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DNA POLYMERASES IN ISOLATED 'NUCLEAR MATRIX' OF EHRLICH ASCITES CELLS

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Isolated nuclei from Ehrlich ascites tumor cells continue a replicative-like in vitro DNA synthesis. Polymerase α is the major dNTP polymerizing enzyme in nuclei. Following complete achromatinization dNTP polymerizing activities are still associated with the residual structure termed 'nuclear matrix'. In contrast to DNA synthesis in native nuclei, 'nuclear matrix' DNA synthesis is mainly due to polymerase β-like activity.

Since the small amount (< 1 %) of residual DNA in 'nuclear matrix' isolated from pulse labelled cells is enriched in newly synthesized DNA [1-4] and since it could be demonstrated by autoradiography that DNA replication sites are likely to be anchored in the 'nuclear matrix' [4] it was suggested that it represents the major intranuclear site for eukaryotic DNA replication. Moreover, other nuclear functions depending on DNA synthesis (e.g. repair and recombination [4]) were also discussed in relation to 'nuclear matrix'. This gives rise to the question whether these functions are still associated with isolated 'nuclear matrix'. The presence of polymerase α activity in 'nuclear matrix' of regenerating rat liver cells has been shown previously [5,6]. In this paper we describe polymerase α and polymerase β activities in 'nuclear matrix' of Ehrlich ascites cells. In this system it has been shown recently that polymerase β is eightfold stimulated during autodigestion of DNA in nuclei which is the first step in 'nuclear matrix' isolation [7].

Material and Methods

Cells and Isolation of Nuclei: Ehrlich ascites cells were propagated and harvested as described [8,9]. The method of Mamaril et al. [10] was used to purify nuclei.

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Preparation of 'Nuclear Matrix': Isolated nuclei were digested with DNase I and extracted three times at 0°C with low-salt buffer, high-salt buffer and once with 1 % Triton X-100 in LS buffer. This was followed by washes in LS buffer [11].

Assay Conditions for DNA Synthesis: Isolated nuclei ($\sim 2 \times 10^7$) or 'nuclear matrices' (corresponding to 4×10^7 nuclei) were assayed in the standard assay mixture described recently [7]. ATP and inhibitors were present in concentrations indicated in the text. Rates of incorporation correspond to cold acid insoluble material of 50 µl aliquots ($\hat{=}$ 3.3×10^6 nuclei, 6.6×10^6 'nuclear matrices').

Density Shift Experiments: Cells suspended at $10^6 \times ml^{-1}$ in HBSS containing 10 µM BrdUrd and 10 µM FdUrd were incubated for 60 min at 37°C, centrifuged and resuspended in fresh HBSS containing BrdUrd/FdUrd and incubated for another 60 min. (^{3}H)-Labelled heavy marker DNA was prepared by introducing (^{3}H)BrdUrd (1 µCi x ml^{-1}, 33 Ci/mmole) during the second incubation. 'Nuclear matrices' were prepared from BrdUrd/FdUrd treated cells and submitted to the standard assay for DNA synthesis followed by lysis in buffer containing 0.25 % SDS, 20 mM EDTA, 150 mM $NaHCO_3$, pH 8.0, 1 mg x ml^{-1} of proteinase K (Merck, Darmstadt, F.R.G.). After incubation for 3 hours at 37°C the mixture was dialyzed for 48 hours against 0.1 N sodium hydroxide and sheared by ultrasonic power. Aliquots of 4 ml were mixed with 2.6 g of Cs_2SO_4. Centrifugation was in a 50 Ti rotor for 48 hours at 35,000 rev x min^{-1}. Cells containing the (^{3}H)-labelled heavy marker DNA were processed as described for 'nuclear matrices'. Marker DNAs were analyzed in parallel gradients. Fractionation of the gradients and scintillation counting was done as described previously [12].

Other Materials: Radiolabelled substances were products of the Radiochemical Center, Amersham, U.K. Deoxyribonucleoside triphosphates (dNTPs) were from Serva, Heidelberg, F.R.G. Di-deoxythymidine triphosphate (ddTTP) was from Boehringer, Mannheim, F.R.G., and N-ethylmaleimide (NEM) from Sigma, Munich, F.R.G. Polydeoxyadenylic deoxythymidylic acid (poly d(AT)) was a product of Miles, and 'activated' DNA was prepared according to [13].

Results

DNA Synthesis in Isolated Nuclei

Nuclei retain their capacity to incorporate (^{3}H)dTMP into DNA. The system is briefly characterized in Table I. ATP and the four dNTPs are prerequisites for optimal DNA synthesis. Addition of ddTTP which is a strong inhibitor for polymerases β and γ [reviewed: 14] has low inhibitory effect. In contrast, NEM which inhibits polymerases α and γ [reviewed: 14] strongly inhibits the rate of synthesis indicating that polymerase α is the major dNTP polymerizing enzyme in isolated nuclei which is consistent with a replicative-like DNA synthesis [8,9]. Fig. 1 shows the incorporation of (^{3}H)dTMP into the acid

Table I
Characteristics of Replicative-like DNA Synthesis in Isolated Nuclei

Complete System:	Percent of Control (Complete System)
ATP 4 mM; dNTPs 100 µM (except dTTP); (^{3}H)dTTP 0.33 µM; 15 min, 37°C	100
-ATP	36
-dNTPs	15
+ddTTP 50 µM	62
+ NEM 5 mM	12

precipitable material of freshly isolated nuclei and nuclei stored at 0°C in the absence of ATP and dNTPs for three days. The rate of DNA synthesis in nuclei stored at 0°C is even higher than that observed in freshly isolated nuclei. This shows that polymerase activity is stimulated by endonucleolytic processes [7,15] which points to an activation of polymerase ß driven DNA synthesis [7].

DNA Synthesis in Isolated 'Nuclear Matrix'

The characteristics of completely achromatinized nuclei ('nuclear matrix') from Ehrlich ascites cells has been described in detail else-

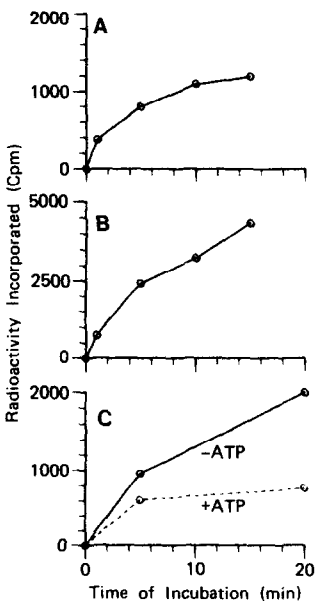


Figure 1
ATP stimulated DNA synthesis in freshly isolated nuclei (A); in isolated nuclei stored at 0°C for three days (B). DNA synthesis in extracted nuclei ('nuclear matrix') (C).

where [11]. The rate of incorporation of (^{3}H)dTMP per 'nuclear matrix' is in the same order of magnitude as observed in isolated nuclei (Fig. 1C). The characteristics of the dNTP polymerizing complex in 'nuclear matrix' is summarized in Table II. In contrast to freshly isolated nuclei ATP inhibits the rate of DNA synthesis in 'nuclear matrix'. The stronger inhibition of DNA synthesis by ddTTP and the weaker inhibition by NEM indicates that in 'nuclear matrix' more of the newly made DNA is synthesized by polymerase β than by polymerase α (Table II) although the salt conditions in the assay (100 mM) are suboptimal for polymerase β [6]. Addition of exogenous primer/template ('activated' DNA, poly d(AT)) stimulates DNA synthesis in 'nuclear matrix' only slightly (Table II). The relative activities of polymerases α and β are not significantly changed by this stimulation (Table II). This shows that 'nuclear matrix' polymerases are 'saturated' by endogenous primer/template.

Heat Stability of DNA Polymerases in Isolated 'Nuclear Matrix'

Isolated DNA polymerases α and β are rather sensitive to elevated temperature [16]. However, temperature sensitivity is significantly

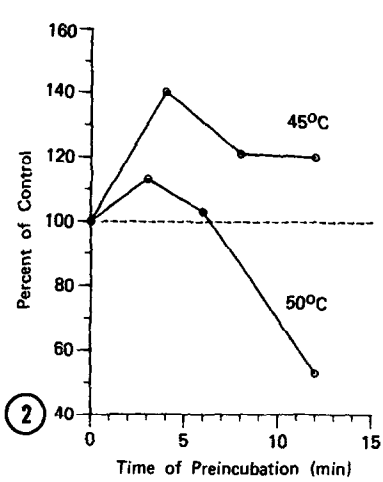
Table II
Characteristics of DNA Synthesis in Extracted Nuclei ('Nuclear Matrix')

Complete System: dNTPs 100 μM (except dTTP); (^{3}H)dTTP 0.33 μM; 15 min, 37°C	Percent of Control (Complete System) 100
+ DNase I (170 μg x ml^{-1})	25
+ATP 4mM	47 ± 12 (n = 4)
+ddTTP 50 μM	22 ± 16 (n = 6)
+ NEM 5 mM	46 ± 8 (n = 5)
+NEM (5 mM) + ddTTP (50 μM)	15
+NEM (5 mM) + ddTTP (50 μM) + ATP (4 mM)	0
+poly d(AT) (12 μg x ml^{-1})	126
+poly d(AT) (12 μg x ml^{-1}) + ddTTP (50 μM)	27
+poly d(AT) (12 μg x ml^{-1}) + NEM (5 mM)	36
+ 'activated' DNA (70 μg x ml^{-1})	125
+ 'activated' DNA (70 μg x ml^{-1}) + ddTTP (50 μM)	19
+ 'activated' DNA (70 μg x ml^{-1}) + NEM (5 mM)	41

reduced when polymerases are bound to 'activated' DNA [16]. After short (0-6 min) preincubations of 'nuclear matrix' at 45°C and 50°C respectively the DNA synthesis is even stimulated (Fig. 2). After preincubation at 45°C for more than 6 min the rate of synthesis remains also higher than in controls. Preincubations at 50°C for 12 min are needed to obtain temperature-induced inhibition in the order of 50 % (Fig. 2). This relative insensitivity of DNA polymerase activity associated with 'nuclear matrix' points to an altered enzyme characteristic due to complex formation with either 'activated' DNA or other protecting structures in the 'nuclear matrix'.

Nature of DNA Synthesis in 'Nuclear Matrix'

DNA strands growing in vivo were heavyendlabelled with BrdUrd. Since replicatively active DNA is preferentially co-isolated with 'nuclear matrix' [1-4] at least a fraction of these in vivo heavyendlabelled molecules must be present also in isolated 'nuclear matrix'. Thus, it could be investigated whether the radioactive precursor for in vitro DNA synthesis in 'nuclear matrix' is incorporated at 3'OH-ends in 'nicked' or 'gapped' 'nuclear matrix' bound bulk DNA (repair synthesis) and/or at heavy ends of DNA strands which were growing in vivo (continuation of in vivo replicative-like DNA synthesis). As shown in Fig. 3B the radioactive precursor is incorporated during in vitro DNA synthesis in the absence of inhibitors into the whole spectrum of DNA molecules separated according to their different degree of density-labelling in vivo. Since most of the radioactive precursor is incorporated into light bulk DNA and since the polymerase ß inhibitor ddTTP reduces the rate of incorporation into light chain DNA significantly (Fig. 3B) this shows that most of 'nuclear matrix' DNA synthesis reflects repair synthesis. This correlates with the expected role of DNA polymerase ß and with the fact that polymerase ß is the most active polymerase in 'nuclear matrix'



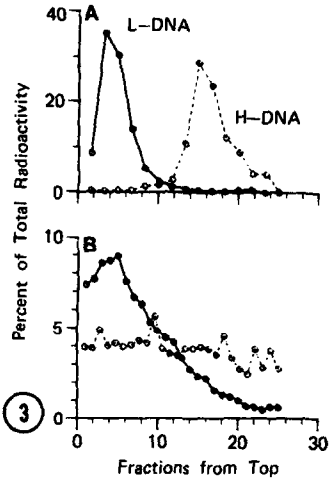


Figure 2

Effect of preincubation of 'nuclear matrix' at elevated temperature on the rate of incorporation of (^{3}H)dTMP into the cold acid insoluble material. Suspensions of 'nuclear matrices' (in LS buffer) were first incubated for various periods at elevated temperature (45°C and 50°C respectively) before they were submitted to the standard assay for in vitro DNA synthesis (Table II, complete system). Matrices which were only incubated under standard assay conditions for in vitro DNA synthesis served as 100 percent controls.

Figure 3

Experiments showing that most of the DNA synthesis in 'nuclear matrix' is due to polymerase β induced repair synthesis. (A) Position of light marker DNA (L-DNA) and in vivo heavy-endlabelled DNA (H-DNA) on alkaline Cs_2SO_4 density gradients. (B) 'Nuclear matrices' were isolated from BrdUrd prelabelled cells. DNA synthesis was stimulated in vitro by incubation in the complete system (see Table II) either in the absence of inhibitor (o—o) or in the presence of the polymerase β inhibitor ddTTP (o----o). Note that in the absence of ddTTP most of the radiolabel was incorporated into light 'nuclear matrix' DNA (o—o). Some radiolabel is shifted into molecules of higher density (strands which were growing in vivo) when polymerase β was blocked by ddTTP (o----o).

(Table II). Incorporation of radioactive precursor in the presence of the polymerase β inhibitor could be due to polymerase α-induced repair synthesis. However, some newly made DNA is density-shifted, indicating (^{3}H)dTMP addition at heavy ends. Thus it cannot be excluded that a portion of 'nuclear matrix' DNA synthesis is due to a continuation of strand growth initiated in vivo (replicative-like DNA synthesis) (Fig. 3B).

Discussion

The results indicate that dNTP polymerizing activities can be co-isolated with the 'nuclear matrix' complex of Ehrlich ascites cells. In contrast to the rat liver system [5,6] in vitro DNA synthesis in Ehrlich ascites cell 'nuclear matrix' is mainly due to polymerase β.

It is unlikely that the polymerase activities are unspecifically trapped in the complex during achromatinisation of nuclei: (a) Since soluble proteins are well extracted (e.g. 100 % of the histones [11]) the binding of the active polymerase molecules cannot be unspecific. (b) Since no primer DNA has to be added to detect the polymerase activity polymerase molecules in 'nuclear matrix' must be directly bound to these primer sites. Otherwise it would be difficult to explain how these sites could be extended by 'insoluble' polymerase molecules. (c) The altered enzyme characteristics of 'nuclear matrix' associated DNA polymerases (heat stability) point to their tight binding to either 'activated' DNA molecules or other macromolecules of the 'nuclear matrix'. Thus, from a-c it can be suggested that specific complexes comprising DNA primer/template and polymerase molecules are co-isolated with 'nuclear matrix'. Such complexes may involve genuine replication complexes and/or repair complexes formed during auto-digestion of DNA at newly generated 3'OH ends which is an inevitable process in the isolation procedure for 'nuclear matrix'.

References

1. Berezney, R. and Buchholtz, L. (1980) Exp.Cell Res. 132, 1-13.
2. Berezney, R. and Coffey, D.S. (1975) Science 189, 291-293.
3. Pardoll, D.M., Vogelstein, B. and Coffey, D.S. (1980) Cell 19, 527-536.
4. Vogelstein, B., Pardoll, D.M. and Coffey, D.S. (1980) Cell 22, 79-85.
5. Smith, H.C. and Berezney, R. (1980) Biochem.Biophys.Res.Commun. 97, 1541-1547.
6. Smith, H.C. and Berezney, R. (1982) Biochemistry 21, 6751-6761.
7. Shi Chanpu and Werner, D. (1982) Biochem.Biophys.Res.Commun. 108, 1624-1630.
8. Hershey, H.V. (1977) Biochim.Biophys.Acta 479, 246-255.
9. Hershey, H.V. (1977) Biochim.Biophys.Acta 479, 256-264.
10. Mamaril, F.P., Dobryjansky, A. and Green, S. (1970) Cancer Res. 30, 352-355
11. Müller, M., Spiess, E. and Werner, D. (1983) Eur.J.Cell Biol. 31, 158-166.
12. Werner, D., Krauth, W. and Hershey, H.V. (1980) Biochim.Biophys. Acta 608, 243-258.
13. Loeb, L.A. (1969) J.Biol.Chem. 244, 1672-1681.
14. Kornberg, A. in DNA Replication, pp. 204-208, W.H. Freeman and Company, San Francisco (1980).
15. Burgoyne, L.A., Waqar, M.A. and Atkinson, M.R. (1970) Biochem. Biophys.Res.Commun. 39, 254-259.
16. Duke, D.K., Kunkel, T.A., Seal, G. and Loeb, L.A. (1979) Biochim. Biophys.Acta 561, 369-382.