Nucleosome structure and positioning modulate nucleotide excision repair in the non-transcribed strand of an active gene

Ralf Erik Wellinger and Fritz Thoma¹

Institut für Zellbiologie, ETH, Hönggerberg, CH-8093 Zürich, Switzerland

¹Corresponding author
e-mail: thoma@cell.biol.ethz.ch

Nucleotide excision repair (NER) is a major pathway to remove pyrimidine dimers (PDs), a class of DNA lesions generated by ultraviolet light. Since folding of DNA into nucleosomes restricts its accessibility and since transcription and DNA repair require access to DNA, nucleosome structure and positioning as well as the transcriptional state may affect DNA repair. We recently determined the chromatin structure of the yeast *URA3* gene at high resolution and found multiple positions of nucleosomes as well as strand- and site-specific variation in DNA accessibility to DNase I (internal protected regions). Here, the same high-resolution primer extension technique was used to investigate NER of PDs in the *URA3* gene of a minichromosome *in vivo*. In the non-transcribed strand (NTS), fast repair correlates with PD locations in linker DNA and towards the 5' end of a positioned nucleosome. Slow repair correlates with the internal protected region of the nucleosome. This repair heterogeneity reflects a modulation of NER by positioned nucleosomes in the NTS. NER in the transcribed strand (TS) is fast, less heterogeneous and shows no correlation with chromatin structure. Apparently, transcription-coupled repair overrides chromatin modulation of NER in the TS. Heterogeneity in NER generated by chromatin structure on the NTS may contribute to heterogeneity in mutagenesis.

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Introduction

Since packaging DNA in nucleosomes and higher-order chromatin structures affects its accessibility to proteins, all DNA-processing reactions including transcription and DNA repair must be intimately coupled to and could even be regulated by structural and dynamic properties of chromatin. Indeed, nucleosomes positioned in promoter regions play a significant role in regulation of transcription, and promoter activation may require remodelling of nucleosome structures, generating a nuclease-sensitive region (NSR; reviewed in Wallrath *et al.*, 1994). Transcription elongation through nucleosomal templates requires a disruption of nucleosomes or a displacement of the histones by RNA polymerase (reviewed in Kornberg and Lorch, 1995). This may lead to a loss of nucleosomes from the transcribed gene (e.g. ribosomal RNA genes, references

in Dammann *et al.*, 1993) or to a rearrangement of nucleosome positions as shown for a gene transcribed from a *GALI* promoter in yeast (Cavalli and Thoma, 1993; Cavalli *et al.*, 1996). Less frequently transcribed yeast genes such as *URA3* or *HIS3* show positioned nucleosomes (Thoma, 1986; Losa *et al.*, 1990; Tanaka *et al.*, 1996). This structure most likely reflects the inactive, major fraction of the genes and suggests that the disruption of the chromatin structure by RNA polymerase II is rapidly reversed and does not require replication.

Irradiation of DNA with ultraviolet light (UV) generates two major classes of pyrimidine dimers (PDs), cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone dimers [(6-4)-PD]. Unless repaired they may lead to transcription blockage, mutations, cell death and cancer (reviewed in Friedberg *et al.*, 1995). The formation of CPDs and (6-4)-PD is dependent on DNA sequence, protein–DNA interactions and chromatin structure (for reviews see Smerdon, 1989; Tornaletti and Pfeifer, 1996). CPD formation is strongly modulated in nucleosomes due to the bending of DNA around the histone surface (Gale *et al.*, 1987; Pehrson, 1989; Pehrson and Cohen, 1992; Schieferstein and Thoma, 1996), while (6-4)-PD are more evenly distributed within nucleosome cores (Gale and Smerdon, 1990).

Nucleotide excision repair (NER) is a ubiquitous multi-step pathway in which numerous proteins are involved to sequentially execute damage recognition, excision of an oligonucleotide with the pyrimidine dimer, and gap repair synthesis (reviewed in Friedberg, 1996). In genes transcribed by RNA polymerase II, the transcribed strand (TS) is faster repaired than the non-transcribed strand (NTS) in higher eukaryotes (e.g. Mellon *et al.*, 1987), bacteria (e.g. Mellon and Hanawalt, 1989) as well as in yeast minichromosomes and chromosomes (Smerdon and Thoma, 1990; Leadon and Lawrence, 1992; Sweder and Hanawalt, 1992; Verhage *et al.*, 1994). This preferential repair of the TS is frequently referred to as 'transcription-coupled repair' (TCR). NER and initiation of transcription share proteins of the general transcription factor TFIIF. However, the molecular mechanism which leads to preferential repair of the TS remains to be elucidated (discussed in Friedberg, 1996). The NTS is repaired more slowly, similar to the genome overall. Mutations in the yeast genes (*RAD7*, *RAD16*) that affect the repair of the NTS also affect the repair of inactive genes and the genome overall (Verhage *et al.*, 1994, 1996; Mueller and Smerdon, 1995). These genetic observations suggest that the NTS could be repaired as a chromatin substrate.

The molecular mechanism of NER in chromatin is barely understood. Early studies in cultured human cells showed that repair synthesis occurs in a rapid early phase and in a slow late phase. At the nucleosome level there is an early phase that may represent refolding of

Fig. 1. Chromatin structure of the minichromosome YRpTRURAP and the *URA3* gene. (A) YRpTRURAP contains the *URA3* gene inserted in the *TRP1* gene of the TRP1ARS1 circle (Thoma, 1986). Indicated are: approximate positions of nucleosomes (circles), genes (arrows) and their 5' and 3' ends, location of primers (numbered arrows), the origin of replication (ARS1), *EcoRI* restriction site (R), dots in 200 bp intervals. (B) Detailed structure of the *URA3* gene (Tanaka *et al.*, 1996). Indicated are: approximate positions of nucleosomes (ellipses 1–6), internal regions protected against DNase I cutting on the top and bottom strand (bars in ellipses), multiple positions of nucleosomes (bars below nucleosomes) and position of linker DNA [numbers, in map units (base pairs from the *EcoRI* site)].

nucleosomes and a prolonged slow phase that may be explained by rearrangement or repositioning of nucleosomes (reviewed in Smerdon, 1989, 1991). Repair synthesis during the early phase showed a bias towards the 5' ends of nucleosome core DNA (Jensen and Smerdon, 1990). This repair effect was accounted for by the preferential formation of CPDs in the 5' ends of core DNA (Smerdon, 1991). Those studies were performed on bulk chromatin containing mixed sequences and did not account for the transcriptional state of the chromatin nor did they address repair of positioned nucleosomes.

To address directly the question of whether and how NER is modulated by transcription and chromatin structure, we used an indirect endlabelling technique to compare CPDs along the DNA sequence with the chromatin structure assayed by nuclease digestion (Smerdon and Thoma, 1990; Bedoyan *et al.*, 1992). These studies were done in a yeast strain containing the minichromosome (YRpTRURAP) with characterized chromatin structure as a model substrate (Thoma, 1986) (Figure 1). They showed fast repair on the TS consistent with TCR, and slow repair in the NTS. Repair was also efficient in a nuclease-sensitive promoter region of the *URA3* gene, but slow in the nuclease-sensitive origin of replication (ARS1). Those studies, however, were limited in resolution and could

not resolve differences in repair of linker DNA and nucleosomes nor differences of repair within positioned nucleosomes.

To study DNA repair within nucleosomes, high-resolution approaches are required for chromatin analysis and for DNA repair assays. We applied a primer extension technique (Axelrod and Majors, 1989) to characterize the chromatin structure of the *URA3* gene at high resolution using MNase and DNase I digestions (Tanaka *et al.*, 1996). The structure of the *URA3* gene is similar in the minichromosome and in the genome. At low resolution, the *URA3* gene showed six positioned nucleosomes flanked by NSRs (Figure 1B; Thoma, 1986). At high resolution, each position resolves into a complex pattern of multiple positions which suggests that nucleosome positions can change (nucleosome mobility). The high-resolution analysis provided important insight into the nucleosomal structure. Reduced DNase I cutting from ~50 bp from the 5' end towards the 3' end was common to all nucleosome regions. This polarity reflects a structural property of nucleosomes (here referred to as 'internal protected region'). Since these 'internal protected regions' only partially overlap on both strands (Figure 1B), they demonstrate differential accessibility of DNA strands on the nucleosome surface. We think that these properties of nucleosome positioning and the internal structure of nucleosomes play an important role in DNA damage recognition and repair.

Here, we have tested this hypothesis using the same primer extension protocol (Axelrod and Majors, 1989; Wellinger and Thoma, 1996) to analyse NER of PDs at high resolution and compared it with the local chromatin structure. We show that a pronounced repair heterogeneity on the NTS correlates with the positioning and the internal structure of nucleosomes. In contrast, repair on the TS is dominated by TCR.

Results

Efficient NER in a minichromosome

FTY23 cells containing the minichromosome YRpTRURAP (Figure 1) were irradiated in suspension with 100 J/m² and NER was allowed to occur for 0–50 min in the absence of photoreactivating light. To calculate CPD damage and repair on the whole minichromosome (overall repair), DNA was cut with *EcoRI*, treated with T4 endonuclease V (T4-endoV) which cleaves at CPDs, or mock treated, fractionated on alkaline agarose gels, blotted to nylon membranes and hybridized to a plasmid-specific probe (Figure 2). Unirradiated DNA (lane 2) and mock-treated samples (odd lanes 1–15) showed intact restriction fragments. In T4-endoV-cleaved samples (even lanes 4–16), the top band (region a) represents the fraction of undamaged DNA, while the smear (region b) shows the fraction of DNA cut at CPDs. With increasing repair time, the smear decreased and the proportion of intact fragment increased, demonstrating efficient repair. After irradiation with 100 J/m², ~48% of the DNA remained undamaged. This corresponds to an initial damage of 0.7 CPDs/plasmid or 0.27 CPDs/kb. After 2.5 h incubation at 30°C, ~80% of the CPDs were repaired by NER (Figure 2B).

Fig. 2. Overall repair of CPDs in YRpTRURAP. (A) Yeast cells were irradiated with 100 J/m² and repair was allowed for 0, 20, 40, 60, 90, 120 and 150 min in the dark (NER). DNA was extracted and cut with *Eco*RI and the fragments were separated by gel electrophoresis on alkaline agarose. Southern blot analysis shows the removal of CPDs after treatment of DNA with T4-endoV (T4-EndoV +, lanes 4, 6, 8, 10, 12, 14 and 16). Non-irradiated DNA was used as a control (lane 2). Mock-treated DNA is shown (T4-EndoV–, lanes 1, 3, 5, 7, 9, 11, 13 and 15). Undamaged and mock-treated DNA migrate on top of the gel (a), whereas DNA cut by T4-endoV at CPDs generates a smear (b). (B) The graph shows the time-dependent removal of CPDs. At time 0 min, 49% of plasmids were cut by T4-endoV which corresponds to an initial damage of 0.7 CPDs per plasmid. Data represent the average of three independent experiments.

High-resolution repair analysis by primer extension

Primer extension (Wellinger and Thoma, 1996) was used to characterize site-specific repair in distinct chromatin regions, namely in two nucleosomal regions of the coding part of *URA3* and in the nuclease-sensitive promoter. *Eco*RI-linearized DNA was treated with T4-endoV to prevent premature stops of *Taq* polymerase at CPDs (Wellinger and Thoma, 1996). The DNA was denatured and annealed to an endlabeled primer which was extended by *Taq* polymerase. *Taq* polymerase is efficiently blocked at CPDs and (6-4)-PD (Wellinger and Thoma, 1996) producing bands which map at pyrimidine dimers or pyrimidine tracts (compare sequencing lanes with lanes of UV-irradiated samples; Figures 3, 5 and 6). In a few cases, *Taq* polymerase was blocked at sites containing CA (TACACA 1019, Figure 3A; TCA 1331, TCA 1340, Figure 5A), possibly by purine photoproducts (Gallagher and Duker, 1986; Bourre *et al.*, 1987). The primer exten-

sion technique used here does not discriminate between CPDs and (6-4)-PD. However, since (6-4)-PD are generated in much lower yields than CPDs (Sage, 1993), the results of this study preferentially relate to CPD repair. Indeed, reversal of CPDs by photolyase prior to primer extension revealed only very weak signals originating from (6-4)-PD. These signals were too weak for a repair analysis (not shown). Non-irradiated DNA yields a very weak background. Stops of the *Taq* polymerase are observed towards the top of the lanes. Hence, accurate repair analysis can be done in the lower part of the gel, ~25–250 bp from the primer. The intensities of bands reflect the frequency of lesions at individual sites or in polypyrimidine tracts. The signal in the whole lane represents the total amount of amplified DNA. Site-specific damage was calculated as the fraction of signal at a lesion divided by the signal of the whole lane after appropriate subtraction of background. After irradiation of cells with 100 J/m², the fraction of DNA damaged at individual sites scattered between 0.001% for weak bands (e.g. CCT1079) and 0.1% for strong bands (e.g. TTTATC 902) (Figure 3A).

DNA repair was obvious as the decrease in band intensities with increasing repair time (Figures 3, 5 and 6). There is, however, substantial strand- and site-specific heterogeneity (see below). Some sites appear to be repaired quickly, while others are much more slowly repaired. To generate repair curves, the damage at each site and at each repair time was measured and normalized with respect to the initial damage (initial damage at time 0 min was set as 100%). Repair curves were calculated for DNA lesions in pyrimidine clusters, rather than for individual dipyrimidines. This is justified, since CPDs in individual clusters were generally repaired with similar rates. The data of three independent repair experiments were averaged and displayed as exponential curves (the curves are similar in all regions and therefore shown for the U2 region only, Figure 4). The $t_{50\%}$, the time at which 50% of the lesions were repaired, was graphically summarized and compared with the local chromatin structure as previously determined (Tanaka *et al.*, 1996) (Figure 7). It is pointed out that quantification of lesions as a fraction of the total DNA in a lane corrects for different amounts of DNA used in different lanes (e.g. lanes 10 and 11 in Figure 5) and allows one to obtain accurate repair data. Moreover, visual inspection of bands may suggest differential repair, but quantification yields similar repair rates (e.g. compare TTTT 996, TTTT 1010, Figures 3B and 4B). This is due to different yields of damage in TTTT 996 (0.016%) and TTTT 1010 (0.012%). Finally, a number of sites were excluded from the quantitative analysis (open boxes in Figures 3, 5 and 6), since the background was too high or the data too scattered.

Repair in the transcribed region

To address how repair occurs in the transcribed region and whether it correlates with chromatin structure or transcription, PD removal was mapped in and around the second and the fourth nucleosomes (U2 and U4) (Figure 1). Nucleosome U2 is best suited for this analysis. It is positioned within a few base pairs, it shows clear internal structure ('internal protected regions') and it contains sufficient pyrimidine dimers and pyrimidine tracts distributed over the whole region on both strands (Figure 3). U4

Fig. 3. Repair of PDs in the region of nucleosome U2 displayed by primer extension. (A) Top strand (non-transcribed strand, NTS) using primer No. 897 (Figure 1). (B) Bottom strand (transcribed strand, TS) using primer No. 896. Indicated are: nucleosome positions (ovals U2, U1, U3) with internal protected regions (bars) according to Tanaka *et al.* (1996); repair times (min, 0 to 150); T4-endoV treatment of DNA (+); UV irradiation conditions for DNA (40 J/m^2 , lane 5) and for cells (chromatin; 100 J/m^2 , lanes 7–13); DNA sequence of the template strand (A, G, C, T); sequence elements with DNA lesions that were quantified (dark boxes, numbers refer to the 5' nucleotide in the YRpTRURAP sequence); sequence elements with DNA lesions that were not quantified due to background or low signal intensities (white boxes); strong background signals (stars).

Fig. 4. Repair curves for lesions in the top strand (Figure 3A) and bottom strand (Figure 3B) of the U2 region respectively. DNA damage for each cluster was set to 100% at time 0 [lane 6 in (A) and (B)]. The average and standard deviation of three experiments (circles) are shown. In a few cases data of two experiments could be used (triangles). The curves represent exponential fits.

Fig. 5. Repair of PDs in the region of nucleosome U4. **(A)** Top strand (non-transcribed strand, NTS) using primer No. 1119. **(B)** Bottom strand (transcribed strand, TS) using primer No. 1118. Description is as in Figure 3.

Fig. 6. Repair of PDs in the *URA3* promoter region. **(A)** Top strand (non-transcribed strand, NTS) using primer No. 1014. **(B)** Bottom strand (transcribed strand, TS) using primer No. 1027.

is more complex, since it is tightly packed together with U3 and U5, and it may occupy multiple positions (Figure 1B). Nucleosomes U3, U5 and U6 are less suited due to the pyrimidine dimer distribution and multiple positioning. Nucleosome U1 lacks pyrimidine tracts on the top strand (Figures 6 and 7).

Fast repair with low heterogeneity on the transcribed strand

In general, repair was much faster on the TS (bottom strand) than on the NTS (top strand). This is obvious from visual inspection of the autoradiographs (Figures 3 and 5), from repair curves (Figure 4) and from the $t_{50\%}$ values

Fig. 7. Summary of repair rates and correlation between DNA-repair and chromatin structure. (A) The *URA3* promoter. (B) Nucleosome U2. (C) Nucleosome U4. The $t_{50\%}$ for each PD cluster is shown as vertical bars for the top and bottom strands respectively. Sites with no repair are shown as bars with dotted ends (this includes two sites possibly containing purine photoproducts, TCA1331, TCA1340). Indicated are the major nucleosome positions (ovals) including the internal protected regions (bars in ovals), promoter elements (white boxes) including the TATA element (dark box). Data are averages of three (B) and two experiments (A and C).

(Figure 7). The average repair of 33 lesions measured on the TS in U2, U4 and in U1 showed a $t_{50\%}$ of 32 ± 8 min. In contrast, the average repair of 21 lesions in the NTS showed a $t_{50\%}$ of 80 ± 41 min [lesions with no repair (Figure 7C) are not included]. Hence, repair in the TS was ~ 2.5 -fold faster. This result is consistent with TCR of the TS. Moreover, average repair in the TS in the U2 and U4 regions is 30 ± 6 min and 31 ± 8 min respectively. Hence, there is no measurable gradient in repair efficiency along the *URA3* gene.

However, a detailed inspection at individual sites and clusters on the TS reveals a moderate heterogeneity of repair rates (Figures 4B and 7). For example, in U2, repair of lesions in CTTTTC 915 ($t_{50\%} = 23$ min) was ~ 2 -fold faster than repair of lesions in TTTTTC 1010 ($t_{50\%} = 43$ min). In U4, repair of lesions in TTTCCTT 1259 ($t_{50\%} = 19$ min) was ~ 2 -fold faster than repair in CCTCTT 1320 ($t_{50\%} = 38$ min). This modulation in the repair rates does not obviously correlate with chromatin structure; in particular, it does not show slow repair in nucleosomes and fast repair in linker DNA nor does it show remarkably slower repair in the 'internal protected region' of U2 and U4 (Figure 7B and C). Hence, the local differences in repair rates could be related to a sequence specificity of the repair process.

Repair heterogeneity on the non-transcribed strand correlates with chromatin structure

In contrast to the TS, a much more pronounced repair heterogeneity was observed on the NTS (top strand, Figures 3A, 4A and 7). For example, repair was fast in TTC 931 ($t_{50\%} = 30$ min), CCT 1079 ($t_{50\%} = 32$ min), TTTATC 902 ($t_{50\%} = 56$ min) and CTTC 940 ($t_{50\%} = 57$ min). Slow repair was observed, e.g. in CCTTTT 1042 ($t_{50\%} = 103$ min). A comparison with the chromatin structure (Figure 7B) shows that this repair heterogeneity correlates well with chromatin structure according to two criteria. First, repair was fast in sites that map in linker DNA, e.g. in linkers between U1 and U2 (Figure 3, site

TTTATC 902), and between U2 and U3 (Figure 3, sites TT 1075, CCT 1079). Second, in U2, repair correlates well with the internal structure described by the accessibility of DNA to DNase I. Fast repair was observed towards the 5' end (sites between TTC 931 and CTT 951, Figures 3 and 7B) where DNase I accessibility is high. Slow repair was recorded in the region towards the 3' end (sites between TT 966 and CCTTTT 1042, including a purine-pyrimidine region TACACA 1019), where DNase I cutting is inhibited ('internal protection', bar in Figures 3A and 7B).

In the NTS (top strand) of the nucleosome U4 region, the correlation between DNA repair ($t_{50\%}$) and chromatin structure was more complex, since nucleosomes U3, U4 and U5 are tightly packed leaving only limited space for linker DNA (Figure 1B). Despite that, lesions observed in the top strand of U4 (sites between TTTTCC 1279 and TT 1373 including purine photoproducts TCA 1331 and TCA 1340) are more slowly repaired than TTTC 1382, TT 1385 and CTC 1389 which map in the contact region of U4 and U5. PDs in the contact region between U3 and U4 (TTGTT 1226) are relatively quickly repaired also (Figures 5A and 7C).

In summary, these results show that repair on the NTS is modulated by chromatin structure, in particular by the internal structure of nucleosomes and nucleosome positioning.

Fast repair in the *URA3* promoter region

The 5' end of the *URA3* gene is characterized by a nuclease-sensitive promoter region and a nucleosome (U1) which may occupy two major and possibly some minor positions (Figures 1 and 7A) (Thoma, 1986; Tanaka *et al.*, 1996). Hence, it is possible that DNA lesions in the NSR could be recognized and repaired more efficiently. The promoter region contains, on both strands, several pyrimidine clusters and in particular long T-tracts on the top strand (Figure 6) (Roy *et al.*, 1990). These T-tracts are hotspots of PD formation (Figure 6A). All pyrimidine

clusters were repaired with $t_{50\%}$ between 30 and 60 min. In contrast to the transcribed region, no obvious differences in repair rates were observed in the top and bottom strand of the promoter region. The repair rates were faster than the average rates in the NTS of the *URA3* gene ($t_{50\%} = 80$ min), which is consistent with an enhanced accessibility of lesions in a NSR. There is a moderate heterogeneity of repair rates on both strands. Previous reports showed enhanced repair rates close to the transcription initiation site of the human *JUN* gene, a property which could be explained by the enhanced concentration of factors (TFIIH) which are involved in repair and transcription initiation (Tu *et al.*, 1996). However, in the *URA3* gene, there are no dramatic DNA repair differences between sites upstream and downstream of the transcription initiation (Figure 7A).

Discussion

Chromatin modulates repair in the non-transcribed strand but not in the transcribed strand

Previous repair studies using indirect endlabelling were restricted to a resolution of approximately ± 20 bp and, hence, did not resolve differential repair in linker DNA and within nucleosomes (Smerdon and Thoma, 1990; Bedoyan *et al.*, 1992; Mueller and Smerdon, 1995, 1996). The primer extension approach used in this work allowed us to determine the location of a lesion at the sequence level and to correlate it with the high-resolution chromatin structure as determined by nuclease digestion (Tanaka *et al.*, 1996). As shown for the U1, U2 and U4 region, this correlation is limited by the pyrimidine distribution in the DNA sequence as well as by the precision and dynamics of nucleosome positioning.

The results on the NTS of the U2 region show a strong correlation between the rate by which pyrimidine dimers are removed and the accessibility of the sequence to micrococcal nuclease and DNase I (Figure 7). Lesions which map in linker DNA and towards the 5' end of nucleosomes were repaired more quickly than lesions that map in the 'internal protected region' of the nucleosome. This correlation holds for the U4 region too, although the nucleosome arrangement is more complex with respect to the tight packaging of nucleosomes U3, U4 and U5 and with respect to multiple positions (Thoma, 1986; Tanaka *et al.*, 1996). Lesions which map in the U3/U4 and U4/U5 boundary are repaired more quickly than lesions which map within nucleosome U4. In contrast, repair on the TS does not correlate with chromatin structure and shows fast repair and moderate repair heterogeneity. Hence, these data strongly suggest that NER in the NTS is modulated by chromatin structure. To our knowledge, this is the first evidence which shows a direct correlation between chromatin structure and DNA repair in the NTS, as well as the first set of data which correlates repair with the internal structure of a positioned nucleosome.

To understand why only repair in the NTS is modulated by chromatin structure, we need to consider the transcriptional properties of the *URA3* gene. Transcription disturbs chromatin structure (e.g. Thoma, 1991; Felsenfeld, 1996). Recent studies of a gene heavily transcribed from the yeast *GAL1* promoter showed that nucleosomes were not lost, but nucleosome positioning was disturbed. This was

consistent with a local disruption of nucleosomes at the site of the RNA polymerase and their rapid reformation behind the polymerase. After gene repression by glucose, nucleosomes were repositioned within a few minutes, demonstrating that nucleosome rearrangement can occur rapidly and without DNA replication (Cavalli and Thoma, 1993; Cavalli *et al.*, 1996). Compared with transcription driven from the *GAL1* promoter, the *URA3* gene in the multicopy YRpTRURAP is rarely transcribed (Smerdon and Thoma, 1990; Bedoyan *et al.*, 1992) and therefore most of the time transcriptionally silent. The presence of positioned nucleosomes (Thoma, 1986; Tanaka *et al.*, 1996) means that this chromatin structure most likely represents the major fraction of inactive genes and that this structure might be only transiently disturbed by transcription. Consequently, modulation of NER in the NTS reflects NER in the inactive gene.

In contrast to the NTS, we found no correlation between NER heterogeneity and chromatin structure in the TS. Current views on NER of transcribed genes agree that RNA polymerase II is blocked at pyrimidine dimers and somehow promotes assembly of the repair complex which results in preferential repair of the TS (TCR, reviewed in Wood, 1996; Friedberg, 1996; Sancar, 1996a). Hence, our data suggest that TCR in the TS overrides modulation of NER by chromatin structure.

DNA repair in nucleosomes

The central topic of this work is the repair of lesions in nucleosomal DNA. In the nucleosome U2 core, DNA repair seems to slow down gradually from the 5' end towards the region of the 'DNase I protection'. This is the first time that such a correlation could be made in a positioned nucleosome *in vivo*. However, similar chromatin and repair studies on other precisely positioned nucleosomes will be required to establish whether the repair properties observed in U2 can be generalized. A previous study analysed the removal of CPDs in mixed sequence genomic nucleosome cores, which were isolated from human cultured fibroblasts after different repair times. In contrast to our observation, CPDs were removed at equal rates from nucleosome core subdomains with no indication for preferential repair towards the 5' end (Jensen and Smerdon, 1990). One explanation could be that nucleosomes and, hence, damage distribution might rearrange as a consequence of the nucleosome isolation procedure. Alternatively, due to the small linker length and small gene size in yeast, yeast nucleosomes might be more precisely positioned and less mobile than nucleosomes in higher eukaryotes (Thoma *et al.*, 1993) which would affect damage recognition on the nucleosome surface.

The correlation between DNA repair heterogeneity in linker and nucleosomal DNA and nuclease accessibility implies a modulation of damage accessibility in the damage recognition step. Hence, some lesions appear to be more readily accessible than others. The fact that even sites in the central protected region are repaired, although at a lower rate, can be explained by dynamic properties of nucleosomes. It is known from the high-resolution data, that nucleosomes in the U2 region are positioned within a few base pairs. The absence of a defined DNase I pattern was consistent with the absence of a defined 'rotational

setting' and, hence, suggested that U2 nucleosomes can move within a few base pairs (Tanaka *et al.*, 1996). A move by half a helical turn (5 bp) flips the 'inner surface' of DNA (facing the histones) outside and thereby alters the accessibility of DNA lesions. Alternatively, a natural dissociation/reassembly of nucleosomes (nucleosome stability) could also make DNA lesions accessible (Thoma *et al.*, 1993). In contrast to NER, where damage recognition cannot be analysed directly *in vivo*, DNA photolyase is an ideal molecular tool to analyse CPD accessibility in chromatin. It binds CPDs and reverts the damage in the presence of photoreactivating light (Sancar, 1996b). We have recently shown that photolyase in yeast preferentially repairs open promoter regions (including the *URA3* promoter) and linker DNA. Photoreactivation in nucleosomes, however, is slow and requires ~2 h (Suter *et al.*, 1997). Hence, these 2 h appear to be a measure for nucleosome stability to make lesions accessible to repair enzymes.

What we would like to know is how lesions are presented on the nucleosome surface to be recognized by repair proteins. This requires nucleosomes with a defined rotational setting. Some regions in the *URA3* gene show a more distinct rotational positioning of nucleosomes (DNase I pattern; Tanaka *et al.*, 1996), but the pyrimidine distribution in these regions is not suited to answer the question of how the rotational setting affects repair *in vivo*. We noticed that site 1030 on the NTS is repaired quickly although it is located in the internal protected region. We may speculate that CPDs at this site could affect nucleosome positioning (rotational setting) in a way that favours damage recognition.

DNA damage recognition by proteins can be 'distributive', which means by 3-dimensional search, or it could be 'processive' by landing on the DNA and translocation along the DNA (linear diffusion). Possible mechanisms for linear diffusion include (i) microscopic dissociation-reassociation between closely spaced sites in the DNA molecule ('hopping'), (ii) intersegment transfer processes between different segments of the DNA molecule, and (iii) sliding along the DNA contour length (Berg *et al.*, 1981). Linear diffusion is a common process for many proteins to speed up target-site recognition. Well-known examples include restriction endonucleases (references in Jeltsch *et al.*, 1996) and the repair endonuclease T4-endoV (Lloyd *et al.*, 1980; for a review see Hanawalt, 1993). *EcoRI* enwraps the DNA helix and follows the helical path during diffusion. The sliding movement is interrupted when other proteins or drugs are bound to DNA (Jeltsch *et al.*, 1994). T4-endoV is processive in 10 mM NaCl, but distributive in 100 mM NaCl (Ganesan *et al.*, 1986; Gruskin and Lloyd, 1986). NER proteins which may have a role in DNA damage recognition in human and *Saccharomyces cerevisiae* cells are XPA (Jones and Wood, 1993) and Rad14p (Guzder *et al.*, 1993) respectively. How these proteins find the lesions is not known. In chromatin, a sliding mechanism would be possible only in regions where DNA is not tightly complexed with proteins, e.g. in some nucleosome-free regions or in linker DNA between nucleosomes. However, *S. cerevisiae* has a nucleosome repeat length of ~160–170 bp, which corresponds to linker lengths between nucleosome cores of only 15–25 bp (reviewed in Thoma *et al.*, 1993). In nucleosomes, the DNA is wrapped in two superhelical coils on the outside

of the histone octamer (Richmond *et al.*, 1993). Hence, sliding on DNA is likely to be sterically restricted by the histones and by the close proximity of the two supercoils. Sliding through nucleosomes would require partial or total disruption of nucleosomes as is the case during transcription by RNA polymerases (Studitsky *et al.*, 1995). In our experiments we observed fast repair at the 5' end of nucleosome U2 and slower repair towards the 3' end. If indeed damage recognition were by a sliding mode, our results would imply a directionality in this process. We rather favour a distributive process of damage recognition in nucleosomes or a 'hopping' process of linear diffusion. The close proximity of intranucleosomal supercoils as well as packaging of DNA into chromatin fibres could also support an intersegment transfer mechanism.

Although the experiments presented here suggest that damage recognition is a limiting step, it is also possible that later steps are modulated by chromatin structure. The human exinuclease complex requires an ~100 bp DNA stretch to remove a 27–29 bp patch with the lesion (Huang and Sancar, 1994). This would also suggest that nucleosome disruption is required. Indeed, repair patches were found to be nuclease sensitive immediately after DNA synthesis which supports a disruption of nucleosomes during DNA repair (for references, see Smerdon, 1989). Nucleosome disruption is a well known phenomenon in activation of some eukaryotic promoters, and protein complexes involved in that process have been identified (Peterson and Tamkun, 1995). Whether NER requires a similar activity is not known. Candidate genes are *RAD7* (Verhage *et al.*, 1994, 1996; Mueller and Smerdon, 1995) and *RAD16* (Verhage *et al.*, 1994, 1996), since both genes are required for repair of the NTS.

Repair in the transcribed strand

Repair of the TS was on average faster than repair of the NTS, which is consistent with TCR. The fact that we found only a 2-fold difference rather than a 5-fold difference observed previously (Smerdon and Thoma, 1990; Bedoyan *et al.*, 1992) could be due to different reasons. First, the high-resolution analysis was done only on a fraction of the gene, while the low-resolution analysis accounts for the whole gene. Second, PhosphorImager analysis of the high-resolution data might have detected weak lesions which escaped low resolution mapping by X-ray film analysis.

An important point is repair heterogeneity on the TS. The low- and high-resolution data showed repair heterogeneity in the TS of *URA3* (Figure 7; Smerdon and Thoma, 1990; Mueller and Smerdon, 1996). Repair rates were also highly variable in both strands of the human *p53* gene (Tornaletti and Pfeifer, 1994). In contrast, however, repair rates were homogeneous in both strands of the yeast *RPB2* gene (Tijsterman *et al.*, 1996). It is possible that this discrepancy depends on different transcription rates. If a stalled RNA polymerase is a key step in damage recognition and initiation of repair, the TS would show homogeneous repair due to stalled polymerases. Less frequently transcribed genes such as the *URA3* gene will show homogenous repair of the TS, if the half-life of stalled complexes is long. Indeed, *in vitro* experiments showed half-lives of 20 h for human RNA polymerases stalled at CPDs (Selby *et al.*, 1997). If RNA

polymerase falls off or retracts from the lesion and chromatin gets a chance to reorganize into nucleosome structures before new polymerases hit the lesions, we would expect that repair on those lesions would be modulated by the chromatin structure. Although a mild repair heterogeneity was observed on the TS, this heterogeneity did not correlate with chromatin structure. This heterogeneity might depend on the DNA sequence or reflect altered chromatin structure around the stalled polymerase.

Fast repair in the *URA3* promoter

In both strands of the *URA3* promoter, DNA repair was relatively fast for all lesions ($t_{50\%} = 41 \pm 8$ min in the top strand and $t_{50\%} = 50 \pm 6$ min in the bottom strand). Repair rates at the *URA3* promoter were found to be similar to that of the TS of *URA3* and more homogeneous than the repair of the NTS. This can be explained in different ways. First, the promoter region is nuclease sensitive. Hence, fast repair data suggest that damage recognition proteins find access to both strands and promote rapid repair. Second, transcription from *TRP1* promoter through the promoter region into *URA3* may explain fast repair rates on the bottom strand. Whether the top strand of the promoter is also transcribed is not known (Bedoyan *et al.*, 1992). Third, fast repair rates seen near the transcription initiation site may be explained by increased local concentrations of DNA repair factors that are associated with general transcription factors (e.g. TFIIF) functioning in transcription initiation (Tu *et al.*, 1996). So far we cannot discriminate between those possibilities.

Concluding remarks

Heterogeneity of DNA repair has been observed at different levels of genomic organization, including chromatin domains, expressed genes and DNA strands. Slow repair rates may generate high mutation frequency (Tornaletti and Pfeifer, 1994). Hence, the intragenic heterogeneity underlines the significance of chromatin structure and nucleosome positioning for DNA repair and mutagenesis. Although the results presented here provide insight into nucleosome repair *in vivo*, the identification of the molecular mechanisms of DNA repair in nucleosomes remains a complex but challenging topic for the future.

Materials and methods

UV irradiation

Saccharomyces cerevisiae strain FTY23 (Thoma, 1986) was grown in minimal medium SD [0.67% yeast nitrogen base without amino acids (Difco), 2% dextrose] supplemented with 20 mg/l histidine and 20 mg/l tryptophan at 30°C to an A_{600} of 1–2. Cells were collected by centrifugation, resuspended in SD to a concentration of 1.5×10^7 to 3×10^7 cells/ml, transferred to trays to form thin layers (6 mm) and irradiated with 100 J/m² using four Sylvania G15T8 germicidal lamps (predominantly 254 nm at 1 mW/cm² for 10 s as measured using a UVX radiometer, UVP Inc., CA). Histidine and tryptophan were added to a final concentration of 20 mg/l and the cells were incubated at 30°C for various repair times in a room equipped with yellow light (Sylvania GE 'Gold', fluorescent light). 100 ml aliquots were collected by centrifugation (1500 g, 5 min, room temperature) and chilled on ice.

Purification of DNA and enzyme digestions

Preparation of DNA was as described (Smerdon and Thoma, 1990). The cells were converted to spheroplasts by incubation in 10 ml Zymolyase

buffer (1 M sorbitol, 0.25 M EDTA pH 8.0, 3 µl/ml β-mercaptoethanol, 1 µl/ml 0.5 M phenylmethane-sulfonyl fluoride (PMSF) in dimethylsulfoxide (DMSO), 1 mg/ml Zymolyase (100 000 units/g; Seikagaku Kogyo Co., Tokyo, Japan) at 30°C for 45 min. Spheroplasts were harvested by centrifugation [10 000 r.p.m., SS34 rotor (Sorvall), 4°C, 5 min] and resuspended in 10 ml pre-chilled wash buffer (1 M sorbitol, 0.1 M EDTA pH 8.0). The washing step was repeated. Spheroplasts were lysed by resuspension in 2 ml lysis buffer (1% dextrose, 10 mM EDTA pH 8.0, 25 mM Tris-HCl pH 8.0), 4 ml 0.2 N NaOH and 1% SDS were added prior to incubation on ice (15 min). After addition of 3 ml 3 M sodium acetate (pH 5.2) and incubation for 15 min, the lysate was centrifuged (at 10 000 r.p.m., 4°C, 20 min). The DNA was purified from the supernatant by phenol extractions, RNase treatment phenol extractions and finally dissolved in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). Yields of plasmid DNA were ~300–400 ng DNA/10⁹ cells (estimated by ethidium bromide staining in an agarose gel). Aliquots of ~150 ng of DNA were digested with *EcoRI* (Boehringer Mannheim), purified on Nucleobond AX5 columns (Machery-Nagel, D-52313 Düren) and dissolved in 100 µl TE-buffer. DNA (75 ng) was treated with T4-endoV (a gift of L. Mullenders) or mock-treated in 60 µl T4-endoV buffer (10 mM Tris-HCl pH 8.0, 80 mM NaCl, 10 mM EDTA pH 8.0) at 37°C for 2 h. DNA was repurified using Nucleobond AX5 columns and dissolved in 50 µl TE.

Irradiation of DNA

DNA was purified from unirradiated cells, digested with *EcoRI*, repurified and dissolved in TE as described above. 10 µl droplets containing ~1–5 ng plasmid DNA were irradiated with 40 J/m², digested with T4-endoV in 20 µl T4-endoV buffer, repurified and dissolved in 10 µl TE as described above.

Analysis of plasmid repair

Electrophoresis was in 1% alkaline agarose gels (BRL 20 cm×25 cm) (Maniatis *et al.*, 1982) at 4°C and 50 V for 14–17 h with circulation of electrophoresis buffer (50 mM NaOH, 1 µM EDTA). DNA was blotted onto nylon membranes (PallB, Pall) using an alkaline blotting protocol (Bio-Rad bulletin 234 890 90-0891) and hybridized to a radioactively labelled DNA fragment which contained the *TRP1ARS1* sequence. Radioactive probes were prepared using oligolabelling kits (Pharmacia). Radioactive bands were detected using Fuji X-ray films and Phosphor-Imager (Molecular Dynamics, 176 u pixel size). Full-length restriction fragments migrate as a top band ('a', Figure 2). CPD-cleaved DNA generates a smear ('b', Figure 2). In each lane, the signals of the top band and the signal of the whole lane were measured and background obtained from an empty lane was subtracted. The signals of the top band were normalized with the signal of the whole lane. The average number of dimers per fragment was calculated by using the Poisson expression $[-\ln(\text{normalized top band of the } +T4 \text{ lane})/(\text{normalized top band of } -T4 \text{ lane})]$ (Mellon *et al.*, 1987).

Primer extension

The primers used for mapping the top strand were [map units (MU) are nucleotides on YRpTRURAP; T_m is the annealing temperature]: No. 1014 (5'-TGATTATCTTCGTTTCCTGC-3'; 817 to 795 MU; T_m = 55°C); No. 897 (5'-GCAATTTGACTGTATTACCAATGTCAGC-3'; 1142 to 1114 MU; T_m = 60°C); No. 1119 (5'-AATGCGTCTCCCTTGT-CATCTAAACC-3'; 1474 to 1449 MU; T_m = 58°C). The primers used for mapping the bottom strand were: No. 1027 (5'-GGAAGGCAAGA-GAGCCCCGAAAGC-3'; 595 to 618 MU; T_m = 60°C); No. 896 (5'-GCAGGAAACGAAGATAAATCATGTGCG-3'; 812 to 837 MU; T_m = 58°C); No. 1118 (5'-GTAATACAGTCAAATTCGAGTACTC-3'; 1129 to 1153 MU; T_m = 58°C). Primer labelling and primer extension were done as described in Wellinger and Thoma (1996). Ten picomoles primer were labelled at the 5' end using T4-polynucleotide kinase (BioLabs) and 10 pmol [γ -³²P]ATP, purified on a G50 column (Pharmacia), precipitated and redissolved in 500 µl TE. Ten microlitres of DNA (1–5 ng) were mixed with 20 µl primer (0.4 pmol; 2×10^5 to 1.2×10^6 c.p.m.), 4 µl 10× *Taq* buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 30 mM MgCl₂), 1.2 µl of nucleotide mixture (5 mM each of dATP, dCTP, dGTP, dTTP; Pharmacia) and overlaid with 25 µl paraffin. Samples were heated at 95°C for 5 min, then chilled in ice water. *Taq* DNA polymerase (1 unit in 5 µl of 1× *Taq* buffer; Cetus, Perkin Elmer) was added and the samples were subjected to 30 cycles of denaturation at 94°C for 45 s, annealing at primer-specific temperatures (T_m) for 4.5 min, and extension for 2 min at 72°C. The DNA was precipitated by 0.1 volumes of 3 M sodium acetate and 4 volumes of ethanol and dried. DNA electrophoresis was done in 5% (w/v) polyacrylamide gels

containing 50% urea and TBE (Maniatis *et al.*, 1982). The gels were dried on Whatman DE 81 papers and exposed on X-ray films (Fuji RX) or quantified using a PhosphorImager (Molecular Dynamics, at 176 u pixel size). Sequencing reactions were done by the chain-termination method using *Taq* DNA polymerase (Wellinger and Thoma, 1996). To obtain the fraction of molecules that contain a PD at a defined site, the signal of each band was measured and divided by the signal of the whole lane. The same procedure was done for the lane containing non-irradiated DNA. This value was used to subtract gel background and signals from unspecific *Taq* polymerase blockage. The values at each repair time were normalized with respect to the signals obtained with no repair (no repair = 100%). Exponential fit was used to generate repair curves. $t_{50\%}$ is the time at which 50% of the damage is repaired.

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