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OCCURRENCE OF A TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-LIKE ACTIVITY IN N-2-FLUORENYLACETAMIDE-TREATED RAT LIVER

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ABSTRACT

A new enzyme activity that resembles terminal deoxynucleotidyl transferase (TdT-like activity) was found in rat liver. Upon treatment of rats with a hepatocarcinogen, N-2-fluorenylacetamide, the TdT-like activity increased in parallel with DNA polymerase α and γ. By phosphocellulose column chromatography, the TdT-like activity was separated from DNA polymerase α, β, and γ. With a poly(dA)-initiator, dGTP or dCTP was the most efficient substrate in the presence of Mg^{2+} or Mn^{2+}, respectively. In the presence of Mg^{2+}, the enzyme utilized poly(dC) as an initiator most efficiently. The TdT-like activity differed from previously described terminal deoxynucleotidyl transferase (E.C.2.7.7.31.) in its Km value for dGTP and in its sensitivity to ATP or to an antibody against calf thymus terminal deoxynucleotidyl transferase.

Keywords: Terminal deoxynucleotidyl transferase, Fluorenylacetamide, Rat liver, DNA polymerases

INTRODUCTION

Previously, we showed that terminal deoxynucleotidyl transferase (TdT) of calf thymus can use the 3'OH-end of growing chains as an initiator when the reaction of DNA polymerase α is blocked at or near the pyrimidine-dimers on the template strands and suggested that the single-stranded chains formed by TdT may reassociate with template strands beyond the lesions and may be further extended by 10S DNA polymerase α.[1] Since the reaction products *in vitro* of this dual-enzyme system contained a large amount of mismatched deoxynucleotides, it was suggested that TdT can help DNA polymerase α bypass thymine-dimers by inserting mismatched deoxynucleotides at the positions opposite to the DNA lesions.[1] Thus, TdT has a biochemical capacity for acting as a "mutator polymerase" *in vitro*. However, it is well known that in normal adult mammals, terminal deoxynucleotidyl transferase is strictly localized in the thymus gland and bone marrow.[2] Here, we tested the possible existence of terminal deoxynucleotidyl transferase activity in rat liver treated with a hepatocaracinogen, N-2-fluorenylacetamide.

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MATERIALS AND METHODS

Chemicals and reagents

N-2-fluorenylacetamide (FAA) was obtained from Nakarai Chemicals, Ltd., Japan. Deoxyhomopolymers, poly(dT), poly(dA), poly(dC) and oligo(dG), were obtained from P-L Biochemicals Inc., U.S.A. Affinity purified antibody (IgG) against calf thymus TdT was prepared as described previously.[3]

FAA-treatment of rats

Male Wister rats (6 weeks old) were fed with a standard diet mixed with FAA at 0.05%[4] for 3, 7, 14, 21, and 28 days and the livers were taken and kept frozen at −65°C until used. In order to prepare the hyperplastic nodules, a feeding cycle, feeding with FAA for 2 weeks followed by 1 week with regular meal, was repeated four times and the hyperplastic nodules were dissected and pooled. Among rats treated with FAA, some developed hepatoma after several months. The cancer nodules were also dissected and kept frozen. Normal regenerating livers were obtained from two-thirds partially hepatectomized rats (6 weeks old) at 48 hr after operation.

Enzyme extraction and partial purification

Four grams of each liver tissue were homogenized in a teflon homogenizer in 10 volumes of buffer A (50 mM Tris. HCl, pH 7.5, 0.1 mM ethylenediamine tetraacetate, 0.5 mM dithiothreitol, 10% glycerol) containing 0.5 M KCl and three kinds of protease inhibitors, i.e., 1.0 mM phenylmethylsulfonylfluoride (PMSF), 2 mM benzamidine, and 0.2 mg/ml ovomucoid.[3] The homogenate was mixed with Triton-X 100 (0.1% in the final concentration) and kept for 30 min at 4°C. The homogenate was centrifuged at 16000 × *g* for 20 min at 0°C and the supernatant was collected and dialyzed against buffer A. The dialyzed extract was applied on a phosphocellulose column (Whatman P-11, 0.9 × 15 cm) equilibrated with buffer A. After washing with the same buffer, the column was eluted with a 100-ml linear gradient of KCl (0–1.0 M). The aliquots of fractions were assayed for DNA polymerase α, β, γ and TdT as follows.

Assay of DNA polymerases

Reaction mixture for the assay of DNA polymerase α (62.5 μl) contained 40 mM potassium phasphate (pH 7.2), 5 μg activated calf thymus DNA,[5] 64 μM each of dATP, dGTP, dCTP and 33 μM (^{3}H)dTTP (0.5 μCi/mmol), 8 mM $MgCl_2$, 4 mM 2-mercaptoethanol, and the enzyme fraction. Reaction mixture for the assay of DNA polymerase β (62.5 μl) contained 80 mM Tris. HCl (pH 9.0), 1.5 μg of poly(dA). $(dT)_{12-18}$ (base ratio of A: T=10:1), 32 μM (^{3}H)dTTP (0.5 μCi/nmol), 80 mM NaCl, 3.2 mM N-ethylmaleimide, 0.5 mM $MgCl_2$, and the enzyme fraction. Reaction mixture (62.5 μl) for the assay of DNA polymerase γ contained 80 mM Tris.HCl (pH 7.9), 1.25 μg poly(rA). oligo$(dT)_{12-18}$ (base ratio of A: T=5:1), 0.5 mM $MnCl_2$, 50 mM KCl, (^{3}H)dTTP (64 μM), and the enzyme fraction.

Assay of TdT-like activity

Reaction mixture for the assay of TdT or TdT-like activity (62.5 μl) contained 80 mM Tris. HCl (pH 7.9), 1.25 μg poly(dA), 64 μM (^{3}H)dGTP (0.5 or 1.25 μCi/nmol), 1.25 mM dithiothreitol, 40 mM KCl, 0.5 mM $MgCl_2$, and the enzyme fraction. The primers, substrates, and divalent cations were changed as indicated. All these reactions were carried out at 37°C for 30 min and the acid-insoluble radioactivity was measured as described previously.[5] One unit of enzyme was defined as the amount which catalizes the incorporation of one nanomole of deoxynucleotides in 60 min under the conditions described above.

RESULTS

Effects of FAA-treatment on DNA polymerases and TdT-like activity

In the liver extract of normal rats (6 weeks after birth), there was a small but significant amout of activity which was measured by the TdT assay (Materials and Methods). This activity was tentatively designated as TdT-like activity. Fig. 1A shows an elution profile of DNA polymerases from the phosphocellulose column. The order of elution was DNA polymerase α, γ and β. The TdT-like activity was eluted as heterogeneous peaks ahead of DNA polymerase α. The TdT-like activity was also detected in the regenerating liver. In this case, however, the amount to TdT-like activity stayed at the control level even though DNA polymerase α increased (Table 1).

The effect of FAA-treatment on rat liver was then examined with respect to the levels of DNA polymerases including TdT-like activity. Six-week-old rats were fed a FAA-containing diet as described in Materials and Methods. Fig. 1B and C show typical elution profiles of extracts from livers treated with FAA for 1 and 2 weeks. The elution profile of enzymes from cancer nodules is shown in Fig. 1D. Table 1 summarizes the levels of DNA polymerases and TdT-like activity per wet weight of liver tissue throughout the course of FAA-treatment. After two weeks, the levels of both DNA polymerase α and γ started to increase and the TdT-like activity also increased in a similar way. On the 28th day, DNA polymerase α had increased about 2-fold, DNA polymerase γ, about 3-fold, and the TdT-like activity had increased about 4-fold.

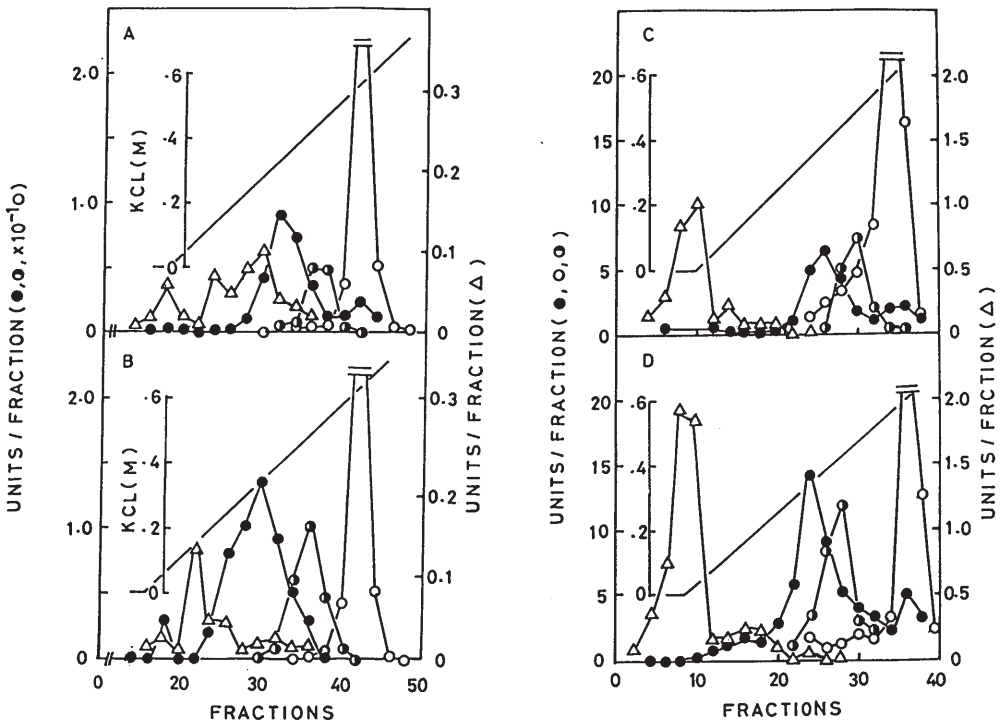

Fig. 1 Phosphocellulose column chromatography of normal and FAA-treated rat liver extracts. DNA polymerases were extracted from 4 grams of liver samples and separated from each other on the phosphocellulose column as described in Materials and Methods. Assays were performed for TdT-like activity (△), DNA polymerase α(●), β(○) and γ(◑). The activity was expressed as units/fraction (2 ml each). A, Normal rat liver; B, liver of FAA-treated for 7 days; C, liver of FAA-treated for 14 days; D, cancer nodules.

Table 1. Effects of FAA-treatment on DNA polymerases and TdT-like activity in rat liver.

FAA Treatment Days	DNA polymerase α	DNA polymerase β	DNA polymerase γ	TdT-like Activity
0	7.5	160	1.7	0.45
3	6.7	n.t.	1.3	0.20
7	2.3	186	1.2	0.18
14	6.7	220	2.1	1.00
21	14.8	169	6.6	0.60
28	11.5	186	5.8	2.2
Hyperplastic nodules	24.3	n.t.	3.1	1.3
Cancer nodules	20.3	186	11.6	3.2
Regenerating liver	23.9	200	1.79	0.48

Activities were expressed as unit/g tissue. Amounts of DNA polymerase α, γ and TdT-like activity were calculated from the elution profiles of column chromatography. Amount of DNA polymerase β was obtained by direct assay of crude extract. Other conditions were described in Materials and Methods.
The values are the means of two independent experiments.
n.t.: not tested.

Properties of the TdT-like Activity

The TdT-like activity was routinely measured by the poly(dA)-dependent dGMP incorporation. This activity was tested with other synthetic polymers in combination with four kinds of deoxynucleoside triphosphates (Table 2). With poly(dA), dGTP was used efficietly in the presence of Mn^{2+} (optimum concentration, 0.5 mM) but dCTP was used more efficiently at a relatively high concentration (160 μM). The enzyme showed the highest activity with poly(dC) in incorporating dGMP. With poly(dC), Mg^{2+} gave much higher incorporation than Mn^{2+} as a cofactor. Oligo(dG) also served as a primer for the polymerization of all four deoxynucleoside triphosphates. The complementarity between primer sequences and the substrates was further tested with a natural DNA and alternating co-polymers (Table 3). With the activated calf thymus DNA, low levels of activity were observed with dGTP, dTTP and dATP. The incorporation of (^{3}H)dGMP was not enhanced but rather suppressed by the addition of the other three unlabeled dNTPs. Similar results were obtained with poly(d(G-C)) and poly(d(A-T)). With these alternating co-polymers, the incorporations should be greatly enhanced by adding another complemen-

Table 2. Primer specificity of TdT-like activity from rat liver.

Primer: Cofactor	Substrate dATP	dGTP	dTTP	dCTP
Poly(dA): Mg^{2+} (5 mM)	228	716	0	271
Poly(dA): Mn^{2+} (0.5 mM)	265	1091	541	2338
Poly(dC): Mg^{2+}	645	10317	105	605
Poly(dC): Mn^{2+}	0	1534	350	16
Oligo$(dG)_{10}$: Mg^{2+}	668	2474	1028	569

Activities were measured with the 2nd phosphocellulose fraction of TdT-like activity as described in Materials and Methods except that primers, divalent cations, and substrates (160 μM, 0.5 μCi/nmol) were changed as indicated. Activity was expressed as cpm incorporated in 30 min at 37°C.

Table 3. Effects of base complementarity between primers and substrates on the TdT-like activity from rat liver.

Primers: Cofactors	Substrates (80 μM) Labeled (^{3}H)	Unlabeled	Incorporation (cpm)
Activated DNA:	dATP	–	633
Mn^{2+} (0.5 mM)	dGTP	–	680
	dGTP	dATP, dTTP, dCTP	140
	dTTP	–	668
	dCTP	–	30
Poly (dG-dC).	dGTP	–	1124
Poly (dG-dC):	dGTP	dCTP	950
Mg^{2+} (5 mM)	dCTP	–	277
	dCTP	dGTP	100
Poly (dA-dT).	dTTP	–	1700
Poly (dA-dT): Mg^{2+}	dTTP	dATP	1000

Reaction was performed as described in Materials and Methods except that ^{3}H-labeled or unlabeled dNTPs (80 μM), primers, and divalent cations were changed as indicated. Activity was expressed as cpm.

tary deoxynucleoside triphosphate, if the reaction is a DNA polymerase type which copies the template sequences. These results indicate that the reaction was not the replicative type but an end-addition type.

Relationships between the TdT-like activity and the previously reported DNA polymerases

As shown in Fig. 1, the TdT-like activity was eluted from the phosphocellulose column at a position which differed from DNA polymerase α, β and γ. DNA polymerase α activity which partially overlapped with TdT-like activity was separated completely on a second phosphocellulose column (Fig. 2). The TdT-like activity may be different from thymus TdT because it was entirely resistant to the inhibition by ATP at the concentrations up to 0.4 mM which completely inhibit thymic TdT.[3] Furthermore, an antibody against calf thymus TdT[3] did not neutralyze the TdT-like activity under the conditions in which calf thymus TdT was inhibited by 99% and rat thymus TdT, by 65% (Table 4). The apparent Km value for dGTP of the TdT-like activity was 3.5 μM, which is much lower than that of thymic TdT (30–40 μM, Ref. 6).

Table 4. Effect of anti-TdT antibody on the TdT-like activity from rat liver.

Anti-TdT IgG (μg)	TdT-like activity of rat liver	TdT of [1] rat thymus	TdT of [1] calf thymus
0	100%	100%	100%
1	102	50	3.2
5	95	35	0.8

An affinity-purified antibody against calf thymus TdT was added to the 2nd phosphocellulose column fraction of the TdT-like activity and then mixed with the reaction mixture. 100% of the TdT-like activity corresponded to 3110 cpm incorporated. Purified TdT of calf thymus (0.2 unit) or rat thymus (0.6 unit) was used.

1) Values taken from Ref. 3.

Other properties

The reaction of TdT-like activity was linear up to 40 min. Optimum concentration of Mn^{2+} was 0.5 mM. The reaction was stimulated about 2-fold by 1 mM dithiothreitol and was completely inhibited by 3 mM N-ethylmaleimide. The TdT-like activity catalyzed the incorporation of dGMP in the presence of poly(2'-0-methylcytidylate).$(dG)_{10}$ (7) at an efficiency of 1/12 with poly(dC) with either Mn^{2+} or Mg^{2+} and the amount of incorporation was comparable to that with oligo(dG).

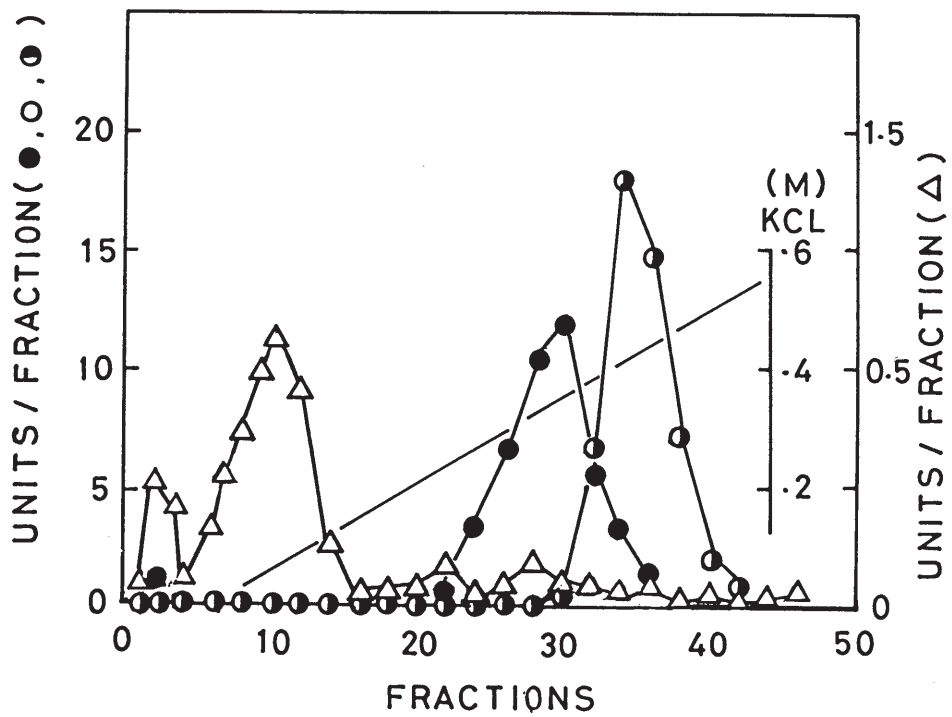

Fig. 2 Rechromatography of TdT-like activity on phosphocellulose column. The first phosphocellulose column fractions of the TdT-like activity from rat liver treated with FAA for 14 days (Fig. 1) were pooled and rechromatographed on a second phosphocellulose column (0.5 x 15 cm). The column was eluted as described in Materials and Methods except that 50 ml of 0–1.0 M KCl gradient was used. △, TdT-like activity; ●, DNA polymerse α; ○, DNA polymerase β; ◑, DNA polymerase γ.

DISCUSSION

Occurrence of terminal deoxynucleotidyl transferase activity has been reported using various tissues, i.e., human brain,[8] rooster sperm,[9] or embryo of *Xenopus laevis*.[10] These activities might be involved in unidentified functions of a wide variety of cells. But, at present, it is not known whether or not these enzyme activities, including our rat liver TdT-like activity, are derived from the same enzyme species due to limited information on their respective characteristics. The liver TdT-like activity presented here differs from thymic TdT in its sensitivity to inhibition by ATP and anti-TdT antibody, and in its apparent Km value for a substrate. This enzyme activity also differs from retrovirus-associated TdT-like activity[11] in its preference for both primers and substrates. Since the TdT-like activity in rat liver increased in parallel with DNA polymerase α and γ by treatment of the animals with FAA, the TdT-like activity may be involved in DNA

replication of FAA-treated rat liver cells. It is tempting to speculate that the TdT-like activity helps DNA polymerase α bypass DNA lesions generated by a carcinogen, FAA, as was observed with calf thymus TdT,[1] but at present, we have not succeeded in proving this because of the instability and small amount of its quantity. Further study of this enzyme may shed new light on the issue of the lowered fidelity of DNA polymerases in carcinogen-treated liver[12] or in the senesent cells.[13,16]

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