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Taq DNA polymerase blockage at pyrimidine dimers

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The most stable DNA-lesions generated by irradiation with ultraviolet light (254 nm) are two kinds of pyrimidine dimers (PDs), cylobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PP) (1). Blockage of DNA-polymerase from *Thermus aquaticus* (*Taq* polymerase) at PDs has been applied in primer extension assays for photofootprinting (2) and for mapping PDs at nucleotide resolution (3,4). These assays depend on an efficient and precise blockage at PDs. Using plasmid DNA with a site specific CPD or 6-4PP and irradiated yeast DNA, we report here that *Taq* DNA polymerase is indeed completely blocked at PDs. Furthermore, we show that the signal depends on the local sequence and on whether the DNA was pretreated with T4-endonuclease V (T4-endoV) or with T4-endoV and DNA-photolyase. These results justify the use of primer extension protocols for quantitation of PDs and they point out some technical limitations of this approach.

M13mp18TT-CPD is plasmid DNA containing a unique *cis–syn* cyclobutane thymine dimer (5). The DNA was cut with *Hin*dIII and aliquots were treated with (i) T4-endoV, which cuts at the CPD, (ii) DNA-photolyase, which reverts CPDs or (iii) T4-endoV and then DNA-photolyase. The DNA samples were denatured, an oligonucleotide labeled at the 5′-end with ^{32}P was hybridized and primer extension by *Taq* DNA polymerase was done for 30 cycles. The reaction products were displayed on a sequencing gel and compared with sequencing lanes obtained by dideoxy sequencing using the same primer extension protocol (Fig. 1a). With untreated DNA, 93 ± 5% of the signal (average of three experiments) accumulated in front of or at the CPD (lane 5, region B) and 7% was detected at the *Hin*dIII site (region A). Pretreatment with T4-endoV (lane 6) or T4-endoV and photolyase (lanes 7) changed the signal in the B and A regions by <2% in individual experiments (see legend to Fig. 1). This result shows that blockage at the CPD site was complete and that the signal at the *Hin*dIII site corresponds to a fraction of plasmid DNA without a CPD and was not due to read through at the CPD site. After photolyase treatment, all signal was found at the *Hin*dIII site (lane 8), demonstrating that blockage in lanes 5–7 was indeed due to a CPD.

Blockage at the CPD results in two major bands migrating at the position of the 3′T and 3′G, and a few minor bands which indicate premature stops (lane 5, region B). T4-endoV cleavage results in a major band at the 3′T and a minor band at th 5′T (lane 6). Additional removal of the overhanging 5′T by photolyase results in a minor band at the 3′T and major band at the site of the removed 5′T (lane 7). Since *Taq* polymerase is known to frequently add a non-template 3′ adenosine residue (6), the results

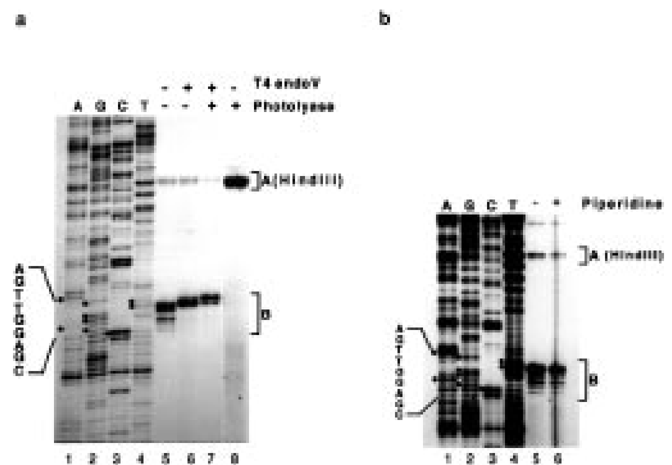

Figure 1. Blockage of *Taq* DNA polymerase at a CPD (a) and 6-4PP (b). (**a**) Primer extension is shown for M13mp18TT-CPD DNA cut with *Hin*dIII (lane 5), and treated with T4-endoV (lane 6), T4-endoV then photolyase (lane 7) or photolyase only (lane 8). Dideoxy sequencing lanes are also shown (lanes 1–4). The relevant part of the sequence is shown (5′-AGTTGGAGC-3′). Boxes A indicate elongation to the *Hin*dIII cut. Boxes B represent blockage in the thymine dimer region. The signals in region B were almost constant in lanes 5, 6 and 7: 95, 95 and 96% (experiment 1); 98, 99 and 96% (experiment 2); 88, 86 and 89% (experiment 3; the values were lower than in experiments 1 and 2 due to higher gel background). Region A plus region B is 100%. (**b**) Primer extension is shown for M13mp18TT-64 DNA, cut with *Hin*dIII (lane 5) and treated with piperidine (lane 6). The sequence is shown in lanes 1–4. The signals in region B of untreated samples (lanes 5) were 84 ± 1% and for piperidine treated samples 78 ± 10% (average of three experiments).

in lane 7 are best explained by DNA synthesis to the 3′T followed, in most cases, by addition of a non-templated nucleotide. Without removal of the overhanging T, addition of a non-template nucleotide is less efficient (lane 6). In the untreated template, one major band appears to represent a stop at the 3′G. The band at the 3′T may represent a template based synthesis or non-template addition of a nucleotide to the DNA stopped at the 3′G.

M13mp18TT-64 is a double-stranded DNA containing a unique (6-4) thymine–thymine photoproduct (5). The DNA was cleaved with *Hin*dIII and an aliquot was treated with piperidine to cleave at the (6-4)PP (7). The major stop was observed at the 3′T of the lesion and some premature stop sites were also present (Fig. 1b). The signals in region B were between 84 ± 1% for untreated DNA and 78 ± 10% for piperidine treated DNA. The values for piperidine were never higher than those for untreated

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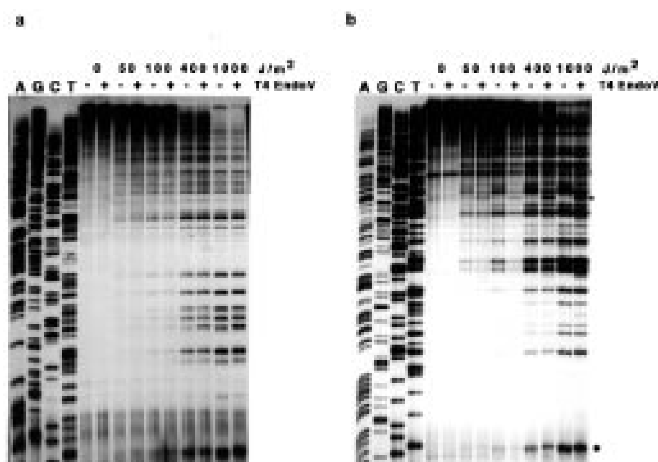

Figure 2. *Taq* polymerase blockage at PDs in the yeast URA3 gene. DNA of a yeast FTY23 containing the URA3 gene in a minichromosome, YRpTRURAP (8), was irradiated with 0–1000 J/m^2, cleaved with *Eco*RI and treated (+) or mocktreated (–) with T4-endoV. Primer extension was done with primer #547 to display a region of the transcribed strand (**a**) and with primer #544 to display a region of the non-transcribed strand (**b**). Some differences between (+) and (–) T4 endoV treatment are indicated (star, dot).

DNA which is consistent with a complete blockage at the (6-4)TT.

We wondered whether premature stops are a general feature of *Taq* polymerase blockage on damaged DNA, which was not cut at CPDs, or whether the premature stop relates to the plasmid sequence context. Therefore, yeast DNA was irradiated with 0–1000 J/m^2, and treated or mocktreated with T4-endoV prior to primer extension (Fig. 2). The intensity of bands generated by UV irradiation increased with increasing dose and, simultanously, the signal for full length extension on top of the gel decreased. The bands obtained for samples treated or mocktreated with T4-endoV were similar at most of the sites and, hence, did not reveal premature blockage of *Taq* polymerase. Significant differences were observed only at a few sites (star, dot in Fig. 2b). One additional band was generated by T4-endoV digestion (star). A doublet of bands (dot) was converted to one band by T4-endoV cleavage as observed with the site specific CPD lesion (Fig. 1). In conclusion, T4-endoV digestion and photolyase treatment are not required for PD analysis at most of the sites.

Plasmid DNA was digested with *Hin*dIII. T4-endoV digestions were done in 10 mM Tris pH 8.0, 80 mM NaCl and 10 mM EDTA at 37°C. Photoreversal was with *Escherichia coli* photolyase in 50 mM Tris pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol and 50 µg/ml BSA using Sylvania 15W F15T8 blacklights for 90 min. The DNA was purified by phenol/chloroform extractions, precipitated with ethanol and dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). For piperidine cleavage, 10 µl piperidine (Fluka) was added to ~5 ng of M13mp18TT-64 DNA in 90 µl TE, incubated at 90°C for 30 min, chilled on ice.The DNA was precipitated with 10 vol of ice cold ethanol.

Primer extension was done in 40 µl solution containing ~0.5 ng M13mp18TT or M13mp18TT-64, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 6 mM each dNTP and 0.4 pmol 5′-^{32}P-end-labeled primer (5′-AGCGGATAACAATTTCACA-CAGGA-3′). The samples were denatured for 5 min at 95°C and chilled on ice. *Taq* DNA polymerase (1 U) (Perkin Elmer) was added and primer extension was done in 30 cycles using the Perkin Elmer PCR thermocycler (denaturation at 94°C for 1 min, primer annealing at 60°C for 5 min, and extension at 72°C for 3 min). The DNA was analyzed on a 6% sequencing gel and quantified using a PhosphoImager (Molecular Dynamics).

Yeast DNA was purified from strain FTY23 [containing a minichromosome YRpTRURAP (8)], by CsCl gradients (9), digested with *Eco*RI in 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT. Droplets of DNA (100 µl, ~2 µg) were irradiated using four germicidal lamps (Sylvania G15T8) at 1.2 mW/cm^2 (measured with a UVX radiometer UVP Inc., CA). The DNA was purified by phenol extractions and dissolved in TE. DNA (1 µg) was either mock-treated or digested with T4-endoV at 37°C over night and repurified. Oligonucleotide #544 (5′-CCATAACAATCGCCAAACTTCGTCCGCCG-3′) hybridizes at nucleotides 1251–1223 to the non-transcribed strand of the URA3 gene in YRpTRURAP and oligonucleotide #547 (5′-GGAGCACAGACTTAGATTGGTATATATACGC-3′) anneals at nucleotide 724–754 to the transcribed strand (8). Thirty cycles of primer extension were done with ~200 ng DNA, 94°C for 45 s, 65°C for 1 min 30 s, 72°C for 2 min. A final cycle was added with 5 min extension at 72°C.

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REFERENCES

1 Friedberg, E. C., Walker, G. C. and Siede, W. (1995) *DNA Repair and Mutagenesis*. ASM Press, Washington, D.C.
2 Axelrod, J. D. and Majors, J. (1989) *Nucleic Acids Res*., **17**, 171–183.
3 Thoma, F., Cavalli, G. and Tanaka, S. (1993) In Broda P.M.A., Oliver S.G. and Sims P.F.G. (eds), *The Eukaryotic Genome*, Cambridge University Press, Cambridge, UK, pp. 43–72.
4 Chandrasekhar, D. and Van Houten, B. (1994) *J. Mol. Biol*., **238**, 319–332.
5 Szymkowski, D. E., Lawrence, C. W. and Wood, R. D. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 9823–9827.
6 Clark, J. M. (1988) *Nucleic Acids Res*., **16**, 9677–9686.
7 Lippke, J. A., Gordon, L. K., Brash, D. E. and Haseltine, W. A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3388–3392.
8 Thoma, F. (1986) *J. Mol. Biol*., **190**, 177–190.
9 Rickwood, D. and Chumbers, J. A. A. (1984) *Centrifugation (2nd edition)*, IRL Press, Inc., Oxford.