ISOLATION OF A RNA-DEPENDENT RNA POLYMERASE FROM VIRUS INFECTED MYELOBLASTS*

by

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SUMMARY

Evidence is given for the presence of a RNA polymerase in virus-infected myeloblasts which is absolutely dependent upon the presence of exogenous RNA template for enzyme activity. In the presence of polyvinyl sulfate, a ribonuclease inhibitor, the high molecular weight virus RNA fraction is the most effective template for RNA synthesis with the RNA-dependent RNA polymerase. Nearest neighbor analysis of the product indicates synthesis of a RNA heteropolymer.

INTRODUCTION

Replication of the nucleic acid component of many small animal viruses containing RNA[1-4] and bacteriophage RNAs[5-7] has been shown to be mediated by a virus-induced RNA polymerase. Of special significance was the isolation of RNA-dependent RNA polymerases from E. coli infected with the RNA bacteriophages MS2[5] and Qβ.[7] Not only was it demonstrated that these enzymes had an absolute requirement for exogenous RNA, but each polymerase specifically required its own viral RNA for enzymatic activity.

At the present time, very little is known concerning viral RNA replication in animal cells infected with tumor viruses. Detection of RNA-dependent RNA polymerase activity in chick embryo cells infected with Rous-associated virus was unsuccessful.[8] It was the purpose of this study to demonstrate the presence of a RNA-dependent RNA polymerase in myelo-

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blast cells infected with BAI strain A myeloblastosis virus. A preliminary report was presented earlier.[9]

MATERIALS AND METHODS

BAI strain A myeloblastosis virus and virus-infected myeloblasts were obtained from the blood of chicks in the terminal stage of myeloblastic leukemia by methods previously described.[10] The virus RNA and membrane-associated myeloblast cell RNA were extracted by a sodium dodecyl sulfate-phenol procedure.[11] Only the 64S RNA fraction from the myeloblastosis virus was used as template for synthesis. Qβ bacteriophage RNA and *E. coli* 23S ribosomal RNA were isolated as described by Olson *et al.*[12]

Standard assays for RNA-dependent RNA polymerase activity were incubated 10 minutes at 37°C according to Haruna and Spiegelman[7], with each tube containing the following in 0.25 ml: 20 μmoles of Tris-HCl (pH 8.5); 2 μmoles of $MgCl_2$; 50 mμmoles each of the four ribonucleoside triphosphates, one of which was radioactively-labeled; 15-40 μg of template RNA; and 50-300 μg of polymerase enzyme protein. Thirty μg of salmon sperm DNA was used as template in assays testing for the presence of the normal cellular DNA-dependent RNA polymerase.

RESULTS

All enzyme fractionation steps were carried out at 0 - 4°. One hundred grams of frozen myeloblast cells were suspended in 100 ml of 0.1M Tris buffer, pH 7.4, containing 0.01M $MgCl_2$ and 0.001M reduced glutathione. After homogenization with a VirTis 45 blender for one minute at high speed, the mixture was centrifuged 30 minutes at 30,000g. The supernatant (S-0) was removed, made 5% v/v in glycerol, and treated with protamine sulfate (Eli Lilly Co.) to a level of 0.04 mg per A_{260} unit of supernatant. After 15 min, the suspension was centrifuged at 23,500g for 15 min. The supernatant (S-1) was concentrated to one-third volume in dialysis tubing using polyethylene glycol 6000 (S-2) and then applied to a column of CM-

Sephadex C-50 (2 x 25 cm) previously equilibrated with 0.01M potassium phosphate buffer, pH 8, containing 5% v/v glycerol. After washing the column extensively with the 0.01M potassium phosphate buffer, the polymerase activity was eluted with 0.3M potassium phosphate buffer, pH 8, containing 0.001M reduced glutathione and 5% v/v glycerol. Five ml fractions were collected and the protein concentration of each fraction was determined.[13] The fraction with the highest polymerase activity was designated as (S-3) fraction.

The elution profile of the RNA-dependent RNA polymerase from the CM-Sephadex column is shown in Fig. 1. Fractions having the enzyme with the highest specific activity were eluted later than the majority of the

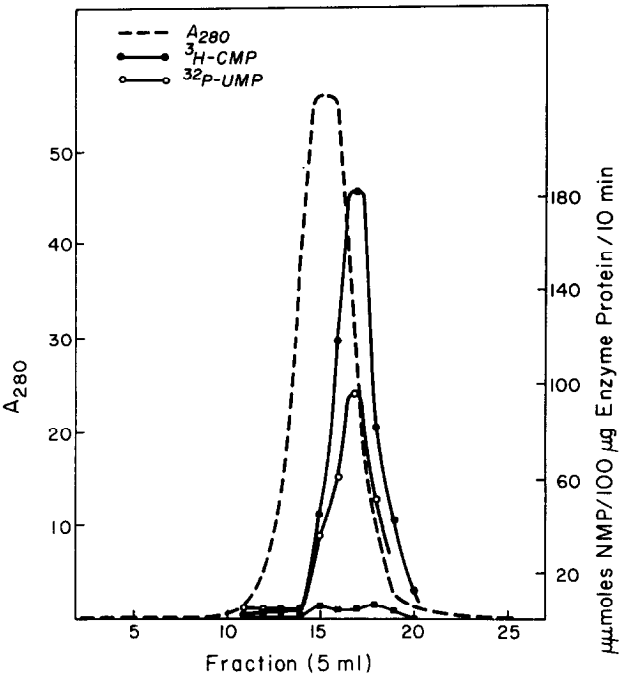

Figure 1. CM-Sephadex chromatography of RNA-dependent RNA polymerase. Enzyme activity was determined by the standard assay procedure. Myeloblast membrane RNA was used as template. ^{3}H-CTP and α-^{32}P-UTP were used as radioactively-labeled nucleoside triphosphates at specific activities of 5 cpm/μμmole and 10 cpm/μμmole, respectively. Control assays (no RNA template) were carried out using ^{3}H-CTP as substrate and are indicated by the closed squares.

protein. When RNA was omitted from the reaction mixture, essentially no polymerizing activity was observed in any of the enzyme fractions from the column.

A summary of the RNA polymerase purification is given in Table 1. Only RNA-dependent enzyme activity was reported in this table. The S-0 fraction shows only a slight dependence on RNA added to the assay mixture. After titration of the S-0 fraction with protamine sulfate solution, the enzyme fraction (S-1) becomes, for the most part, dependent on RNA and a very large increase in total enzyme activity was observed. Titration with protamine sulfate not only facilitated removal of endogenous RNA template, but was very effective in removing the cellular DNA-dependent RNA polymerase. Table 2 shows that negligible DNA-dependent RNA polymerase activity remains in the S-1 fraction after addition of 0.04 mg of protamine sulfate per A_{260} absorbancy unit of the protein solution.

An enzyme activity similar to that reported by August et al.[14] was observed in the S-2 fraction but was reduced to an insignificant level in the S-3 fraction (Table 3). Washing the CM-Sephadex column with 0.01M potassium phosphate buffer, pH 8 after sample application, separated this AMP-incorporating enzyme which required a poly A template. Interference by RNA phosphorylase activity was avoided by utilizing nucleoside triphosphates as substrates for polynucleotide synthesis. Also, the nucleoside diphosphates were not utilized as substrates by the S-3 enzyme fraction.

Table 4 clearly shows that the observed polymerase activity in the S-3 fraction was completely dependent upon the addition of exogenous RNA to the reaction mixture and ribohomopolymers did not act as template for the polymerase. It seems, therefore, that enzyme activity is dependent on a heteropolymer form of RNA and the availability of a 3'-OH end on the ribohomopolymers is insufficient for stimulation of RNA synthesis. Investigation of RNA from different sources indicated that RNA from myeloblastosis virus was the most effective template. Table 5 shows the results of

Table 1. Purification of RNA-dependent RNA polymerase from virus-infected myeloblasts.

Enzyme Fraction	Specific Activity	Total Activity μμmoles ^{3}H-CMP/10 min
S-0	1.2	7,600
S-1	484	1,380,000
S-2	462	826,000
S-3	5,950	101,000

Enzyme fractions were prepared as described previously. The standard assay procedure was used including 40 μg of myeloblast membrane RNA as template. The labeled ribonucleoside triphosphate was ^{3}H-CTP at a specific activity of 6 cpm/μμmole. Specific activity is defined as μμmoles ^{3}H-CMP/mg protein/10 min.

Table 2. Protamine sulfate precipitation of DNA-dependent RNA polymerase.

Enzyme Fraction	Template	Net CPM (^{3}H-UMP) incorporated
S-0	Myeloblast RNA	115
"	Salmon Sperm DNA	1818
S-1 (after addition of protamine sulfate)	Myeloblast RNA	226
	Salmon Sperm DNA	10

Subtraction of control (no template added) reaction CPM results in net CPM. Control reaction for S-0 fraction incorporated 539 CPM and the control reaction for S-1 fraction incorporated 372 CPM. Fifty μg of myeloblast membrane RNA and 30 μg of Salmon Sperm DNA were added to standard reaction mixtures. Enzyme protein was added at a level of 100 μg and 1 x 10^6 cpm of ^{3}H-UTP was added as labeled substrate.

Table 3. Removal of polyadenylate-dependent AMP incorporating activity.

Enzyme Fraction	Template	CPM (^{3}H-AMP) incorporated
S-2	Poly A (6 μg)	5070
	Poly A (30 μg)	11080
	None	340
S-3	Poly A (6 μg)	210
	Poly A (18 μg)	255
	Myeloblast RNA (30 μg)	1400
	None	180

Reactions were carried out under standard assay conditions using 100 μg of enzyme protein. The labeled ribonucleoside triphosphate was ^{3}H-ATP at a specific activity of 6 cpm/μμmole.

this study. Reactions were carried out in the presence of polyvinyl sulfate which inhibited ribonuclease activity present in the polymerase frac-

Table 4. RNA template requirement for RNA polymerase activity.

Nucleic Acid Template	μμmoles ^{3}H-CMP or ^{3}H-AMP+ incorporated
Myeloblastosis virus RNA	121
Poly C	26
Poly G	8
Poly G, I	17
Poly U	10+
Poly A	23+
DNA	6
None	10

Reactions were carried out under standard assay conditions using 72 μg of S-3 enzyme fraction and 30 μg of template. The labeled ribonucleoside triphosphates used were ^{3}H-CTP and ^{3}H-ATP at specific activities of 6 and 7 cpm/μμmole, respectively.

Table 5. RNA template specificity by RNA-dependent RNA polymerase.

RNA Template	μμmoles ^{32}P-UMP incorporated
Myeloblastosis virus RNA	138
Qβ bacteriophage RNA	69
E. coli 23S rRNA	104
Myeloblast membrane RNA	41
None	7

Reactions were carried out under standard assay conditons using 92 μg of an S-3 enzyme fraction and 40 μg of each RNA template. The radioactively-labeled ribonucleoside triphosphate was α-^{32}P-UTP with a specific activity of 63 cpm/μμmole.

Table 6. ^{32}Phosphate content of mononucleotides isolated after alkaline degradation of enzymatically synthesized RNA.

Labeled substrate	Distribution of Radioactivity Percent of Total CMP			
	UMP	AMP	CMP	GMP
Uridine - ^{32}P - PP	36.9	30.2	13.0	19.7
Cytidine - ^{32}P - PP	8.4	43.1	33.4	14.9
Guanosine - ^{32}P - PP	18.6	36.9	17.0	27.4

All reaction mixtures were incubated 5 minutes under standard assay conditions with the addition of 1 μg of polyvinyl sulfate, 111 μg of S-3 enzyme fraction, and 15 μg of Myeloblastosis virus RNA. Specific activities of α-^{32}P-UTP, α-^{32}P-CTP, and α-^{32}P-GTP were 100 cpm/μμmole, 150 cpm/μμmole, and 67 cpm/μμmole, respectively. Nearest neighbor analysis followed the procedure described by Hayashi and Spiegelman.[16]

tion. Under the same conditions, transfer RNA did not act as template in the polymerase reaction.

Examination of the product of the polymerase reaction using nearest neighbor analysis[15] demonstrated that ribonucleotides were being incorporated into the internucleotide linkages of a heteropolymer. Table 6 shows the distribution of radioactivity using α-^{32}P-UTP, α-^{32}P-CTP, and α-^{32}P-GTP separately as radioactively-labeled substrates.

These results show clearly that a RNA-dependent RNA polymerase has been partially purified from virus infected chick myeloblasts and that it displays an absolute requirement for a RNA template for enzymatic activity. The existence of a RNA-dependent enzyme in the virus-infected myeloblasts, utilizing myeloblastosis virus RNA as a template, supports the view that the RNA genome of the tumor virus is replicated in a manner similar to other RNA viruses. However, the requirement of the enzyme for homologous RNA is not absolute as reported for Qβ and MS2 RNA replicases. It is possible that the specificity of the enzyme for myeloblastosis virus RNA will improve upon further purification of the enzyme.

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