithiothreitol) containing 0.5% Nonidet P40 (Shell Chemical Co.) and 0.05 M KCl; subsequent centrifugation at 1,000 X g for 10 min sedimented the nuclear pellet and left the membranous components in the supernatant. The microsomal fraction was eluted with glycerol medium containing 0.25 M KCl to release ribosome-bound DNA polymerase (8) from the microsomal pellet. All subcellular fractions, except the 164,000 X g supernatant, were adjusted to approximately 10 mg protein per ml in a glycerol medium con-

TABLE 1

Distribution of DNA polymerase activities in subcellular fractions of control spleens, 21-day infected spleens, and a 1:1 mixture of control and infected spleens

Subcellular fractions	Subfraction	Spleen source	Protein (mg/g spleen)	Polymerase activities* with $(rA)_n \cdot (rU)_n$	ΔDNA	Act. DNA
Nuclear	Pellet	Control	24.07	0.2	0.0	4.9
		Infected	22.07	0.3	0.8	14.8
		Mixture	23.32	0.2	0.4	10.2
	Membranous	Control	48.69	0.1	0.1	1.2
		Infected	70.99	2.5	0.2	3.6
		Mixture	63.57	2.1	0.1	2.4
Mitochondrial	-	Control	18.76	0.1	0.1	0.4
		Infected	7.59	34.5	0.7	8.3
		Mixture	11.89	25.2	0.3	4.4
Microsomal	KCl-eluate	Control	6.23	0.2	2.6	23.6
		Infected	2.82	4.6	1.3	28.2
		Mixture	4.18	2.5	2.2	25.8
	Pellet	Control	9.17	0.2	0.2	0.2
		Infected	3.72	56.2	0.0	24.3
		Mixture	5.92	42.0	0.0	12.5
Supernatant	-	Control	56.55	0.0	4.7	8.9
		Infected	36.16	0.0	6.2	8.9
		Mixture	44.32	0.0	5.9	9.2
Plasma virus (RPV-HL-68-4)			0.015**	229.5	0.3	83.3

*The reaction mixture (9) contained 25 mM Tris-Cl at pH 8.0, 50 mM KCl, 0.17 mg/ml bovine serum albumin, 2 mM dithiothreitol, 0.1 mM thymidine triphosphate, 35 μCi/ml [^{3}H]thymidine triphosphate (17.5 Ci/mmole), and the following individual additions: (a) 0.01 mg/ml $(rA)_n \cdot (rU)_n$ (A/U=2), 0.5 mM $MnCl_2$, and 2.0 mM $MgCl_2$; (b) 0.1 mg/ml "act. DNA," calf thymus DNA activated by DNase (10), 10 mM $MgCl_2$, and 0.25 mM other deoxyribonucleotide triphosphates. Reactions were also done with "ΔDNA," heat-denatured by 3 min boiling at 100°C, in the place of "act. DNA." All subcellular fractions, except supernatant and KCl eluate of microsomal fraction, were first solubilized by treatment with high salt and detergent. Protein, 0.5–5 μg, was included in 0.1 ml reaction mixture at 37°C. At different time intervals, 0.02 ml was assayed for incorporation by the paper disk method (11). Protein was determined by the method of Lowry et al. (12). Polymerase activity is expressed as pmoles of dTMP incorporated by 1 μg protein of the preparation in the 30-min interval from 30 to 60 min of reaction.

**Protein content of isolated virus from 1 ml of plasma.

taining 0.5 M KCl and 0.5% Nonidet P40, incubated 10 min at 37°C, chilled in an ice bath, and centrifuged at 164,000 X g for 90 min. This solubilized the polymerase activity into the supernatant, which could be stored at -70°C without appreciable loss of activity

for at least a month.

RESULTS AND DISCUSSION

Results of subcellular fractionation experiments (Table 1) demonstrate that the RNA-directed DNA polymerase present in virus-infected spleens can be localized and also separated from other DNA polymerases of the cell. The cellular DNA polymerases, which were distinguished by their template specificity as well as their presence in the control noninfected spleens, were found mainly in the nuclear pellet, in the 0.25 M KCl eluate of the microsomal fraction, and in the 164,000 X g supernatant — as has been found in the liver (7). The RNA-directed DNA polymerase, measured in this experiment by its ability to utilize $(rA)_n \cdot (rU)_n$ as template–primer, is detected mainly in the microsomal fraction, in the mitochondrial fraction, and in the membranous components of nuclear fraction of the infected spleens, but not in the same fractions from control spleens. Fractionation experiments with mixtures of control and injected spleens showed that no inhibitory activity of the polymerase was present in the control spleens to mask the detection of this enzyme. The RNA-directed DNA polymerase is not eluted from microsomal sediments by 0.25 M or 0.5 M KCl; this provides a convenient way of separating it from the ribosome-bound DNA polymerase of the cell. Thus, by first washing with a medium containing 0.5 M KCl and then eluting with a medium containing both 0.5% Nonidet P40 and 0.5 M KCl, it is possible to obtain a relatively clean preparation, suitable for subsequent purification, from microsomal fraction of infected spleens. Such a preparation showed a lower specific activity (about 4-fold) but a much greater yield (about 60-fold on the basis of gram spleen to ml plasma) of RNA-directed DNA polymerase than was found in preparations of Rauscher leukemia virus isolated from mouse plasma. Since one usually obtains on the average 0.6 ml of plasma and 2.0 grams of spleen from an infected mouse, we conclude that the spleens of Balb/c mice infected with Rauscher leukemia virus are a good source for the purification of the RNA-directed DNA polymerase.

Our initial attempts to chromatograph the extracted enzyme preparation in Sephadex gel columns and DEAE–cellulose columns, using low salt media and low-to-high salt gradi-

TABLE 2

Solubilization of polymerase activity

Material	Nonidet P40 (%)	KCl (M)	Solubilized (%)
Virus	0.2	0.00	25
	0.2	0.05	36
	0.2	0.10	56
	0.2	0.20	75
	0.2	0.50	98
	0.0	0.50	0
Microsomal extract	0.5	0.50	93
	0.5	0.25	32

Isolated plasma Rauscher leukemia virus (0.075 mg protein) was suspended in 1 ml of solution containing 0.01 M Tris–Cl at pH 8.0, 0.01 M NaCl, 0.004 M $MgCl_2$, 0.002 M dithiothreitol, and 15% glycerol, in addition to the listed concentrations of Nonidet P40 and KCl. After standing in an ice bath for 15 min, the suspension was spun at 164,000 X g for 30 min. Supernatants thus obtained were adjusted to contain 0.2% Nonidet P40 and 0.5 M KCl and assayed for polymerase activity.

Microsomal extract was a preparation from 0.25 M KCl-washed microsomal pellet of infected spleens, solubilized with detergent and high salt solutions and prepared as described in MATERIALS AND METHODS, containing 2.6 mg protein per ml. It was diluted 2-fold to contain, respectively, 0.50 and 0.25 M KCl in the glycerol medium and subsequently spun at 164,000 X g for 15 hours. Activity remaining in the supernatant was then determined.

ents, resulted in nearly complete loss of polymerase activity. A careful examination of the conditions required for enzyme solubilization (Table 2) showed that, in addition to detergent, 0.5 M KCl was essential for a complete release of the polymerase into solution. Furthermore, the polymerase activity from the microsomal fraction of infected spleens, which was previously solubilized by 0.5% Nonidet P40 and 0.5 M KCl, became sedimentable when the KCl concentration of the medium was lowered to 0.25 M. Considerable stability of the polymerase activity was also observed with the use of high salt media. Thus, in order to maintain both the stability and solubility of RNA-directed DNA polymerase, we decided to perform the initial purification steps in the presence of 0.5 M KCl and 0.2% Nonidet P40. This was tried with Sephadex G100 gel filtration and various types of ion-exchange column chromatography. With high salt and detergent in the glycerol medium, recovery

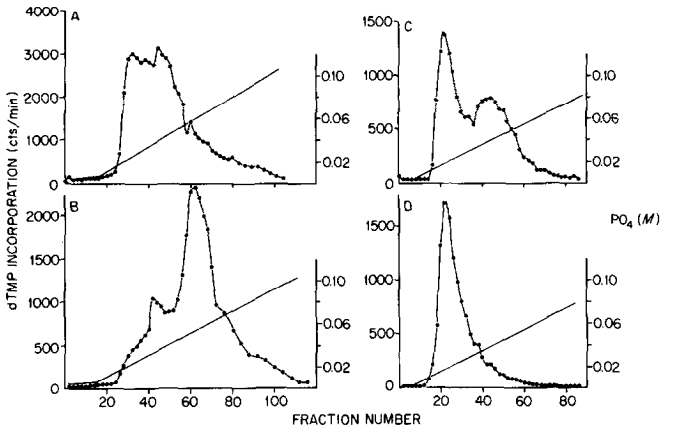

FIG. 1. Hydroxyapatite column chromatography. Extracts from membranous components of the nuclear fraction (A) and the 164,000 X g supernatant (B) from infected spleens were applied to a 2- X 50-cm column and eluted with 1 liter of 5–100 mM phosphate (pH 7.5) gradient containing 15% glycerol, 500 mM KCl, 4 mM $MgCl_2$, 0.4 mM EDTA, and 2 mM dithiothreitol, at a pumping flow rate of 8.0 ml/6 min/fraction. Extracts from mitochondrial fraction (C) and from 0.25 M KCl-washed microsomal pellet (D) were applied to a 0.9- X 120-cm column and eluted with 1 liter of the same phosphate gradient medium, at a pumping flow rate of 10 ml/10 min/fraction. Both columns were operated at 4°C, and for (A), (C), and (D) 0.2% Nonidet P40 was included in the gradient medium. Polymerase activity was measured using DNase-I activated calf thymus DNA as template-primer in a 0.025-ml reaction (as in Table 1) with 0.005 ml of the fraction.

of the polymerase activity from the Sephadex column was invariably above 80%, in contrast to 0–8% obtained with 0.05 M KCl in the same glycerol medium. Among the ion exchangers tested, hydroxyapatite gave the best results by its ability to adsorb all the DNA polymerases in the presence of 0.5 M KCl and 0.2% Nonidet P40. Figure 1 shows different profiles of DNA polymerases obtained by hydroxyapatite chromatography of extracts from different subcellular fractions of the infected spleens. The first peak, eluted at about 0.025 M phosphate concentration, was identified as the RNA-directed DNA polymerase. After the two purification steps by hydroxyapatite column chromatography and Sephadex gel filtration, which presumably removes most of the co-precipitable structural proteins in the initial crude extract, the polymerase preparation can be put into a 0.1 M KCl medium for subsequent chromatography on phosphocellulose without appreciable loss of the polymerase activity.

Employing the procedures described here, we have purified RNA-directed DNA polymerase in high yield from the microsomal pellet as well as the membranous components of

the nuclear fraction of infected spleens. An attempt to purify this enzyme from the mitochrondrial fraction was not successful, possibly due to a high content of lysosomal enzymes in the extract. The final products, approximately 800- to 1400-fold purified from the extract of microsomal pellet, appeared identical to the plasma Rauscher virus polymerase with respect to enzyme kinetics, template specificity, divalent metal requirements, chromatographic behavior, and glycerol gradient sedimentation rate. A complete account of these results will be published in a subsequent paper.

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