Site-specific repair of cyclobutane pyrimidine dimers in a positioned nucleosome by photolyase and T4 endonuclease V *in vitro*

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Since genomic DNA is folded into nucleosomes, and DNA damage is generated all over the genome, a central question is how DNA repair enzymes access DNA lesions and how they cope with nucleosomes. To investigate this topic, we used a reconstituted nucleosome (HISAT nucleosome) as a substrate to generate DNA lesions by UV light (cyclobutane pyrimidine dimers, CPDs), and DNA photolyase and T4 endonuclease V (T4-endoV) as repair enzymes. The HISAT nucleosome is positioned precisely and contains a long polypyrimidine region which allows one to monitor formation and repair of CPDs over three helical turns. Repair by photolyase and T4-endoV was inefficient in nucleosomes compared with repair in naked DNA. However, both enzymes showed a pronounced site-specific modulation of repair on the nucleosome surface. Removal of the histone tails did not substantially enhance repair efficiency nor alter the site specificity of repair. Although photolyase and T4-endoV are different enzymes with different mechanisms, they exhibited a similar site specificity in nucleosomes. This implies that the nucleosome structure has a decisive role in DNA repair by exerting a strong constraint on damage accessibility. These findings may serve as a model for damage recognition and repair by more complex repair mechanisms in chromatin.

Keywords: DNA damage/DNA repair/nucleosome/photolyase/T4 endonuclease V

Introduction

UV-induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts [(6–4)PDs] are the major DNA lesions generated by UV light. In most organisms, these UV photoproducts are repaired by the multistep nucleotide excision repair (NER) pathway which also removes other bulky DNA lesions (Friedberg *et al.*, 1995). Many organisms have developed additional, more specific repair systems to remove UV-induced lesions, such as photolyases which directly revert UV photoproducts in the presence of photoreactivating light and UV damage-specific endonucleases (Sancar and Sancar, 1988; Sancar, 1994; Wood, 1996). Eukaryotic repair enzymes face the problem that genomic DNA is compacted into a structural hierarchy of nucleosomes and higher-order chromatin structures. To understand the influence of chromatin structure on DNA repair, it is

important to know how DNA lesions are distributed in chromatin and how their accessibility is affected by the structural and dynamic properties of chromatin.

The primary repeating unit of chromatin is the nucleosome core, which consists of 145 bp of DNA wrapped around a histone octamer in 1.8 left-handed superhelical coils. The inner and outer surface of nucleosomal DNA, which face the histones and the solution, respectively, define its 'rotational setting'. The path of DNA does not wrap uniformly around the core histones but it suffers severe distortions (Richmond *et al.*, 1984). Mapping of the CPD distribution in mixed sequence nucleosomes revealed a modulation of damage formation, with CPDs forming preferentially at sites where the minor groove faces the solution (Gale *et al.*, 1987; Gale and Smerdon, 1988). However, the CPD distribution in a defined sequence nucleosome can substantially differ from that observed in mixed sequence nucleosomes (Schieferstein and Thoma, 1996). In contrast to CPDs, (6–4)PDs are more randomly distributed in nucleosome cores (Gale and Smerdon, 1990).

Structural parameters may affect damage accessibility in nucleosomes: (i) the DNA double helix exhibits substantial distortions and its flexibility is restricted; (ii) the core histones conceal part of the inner DNA surface; and (iii) the adjacent superhelix of DNA restricts accessibility. In addition, folding of nucleosomes into higher-order structures may introduce further steric hindrance. On the other hand, dynamic properties of chromatin may facilitate access to nucleosomal DNA. Nucleosomes may undergo disassembly/reassembly, unfolding or sliding along the DNA (nucleosome mobility), and different positions of nucleosomes may exist in a dynamic equilibrium (reviewed in Thoma, 1992). CPDs can change the rotational setting of DNA during nucleosome assembly (Suquet and Smerdon, 1993; Schieferstein and Thoma, 1996). Whether CPDs may generate similar effects on pre-assembled nucleosomes remains to be seen. At least, CPD formation in a defined sequence nucleosome had no effect on the rotational setting (Schieferstein and Thoma, 1996).

Different approaches were taken to address repair in nucleosomes. NER studies were performed on nucleosome cores isolated from bulk chromatin of UV-irradiated cultured human cells. Analysis of the CPD distribution implied that CPDs were repaired with equal efficiency from inner and outer surfaces of the DNA helix relative to the histone surface. On the other hand, analysis of DNA repair patches revealed a bias towards the 5' ends of nucleosomes at early repair times. This repair bias was accounted for by the non-uniform distribution of DNA lesions (Jensen and Smerdon, 1990; Smerdon, 1991). Alternatively, a yeast strain containing a minichromosome with genes having well-characterized chromatin structures and nucleosome positions was used to compare site-

specific repair with chromatin structure. No dramatic effects were resolved between NER of CPDs in non-nucleosomal regions and nucleosomes (Smerdon and Thoma, 1990). However, a high resolution study indicated preferential repair in linker DNA and a modulation of repair within a positioned nucleosome in the non-transcribed strand of the yeast *URA3* gene (Wellinger and Thoma, 1997). In NER, damage recognition is followed by DNA incisions, removal of the damaged oligonucleotide, gap repair synthesis and ligation (Friedberg *et al.*, 1995). Hence, the observation of damage removal allows only limited interpretation with respect to damage recognition. In the photoreactivation process, however, CPD recognition and repair are performed by a single enzyme, CPD photolyase. Indeed, photoreactivation in yeast minichromosomes was tightly modulated by chromatin structure. CPDs in non-nucleosomal and linker DNA were quickly repaired, whereas CPDs in nucleosomes appeared to be more resistant to photolyase (Suter *et al.*, 1997). The *in vivo* repair studies were complemented by several investigations *in vitro*. Only a fraction of CPDs were substrates for T4 endonuclease V (T4-endoV) in SV40 minichromosomes (Evans and Linn, 1984). Human cell extracts were reported to be less active on SV40 minichromosomes and plasmid DNA assembled into chromatin, as compared with repair in naked DNA (Wang *et al.*, 1991; Sugasawa *et al.*, 1993). These *in vitro* studies did not discriminate between DNA lesions in nucleosomes, linker DNA or non-nucleosomal regions.

In summary, DNA damage recognition and repair in nucleosomes is barely understood. We therefore reconstituted a defined sequence nucleosome (HISAT nucleosome) which can be used as a model substrate to investigate the mutual effects of DNA damage formation, repair and nucleosome structure. We have shown previously that the HISAT sequence, which is a 134 bp DNA fragment of the yeast DED1 promoter, forms a positioned nucleosome *in vitro* (Losa *et al.*, 1990). This sequence contains a polypyrimidine region which allows us to induce CPDs over three helical turns in the nucleosome. CPD formation did not affect nucleosome stability or the rotational setting (Schiefelstein and Thoma, 1996). To address damage accessibility directly, we used in this study two enzymes, *Escherichia coli* photolyase and T4-endoV, which have different mechanisms but act on the same DNA lesions. We show that both enzymes can process DNA lesions on the nucleosome surface, but the nucleosome structure exerts a dominant constraint over damage accessibility. These findings may serve as a paradigm for damage recognition in chromatin *in vivo* and imply the necessity for chromatin transitions and/or chromatin dynamics to allow repair in nucleosomal DNA.

Results

Nucleosome structure and the rotational setting are preserved during photoreactivation

End-labelled HISAT DNA (Figure 1A) was reconstituted into nucleosomes by histone octamer transfer from chicken erythrocyte core particles and irradiated with UV light at a dose of 750 J/m² to yield an average of 1.1 CPDs per nucleosome (Schiefelstein and Thoma, 1996). These nucleosomes were incubated with *E.coli* DNA photolyase

in the presence of photoreactivating light for up to 180 min at room temperature. Band shift gels showed efficient incorporation of labelled DNA into nucleosomes (>93%). This fraction remained unchanged by UV irradiation and photoreactivation (Figure 1B). Hence, neither UV irradiation nor photolyase disrupted nucleosomes.

To test whether photolyase affected the rotational setting, nucleosomes were digested with DNase I. Unirradiated nucleosomes yielded a characteristic '10 bp ladder', showing that the DNA has a defined rotational setting on the octamer surface (Figure 1C, lanes 5–8; schematically summarized in Figure 1A) (Schiefelstein and Thoma, 1996). We consistently observed cutting at map unit (MU) 1353 (Figure 1C, arrowheads) where the DNA minor groove is expected to face towards the histone octamer and therefore should be resistant to DNase I digestion. This indicates a structural anomaly of the HISAT nucleosome at position 1353. The digestion patterns observed after UV irradiation and photoreactivation for 180 min (Figure 1C, lanes 11–14 and lanes 17–20, respectively) were indistinguishable from those of untreated nucleosomes. It is conceivable that UV irradiation and photoreactivation might transiently alter the nucleosome structure, which could not be detected by our assay. However, neither treatment resulted in a persistent change of the rotational setting.

Photoreactivation is partially inhibited on the nucleosome surface

To measure CPD repair in nucleosomal and naked DNA having the same damage distribution, either UV-irradiated nucleosomes or DNA isolated from these irradiated nucleosomes were treated with photolyase for various times. DNA was then purified and the remaining CPDs were detected by T4-endoV digestion and gel electrophoresis (Figure 2). Each band was quantified and used to calculate overall repair and site-specific repair (Figure 3). The initial CPD distribution generated in nucleosomes exhibited high yields in all T-tracts (T₃, ¹T₅, T₆, ²T₅ and T₉) which corresponded to thymine–thymine (TT) dimer sites, with local CPD maxima found in the T₆ and the T₉ tracts (Figure 2, lane 2; Figure 3B, white bars). The T-tracts were separated by much weaker bands corresponding to C-containing dimers (CT, TC and CC), in agreement with previous results (Schiefelstein and Thoma, 1996).

Decreasing band intensities with increasing photoreactivation times reflect site-specific repair by photolyase. CPD repair in naked DNA was fast. Photolyase removed >90% of CPDs from the polypyrimidine region within 32 min (Figure 2, lanes 6–10; Figure 3A, white circles). Repair at individual sites was not equally efficient but showed heterogeneity. Most repair-resistant sites (Figure 2, lane 9, black dots) include C-containing CPDs (MU 1339, 1347 and 1360) which are repaired less efficiently than most TT dimers. This is consistent with previous observations (Kim and Sancar, 1991). A TT dimer (MU 1363) is also repaired slowly. This site is located towards the 5' end of the T₉ tract which is known to form a T-tract structure (Schiefelstein and Thoma, 1996). It is possible that the unusual structure interferes with photolyase binding to naked DNA and reduces repair efficiency at this site (Wang and Taylor, 1991). Figure 3B displays initial damage (white bars) and remaining CPDs after 8 min

In contrast to repair in naked DNA, repair on the nucleosome surface was generally slow and inefficient (Figure 2, lanes 3–5). This is illustrated quantitatively by the initial lesions (white bars) and the CPDs remaining after 120 min (black bars, Figure 3D). On average, photolyase removed only 29% of the total CPDs from nucleosomes in 120 min (Figure 3A, white squares), and this value did not increase significantly by incubation for 180 min. Reconstituted samples contained a small fraction

The central question is whether folding of DNA on the nucleosome surface leads to site-specific repair by photolyase. Inspection of the gels reveals that CPDs in nucleosomes appeared to be repaired more efficiently at some sites of the T₃ and both T₅ tracts and, in particular, at the 3' end of the polypyrimidine region (Figure 2, lane 4, open bars). To assess this observation quantitatively, the percentage of site-specific repair (after 120 min) was

Fig. 2. Photoreactivation in nucleosomes and naked DNA. UV-irradiated nucleosomes and DNA isolated from these nucleosomes were treated with photolyase for the indicated times (min). DNA was purified, digested with T4-endoV and analysed on sequencing gels. Lane 2, initial CPD distribution; lanes 3–5, photoreactivation in nucleosomes; lanes 6–10, photoreactivation in naked DNA. Note that for the same amount of total DNA (3.6 μ g), 0.2 μ g of photolyase were used for nucleosomes but only 0.1 μ g for naked DNA. A/G is a sequencing marker. Open bars (lane 4) indicate efficiently repaired sites (see Figure 3E). Due to the additional pyrimidine base at the 5' end, T4-endoV-cleaved DNA migrates more slowly than the corresponding DNase I cleavage product. Hence, the open dots which indicate nucleosome-specific DNase I cutting sites are shifted by about one base relative to the CPD bands having the same MU. Black dots (lane 9) mark slowly repaired CPDs in naked DNA at MU 1339, 1347, 1360 and 1363.

calculated at individual sites (Figure 3E) and compared with the average repair of the whole region (29%, dashed line). [CPD yields at CT and CC dipyrimidine sequences (Figure 3B, asterisks) were very low and were excluded from interpretation (asterisks in Figures 3B and D and 4C).] The most efficient repair (up to 60%) was observed in the 1T_5 tract (MU 1351/52) and at the 3' end of the polypyrimidine region (MU 1370–1374). Repair in the 2T_5 tract was close to or slightly above the average at most sites. Site-specific repair efficiencies were lowest in the T_6 and T_9 tracts at the sites of high initial CPD damage (MU 1344 and 1364–1366). Most importantly, the repair patterns observed in nucleosomes and naked DNA are different (compare Figures 3C and E). For example, repair at site 1344 was fast in DNA but slow in nucleosomes. There was a decrease in repair efficiencies from site 1369 to 1364 in nucleosomes, while the same sites were repaired at similar rates in DNA. Also, the site-specific repair pattern in the 2T_5 tract was different for nucleosomes and DNA. Thus, the nucleosome structure modulated the site-specific repair by photolyase. Furthermore, repair did not correlate with the amount of initial damage.

Since the reconstituted samples contain a fraction of free DNA (<7%) and since free DNA is completely

repaired in 2 h, it is of interest how repair of the free DNA influences the repair pattern (Figure 3E). However, subtraction of the repair signal of free DNA by taking into account the different yields of CPDs in DNA and nucleosomes generated a pattern very similar to that shown in Figure 3E (not shown). Hence, the site-specific repair heterogeneity originates from repair on the nucleosome surface.

If the outer surface of nucleosomal DNA were preferentially accessible to photolyase, efficiently repaired sites should co-localize with preferential DNase I cutting sites. However, when compared with the DNase I cutting pattern (Figure 2, white circles), the sites which showed efficient repair were located between two DNase I cutting sites (sites 1351/52, open bars) or 3' thereof (sites 1358 and 1368–1374). This comparison shows that no simple correlation exists between the rotational setting measured by DNase I and the accessibility of CPDs to photolyase.

Removal of the N-terminal histone tails does not substantially alleviate inhibition of photoreactivation nor alter the site-specific repair
N-terminal tails of histones might enhance the structural stability of nucleosomes, reduce site-specific flexibility

and accessibility of DNA and, thereby, affect DNA damage formation and repair. To investigate this topic, nucleosomes were reconstituted with trypsinized nucleosome core particles lacking histone tails (Figure 4A), irradiated

with 750 J/m^2 and photoreactivated. In nucleoprotein gels, trypsinized nucleosomes migrated further than intact nucleosomes due to the reduced mass of the nucleoprotein complex and the lack of the neutralizing positively charged amino acid residues of the N-terminal histone tails (Figure 4B). Reconstitution efficiency was $\sim 86\%$, compared with 93% for intact nucleosome cores. This fraction remained unchanged after UV irradiation and photoreactivation. Trypsinized nucleosomes retained the characteristic DNase I pattern of the HISAT nucleosome (Figure 4C, lanes 9–11). Minor changes in cutting frequencies might be due to the increased fraction of free DNA which yields a DNase I pattern different from nucleosomes or might reflect subtle changes in nucleosome structure due to the removal of the histone tails. The DNase I pattern of trypsinized nucleosomes did not change after UV irradiation and photoreactivation for 120 min (Figure 4C, lanes 14–16 and 17–19, respectively). Thus, the removal of histone tails did not destabilize nucleosomes nor affect the rotational setting under our assay conditions.

DNase I is sensitive to the width of the minor groove and DNA bendability (Drew and Travers, 1984; Lahm and Suck, 1991), whereas UV damage formation is affected by the flexibility of adjacent pyrimidine bases (Becker and Wang, 1989). Thus, different kinds of information about the DNA structure are obtained by DNase I and UV light. Comparison of the CPD yields and the CPD distribution in intact and trypsinized nucleosomes showed that they were very similar (compare Figure 2, lane 1, and Figure 4C, lane 5). Hence, not only the conformation of the DNA backbone but also the local flexibility of bases appeared to be unaffected by the removal of the histone tails.

Irradiated trypsinized nucleosomes were photoreactivated for 30, 60 and 120 min (Figure 4C, lanes 6–8) and repair was quantified as described for intact nucleosomes (gels not shown). Overall repair reached 37% after 120 min (Figure 3A, black squares). The increase in overall repair from 29% in intact nucleosomes to 37% in trypsinized nucleosomes after 120 min can be accounted for by the larger fraction of free DNA in the experiments with trypsinized core particles (14% compared with 7% for intact core particles) (compare Figures 1B and 4B). Site-specific CPD repair after 120 min was, in general, very

Fig. 3. Quantification of CPD repair by photolyase. UV-irradiated (750 J/m^2) nucleosomes or DNA isolated from these nucleosomes were treated with photolyase for various times. CPDs were then mapped as described in Figure 2. (A) Time course of overall repair in nucleosomes and DNA. N_i , repair in intact nucleosomes (□); N_t , repair in trypsinized nucleosomes (■); D_i , repair in DNA isolated from intact nucleosomes (○); D_t , repair in DNA isolated from trypsinized nucleosomes (●). (B) Photoreactivation in naked DNA. Initial CPD yields (white bars); CPD left after 8 min photoreactivation (black bars). The amount of site-specific CPDs is represented as the fraction of DNA molecules with an incision at that site. The map unit is given for the 5' pyrimidine of a CPD. (C) Site-specific repair in DNA is shown as the fraction of CPDs removed in 8 min photoreactivation (complete repair of initial damage is 100%). The dashed line indicates an average of all sites (overall repair, 57%). (D) Photoreactivation in intact nucleosomes. Initial CPD yields (white bars); CPDs left after 120 min photoreactivation (black bars). (E) Site-specific repair in intact nucleosomes. Repair is shown as the fraction of CPDs removed from nucleosomes by 120 min photoreactivation. Overall repair (29%) is indicated by the dashed line. Asterisks mark sites that have been excluded from interpretation (see text and Figure 7).

Fig. 4. Photoreactivation in trypsinized nucleosomes. (A) The proteins are shown by SDS-PAGE and Coomassie blue staining. Lane 1, native cores; lanes 2–5, the time course of trypsinization; lane 6, the trypsinized cores used for reconstitution. H2A, H2B, H3 and H4 are core histones; P1–P5 are tryptic peptides of core histones (Böhm and Crane, 1984). (B) DNA band shift analysis. DNA (lane 1) was reconstituted into nucleosomes (Rec, lane 2), irradiated with 750 J/m² (Rec→UV, lane 3) and photoreactivated for 30, 60 and 120 min (Rec→UV→P, lanes 4–6, respectively). Bands represent naked DNA (DNA) and nucleosomes (Nucl). (C) DNase I footprinting. Nucleosomes (Nucl lanes) were digested with DNase I (DI) after reconstitution (Rec, lanes 9–11), UV irradiation (Rec→UV, lanes 14–16) and photoreactivation for 120 min (Rec→UV→P, lanes 17–19); digestion of DNA (D lanes) isolated from untreated or irradiated nucleosomes (lanes 12–13 and 20–21, respectively). Lane 1, untreated DNA; lane 2, DNA isolated from UV-irradiated nucleosomes; lanes 3 and 4, sequencing markers A/G and T/C. Open dots mark nucleosome-specific DNase I cutting sites; numbers show the map unit. Lane 5, initial CPD distribution; lanes 6–8, CPD distribution after photoreactivation for 30, 60 and 120 min. Open bars (lane 8) indicate efficiently repaired sites.

similar in intact and trypsinized nucleosomes (compare Figures 2 and 4C). In summary, removal of the histone tails did not dramatically affect site-specific repair by photolyase and did not substantially alleviate inhibition of photoreactivation.

Inhibition and modulation of T4 endonuclease V activity in nucleosomes

The experiments were repeated with T4-endoV. Whereas photolyase directly reverts CPDs, T4-endoV introduces nicks in DNA by cutting the *N*-glycosidic bond at the 5' side of a pyrimidine dimer and cleavage of the apyrimidinic phosphodiester bond (Gordon and Haseltine, 1980). The stability of both intact and trypsinized nucleosomes was not affected by UV irradiation and T4-endoV digestion, and the rotational setting remained preserved during T4-endoV digestion for up to 105 min at 37°C (data not shown).

Either UV-irradiated nucleosomes or DNA isolated from these irradiated nucleosomes were digested with T4-endoV. Following digestion, both intact and trypsinized nucleosome samples were run on a preparative nucleosome-protein gel in order to separate nucleosomes and free DNA. This gel purification step was necessary, since overall incision on nucleosomes by T4-endoV was very low. Gel-purified nucleosomal DNA was then analysed directly on sequencing gels (Figure 5), and a quantitative summary is given in Figure 6. T4-endoV efficiently incised

CPDs in naked DNA (Figure 5A and B, lanes 7–4). Within 60 min, >85% of the total CPDs in DNA were incised, and this value increased to 95% after 105 min digestion (Figure 6A, circles). In contrast, cutting of CPDs in intact nucleosomes was strongly inhibited (Figure 5A, lanes 1–2; Figure 6A, white squares). Overall incision in nucleosomes by T4-endoV increased from 9% after 60 min to 13% after 105 min. Very similar results were obtained with trypsinized nucleosomes except that incision was slightly higher (19%) (Figure 5B, lanes 1–2; Figure 6A, black squares). Hence, nucleosome structure severely inhibited T4-endoV cleavage on the nucleosome surface. T4-endoV activity could not be enhanced by removal of histone tails.

Despite this low activity, T4-endoV incision showed a pronounced site specificity (Figure 5A, lanes 1–2). The site-specific incision pattern in nucleosomes was clearly different from the pattern observed in naked DNA, showing that the nucleosome structure modulated T4-endoV cutting. The nucleosomal incision pattern was also clearly different from a pattern generated by irradiation of naked DNA (lane 3) and, hence, cannot be derived from a minor fraction of free DNA in the nucleosomal sample. In the nucleosome pattern, relatively efficient cutting was observed at a few sites in both T₅ tracts, in the T₉ tract and at the 3' end of the polypyrimidine region (Figure 5A and B, lanes 1 and 2). In Figure 6C, repair was calculated with respect to initial damage (Figure 6B) and

Fig. 5. CPD incision in nucleosomes and naked DNA by T4 endonuclease V. UV-irradiated nucleosomes and DNA isolated from these nucleosomes were digested with T4-endoV for the indicated times (min). Nucleosome samples were run on a gel to separate reconstituted DNA from free DNA. Gel-purified nucleosomal DNA was then analysed directly on sequencing gels. **(A)** CPD incision in intact nucleosomes. Initial CPD yields (lane 4); CPD incision in nucleosomes (lanes 1–2) and naked DNA (lanes 5–7). For comparison, the CPD distribution in UV-irradiated (750 J/m^2) naked DNA is shown (lane 3). A/G and T/C are sequencing markers. Open bars (lane 2) indicate efficiently incised sites. **(B)** CPD incision in trypsinized nucleosomes. Details as in (A).

compared with the average incision in the whole region (13%, dashed line). The most efficient incision ($>20\%$; open bars in Figure 5) occurred within both T_5 tracts and at the 3' end of the polypyrimidine region. In contrast, sites in the T_6 and the T_9 tracts at which the initial CPD yields were high (MU 1344 and 1364–1366) exhibited incision minima (Figure 6C). (CPD yields at CT and CC dipyrimidine sequences were low and were excluded from interpretation.) Similar results were obtained by T4-endoV digestion of trypsinized nucleosomes (Figure 5B). Hence, as observed in the photolyase experiments, removal of histone tails failed to substantially increase CPD accessibility in nucleosomes. Interestingly, the modulation of repair by T4-endoV on the nucleosome surface was similar to that observed for photolyase (compare Figures 3E and 6C).

Discussion

By using two different repair enzymes, photolyase and T4-endoV, and the HISAT nucleosome as a model system, we showed that DNA damage can be recognized and repaired on the nucleosome surface. Moreover, the nucleosome structure exerts site-specific constraints on the repair process by both enzymes. These findings may serve as a model for damage recognition and repair by more complex repair mechanisms in chromatin.

The HISAT nucleosome as a model system

The HISAT sequence contains several T-tracts which form an unusual structure in solution. This unusual structure, however, is abolished when the sequence is packaged

into a nucleosome (Schieferstein and Thoma, 1996). In agreement with this, it has been shown by hydroxyl radical footprinting that T-tracts behave very similarly to mixed sequences when associated with a histone octamer (Hayes *et al.*, 1991a). The HISAT nucleosome shows an unusual DNase I cutting site (Figure 1C) which is unexpected with respect to the studies of mixed populations of nucleosomes. However, similar differences have been reported for the 5S rDNA nucleosome (Simpson and Stafford, 1983). Even a DNA sequence with 2-fold symmetry can reconstitute into an asymmetric nucleosome (Luger *et al.*, 1997). Hence, each nucleosome has, to some extent, specific properties and there is no such thing as a representative 'average' nucleosome. We therefore think that the HISAT nucleosome is a genuine model to study the relevance of chromatin structure for DNA repair.

Site-specific CPD repair in nucleosomes by photolyase

An essential determinant for substrate recognition by photolyase is the CPD structure itself, with additional specificity being provided by the unique conformation of the phosphodiester backbone (Sancar, 1994). Photolyase contacts the phosphodiester bond immediately 5' and the three phosphodiester bonds immediately 3' to the dimer on the damaged strand (Husain *et al.*, 1987). These contacts coincide with the major DNA distortions induced by a CPD (Taylor *et al.*, 1990). How does such an enzyme cope with the structural constraints imposed on DNA in nucleosomes? Histone–DNA contacts in the nucleosome core result in substantial deformation of B-form DNA (Richmond *et al.*, 1984). These interactions exert a domin-

Fig. 6. Quantification of CPD incision by T4 endonuclease V. (A) Time course of overall incision in nucleosomes and DNA. N_i , incision in intact nucleosomes (□); N_t , incision in trypsinized nucleosomes (■); D_i , incision in DNA isolated from intact nucleosomes (○); D_t , incision in DNA isolated from trypsinized nucleosomes (●). (B) CPD cleavage in intact nucleosomes. Total CPD yields (white bars); CPD incised after 105 min digestion (black bars). (C) Site-specific T4-endoV cleavage in nucleosomes shown as the fraction of CPDs incised in 105 min (initial damage is 100%). The dashed line indicates an average of all sites (overall repair, 13%). Asterisks mark sites that have been excluded from interpretation (see text).

ant constraint over rigid DNA structures such as T-tracts and tolerate DNA distortions induced by CPDs without changing the rotational setting (Schieferstein and Thoma, 1996). A graphic representation of the HISAT nucleosome summarizing the footprinting and photoreactivation data is shown in Figure 7A. The location of the polypyrimidine region in the nucleosome is based on the DNase I digestion pattern (Figure 1A; for a discussion see Schieferstein and Thoma, 1996).

Fig. 7. Schematic summary of DNA repair in a nucleosome. This representation corresponds to a view on the top turn of nucleosomal DNA. The underlying lower turn is not shown (adapted from Richmond *et al.*, 1984). The polypyrimidine region (MU 1336–1375) is indicated by circles (T bases) and squares (C bases) with a gap at the position of the single G base (MU 1354). Pyrimidines at MU 1353 and 1375 (black circles) cannot form CPDs with the flanking purines. Angles indicate the high yields of CPD at MU 1344 and 1364–1366. Map units at the outside mark DNase I cutting sites. The putative nucleosome centre is indicated by the arrowhead. The arcs indicate putative DNA-tetramer contacts. The location of the T-tracts is shown on the inside. Sites coloured in blue (N.D.) were excluded from analysis. (A) Removal of CPDs by photolyase was classified as efficient (>34% repair; red), moderate (34–24% repair; yellow) or inefficient (<24%; white) according to Figure 3E. (B) Incision of CPDs by T4-endoV was classified as efficient (>18% incision; red), moderate (18–8% incision; yellow) or inefficient (<8%; white) according to Figure 6C.

We expected that site specificity of photoreactivation should correlate with the DNase I cutting sites where the minor groove faces away from the histone surface and therefore is exposed and accessible to enzymes. This was not observed. Only a few moderately and efficiently repaired sites correlate with DNase I sites (at MU 1337, 1345/46 and 1367/68). Hence, DNase I and photolyase (and T4-endoV, see below) recognize different features of nucleosomal DNA.

Severe inhibition of photoreactivation occurred at sites 1343–1344 and 1363–1366 (white symbols). At these sites, the sugar–phosphate backbone of the damaged strand and the minor groove face the underlying superhelical DNA turn (not depicted in Figure 7A). Hence, binding of photolyase at these positions might be sterically hindered by the adjacent DNA turn. On the other hand, efficiently repaired sites were found at MU 1351–1352 (1T_5 tract) and MU 1367–1374 (red symbols). In those regions, the DNA backbone is exposed on the top of the nucleosome, which could favour interactions with photolyase. In contrast, CPDs in the regions of MU 1338–1342 are only slowly repaired, although the DNA is exposed. Hence, exposure on the top turn cannot be a sufficient criterion for efficient repair. Slow repair at 1338–1342 could be related to a more rigid DNA structure close to the dyad of the particle.

Core particle DNA is divided into a central region of ~100 bp that is firmly bound by histones and two more labile end regions of ~20 bp (van Holde, 1989). From MU 1374 towards the 3' end, there was no significant difference in the DNase I digestion pattern of nucleosomes and naked DNA (Figures 1C) (Schieferstein and Thoma, 1996), suggesting that this part of the HISAT sequence might be weakly bound by the histones. It seems possible that efficient repair at the 3' end of the polypyrimidine region is due to weaker histone–DNA interactions at the periphery of the nucleosome. DNA sequences at the edge of nucleosomes are more accessible to transcription factors and restriction endonucleases than those towards the centre of the nucleosome (for a review, see Steger and Workman, 1996).

The histone (H3/H4)₂ tetramer is known to have a crucial role in organizing the central part of nucleosome cores (for a review, see Pruss *et al.*, 1995). In the HISAT nucleosome, the 5' ends of the T₆ and the T₉ tracts would coincide with DNA–tetramer contacts (Figure 7A, arcs). Thus, restricted DNA flexibility in these T-tracts due to interactions with the tetramer might contribute to the inhibition of photoreactivation at these positions. This explanation is consistent with our observation that removal of the histone tails, which extend outside of the core particle, failed to enhance repair.

The 1T_5 tract (MU 1349–1353) is the only T-tract in which CPD yields are significantly reduced in nucleosomes compared with irradiation of naked DNA (Schieferstein and Thoma, 1996). Since torsional flexibility of DNA is important for UV damage formation (Becker and Wang, 1989), reduced CPD yields in the 1T_5 tract might be due to histone–DNA interactions that restrict DNA flexibility. On the other hand, we noticed efficient repair by photolyase at MU 1351–1352 and efficient cutting by DNase I at MU 1353. At this site, the minor groove faces inside and DNA should be protected against cutting. Hence, efficient repair

and DNase I cutting indicate an unusual DNA structure in that region. These results emphasize that defined sequence nucleosomes may have unique structural properties which are decisive for damage recognition by repair enzymes, and that these structural properties are not predicted from structural models of bulk nucleosomes.

CPD incision in the HISAT nucleosome by T4 endonuclease V

The crystal structure of a T4-endoV–DNA complex revealed that the protein induced a sharp DNA kink at the position of the TT dimer. T4-endoV recognizes the deformed DNA backbone in the close vicinity of the CPD. Upon binding of the enzyme, the adenine opposite the 5' moiety of the dimer is flipped out of the DNA helix (Vassilyev *et al.*, 1995). The different interactions of T4-endoV and photolyase with naked DNA substrates suggested their repair patterns in nucleosomes to be different too. Indeed, the overall repair of CPDs by T4-endoV was lower than CPD reversal by photolyase. However, the site-specific repair pattern of T4-endoV was surprisingly very similar to that of photolyase (compare Figure 7A and B). This implies that not all of the different interactions of T4-endoV and photolyase with naked DNA substrates can be essential for damage recognition in nucleosomes. Our results support the idea that the pyrimidine dimer and the unique DNA conformation around the CPD are crucial structural determinants for damage recognition on the nucleosome surface. The distortions of nucleosomal DNA required for bending around the octamer will interfere with conformational constraints required for binding and repair.

Implications for damage recognition and repair in chromatin.

Previous work showed that a fraction CPDs in SV40 minichromosomes are resistant to T4-endoV cleavage (Evans and Linn, 1984). Similarly, NER of DNA by human cell extracts was suppressed in plasmid DNA which was pre-assembled into chromatin *in vitro* (Wang *et al.*, 1991). Although these studies did not discriminate between linker DNA, nucleosomes or nucleosome-free regions, they suggest an inhibitory role for nucleosomes in DNA repair. Here, we found a partial and site-specific inhibition of photolyase and T4-endoV at the level of a defined sequence nucleosome. Moreover, we noticed that even at sites with efficient repair, the maximal levels achieved were only 60–70% (for photolyase) and never 100%. One explanation for this incomplete repair is heterogeneity in the nucleosome population, e.g. with respect to nucleosome positioning. Although we used a short DNA fragment for reconstitution which limits nucleosome mobility (Losa *et al.*, 1990), it cannot be excluded that a minor fraction of nucleosomes adopt different positions and, hence, alter damage accessibility. For example, an efficiently repaired site (e.g. 1352) could be moved into an inefficiently repaired position (e.g. 1342) by a translational change of the nucleosome position by 10 bp. Such a change would not alter the general rotational setting of the DNA.

How does repair on reconstituted nucleosomes relate to DNA repair *in vivo*? First of all, *in vivo*, the HISAT sequence is located in a nuclease-sensitive region and

hence not packaged in a nucleosome (Losa *et al.*, 1990). We recently found that photoreactivation by CPD photolyase in yeast is tightly regulated by chromatin structure (Suter *et al.*, 1997). CPDs in nuclease-sensitive regions and in linker DNA between nucleosomes were rapidly removed within 15 min. This includes DNA repair in the HISAT sequence. However, ~2 h were required to repair CPDs in nucleosomes *in vivo* (Suter *et al.*, 1997). This observation is in agreement with our *in vitro* study and consistent with a severe inhibition of photolyase *in vivo* by folding of DNA in nucleosomes. Furthermore, the current study implies that photoreactivation in cells may also be modulated in nucleosomes.

In contrast to our model system, repair goes to completion in cells, indicating that this inhibition can be relieved. This can be explained by dynamic properties of nucleosomes *in vivo*. It is well known that nucleosomes are mobile and can shift position *in vitro*. Multiple overlapping positions of nucleosomes found *in vivo* are consistent with nucleosome mobility (Thoma, 1992; Tanaka *et al.*, 1996, and references therein). Hence, alterations in nucleosome positions may affect damage recognition (Suter *et al.*, 1997). Alternatively, we cannot exclude that nucleosome remodelling activities might be required to facilitate damage recognition by photolyase in nucleosomes. Nucleosome rearrangements or disruptions which could be a consequence of nucleosome remodelling have been reported in the process of NER (reviewed in Smerdon, 1991). The HISAT nucleosome provides a model substrate to address the importance of chromatin dynamics for repair in future.

Materials and methods

Preparation of end-labelled DNA and nucleosome core particles

End-labelled DNA and nucleosome core particles were prepared as described (Schierstein and Thoma, 1996). For the preparation of trypsinized nucleosome core particles, core particles [2.1 mg/ml in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 10 mM NaCl] were adjusted to 50 mM NaCl, mixed with trypsin (500 µg/ml) (type XIII, TPCK-treated, Sigma) to yield a trypsin concentration of 6 µg/ml and digested for 24 min at room temperature. Digestion was stopped by addition of trypsin inhibitor (1 mg/ml) (from chicken egg white, Boehringer) to a final concentration of 60 µg/ml (Ausio *et al.*, 1989; Hayes *et al.*, 1991b). The proteins were analysed on SDS-PAGE. Trypsinized core particles (2 mg/ml) were stored in aliquots at -70°C.

Nucleosome reconstitution

Reconstitution was by histone octamer transfer as described (Schierstein and Thoma, 1996). For reconstitution with trypsinized cores, the dilution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) contained trypsin inhibitor (10 µg/ml). The final DNA concentration was ~120 ng DNA/µl in 93 mM NaCl. Reconstitution efficiency was analysed on 0.7% agarose gels (15 cm×10 cm) in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1.25 mM EDTA, pH 8.3). Reconstituted samples were mixed with 0.5 vol. of 30% glycerol and electrophoresed for 90 min at 30 mA and room temperature. The gel was dried onto chromatographic paper (DE81, Whatman) and exposed to PhosphorImager screens (Molecular Dynamics) or X-ray films (Fuji) at -70°C using enhancer screens.

DNase I footprinting

Nucleosomal samples and naked DNA were treated with DNase I (grade I, Boehringer) as described (Schierstein and Thoma, 1996), except that reconstituted samples were digested at a final concentration of 2.4 U/µg of DNA. DNase I digestion of DNA isolated from trypsinized nucleosomes was in the presence of trypsin inhibitor (10 µg/ml) which had no effect on the cutting pattern.

UV irradiation

Reconstituted samples and naked DNA were irradiated as described (Schierstein and Thoma, 1996), using six germicidal lamps (G15WT8, Sylvania, emitting predominantly 254 nm) with 750 J/m² at a fluence of 15 W/m². UV fluence was measured using a UVX radiometer (UVP, San Gabriel, CA) equipped with a 254 nm photocell (model UVX-25, UVP, San Gabriel, CA).

Photoreactivation

All experiments were done in a darkroom equipped with yellow safety light (GE 'Gold' fluorescent light, Sylvania). Seventy µl of UV-irradiated nucleosomes were mixed with *E. coli* photolyase (0.1 µg/µl; a gift of A. Sancar) to yield a ratio of 1.8 µg DNA/1 µg photolyase. DNA isolated from UV-irradiated nucleosomes was dissolved in TE (pH 7.5), 92 mM NaCl, at a concentration of 120–200 ng/µl and mixed with photolyase (0.1 µg/µl) to yield a ratio of 3.6 µg DNA/1 µg photolyase. After incubation at room temperature for 5 min in the dark, samples were pipetted into inverted Eppendorf tube caps, covered with a microscopic slide and irradiated with six fluorescent lamps (15W F15T8 BLB, Sylvania, peak emission at 375 nm) at ~26°C (17–24 W/m² for nucleosomes, 18 W/m² for DNA). The fluence was measured using a UVX radiometer (UVP, San Gabriel, CA) with a 365 nm photocell (model UVX-36, UVP, San Gabriel, CA). Aliquots were taken after various repair times and photolyase was inactivated immediately by phenolization. Purified DNA was then digested with T4-endoV to map the remaining CPDs. DNA isolated from trypsinized nucleosomes was photoreactivated in the presence of trypsin inhibitor (10 µg/ml) which had no detectable effect on photolyase.

T4-endoV digestion

Seventy-five µl of UV-irradiated nucleosomes were incubated at 37°C for 5 min and mixed with T4-endoV (4 ng/µl) (a gift of R.S. Lloyd) to yield a final ratio of 0.5 µg DNA/4 ng T4-endoV. Samples were taken after 60 and 105 min. Following digestion, reconstituted and free DNA were separated on a 5% polyacrylamide gel (17.5 cm×12 cm×0.1 cm) in 1× TBE. Samples were mixed with 0.5 vol. of 30% glycerol and electrophoresed at 200 V until a bromophenol blue marker, loaded in a separate lane, migrated out of the gel. Nucleosomal bands were localized by autoradiography, cut out of the gel and crushed. DNA was eluted with 0.5 M ammonium acetate, 1 mM EDTA (pH 8.0) (Maxam and Gilbert, 1980). For T4-endoV analysis of naked DNA, DNA was isolated from UV-irradiated nucleosomes and dissolved in TE (pH 7.5), 100 mM NaCl, at a concentration of ~120 ng/µl. Digestion with T4-endoV was as described for nucleosomes. Aliquots were taken after 30, 60 and 105 min. To ensure complete incision, fresh enzyme was added to the remaining sample and digestion proceeded for another 120 min. Purified DNA was then analysed on 9% denaturing sequencing gels. DNA isolated from trypsinized nucleosomes was digested in the presence of trypsin inhibitor (10 µg/ml) which had no detectable effect on T4-endoV.

Mapping of CPDs

Purified DNA samples from photoreactivation experiments were dissolved in TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)], 100 mM NaCl, and incubated at 37°C for 5 min. T4-endoV was added and incubation continued for 60 min. Fresh enzyme was added after 15, 30 and 45 min to ensure complete digestion. Purified digestion products were electrophoresed on denaturing 9% polyacrylamide gels (Maxam and Gilbert, 1980). Gels were dried and exposed to X-ray films (Fuji) and to a PhosphorImager screen (Molecular Dynamics).

Quantification of CPDs

Each band was quantified, and background was subtracted. The sum of all CPD-specific bands and the band representing the intact fragment represents the total amount of DNA loaded per lane (100%). The value of each band was expressed as a fraction of the total lane (Schierstein and Thoma, 1996). To calculate repair of a specific site, the amount of CPDs at a given repair time was expressed as a fraction of the initial damage at that site.

Sequencing

End-labelled DNA was sequenced according to Maxam and Gilbert (1980) with a minor modification. Instead of cleaving the purine residues of end-labelled DNA with piperidine formate, we used 0.5 M acetic acid and incubated samples at 45°C for 20 min.

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