POLY rC-OLIGO dG SPECIFIC TEMPLATE ACTIVITY OF MOUSE VIRAL DNA POLYMERASE DETECTED BY ISOELECTRIC FOCUSING

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SUMMARY

An acid fraction from polyacrylamide gel isoelectric focusing contained only DNA polymerase template activity for $(rC)_n$-$(dG)_{12-18}$ with little or no activity with $(rA)_n$-$(dT)_{12-18}$. A factor which stimulated $(rC)_n$-$(dG)_{12-18}$ activity was also detected in low molecular weight fractions from G-100 Sephadex chromatography of the purified enzyme. Since $(rC)_n$-$(dG)_{12-18}$ acitivty is thought to be descriptive of viral DNA polymerase, this stimulation is proposed to be significant for the regulation and specificity of viral DNA synthesis.

INTRODUCTION

Viral DNA polymerase of RNA tumor viruses utilizes $(rA)_n$-$(dT)_{12-18}$ and $(rC)_n$-$(dG)_{12-18}$ well as template-primers. However, cellular DNA polymerases copy $(rA)_n$-$(dT)_{12-18}$ much more efficiently than $(rC)_n$-$(dG)_{12-18}$ (1). It is not clear whether this difference in template specificity of cellular and viral polymerases is due to an inherent difference in the enzymes, or to the presence of factors donated by the virus or by the cell. A regulator present in the cell or virus that directs the template specificity of the enzyme would offer a mechanism for regulation of virus infectivity and a site at which inhibition of the enzyme activity may be achieved.

METHODS

Viruses. The Moloney strain of murine sarcoma and leukemia virus propagated in NIH/3T3 mouse cells was purchased from Electro-Nucleonics (Bethesda, MD). Virus (1.2 mg) was disrupted with 5 ml of virus disruption buffer (VDB; 50 mM Tris-HCl pH 8.0, 0.5 M KCl, 50 mM NaCl, 0.1 mM EDTA, 0.25% Triton X-100, 10 mM dithiothreitol and 10% glycerol). After disruption for 2 hours, debris was removed by centrifugation. The supernate was dialyzed against Buffer A (0.05 M Tris-HCl pH 8.0, 0.05% Triton X-100, 1 mM 2-mercaptoethanol and 10% glycerol) and passed through a 4 ml DEAE-cellulose column, phospho-cellulose, poly rC-agarose, and finally a G-100 Sephadex column (manuscript in preparation).

Viral DNA polymerase assay. Assay of viral DNA polymerase was done by the usual methods (1). When $(rA)_n$-$(dT)_{12-18}$ was used as template (0.02 mg/ml), 1 mM $MnCl_2$ was present in the assay mix. When $(rC)_n$-$(dG)_{12-18}$ served as

template, 1 mM $MnCl_2$ and 5 mM $MgCl_2$ were present. Activity is expressed as pmoles of [^{3}H]-XMP incorporated per hour for 10 µl of enzyme extract where the specific activity of [^{3}H]-TTP and [^{3}H]-dGTP were both 500 cpm/pmole.

Polyacrylamide gel isoelectric focusing. A variation of the procedure used by Catsimpoolis was used to focus extracts (2). The gel is polymerized in the presence of 3.4 µl of N,N,N',N'-tetraethylenemethylenediamine (TEMED), 1.4 mg of ammonium persulfate, 50 µl of 40% carrier ampholytes (LKB) pH 3-10, 0.66 ml of 22.5% acrylamide solution (22.2 g acrylamide and 0.3 g methylene-bis-acrylamide in 100 ml of H_2O) and 1.3 ml of protein solution. Each sample was added to a 5 mm i.d. x 130 mm glass tubes that fit a Hoefer Scientific (San Francisco, CA) gel electrophoresis apparatus. After polymerization (45 min), 0.08 M NaOH was used to fill the lower reservoir (cathode) and 1.4 M H_3PO_4 filled the upper reservoir (anode).

Focusing was performed at a constant current of 2.5 mA per tube until the voltage reached 250 volts, whereupon the voltage was allowed to remain constant and the current was allowed to decrease. Total time of focusing was 18 hours at 4° C.

After focusing, gels were removed from the tubes and sliced into 2 mm sections for enzyme assay or pH measurement. The gel slices were incubated in 150 µl of H_2O for 2 hours before the pH was measured with a combination micro-electrode. The 2 mm sections of the gel were assayed for enzyme activity by incubating the gel slice in 0.1 ml of standard reaction mixture for 3 hrs at 37° C. Aliquots (60 µl) of each assay mix were then added to trichloroacetic acid and precipitable radioactivity measured as usual.

Ribonuclease treatment of virus extracts. Treatment of disrupted virus preparations with both ribonuclease A and T_1 was done according to procedures used by Kacian and Spiegelman (3). Virus preparation in VDB (1 ml) was diluted to 2 ml with 50 mM Tris-HCl pH 8.0 and 10 mM dithiothreitol and made 10 µg/ml with ribonuclease A and T_1 each for 30 min at 37° C. An identical sample without ribonuclease and the treated sample (0.4 ml) were analyzed by isoelectric focusing as usual.

No attempts were made to remove the salts present in the sample prior to focusing. During focusing the ions move to either end of the pH gradient, but the pH gradient itself has little ionic strength except for the ampholytes and proteins. With salt present at the outset of focusing, the effect is to steepen the pH curve, but the proteins still focus at their respective pI. Resolution if lost, but the efficacy of the method is the same.

RESULTS

Polyacrylamide gel isoelectric focusing revealed an acid focusing activity specific for $(rC)_n$-$(dG)_{12-18}$ when crude extracts were focused for 18 hours (figure I). Multiple other peaks of activity were detected with both templates in the neutral and slightly acidic regions of the gel. The multiple peaks and the acid peak are also characteristic of the purified enzyme (manuscript in preparation). With the purified enzyme the pH 5.5 and the pH 6.6 peaks predominate. Focusing experiments with other DNA polymerases (E. coli and M. luteus DNA polymerase I) under similar conditions produced single sharp peaks of activity. The heterogeneity is most likely the result of the interaction of the viral DNA polymerase with other viral constituents. This proposal is

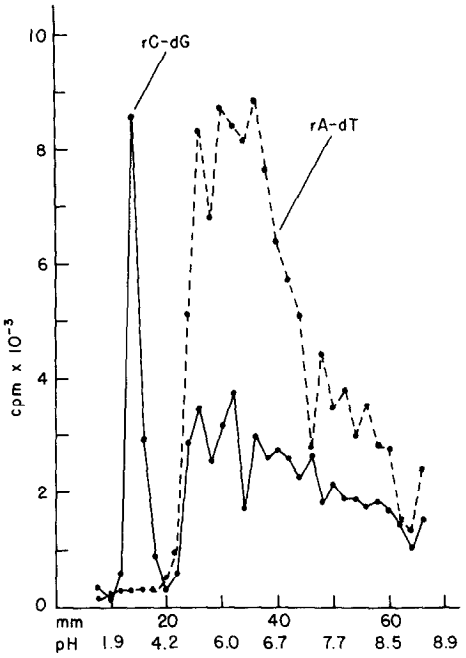

Figure I. Polyacrylamide gel isoelectric focusing of disrupted Mo-MSV(MLV) according to procedure in Methods. Activity in the gel slices was measured for $(rC)_n$-$(dG)_{12-18}$ (●——●) and $(rA)_n$-$(dT)_{12-18}$ (●----●)

offered considering the known interaction of viral DNA polymerase with transfer-RNA and the phosphoprotein, p12, which has viral RNA binding properties (4). Further experiments to confirm these interactions are in progress.

If the binding proteins are specific for certain template-primers and are attached to the viral RNA so that they are not available for exogenously added template, then the liberation of the binding proteins by degrading the RNA with ribonuclease A and T_1 should allow a greater interaction with exogenously added template. The specific activity of the enzyme for a particular template-primer should increase. After ribonuclease treatment and isoelectric focusing which concentrated the ribonuclease and removed it from the areas of the gel that contained much of the DNA polymerase, gel slice assays with added template-primer showed that the $(rA)_n$-$(dT)_{12-18}$ activity was unaffected, but the

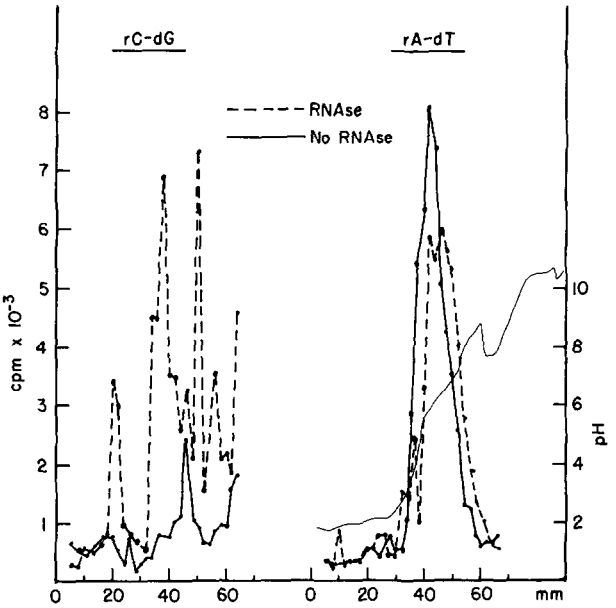

Figure II. Gel focusing of Mo-MSV(MLV) extracts after treatment with both ribonuclease A and T_1. Left panel represents gel slices that were assayed with $(rC)_n$-$(dG)_{12-18}$. Right panel shows assay with $(rA)_n$-$(dT)_{12-18}$. The pH profile indicated by the thin line in the right panel was virtually the same for all gels. Ribonuclease A focused at pH 7.8 and was most likely responsible for the diminished activity of the polymerase since $(rC)_n$-$(dG)_{12-18}$ also served as substrate for the focused ribonuclease at this pH.

$(rC)_n$-$(dG)_{12-18}$ activity increased over the control. Also, in this particular experiment, the preparation did not exhibit any acid focusing $(rC)_n$-$(dG)_{12-18}$ activity before treatment (control) but did produce an acid focusing activity for $(rC)_n$-$(dG)_{12-18}$ after treatment (figure II).

It should be noted that the presence of the factor specific for $(rC)_n$-$(dG)_{12-18}$ varied from one preparation to another. It was also extremely labile and could not be characterized further. However, in 24 separate runs on gel focusing with several different enzyme preparations, the $(rC)_n$-$(dG)_{12-18}$ specific template activity was observed in varying degrees 19 times. The size of the peak and the amount of the activity varied greatly after different runs from greater than 90% of the total $(rC)_n$-$(dG)_{12-18}$ activity detected on the gel

to amounts that were barely detectable over background. The activity was also seen by other analytical methods with a similar degree of reproducibility. For example, using glycerol density gradient isoelectric focusing (that uses a density gradient to stabilize against convection instead of a gel), the specific factor was detected in 10 out of 16 experiments. It was observed that the phosphoric acid concentration of the electrode solution might be important for the reproducibility of the acid focusing peak.

An activity specific for $(rC)_n$-$(dG)_{12-18}$ was also detected in the low molecular weight fractions from G-100 Sephadex chromatography during the purification. This activity, which was also variable, was detected 5 out of 7 trials with enzyme preparations of various degrees of purity. A specific template activity for $(rA)_n$-$(dT)_{12-18}$ was never detected with any of the methods, and curious peaks were never detected with DNA polymerases from other sources. Previously, workers have neglected in most cases to routinely use $(rC)_n$-$(dG)_{12-18}$ to monitor enzyme activity during purification and, therefore, may have overlooked the specific template activity.

Preliminary evidence has also been obtained to show that this factor is phosphorylated and binds strongly to single-stranded DNA-cellulose. The above information suggests that the binding proteins allow a specific interaction between enzyme and viral RNA and may be instrumental in determining the template specificity and species specificity of the viral DNA polymerase.

DISCUSSION

The multiple peaks of enzyme activity seen after isoelectric focusing are most likely due to the interaction of the enzyme with other functional viral components such as viral RNA and the phosphorylated RNA binding protein, p12, recently described by Todaro et al. (4). The increase in enzyme activity for $(rC)_n$-$(dG)_{12-18}$ after ribonuclease treatment was probably due to the degradation of the nucleic acid associated with the enzyme-binding protein complex which allowed an increased interaction with exogenous template. This effect was more pronounced with $(rC)_n$-$(dG)_{12-18}$ as compared with $(rA)_n$-$(dT)_{12-18}$. The

significance of an $(rC)_n$-$(dG)_{12-18}$ stimulator is underscored by the fact that rC rich regions serve as initiation sites for DNA synthesis in bacterial systems (5) and that a similar system might be responsible for RNA tumor virus initiation of replication.

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