Human DNA Polymerase III (R-DNA Polymerase): Distinction from DNA Polymerase I and Reverse Transcriptase

Abstract. *DNA polymerase III is an enzyme activity in eukaryotic cells which under certain conditions shows strong preference for polyadenylic acid as template when primed by oligodeoxythymidylate. Its first complete separation from other DNA polymerases in human lymphoblasts is reported. This enzyme is biochemically and immunologically distinct from DNA polymerase I and from viral reverse transcriptase from a primate type C virus.*

We have characterized two DNA polymerases in phytohemagglutinin (PHA)-stimulated human blood lymphocytes—DNA polymerases I and II (*1*). Both enzymes preferentially used DNA as a template and showed several properties identical to those of the two mammalian cell DNA polymerases described and extensively characterized first by Chang and Bollum (*2*) and later by others (*3*). In addition, Fridlender *et al.* (*4*) discovered another DNA polymerase activity in HeLa cells and later in PHA-stimulated human lymphocytes. They named it R-DNA polymerase [DNA polymerase III (*5*)] because under certain assay conditions it best utilized polyadenylic acid [$(A)_n$] as a template in synthesizing polydeoxythymidylate [$(dT)_n$] a property it shared with viral reverse transcriptase (*6*) and with a reverse transcriptase in human leukemic cells (*7*). This overlap in template utilization made it important to determine the relation of reverse transcriptase to DNA polymerase III.

We found [see (*8*) for a study of the separation of all the DNA polymerases in human lymphoblast cells] that the DNA polymerase III reported in human lymphocytes (*4, 9*) remained mixed with DNA polymerase I after its passage over either a single diethylaminoethyl (DEAE)-cellulose (*4*) or phosphocellulose column (*9*). Therefore, in order to study DNA polymerase III free from contamination with other DNA polymerases, we separated DNA polymerases I and III from extracts of cultured human lymphoblasts derived from a normal donor. We then showed that DNA polymerase III had biochemical and immunologic properties that further differentiated it from DNA polymerase I. Finally, a comparison of specific primer-template utilization and immunologic properties served to distinguish DNA polymerase III from reverse transcriptase of a primate type C virus.

Ten grams of uninfected human lymphoblast culture cells [NC37 cell line (*10*)], were homogenized and extracted with a salt solution of high concentration and a detergent (*8*). Nucleic acids were removed by ultracentrifugation and by passage of the extract over a fibrous DEAE-cellulose column. During chromatography of the material on a second, microgranular DEAE-cellulose column, DNA polymerase II (*5*) eluted at low salt strength (0.05*M* KCl), while an 0.3*M* KCl washing removed DNA polymerases I and III. Attempts to adequately separate DNA polymerases I and III from each other with a linear salt gradient instead of step elution on DEAE-cellulose columns were unsuccessful (*8*). This is an important point because previous studies (*4, 9*) have not shown that the most purified fraction of the R-DNA polymerase obtained was free of DNA polymerase I.

Further chromatography of the 0.3*M* KCl washing from the DEAE-cellulose on phosphocellulose columns still gave incomplete separation of the two DNA polymerases. They eluted as overlapping peaks between 0.25*M* and 0.4*M* KCl (*8*). However, when this 0.25*M* to

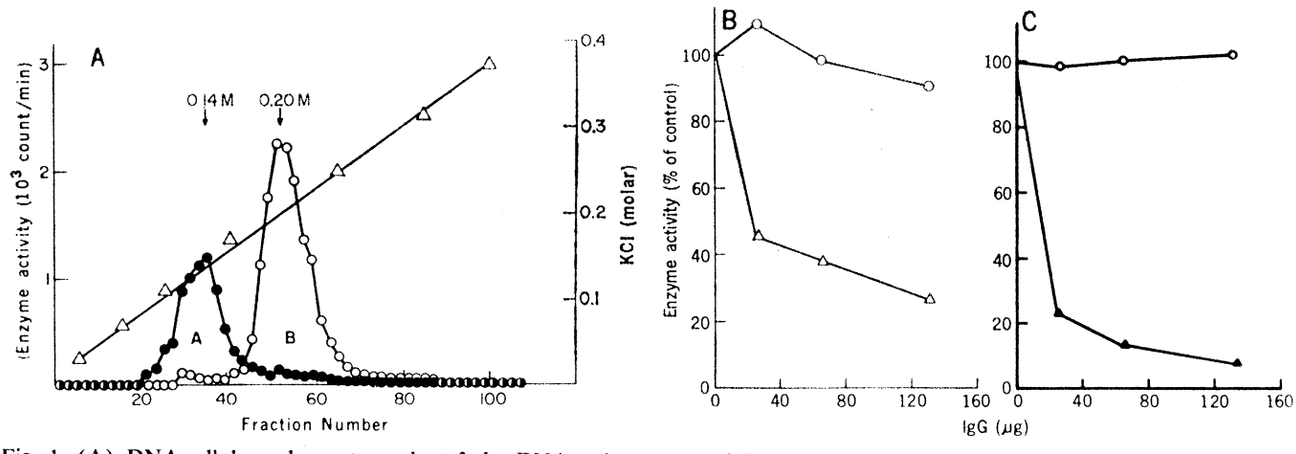

Fig. 1. (A) DNA-cellulose chromatography of the DNA polymerase activities eluting between 0.25*M* and 0.4*M* KCl on a phosphocellulose column. (The portion fractionated on the phosphocellulose column had been passed over DEAE-cellulose.) Denatured calf thymus DNA was adsorbed to Munktells 410 cellulose (Bio-Rad) and utilized as described in (*15*). The salt gradient was 0.05*M* to 0.4*M* KCl (open triangles). (Peak A) Acid precipitable radioactivity in assays with "activated" DNA as a primer template. (Peak B) Acid precipitable radioactivity with $(dT)_{12\text{-}18} \cdot (A)_n$ as a primer template. Every other fraction was assayed, as in legend to Table 1. (B) Effect of antibody to DNA polymerase I on DNA polymerases in peaks A and B. Ten microliters of enzyme (protein concentration < 1 μg/ml) were mixed with immunoglobulin G (IgG) in a volume of 20 μl and incubated at room temperature for 10 minutes. The polymerase was assayed as described in Table 1 except that reaction mixtures (50 μl) also contained 40 μg of bovine serum albumin. DNA cellulose peak A (open triangles): The primer template for the reaction was "activated" DNA; reaction mixtures contained 0.01*M* $MgCl_2$ and 0.06*M* KCl; incubation was at 37°C. DNA cellulose peak B (open circles): The primer template was $(dT)_{12\text{-}18} \cdot (A)_n$; reaction mixtures contained 0.001*M* $MnCl_2$ and 0.125*M* KCl; incubation was at 30°C. The data represent the averages of two experiments, each performed in duplicate. (C) Effect of rat antibody to reverse transcriptase from simian sarcoma virus on the enzyme in peak B (open circles) and on simian sarcoma virus reverse transcriptase (solid triangles) [purified (*16*) and stored at a protein concentration of 47 μg/ml prior to use]. Enzyme neutralization was assayed with 10 μl of enzyme, and polymerase reaction conditions matched those for DNA-cellulose peak B as described above.

Table 1. Comparison of DNA polymerase activities in DNA-cellulose peaks A and B (Fig. 1A) with different primer templates. The results represent the ratio of the tritated nucleotide incorporated in a DNA polymerase assay with a given primer template to that incorporated in an assay with the optimum primer template. For peak A, "activated" DNA is the optimum primer template and yields 4.5 pmole of [^{3}H]thymidylic acid incorporation in a reaction. For peak B, $(dT)_{12\text{-}18} \cdot (A)_n$ is optimum, giving 0.83 pmole of [^{3}H]thymidylic acid incorporation. The relative amounts of polymerase activity in peak A compared to peak B varied from experiment to experiment (*8*). Assay mixtures contained 10 μl of enzyme (at a concentration of less than 1 μg per milliliter of protein) and were initiated by the addition of 40 μl of a mixture which gave final concentrations of 0.05*M* tris·Cl, *p*H 7.5, 0.001*M* dithiothreitol, 80 μmole each of unlabeled deoxynucleoside triphosphate [deoxyadenosine triphosphate (dATP) for $(dT)_{12\text{-}18} \cdot (A)_n$ assays; deoxycytidine triphosphate (dCTP) for $(dG)_{12\text{-}18} \cdot (C)_n$; dATP, dCTP, and deoxyguanosine triphosphate (dGTP) for "activated" DNA; and none for $(dT)_{12\text{-}18}$], 6 μmole of [^{3}H]thymidine triphosphate (6600 count/min per picomole) [except with $(dG)_{12\text{-}18} \cdot (C)_n$ where 6 μmole of [^{3}H]dGTP (7900 count/min per picomole) was used], and 25 μg of primer template per milliliter. With "activated" DNA, assay mixtures contained 0.06*M* KCl and 0.01*M* $MgCl_2$, and incubations were performed at 37°C. With the other primer templates, reaction mixtures contained 0.125*M* KCl and 0.001*M* $MnCl_2$, and incubations were performed at 30°C [assaying peak A with $(dT)_{12\text{-}18} \cdot (A)_n$, $(dG)_{12\text{-}18} \cdot (C)_n$, and $(dT)_{12\text{-}18}$ at 37°C in the presence of 0.06*M* KCl and 0.01*M* $MgCl_2$ did not augment its activity]. All incubations were for 20 minutes (incorporation was a linear function of time for at least this long) and assays were terminated and processed for counting as described (*1*). Assays for transcription of the 70*S* RNA of avian myeloblastosis virus (AMV) were performed as described [table 1 in (*12*)], with added $(dT)_{12\text{-}18}$ as a primer and [^{3}H]dGTP (7900 count/min per picomole) as the label. Less than 0.002 pmole of [^{3}H]deoxyguanylic acid was incorporated by DNA polymerase III under these assay conditions; the leukemic enzyme in (*12*) incorporated 5.1 pmole, while normal leukocyte enzymes converted less than 0.05 pmole into acid insoluble material.

Enzyme	Primer template				
	Activated DNA	$(dT)_{12\text{-}18} \cdot (A)_n$	$(dG)_{12\text{-}18} \cdot (C)_n$	$(dT)_{12\text{-}18}$	AMV 70*S* RNA
DNA-cellulose peak A	1	0.01	< 0.01	< 0.01	< 0.01
DNA-cellulose peak B	0.6	1	< 0.01	< 0.01	< 0.01

0.4*M* KCl region from the phosphocellulose columns was chromatographed on a DNA-cellulose column, two distinct areas of DNA polymerase activity were obtained (Fig. 1A). Peak A, eluting at 0.14*M* KCl, was detected in DNA polymerase assays containing "activated" DNA (*11*) as a primer template, while peak B, eluting at 0.2*M* KCl, was outlined by assays containing $(dT)_{12-18} \cdot (A)_n$, the preferred primer template under our assay conditions (Table 1).

The two peaks were separately pooled for further analysis. The primer-template preferences of the polymerase activity in each pool are shown in Table 1. The DNA polymerase in peak A had the characteristics of DNA polymerase I from PHA-stimulated blood lymphocytes (*1*). It best utilized "activated" DNA as a primer template with Mg^{2+} as the divalent cation and showed almost no activity with $(dT)_{12-18} \cdot (A)_n$. The material in peak B, however, while using "activated" DNA in the presence of Mg^{2+}, was most active with $(dT)_{12-18} \cdot (A)_n$ and Mn^{2+}, as has been reported for other R-DNA polymerases (*4*). Both viral and leukemic reverse transcriptase transcribe heteropolymeric regions of 70*S* RNA of RNA tumor virus and use $(dG)_{12-18} \cdot (C)_n$ and $(dT)_{12-18} \cdot (A)_n$ as primer templates (*7, 12, 13*) (dG is deoxyguanylic acid and C is cytidylic acid); DNA polymerase III, while it used $(dT)_{12-18} \cdot (A)_n$, did not share the other primer-template affinities shown by the reverse transcriptases (Table 1).

Further differentiation of DNA polymerases I and III was achieved with antibody prepared in rats against DNA polymerase I from human lymphoblast culture cells (*14*). Under optimum conditions for DNA polymerase activity, the enzyme in peak A was inhibited by almost 80 percent by antibody to DNA polymerase I (Fig. 1B). The material in peak B remained virtually unaffected. Thus, in their catalytic and antigenic properties, the enzymes in peaks A and B are distinct from each other and correspond to DNA polymerases I and III, respectively.

DNA polymerase I is virtually unable to transcribe $(A)_n$ under these conditions (Table 1). Previously (*1*) we observed significant transcription of $(A)_n$ by this enzyme. It is now apparent that such transcription was due to contaminating DNA polymerase III. In support of this interpretation, Sedwick *et al.* (*3*) have obtained highly purified DNA polymerase I from KB cells, which does not transcribe $(A)_n$.

While DNA polymerase III (R-DNA polymerase) is found in a variety of eukaryotic cells (*4*), its role in DNA metabolism and information transfer is unknown. Because of its preference in vitro for copying a synthetic RNA template, DNA polymerase III could be confused with a viral reverse transcriptase in neoplastic tissue. Conversely, any candidate viral-like enzyme might simply be some form of DNA polymerase III. In studying the DNA polymerases of human leukemic cells, we and our colleagues in our laboratory have isolated enzymes closely related biochemically and immunologically to the reverse transcriptases of primate type C animal RNA tumor viruses (*7, 12, 13*). Unlike DNA polymerase III, these leukemic enzymes can transcribe both C_n and the heteropolymeric regions of viral 70*S* RNA (*7, 12*). In addition, we (*13*) have shown that DNA polymerase III, isolated from human leukemic cells by the technique described above, is not inhibited by antibodies to reverse transcriptases from primate type C viruses. However, the viral-like reverse transcriptase in these same cells is inhibited by these antibodies. Likewise, when DNA polymerase III isolated in our experiments from NC37 cells is reacted with antibody to the reverse transcriptase of a primate type C RNA tumor virus (simian sarcoma virus), it is not inhibited in a DNA polymerase assay (Fig. 1C). Thus the reverse transcriptases in leukemic cells and RNA tumor viruses have significant structural differences from DNA polymerase III. We conclude that DNA polymerase III is biochemically and immunologically distinct from both DNA polymerase I and from reverse transcriptase.

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References and Notes

1. R. G. Smith and R. C. Gallo, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2879 (1972).
2. L. Chang and F. Bollum, *J. Biol. Chem.* **246**, 5835 (1971); *Biochemistry* **11**, 1264 (1972).
3. W. Sedwick, T. Wang, D. Korn, *J. Biol. Chem.* **247**, 5026 (1972); E. Baril, O. Brewer, M. Jenkins, J. Laszlo, *Biochemistry* **10**, 1981 (1971).
4. B. Fridlender, M. Fry, A. Bolden, A. Weissbach, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 452 (1972); A. Bolden, M. Fry, R. Muller, R. Citarella, A. Weissbach, *Arch. Biochem. Biophys.* **153**, 26 (1972).
5. In the nomenclature used here, DNA polymerase I is the high-molecular-weight enzyme (6*S* to 8*S*) found in the cytoplasm of proliferating cells. DNA polymerase II is the low-molecular-weight (3.3*S*) enzyme found in resting and dividing cells in both the nucleus and cytoplasm [see L. Chang and F. Bollum, *J. Biol. Chem.* **247**, 7948 (1972) and (*9*)]. We are calling R-DNA polymerase cellular DNA polymerase III.
6. D. Baltimore and D. Smoler, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1507 (19 → N. Goodman and S. Spiegelman, *ibid.*, p. 2203; M. Robert, R. Smith, R. Gallo, P. Sarin, J. Abrell, *Science* **176**, 798 (1972).

7. M. Sarnghadaran, P. Sarin, M. Reitz, R. Gallo, *Nat. New Biol.* **240**, 67 (1972).
8. B. Lewis, J. Abrell, R. Smith, R. Gallo, in preparation.
9. R. McCaffrey, D. Smoler, D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 521 (1973).
10. R. Gallo and S. Pestka, *J. Mol. Biol.* **52**, 195 (1970).
11. "Activated" DNA is native salmon sperm DNA partially digested by deoxyribonuclease I (A. Schrecker, R. Smith, R. C. Gallo, *Cancer Res.*, in press).
→ G. Todaro and R. Gallo, *Nature (Lond.)* **244**, 206 (1973).
13. R. Gallagher, G. Todaro, R. Smith, D. Livingston, R. Gallo, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
14. G. Smith, B. Lewis, J. Abrell, R. Gallo, in preparation.
15. B. Alberts and G. Herrick, *Methods Enzymol.* **21**, 198 (1971).
16. J. Abrell and R. Gallo, *J. Virol.* **12**, 431 (1973).
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Septal Tryptophan-5-Hydroxylase: Divergent Response to Raphe Lesions and Parachlorophenylalanine

Abstract. *A soluble form of tryptophan-5-hydroxylase activity was found to be present in areas rich in serotonergic terminals (colliculi, hippocampus, septal area, and remaining telencephalon) as well as in brainstem, an area rich in cell bodies. The enzymatic activity in all brain regions, except the septal area, was inhibited to varying degrees following administration of parachlorophenylalanine. Destruction of the raphe nuclei in the midbrain led to a large and comparable decrease in both serotonin content and tryptophan hydroxylase activity of the hippocampus. In contrast, these lesions did not significantly affect the enzymatic activity of the septal area although the serotonin content was decreased by 72 percent. These findings suggest that the major portion of the tryptophan hydroxylase activity of the septal area is uniquely different from that found in other telencephalic areas in that it is not localized in serotonergic nerve terminals nor is it inhibited by parachlorophenylalanine.*

It has been demonstrated that the serotonin content of the telencephalon is exclusively localized in axons whose cell bodies lie primarily in the raphe nuclei of the brainstem (*1*). Destruction of the cell bodies in the raphe nuclei or of the fibers of passage in the medial forebrain bundle leads to a parallel loss of serotonin fluorescent terminals, synaptosomal uptake of serotonin, serotonin content, and activity of the rate-limiting enzyme, tryptophan hydroxylase (*2*). It has therefore been concluded that serotonin and tryptophan hydroxylase are uniquely localized in telencephalon within the axon terminals of serotonergic neurons.

Injection of parachlorophenylalanine (*p*-CP) produces an inhibition of tryptophan hydroxylase activity in brain (*3*). Recently, however, it has been reported that *p*-CP does not inhibit the tryptophan hydroxylase activity of the septal area (*4*). This finding suggests a unique characteristic of the septal enzyme which might reflect a difference in its compartmentalization. The activity of septal tryptophan hydroxylase has not been measured following brain lesions. We have therefore conducted the appropriate experiments utilizing brain lesions and *p*-CP. Our results suggest that the bulk of the soluble tryptophan hydroxylase activity of the septal area differs from that found in other telencephalic areas in that it is not inhibited by *p*-CP and is not localized within serotonergic nerve terminals.

Male albino rats (Holtzman), 70 to 80 days of age, were housed two per cage and maintained on Purina Lab Chow and tap water. In the first experiment animals were injected intraperitoneally with a suspension of DL-*p*-CP (300 mg/kg) or an equivalent volume (3 ml/kg) of vehicle (*5*).

Table 1. Effect of *p*-CP (300 mg/kg) on soluble tryptophan-5-hydroxylase activity.

Brain region	Enzyme activity (nmole/mg/hr)*	Change from control (%)
Brainstem		
Control	5.98 ± 0.49 (9)	
p-CP	1.09 ± 0.29 (3)	−82†
Colliculus		
Control	4.46 ± 1.10 (9)	
p-CP	2.01 ± 0.68 (3)	−55†
Hippocampus		
Control	0.56 ± 0.12 (6)	
p-CP	0.32 ± 0.01 (5)	−43†
Telencephalon		
Control	1.22 ± 0.13 (6)	
p-CP	0.12 ± 0.01 (6)	−90†
Septal area		
Control	1.55 ± 0.23 (12)	
p-CP	1.27 ± 0.32 (8)	−18

* Values are given as means ± S.E.M. The number of determinations is shown in parentheses. † $P < .05$; *t*-test, two tailed.

Animals were decapitated 3½ days later, and the brains were removed and dissected on ice. The following brain regions were obtained: brainstem, including midbrain and pons but excluding cerebellum; colliculus, including both superior and inferior colliculi; hippocampus (both hippocampi were combined); septal area, the region lying between the lateral ventricles, and bounded by the corpus callosum dorsally, the anterior commissure ventrally, the genu of the corpus callosum rostrally, and the columns of the fornix caudally; and telencephalon, all of the telencephalon except for the septal area and hippocampi. Soluble tryptophan-5-hydroxylase activity was measured by the method of Gál and Patterson (*6*) which utilizes 2-amino-4-hydroxy-6-methyltetrahydropterin as the cofactor.

All brain regions exhibited the presence of a soluble tryptophan hydroxylase (Table 1). Enzymatic activity was highest in both the brainstem, an area rich in serotonergic cell bodies, and in the colliculus, an area rich in their terminals. Telencephalic areas contained lower levels of enzyme activity. Injection of *p*-CP led to a large and equivalent inhibition of enzymatic activity in both telencephalon and brainstem, and a smaller although significant inhibition in hippocampus and colliculus. In contrast, there was no inhibition of tryptophan hydroxylase activity in the septal area. The absence of enzyme inhibition in the septal area might be due to an insufficient quantity of drug reaching this brain region. We therefore injected a group of animals with DL-[2-^{14}C]-*p*-CP (90 μc/kg) with a specific activity of 3.27 μc/mg (*7*). Five animals were decapitated 1 day and seven 3 days after injection, and the radioactivity within each brain region was determined by liquid scintillation counting (*8*). The brainstem, colliculus, and hippocampus demonstrated essentially the same amount of radioactivity at 1 day after injection, with a mean ± S.E.M. of 26,337 ± 1,521 disintegrations per minute (DPM) per gram. This decreased to 10,356 ± 426 DPM/g by 3 days. The septal area did not differ from other brain regions, demonstrating 27,974 and 9,872 DPM/g at 1 and 3 days, respectively, after injection.

Knapp and Mandell (*4*) have suggested that there are two forms of tryptophan hydroxylase in brain. Homogenates of various brain regions in 0.35*M* sucrose yielded a soluble and a