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TATA-binding protein promotes the selective formation of UV-induced (6-4)-photoproducts and modulates DNA repair in the TATA box

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DNA-damage formation and repair are coupled to the structure and accessibility of DNA in chromatin. DNA damage may compromise protein binding, thereby affecting function. We have studied the effect of TATA-binding protein (TBP) on damage formation by ultraviolet light and on DNA repair by photolyase and nucleotide excision repair in yeast and *in vitro*. *In vivo*, selective and enhanced formation of (6-4)-photoproducts (6-4PPs) was found within the TATA boxes of the active *SNR6* and *GAL10* genes, engaged in transcription initiation by RNA polymerase III and RNA polymerase II, respectively. Cyclobutane pyrimidine dimers (CPDs) were generated at the edge and outside of the TATA boxes, and in the inactive promoters. The same selective and enhanced 6-4PP formation was observed in a TBP–TATA complex *in vitro* at sites where crystal structures revealed bent DNA. We conclude that similar DNA distortions occur *in vivo* when TBP is part of the initiation complexes. Repair analysis by photolyase revealed inhibition of CPD repair at the edge of the TATA box in the active *SNR6* promoter *in vitro*, but not in the *GAL10* TATA box or in the inactive *SNR6* promoter. Nucleotide excision repair was not inhibited, but preferentially repaired the 6-4PPs. We conclude that TBP can remain bound to damaged promoters and that nucleotide excision repair is the predominant pathway to remove UV damage in active TATA boxes.

Keywords: DNA damage/DNA repair/photolyase/TATA-binding protein/transcription

Introduction

DNA is continuously damaged by intra- and extracellular DNA-damaging agents, which, unless repaired, may lead to mutations, cell death and cancer (Lindahl, 1993). To ensure efficient repair and maintenance of the genomic integrity, repair processes are integrated in a network with transcription, gene expression, replication and cell cycle control. Since DNA lesions are generated all over the genome, including active and inactive genes as well as in DNA elements necessary for regulation of gene expression or replication, understanding of DNA repair processes requires investigations of protein–DNA interactions, DNA-damage formation and repair at specific sites. Here we study how a key protein involved in transcription initiation by all nuclear RNA polymerases, the TATA-binding protein (TBP), affects formation of DNA damage by ultraviolet (UV) light and repair of UV lesions by photolyase and nucleotide excision repair (NER).

UV light introduces two major stable forms of mutagenic photoproducts, cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). CPDs and 6-4PPs distort DNA structure (Wang and Taylor, 1991; Kim and Choi, 1995). The yields and type of damage depend on the sequence and structure of DNA (Lippke *et al*., 1981; Brash and Haseltine, 1982; Lyamichev *et al*., 1990, 1991), DNA-bending (Pehrson and Cohen, 1992) and folding of DNA into nucleosomes (Gale *et al*., 1987; Pehrson, 1989; Gale and Smerdon, 1990; Brown *et al*., 1993; Suquet and Smerdon, 1993; Schieferstein and Thoma, 1996, 1998; Mann *et al*., 1997; for reviews see Sage, 1993; Tornaletti and Pfeifer, 1996; Smerdon and Thoma, 1998). A modulation of damage formation was observed *in vivo* at binding sites of sequence-specific transcription factors suggesting that those factors alone or in combination with other chromatin proteins modulated DNA structure (e.g. Becker and Wang, 1984; Selleck and Majors, 1987b, 1988; Becker *et al*., 1989; Pfeifer *et al*., 1992; Gao *et al*., 1994; Tornaletti and Pfeifer, 1995). DNA lesions affect gene expression by blockage of elongating RNA polymerases *in vitro* (reviewed in Selby and Saucar, 1994; Donahue *et al*., 1994; Livingstone-Zatchej *et al*., 1997; Suter *et al*., 1997; Aboussekhra and Thoma, 1998). Alternatively, DNA lesions could affect transcription initiation and regulation by compromising binding of transcription factors or set up of the initiation complex. In support of this hypothesis, *in vitro* experiments with cellular extracts (Tommasi *et al*., 1996) and TFIIIA binding to 5S rDNA (Liu *et al*., 1997) showed that DNA damage prevents binding of specific transcription factors. Whether and how factor binding is compromised by UV-induced DNA lesions *in vivo* is unknown, and we do not know whether or how transcription initiation complexes affect and are affected by damage formation.

CPDs and 6-4PPs can be repaired by photoreactivation and nucleotide excision repair. During photoreactivation, a damage-specific enzyme [CPD-photolyase or (6-4)-photolyase] binds to the photoproduct and reverts the damage in a light-dependent reaction, restoring the bases to their native form. CPD-photolyases were isolated from many organisms including *Escherichia coli* and *Saccharomyces cerevisiae*, while (6-4)-photolyases were found in *Drosophila*, *Xenopus laevis* and rattle snakes (reviewed in Yasui *et al*., 1994; Sancar, 1996b). In contrast to photoreactivation, NER is a highly conserved multistep mechanism which repairs a broad range of DNA damage

including CPDs and 6-4PPs. During NER, the lesions are recognized and excised as a fragment of DNA, and the resulting gap is filled in by a DNA polymerase (reviewed in Sancar, 1996a; Wood, 1996). NER repairs 6-4PPs more rapidly than CPDs (Mccready and Cox, 1993; Szymkowski *et al*., 1993; Galloway *et al*., 1994; Suquet *et al*., 1995), which may result from a enhanced recognition of 6-4PPs due to greater DNA distortions.

Like DNA-damage formation, both DNA repair mechanisms are modulated by chromatin structure and transcription. Photoreactivation is fast in nucleosome free regions and slow in positioned nucleosomes *in vivo* (Suter *et al*., 1997) and inefficient in reconstituted mononucleosomes *in vitro* (Schieferstein and Thoma, 1998). Moreover, photoreactivation is slow on the transcribed strand of genes transcribed by RNA polymerase II (RNAPII) and RNA polymerase III (RNAPIII), suggesting that polymerases stalled at DNA lesions inhibit accessibility of CPDs to photolyase (Livingstone-Zatchej *et al*., 1997; Suter *et al*., 1997; Aboussekhra and Thoma, 1998). NER is also affected at sites which are known to interact with transcription factors (Gao *et al*., 1994; Tu *et al*., 1996) or positioned nucleosomes (Wellinger and Thoma, 1997). NER shows rapid repair of the transcribed strand in RNAPII-transcribed genes (for a review see Friedberg, 1996), but not in RNAPI and RNAPIII genes (Christians and Hanawalt, 1993; Fritz and Smerdon, 1995; Dammann and Pfeifer, 1997; Aboussekhra and Thoma, 1998). This NER strand bias in RNAPII genes is possibly linked to the dual role of the general transcription factor TFIIH in transcription initiation and DNA repair (for a review see Friedberg, 1996). Fast NER at the start of transcription may indicate that recruitment of TFIIH for transcription initiation leads to an increased local concentration of repair factors (Tu *et al*., 1996; Teng *et al*., 1997).

Among the transcription factors which might have a direct effect on DNA damage formation and repair is TBP. This protein is an essential component for transcription initiation by all three nuclear RNA polymerases (Hernandez, 1993). In RNAPII genes, TBP is part of the general transcription factor TFIID, binds to the TATA box and recruits the other general factors and RNAPII (reviewed in Orphanides *et al*., 1996). In RNAPIII genes, TBP is part of TFIIIB and is required for transcription of the TATA-less genes as well as for the yeast *SNR6* gene which contains a TATA box (reviewed in Geiduscheck and Kassavatis, 1995). Crystal studies have shown that TBP makes contact with 8 bp of the TATA elements and dramatically alters DNA structure. The DNA is untwisted, sharply bent towards the major groove and exposes a wide, shallow minor groove to which TBP is bound (Kim *et al*., 1993a,b; reviewed in Patikoglou and Burley, 1997). The highest level of structural analysis was achieved with the ternary complexes of TBP–TFIIA–DNA (Geiger *et al*., 1996; Tan *et al*., 1996) and TBP–TFIIB–DNA (Nikolov *et al*., 1995). But an important question is whether similar distortions occur in the living cell when TBP is part of the initiation complex and whether the distortions are similar in the initiation complex of RNAPII and RNAPIII genes. A first indication for an altered DNA structure *in vivo* was the observation of enhanced UV photoproduct formation in the TATA elements of the transcriptionally active *GAL1* and *GAL10* genes in yeast (Selleck and Majors, 1987a,b). However, the nature of the photo-products was not identified and it remained open whether TBP was involved and sufficient for enhanced damage formation.

To address this question, we have investigated UV-damage formation in yeast in the TATA boxes of a gene transcribed by RNAPIII, *SNR6*, and in a gene transcribed by RNAPII, *GAL10*. Since TBP is the only common factor in RNAPII and RNAPIII initiation complexes, this genetic approach allows identification of the effect of TBP on the TATA box structure *in vivo*. The studies are complemented by studies of the TBP–TATA box complex *in vitro*. We demonstrate that TBP is sufficient *in vivo* and *in vitro* to generate a specific DNA lesion (6-4PP) within the bent part of the TATA box, indicating that the same DNA structure occurs in living cells and *in vitro*.

A recent study suggested that TFIID–TBP might be involved in DNA-repair processes. It was shown that TBP–TFIID binds selectively to cisplatin- or UV-damaged DNA. Cisplatin-treated or UV-irradiated DNA could be used as a competing binding site which may lure TBP–TFIID away from its normal promoter sequence (Vichi *et al*., 1997). This study brings up a set of important questions. What happens at the TATA box? Do TBP–TFIID proteins remain bound after damage formation in the TATA box, or are they displaced? How are DNA lesions repaired at the TATA box? Does TBP affect repair at the TATA box? Here, we use photolyase *in vitro* and in yeast to demonstrate that TBP and proteins of the RNAPIII initiation complex can remain bound to a damaged promoter element of the *SNR6* gene and inhibit photoreactivation. In contrast, NER is not inhibited indicating differential roles of NER and PR at this promoter element. Moreover, neither photoreactivation nor NER are inhibited in the *GAL10* gene, indicating differential stability of RNAPIII and RNAPII initiation complexes *in vivo*.

Results

TBP enhances pyrimidine dimer (PD) yields in the TATA box in vivo

The *SNR6* gene has a TATA box at position –30, an A-box within the transcribed region and an essential B-box located downstream of the termination signal (Brow and Guthrie, 1990). TFIIIC binds to A- and B-boxes and recruits TBP as part of TFIIIB to the TATA box (Burnol *et al*., 1993; Gerlach *et al*., 1995). TFIIIB protects ~40 bp in footprinting experiments *in vitro* (Gerlach *et al*., 1995; Colbert *et al*., 1998) (Figure 1E). At this position, a similar sized footprint was observed *in vivo* (Marsolier *et al*., 1995), indicating that it was generated by the TBP–TFIIIB complex. We therefore refer to the TBP–TFIIIB complex, although realizing that additional proteins may be involved. The footprint is missing and the TATA region is accessible to nucleases in a transcriptionally silent mutant which has a 2 bp deletion in the B-box (*snr6Δ2*) (Marsolier *et al*., 1995). The protected TATA-box region contains three pyrimidine clusters (PCs): PC –21 in the bottom strand, which includes the TBP binding site, and PC –18 and PC –41 localized at both sides of the TATA box on the top strand.

To investigate the effect of the TATA binding complex on UV-damage formation, the *S.cerevisiae* cells AAY1 (*SNR6*, *rad1*Δ) and AAY2 (*snr6Δ2*, *rad1*Δ) were UV-irradiated and the yields of PDs were analysed by primer extension. Figure 1A reveals two strong bands at position PC –21A and PC –21B in the wild-type *SNR6*

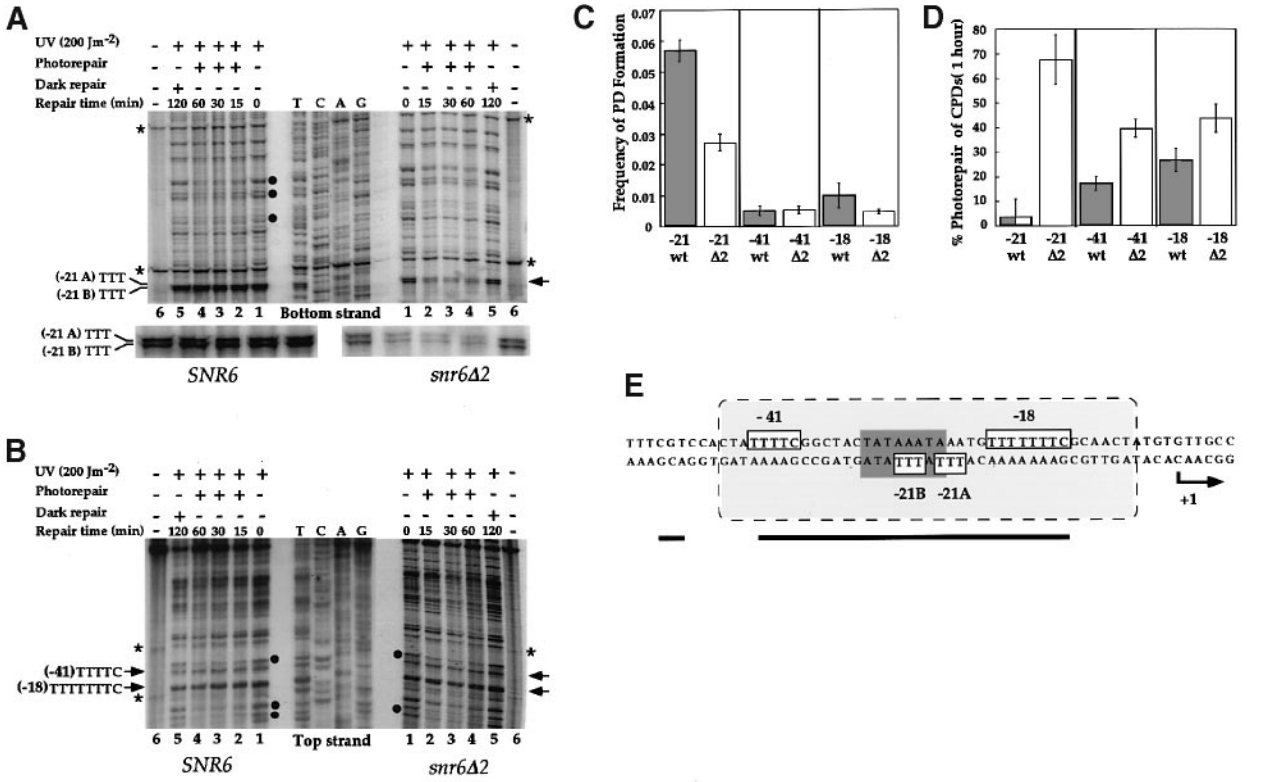

Fig. 1. UV-damage formation and photoreactivation in the SNR6-TATA box region. Liquid cultures of AAY1 (*SNR6, rad1*Δ) and AAY2 (*snr6*Δ*2, rad1*Δ) were UV-irradiated (UV+, lanes 1–5) reincubated under yellow light (dark repair, lane 5) or photoreactivating light (photorepair, lanes 2–4) for the indicated repair times and DNA damage (CPDs and 6-4PPs) was analysed by a *Taq*-polymerase blockage assay using primer extension. (**A**) Primer extension products in the bottom strand. (**B**) Primer extension products in the top strand. The bands do not resolve individual PDs, since the primers had to be chosen more than 350 bp upstream and downstream of the TATA element (see Materials and methods) (Marsolier *et al*., 1995). (–21A)TTT, (–21B)TTT, (–41)TTTT, (–18)TTTTTTT indicate the position of the PCs. Lane 6 is DNA of non-irradiated cells. Asterisks indicate non-specific *Taq* polymerase arrests. Dots refer to sites which are repaired rapidly. T, C, A and G are sequencing lanes. Bottom panels are enlargements of the TATA-box region of lanes 1–5. (**C**) Quantitative analysis of PD yields in the wild-type *SNR6* (wt, dark bars) and in the mutant *snr6*Δ*2* (Δ2, white bars). –21, –41 and –18 refer to the PCs. Averages and standard deviations of three experiments are shown. (**D**) The fraction of PDs (%) removed after 1 h photoreactivation (photorepair). (**E**) The sequence of the *SNR6* promoter, the region protected from DNase I digestion *in vitro* with purified TFIIIB (bars) (Gerlach *et al*., 1995), the region protected from micrococcal nuclease digestion *in vivo* (in dashed box) (Marsolier *et al*., 1995). This region is accessible to nuclease in the *snr6*Δ*2* mutant. The dark box refers to the TATA element. –21A, –21B, –41 and –18 show the PCs.

promoter (lanes 1, *SNR6*). A comparison with the other PD sites on the same strand, indicates that PC –21 is a hot spot for PD formation. Two bands were also generated in the *snr6*Δ*2* mutant (Figure 1A, lane 1, *snr6*Δ*2*), but compared with other sites on the same strand, they were less prominent. A quantitative comparison showed that PD yields in PC –21 (TTTATTT) are ~2-fold higher in the wild-type gene than in the mutant *snr6*Δ*2* (Figure 1C). In PC –18 and PC –41, neither an enhancement nor a difference in PD yields was observed between the mutant and wild-type gene (Figure 1B and C). These results show that the enhancement of the PD yield is restricted to the TBP binding site (PC –21) and correlates with the presence of the TBP–TFIIIB complex.

Previous work reported enhanced yields of photoproducts in the TATA box of the transcribed *GAL1* and *GAL10* genes (Selleck and Majors, 1987a). To investigate this observation in detail, AMY3 (*rad1*Δ) cells were grown either in glucose or galactose, then UV-irradiated and the photoproducts were mapped in the *GAL10* promoter. The presumed *GAL10* TATA region (Selleck and Majors, 1987a) contains two pyrimidine clusters PC –109 (5′CTT), which belongs to the canonical TATA box, and a flanking PC –104 (5′TCTT) (Figure 2B). UV-irradiation generated two bands in PC –109 (Figure 2A, lane 1). The upper thymine–cytosine dimer is induced with a lower yield than the lower thymine–thymine dimer. The overall yields in PC –109 were 2-fold higher in galactose than in glucose (Figure 2A, lane 1, galactose; Figure 2C, lane 1, glucose). Although the sequence of PC –104 and PC –109 are similar, the enhancement of PD yields is restricted to the PC –109. This result, together with previous observations (Selleck and Majors, 1987a,b), strongly suggest that TBP–TFIID enhances PD formation in its binding site (PC –109). The enhancement of PD formation in the *GAL10* TATA box occurs at a similar site as in the *SNR6* TATA box (PC –21) (Figure 1A), which indicates that enhanced photoproduct formation is an intrinsic property of TBP binding and seems to be independent of the promoter and TBP-associated factors.

TBP promotes the selective formation of 6-4PPs within the TATA box in vivo

Since *Taq* polymerase is efficiently blocked at CPDs and 6-4PPs, the primer extension assay used above detected both classes of UV lesions (Wellinger and Thoma, 1996). To analyse the nature of the photoproducts formed in the *SNR6* and *GAL10* TATA elements, damaged DNA extracted from irradiated cells was treated with *E.coli*

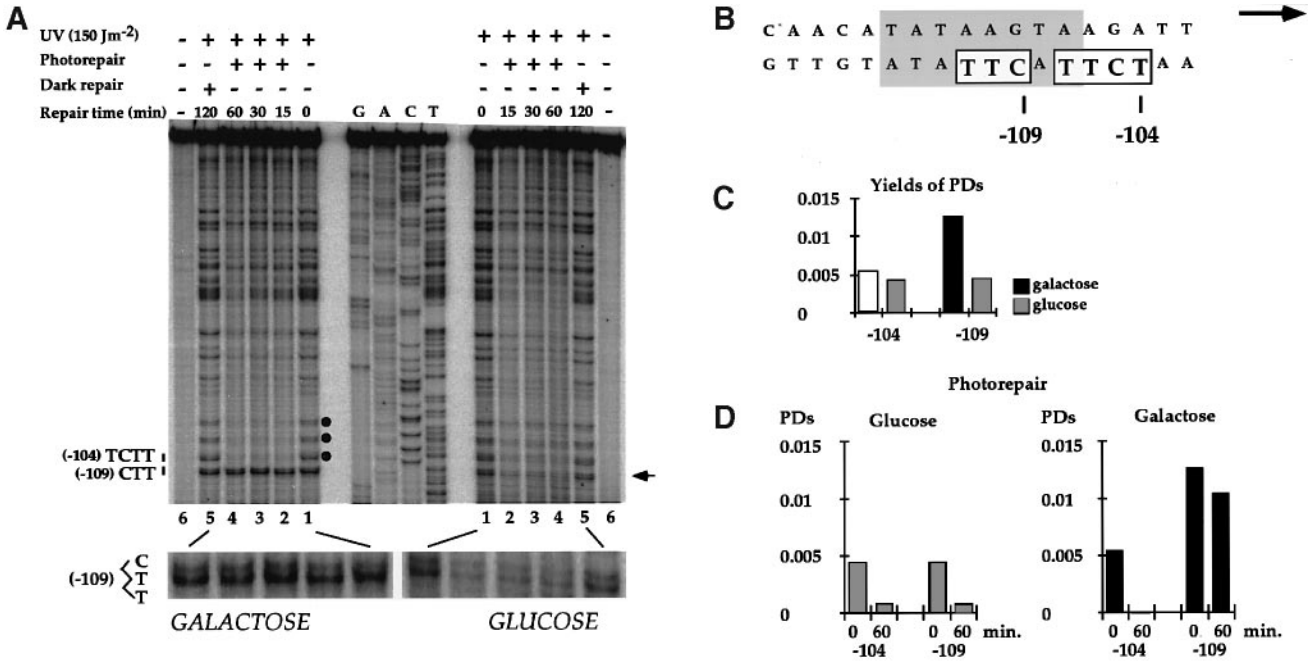

Fig. 2. UV-damage formation and photoreactivation in the GAL10 TATA box region. Liquid cultures of AMY3 (*rad1*Δ) were UV-irradiated with a dose of 150 J/m^2 and treated as in Figure 1. (**A**) Primer extension products. UV-irradiated DNA (lanes 1–5), non-irradiated DNA (lane 6), DNA sequencing (lanes T, C, A and G). (–104)TCTT and (–109)CTT indicate the PCs. The arrow indicates the position of the PDs formed in the TATA box. Bottom panels are enlargements of the (–109)CTT region. (**B**) The *GAL10* TATA box region. The pyrimidine clusters are indicated, (–104)TCTT, (–109)CTT (white boxes), the TATA element (dark box) (Selleck and Majors, 1987b). Transcription initiation is further downstream (arrow). (**C**) Quantification of the fraction of molecules containing a PD in PC –104 and PC –109 (data from lane 1, glucose, and lane 1 galactose). (**D**) The fraction of molecules containing a PD in PC –104 and PC –109 after 0 and 60 min of repair by photolyase (data from lanes 1 and 4, glucose and galactose).

CPD-photolyase *in vitro*, which selectively removes the CPDs. Primer extension was then used to detect the presence of the remaining PDs, the 6-4PPs. In the TATA element and in the flanking region (Figure 3A, dots) of the *snr6*Δ*2* mutant, the bands disappeared after photoreactivation, indicating that most of the PDs were CPDs (Figure 3A, lanes 4 and 5). However, when DNA from *SNR6* wild-type cells was analysed, the bands in PC –21A and in the flanking region (Figure 3A, dot) disappeared, demonstrating that these PD were CPDs. In PC –21B, however, a signal persisted, indicating the presence of 6-4PPs. Hence, compared with the PC –21A and the flanking site, a large fraction of 6-4PPs can be detected in PC –21B only in the wild-type cell, when TBP is present.

A similar result was obtained by analysis of photodimers in the *GAL10* TATA box. PDs induced in transcriptionally inactive state were repaired *in vitro* by photolyase and therefore are CPDs (Figure 3B, lanes 1 and 2, glucose). From the PDs induced in the transcribed state, PDs located in PC –104 were repaired by photolyase and represent CPDs. The signal in PC –109, however, was resistant to photoreactivation (Figure 3B, lanes 3 and 4, galactose) indicating the presence of a large fraction of 6-4PPs in the active *GAL10* TATA box. These results show that the TBP-associated complexes in RNAPII and RNAPIII transcription systems promote a selective formation of 6-4PPs in their respective TATA boxes. Since TBP is the only protein shared between the RNAPIII and RNAPII initiation complex, these results suggest that TBP is responsible for 6-4PP formation.

If TBP promotes the formation of 6-4PPs, the fraction of 6-4PPs formed in the TATA box would indicate the fraction of promoters loaded with TBP. Quantification of the photoreactivated samples, however, was limited by formation of additional stops for *Taq* polymerase (Figure 3A, stars) and a signal enhancement due to CPD removal between primer and 6-4PPs can not be excluded, although on average only 0.3 CPDs/kb were formed. Our estimations of the fraction of 6-4PPs in –21B yield a maximum of >90% and a minimum of 35%. This fraction was larger than in the immediately flanking regions (–21A; Figure 3A, dots). The high levels of 6-4PPs are consistent with the strong footprint observed over the TATA box (Marsolier *et al.*, 1995).

TBP promotes the selective formation of 6-4PPs and increases PD yields in vitro

To directly test whether TBP alone is responsible for enhanced PD yields and preferential formation of 6-4PPs, the effects of TBP binding to DNA from *SNR6* promoter (–60 to +1) was studied *in vitro* (Figure 4). The evolutionary conserved C-terminal domain of the *S.cerevisiae* TBP (cyTBP) (Tan *et al.*, 1996) which contains the DNA binding specificity was used. Free DNA labelled at one end, and DNA complexed with TBP were UV-irradiated with 1 kJ/m^2. DNA was purified and pyrimidine dimers were analysed using either T4 endonuclease V (T4-endoV), which cuts specifically at CPDs, or the *Neurospora crassa* UV-induced dimer endonuclease (UVDE), which cuts both at CPDs and 6-4PPs (Yajima *et al.*, 1995). Analysis of PD-formation in both PC –18 and PC –41 revealed the same CPD patterns in presence or absence of TBP (Figure 4, lanes 2 and 5). Hence, TBP had no detectable effect

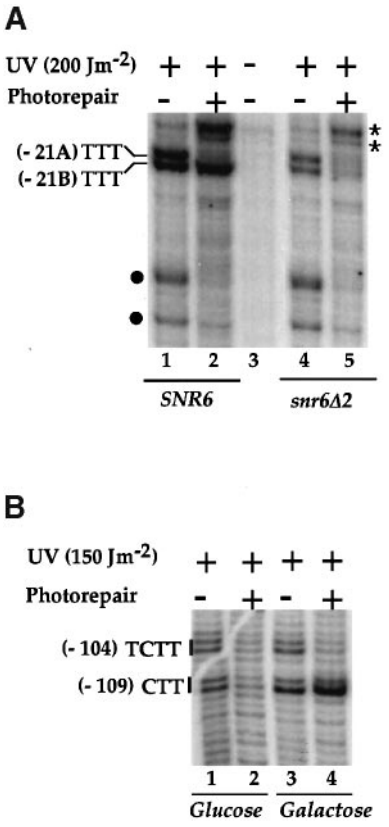

Fig. 3. Formation of 6-4PPs in the TATA box of transcribed *SNR6* and *GAL10* genes. (**A**) Photoproducts formed in the *SNR6* TATA box. FTY113 (*SNR6*) and FTY115 (*snr6Δ2*) were irradiated with 200 J/m^2 (UV+) and the DNA was purified. DNA damage (CPDs and 6-4PP) is shown in lanes 1 and 4. An aliquot of damaged DNA was treated with *E.coli* photolyase to remove CPDs (photorepair+). The remaining photoproducts (6-4PP) are shown in lanes 2 and 5. The PCs are indicated (–21A)TTT, (–21B)TTT (as in Figure 1). Dots indicate bands outside the TATA box which are removed by photolyase. Stars refer to additional *Taq* polymerase blocks after photoreactivation. (**B**) Photoproducts formed in the *GAL10* TATA box. W303-1a cultures grown in galactose or glucose were irradiated with 150 J/m^2 (UV+). DNA was analysed as in (A). (–104)TCTT and (–109)CTT are indicated (as in Figure 2).

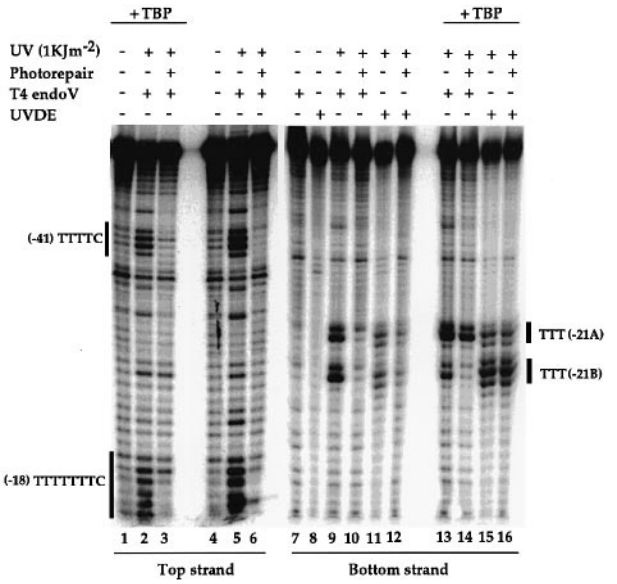

Fig. 4. Formation and photorepair of pyrimidine dimers in the *SNR6* promoter region *in vitro*. Top and bottom strands were annealed, and the DNA was radioactively labelled at one end. Aliquots were complexed with ycTBP (+TBP), irradiated with UV light (UV+), and incubated with *E.coli* photolyase in presence of photoreactivating light (Photorepair+). The DNA was repurified and cut with T4-endoV at CPDs (T4-endoV+) or with UVDE at CPDs and 6-4PPs (UVDE+). The pyrimidine clusters are indicated (as in Figure 1). (Note that the signals of in –21A and –21B of lane 11 were slightly weaker than in lane 9, indicating that CPDs were not completely cut by UVDE. Longer incubations were avoided due to an additional nicking activity.)

on CPD formation on both flanking regions PC –18 and PC –41.

In contrast, analysis of the bottom strand revealed specific effects of TBP on damage formation in the TATA-element. Irradiation of free DNA generated similar yields of CPDs in PC –21A (8%) and PC –21B (9%), detected by both T4-endoV cleavage and by UVDE cleavage [6% (PC –21A), 5% (PC –21B); Figure 4, lanes 9 and 11]. In presence of TBP, however, the yield of photoproducts (CPDs and 6-4PPs) was about three times higher in PC –21B (18%) and slightly higher in PC –21A (8%; Figure 4, lane 15). This demonstrates that TBP enhances photoproduct formation.

The enhanced signal in PC –21B compared with PC –21A (Figure 4, lane 15) and similar signals in PC –21B and PC –21A in absence of TBP (lane 11) clearly support the formation of 6-4PPs in PC –21B in presence of TBP. A comparison of PC –21B in lane 13 and 15 reveals low levels of CPDs but high levels of both photoproducts (CPDs and 6-4PP). The CPDs in PC –21B probably reflect the fraction of DNA not (or not properly) bound to TBP. In summary, both enhancement and selective formation of 6-4PPs in the TATA element reproduced the observations made *in vivo* and show that binding of TBP to DNA is sufficient to enhance PD formation and restrict the photodimer formation within the TATA box to the 6-4PPs class.

The 6-4PP formation *in vivo* and *in vitro* occurs at the sites where crystal structure studies revealed bent DNA. Hence, these UV photofootprints provide direct evidence that these structural distortions in the complex are responsible for the preferential formation of 6-4PPs. Moreover, TBP in RNAPII and RNAPIII initiation complexes *in vivo* generates DNA distortions very similar to those observed *in vitro*.

TBP remains bound after damage induction and inhibits CPD repair by photolyase in vitro

A central question is whether the proteins remain bound after damage induction and how they affect repair. To address this question *in vitro*, free DNA and DNA–TBP complexes were UV-irradiated and treated with *E.coli* CPD photolyase *in vitro*. The remaining photoproducts were analysed by T4-endoV and UVDE digestion. CPDs in free DNA were repaired within 5 min (Figure 4, lanes 10 and 12). In presence of TBP, the CPDs in PC –21B were efficiently photorepaired, and indeed may reflect a fraction of DNA not bound to TBP. However, CPDs in PC –21A were resistant to repair (Figure 4, lane 14). This demonstrates that TBP remains bound to DNA, which is damaged at the edge of the TATA box in PC –21A, and inhibits photorepair, possibly by preventing access of CPDs to photolyase. In contrast, TBP binding did not

affect photorepair in PC –18 and PC –41 (Figure 4, lane 6). This shows that TBP-mediated inhibition of photolyase is TATA-box specific.

TBP complexes can remain bound to the damaged SNR6 TATA box and inhibit photorepair of CPDs in vivo

DNA-repair by photolyase is a major pathway for CPD repair in many organisms including the yeast *S.cerevisiae*. The action of photolyase was shown to be restricted by folding of DNA into nucleosomes and by RNA polymerases blocked at CPDs, but photoreactivation was very efficient in open promoters which are not folded in nucleosomes (Livingstone-Zatchej *et al*., 1997; Suter *et al*., 1997). Here we analysed whether the TBP containing initiation complexes remain bound after damage induction *in vivo* and how photolyase can repair damaged promoter elements. We used yeast strains deficient in NER: AAY1 (*SNR6*, *rad1*Δ) and AAY2 (*snr6*Δ*2*, *rad1*Δ). After damage induction, yeast cultures were exposed to photoreactivating light and the remaining PDs were analysed (Figure 1). In the *snr6*Δ*2* mutant, in the absence of the TFIIIB footprint, ~70% of the lesions were photorepaired in PC –21 (–21A and –21B) within 1 h (Figure 1A and D). The repair efficiency was similar to that of other sites on the same strand (Figure 1A). However, in the wild-type *SNR6* promoter, repair of CPDs by photolyase was strongly inhibited in PC –21. In 1 h, only ~5% of the lesions were photorepaired (both bands; Figure 1D, –21wt). The enlargement in Figure 1A, *SNR6*, shows no decrease in signal intensity in PC –21A with increasing repair time. (The 6-4PPs in PC –21B can not be repaired by the photolyase of *S.cerevisiae*.) Other sites in the flanking region were efficiently repaired (Figure 1A, dots). The lack of photorepair in PC –21A of the wild-type *SNR6* strongly suggests that the TBP–TFIIIB complex remains bound after damage induction and inhibits access of photolyase to CPDs. Photorepair of CPDs in PC –18 and PC –41 was also reduced in wild-type promoter, but <2-fold compared with the mutant promoter (Figure 1B and D). This indicates that the TBP–TFIIIB complex inhibits DNA repair by photolyase within the *SNR6* promoter and that this inhibition is most tightly restricted to the TBP cognate site. This is direct evidence that transcription factors can inhibit the accessibility of DNA damage to repair enzymes and prevent activity of a major repair pathway.

In the *GAL10* promoter the situation is distinct. In the inactive promoter (Figure 2A, glucose) photorepair was efficient at all sites (Figure 2A, compare lanes 2–4 with lanes 1 and 5; Figure 2D). In the active promoter, DNA damage in PC –109 can not be photorepaired, since it consists of 6-4PPs (Figure 2A and D, galactose). However, CPDs in PC –104 were efficiently repaired and as efficient as many other sites on the same strand (Figure 2A, dots). Hence, photorepair of CPDs in PC –104 is not inhibited. Apparently photolyase finds access to CPDs suggesting that the TBP initiation complex was displaced by UV damage. This is in contrast to the *SNR6* promoter, and may indicate differential stability of the RNAPII and RNAPIII initiation complexes.

Preferential nucleotide excision repair of 6-4PPs in the SNR6 and GAL10 TATA boxes

NER is the second repair pathway for UV-lesions. It removes 6-4PPs as well as CPDs and it shares proteins of the general transcription factor TFIIH with the RNAPII transcription system. Since TBP is involved in recruiting TFIIH to the promoter, it is attractive to speculate that TBP might also play a role in TATA box repair. Primer extension technique was used to investigate the effect of the TBP-associated complex on NER. For the *SNR6* gene FTY113 (*SNR6*, *RAD1*) and FTY115 (*snr6*Δ*2*, *RAD1*) cells were UV-irradiated and re-incubated in the dark for different repair times (Figure 5A shows an autoradiograph). In comparison with photoreactivation, NER is relatively inefficient. Quantitative analysis showed that NER was very similar in PC –21A and PC –21B of the *snr6*Δ*2* mutant and in PC –21A of the wild-type *SNR6* promoter (Figure 5B). Up to 40% of PDs were repaired within 4 h (Figure 5B). Similar results were obtained for PC –18 and PC –41 (not shown). In contrast to photoreactivation, there was no evidence that TFIIIB inhibits NER of CPDs at the *SNR6* TATA-box (PC –21A).

Interestingly, the 6-4PPs in PC –21B of *SNR6* are more rapidly excised than the CPDs formed in PC –21A (Figure 5A and B, compare band intensities of –21A and –21B in enlargements). Since the same repair rates were measured for CPDs in PC –21A and B of the mutant *snr6*Δ*2* (Figure 5B), fast repair of 6-4PPs is not due to DNA sequence, but indicates a preference of NER for 6-4PPs.

Figure 6 shows NER in the *GAL10* TATA-box. In the inactive promoter (glucose), only 10% of PDs were removed from the PC –109 and PC –104 (Figure 6B) in 1 h. This repair rate is similar to that found within the transcriptionally inactive gene or in the non transcribed strand of the *GAL10* gene (data not shown). However, in the active *GAL10* promoter (galactose), there is a differential repair in PC –104 and PC –109, containing CPDs and 6-4PPs, respectively. After damage induction (Figure 6A, lane 1), the signal in PC –109 is stronger than in PC –104. After 1 h NER (Figure 6A, lane 2), the signal intensities are similar at both sites. Approximately 50% of the 6-4PPs were repaired in PC –109 in 1 h, while only ~25% of CPDs were excised from PC –104 (Figure 6B). This result shows that the 6-4PPs in PC –109 are more rapidly repaired than the CPDs in PC –104 and they are more rapidly repaired than CPDs induced in the same sites in the inactive promoter (Figure 6B). Together, these results indicate that for CPDs and 6-4PPs formed in the same sequences, 6-4PPs are more rapidly excised by NER.

Discussion

Selective formation of 6-4PPs in the TATA box reveals similar DNA structures of TBP–DNA complexes in vivo and in vitro

TBP is an essential factor for transcription initiation by all three nuclear RNA polymerases and plays a key role in assembly of the transcription machineries. Its interaction with DNA generates DNA deformations which are well known *in vitro*, while more detailed structural information *in vivo* is scarce (reviewed in Burley and Roeder, 1996;

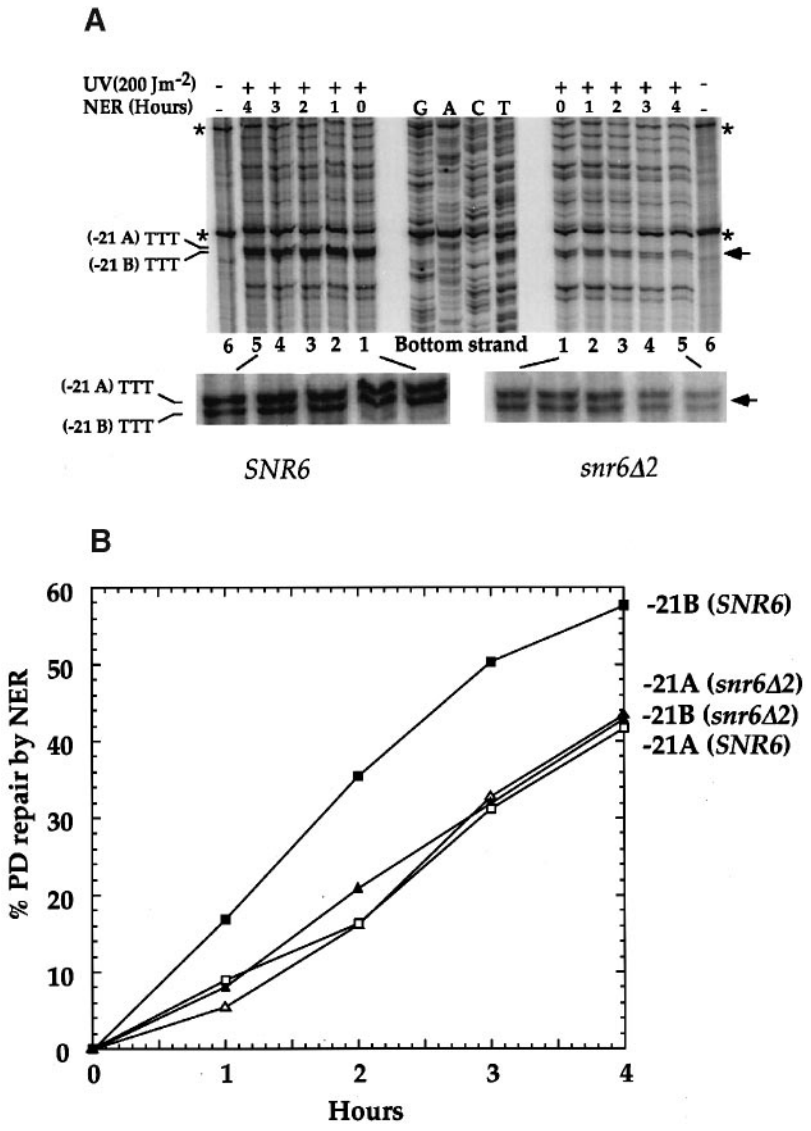

Fig. 5. Nucleotide excision repair in the *SNR6* promoter. FTY113 (*SNR6*) and FTY115 (*snr6Δ2*) were irradiated with 200 J/m^2 (UV+), re-incubated in the absence of photoreactivating light for the indicated time (NER, hours). (**A**) PDs were analysed as in Figure 1. The enlarged panels show the (–21A)TTT, (–21B)TTT region of the same samples, but separated by longer gel electrophoresis. (**B**) Quantitative analysis of PD removal from *SNR6*/*snr6Δ2* promoters. PDs in PC –21A, open symbols; PDs in PC –21B, solid symbols; *SNR6*, squares; *snr6Δ2*, triangles.

Patikoglou and Burley, 1997). Previous work showed enhanced yields of UV photoproducts within the putative 'TATA' boxes of the active *GAL1* and *GAL10* genes. Ultraviolet modification at this site was enhanced only in transcriptionally active promoters indicating a putative involvement of TBP or the whole initiation complex (Selleck and Majors, 1987a,b). Here, we show that this phenomenon is not restricted to genes transcribed by RNAPII, but is detected in an RNAPIII gene with a TATA box (*SNR6*), and in a TBP–TATA box complex *in vitro*. Since TBP is the only common factor between RNAPII and RNAPIII initiation complexes, our data show that TBP is responsible for the enhanced photoproduct formation.

In addition, we have characterized the photoproducts and demonstrate the selective formation of 6-4PPs within the TATA box of the active *SNR6* and *GAL10* genes *in vivo* and in the TBP–TATA complex *in vitro*. No 6-4PPs were detected in the inactive promoters and in the absence of TBP. Therefore, enhanced and specific damage formation is an intrinsic property of TBP–TATA box interactions. The additional factors which associate with TBP *in vivo* in the RNAPII and RNAPIII initiation complex have no observable effect on the DNA structure.

Based on the sequences used for the crystal structure analysis (Kim *et al*., 1993a,b; Tan *et al*., 1996), it was inferred that TBP binds to the *SNR6* and *GAL10* TATA elements as indicated in Figures 1E and 2B, respectively. Consistent with this assumption, the 6-4PPs formation was found to be tightly restricted to the 'internal' pyrimidine cluster located within the 8 bp of presumed TBP contacts in the TATA box (PC –21B in *SNR6* and PC –109 in *GAL10*). All other pyrimidine clusters formed CPDs in presence and absence of TBP. All structural studies *in vitro* on TBP–DNA, TFIIA–TBP–DNA and TFIIB–TBP/DNA complexes revealed significant distortions of the DNA structure: a sharp bend towards the major groove, a flattened and broadened minor groove and partially unwound DNA flanked by kinks at either

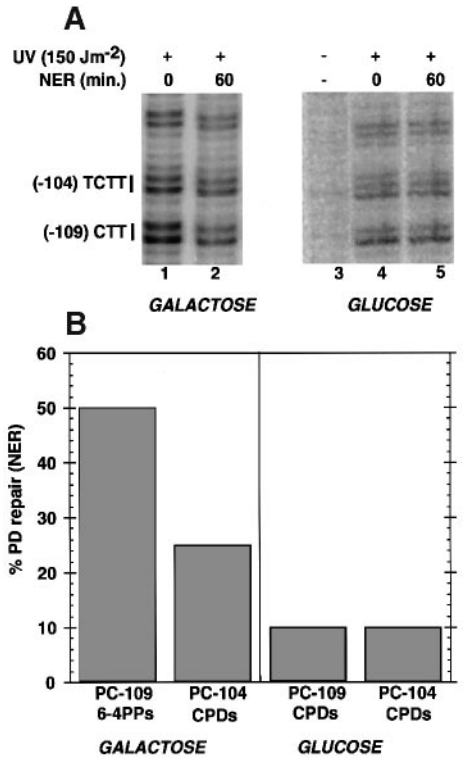

Fig. 6. Nucleotide excision repair in the *GAL10* promoter. Yeast cells (W303-1a) grown either in glucose or galactose were UV-irradiated (150 J/m^2) and incubated for NER as in Figure 5. PD were analysed as in Figure 2. (**A**) Enlargement of the TATA region for 0 and 60 min repair (lanes 1, 2, 4 and 5) and undamaged DNA (lane 3). (–104)TCTT, (–109)CTT are the pyrimidine clusters (as in Figure 2). (**B**) Quantification of repair after 60 min in PC –104 and PC –109. The site specific photoproducts are indicated (CPDs, 6-4PP).

end of the TATA sequence (Kim *et al*., 1993a,b; Geiger *et al*., 1996; Tan *et al*., 1996). It is known that photoproducts form more easily on melted and bent DNA (Becker and Wang, 1989) and in structurally distorted DNA of the *Eco*RI endonuclease–DNA complex (Becker *et al*., 1988). CPDs bend DNA by ~7° (Ciarrocchi and Pedrini, 1982; Wang and Taylor, 1991) while 6-4PPs introduce a sharp kink of ~44° (Kim and Choi, 1995). The selective and enhanced formation of 6-4PPs within the TATA box strongly suggests that the TBP induced DNA distortions more closely resemble DNA distortions in the 6-4PPs than in CPDs. The enhanced and selective formation of 6-4PP in the *SNR6* and *GAL10* promoters therefore demonstrates that TBP induces the same DNA distortions when complexed in a large initiation complex in living cells. Moreover, our *in vivo* data emphasize the structural similarity of DNA in the RNAPIII and RNAPII complexes which appears to be relevant for the transcription initiation process.

TBP and TBP–TFIIIB inhibit photorepair, suggesting that the proteins remain bound to the UV-damaged TATA box

An important question is whether proteins remain bound to their damaged binding sites or whether they are displaced. Dissociation of the complex would inevitably disrupt function, in this case initiation of transcription. On the other hand, binding might prevent repair and enhance mutagenesis. *In vitro* band shift experiments with cellular extracts and oligos containing binding sequences for specific transcription factors as well as experiments with TFIIIA binding to 5S rDNA revealed that DNA lesions prevent factor binding (Tommasi *et al*., 1996; Liu *et al*., 1997). In contrast to those results, our *in vitro* experiments have shown that TBP alone inhibits photorepair of CPDs in PC –21A, but not in PC –18 and PC –41. This is consistent with the observation that TBP bound to DNA protects only about 20 bp against DNase I digestion (Auble and Hahn, 1993). *In vivo*, CPDs in PC –21A of *SNR6* were also completely resistant to photoreactivation, while the same site was efficiently repaired in the *snr6Δ2* mutant. These data demonstrate that TBP *in vitro* as well as the TBP–TFIIIB complex *in vivo* remain bound after the PC –21A promoter element was damaged and prevent accessibility of CPDs to photolyase. Hence, CPD induced DNA distortions at the site where the DNA leaves the TBP complex have no remarkable effect on TBP binding *in vitro* and do not disrupt the complex *in vivo*. This offers the possibility that the *SNR6* gene can maintain transcriptional acitvity, despite of a damage in the TATA box. In addition, the inhibition of photorepair by TBP shown here provides evidence that a transcription factor can inhibit a simple DNA repair process *in vivo* and it shows a strict modulation of a repair process by site specific protein interactions. Moreover, this result implies that alternative repair pathways must exist that repair those lesions which are not accessible to photolyase.

Photoreactivation in PC –18 and PC –41 was ~2-fold slower in the wild-type *SNR6* than in the *snr6Δ2* mutant (Figure 1D) and complete *in vitro*. This is an indication that TBP–TFIIIB also modulates repair in the TATA-box flanking regions. Unlike the *SNR6* TATA element, the CPDs induced in the vicinity of the *GAL10* TATA box (PC –104) were not resistant to photorepair *in vivo*. TFIIIB forms a stable complex with DNA and can promote multiple rounds of initiation by polymerase (Kassavetis *et al*., 1992). We therefore take our repair observations as an indication for differential stability or different initiation frequencies of the RNAPII and RNAPIII initiation complexes.

Nucleotide excision repair is the major pathway to remove UV-damage in active TATA boxes

Much to our surprise, NER repaired CPDs in PC –21A of the wild-type *SNR6* promoter and in the *snr6Δ2* mutant with similar rates (Figure 5B). In contrast to photolyase, NER was not inhibited in the wild-type *SNR6* promoter. Moreover, the 6-4PPs of the *SNR6* and *GAL10* TATA boxes were repaired faster than the CPDs induced in the same site. Therefore, nucleotide excision repair is the predominant pathway to remove UV damage in active TATA boxes.

The reasons for similar rates of CPD repair in the wild-type *SNR6* (PC –21A) and in the *snr6Δ2* mutant are not fully understood. Two explanations are proposed: (i) it is conceivable that TBP–TFIIIB is unstable on damaged DNA and dissociates. Hence, the situation will be similar in the wild type and the mutant, allowing similar access to damage recognition proteins, and therefore generate similar repair rates. A preference of damage recognition proteins for 6-4PP as shown for Rad14p *in vitro* (Guzder *et al*., 1993) would explain the enhanced NER efficiency

of 6-4PPs. However, the affinity of TBP for UV damaged DNA (Vichi *et al*., 1997) and the strong inhibition of photorepair in PC –21 of *SNR6* argues against a TBP–TFIIIB instability on UV-damaged TATA box. (ii) Photoreactivation in yeast generally repairs CPDs faster than NER. Photolyase removes CPDs in open chromatin regions in 15–30 min and in nucleosomes in 2 h (Suter *et al*., 1997), while NER takes several hours (Wellinger and Thoma, 1997). This is also true for the *SNR6* and *snr6Δ2* promoters, where photoreactivation removed up to 40% of CPDs from the PC –41 and PC –18 in 1 h, while NER required 4 h (Figure 1D and 5B). It therefore seems conceivable that within the first hour after damage formation, the CPD sites in *snr6Δ2* remain accessible to photolyase. With some delay, damaged sites might recruit TBP in its function as a damage recognition protein (Vichi *et al*., 1997). The recruitment of TBP could explain why CPDs in *snr6Δ2* are not completely repaired by photolyase in 1 h. In a next step, these complexes will recruit the NER machinery through the affinity of TBP with TFIIH. Therefore, repair will be similar for CPDs in *SNR6* and in the *snr6Δ2* mutant.

Why are the 6-4PP in the *SNR6* and *GAL10* TATA boxes repaired more rapidly than the CPDs at the edge of the TATA boxes? This effect could be explained if 6-4PP formation in the internal pyrimidine cluster destabilizes TBP binding more efficiently than CPD formation at the edge of the TATA box. Although our results do not provide direct information on whether TBP remains bound after 6-4PP induction, the enhanced and selective formation of 6-4PPs strongly suggests that these photoproducts can be accommodated in a TBP–DNA complex, and hence argues in favour of a stable 6-4PP–TBP complex. Given that TBP remains bound, preferential repair of 6-4PP can be explained in two ways: (i) the 6-4PP–TBP complex is more stable than the CPD–TBP complex and may therefore more efficiently recruit NER machinery; and (ii) preferential repair is independent of TBP, but reflects a difference in damage recognition and processing by NER.

Concluding remarks

We have shown that similar structural distortions occur in the TBP–DNA complex *in vitro* and *in vivo*. Hence, engagement of the TBP in transcription initiation complex with its numerous additional proteins does not disturb the basic structural information. Moreover, UV footprinting results for other RNAPII promoters show a similar enhanced photoproduct formation [*SUC2*, *GAL1* (Selleck and Majors, 1987a) and *DED1* (F.Thoma, unpublished data)], indicating that this is a general property of TATA boxes engaged in transcription initiation.

We have shown that TBP can remain bound to TATA boxes damaged at the edge by a CPD *in vitro* and *in vivo*. The TBP-dependent specific 6-4PP formation and its binding to UV-irradiated and cisplatin-damaged plasmid DNA (Vichi *et al*., 1997) predict that TBP remains bound to TATA boxes damaged in the internal pyrimidines. An important issue to answer in the future is whether those damaged promoters are still productive in transcription initiation. The fact that the *GAL10* and *SNR6* promoters behave differently indicates that the answer to this question will be promoter dependent. This adds an other element to the heterogeneity in DNA repair processes and to the topic of how functional properties of protein–DNA interactions are compromised by DNA lesions.

Finally, photolyase but not NER is inhibited by TBP and associated factors bound to a damaged TATA box. Only NER removes the 6-4PPs. In this situation, NER is the predominant repair pathway to repair UV lesions in active TATA boxes. However, photolyase is more efficient in CPD repair outside of the TATA box, in inactive promoters, and in open promoters that are not folded in nucleosomes (Suter *et al*., 1997). Photolyase preferentially repairs the untranscribed strand of active genes (RNAPII and RNAPIII genes; Livingstone-Zatchej *et al*., 1997; Suter *et al*., 1997; Aboussekhra and Thoma, 1998), while NER preferentially repairs the transcribed strand of RNAPII genes (reviewed in Sancar, 1996a; Wood, 1996). All these observations together illustrate the complementary role of these two DNA repair pathways at the level of site-specific and gene-specific repair with the consequence to ensure survival and maintenance of genomic integrity when the organism is exposed to the damaging effects of sunlight.

Materials and methods

Yeast strains

FTY113 (*MATα, ade2-102, ura3-52, lys2-801, his3Δ200, leuΔ1, trp1Δ63*/pRS314+U6 TRP1, *SNR6*); FTY115 (*MATα, ade2-102, ura3-52, lys2-801, hisΔ200, leuΔ1, trp1Δ63*/pRS314+U6 TRP1, *snr6Δ2*) (Marsolier *et al*., 1995); W303-1a (*MAT***a***, ade2-1, ura3-1, trp1-1, his3-11,15, leu2-3, trp1-1, can1-100*). AAY1, AAY2 and MEY3 are *rad1Δ* deletion strains derived from FTY113, FTY115 and W303-1a, respectively, generated by gene replacement technique.

Culture and UV-irradiation of yeast cells

Three litres of yeast cells were grown at 30°C in minimal medium (2% dextrose, 0.67% yeast nitrogen base without amino acids; Difco) (Sherman *et al*., 1986) supplemented with the appropriate amino acids to a final density of ~10^7 cells/ml. The cells were then collected by centrifugation and resuspended in 1 l of water to a concentration of between 1.5×10^7 and 3×10^7 cells/ml. Aliquots (250 ml) were transferred to a 22×31.5 cm plastic tray and UV-irradiated at room temperature with a dose of 150–200 J/m^2, using 4 Sylvania G15T8 germicidal lamps (predominantly 254 nm) at 1 mW/cm^2 (measured using a UVX radiometer, UVP Inc., CA). All steps from UV irradiation to DNA extraction were done in yellow light (Sylvania GE 'Gold', fluorescent light) to prevent undesired photoreactivation.

Photoreactivation

After UV-irradiation, samples of 250–500 ml were photoreactivated in water using Sylvania type F15 T8/BLB bulbs (emission peak at 366 nm) at 1.5 mW/cm^2 for 15–60 min. Cells (250 ml) were collected and chilled on ice.

Dark repair

After UV irradiation, minimal medium supplemented with the appropriate amino acids was added, and the cells were incubated at 30°C (in the dark) for various repair times. Repair was arrested by collecting cells (by centrifugation) and chilling them immediately on ice.

DNA preparation and enzyme digestions

Genomic DNA was prepared using Qiagen tips and protocols (Qiagen Genomic DNA Handbook).

Primer extension analysis

Primer labelling and primer extension were performed as described previously (Aboussekhra and Thoma, 1998) using the following PAGE-purified primers:

SNR6 top strand: Nr 716: 5′-CGTACCATTGCATAGCTGTAACAA-TATTC-3′

SNR6 bottom strand: Nr 717a: 5′-TATATTGCTACCATGACTGTCT-GAG-3′

GAL10 bottom strand: Nr 848: 5′-GGCTTCTAATCCGTACTTCAA-TATAG-3′
The primers were chosen to anneal about 350 bp outside of the *SNR6* gene to avoid cross-hybridization with a plasmid containing the *SNR6* gene (Marsolier *et al*., 1995).

DNA labelling and DNA–TBP complex formation

Two primers were annealed to generate a 60 bp DNA with 5′ overhangs. The DNA ends were filled in with either [α-^{32}P]dATP (bottom strand) or [α-^{32}P]dCTP (top strand) (Amersham) using Klenow fragment (Boehringer Mannheim). Radiolabelled DNA was mixed with cyTBP (61–240, C-terminal part) (Tan *et al*., 1996) in 1× buffer A (10% glycerol, 20 mM Tris pH 8, 5 mM $MgCl_2$, 60 mM KCl, 1mM DTT, 0.01% Nonidet P-40). The mixture was incubated for 30 min at room temperature. The DNA duplex is:

```
5′-TTTCGTCCACTATTTTCGGCTACTATAAATAAATGTTTTTTTCGC-
AACTATGTGTTCG-3′
3′-AGCAGGTGATAAAAGCCGATGATATTTATTTACAAAAAAAGCGTT-
GATACACAAGCGG-5′
```

UV irradiation and photoreactivation in vitro

DNA and DNA–TBP complexes were UV-irradiated with a dose of 1 kJ/m^2 in a volume of 20 µl on Parafilm. One microlitre of *E.coli* photolyase (0.1 µg/µl) was immediately added to the UV-damaged DNA or DNA–TBP complex and incubated for 5 min under photoreactivating light.

Mapping of PDs with T4 endoV and UVDE

Purified DNA samples were dissolved in 9 µl 1× T4-endoV buffer (20 mM Tris pH 7.4, 10 mM EDTA, 0.1 M NaCl, 0.1 mg/ml BSA) or UVDE buffer (50 mM Tris–Cl pH 8.0, 100 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT and 1mg/ml BSA) and incubated at 37°C for 30 min. T4-endoV (a gift from R.S.Lloyd) or UVDE (a gift from A.Yasui) was added and incubation continued for 90 min. Fresh enzyme was added after 30 min to ensure complete digestion. DNA was purified and then electrophoresed on denaturing 8% polyacrylamide gels. Gels were dried and exposed to X-ray films (Fuji) and to a PhosphorImager screen (Molecular Dynamics).

Quantification

The sequencing gels were used to calculate the relative repair of the lesions. A volume box was laid around each band obtained from a lesion and the corresponding gel background was subtracted using a volume box of the same size outside of the loaded lanes. The number obtained was then divided by the value obtained by a volume box which covered the whole lane to correct for loading differences (Wellinger and Thoma, 1997). The values obtained for the non-irradiated DNA were then subtracted in order to correct for unspecific background signal due to DNA nicking or unspecific Taq polymerase blockage. For standardization, the corrected values obtained at t_0 (no repair) were defined as 100% damage.

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