The elongation of mismatched primers by DNA polymerase α from calf thymus

Bernd Reckmann, Frank Grosse and Gerhard Krauss

Zentrum Biochemie, Abteilung Biophysikalische Chemie, Medizinische Hochschule Hannover,
Konstaty-Gutschow-Strasse 8, 3000 Hannover, FRG

Received 24 May 1983; Revised 13 July 1983; Accepted 13 August 1983

ABSTRACT

The ability of the 9S and 5.7S DNA polymerase α subspecies from calf thymus in elongating a mismatched primer terminus has been investigated. With poly(dA) as template, the elongation rate for (dT)8dG, (dT)8dC and (dT)1GdT is 20-fold lower for the 9S enzyme and 5-fold lower for the 5.7S enzyme as compared to (dT)10. The presence of a second mismatch at the primer terminus reduces the elongation rate further by a factor of two. Exonucleolytic excision of the mismatches can be excluded. With (dT)8dG (dT)n as primer we show, that at least five T-residues have to follow the mismatch in order to establish the elongation rate of a perfectly paired primer. The KM value for (dT)10dG as primer is 400 nM as compared to 10 nM for (dT)10. Addition of Mn2+ increases the relative efficiency of elongation of the mismatched primers.

INTRODUCTION

The mechanism by which cells achieve a high accuracy of DNA replication is of considerable interest for studies of the biochemical basis of mutagenesis. For procaryotes, it could be shown that the error rates of reconstituted in vitro replication systems are of nearly the same magnitude as the spontaneous mutation rates in vivo (1,2). An important contribution to the high fidelity of the procaryotic replication systems comes from the 3'-5' exonucleases proofreading activity of the DNA polymerases, that provides a mean of excising erroneously incorporated nucleotides (3). The situation in eucaryotic systems is less well understood. Eucaryotic DNA polymerases do not contain a proofreading activity. Consequently the fidelity of this enzyme class has been determined to be lower than that of the procaryotic systems (4,5). An important aspect of the error production by eucaryotic DNA polymerases is the way these enzymes deal with erroneously incorporated nucleotides. Two alternative pathways could operate after
A misinsertion has occurred. The enzyme may dissociate from the template or elongation of the error may occur during the processive action of the polymerase. If dissociation occurs after misincorporation, the enzyme will have to reinitiate synthesis on a mismatched primer terminus. In the present paper we have studied the elongation of synthetic mismatched primers by the 9 S and 5.7 S DNA polymerase α from calf thymus (6,7). The latter enzyme has been shown earlier to be more inaccurate in copying homopolymeric templates (8). We show that elongation of a mismatched primer can occur by both enzyme species, the 5.7 S enzyme being more efficient, however.

MATERIALS

The 9 S and 5.7 S DNA polymerase α subspecies were purified from calf thymus as outlined earlier (6,7). Terminal deoxynucleotidyl transferase (TdT) was prepared from the same source. Ribonuclease U2 and alkaline phosphatase were from Boehringer. Poly(dA) (500-1000 nucleotides chain length), (dT) 10, (dT) 8G, (dT) 8C, (dT) 10dGdT, and (dT) 10dCdT were purchased from PL-Biochemicals. The oligonucleotides were checked prior to use by HPLC-chromatography for homogeneity. All mismatched primers used were homogeneous to at least 98%. (dA) 40 was prepared from (dA) 6 by elongation with TdT. Radiocative nucleotides were from Amersham. DE-81 and GF-C paper was from Whatman. All other reagents used were of the highest commercially available purity.

METHODS

The elongation of mismatched primers was assayed in 50-150 μl of 50 mM Tris-HCl pH 7.8, 2 mM dithioerythritol, 2.5 mM MgCl₂, 1.6% Ampholine, 50 μM poly(dA) (nucleotide concentration), 0.7-1.0 μM primer molecules, 50 μM ³H-dTTP (10-30 cpm/pmol) and 2-10 units of DNA polymerase α (one units is defined as the amount of enzyme which incorporates one nmole dTMP in one hour at 37°C with activated DNA as template primer). Following incubation at 37°C, aliquots were withdrawn and pipetted onto GF-C and DE-81 paper. With GF-C paper circles the acid precipitable radioactivity was determined as outlined by Fasiolo et.al (9). DE-81 paper circles were washed with 0.2 ml of 0.4 M NH₄HCO₃ (3 times) and then dried.
and counted.

\((dT)_{10}^nGnG\) was synthesized by the terminal addition of GTP to
\((dT)_{10}^n\) by TdT. The reaction mixture contained 100 mM K-cacodylate
1 mM CoCl₂, 0.2 mM dithioerythritol, 100 μM \((dT)_{10}^n\), 1 mM GTP, and
50 units of TdT. Following incubation for 30' at 37°C, the re-
action products were separated by HPLC (Whatman Partisil 10 SAX
column) using a gradient from 0.001 to 0.3 M K-phosphate pH 6.3
in 60% formamide.

\((dT)n^32P(dT)n\) was obtained by the terminal addition of
\([\alpha^32P]dTTP\) (1000 μM) to \((dT)n^8dG\) (100 μM) by TdT. The reaction
was stopped by heating to 80°C for 3' as soon as 2-3 T residues
had been incorporated. Poly(dA) was then added and the reaction
mixture was precipitated by the addition of 3 volumes of ethanol.
The poly(dA) \cdot (dT)n^8dG(dT)n was dissolved in H₂O and was replicated
by the 9 S enzyme (5 units) with cold dTTP as substrate (see
above).

After incubation at 37°C the reaction was stopped and the sample
was electrophoresed with 20 V/cm in 20% polyacrylamide gels con-
taining 8 M urea (10). Products were identified by autoradio-
graphy on Kodak XAR 5 x-ray film using an intensifying screen.

\((dT)n^8[32P]rnG\) was obtained by the addition of \([\alpha^32P]rGTP\) (40 μM)
to \((dT)n^8(80 μM)\) by TdT following RNAse U₂ treatment and incuba-
tion with alkaline phosphatase.

RESULTS

Kinetics of mismatch elongation

The assay system for the elongation of mismatched primers con-
tained poly(dA) as template and oligodeoxythymidylates with
various noncomplementary bases at or near the 3' -OH end. The
time course of elongation of \((dT)n^8dG\) by the 9 S enzyme as
compared to the elongation of \((dT)n_{10}^n\) is given in fig. 1. The
elongation of the mismatched primer occurs at a low, but easily
detectable rate. The elongation is linear with the time for the
first 30'. The elongation rate is about 5% of that observed for a
correctly paired primer. A plateau is reached after about 60'.
Upon subsequent addition of further enzyme, the incorporation of
dTTP is only slightly increased (fig.1). Addition of \((dT)n_{10}^n\)
however, leads to a burst of dTMP incorporation, indicating that
the enzyme has not been inactivated during incubation.
The size of the reaction products was determined by denaturing polyacrylamide gel electrophoresis. The elongation with poly(dA) 
(dT)_{10} show a chain length of the products of 40-70 nucleotides. 
This corresponds to the average gap size indicating that the product length is inversely proportional to the primer to template ratio. In contrast, using poly(dA)·(dT)_{8}dG as template primer, product molecules of 500 to 1000 nucleotides length are observable independent from varying template to primer ratios 
(data not shown).
This indicates that only few mismatched primers are utilized by the enzyme. Those are elongated until the template strand is fully copied. Utilization of further mismatches is then inhibited. 
Elongation of different mismatched primers
The elongation rates of different mismatched primers are summarized in table I. There is no difference whether the noncomplementary nucleotide at the 3'-OH terminus is dGMP, rGMP or dCMP. 
The presence of two noncomplementary nucleotides at the 3'-end reduces the elongation rate further by a factor of about two. Furthermore, the elongation rate of a mismatched primer is not increased, when a complementary nucleotide is present at the
Table I: Elongation of mispaired primer termini by the 9 S and 5.7 S DNA polymerase α.

| Primer          | Enzyme species | 9 S | 5.7 S |
|-----------------|----------------|-----|-------|
|                 |                |     |       |
| (dT)₁₀          |                | 100 | 100   |
| (dT)₈dC        |                | 5±1 | 20±3  |
| (dT)₈dG        |                | 5   | 20    |
| (dT)₁₀dGdT     |                | 5   | 20    |
| (dT)₁₀dCdT     |                | 5   | 20    |
| (dT)₁₀rG       |                | 5   | 20    |
| (dT)₁₀rGrG     |                | 2±0.5 | 10±2 |

a determined in the standard assay from the initial linear part of the incorporation kinetics. The elongation rate of (dT)₁₀ was set to 100%.

b variation in the elongation rates as obtained for different enzyme batches.

3'-side of the mismatch.

The influence of correct base pairs flanking the 3'-side of the mismatch has been investigated as follows: (dT)₈dG was elongated with [³²P]dTTP to form a primer of the structure (dT)₈dG[³²P](dT)ₙ, where n was 2-10. This primer was used in the replication assay as substrate for the 9 S polymerase. Fig. 3 demonstrates that primers where n>5 were completely elongated by the polymerase to yield products the length of which corresponds to the length of the template molecule. Those primers with n=1-5, however, were not elongated. This result shows that more than five correctly paired bases have to follow the mismatch in order to be elongated as efficiently paired primer. The observation, that during longer incubation times, the primers with n=1-5 are not elongated, is ascribed to a low affinity of the enzyme to these primers: The Kₘ-value for (dT)₁₀ is 10 nM, that for (dT)₈dG is about 400 nM (data not shown).

It is critical to the elongation of mismatched primers to exclude
the presence of a nuclease and of terminal deoxynucleotidyl transferase activity in the DNA polymerase α preparations used. We can safely exclude the presence of both enzyme activities in our polymerase preparations. When poly(dA) is omitted from the reaction mixture, incorporation of dTMP into acid precipitable material is neither observable with matched nor with mismatched primer. Furthermore, the incorporation of dTMP is not influenced by the presence of rATP at concentrations up to 1 mM. rATP has shown to be an inhibitor of TdT (11). The interference of a nuclease activity with our experiments was excluded by nearest neighbour analysis (12) and by the observation that the label in (dT)$_8$[$^{32}$P]dG is found in a high molecular weight product after elongation with nonradioactive dTTP by the polymerase (fig 2). This holds for both, the 9 S and 5.7 S DNA polymerase α. Fig. 2 shows again that only few mismatched primers are utilized by the enzyme and that the elongation leads to full length copies of the template (dA)$_{40}$, exactly as was shown for poly(dA) (see above).

9 S DNA polymerase α vs 5.7 S DNA polymerase α.

We have previously shown that a 5.7 S DNA polymerase α subspecies can be isolated from calf thymus, that has a decreased fidelity in copying synthetic templates (8). The data in table I show that the 5.7 S enzyme has a strongly increased ability in elongating mismatched primers as compared to the 9 S enzyme.

Influence of Mn$^{2+}$

The influence of Mn$^{2+}$ on both the elongation of a correct primer and on the mispaired primers is illustrated for the 9 S enzyme in fig 4. For (dT)$_{10}$, the dependence on the Mn$^{2+}$ concentration
Fig. 3: Elongation of \((dT)_8dG\) \((dT)_n\).
Electrophoresis of the reaction products \((dT)_8dG^{[32P]}\) \((dT)_n\) was prepared from \((dT)_8dG\) with TdT and was then elongated by the 9 S enzyme in the standard assay. Products were analyzed on a denaturing polyacrylamide gel.

(A) \((dT)_8dG^{[32P]}\) \((dT)_n\) as prepared by the TdT reaction.
(B)-(E) Products after 15', 30', 60' and 90' incubation. These assays contained only one fifth of the radioactively labeled primer applied to lane A.

follows a distinct maximum at about 0.6 mM Mn\(^{2+}\), with a significantly lower elongation rate at Mn\(^{2+}\) \(\gg\) mM.

A similar dependence is not observed for the mismatched primers. It is remarkable that the elongation of poly (dT)\(_{10}\)dGdT at Mn\(^{2+}\) concentrations \(\gg\) mM is nearly as efficient as that of (dT)\(_{10}\). Furthermore, a difference in the elongation rates between (dT)\(_{10}\)dGdT and (dT)\(_{10}\)dCdT is observable, in contrast to the situation in the presence of Mn\(^{2+}\) (table I). Essentially the same results are obtained when 2 mM Mg\(^{2+}\) is present in addition to Mn\(^{2+}\).

DISCUSSION

We have shown that DNA polymerase \(\alpha\) is able to initiate replication on mismatched primers. As compared to perfectly paired primers, the elongation rate is considerably slower. Only few of the mismatched primers are utilized in the elongation reaction and saturation of elongation is achieved soon. Further utilization of the remaining mismatched primers is then strongly suppressed. This behaviour is ascribed to a weak affinity of the polymerase for a mismatched primer. The \(K_M\) value for a mismatched primer is by nearly two orders of magnitude higher as compared
Fig. 4: Elongation of mismatched primers by the 9 S enzyme in the presence of Mn$^{2+}$. The elongation of the primers given was investigated in the standard assay, by replacing Mg$^{2+}$ by Mn$^{2+}$.

to a perfectly paired primer. We suppose that those template strands, where elongation of a mismatch has occurred, have been completely copied at the beginning of the saturation phase. This process may provide primer termini that cannot be elongated further, that however still represent strong binding sites for the polymerase. Those primer termini could thus provide a sink for the polymerase. The elongation rate measured for the mismatched primers is a composite of the rate constants for the addition of the first nucleotide to the mismatch and of the rate constants for the addition of further nucleotides. Most probably the addition of the first nucleotide(s) to the mismatch is the rate limiting step of the elongation reaction observed. Once the mismatch has been elongated by 5-6 nucleotides, the presence of a mismatch is no longer recognized by the enzyme and elongation will then proceed with an efficiency corresponding to the elongation of a perfectly paired primer. The data in table I show that one correct nucleotide following the mismatch is not sufficient to increase the elongation rate. Rather at least 5-6 complementary nucleotides have to follow the mismatch in order to provide a substrate.
for elongation that is utilized as efficiently as a perfectly paired primer. This observation reflects either the requirement at the active site of the enzyme for 5-6 perfect basepairs in the template primer complex, or the requirement of a certain thermodynamic stability of the template primer complex near the 3'-OH end.

The elongation of a mismatch by correct bases leads to a substrate that allows a faster incorporation of further correct bases. Thus one would expect an initial sigmoidal curve in fig. 2. The failure to detect a sigmoidal behaviour may be either due to an insufficient sensitivity of our method in following the first steps of mismatched primer elongation or due to the fact that every initiation event leads to a processive step with an incorporation of 10-20 nucleotides. Studies of the incorporation of the first nucleotide have to be performed with fast kinetic techniques e.g. quenched flow (work in progress).

The 5.7 S enzyme elongates a mismatch much more efficiently than the 9 S enzyme. We have shown earlier, that the 5.7 S enzyme is more error prone in copying synthetic templates as compared to the 9 S enzyme (8). The present results are in accordance with our earlier observations. Evidently the 5.7 S enzyme exhibits a relaxed specificity both towards the newly incoming nucleotide and towards the primer end to be elongated. The substitution of Mg$^{2+}$ by Mn$^{2+}$ has a profound effect on the relative efficiency of elongation of the mismatched primers. Whereas the elongation of a perfectly paired primer is strongly decreased upon increasing the Mn$^{2+}$ concentration, a similar effect is not observable for the mismatched primers. It is noteworthy that the presence of a mismatch in (dT)$_{10}$dGdT is no longer recognized by the enzyme at high Mn$^{2+}$. This observation supports earlier results on the influence of Mn$^{2+}$ on the fidelity of DNA polymerases (13,14). Mn$^{2+}$ seems to release the stereochemical constraints at the active site of the enzyme with respect to proper base pairing between primer and template. It is important to note that this influence of Mn$^{2+}$ is observed also in the presence of Mg$^{2+}$. In conclusion, the mutagenic potential of Mn$^{2+}$ is expressed also in an increased ability to elongate a mismatched primer.

A main conclusion concerning the mechanisms of error production
by DNA polymerase α is that this enzyme is indeed capable to initiate synthesis on a mismatched primer. However, if enzyme is limiting and if correctly paired primer termini are also available, the elongation of the mismatch is unlikely to occur. This is due to the low affinity of the enzyme for the mismatched primer terminus. Errors during replication by DNA polymerase α will mainly occur during processive synthesis, where, following incorporation of a noncomplementary nucleotide, the enzyme does not dissociate, but elongates the error within a processive reaction cycle (5). If, however, dissociation follows misincorporation, elongation is much more unlikely. In this case, repair of the mismatched primer terminus might be favoured.

ACKNOWLEDGEMENTS

The expert technical assistance of Maria Wehsling and Kornelia Fischer is gratefully acknowledged. This work was supported by a grant of the Deutsche Forschungsgemeinschaft to G.K.

REFERENCES

1) Fersht, A.R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4946 - 4950
2) Sinha, N.K. & Haimes, M.D. (1981) J. Biol. Chem. 256, 10671 - 10683
3) Kunkel, T.A., Eckstein, F., Mildvan, A.S., Koplitz, R.M. & Loeb, L.A. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6734-6738
4) Kunkel, T.A. & Loeb, L.A. (1981) Science 213, 765 - 767
5) Grosse, F., Krauss, G., Knill-Jones, J.W. & Fersht, A.R. (1983) EMBO J. 2, 1515 - 1519
6) Grosse, F. & Krauss, G. (1981) Biochemistry 20, 5470 - 5475
7) Grosse, F. & Krauss, G. (1980) Nucleic Acids Res. 8, 5703-5713
8) Brosius, J., Grosse, F. & Krauss, G. (1983) Nucleic Acids Res. 11, 193-202
9) Fasiolo, F., Befort, N., Boulanger, Y. & Ebel, J.P. (1970) Biochim. Biophys. Acta 217, 305 - 317
10) Maniatis, T., Jeffrey, A. & v.d.Sande, H. (1975) Biochemistry 14, 3787 - 3790
11) Bhalla, P.B., Schwartz, M.K., modal, M.J. (1977) Biochem. Biophys. Res. Commun. 76, 1056 - 1061
12) Conoway, R.C. & Lehman, I.R. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 4585 - 4588
13) Sirover, A.M., Dube, D.K. & Loeb, L.A. (1979) J. Biol. Chem. 254, 107 - 111
14) Goodman, M.F., Kenner, S., Guidotti, S. & Branscomb, E.W. (1983) J. Biol. Chem. 258, 3469 - 3475