JOURNAL OF VIROLOGY, June 1975, p. 1507-1510
Vol. 15, No. 6
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Isolation of DNA Polymerase γ from an Adenovirus 2 DNA Replication Complex

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Received for publication 13 December 1974

The major DNA polymerase in a nuclear membrane complex that is capable of synthesizing viral DNA sequences in vitro has been purified about 900-fold from adenovirus 2-infected KB cells. The enzyme was characterized as belonging to the class of mammalian DNA polymerases (DNA polymerase γ) that can utilize poly(A)·oligo(dT) as template primer.

Several recent studies have provided considerable new information on the replication of the linear adenovirus DNA genome (4, 8, 13–17). However, the exact mechanism of adenovirus DNA replication and the enzymes involved remain largely obscure. Recently we have described the properties of a nuclear membrane fraction isolated from adenovirus 2-infected KB cells that has some properties of a functional DNA replication complex. This complex, isolated by sedimenting sonically disrupted nuclei in a discontinuous sucrose gradient, contains newly synthesized adenovirus DNA sequences that can be chased into genome length viral DNA (18). In vitro, the complex synthesizes exclusively adenovirus DNA sequences (T. Yamashita, M. Arens, and M. Green, J. Biol. Chem., in press) and contains (i) DNA polymerase activity, (ii) endonuclease activity, and (iii) two early proteins of molecular weight 75,000 and 45,000 that bind to single-stranded DNA cellulose (11). We describe in this report the purification and some properties of the DNA polymerase present in the nuclear membrane complex of KB cells at 18 h after infection with adenovirus 2, a time when only viral DNA is synthesized (9). The enzyme is characterized as belonging to the class of mammalian DNA polymerases that can utilize certain synthetic polyribonucleotides as template (3, 5, 7, 12).

Adenovirus 2-infected KB cells were fractionated into cytoplasm, nucleoplasm, and nuclear membrane complex. Assay of each fraction for DNA polymerase activity using activated calf thymus DNA as template primer in the presence of 20 mM Mg^{2+} showed that 10% of the total DNA polymerase activity was present in the replication complex.

The steps and yields of enzyme activity during a typical purification of DNA polymerase from the replication complex are briefly described in the legends to Fig. 1 and 2 and are summarized in Table 1. The DNA polymerase activity present in the adenovirus 2 DNA replication complex, as measured with activated calf thymus DNA as template primer, was quantitatively extracted with 0.5% Triton X-100, 0.5 M KCl (Table 1, left column). The apparent loss and regain of activity that we reproducibly find with the synthetic template primer may be due to the presence of DNA which binds the enzyme but cannot efficiently direct incorporation in the presence of only one deoxynucleoside triphosphate. Better recoveries of activity from glycerol gradients were obtained when samples with higher protein concentrations were used.

The pattern of elution from phosphocellulose of the DNA polymerase of the Triton-high salt extract is shown in Fig. 1. Fractions 1 to 8 were the column flow through fractions which contained about 50% of the protein (6) but no DNA polymerase activity. The major peak eluted at about 0.18 M KPO_4 and constituted 85% of the total DNA polymerase activity eluted from the column. The minor peak, which eluted at about 0.11 M KPO_4, was variable in amount and was perhaps due to cytoplasmic contamination of the membrane preparation (12). The column was further washed with 1 M KPO_4 buffer but no additional DNA polymerase activity was eluted. These observations support the contention that virtually all of the DNA polymerase activity present in the replication complex is displayed in the profile of Fig. 1. Both the major and minor peak activities utilize poly(A)·$(dT)_{12-18}$ with about five times the efficiency of activated calf thymus DNA. We were unable to detect any DNA-dependent DNA polymerase activity, α or β, which would elute at about 0.21

and 0.25 M KPO_4 (10), respectively, and would not respond to the poly(A)·$(dT)_{12-18}$ template primer. (DNA polymerase α is the high-molecular-weight enzyme found predominantly in the cytoplasm; DNA polymerase β is the low-molecular-weight enzyme reported in both nucleus and cytoplasm [1, 3, 5, 7, 12].)

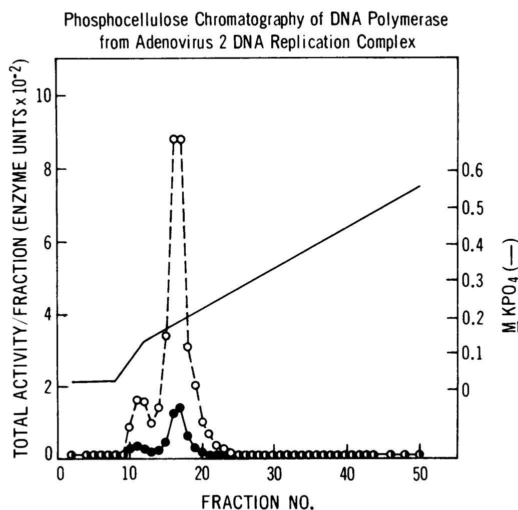

FIG. 1. *Phosphocellulose column chromatography of DNA polymerase isolated from the adenovirus 2 DNA membrane replication complex. The nuclear membrane complex was prepared by the discontinuous sucrose gradient technique as described (18). The DNA polymerase activity was solubilized by treatment of the complex with Triton X-100 and 0.5 M KCl (Ito, Arens, and Green, manuscript in preparation) and centrifuged at 100,000 × g for 1 h. The supernatant fluid was dialyzed against 4 liters of 20 mM potassium phosphate buffer (pH 7.0), 20% glycerol, 0.1 mM EDTA and 3 mM DTT overnight and then adsorbed to phosphocellulose. The enzyme was eluted with a linear gradient of 0.02 to 0.6 M potassium phosphate (pH 7.2). Each fraction was assayed for DNA polymerase activity using activated calf thymus DNA or poly(A)·$(dT)_{12-18}$ as template primer. The standard reaction (90 µl) contained 50 mM Tris-hydrochloride (pH 7.2), 90 µM each of dATP, dCTP, and dGTP, 1 µCi of [methyl-^{3}H]TTP (41.7 Ci/mmol), 20 mM KCl, and 20 µg of activated calf thymus DNA. For assays with poly(A)·oligo(dT) as template primer, the reaction mixture (90 µl) contained 50 mM Tris-hydrochloride (pH 7.2), 1 µCi of [methyl-^{3}H]TTP (41.7 Ci/mmol), 0.8 mM $MnCl_2$, 3 mM dithiothreitol, 0.02% NP-40, 72 mM KCl, and 30 nmol of poly(A)·$(dT)_{12-18}$ (Table 1, Fig. 1 and Fig. 2) or poly(A)·$(dT)_{10}$ (Table 2). The reaction was terminated by the addition of 10 µl of 0.25 M EDTA. Eighty microliters of each assay mixture was spotted on a Whatman DE81 filter disk. The disks were washed four times with 5% Na_2HPO_4 and twice with water, dried, and counted in a toluene-based scintillation fluid. Activated calf thymus DNA as template-primer (●); poly(A)·$(dT)_{12-18}$ as template-primer (○).*

The major phosphocellulose peak II enzyme was further purified on DEAE-cellulose where it eluted as a single peak of activity at 0.1 M KCl. At this stage of purification the enzyme sedimented as a single symmetrical peak in a glycerol gradient (Fig. 2) with both calf thymus and synthetic polymer activities. The enzyme

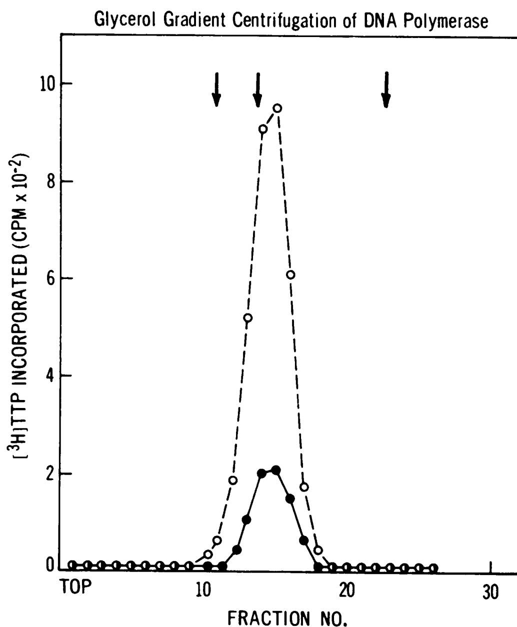

FIG. 2. *Glycerol gradient sedimentation of DNA polymerase γ after DEAE-cellulose column chromatography. The peak II enzyme from phosphocellulose (comprising 80 to 90% of the total activity) was adsorbed to DEAE-cellulose and eluted with a linear gradient of from 0 to 0.6 M KCl in 0.05 M Tris-hydrochloride (pH 7.2), 10% glycerol, 0.1 mM EDTA and 3 mM dithiothreitol. The peak of enzyme activity was eluted at about 0.1 M KCl. The pooled active fractions were concentrated by dialysis against 60% glycerol in 0.05 M Tris-hydrochloride (pH 7.2) containing 0.2 M KCl and then dialyzed against 10% glycerol in the same buffer before layering onto gradients of 20 to 40% glycerol in 0.05 M Tris-hydrochloride (pH 7.2), 0.2 M KCl, 0.1 mM EDTA and 3 mM dithiothreitol. Centrifugation was for 24 h at 46,000 rpm in the Spinco SW50.1 rotor (2 C). Each fraction from the gradients was assayed with activated calf thymus DNA in the standard assay and with poly(A)·$(dT)_{12-18}$ in the presence of Mn^{2+} as described in the legend to Fig. 1. Activated calf thymus DNA as template primer (●); poly(A)·$(dT)_{12-18}$ as template primer (○). Markers were BSA (4.4S), Escherichia coli alkaline phosphatase (6.3S) and beef liver catalase (11.3S) which were centrifuged in parallel gradients and fractionated in exactly the same manner as the sample gradients.*

has a sedimentation coefficient of about 6.7S which is equivalent to a molecular weight of 125,000 for a globular protein. In several different preparations, the glycerol-purified enzyme was purified about 90-fold from the Triton extract, which would correspond to approximately a 900-fold purification from the initial cell pellet.

Characterization of the enzyme purified through the glycerol gradient step with regards to template-primer preference is shown in Table 2. The DNA polymerase of the adenovirus 2 DNA replication complex has greater than twofold higher activity with poly(A)·$(dT)_{10}$ as template-primer in the presence of Mn^{2+} than with activated calf thymus DNA in the presence of Mg^{2+}. This distinguishes this DNA polymerase from DNA polymerase α and β present in the cytoplasm and in the nucleus of KB cells (10). The KB cell enzymes were reported to utilize the polyribonucleotide strand of poly(A)·oligo(dT) with less than 1% the efficiency of activated DNA under the same conditions (10). A recent report has indicated the β DNA polymerase from several tissues can copy poly(A)·oligo(dT) under appropriate conditions (2). However, the enzyme from the adenovirus DNA replication complex is distinguished from DNA polymerase β by molecular weight and column elution characteristics (12) and can clearly be classified as DNA polymerase γ. DNA polymerase γ is the enzyme that responds to poly(A)·oligo(dT) as template primer in the presence of Mn^{2+} [3, 5, 7, 12]).

TABLE 1. *Recovery of DNA polymerase activity from adenovirus 2 DNA replication complex*

Fraction[a]	Total Activity[b] (pmol of [^{3}H]TTP incorporated)	
	Activated calf thymus DNA	$(rA)_n \cdot (dT)_{12\text{-}18}$
Replication complex	812	1,740
Triton-high salt extract	984	779
Phosphocellulose peak II	372	2,410
DEAE-cellulose	110	950
Glycerol gradient centrifugation	17	88

[a] The starting material was 7 g (wet weight) of KB cells harvested at 18 h after infection with adenovirus 2.

[b] Assays were performed as described in the legend to Fig. 1.

TABLE 2. *Template-primer preference of glycerol gradient-purified DNA polymerase from the adenovirus 2 DNA nuclear membrane complex*

Template primer[a]	Substrate	Relative DNA polymerase activity (%)	
		20 mM Mg^{2+}	0.8 mM Mn^{2+}
Activated calf thymus DNA (20 μg)	[^{3}H]dTTP, dATP dCTP, dGTP	100	0
Poly(A)·$(dT)_{10}$ (30 nmol)	[^{3}H]TTP	5	214
Poly(C)·$(dG)_{12\text{-}18}$ (30 nmol)	[^{3}H]dGTP[b]	1	1

[a] Assays were performed as described in the legend to Fig. 1. In each assay mixture only the nucleotide or nucleotides which are complementary to the template were present. All unlabeled nucleotides were present at a concentration of 90 μM and all labeled nucleotides were present at 1 μCi/reaction.

[b] The specific activity of the [^{3}H]dGTP was 6.51 Ci/mmol.

The data of Table 2 also show that the DNA polymerase γ from the adenovirus 2 nuclear membrane complex had no detectable activity with poly(C)·$(dG)_{12\text{-}18}$ as template primer in the presence of Mn^{2+}. This agrees with the report of Bolden et al. (1) that partially purified DNA polymerase γ does not utilize poly(C)·$(dG)_{12\text{-}18}$ as template primer. More recent studies, however, have provided evidence for the presence of two and perhaps three DNA polymerase γ activities in HeLa cells (12) which upon further purification showed altered template-primer specificities. The DNA polymerase γ found in the nucleus utilized poly(A)·$(dT)_{12\text{-}18}$ about threefold more efficiently than activated DNA, but could also utilize poly(C)·$(dG)_{12\text{-}18}$ (12). One of the DNA polymerase γ activities in the cytoplasm showed less activity with poly(A)·$(dT)_{12\text{-}18}$ than with activated DNA, and did not respond to poly(C)·$(dG)_{12\text{-}18}$. The second cytoplasmic activity closely resembled the nuclear DNA polymerase in template preference. Further characterization of DNA polymerase from the nuclear membrane complex from infected and uninfected KB cells may provide additional insight into the number of DNA polymerase γ species and their utilization of synthetic RNA template primers.

The possibility that the viral DNA synthesizing capacity of the adenovirus 2 DNA replication complex is due to the presence of an extremely active, specific, and unstable DNA polymerase which is present is small quantities cannot be ruled out. The complete recovery of activity by the Triton-high salt extraction, the

apparent lack of DNA-dependent DNA polymerase activity throughout the purification procedure, and the consistently high ratio of activity with poly(A)·oligo(dT) versus activated calf thymus DNA strongly indicate that the only DNA polymerases present are γ DNA polymerases. The information provided herein may shed light on the enzymology of adenovirus replication and may also indicate a functional role for the poorly understood γ DNA polymerases.

This investigation was supported by Public Health Service grant AI-01725 from the National Institute of Allergy and Infectious Diseases and by research contract NO1 CP 43359 within the Virus Cancer Program of the National Cancer Institute. M. G. is a Research Career Awardee of the National Institutes of Health (5K6-A14739).

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