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Effect of 8-oxo-guanine and abasic site DNA lesions on *in vitro* elongation by human DNA polymerase ϵ in the presence of replication protein A and proliferating cell nuclear antigen

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Short (page heading) title

Replication of 8-oxo-guanine and abasic sites by human DNA pol ϵ

Key words

DNA replication, DNA polymerase, DNA damage, RPA, PCNA

SYNOPSIS

DNA polymerase (pol) ϵ is thought to be the leading strand replicase in eukaryotes. Here we show that human DNA pol ϵ can efficiently bypass an 8-oxo-G lesion on the template strand by inserting either dCMP or dAMP opposite to it, but it cannot bypass an abasic site. During replication DNA polymerases associate with accessory proteins that may alter their bypass ability. We investigated the role of the human DNA sliding clamp PCNA and of the human single-stranded DNA binding protein RPA in the modulation of the DNA synthesis and translesion capacity of DNA pol ϵ . RPA inhibited the elongation by human DNA pol ϵ on templates annealed to short primers. PCNA did not influence the elongation by DNA pol ϵ and had no effect on inhibition of elongation caused by RPA. RPA inhibition was considerably reduced when the length of the primers was increased. On templates bearing the 8-oxo-G lesion, this inhibitory effect was more pronounced on DNA replication beyond the lesion, suggesting that RPA may prevent extension by DNA pol ϵ after incorporation opposite an 8-oxo-G. Neither PCNA nor RPA had any effect on the inability of DNA pol ϵ to replicate past the AP site, independent of the primer length.

INTRODUCTION

7,8-dihydro-8-oxo-guanine (8-oxo-G) and abasic sites (AP site) are two of the most frequent endogenous DNA lesions generated during normal cell growth. Based on structures of 8-oxodG:dC pair in double-stranded DNA, 8-oxo-G does not appear to cause a serious distortion in the overall helical structure of the DNA [1, 2] and this seems also to hold true when the structure of the miscoding base pair 8-oxodG:dA is investigated [3, 4]. The 8-oxodG:dA mismatch can give rise to G-C→T-A transversion during DNA replication with adverse consequences for the cell. To obviate this potent source of mutagenesis cells have evolved two different base excision repair (BER) systems that insure repair of the 8-oxo-G lesion. The first is an OGG1-dependent pathway, which targets the 8-oxodG:dC pair, removes the lesion and leaves an intact DNA strand to act as template for the re-synthesis step [5]; the second is a MUTYH-dependent pathway which targets the 8-oxodG:dA mismatch and removes the adenine [6]. However these repair systems are not unfailing so that 8-oxo-G in the template strand is sometimes encountered by DNA polymerases (pols) during DNA replication or by re-synthesis associated with DNA repair. Indeed, a number of eukaryotic DNA pils can bypass *in vitro* the 8-oxo-G lesion in a mutagenic way by incorporating adenine and cytosine during the bypass. These enzymes do not exclusively belong to the Y family polymerases, specialized for translesion synthesis (TLS), but include also replicative DNA pils such as DNA pils α and δ [7, 8].

Abasic sites arise frequently by spontaneous hydrolysis of purines in the DNA and represent a common intermediate of BER systems. Compared to the 8-oxo-G lesion, an abasic site poses a more serious problem to the advancement of a DNA pil since the modified base has lost its coding capacity. Accordingly, its replication requires the intervention of one or more TLS DNA pils and often results in incorporation of dAMP opposite the lesion ([9] and ref therein). However, it has been reported that an AP site can also be bypassed *in vitro*, albeit inefficiently, by replicative polymerases DNA pil α [10] and by DNA pil δ in the presence of the processivity factor PCNA [11].

Efficient and accurate replication of the eukaryotic genome requires not only DNA pils α and δ but also DNA pil ϵ . Recent work in yeast supports a model wherein, during normal DNA replication, DNA pil ϵ is primarily responsible for copying the leading-strand and DNA pil δ primarily responsible for copying the lagging strand [12]. Although much information is already available in the literature concerning the capacity of DNA pil δ to deal with 8-oxo-G and AP lesions, such information is still scarce concerning DNA pil ϵ , particularly for the human enzyme.

Therefore, we investigated the effect of 8-oxo-G or AP site lesions on *in vitro* DNA elongation catalyzed by human DNA pil ϵ . To this end we used primer extension assays of DNA templates containing either an 8-oxo-G lesion or a tetrahydrofuran moiety mimicking an abasic site. We performed experiments under both running and standing start conditions and our results indicate that DNA pil ϵ can efficiently bypass the 8-oxo-G lesion by inserting either dCMP or dAMP opposite to it, while its primer elongation activity is arrested by the synthetic abasic site. Furthermore, since replicative DNA pils associate with accessory factors during DNA replication that affect their properties and may alter their bypass ability, we investigated the role of the processivity factor PCNA and of the single stranded DNA binding protein RPA in modulating the DNA synthesis and translesion capacity of DNA pil ϵ .

EXPERIMENTAL

Proteins

Recombinant His-tagged human heterotrimeric RPA, PCNA and heterotetrameric DNA pol δ were expressed and purified as described [13-15]. Human DNA pol ϵ was purified from HeLa cells through six purification steps as described [16]. Briefly, 90 liters of HeLa cells in suspension at $5-8 \times 10^5$ cells/ml were harvested and homogenized and then DNA pol ϵ purified by conventional methods. Steps included $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion exchange chromatography on DEAE-Sephacel and phosphocellulose, adsorption chromatography on hydroxylapatite, and glycerol gradient centrifugation. A DNA pol assay using poly(dA)/oligo(dT) as template/primer [17] as well as Western blotting against DNA pols α , δ and ϵ were used to monitor the purification. The antibodies used were: mouse monoclonal antibodies 1ct102 and 1ct25 against the catalytic subunit of DNA pol α [18]; rat monoclonal antibody 1E8 or rabbit polyclonal antibodies K30, K31, K32 and K33 against the catalytic subunit of DNA pol δ [18]; mouse monoclonal antibodies 93G1A, 93H3B and 93E24A against DNA pol ϵ p262 [19]; mouse monoclonal antibodies 102E2 and 102F1 against DNA pol ϵ p59 were raised against recombinant p59 expressed in *E. coli*; rabbit polyclonal antibodies p12CR1 and p12CR2 as well as p19DH1 and p19DH2 were raised against recombinant p12 and p19, respectively, expressed in *E. coli*. Proteins for antibody production were purified by combination of conventional purification and preparative SDS-PAGE. The hydroxylapatite fraction (HA) had a specific activity of 3,900 units/mg while the glycerol gradient fraction (GG) had a specific activity of 24,000 units/mg. DNA pol ϵ consisted of all four subunits as can be seen by Western blot analysis (Supplementary Fig. 1). The protein content of the HA fraction was estimated to be 50 $\mu\text{g}/\text{ml}$ by standard protein measurement, of which an estimated 5 $\mu\text{g}/\text{ml}$ (13.5 fmol/ μl) constituted DNA pol ϵ (~ 10 % purity). The HA fraction was free of immunoreactive DNA pol α that eluted at higher salt from hydroxylapatite, and essentially free of immunoreactive DNA pol δ , that was mainly separated in the previous columns, and also eluted prior to DNA pol ϵ from hydroxylapatite. The concentration of the GG fraction was estimated to be 5.5 $\mu\text{g}/\text{ml}$ (15 fmol/ μl) by quantifying different fractions from fluorescently stained gels using the average intensity of the MW marker bands as calibrator. Purity was estimated to be > 50 % and the fraction was devoid of other replicative DNA pols.

DNA Substrates

All oligonucleotides were from Eurogentec and purified from polyacrylamide denaturing gel. The 100 mer templates, either undamaged or containing an 8-oxo-G or a synthetic AP site (tetrahydrofuran moiety), and the primers were further purified on a 12% (wt/vol) polyacrylamide, 7M urea, 30% formamide gels. After elution and ethanol precipitation, their concentrations were determined spectrophotometrically. The sequence of DNA templates and primers is indicated in Fig.1. Primers were 5'-labelled with T4 polynucleotide kinase (New England Biolabs) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ according to the manufacturer's protocol. Each primer was mixed with the complementary template oligonucleotide at a 1/1 (M/M) ratio in the presence of 20mM Tris HCl pH 8 and 50mM KCl, heated at 90°C for 5 minutes and then slowly cooled.

Primer extension assays

Reactions of 10 μ l were incubated at 37°C for 30 minutes and contained 0.1 pmoles of DNA templates, 50mM Hepes pH 7.5, 5mM MgCl₂, 1mM DTT, 200 μ g/ml of BSA and 100 μ M of each dATP, dCTP, dGTP and dTTP, and 23 fmoles of HA fraction of DNA pol ϵ unless otherwise indicated in the figure legends. The amount of RPA and PCNA used is indicated in the figures and figure legends. The reactions were stopped by adding 0.5 reaction volume of stop solution containing 0.1% xylene cyanol and 0.1% bromophenol blue in 90% formamide. Before loading onto the gel, the reactions were denatured by heating at 100°C for 3 min. The reaction products were resolved on denaturing polyacrylamide gel electrophoresis (7M urea, 10-15% acrylamide, 30% formamide) and then visualized and quantified using Molecular Dynamics PhosphorImager and ImageQuant software. The percentage of extension was calculated as the ratio of the intensity of the bands greater than the primer to the intensity of all bands, including the primer.

RESULTS

Bypass of 8-oxo-G by DNA polymerase ϵ under running start conditions, influence of PCNA and RPA

To carry out this study we used either the HA or the highly purified GG fractions of the DNA pol ϵ purified as described in Experimental. Initial titration experiments with the HA fraction indicated that at least 15 fmoles of DNA pol ϵ were required to obtain significant extension (40 to 60 %) of the DNA template-primers employed (data not shown).

To investigate the capacity of DNA pol ϵ to replicate past the 8-oxo-G lesion when acting alone or in the presence of PCNA we first used as DNA template-primer the 100 mer A shown in Fig. 1 annealed to its corresponding 17 mer primer. The 3'OH of the primer is 22 nucleotides upstream from the 8-oxo-G lesion and its elongation is monitored under conditions defined as running start. As it can be seen by comparing lanes 1 and 5 of Fig.2A, the enzyme paused at the 8-oxo-G and at the nucleotide preceding it, as indicated by the appearance of a distinct doublet in the gel; however, longer products up to template full length were also easily detected. By comparing lanes 1 and 5, the bypass efficiency of the 8-oxo-G lesion was estimated to be 90%, indicating that, after an initial pausing, the lesion is efficiently bypassed by DNA pol ϵ . Next we asked if addition of the sliding clamp PCNA had any effect on the capacity of DNA pol ϵ to replicate past the 8-oxo-G lesion. Increasing concentrations of human PCNA did not significantly affect the bypass efficiency of the 8-oxo-G lesion by DNA pol ϵ as it can be seen in lanes 2-4 and 6-8 of Fig. 2A and from Fig. 2C. In a parallel control experiment we found that 2.8 picomoles of PCNA caused roughly 4 fold stimulation of DNA synthesis performed by human DNA pol δ while, under identical experimental conditions with the same undamaged DNA template primer, no stimulation was observed for DNA pol ϵ (Supplementary Fig. 2). This shows that the PCNA utilized in this study is functionally active. Moreover, since PCNA dynamically slides on and off of linear DNA templates, we used the highest tested PCNA concentration (2.8 picomoles) for all subsequent experiments.

Next the effect of the single stranded DNA binding protein RPA on the extension capacity of DNA pol ϵ was investigated. Biochemical studies have shown that human RPA binds *in vitro* to single-stranded DNA in at least two modes that interact with 8-10 nucleotides or with 30 nucleotides, respectively [20, 21]. As seen in Figs. 2B and 2D, increasing concentrations of human RPA resulted in increased inhibition of primer extension on both undamaged and damaged 100 mer template A annealed to a 17 mer primer. With 0.1 picomoles of this template-primer, 0.28 picomoles of RPA corresponds to one molecule of RPA for 30 nucleotides of single

stranded DNA while 0.96 picomoles correspond to one molecule of RPA per 8 nucleotides. It is worth noting that, at the highest concentration of RPA, the inhibition of the synthesis seemed to be more pronounced for the reaction products beyond the lesion when compared to the products prior to the lesion (compare lower and upper part of lanes 5 to 8 in Fig. 2B and see also Fig. 2E and Supplementary Fig.3). For all subsequent experiments, RPA concentration was kept to 0.96 picomoles corresponding to 1 RPA for 8-10 nucleotides of single stranded DNA.

We then asked whether the inhibitory effect of RPA is affected by PCNA. To this aim we monitored the effect of RPA and PCNA together on the activity of DNA pol ϵ HA fraction using the same template-primer either intact or damaged (Fig. 2E, quantified in Fig. 2G). PCNA did not influence the inhibitory effect of RPA in a detectable manner (compare lanes 2 to 4 and 6 to 8 of Fig. 2E). Like in the absence of PCNA (Fig. 2B), RPA inhibition is stronger for the synthesis beyond the 8-oxo G lesion also in the presence of PCNA. We varied PCNA concentration from 0.4 to 2.8 picomoles and found no influence on the RPA inhibition of synthesis on the damaged template (data not shown). Comparable results were obtained when the experiment depicted in Fig. 2E was repeated with the highly purified GG fraction of DNA pol ϵ (see Supplementary Fig. 3). These results indicate that, under our experimental conditions, the presence of PCNA did not relieve the inhibitory effect of RPA and confirmed that inhibition of translesion synthesis past 8-oxo-G by RPA is more pronounced in the presence of both proteins.

In summary our results show that human DNA pol ϵ efficiently replicates past the 8-oxo-G lesion. PCNA influenced primer extension of neither intact nor damaged templates. High concentrations of RPA inhibited synthesis by DNA pol ϵ on both templates. Moreover, inhibition by RPA appeared to be more pronounced for reaction products representing elongation past the 8-oxo-G lesion. PCNA did not relieve the inhibitory effect of RPA in any experimental conditions.

Bypass of 8-oxo-G by DNA polymerase ϵ under standing start conditions, influence of PCNA and RPA

In order to further investigate the effect of RPA and PCNA on the 8-oxo-G bypass by DNA pol ϵ , we examined the effect of these accessory proteins in a standing start reaction, where the 3'OH of the primer is situated in the immediate vicinity before the lesion. For this purpose we used the 100 mer template A annealed to the 39 mer primer (Fig.1).

Overall, the results of such experiments (Fig. 2F, quantified in Fig. 2H) appeared comparable to those performed under running start conditions. The lesion did not affect the activity of DNA pol ϵ , and PCNA had no influence on this. RPA had only a modest inhibitory effect which could not be reverted by PCNA. Clearly, the inhibitory effect of RPA on the overall primer extension is not that strong as it is in running start conditions (Fig. 2E and 2G). This difference may be due to the shorter primer used in running start reactions (see Discussion). We have repeated the experiment in Fig. 2F with the GG fraction of DNA pol ϵ and obtained comparable results (see Supplementary Fig. 4).

Taken together these results indicate that in standing start conditions the 8-oxo-G lesion did not affect the activity of DNA pol ϵ . Like in running start reactions, primer extension by DNA pol ϵ on a template, either undamaged or bearing the 8-oxo-G lesion, appears to be inhibited by RPA, although to a sensibly less extent than under running start conditions. This inhibition is not affected by PCNA.

Nucleotide incorporation opposite the 8-oxo-G lesion by DNA polymerase ϵ .

Next we asked which nucleotide was incorporated opposite the 8-oxo-G by DNA pol ϵ and if RPA and PCNA would influence such reaction. To this end a standing start assay with the 100 mer template A annealed to the 39 mer primer was employed (Fig.1) and the insertion of a single nucleotide opposite the lesion was monitored. Fig. 3A shows that on the undamaged template the enzyme incorporated only the correct dCMP opposite the undamaged G, as expected (lanes 2 to 5). Since the G implicated in the lesion is followed by a second one, the position of the main radioactive band corresponded to the position of the second G (lane 3). Some incorporation was also detected at a subsequent G that may be the result of a frameshift of the C nucleotide located between two Gs (see sequence on the left of Fig. 3A). With the 8-oxo-G incorporation of A but not of G and T was detected in addition to C (lanes 7 to 10). We then monitored the effect of RPA and PCNA (Fig. 3B). Inclusion of RPA or PCNA separately, or both together did not change the pattern of incorporation by DNA pol ϵ .

Taken together these data show that DNA pol ϵ inserts both C and A opposite the 8-oxo-G lesion and that RPA and PCNA do not change the pattern of single nucleotide insertion catalyzed by the polymerase.

Bypass of an AP site by DNA polymerase ϵ , and influence of PCNA and RPA

Next we investigated the primer extension capacity of DNA pol ϵ on a template containing a specific AP site. For our initial experiments we used the 100 mer template B annealed to its corresponding 17 mer primer with the 3'OH at a distance of 22 nucleotides from the AP site (see Fig. 1). Time course experiments of 30, 60 and 90 minutes indicated that DNA pol ϵ was severely blocked at the nucleotide preceding the AP site, although some incorporation opposite the lesion was also detected (data not shown). Moreover, contrary to what was observed with the 8-oxo-G lesion, no synthesis was detectable past the AP site. Experiments performed in the presence of PCNA gave similar results (data not shown).

We then analyzed the effect of the AP site on the elongation capacity of DNA pol ϵ using longer primers and with RPA and PCNA present. As templates for these experiments we used the 100 mer template B annealed either to a 44 mer primer 22 nucleotides from the AP site (running start) or to a 45 mer primer where the 3'OH of the primer is situated at the nucleotide preceding the lesion (standing start) (see Fig.1). RPA and PCNA, separately or together (Figs. 4A and B), had no detectable effect on the synthesis by DNA pol ϵ with the undamaged 100/44 and 100/45 template/primers (Figs. 4A and B, lanes 1-4), showing that the inhibitory effect of RPA is influenced by the length of the primers used (see Discussion). Inclusion of the two accessory factors did not change the replication of the damaged template by DNA pol ϵ under running start conditions, which appeared to be mainly arrested at the nucleotide preceding the AP site, with some incorporation in front of the lesion (Fig. 4A, lanes 5-8). The same results were obtained with the standing start reaction (Fig.4B). We also investigated the effect of the AP site using the GG fraction of DNA pol ϵ under the experimental conditions of Figs. 3A and B and obtained comparable results (see Supplementary Fig. 5 A and B).

While this work was in progress it was reported that yeast DNA pol ϵ was capable of translesion synthesis past an abasic site [22]. Notably, with a 76/58 template/primer in which the 3'OH was placed 3 bases from the lesion, the bypass efficiency was estimated to be up to 17%. We therefore decided to compare, under the same experimental conditions, the capacity of the human DNA pol ϵ preparations used in our study to replicate the AP site placed on our template/primers and on the same DNA template/primer used by Sabouri et al (template/primer C/C in Fig. 1). We

found that synthesis by both the HA fraction (Fig. 5) and the GG fraction (data not shown) of the DNA pol ϵ was arrested at the AP site on all the templates tested, and no synthesis was detectable beyond the AP site.

Taken together these results indicate that, under our experimental conditions, human DNA pol ϵ cannot synthesize past an AP site but is arrested mainly at the base preceding the lesion with minor incorporation opposite the lesion. This inhibitory effect of the AP site was observed with both running and standing start reactions, and with different DNA template/primers. RPA and PCNA had no effect on the inability of the DNA pol ϵ to bypass the AP site.

DISCUSSION

We have investigated the capacity of the human replicative DNA pol ϵ to bypass the 8-oxo-guanine lesion and a model abasic site in oligonucleotides DNA substrates. The influence of the accessory replicative proteins PCNA and RPA on the bypass capacity of DNA pol ϵ was also studied. DNA pol ϵ was purified from HeLa cells through six purification steps (see Experimental and Supplementary Fig. 1 for a protein gel image of the last two steps). Given the relative paucity of the material obtained at the last step, most of the experiments were conducted with the fraction preceding it (hydroxylapatite) that had a specific activity of 3,900 units /mg and was essentially devoid of immunoreactive DNA pols α and δ . Key experiments were repeated with the most purified glycerol gradient fraction and led to the same conclusions as those obtained with the hydroxylapatite fraction (Supplementary Figs. 3-5).

8-oxo-G is a non-distorting DNA lesion that has high mutagenic miscoding potential and can be bypassed *in vitro* by the replicative DNA pols α and δ by incorporating both dCMP and dAMP opposite the lesion [7, 8]. The efficiency of DNA pol δ to bypass of a 8-oxo-G, compared to bypass of the undamaged G, can vary from 15 to 90 %, depending whether the assays are performed under single or multiple-hit conditions [23-25].

By monitoring the replication of 8-oxo-G by the human DNA pol ϵ under running start conditions we found that, after an initial pause both at the base preceding and across from the lesion, DNA pol ϵ efficiently replicated the damaged substrate (Figs. 2A, B and E). Data in Fig. 2A also indicate that increasing concentrations of human PCNA did not significantly affect either the extent of synthesis or the bypass capacity of DNA pol ϵ . It should be noted that, although DNA pol ϵ was originally isolated as a large form of DNA pol δ insensitive to stimulation by PCNA [16, 26], PCNA was later found to stimulate [27, 28] or not [17, 29] the *in vitro* processivity of DNA pol ϵ , possibly depending on the type of DNA substrates and experimental conditions used. However, to insure that the PCNA we used was functionally active we have performed a control experiment showing that PCNA stimulated synthesis by DNA pol δ under our experimental conditions (Supplementary Fig. 2)

We then monitored the effect of human RPA on the extension and the 8-oxo-G bypass capacity of the human DNA pol ϵ . Increasing the concentration of RPA resulted in increased inhibition of primer extension on both undamaged and damaged templates (Fig. 2B and D). However, in experiments with DNA primers longer than the 17 mer used in Figs. 2A, B and E, the inhibitory effect of this RPA concentration was nearly undetectable (Figs. 2F, H and 4 and Supplementary Fig. 4). We further confirmed this by performing the experiment depicted in Fig. 6, showing that,

with the same template, increasing the length of the primer from 17 to 44 mer nearly abolishes the RPA inhibition.

Since RPA has been shown to possess a strand displacement activity [30], a trivial explanation for this observation could be that a short primer is more easily displaced than a longer one, resulting in reduction of DNA synthesis. However this displacement activity is abolished at a concentration of $MgCl_2$ as low as 1mM and almost undetectable under DNA polymerization conditions [30]. Therefore a perhaps more appealing explanation could come from the elegant work by the Johansson laboratory [31], who showed that a minimal length of double-stranded template/primer ($\approx 40bp$) is required to maximize processivity of the yeast DNA pol ϵ , possibly by reducing polymerase dissociation. Therefore, it may be that, with the 17 mer primer, binding of RPA in the vicinity of the primer reduces re-association by the DNA pol ϵ , in this way decreasing the net amount of synthesis.

We also observed that RPA seemed to preferentially inhibit synthesis past the 8-oxo-G lesion in the running start reaction (Fig. 2E and Supplementary Fig. 3). Here too, the pausing of the enzyme at the lesion could transiently increase its dissociation from the primer so that the inhibitory effect of RPA becomes more evident. These results may indicate that extension past the 8-oxo-G lesion by the human DNA pol ϵ is the limiting step in the presence of RPA. Experiments performed to monitor the 8-oxo-G bypass capacity of DNA pol ϵ under standing start conditions (Fig. 2F) gave results overall similar to those obtained with running start conditions, except for a diminished inhibitory effect of RPA on the overall DNA extension, as discussed above. Finally, results of experiments performed with the GG fraction of the human DNA pol ϵ paralleled those observed with the hydroxyapatite fraction (Suppl. Figs 3 and 4).

Next we addressed the question of which nucleotide was incorporated opposite the 8-oxo-G. The experiment of single nucleotide insertion shown in Fig. 3A indicates that, as expected, only C was incorporated on the undamaged template while both C and A, but not T and G, were incorporated opposite the lesion. Addition of RPA or PCNA separately or together did not change the pattern of nucleotide insertion obtained with the polymerase alone (Fig. 3B). Crystal structures of the T7 DNA polymerase with 8-oxo-G containing templates revealed that incorporation of either dC or dA in front of this lesion induces local perturbation of the DNA backbone or formation of a Hoogsteen-like base pair, respectively [32]. Such alterations of the DNA structure could interfere with the binding of DNA pol ϵ onto DNA and account for its reduced bypass efficiency of 8-oxo-G in the presence of RPA.

In this study we also report that human DNA pol ϵ is unable to replicate past a synthetic abasic site and stopped mainly at the base preceding the lesion, with some residual incorporation opposite it. Some incorporation opposite an abasic site has been observed also with the yeast DNA polymerase δ alone [33] while the replicative T4 DNA polymerase was shown to completely stop at the base preceding the lesion [34]. This different behaviour may be related to the higher efficiency of the 3'-5' exonuclease activity associated to the T4 polymerase. The inability of DNA pol ϵ to bypass an AP site was observed with all primers tested, spanning from 17 to 58 mers, upon addition of RPA or PCNA, separately or in combination, and under running and standing start conditions (Figs. 4 and 5 and Suppl. Fig. 5).

Unlike to what reported with the yeast enzyme in the experiments performed by Sabouri et al. (22), we did not detect synthesis past the AP site with the human DNA pol ϵ , even with the template primer they used, with both the hydroxylapatite (Fig 5) and the GG fraction (data not shown). Therefore, although we cannot rule out that different experimental conditions may play a role, we show that this discrepancy is not dependent on the use of different substrates and our

results may reflect a different bypass capability of an AP site for the yeast and human form of DNA pol ϵ .

If proven to be physiologically relevant, the documented arrest of the synthesis by the human DNA pol ϵ in the presence of accessory proteins at the endogenously frequent AP site lesions could greatly contribute to the activation of DNA-damage checkpoints.

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LEGENDS

Fig.1. DNA templates used in this study

Fig.2. Effect of PCNA and RPA on the primer extension activity of DNA pol ϵ with DNA templates undamaged or containing a single 8-oxo-G residue.

A) Effect of PCNA on primer extension by DNA pol ϵ under running start conditions. DNA template A, annealed to the 17 mer primer, was either undamaged (lanes 1-4) or contained an 8-oxo-G residue (lanes 5-8). Reactions were analyzed on a denaturing 15% polyacrylamide gel. DNA pol ϵ was incubated alone (lanes 1 and 5) and in the presence of 0.7 pmol (lanes 2 and 6), 1.4 pmol (lanes 3 and 7) and 2.8 pmol (lanes 4 and 8) of PCNA. Lane C, control reaction in the absence of proteins. The positions of the 17 mer primer, of the 100 mer full length products and of the 8-oxo-G lesion are indicated on the left side of the gel. T/P means template/primer. **C)** Mean values of 3 independent experiments performed as indicated for Fig. 2A, expressed as percentage of primer extension (% extension) on DNA templates either undamaged (white bars) or containing a single 8-oxo-G residue (gray bars). **B)** Effect of RPA on primer extension by DNA pol ϵ under running start conditions. DNA template A, annealed to the 17 mer primer, was either undamaged (lanes 1-4) or contained an 8-oxo-G residue (lanes 5-8). Reactions were analyzed on a denaturing 15% polyacrylamide gel. DNA pol ϵ was incubated alone (lanes 1 and 5) and in the presence of 0.28 pmol (lanes 2 and 6), 0.57 pmol (lanes 3 and 7) and 0.96 pmol (lanes 4 and 8) of RPA. The positions of the 17 mer primer, of the 100 mer full length products and of the 8-oxo-G lesion are as indicated for Fig 1A. **D)** Mean values of 3 independent experiments performed as indicated for Fig. 2B, expressed as percentage of primer extension (% extension) on DNA templates either undamaged (white bars) or containing a single 8-oxo-G residue (gray bars). **E)** Effect of PCNA and RPA on primer extension by DNA pol ϵ under running start conditions. DNA template A, annealed to the 17 mer primer, was either undamaged (lanes 1-4) or contained an 8-oxo-G residue (lanes 5-8). Reactions were analyzed on a denaturing 15% polyacrylamide gel. DNA pol ϵ was incubated alone (lanes 1 and 5); in the presence of 0.96 pmol of RPA alone (lanes 2 and 6); in the presence of 2.8 pmol of PCNA alone (lanes 3 and 7) or in the presence of RPA and PCNA together (lanes 4 and 8). Lane C, control reaction in the absence of proteins. The positions of the 17 mer primer, of the 100 mer full length products and of the 8-oxo-G lesion are indicated on the left side of the gel. T/P means template/primer. **G).** Mean values of 3 independent experiments performed as indicated for Fig. 2E. Activity, is expressed as percentage of primer extension (% extension) on DNA templates either undamaged (white bars) or containing a single 8-oxo-G residue (gray bars). Error bars represent \pm SD values. **F)** Effect of PCNA and RPA on primer extension by DNA pol ϵ under standing start conditions. DNA template A, annealed to the 39 mer primer, was either undamaged (lanes 1-4) or contained an 8-oxo-G residue (lanes 5-8). Reactions were analyzed on a denaturing 10% polyacrylamide gel. DNA pol ϵ was incubated alone (lanes 1 and 5); in the presence of 0.96 pmol of RPA alone (lanes 2 and 6); in the presence of 2.8 pmol of PCNA alone (lanes 3 and 7) or in the presence of RPA and PCNA together (lanes 4 and 8). Lane C, control reaction in the absence of proteins. The positions of the 39mer primer, of the 100 mer full length products and of the 8-oxo-G lesion are indicated on the left side of the gel. **H).** Mean values of 3 independent experiments performed as indicated for Fig. 2F. Activity, is expressed as percentage of primer extension (% extension) on DNA templates either undamaged (white bars) or containing a single 8-oxo-G residue (gray bars). Error bars represent \pm SD values.

Fig. 3. Nucleotide incorporation opposite the 8-oxo-G lesion by the DNA pol ϵ alone or in the presence of PCNA and RPA.

A) Incorporation by the DNA pol ϵ alone. Single nucleotide insertion reactions were conducted using DNA template A annealed to the 39 mer primer. The template was either undamaged (lanes 1-5) or contained an 8-oxo-G residue (lanes 6-10). Reactions were analyzed on a denaturing 12% polyacrylamide gel. DNA pol ϵ was incubated with 100 μ M of dATP (lanes 2 and 7), dCTP (lanes 3 and 8), dGTP (lanes 4 and 9) or dTTP (lanes 5 and 10). Lanes 1 and 6: reactions in the presence of the four nucleotides with either undamaged or 8-oxo-G containing DNA template respectively. Lane C, control reaction in the absence of DNA pol. T/P means template/primer.

B) Incorporation by the DNA pol ϵ in the presence of PCNA and RPA. Single nucleotide insertion reactions were conducted using DNA template A annealed to the 39 mer primer. The template was either undamaged (lanes 1-4) or contained an 8-oxo-G residue (lanes 5-12). Reactions were analyzed on a denaturing 12% polyacrylamide gel. DNA pol ϵ was incubated with 100 μ M of dCTP (lanes 1-4 and 9-12) or dATP (lanes 5-8). DNA polymerase ϵ was incubated alone (lanes 1, 5 and 9); in the presence of 0.72 pmol of RPA alone (lanes 2, 6 and 10); in the presence of 2.8 pmol of PCNA alone (lanes 3, 7 and 11) or in the presence of RPA and PCNA together (lanes 4, 8 and 12).

The nucleotides sequence downstream of the primer junction is shown on the left and the position of the 8-oxo-G lesion (+1) is indicated on the right side of the Figure. The position of full length 100 mer product is indicated on the left. Fig. 3B is based on two separate experiments; the first includes lanes 1 to 8 and the second lanes 9 to 12.

Fig. 4. Effect of RPA and PCNA on the primer extension activity of DNA pol ϵ with DNA templates undamaged or containing a single AP site.

A) Effect of PCNA and RPA on primer extension by DNA pol ϵ under running start conditions. Primer extension assays were conducted using DNA template B annealed to a 44 mer primer. The template was either undamaged (lanes 1-4) or contained an AP site (lanes 5-8). Reactions were analyzed on a denaturing 10 % polyacrylamide gel. DNA pol ϵ was incubated in the absence of RPA and PCNA (lanes 1 and 5); in the presence of 0.5 pmol of RPA alone (lanes 2 and 6); in the presence of 2.8 pmol of PCNA alone (lanes 3, and 7) or in the presence of both RPA and PCNA (lanes 4 and 8). The position of the full length product (100 mer) and the position of the primer (44 mer) are indicated on the left side of the gel while the position of the AP site is indicated on the right side of the gel. Lane C, control reaction in the absence of proteins. T/P means template/primer.

B) Effect of PCNA and RPA on primer extension by DNA pol ϵ under standing start conditions. Primer extension assays were conducted using DNA template B annealed to a 45 mer primer. The template was either undamaged (lanes 1-4) or contained an AP site (lanes 5-8). Reactions were analyzed on a denaturing 10 % polyacrylamide gel. DNA pol ϵ was incubated in absence of RPA and PCNA (lanes 1 and 5); in the presence of 0.5 pmol of RPA alone (lanes 2 and 6); in the presence of 2.8 pmol of PCNA alone (lanes 3, and 7) or in the presence of both RPA and PCNA (lanes 4 and 8). The positions of the full length product (79 mer), of the primer (45 mer) and of the AP site are indicated on the right side of the gel. Lane C, control reaction in the absence of proteins.

Fig. 5. Extension activity of DNA pol ϵ on different DNA templates either undamaged or containing a single AP site. Primer extension assays were conducted using DNA template B, annealed either to a 44 mer primer (running start; lanes 1-3) or a 45 mer primer (standing start; lanes 4-6), and DNA template C annealed to a 58 mer primer (lanes 7-9). The templates were either undamaged (lanes 2, 5 and 8) or contained an AP site (lanes 3, 6 and 9). Lanes 1, 4 and 7 are control reactions in the absence of DNA pol. Reactions were analyzed on a denaturing 10 % polyacrylamide gel. Positions of full length products, primers and AP sites are indicated on both sides of the Figure.

Fig. 6. Differential inhibitory effect of RPA on the extension activity by DNA pol ϵ on an undamaged DNA template with primers of different lengths.

A) Primer extension assays were conducted using undamaged DNA template B annealed to the 17 mer primer. Reactions were analyzed on a denaturing 15% polyacrylamide gel. DNA pol ϵ was incubated alone (lane 1) or in the presence of 0.96 pmol of RPA (lane 2). Lane C, control reaction in the absence of proteins. The positions of the 17 mer primer and of the 100 mer full length products are indicated on the left side of the gel.

B) Primer extension assays were conducted using undamaged DNA template B annealed to the 44 mer primer. Reactions were analyzed on a denaturing 10% polyacrylamide gel. DNA pol ϵ was incubated alone (lane 1) or in the presence of 0.6 pmol of RPA (lane 2). Lane C, control reaction in the absence of proteins. The positions of the 44 mer primer and of the 100 mer full length products are indicated on the left side of the gel. T/P means Template/Primer.

C) Quantification of the DNA pol activity shown in A and B, expressed as percentage of primer extension (% Extension) of DNA templates either in the absence (white bars) or in the presence of RPA (gray bar). 100 % is the primer extension obtained with the polymerase alone.

DNA templates: undamaged or containing a single 8-oxo-G lesion and respective primers

<p>100merA / 17merA</p> <p>5'-CTAATCGTGATTATCGTATCTCTCTATGCTACTGCCGGTCACGGCAATTGAGGCCGAGCG<u>G</u>TGTGAATTCCTCACTGCCCGTCGTATGCTCTTGTTGTA-3'</p> <p>3'-CATACGAGAACCAACAT-5'</p>
<p>100merA / 39merA</p> <p>5'-CTAATCGTGATTATCGTATCTCTCTATGCTACTGCCGGTCACGGCAATTGAGGCCGAGCG<u>G</u>TGTGAATTCCTCACTGCCCGTCGTATGCTCTTGTTGTA-3'</p> <p>3'-ACACTTAAGGAGT GACCGGCAGCATACGAGAACCAACAT-5'</p>

The guanine, either undamaged or bearing the 8-oxo-G lesion, is underlined.

DNA templates: undamaged or containing an AP site and respective primers

<p>100mer B / 17mer B</p> <p>5'-TAAGCTAGTAGTATTATAAAATTATGGAGGTTT<u>T</u>ACTGCCAAATAAAATATAGTAAAGATTATTAGGATTGAAAT TATGTAATTGAAACTAAATGCTACT-3'</p> <p>3'-TCTAATAATCCTAAACT-5'</p>
<p>100mer B / 44mer B</p> <p>5'-TAAGCTAGTAGTATTATAAAATTATGGAGGTTT<u>T</u>ACTGCCAAATAAAATATAGTAAAGATTATTAGGATTGAAAT TATGTAATTGAAAGTAAATGCTACT-3'</p> <p>3'-TCTAATAATCCTAAACTTTAATACATTAACCTTCATTACATCA-5'</p>
<p>100mer B / 45mer B</p> <p>5'-TAAGCTAGTAGTATTATAAAATTATGGAGGTTT<u>T</u>ACTGCCAAATAAAATATAGTAAAGATTATTAGGATTGAAAT TATGTAATTGAAACTAAATGCTACT-3'</p> <p>3'-TCACCCCTTATTTTATATCATTCTAATAATCCTAAACTTTAATA-5'</p>
<p>76mer C / 58mer C</p> <p>5'-TGATCGATCGTAAC<u>G</u>TAGCAGGATCGAATCGTCCTTAGAGGATCTATCGAATCCGTC TAATGGTTACACTGTCAG-3'</p> <p>3'-GTCCTAGCTTAGCAGCAATCTCCTATGATAGCTTACGCACATTACCAATGTGACAGTC-5'</p>

The guanine, either undamaged or substituted with the synthetic abasic site, is underlined.
Letters A, B, C and D identify the templates with the respective primers.

Figure 1

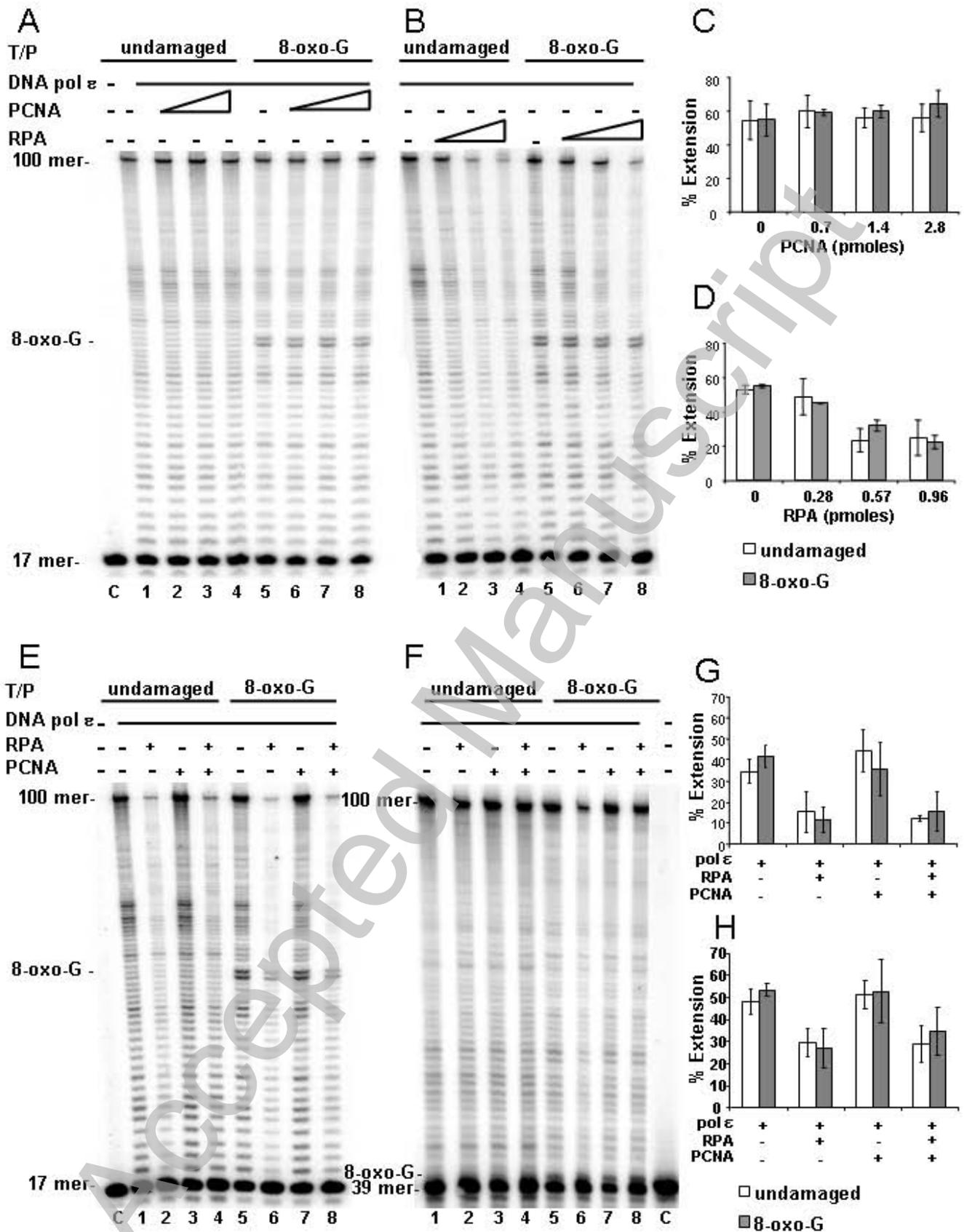


Figure 2

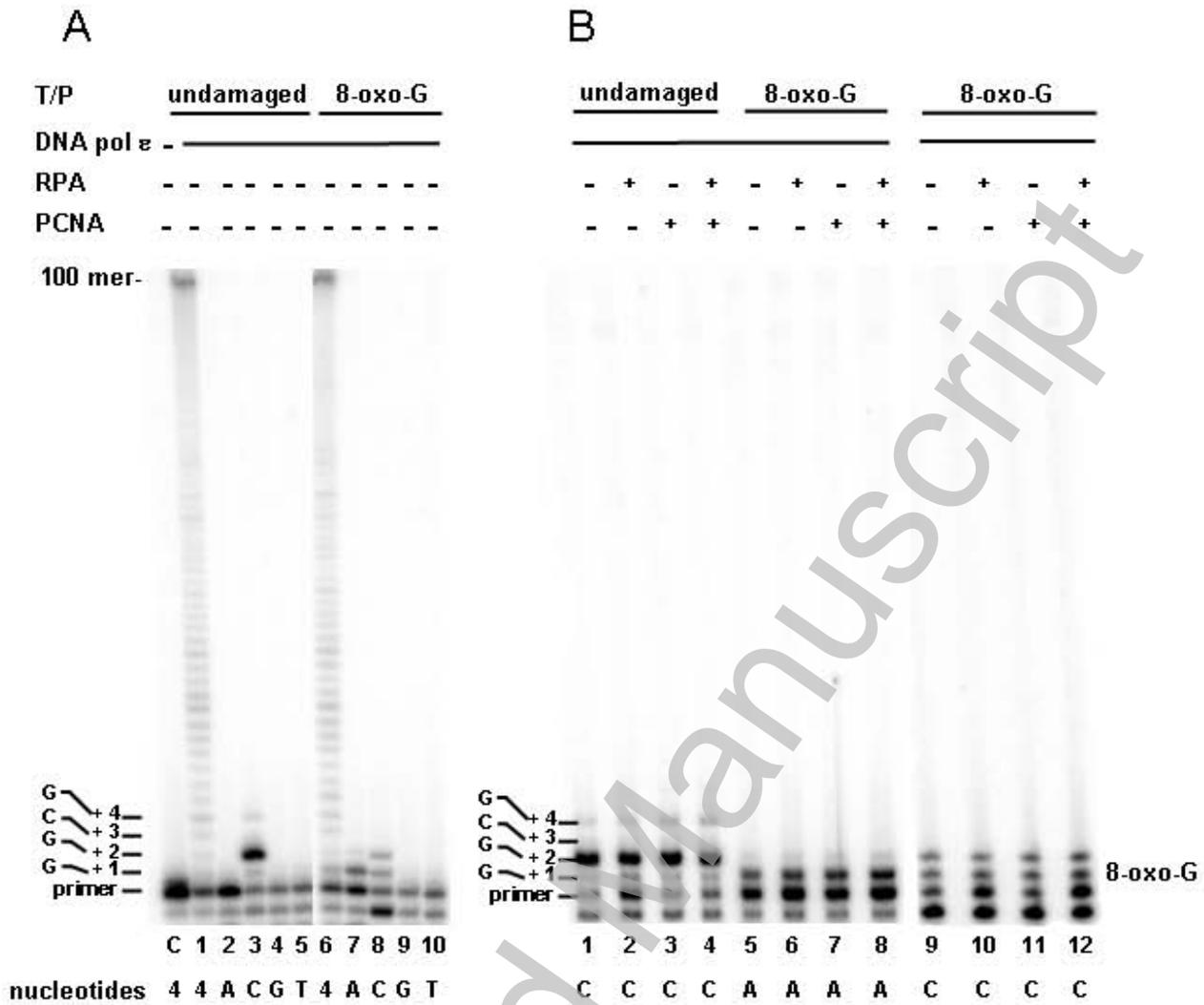


Figure 3

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20100405

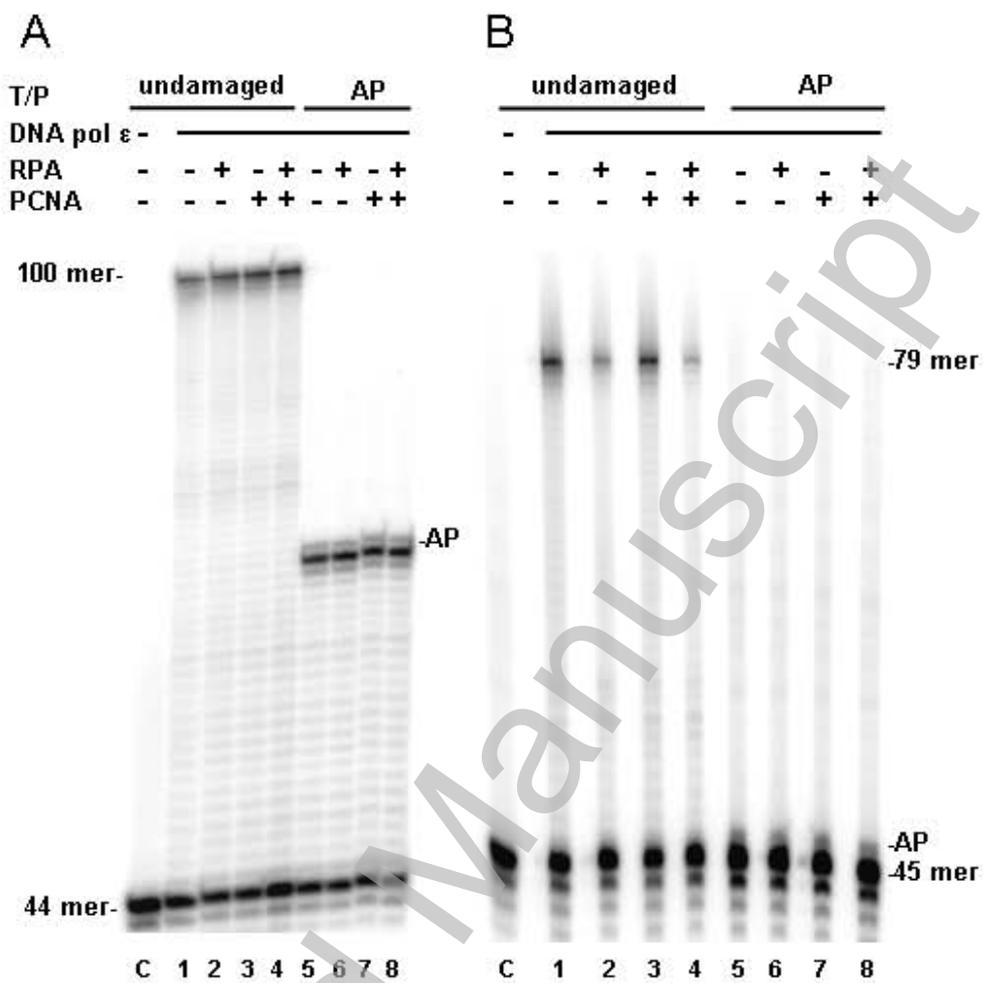


Figure 4

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20100405

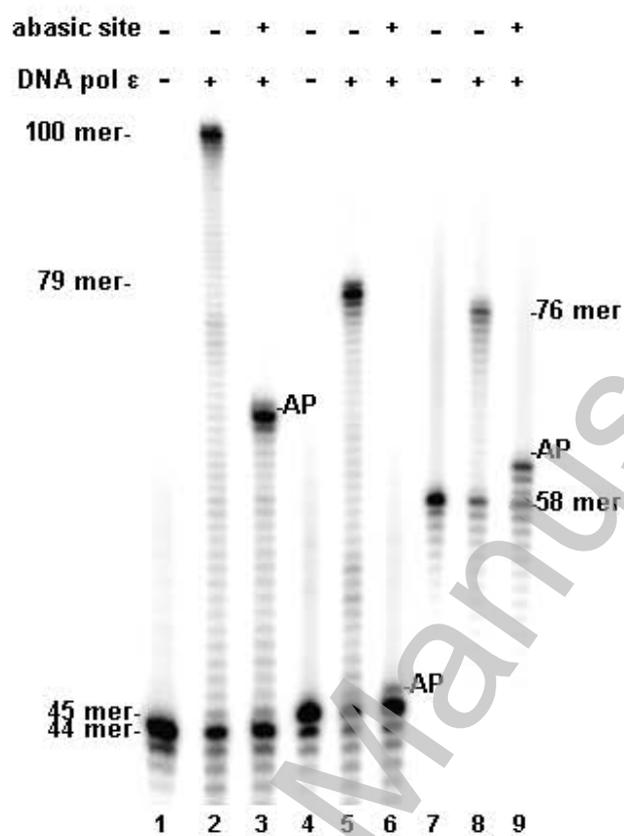


Figure 5

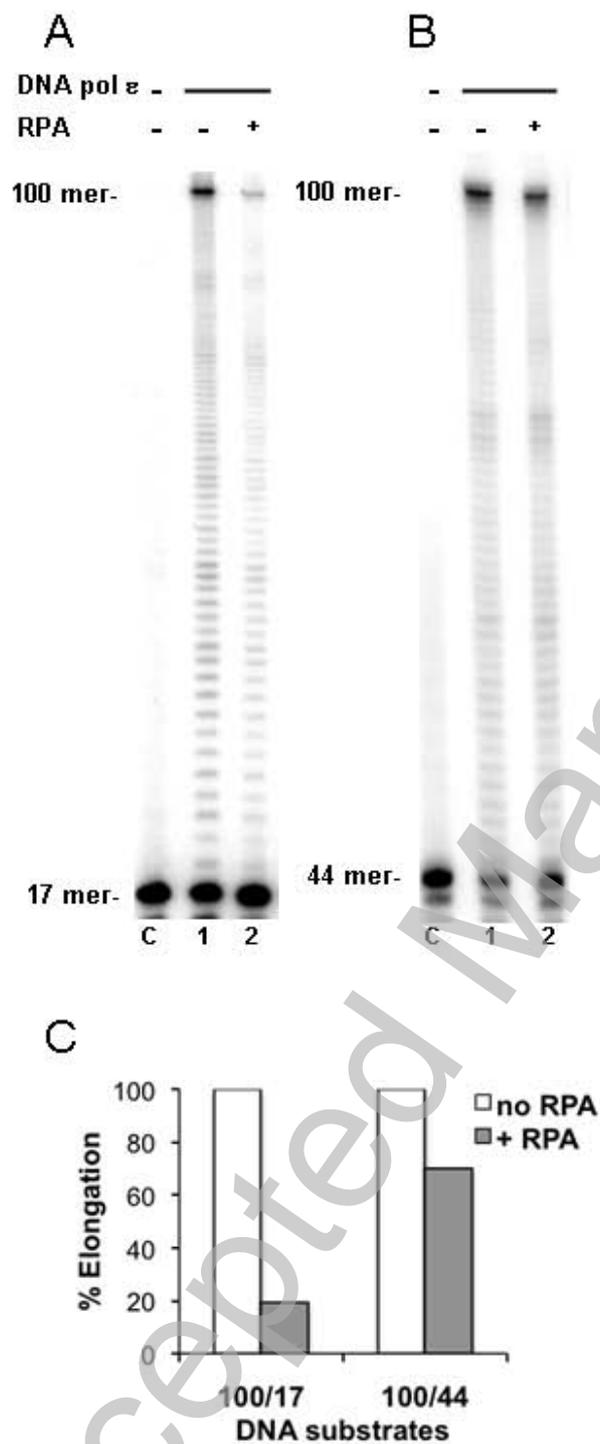


Figure 6