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On the fidelity of DNA polymerase α: the influence of α-thio dNTPs, Mn^{2+} and mismatch repair

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ABSTRACT

The Φ X174am16 revertant system has been used to investigate the influence of α-thio-dNTPs and of Mn^{2+} on the fidelity of the 9S DNA polymerase α from calf thymus. Upon substituting dGTP by α-thio-dGTP during the in vitro replication, a nearly tenfold decrease in the frequency of G:G and G:T mispairs is observed. The formation of all other mispairs is not changed in the presence of the corresponding α-thio-dNTP. Mn^{2+} at concentrations of 0.5 mM does not influence the frequencies of the mispairs. The expression rate of errors formed during in vitro replication in the (-) strand has been determined for all mispairs detectable in the Φ Xam16 system. The (-) strand expression of G:T, T:T and C:T mismatches is about 50%, whereas for A:G, G:G and C:A mismatches it is clearly below 50%. We conclude that the different base-base mismatches are repaired with different efficiencies.

INTRODUCTION

The reversion frequencies at the amber codon TAG of mutant Φ X174 DNA have been frequently utilized for measuring the frequencies of errors produced during in vitro replication of Φ X174 DNA by isolated DNA replication systems (for a review see (1)). From this assay system, a rather detailed picture of the fidelity of DNA polymerases has been achieved. The procaryotic DNA polymerases could be shown to replicate Φ X174 single-stranded DNA with high fidelity (2,3). The inclusion of α-thio-dNTPs during the in vitro replication made it possible to determine the contribution of the 3'-5' exonuclease activity to the overall replication fidelity (4). The eucaryotic DNA polymerases have been found to be rather error prone, due to a lack of a 3'-5' exonuclease proofreading activity (5,6).

The frequencies of specific mispairs formed during in vitro replication can be obtained best from the X174 am16 revertant

assay that has been introduced by Fersht & Knill-Jones (7). Due to the existence of different reversion pathways, the frequencies of seven mutations arising from G:T, G:G, G:A, T:T,T:C,C:A and A:G mispairs can be determined in this system. In an earlier work we have shown that the various mispairs are formed with strongly different frequencies during the in vitro replication by the 9S DNA polymerase α from calf thymus (6). We now have used the am16 revertant system to study the influence of Mn^{2+}, pyrophosphate and α-thio-dNTPs on the fidelity of the 9S DNA polymerase α. Furthermore, we have determined the expression rates for the different mispairs in the E.coli host.

MATERIALS AND METHODS

The 9S DNA polymerase α was purified from calf thymus glands as outlined earlier (8). Its specific activity was about 50 000 units/mg. One unit is defined as the amount of enzyme, that catalyzes the incorporation of 1 nmol of dTMP into acid insoluble material at 37°C in 1 h with activated calf thymus DNA as template primer. The ΦX174am16 phage and the host strains E.coli C(F^-), CQ_2(F^-,supF) and C600 (F^-,thr,leu,thi,lacY,tonA,supE) were gifts from A.Fersht (Imperial College London). The plus strand of ΦX174am16 phage was purified as described (9). Unlabeled deoxy- and ribonucleoside triphosphates were products of Boehringer (Mannheim). The oligonucleotides 5'-CCCAGCCTGAATCT-3', 5'-CCCAGCCTCAATCT-3', 5'-CCCAGCCTTAATCT-3', 5'-CCCAGCCGAAATCT-3', 5'-CCCAGCATAAATCT-3', 5'-CCCAGCGTAAATCT-3', 5'-CCCAGCCCAAATCT-3' were synthesized as described (10). α-thio dNTPs were a kind gift of Prof.F.Eckstein, Göttingen.

Replication of ΦX174 single-stranded DNA

ΦX174am16 single-stranded DNA (0.1-1.0 pmol) was replicated for 90 min at 37°C in a 50 µl reaction mixture, containing 20mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 60 mM potassium acetate, 2.5 mM dithiothreitol, bovine serum albumine (0.1 mg/ml), 5-20 units of DNA polymerase α and 100 µM each of dATP, dGTP, dCTP and dTTP. Replication under pool bias conditions was carried out as described (6). When the replication was initiated by the primase activity, the replication mixture contained additionally 0.1 mM of each of the four NTPs.

The oligonucleotides spanning the am16 sequence were annealed to the ΦX174 single-stranded DNA at a tenfold molar excess at 45°C. $d(GC)_3$ was annealed at a 500-fold excess at 37°C. The extent of replication was followed by neutral and alkaline agarose gel electrophoresis of the replication products as outlined earlier (11). Furthermore, $[^{32}P]$ labeled dTTP was included in a parallel assay and the acid insoluble radioactivity was measured.

Fidelity measurements were performed as described by Fersht & Knill-Jones (7) and by Grosse et al. (6). Following replication, the DNA polymerase α was inactivated by heating to 60°C for 10 min. Spheroplasts of E.coli C600 were transfected with 0.05-0.2 fmol and 50-100 fmol of replicated DNA to measure the expression of the progeny and revertant phages, respectively. After incubation at 30°C for 10 min, the spheroplasts were mixed with soft agar containing E.coli CQ2 for the expression of the unchanged progenies, and with soft agar containing E.coli C for the expression of the revertants. The mixtures were plated on L-agar. E.coli CQ2 was incubated at 37°C and E.coli C at 30°C.

The reversion frequencies of in vitro replication assays in the absence of a nucleotide pool bias were evaluated by characterizing and counting the individual phenotypes on the revertant plates. Plaques were taken from the revertant plates and were replated on E.coli C at different temperatures. Incubation at 39°C allows to differentiate between wildtype-like phages (large plaques), ts 38 (small plaques) and ts 34/35 (no plaques). The wildtype-like phages were replated again at 42.5°C. Wildtype and pseudo-wildtype phages yield large plaques, ts 43 phages yield small plaques and ts 42 phages do not grow under these conditions. Phages of the ts 34/35 group were replated at 34.5°C. The ts 35, but not the ts 34 phages will grow at this temperature.

The specific reversion frequencies were calculated by relating the numbers of the specific revertant plaques to the numbers of plaques scored upon plating on E.coli CQ_2. For the rarest errors, the reversion frequencies determined are based on the scoring of at least five specific revertant plaques.For the more frequent errors, about 20 plaques were scored. The reversion frequencies were corrected for the expression of the specific mispairs in order to obtain the error rates of the in vitro replication.

Expression of errors in the (-) strand

The seven oligonucleotides listed in the Materials section were hybridized to ΦX174 single-stranded DNA and were then elongated either by DNA polymerase α or E.coli DNA polymerase I (Klenow fragment) to yield the RFII form. The seven heteroduplexes were transfected and propagated in E.coli C600. The progenies were then used to transfect E.coli C and E.coli CQ_2. Plaques were scored on the suppressor and wildtype host and the expression rate of the mispair, corresponding to the specific revertant, is then calculated from the ratio revertant/(am16 + revertant).

RESULTS

Accuracy under conditions of biased and unbiased nucleotide pools

The replication of the single-stranded ΦX174 DNA was initiated either by the primase activity of the DNA polymerase (12) or by a $d(GC)_3$ primer. In the latter case, a complete conversion to the RFIII form was observed in the presence of a 500 fold molar excess of the primer over the template strand. The choice of $d(GC)_3$ as a primer was due to the presence of a complementary sequence that is located 69 nucleotides apart from the am16 codon. For all experiments described in the present work we used freshly prepared DNA polymerase for the in vitro replication. With freshly prepared enzyme we observed a linear increase of the reversion frequency with increasing pool bias only up to biases of 3:1. With higher biases, a saturation behaviour shows up (fig.1). This is in contrast to our earlier work, where we had obtained with a three month old polymerase a linear relationship between reversion frequency and nucleotide pool for biases up to 30:1. The processivity of the DNA polymerase α used was in the range of 10-20 as determined both for ΦX174 and poly(dT) templates. Increasing the the pool bias influences the processivity only marginally (Frank Grosse, submitted to publication). Thus processivity cannot account for the poor pool bias response.
We thus were obliged to characterize the temperature sensitivity of the revertant phages obtained from in vitro replication at equimolar concentrations of all four nucleotides. The uncertainty in the error rates determined this way is larger as compared to the pool bias method. Furthermore, only the sum of the fre-

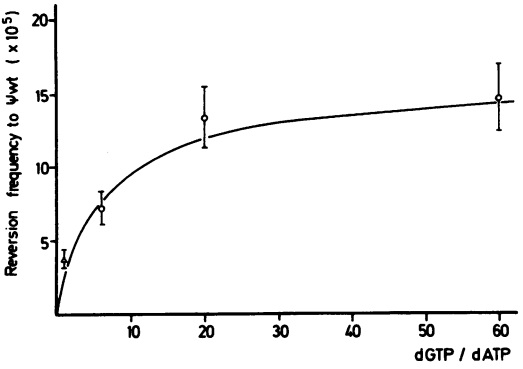

Fig.1: Dependence of the phage reversion frequency to ψwt on the bias of dGTP over dATP during DNA replication
The bars indicate the experimental error estimated from four experiments at the same bias.

quencies of G:T and C:T mispairs can be determined since pseudowildtype and wildtype do not differ in their temperature sensitivity. The reversion frequencies as obtained from the classification of the revertant phenotypes are summarized in table I.

In order to obtain the error rates of the in vitro replication,

Table I: The specificity of error production by the 9S DNA polymerase α: Reversion frequencies, expression rates and error frequencies.

mispair	reversion frequency[a] (x 10^{-5})	expression rate[b] of errors in the (-) strand	error rate (x 10^{-5})
G:T	} 4.4	54 %	} 8.4
C:T		42 %	
T:T	< 0.2	47 %	< 0.4
G:A	< 0.25	29 %	< 0.85
A:G	1.1	18 %	6.3
C:A	< 0.2	17 %	< 1.2
G:G	1.9	14 %	13.6

[a]The reversion frequencies were determined by a characterization of the various temperature sensitive revertants. The in vitro replication was performed at 1:1 concentrations of the dNTPs.
[b]The expression rate of the errors in the (-) strand was determined as described in the methods section.

the reversion frequencies have been corrected for the (-) strand expression (see below and table I). The error rates given in table I differ slightly from those reported earlier for this enzyme, due to the different expression rates for the various mispairs determined in the present work. The general picture of the specificity of error production by the DNA polymerase α is however unchanged. G:T, G:G and A:G mispairs are the most frequent errors, the G:A, C:A and T:T mispairs are formed at a much lower frequency.

The influence of α-thio-dNTPs on the fidelity

The frequencies of formation of the various mispairs in the presence of the thio-analogues are given in table II. In these experiments, only one of the four deoxynucleotides was substituted by its thio-derivative. Substitution of all four deoxynucleotides resulted in a strong inhibition of the nucleotide incorporation and a conversion to the RFII form was not possible. Substitution of only one deoxynucleotide resulted in a two-fold decrease in the incorporation rate and a conversion to the RFII form could be achieved, as judged by agarose gel electrophoresis. In the presence of α-thio-dGTP , the formation of G:G and G:T mispairs is significantly lowered, whereas the formation of all other mispairs is not changed significantly in the presence of the corresponding α-thio-derivative. The results show that DNA polymerase α does not possess a cryptic exonuclease activity, they indicate however an influence of the thio-substitution on the formation of specific mispairs. In contrast to our present results, Abbotts & Loeb (13) recently have reported an increased error rate in the presence of α-thio-dCTP. The am3 revertant system used by these authors however does not allow to extract the frequencies of so many mispairs as does the am16 system.

Fidelity in the presence of Mn^{2+} and pyrophosphate

Mn^{2+} has been shown to decrease the fidelity of DNA polymerases in a concentration dependent manner (14,15). When we replaced Mg^{2+} by Mn^{2+}, a complete conversion of the ΦX single-stranded DNA to the RFII form could only be achieved at a Mn^{2+} concentration of about 0.5 mM. Other concentrations were strongly inhibitory and a production of the RFII form was no longer possible.The

Table II: Influence of α-thio-dNTPs, Mn^{2+} and pyrophosphate on the fidelity of DNA polymerase
The data have been corrected for the (-)strand expression according to table I.

mispair	Error rates ($x10^{-5}$) in the presence of α-thio-dNTP[a]	0.5 mM Mn^{2+}	1 mM pyrophosphate
G:T (+C:T)	0.27	4.8	7.0
T:T	< 0.5	< 1.0	< 0.5
G:A	< 0.6	< 0.6	< 0.7
A:G	6.0	3.3	4.0
C:A	< 1.1	< 1.3	< 0.8
G:G	2.7	21	13.8

[a]The α-thio-dNTP (100 μM) given was used to replace the corresponding unmodified nucleotide during in vitro replication.

data in table II show that the frequency of formation of the various mispairs is only marginally influenced upon replacing Mg^{2+} by Mn^{2+}. Table II also includes data on the fidelity of replication in the presence of pyrophosphate. Pyrophosphate at 1 mM does not alter the fidelity of the 9S enzyme. This finding supports recent data by Abbotts & Loeb (13).

Expression of errors in the (-) strand

The reversion frequencies measured with the phage replication system are influenced by mismatch repair processes in the E.coli host. Hence, only a fraction of errors produced during in vitro replication will be expressed. An essential component of the mismatch repair system in E.coli is the dam-methylase which methylates GATC sequences at A (16). ΦX174 DNA does not contain a GATC site and thus will not be subject to methyl-A directed mismatch repair (17). In the absence of directed repair, the correction of the mismatch should occur to the same extent in the (+) and in the (-) strand and thus 50% of the errors should be expressed. It is not known however, to what extent the methylation independent mismatch correction shows a specificity towards certain types of mismatches. Therefore it is essential to get quantitative data on the expression rate of all the different mispairs that are considered in the am16 revertant system. To this purpose we primed the in vitro replication of ΦX174 single-

stranded DNA with different pentadacanucleotides that span the am16 sequence. These oligonucleotides carried different base changes in the sequence complementary to the am16 codon and thus created each of the different heteroduplexes. The primers were elongated by DNA polymerase α or DNA polymerase I (Klenow fragment) to produce the RFII form. Following transfection, the expression of the various mutant phenotypes was scored. Table I shows that the mispairs are repaired with different efficiencies. The repair efficiency of the py-py mismatches is in the range of the theoretically expected value of 50%. By contrast, the purine-purine mismatches and the C:A mismatch are repaired mainly on the newly formed (-) strand.

DISCUSSION

The frequency of specific mispairs formed during in vitro replication of ΦX174 single-stranded DNA by the 9 S DNA polymerase has been determined by scoring different mutant phenotypes that can arise from reversion at the am16 codon. It was not possible to determine error frequencies from nucleotide pool bias studies since freshly prepared enzyme showed only a weak dependence of the reversion frequencies on the nucleotide pool bias. However, at equal concentrations of nucleotides essentially the same error frequencies were measured as determined in an earlier study from pool bias measurements. The lack of a pool bias dependence is difficult to explain since mechanistic details of the chain elongation reaction are not known for this enzyme up to now. However, a similar finding has been reported for a high-molecular weight DNA polymerase α from Drosophila (18).

Our studies with thio-dNTPs indicate a strong influence of even subtle changes in the nucleotide structure on the error rates. We observe a nearly tenfold decrease in the error rates for G:G and G:T mispairs. According to model considerations of Topal & Fresco (19), a G:G mispair requires a G_{syn}: $G_{enol\text{-}imino}$ configuration. It is conceivable that the substitution of the oxygen by sulfur will shift the syn-anti equilibrium of the incoming dGTP in favour of the anti-form. The frequencies of G:T mispairs which may be formed via the $G_{enol\text{-}imino}$ form (19), may be decreased due to a shift in the tautomerism of G, as a conse-

quence in the shift of the syn-anti equilibrium. In any case, our data reveal that one has to be cautious in extrapolating error rates obtained in the presence of α-thio-dNTPs to those obtained with unmodified nucleotides and vice versa. Such extrapolations have been made e.g. in studies on the contribution of the proofreading exonuclease activity of procaryotic DNA polymerases to the overall replicational fidelity (4).

Mn^{2+} at concentrations that allow effective DNA synthesis, does not alter the fidelity of DNA polymerase α significantly. This indicates that in the presence of Mn^{2+}, a proper recognition of the cognate nucleotide is still possible. The mutagenic effect of Mn^{2+} at higher concentrations may be due to the reduction of the incorporation rate observed under these conditions. We have shown earlier that a reduced rate of DNA synthesis can lead to a decrease in fidelity (15).

The erroneous products of the in vitro replication are transfected to the E.coli host as heteroduplexes with a single base-mismatch. The fate of the heteroduplexes depends on the timely order with which repair or further replication will occur. In the case of replication without prior repair, the information of the (-) strand is expected to be expressed to a greater extent as compared to the (+) strand, since further multiplication of the Φ X174 DNA is supposed to occur via a rolling circle replication of the (-) strand (20). Under these circumstances one should observe an expression rate of the (-) strand of nearly 100%. If repair precedes replication, the expression rate of the (-) strand should be about 50%. The ΦX174 genome is not subject to dam - methylation and thus repair of the mismatches should be undirected and should occur to the same extent on the (-) and the (+) strand. Our data reveal a (-) strand expression in the range of 19-50% with a significant dependence on the type of mismatch involved. The (-) strand expression of the A:G, G:G and C:A mispairs is clearly below 50% which has to be interpreted in terms of a repair that prefers the newly synthesized (-) strand. It could be argued that this is due to a preferential loss of the newly synthesized strand. However, it is difficult to see, why this should occur in a mismatch dependent manner. Therefore we conclude that (i) different base-base mismatches are repaired

with different efficiencies and that (ii) the gaps in the newly replicated strand may be sufficient for the recognition of the daughter strand by the mismatch repair system of E.coli. The efficiency of repair is poor for the G:T, T:T and C:T mispairs, it is higher for the A:G, G:A, G:G and C:A mismatches. Somewhat different efficiencies of mismatch repair have been reported recently for two methyl-directed systems (21,22). However, in both cases the repair efficiency for py-py mismatches is lower as compared to the pu-pu mismatches. These authors have used fully ligated and GATC containing DNA as repair substrates while the present study was intended to provide for a correction for repair processes occurring on unligated *in vitro* products. The difference in the repair substrates may account at least partially for the different results.

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