Volume 11 Number 1 1983 Nucleic Acids Research

Subspecies of DNA polymerase α from calf thymus with different fidelity in copying synthetic template-primers

Sabine Brosius[1], Frank Grosse and Gerhard Krauss*

Zentrum Biochemie, Abteilung Biophysikalische Chemie, Medizinische Hochschule Hannover, Konstanty-Gutschnow Strasse 8, 3000 Hannover, FRG

Received 16 November 1982; Accepted 4 December 1982

ABSTRACT

Three different subspecies of DNA polymerase α from calf thymus sedimenting at 9 S, 7 S and 5.7 S have been investigated with respect to their accuracy of in vitro DNA synthesis on poly(dA)(dT)$_{16}$ and poly d(AT) as template-primers. Our results indicate that the structure of DNA polymerase α has a strong influence on the accuracy of DNA synthesis. The 9 S enzyme shows a misincorporation frequency of about 1:100 000. An error rate of 1:15 000 is measured for the 7 S species. The 5.7 S enzyme for which an error rate of 1:3 000 is determined, has to be considered as error prone. Lowering the rate of DNA synthesis leads to a decrease in fidelity. The single stranded DNA binding protein from E.coli increases the accuracy of the 5.7 S and the 7 S enzyme by a factor of two. Mn^{2+} decreases the fidelity of all three subspecies in a concentration dependent manner.

INTRODUCTION

It can be estimated from the rates of spontaneous mutations in eucaryotic cells that the stable misincorporation of a base during DNA replication occurs at a frequency of 10^{-8} to 10^{-11} per base pair replicated (1). Although the respective contributions of DNA replication and of repair processes are not known, it is reasonable to assume that the fidelity of the replicative DNA polymerases is an important factor for maintaining the high accuracy of information transfer during cell divisions. In higher eucaryotes DNA polymerase α is responsible for the replication of the cellular DNA. The error rate of DNA polymerase α as measured by the in vitro replication of X 174 DNA or by the replication of synthetic homopolymeric deoxynucleotides is in the region of 10^{-3} to 10^{-5} per base pair replicated (2-5). From measurements of the reversion frequency of ΦX 174 amber mutants, the in vitro error frequency of reconstituted phage and E.coli

0305-1048/83/1101-0193$2.00/0

replication systems has been determined to be much lower (6-8). The error frequency of 10^{-7} - 10^{-8} measured for the E.coli DNA polymerase III replication system is of a similar magnitude as the spontaneous mutation rate in E.coli (6,9). Important factors for maintaining the high accuracy in these systems are the proofreading exonuclease activity of DNA polymerase III and the presence of accessory proteins of replication such as the single stranded DNA binding protein (SSB). Eucaryotic DNA polymerase α, however, does not possess a proofreading exonuclease activity. Furthermore, accessory proteins with precisely defined functions in eukaryotic DNA replication have not yet been detected.
Most biochemical investigations on the catalytic properties of DNA polymerase α have been hampered by the marked heterogeneity of this class of enzymes. Proteolytic breakdown and loss of subunits have made it difficult to establish the subunit composition of DNA polymerase α unequivocally. We have recently described the purification of two DNA polymerase α species from calf thymus to near homogeneity. A DNA polymerase α sedimenting at 9 S has a molecular weight of about 500 kd and contains polypeptides of 148 kd, 59 kd, 55 kd and 48 kd (10). Enzymes of similar subunit composition have been purified earlier from Drosophila (11), from rat liver (12) and from Hela cells (13). The 5.7 S enzyme is obtained as a mixture of polypeptides of 134 kd and 123 kd (14). Most probably these polypeptides are derived from the large subunit of the 9 S enzyme by proteolytic degradation. We also observe a 7 S DNA polymerase α, the subunit composition of which is not yet clear.

MATERIALS AND METHODS

Unlabeled polydeoxynucleotides were purchased from PL-Biochemicals. Unlabeled deoxynucleotides were from Boehringer-Mannheim. ^{3}H - labeled deoxynucleotides were from Amersham. GF-C filters were purchased from Whatman and from Machery-Nagel. DNAse I was from Worthington. The 9 S DNA polymerase α and the 7 S polymerase α were obtained from preparative ultracentrifugation runs as outlined in the purification procedure of Grosse & Krauss (10). The 5.7 S enzyme was prepared as described earlier (14). The SSB-protein from E.coli was prepared according to Krauss et al. (15).

The activity of the enzymes was assayed on activated calf thymus DNA as template-primer (14). One enzyme unit is defined as the amount of enzyme required to convert 1 nmole of dTTP into acid precipitable product in one hour at 37°C. The misincorporation assay with poly(dA)·$(dT)_{16}$ as template-primer contained 60 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 1 mM dithioerythritol, 0.3 mg/ml bovine serum albumine, 0.8% ampholine (LKB, pH 3.5-10), 30 µM dGTP, 30 µM dTTP, and 60 µM (nucleotide concentration) of poly (dA)·$(dT)_{16}$ (A:T = 5:1). dTMP and dGMP incorporation was measured by including ^3H - dTTP (40-60 cpm/pmol) or ^3H - dGTP (3000 - 5000 cpm/pmol), respectively, in parallel assays. The assay mixture was filtered prior to the assay through a syringe containing 8 layers of GF-C paper in order to obtain low blank values. 5 ul of enzyme (5-30 units) were incubated with 30-70 µl of the assay mixture at 37°C for a given time. Aliquots were then pipettet onto GF-C filter discs. Prior to use, the filter discs had been soaked in 0.1 M $Na_4P_2O_7$ for 15 minutes and dried. Filter discs were immersed in 10% cold trichloroacetic acid, 10 mM $Na_4P_2O_7$ for 10 minutes. Washing was continued with 5% trichloroacetic acid (20 x 2 ml) and ethanol (5 x 1 ml) on a small funnel. Discs were dried and counted. Misincorporation assays with poly d(AT) as template-primer contained poly d(AT) at a concentration of 20 µg/ml and 30 µM of dATP, dTTP, and dGTP each. Background radioactivity in the misincorporation assays was dependent on the batch of ^3H-dGTP used. Within one batch, blank values were reproducible within 10%.

RESULTS

The 9 S and 5.7 S DNA polymerase α species described previously (10,14) had specific activities of 65000 and 80000 units/mg, respectively. Another species, sedimenting at 7-7.5 S, has not yet been characterized with respect to its subunit composition. As judged from its specific activity of 25000 units/mg the 7 S species is 20 - 30 % pure. In the present study we have compared the fidelity of DNA synthesis of the three polymerase α species. We have used poly(dA)·$(dT)_{16}$ and poly d(AT) as template-primer systems. Misincorporation was determined by comparing the incorporation of the correct nucleotide(s) to that of the incorrect nucleotide dGMP. The results are summarized in table I. In these

Table I: Misincorporation frequencies of DNA polymerase α subspecies

	poly (dA)·(dT)$_{16}$			poly d(AT)		
	incorporation[a]			incorporation[a]		
enzyme	dTMP (pmol)	dGMP (fmol)	error frequency	dAMP and dTMP (pmol)	dGMP (fmol)	error frequency
9 S	697	7	1/100 000	1210	20	1/60 000
[b] + SSB (E.coli)	421	3	1/100 000			
7 S	1117	74	1/16 000	688	50	1/14 000
+ SSB (E.coli)	1035	33	1/31 000			
5.7 S	423	144	1/3 000	1170	280	1/4 200
+ SSB (E.coli)	334	55	1/3 000			

[a] With the same batches of enzyme subspecies, errors in the misincorporation frequencies are estimated to be ±15 %.
[b] SSB protein was present at 6 µg/ml.

experiments the correct and incorrect nucleotides were present at equimolar concentrations. The presence of the incorrect nucleotide had only a small effect on the incorporation rate of the correct nucleotide: For poly(dA)·(dT)$_{16}$, the K_M of dTTP is 8 µM, the K_I of dGTP is 100 µM.

The 9 S enzyme and the 5.7 S enzyme utilize poly(dA)·(dT)$_{16}$ with comparable efficiency (10,14). Utilization of poly(dA)·(dT)$_{16}$ by the 7 S enzyme was dependent on the batch of enzyme. In general, the 7 S enzyme had a stronger preference for poly(dA)·(dT)$_{16}$, probably due to the presence of the "D-enzyme" species described by Hesslewood et al. (16).

We performed several control assays to make sure that the misincorporation is really caused by the DNA polymerase α. The data in Table II show that the misincorporation is template dependent. Omission of (dT)$_{16}$ or poly(dA) did not lead to an incorporation that was above the background radioactivity level. Furthermore the radioactivity incorporated in the misincorporation assays

Table II: Control assays on the misincorporation of the 5.7 S enzyme

	poly (dA)·(dT)$_{16}$ acid precipitable radioactivity	
	^{3}H-dTMP (cpm)	^{3}H-dGMP (cpm)
standard assays	22004	694
- enzyme	71	221
+ 1 mM rATP	23100	670
+ 1 mM Ap_4A	21809	705
- poly (dA)	81	231
- (dT)$_{16}$	78	218
DNAse I treatment[a]	103	211

[a] Following incubation in the standard assays (see table I), 1µg of DNAse I was added and incubation was continued for further 20 min at 37°C.

was DNAse I sensitive. Contamination of the DNA polymerase α preparations with Terminal Deoxynucleotidyl Transferase (TdT) could seriously interfere with the misincorporation assays, leading to an apparent low accuracy. However, we can safely exclude the presence of TdT : The misincorporation is template - dependent, whereas incorporation catalyzed by TdT does not require a template. Furthermore, the misincorporation is not influenced by the presence of rATP or Ap_4A at concentrations up to 1 mM. Both compounds are inhibitors of TdT (17,18).

Our results clearly show that the smaller enzyme species are much less accurate than the large 9 S enzyme. The 5.7 S enzyme and the 9 S enzyme differ in their accuracy by more than one order of magnitude. With freshly prepared 9 S enzyme we always measure misincorporation rates of 1 : 10^5, which is at the limit of detection of our assay. Even at a tenfold excess of dGTP over dTTP the misincorporation frequency of the 9 S enzyme was found to be 1:30000 - 1:50000. Extrapolating back to equal concentrations of correct and incorrect nucleotide indicates an error rate of even less than 1:10^5 for poly(dA)·(dT)$_{16}$ as template-primer. Upon prolonged storage of the 9 S enzyme its accuracy drops to 1:30000 - 1:50000. The 7 S enzyme shows an intermediate accuracy. For different batches of the 7 S enzyme we measured error rates of 1:10000 - 1:30000. NaDodSO$_4$ gel

analysis of the 7 S enzyme reveals the presence of most of the polypeptides found also in the 9 S enzyme, the smaller ones being present in lesser amounts. Due to the impurity of the 7 S subspecies we could not quantitate the amount of these polypeptides. To our opinion the rather broad scatter of the error rates reflects the ill defined composition of this subspecies, since different batches may contain varying amounts of subunits. The 5.7 S enzyme has to be considered as an error prone polymerase. Since we have indications that the 5.7 S subspecies arises from the 9 S enzyme by proteolysis (10), we have tried to convert the purified 9 S enzyme to the 5.7 S species by treatment with trypsin. However, these experiments have not been successful. Most probably specific proteases are involved in the breakdown of the 9 S enzyme.

Dependence on dNTP concentration

We have performed one set of misincorporation assays with poly(dA)·$(dT)_{16}$ as template-primer, where the dTTP concentration was below the K_M values of 8 uM. At 2 uM of dGTP and dTTP each, the fidelity of both the 5.7 S and 7 S enzymes decreased (table III). Due to the lower incorporation levels measured at the low nucleotide concentrations we could only determine an upper limit of the error rate of the 9 S enzyme. Our results indicate that at least for the 5.7 S and 7 S subspecies a decreased rate of DNA synthesis results in an increased error frequency.

We also have investigated the influence of the concentration ratio of correct to incorrect nucleotide on the accuracy of the 5.7 S enzyme (table III). The data indicate a roughly linear dependence of the error rate on the nucleotide pool bias.

Furthermore we have looked for a possible turnover of the incorrect nucleotide during the misincorporation assay, which would be an indication of a proofreading nuclease activity. Following a 30 minutes incubation with the 5.7 S enzyme, the assay mixture was analyzed on PEI-sheets for the production of dGMP (data not shown). We did not observe a hydrolysis of dGTP to dGMP in the poly(dA)·$(dT)_{16}$ systems. These experiments would have allowed us to detect a breakdown of 0.5% of the dGTP present.

Influence of SSB protein from E.coli

It has reported earlier that the presence of the SSB protein enhances the fidelity of DNA polymerase by a factor of 2 - 20

Table III: Influence of dNTP concentrations on the misincorporation frequencies

DNA polymerase subspecies	poly (dA)·(dT)$_{16}$ Concentration of dNTPs in the misincorporation assay		[a]error frequency
	dGTP (uM)	dTTP (uM)	
5.7 S	180	30	1/300
	90	30	1/800
	30	30	1/3000
	30	300	1/20 000
5.7 S	2	2	1/800
7 S	2	2	1/3000
9 S	2	2	1/10 000

[a] determined in the standard misincorporation assay

(19). We could detect a two-fold increase in fidelity for the 7 S and 5.7 S enzyme in the presence of SSB protein (table I), Due to the high fidelity of the 9 S enzyme and the detection limit of our assay we were not able to measure a further decrease of the error rate upon addition of SSB protein.

Influence of Mn^{2+}

The three DNA polymerase α species do not differ in their sensitivity against the presence of Mn^{2+} (table IV). Mn^{2+} decreases

Table IV: Influence of Mn^{2+} on the misincorporation frequencies

	poly (dA)·(dT)$_{16}$			
	[a]0.2 mM Mn^{2+}		1 mM Mn^{2+}	
polymerase subspecies	incorporation of dTMP (pmol)	error frequency	incorporation of dTMP (pmol)	error frequency
9 S	445	1/70 000	56	1/15 000
7 S	312	1/10 000	33	1/5000
5.7 S	368	1/4000	44	1/800

[a] determined in the standard misincorporation assay with Mn^{2+} instead Mg^{2+}

the fidelity of the three enzyme subspecies. The effect is concentration dependent. At a concentration of 0.4 mM Mn^{2+}, where the rate of DNA synthesis is optimal, the fidelity decreases by only a factor of two. A stronger decrease in fidelity is observed at 1 mM Mn^{2+}, where the polymerization rate is decreased. These results confirm earlier observations of Sirover et al. (20).

DISCUSSION

Error rates ranging from 1/3000 to 1/150000 have been reported for DNA polymerase α (21). The DNA polymerase α preparation used in these studies, however, were not pure and have not been characterized with respect to their subunit composition. Our present results with two homogeneous subspecies of α-polymerase demonstrate that structure and subunit composition of this enzyme have a strong influence on the accuracy of DNA synthesis. We have shown in this paper that different subspecies of DNA polymerase α exhibit different abilities in faithfully copying synthetic template-primer systems. The existence of small DNA polymerase α species with decreased fidelity may be an explanation for the wide range of error rates measured up to now for this class of enzymes. Thus our results emphasize the necessity of characterizing the molecular structure of DNA polymerase α subspecies for the study of accuracies.

The 9 S enzyme is a remarkably accurate enzyme. The error rate of about $1:10^5$ found for this enzyme species is comparable to that determined for DNA poymerase I from E.coli with synthetic template-primer systems (21). This high accuracy of the 9 S enzyme is of considerable mechanistic interest, since the 9 S enzyme does not contain a proofreading exonuclease activity as do the DNA polymerases of E.coli. The mechanisms by which DNA polymerases can achieve high accuracy without proofreading are still a matter of speculation. Kinetic models have been proposed (22) as well as models including increase of nucleotide selection and base pairing specificity (23-25). Since none of these models has been verified experimentally as yet, it is difficult for us to explain the different accuracies of the DNA polymerase α subspecies on a molecular level. We assume that the degradation of the 148 kd subunit of the 9 S enzyme to the 134 kd and 123 kd

polypeptides contributes to the decrease of accuracy, possibly by impairing base selection. The loss of the smaller polypeptides may also decrease the fidelity. Little is known about the function of the smaller polypeptides. There is evidence that the small polypeptides help to sustain the processivity of DNA synthesis (26). For DNA polymerase α species from Drosophila it could be shown that different subspecies exhibit different processivity, the high molecular weight polymerase being the most processive one (27). Hopfield's energy relay model links processivity and accuracy, predicting that processive synthesis is more accurate than a distributive synthesis (22).
A central question arising from our findings relates to a possible in vivo occurence of the smaller polymerase species. It remains to be shown, whether the 7 S and 5.7 S enzyme subspecies only arise from uncontrolled proteolysis during the preparation or whether they can be also created by genetically programmed proteolysis during specific in vivo situations. Several reports in the literature point to the occurrence of error prone DNA polymerase α species in vivo. It has been speculated that during SOS-like repair (28), carcinogenesis (29), and cell senescence (30,31) error prone DNA polymerase α species could function as mutator enzymes. In the studies on ageing fibroblast cells a small 5 S polymerase α species with decreased fidelity of DNA synthesis has been shown to accumulate in late passage cells. This 5 S polymerase has a similar salt dependence of DNA synthesis as the 5.7 S subspecies from calf thymus.

ACKNOWLEDGEMENTS

We thank Dr. Jörg Langowski for critically reading the manuscript. The expert technical assistance of Maria Wehsling is gratefully acknowledged.

[1]Present address: Institut für Mikrobiologie, Ruhruniversität Bochum, D-4630 Bochum, FRG
*To whom reprint requests should be sent

REFERENCES

1) Drake,J.W., (1969) Nature 221, 1132
2) Seal,G., Shearmann,G.W. and Loeb,L.A. (1978) J.Biol.Chem. 254, 5229-5237

3) Salisbury,J.G., O'Conner,P.J. and Saffhill,R. (1978) Biochem.Biophys.Acta 517, 181-185
4) Krauss,S.W. and Linn,S. (1980) Biochemistry 19, 220-228
5) Kunkel,T.A. and Loeb,L.A. (1981) Science 213, 765-767
6) Fersht,A.R. (1979) Proc.Acad.Sci.U.S.A. 76, 4946-4950
7) Liv,C., Burke,R.L., Hibner,U., Barry,J. and Alberts,B.M. (1979) Cold Spring Harbor Symp.Quant.Biol. 43, 468-487
8) Sinha,N.K. and Haimes,M.D. (1980) ICN-UCLA Symp.Mol.Cell Biol. 707-723
9) Fowler,R.G., Degnen,G.E. and Cox,E.C. (1974) Mol.Gen.Genet. 133, 179-191
10) Grosse,F. and Krauss,G. (1981) Biochemistry 20, 5470-5475
11) Banks,G.R., Boezi,J.A. and Lehman,J.R. (1979) J.Biol.Chem. 254, 9886-9892
12) Mechali,M., Abadiedabat,J. and de Recondo,A.M. (1980) J.Biol.Chem. 255, 2114-2122
13) Lamothe,P., Baril,B., Chi,A., Lee,L. and Baril,E. (1981) Proc.Natl.Acad.Sci.U.S.A. 78, 4723-4727
14) Grosse,F. and Krauss,G. (1980) Nucl.Acid.Res. 8, 5703-5713
15) Krauss,G., Sindermann,H., Schomburg,U. and Maass,G. (1981) Biochemistry 20, 5346-5352
16) Hesslewood,I.P., Holmes,A.M., Wakeling,W.F. and Johnston,I.R. (1978) Eur.J.Biochem. 84, 123-131
17) Bhalla,R.B., Schwartz,M.K. and Modak,M.J. (1977) Biochem. Biophys.Res.Commun. 76, 1056-1061
18) Ono,K., Iwata,Y., Nakamura,H. and Matsukage,A. (1980) Biochem.Biophys.Res.Commun. 95,34-40
19) Kunkel,T.A., Meyer,R.R. and Loeb,L.A. (1979) Proc.Natl. Acad. Sci.U.S.A. 76, 6331-6335
20) Sirover,A.M., Dube,D.K. and Loeb,L.A. (1979) J.Biol. Chem. 254, 107-111
21) Loeb,L.A. and Kunkel,T.A. (1982) Ann.Rev.Biochem. 51 429-458
22) Hopfield,J.J. (1980) Proc.Natl.Acad.Sci.U.S.A. 77, 5248-5252
23) Kornberg,A. (1969) Science 163, 1410-1418
24) Sloan,D.L., Loeb,L.A., Mildvan,A.S. and Feldman,R.J. (1975) J.Biol.Chem. 250, 8913-8920
25) Gillan,F.D. and Nossal,N.G. (1976) J.Biol.Chem. 251, 5225-5232
26) Hockensmith,J.W. and Bambara,R.A. (1981) Biochemistry 20, 227-232
27) Villani,G., Fay,P.J., Bambara,R.A. and Lehman,I.R. (1981) J.Biol.Chem. 256, 8202-8207
28) Sarasin,A.R. and Hanawalt,P.C. (1980) J.Mol.Biol. 138, 299-319
29) Chan,J.H. and Becker,F. (1979) Proc.Natl.Acad.Sci.U.S.A. 76, 814-818
30) Linn,S., Kairis,M. and Holliday,R. (1976) Proc.Natl.Acad. Sci.U.S.A. 73, 2818-2822
31) Murray,V. (1981) Mechanisms of Ageing and Dev. 16, 327-343