Identification of a DNA polymerase β -like form in *Drosophila melanogaster* adult flies

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ABSTRACT

DNA polymerase β is widely distributed in the eukariotes. So far, few examples are known in which a DNA polymerase α -like form alone is reported. Surprisingly, DNA polymerase β was not detected in *Drosophila* embryos, while it is present in the cells of multicellular species from sponge to mammals. In view of the relevance of *Drosophila* as a model biological system for studying the role of the various DNA metabolism enzymes in vivo we have reinvestigated the presence of the DNA polymerase β -like form in *Drosophila* adult flies.

Here we report the occurrence in *Drosophila melanogaster* adult flies of a DNA polymerase activity that, for its NEM¹ resistance, template specificity, sensitivity to ddTTP, sedimentation coefficient and nuclear localization can be classified as a β -like form.

INTRODUCTION

Multiple forms of DNA-dependent DNA polymerase activities have been described in a variety of eukaryotic cells. Two major enzymatic species, designated α and β , have been identified and classified in vertebrates according to their sedimentation coefficient, template specificity and NEM sensitivity (2).

DNA polymerase α , which is ubiquitous in growing cells, seems to be associated with DNA replication since, for example, it increases during the cell cycle in coincidence with the S phase and during liver regeneration (3, 4).

DNA polymerase β is less clearly related to DNA replication. Several lines of evidence suggest that this enzyme participates in the repair of DNA in phytohemagglutinin-stimulated lymphocytes (5), in U.V. irradiated neuronal

nuclei (6) and in the DNA repair synthesis which occurs during meiotic prophase (7). In a phylogenetic study on the distribution of DNA polymerase among various animal and vegetal species (8), L. Chang concludes that this form is widely distributed in multicellular animals from sponge to mammals; but surprisingly, it was not detected in Drosophila embryos (8, 9, 10).

In view of the relevance of this biological system, in which biochemical studies can be coupled to genetical analysis, we have reinvestigated the presence of the β DNA polymerase activity in this animal. Here we described three distinct DNA-dependent DNA polymerase activities in Drosophila melanogaster adult flies. The major form, extracted at low ionic strength and sedimenting at about 7S is NEM and 0.17 M KCl sensitive, uses essentially activated DNA and is slightly inhibited by the nucleotide analog ddTTP, thus resembling the vertebrates α DNA polymerase species. The second enzymatic activity, about 10% of the total activity, can be classified as a β -like form due to its NEM-resistance, template specificity, sensitivity to ddTTP, lower sedimentation coefficient (about 5S), and nuclear localization. A third minor activity, associated to the mitochondrial fraction and sedimenting at about 7S, differs from the α form because it is activated by 0.17 M KCl and from the β form for its sedimentation coefficient, NEM-sensitivity and template specificity.

MATERIALS AND METHODS

One day old wild type Canton S flies were used.

Chemicals

Synthetic polymer poly(dT).poly(rA) was from Miles Laboratories Inc., poly(dA.dT).poly(dA.dT) was from P-L Biochemicals Inc. Salmon sperm DNA was obtained from Sigma. Unlabeled deoxynucleoside-5'-triphosphates were purchased from Schwarz Mann. ^3H dTTP and ^3H dATP were purchased from New England Nuclear, ddTTP was from P-L Biochemicals Inc. Yeast alcohol dehydrogenase and Pancreatic DNase I were obtained from Worthington.

Preparation of "nicked" DNA

"Nicked" salmon sperm DNA was prepared by digestion of native salmon

sperm DNA as previously described (11).

Assay of DNA polymerase activity

Three reaction mixtures were used for the assays. Mixture I contained in a total volume of 300 μ l: 50 mM Tris HCl (pH 8.0), 90 μ g of "nicked" DNA, 6.6 mM KCl, 10 mM $MgCl_2$, 3.3 mM 2-mercaptoethanol, 0.16 mM each of dATP, dCTP, dGTP, 16 μ M 3H dTTP (specific activity of 3 to 5 $\times 10^5$ cpm/nmoles) and 0.1 to 1 unit of enzyme. Mixture II differs from I in that 3H dATP (specific activity of 5 $\times 10^5$ cpm/nmoles) was used as labeled triphosphate, ddTTP was added at a final concentration of 2.56 mM and $MgCl_2$ was 12.56 mM (12). Mixture III contained in a total volume of 150 μ l: 33 mM Tris HCl (pH 8.3), 0.4 mM $MnCl_2$, 13 mM KCl, 33 μ M 3H dTTP (specific activity of 3 to 5 $\times 10^5$ cpm/nmoles), 0.66 μ g of poly(dT).poly(rA), and 0.1 to 1 unit of enzyme. Assays were performed as previously described (11). One unit of enzyme activity is defined as the amount catalyzing the incorporation of 1 nmole of 3H dTTP into an acid insoluble product in 20 min in mixture I. NEM sensitivity was tested after a preincubation of the extracts with 6 mM NEM for 30 min at 4°C.

Preparation of extracts

All the operations were performed at 4°C. One gram of flies was suspended in 5 ml of 0.1 M Tris HCl (pH 7.6), 1 mM EDTA, 4 mM 2-mercaptoethanol, 5% glycerol (buffer A), homogenized in a Potter and sonified 10 sec six times in a Branson sonifier model J-17A using a microtip at maximum power. The homogenate was centrifuged at 12,000 $\times g$ for 20 min and the supernatant collected and centrifuged again in a Spinco Ti 50 rotor at 45,000 rpm for 1 hr (low ionic strength extract). The pellet of the first centrifugation was suspended in buffer A containing 1 M KCl and homogenized by sonication as described above. The homogenate was centrifuged in a Spinco Ti 50 rotor at 45,000 rpm for 1 hr and the supernatant collected (high ionic strength extract).

DEAE batch

The low and high ionic strength extracts were dialyzed over-night against a 0.05 M potassium phosphate buffer (pH 7.4) containing 25% glycerol, 2 $\times 10^{-3}$ M 2-mercaptoethanol and 5 $\times 10^{-4}$ M EDTA (buffer B). After dialysis

each extract was mixed with DEAE cellulose previously equilibrated with buffer B without EDTA (buffer C). The suspension was stirred for 20 min, centrifuged for 20 min at 27,000 x g and the supernatant fluid discarded. DEAE cellulose was resuspended in one volume of buffer C, poured into a glass column and washed with 3 volumes of buffer C. Almost all DNA polymerase activities were recovered from the DEAE with buffer C made 0.3 M in potassium phosphate pH 7.4. The active fractions were pooled, put in a dialysis bag and concentrated against Ficoll.

Preparation of nuclear and mitochondrial fractions

Nuclei were prepared essentially as described by Burckhardt and Birnstiel (13). 3 g of adult flies were ground in a mortar 3 times with 20 ml fractions of buffer B (pH 7.5) containing 100 mM sodium glycerophosphate, 50 mM glycine, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 10 mM 2-mercaptoethanol, 50 mM $NaHSO_3$. The homogenates were filtered through one layer of Miracloth. The nuclei were collected by centrifugation at 1000 x g for 5 min and the supernatant used for mitochondrial preparation. The nuclear pellet was suspended in 20 ml of the same buffer and centrifuged again at 1000 x g for 5 min. The pellet was suspended in 20 ml of buffer A and centrifuged 15 min at 12,000 x g. The pellet was homogenized as described above for whole flies except that 0.4% Nonidet-P40 detergent was added in the extraction at high ionic strength. All steps of nuclei preparation were monitored by phase-contrast microscopy.

The mitochondrial supernatant was centrifuged again twice 15 min at 1000 x g and the pellets discarded. The final supernatant was centrifuged for 15 min at 12,100 x g and the pellet suspended in 20 ml of buffer B and recentrifuged in the above conditions. The pellet was resuspended in 20 ml of buffer A and centrifuged for 15 min at 12,100 x g. The final mitochondrial pellet was homogenized as described for the final nuclear pellet.

Glycerol gradient sedimentation analysis

100 μ l of low and high ionic strength extracts or DEAE batch (diluted 1/3) were layered on a linear (10-30%) glycerol gradient containing in 0.2 M Tris HCl buffer (pH 7.6), 0.5 M KCl or 1 M KCl, 0.5 mM EDTA, 2 mM 2-mercaptoethanol. Yeast alcohol dehydrogenase was added as sedimentation marker and assayed according to Vallee and Hoch (14). Gradients were centrifuged in an SW

65K rotor at 50,000 rpm for 16 hrs. Fractions of 200 μ l were collected and assayed as described above.

RESULTS

DNA polymerase activity was determined in low and high ionic strength extracts of *Drosophila melanogaster* adult flies and analyzed by glycerol gradient centrifugation. Fig. 1 shows the sedimentation profile of DNA polymerase in a low ionic strength extract. Most of the activity sediments at about 7S and is completely inhibited by NEM; however a trace amount of DNA polymerase activity, sedimenting at about 5S and NEM-resistant, is also detectable. Since in many systems the low molecular weight form is more efficiently extracted at high ionic strength (15), the pellet of the first extract was extracted again with the same buffer containing 1 M KCl and then analyzed on glycerol gradient.

Figure 1 - Sedimentation analysis of a low ionic strength extract. 100 μ l sample was layered on a linear (10-30%) glycerol gradient containing 0.5 M KCl (see Methods). 25 μ l sample of each fraction was assayed for DNA polymerase activity in the reaction mixture I (O—O) and in the same mixture after preincubation with NEM (▲---▲) (see Methods). The arrow indicates the position of yeast alcohol dehydrogenase (7.4S) used as a marker. Migration was from right to left.

In the high ionic strength extract, two peaks of DNA polymerase can be separated (Fig. 2A and B). The peak sedimenting at about 7S is NEM-sensitive as is the major activity present in the low ionic strength extract, while the peak sedimenting at about 5S is NEM-resistant (Fig. 2A). The 5S peak uses very efficiently also poly(dT)poly(rA) as template-primer (Fig. 2B). The 5S value was estimated not only from the position of the ADH used as a sedimentation marker in the gradients but also from the comparison with DNA polymerase β from chicken embryos myoblasts and mouse run in parallel gradients (7, 16). The 7S peak for these properties resembles the α -DNA polymerase activity found in vertebrates, while the 5S peak shares some

Figure 2 - Sedimentation analysis of a high ionic strength extract. Experimental conditions were as described in Figure 1.

A) DNA polymerase activity was assayed in mixture I (O—O) and in the same mixture after preincubation with NEM (▲—▲).

B) DNA polymerase was assayed in the reaction mixture I (O—O) and using polydT.polyrA as template primer in mixture III after preincubation with NEM (Δ—Δ).

characteristics with the β -DNA polymerase activity, although its sedimentation coefficient is higher than the values reported for β enzymes in a variety of biological systems (2).

In order to better characterize these two DNA polymerase species their template-primer specificity has been studied. As shown in table 1 both enzymes prefer activated DNA, while native DNA is a very poor template. Synthetic polymers such as poly(dA.dT)poly(dA.dT) and poly(dT)poly(rA) are utilized with higher efficiency by the 5S peak.

Another criterion to distinguish the α and β DNA polymerase species is their differential sensitivity to the ddTTP (12). The effect of this nucleotide analog is tested on the two enzymatic forms fractionated on a glycerol gradient of a high ionic strength whole flies extract (Fig. 3). The α form is relatively resistant to the ddTTP (about 20% inhibition), while the β activity is severely inhibited (about 80% inhibition). Another property which is peculiar for the β -DNA polymerase is its nuclear localization; for this reason the intracellular distribution of the 5S peak has been studied. In Fig. 4A and B, a glycerol sedimentation analysis of extracts of

Table 1
Template specificities

Template	7S peak		5S peak	
	pmoles°			
Activated DNA	36.4	(100%)	29.12	(100%)
Native DNA	2.18	(5.9%)	2.8	(9.6%)
poly(dA.dT).poly(dA.dT)	3.54	(9.7%)	5.82	(19.9%)
poly(dT).poly(rA)	2.7	(7.4%)	9.6	(32.9%)

° total nucleotides incorporated in 20 minutes at 37°C.

The DEAE cellulose eluates (see Methods) from low and high ionic strength extracts were run on glycerol gradients and the most active fractions of the 7S and 5S peaks were pooled and 25 μ l samples were used in the assays for template specificity. The fractions of 5S peak were preincubated with NEM (see Methods) before the assays. Activated and native DNA were assayed in mixture I; poly(dT).poly(rA) in mixture III. Poly(dA.dT).poly(dA.dT) was assayed in a modified mixture III in which 0.66 μ g of poly(dA.dT).poly(dA.dT) substituted poly(dT).poly(rA) and 33 μ M dATP was present.

Figure 3 - ddTTP sensitivity of α and β DNA polymerase species. 100 μ l sample of a high ionic strength extract of whole flies was fractionated in the experimental conditions of Figure 2. DNA polymerase activity was assayed in mixture I (O—O), in the same mixture after preincubation with NEM (Δ — Δ), and in the presence of ddTTP in mixture II (O—O—O).

nuclear and mitochondrial fractions respectively is shown. In the nuclear fraction a peak is detectable sedimenting at about 5S which is NEM-resistant and which uses efficiently poly(dT)poly(rA) as template primer. This enzymatic form is thus identical to that extracted at high ionic strength from the whole flies (Fig. 2). In the mitochondrial fraction a major peak sedimenting at about 7S which uses activated DNA as template primer and NEM-sensitive is present. This activity, however, does not seem identical to the 7S peak extracted at low ionic strength from the whole flies, because the mitochondria-associated enzyme is two-fold stimulated at 0.17 M KCl while the former is more than 90% inhibited at this KCl concentration (data not shown). A trace amount of a 5S peak activity, NEM-resistant and which uses

Figure 4 - Sedimentation analysis of nuclear (A) and mitochondrial (B) extracts. A) 150 μ l sample of a nuclei extract was layered on a linear (10-30%) glycerol gradient containing 1 M KCl (see Methods). 50 μ l sample of each fraction was assayed for DNA polymerase activity in the reaction mixture I (O—O) in the same mixture after preincubation with NEM ($\blacktriangle$ --- $\blacktriangle$) and using polydT.polyrA as template primer in mixture III (Δ -•--- Δ). B) 150 μ l of a mitochondrial extract was analyzed on a glycerol gradient in the same experimental conditions described in panel (A).

poly(dT)poly(rA) as template, is also detectable. This activity is probably due to a nuclear contamination.

It would be noted that in the gradient of Fig. 4A, containing 1 M KCl in order to prevent protein aggregation which in nuclear preparations occurs

in 0.5 M KCl gradients, is not detectable any 7S activity.

On these bases it is possible to exclude in the nuclear preparation the presence of the 7S activity associated with the mitochondrial fraction but it is not possible to exclude the presence of the major 7S α -polymerase, being, as above mentioned, this enzyme almost completely inhibited by the KCl concentration (0.17 M) present in the assays.

DISCUSSION

Few examples are known among eukaryotes in which the presence of only one DNA polymerase activity, generally an α -like form, has been reported (8, 17, 18, 19). Brakel and Blumenthal (10) identified in Drosophila embryos three NEM-sensitive α -like DNA polymerase species sedimenting at 9S, 7.3S and 5.5S and suggest that endogeneous proteolysis converts the 7.3S into the 5.5S form. Data reported in this paper indicate that, in Drosophila adult flies, besides a major DNA polymerase activity, extracted at low ionic strength, that for its sedimentation coefficient, NEM and 0.17 M KCl sensitivity, template specificity and resistance to ddTTP can be classified as an α -like form, two minor activities sedimenting at about 5S and 7S respectively are also detectable.

The 5S peak, extracted preferentially at high ionic strength, is NEM-resistant, ddTTP sensitive, can use poly(dT).poly(rA) as template (although with lower efficiency compared to activated DNA) and seems localized mainly in the nucleus. It is very unlikely that the 5S DNA polymerase form described in this paper in adult flies can originate from the major 7S form as it occurs for the 5.5S species described in Drosophila embryos (10), since the two enzymes differ markedly in their properties, as discussed above. For the same reasons the 5S peak isolated in our experimental conditions in adult flies is different from the 5.5S peak described by Brakel and Blumenthal (10) in Drosophila embryos. Indeed, the 5S peak identified in adult flies shows many characteristics common to the β -DNA polymerase activity described in a variety of eukaryotes.

It is pertinent to note that the sedimentation coefficient of this polymerase form is not very close to the S values reported for other β -DNA

polymerases (2). This 5S value, however, does not change appreciable in all the experimental conditions we used: i.e., 0.5 M and 1 M KCl concentrations in the glycerol gradients; in crude extracts from total flies or after a purification step with DEAE-cellulose chromatography; in nuclear preparations.

Finally, the minor 7S form, distinguishable from the major 7S activity because it is activated by 0.17 M KCl, is found associated to the mitochondrial fraction. The properties of this activity, however, seem not identical, at least in these poorly purified preparations, to those reported in other systems for DNA polymerases γ , which several lines of evidence indicate as the mitochondrial-associated enzyme (20, 21). The present data do not allow us to exclude that this activity could derive from a contamination of spiroplasma which are known to be prokariotic parasites of Drosophila adult flies (22, 23).

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REFERENCES

1. Abbreviations: NEM, N-ethylmaleimide; ddTTP, 2',3' dideoxy-thymidine-5'triphosphate
2. Weissbach, A. (1977) Ann. Rev. Biochem. 46, 25-47
3. Spadari, S. and Weissbach, A. (1974) J. Mol. Biol. 86, 11-20
4. Baril, E.F., Jenkins, M.D., Brown, O.E., Lazlo, J. and Morris, H.P. (1973) Cancer Res. 33, 1187-1193
5. Bertazzoni, U., Stefanini, M., Pedrali-Noy, G., Giuliotto, E., Nuzzo, F., Falaschi, A. and Spadari, S. (1976) Proc. Natl. Acad. Sci. USA 73, 785-789
6. Hübscher, V., Kuenzle, C.C., Limacher, W., Scherrer, P. and Spadari, S. (1978) Cold Spring Harbor Symp. Quant. Biol. 43 in press
7. Grippo, P., Geremia, R., Locorotondo, G. and Monesi, V. (1978) Cell Diff. 7, 237-248
8. Chang, L.M.S. (1976) Science 191, 1183-1185
9. Karkas, J.D., Margulies, L. and Chargaff, E. (1975) J. Biol. Chem. 250, 8657-8663
10. Brakel, C.L. and Blumenthal, A.B. (1977) Biochemistry 16, 3137-3143

11. Grippo, P., Locorotondo, G. and Caruso, A. (1975) FEBS-Letters 51, 137-142
12. Waqar, M.A., Evans, M.J. and Huberman, J.A. (1978) Nucleic Acid Res. 5, 1933-1946
13. Burckhardt, J. and Birnstiel, M.L. (1978) J. Mol. Biol. 118, 61-79
14. Vallee, B.L. and Hoch, F.L. (1955) Proc. Natl. Acad. Sci. USA 41, 327-338
15. Craig, R.K. and Keir, H.M. (1974) in The Cell Nucleus, Busch. I. Ed., Vol. 5, pp. 35-66, Academic Press, London, New York
16. Grippo, P., Cossu, G. and Molinaro, M. (1977) Cell Diff. 6, 9-16
17. Loomis, L.V., Rossomando, E.F. and Chang, L.M.S. (1976) Biochim. Biophys. Acta 425, 469-477
18. Gardner, J.M. and Kado, C.I. (1975) Biochemistry 15, 688-697
19. Chang, L.M.S. (1977) J. Biol. Chem. 252, 1873-1880
20. Bolden, A., Pedrali-Noy, G. and Weissbach, A. (1977) J. Biol. Chem. 252, 3351-3356
21. Hübscher, U., Kuenzle, C.C. and Spadari, S. (1977) Eur. J. Biochem. 81, 249-258
22. C.R.C. Handbook of Microbiology (1977) I, 448
23. Tully, J.G., Whitcomb, R.F., Clark, H.F. and Williamson, D.L. (1977) Science 195, 892-894