Initiation and bidirectional propagation of chromatin assembly from a target site for nucleotide excision repair

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To restore full genomic integrity in a eukaryotic cell, DNA repair processes have to be coordinated with the resetting of nucleosomal organization. We have established a cell-free system using *Drosophila* embryo extracts to investigate the mechanism linking *de novo* nucleosome formation to nucleotide excision repair (NER). Closed-circular DNA containing a uniquely placed cisplatin–DNA adduct was used to follow chromatin assembly specifically from a site of NER. Nucleosome formation was initiated from a target site for NER. The assembly of nucleosomes propagated bidirectionally, creating a regular nucleosomal array extending beyond the initiation site. Furthermore, this chromatin assembly was still effective when the repair synthesis step in the NER process was inhibited.

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Introduction

The dynamic organization of chromatin in the eukaryotic genome not only allows efficient packing of genomic information, but is also essential for the regulation of DNA metabolism. These dynamics can operate at the level of the basic chromatin subunit, a nucleosome, through the regulation of DNA accessibility by assembly and disassembly events (Kornberg and Lorch, 1992; Felsenfeld, 1996; Krude and Elgin, 1996; Wolffe and Pruss, 1996). Nucleosome disruption can be promoted by specific molecular machines including SWI/SNF, NURF, brahma and RSC (Cairns *et al.*, 1996; Steger and Workman, 1996). Access to nucleosomal DNA may be facilitated by histone chaperones such as nucleoplasmin (Chen *et al.*, 1994) as well as post-translational modification of histones including acetylation (Turner and O'Neill, 1995; Roth and Allis, 1996) and poly ADP-ribosylation (Althaus, 1992).

The resetting of nucleosomal structures after a disassembly event is equally important. In the case of nucleotide excision repair (NER), one of the major DNA

repair pathways, nucleosomal organization is assumed to be transiently disrupted so that repair enzymes have access to lesions and can remove them from DNA (Smerdon, 1989). Unfolding during repair could involve chromatin remodelling mechanisms similar to those used for transcription (Friedberg, 1996). A proper nucleosomal organization must then be re-established after repair. Recently, we found that *de novo* chromatin assembly on DNA occurs concomitantly with UV damage-dependent repair synthesis in extracts from *Xenopus* eggs and human cells, suggesting a mechanistic coupling between the two processes. Human chromatin assembly factor 1 (CAF1) was necessary for this repair-associated chromatin formation (Gaillard *et al.*, 1996). CAF1 was first identified as a nucleosome assembly factor associated with DNA replication in human cell-free systems (Stillman, 1986; Smith and Stillman, 1989; Krude and Knippers, 1993; Kaufman *et al.*, 1995; Verreault *et al.*, 1996).

The disruption and reassembly of chromatin during NER is likely to have some parallels with the corresponding sequence of events during DNA replication. However, the precise mechanisms coupling chromatin assembly with DNA repair may be significantly different from those used for replication. Previous studies on chromatin assembly at the replication fork have revealed that nucleosomes are deposited onto newly replicated DNA when a sufficient length of DNA has been synthesized (Annunziato, 1990; Gruss and Sogo, 1992; Kaufman and Botchan, 1994; Krude, 1995). In this case, the chromatin assembly machinery may function in a sequential, stoichiometric fashion. In contrast, during NER, a very different situation arises. Although the extent of disruption of nucleosomal organization at a site of repair has been estimated to reach up to 2 kb (Althaus, 1992), the amount of DNA synthesized is limited to a short patch of ~30 nucleotides (nt) (Edenberg and Hanawalt, 1972; Hansson *et al.*, 1989; Cleaver *et al.*, 1991; Svoboda *et al.*, 1993) and the mechanism of nucleosome reformation after repair has not been defined. It is critical to understand how a process of DNA synthesis limited to such a defined region can be sufficient to ensure the resetting of an extended nucleosomal array. A mechanism ensuring a proper regulation of assembly during NER is essential because of the importance of chromatin organization for DNA metabolism.

DNA molecules containing single, defined sites of damage have proven to be valuable tools for analysis of the NER process (Sancar, 1996; Wood, 1996). Their use in conjunction with a model system in which both NER and chromatin assembly can be followed in parallel represents a powerful way to examine chromatin assembly at a unique site of repair. *Drosophila* preblastoderm embryo extracts in which chromatin assembly is supported by the endogenous pool of histones (Becker and Wu, 1992) are used here for this purpose. We have defined

Fig. 1. Conditions favouring chromatin assembly preferentially on damaged DNA templates in *Drosophila* embryo extracts compared with *Xenopus* egg extracts. Control (–) or UV-irradiated (+) plasmid DNA was incubated with a *Drosophila* embryo extract or *Xenopus* egg extract for 2 h at various equivalent (eq.) KCl concentrations as described in Materials and methods. Radiolabel incorporation due to repair synthesis was visualized by autoradiography (Labeled DNA). The total population of DNA molecules was visualized by ethidium bromide staining of the gel (Total DNA). I, supercoiled form; II, nicked form.

conditions under which it is possible to observe selectively the chromatin assembly pathway coupled to efficient NER, enabling a precise analysis of nucleosome formation at a defined site of repair. The results support a mechanism in which NER initiates nucleosomal formation from the repair site, followed by a bidirectional propagation of chromatin assembly that leads to regular arrays of nucleosomes. Chromatin assembly is still effective when the repair synthesis step in the NER process is inhibited. This novel chromatin assembly mechanism linked to a site of NER may be significant in the response to genotoxic stress.

Results

Selection of a chromatin assembly pathway specifically coupled to repair in a *Drosophila* cell-free system

Extracts from preblastoderm *Drosophila* embryos have a high capacity to assemble chromatin *in vitro* (Becker and Wu, 1992). These extracts contain pools of histones and factors which facilitate the efficient assembly of nucleosomes on DNA (Foe and Alberts, 1983). Early embryos replicate their DNA at high rates (Blumenthal *et al.*, 1974) and at this time of development are expected to rely on efficient repair of damaged DNA. We explored whether such *Drosophila* extracts were capable of simultaneous DNA repair and chromatin assembly. A simple assay, initially set up with *Xenopus* egg extracts, consists of incubating UV-irradiated circular plasmids with a cell-free extract in the presence of radiolabelled dNTPs and analysing both radiolabel incorporation and supercoiling of the purified DNA (Gaillard *et al.*, 1996). The labelling represents DNA repair synthesis after incision of UV photoproducts (Wood *et al.*, 1988) whereas the supercoiling is indicative of chromatin formation on the DNA (Germond *et al.*, 1979).

We analysed the *Drosophila* extract in this way, using our *Xenopus* egg extract as a reference. Preferential incorporation of dNTPs in UV-treated plasmids compared with untreated DNA (Figure 1, top panel, compare lanes

+ and –) indicated that the *Drosophila* extract was competent for UV damage-dependent DNA synthesis. The influence of salt concentration was tested, as it is important for NER reactions *in vitro* (Wood *et al.*, 1988). The contribution from the extracts (in equivalent KCl) was measured by conductimetry and the final concentration was obtained by adding KCl. Comparison of *Drosophila* and *Xenopus* extracts provided two sets of information (Figure 1, 'Labeled DNA'). Firstly, throughout the range of salt conditions tested, the damage-dependent radiolabel incorporation in UV-treated plasmids remained constant with *Drosophila* extract. Secondly, for all salt conditions tested in the *Drosophila* extract, a constant fraction of labelled DNA was supercoiled, suggesting its assembly into chromatin. Limited labelling in the control plasmid indicated the presence of a few nicks in the DNA substrates. In contrast, both the extent of supercoiling and repair synthesis were decreased at higher salt concentrations in the *Xenopus* extract.

When examining the total population of DNA molecules by ethidium bromide staining (Figure 1, 'Total DNA'), formation of supercoiled molecules was also detected, revealing the existence of a repair-independent chromatin assembly pathway in both *Xenopus* and *Drosophila* extracts. This pathway was salt-sensitive, as assessed on unirradiated molecules in which the labelled fraction was very limited (Figure 1, bottom panel, lanes 11, 13, 15, 17 and 19). A time-course analysis using *Drosophila* extract revealed that under high salt conditions only limited supercoiling was observed even after 6 h, as opposed to low salt conditions where significant levels of control DNA were supercoiled after 30 min (Figure 2A, bottom panel). Although the supercoiling assay is a convenient test for chromatin assembly (Germond *et al.*, 1979) it was necessary to confirm that authentic chromatin assembly was taking place. Micrococcal nuclease (MNase) digestion analysis was used to verify that the supercoiled plasmids contained regularly spaced nucleosomes (Figure 2B). At low salt concentrations, nucleosomal ladders were observed for the control and the UV-irradiated plasmid DNA for both labelled and total DNA. In contrast, at higher salt concentration within the physiological range, defined nucleosomal ladders were detected only for the labelled plasmid DNA. This shows that elevated salt concentration suppresses repair-independent chromatin assembly by *Drosophila* embryo extracts. These conditions were used in subsequent experiments for the selective analysis of chromatin assembly tightly linked to DNA repair.

Efficient nucleotide excision repair in *Drosophila* embryo extracts

The DNA repair activity of *Drosophila* preblastoderm embryo extracts has not been reported before, and it was important to analyse further and quantify the repair properties. During a 2 h reaction, radiolabel incorporation increased with the UV-C dose applied to the plasmid DNA (Figure 3A, left). An estimate of the percentage of lesions repaired can be made by assuming that, as in other eukaryotes, UV-induced DNA synthesis is limited to short patches of ~30 nt characteristic of NER. Up to ~45% of lesions were repaired in a plasmid irradiated with 50 J/m² (Figure 3A), a dose that induces ~1.5 pyrimidine

Fig. 2. Repair synthesis and chromatin assembly, at low and high salt concentrations in *Drosophila* embryo extracts. **(A)** Time-course analysis. Control (0 J/m²) or UV-irradiated (500 J/m²) plasmid DNA was incubated with *Drosophila* embryo extract at either 40 (low) or 100 (high) mM equivalent KCl. **(B)** Micrococcal nuclease analysis. Reactions were incubated for 4 h at low and high salt conditions as in (A). Micrococcal nuclease (MNase) digestion of each substrate was performed for 30, 60 or 180 s and processed as described in Materials and methods. A molecular weight marker (123 bp ladder, BRL) was run in parallel. Lengths in base pairs are indicated on the left. Positions of mononucleosome (mono), dinucleosome (di), trinucleosome (tri) and tetranucleosome (tetra) are indicated on the right. Radiolabel incorporation due to repair synthesis was visualized by autoradiography (Labeled DNA). The total population of DNA molecules was visualized by ethidium bromide staining of the gel (Total DNA). I, supercoiled form; Ir, closed circular form; II, nicked form.

dimers per pBS plasmid (Wood *et al.*, 1995). Thus, the *Drosophila* cell-free system exhibits a high repair efficiency. The time-course of repair was followed for a plasmid irradiated at 500 J/m² (Figure 3A, right). At 15 min, ~5% of the lesions were already repaired and up to 38% and 63% of lesions were repaired after 2 and 8 h, respectively. We also tested UV-irradiated DNA from which pyrimidine hydrates had been removed by treatment with Nth protein (endonuclease III) (Wood *et al.*, 1995) and found similar results (data not shown), strongly suggesting that NER is responsible for most of the UV-induced DNA synthesis. This is consistent with a previous report of NER by extracts from a cultured embryonic *Drosophila* cell line (Shimamoto *et al.*, 1995).

For a high-resolution analysis of repair at the site of damage, the *Drosophila* system was further analysed using DNA containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink, a lesion that is avidly repaired by NER

Fig. 3. Nucleotide excision repair catalysed by *Drosophila* preblastoderm embryo extracts. **(A)** Quantitation of UV damage-dependent DNA synthesis. Repair synthesis reactions were carried out with plasmid DNA as described in Materials and methods. Left: Incorporation of dNMPs (in pmol) as a function of UVC dose (J/m²). Right: % of lesions repaired as a function of time for plasmids irradiated with 500 J/m². **(B)** Analysis of dual incisions formed during nucleotide excision repair. Left: schematic of Southern hybridization analysis of incisions formed around a single cisplatin-DNA adduct. After reactions, purified DNA was digested with two restriction enzymes 84 nt apart flanking the site of repair including the 3' and 5' incision sites. A radiolabelled oligonucleotide probe allows detection of dual incision events as oligonucleotides 24 to 34 nt long. Non-incised or fully repaired DNA is detected as 84-mers. Uncoupled 3' incisions are detected predominantly as 48 and 49-mers. Right: DNA substrates with a single cisplatin-DNA adduct were incubated with *Drosophila* embryo extract at 23°C for the times indicated (lanes 1–4). The reaction in lane 5 was performed with a human whole cell extract as described previously (Moggs *et al.*, 1996). Sizes of DNA fragments (in nucleotides) are indicated on the left. **(C)** Analysis of the extent of DNA synthesis during nucleotide excision repair. Left: schematic of DNA substrate containing a single cisplatin-DNA adduct showing the sizes (in bp) of the *Bst*NI restriction fragments flanking the repair site. Right: control DNA substrate (–) or DNA containing a single cisplatin-DNA adduct (+) were incubated with *Drosophila* embryo extract for 2 h at 23°C.

in human cell extracts (Moggs *et al.*, 1996). In mammalian cells, dual incision of the damaged DNA strand proceeds via an open intermediate (Evans *et al.*, 1997) with cleavage of the 3' and 5' single-strand/double-strand DNA junctions by the structure-specific endonucleases XPG and XPF-ERCC1 respectively (Sijbers *et al.*, 1996). With *Drosophila* extract, oligonucleotides 24–34 nt long, predominated by 29- and 30-mers, were detected in an incision assay (Figure 3B, lanes 2–4). The size of the excised fragments is characteristic of authentic NER, but it is interesting that the pattern with *Drosophila* embryo extracts was different in detail from that observed using human cell extracts, where the predominant excised oligonucleotides are 26 nt

long (Figure 3B, lane 5; Moggs *et al.*, 1996). Species-specific differences in the size and shape of the repair proteins or their developmental modification may alter the positions of incisions. We also examined the extent of radiolabel incorporation at the lesion site and, as expected in a typical NER reaction, repair synthesis was limited to a short patch found in the 33, 38 and 68 bp restriction fragments around the site of the cisplatin adduct (Figure 3C). The efficiency of repair of the cisplatin DNA adduct was ~40% after 2 h as measured by radiolabel incorporation into the 33 bp fragment containing the lesion site, consistent with the repair efficiency calculated above for a low dose of UV irradiation. The combination of this high repair efficiency and the ability to select a repair-linked chromatin assembly pathway provides a powerful system for investigating chromatin assembly linked to NER.

Nucleosome formation initiated at a target site for NER

The coupling mechanism between NER and chromatin assembly was investigated under high salt conditions using the DNA containing a single lesion. Formation of nucleosomal arrays was analysed by MNase digestion, including similarly constructed undamaged DNA as a control. As expected, significant radiolabel incorporation was detected only on the platinated DNA (Figure 4A, upper panel, compare lanes – and +). Repair was already associated with mononucleosomal DNA after 40 min. After 2 h the DNA that had undergone repair synthesis was assembled into regular nucleosomal arrays, and progressively larger nucleosomal DNA fragments appeared after 6 h. Analysis of the total population of DNA molecules by ethidium bromide staining (Figure 4A, lower panel) paralleled these observations, with nucleosomal ladders being detectable only for platinated DNA after 2 h. This is consistent with the high repair efficiency and selection of repair-linked chromatin assembly at high salt concentrations in this system. However, in contrast to the labelled DNA, a significant proportion of the total DNA formed a background smear, which is likely to represent the unrepaired fraction (60%). This excludes that the simple presence of a DNA lesion can promote chromatin assembly and rather favours a chromatin assembly mechanism linked to the repair process. Furthermore, proteins such as HMGs which might promote chromatin assembly by binding to DNA distortions (Travers *et al.*, 1994) do not interact with a 1,3-intrastrand cisplatin–DNA crosslink (Pil and Lippard, 1992). For undamaged DNA, MNase-generated fragments migrated essentially at the position of mononucleosomal DNA after 6 h, reflecting a slow, non-specific assembly of bulk DNA into chromatin under these conditions.

We then verified that the labelled DNA organized into chromatin resulted from repair at the cisplatin lesion site (Figure 4B). Undamaged and platinated substrates were incubated in *Drosophila* embryo extracts for 45 min or 6 h, and chromatin was digested to mononucleosomes by extensive MNase treatment. DNA purified after protein extraction was then challenged with two restriction enzymes, *Hind*III and *Xho*I, each recognizing a unique cleavage site near the cisplatin adduct. Mononucleosomal DNA labelled by a specific NER reaction at the lesion

Fig. 4. Nucleotide excision repair and chromatin assembly on single-lesion DNA substrates. (A) Control (–) or platinated (+) substrates were incubated in *Drosophila* embryo extract for the indicated times at 100 mM equivalent KCl. Micrococcal nuclease (MNase) digestion of each substrate was performed for 15, 45 or 90 s and processed as described in Materials and methods. Radiolabel incorporation due to repair synthesis was visualized by autoradiography (Labeled DNA). The total population of DNA molecules was visualized by ethidium bromide staining of the gel (Total DNA). A molecular weight marker (123 bp ladder, BRL) was run in parallel (M). Positions of mononucleosome (mono), dinucleosomes (di), trinucleosomes (tri), tetranucleosomes (tetra), pentanucleosomes (penta) and hexanucleosomes (hexa) are indicated on the right. (B) Digestion of mononucleosomal DNA with enzymes specific to the damaged site. Upper panel: schematic showing the principle of the experiment. Lower panel: autoradiograph showing radiolabelled mononucleosomal DNA. Control (–) or platinated (+) DNA was incubated in *Drosophila* embryo extracts at 100 mM equivalent KCl for either 45 min or 6 h. Extensive micrococcal digestion was then carried out for 4 min in order to obtain mononucleosomes. Mononucleosomal DNA obtained through MNase digestion was purified and half of it was digested with *Hind*III and *Xho*I (H/X digestion). Undigested and digested samples were run in parallel in an agarose gel. The positions of the non-digested (mono) and digested mononucleosomal DNA (cleaved mono) are indicated on the left.

site should be cleavable by at least one of the two enzymes. In contrast, labelled mononucleosomal DNA resulting from random non-specific DNA synthesis elsewhere on the molecule was expected to be resistant to cleavage by both enzymes (Figure 4B, upper panel). Labelled mononucleosomes from reactions performed with platinated DNA were sensitive to both enzymes, reflecting the presence of a nucleosome spanning the repaired site (Figure 4B, lower panel). These results demonstrate that a nucleosome is assembled at the repaired site and that

Fig. 5. Nucleosomal organization at various distances from a defined NER site. (A) Schematic representation of the single-lesion DNA substrate and localization of the probes used to analyse the quality of the chromatin at different locations on the substrate. 'Pt site': damaged site; 'distal': diametrically opposed to repair site ~ 3500 bp away. '+800' and '-800': ~ 800 bp away from either side of the lesion. (B) A single membrane corresponding to the gel in Figure 4A was analysed by Southern hybridization using successively the various probes shown in (A) as described in Materials and methods.

subsequent assembly events lead to extended regular arrays of nucleosomes.

Bidirectional propagation of nucleosomal arrays from a target site for NER

To analyse how NER at a single site, limited to a short, 30 nt region, can lead to nucleosome formation, the quality of the chromatin was examined at various distances from the lesion site. The DNA blot from Figure 4A was probed with specific oligonucleotides (Figure 5A) complementary to either the lesion site (Pt site), to regions located ~ 800 nt 5' and 3' to the lesion (-800, +800) and to a region opposite the cisplatin lesion, ~ 3500 nt away (distal). Using the probe at the Pt site, nucleosomal ladders were observed similar to those in Figure 4A, for the platinated substrate after 2 h, with larger nucleosomal fragments appearing after 6 h (Figure 5B). However, probing the membrane with the 'distal' oligonucleotide gave only a smear of fragments on both the platinated and the undamaged DNA after a 2 h reaction (Figure 5B). This indicates that at a time where regular nucleosomal arrays were detectable at

Fig. 6. Bidirectional propagation of nucleosomes from a defined NER site. (A) Densitometric analysis was performed on the 45 s MNase digestion from each 6 h reaction in Figure 5B as well as from the autoradiograph shown in Figure 4A. Data within each lane were normalized to the maximum peak of intensity and are presented in arbitrary units. (B) Model for nucleosome propagation from the repair site. Initially, mononucleosomes predominantly assemble at a defined target site for NER (left). Subsequently, an ordered chromatin assembly propagates bidirectionally from this site (right).

the repair site, little assembly had occurred ~ 3500 nt from the lesion. After a 6 h reaction the signal detected with the distal probe did not correspond to a regular and extended array of nucleosomes.

Considering the 5' $\rightarrow$ 3' directionality of the repair synthesis step, we wanted to test whether the propagation of nucleosome assembly initiated at the repair site had a corresponding unidirectionality. The membrane was probed successively with the '+800' and '-800' probes (Figure 5A). Nucleosomal ladders were detected with both probes for the platinated substrate only (Figure 5B). Interestingly, the formation of nucleosome arrays proceeded with comparable kinetics 5' and 3' to the lesion, with a nucleosomal ladder detectable after 2 h and longer arrays detectable after 6 h (as observed with the 'Pt site' probe, Figure 5B). Densitometric scanning was carried out to examine more closely the profiles of the nucleosomal ladders at the different sites (Figure 6A). Profiles obtained for the labelled fragments had a peak distribution similar to the profile with the 'Pt site' probe. In addition, the background smear representing the unpaired fraction of single lesion DNA was also detected by the probe. Profiles obtained for the +800 or -800 probes had maximum peaks for the low molecular weight nucleosomal fragments,

Fig. 7. DNA repair synthesis is not required for chromatin assembly from a defined NER site. *Drosophila* embryo extract (400 µg) was preincubated with (+) or without (–) aphidicolin for 15 min on ice before addition of the reaction components including platinated or control DNA substrate. Reactions were then incubated for 4 h at 23°C at 100 mM equivalent KCl. MNase analysis was performed as in Figure 4A. Upper panel (Labeled DNA) shows radiolabel incorporation due to repair synthesis. Lower panel (Probed DNA) shows Southern hybridization of the DNA with the '+800' probe shown in Figure 5A.

consistent with a less extensive assembly than at the site of the lesion. The smeared profile obtained with the distal probe confirmed that only poor assembly occurred far away (~3500 bp) from the lesion site. Interpretation of these data enabled us to propose the scheme in Figure 6B, where chromatin assembly initiates from the NER repair site, leading to the bidirectional propagation of regular nucleosome arrays over a limited distance around the DNA molecule.

DNA synthesis is not required for chromatin assembly from a target site for NER

The unidirectionality (5'→3') of the repair synthesis step in the NER process contrasts with the bidirectional propagation of chromatin assembly. This suggests that the DNA repair synthesis step itself might not be essential for the coupling of NER and chromatin assembly. To test this hypothesis, the formation of nucleosomal arrays was analysed on undamaged and platinated substrates, in the presence of aphidicolin, a potent inhibitor of DNA synthesis. Aphidicolin specifically inhibits DNA repair synthesis without affecting any of the preceding steps of the NER process, including recognition of the lesion and dual incision of the damaged strand (Coverley *et al.*, 1992; Calsou and Salles, 1994; Moggs *et al.*, 1996). We confirmed that dual incisions were formed by *Drosophila* embryo extract in the presence of aphidicolin (data not shown). When aphidicolin was added to the reaction, all label incorporation was eliminated, consistent with an efficient inhibition of DNA synthesis (Figure 7, compare

lanes – and +, Labeled DNA). Despite this inhibition, a nucleosomal ladder was detected on the platinated substrate with an oligonucleotide probe (Figure 7). This ladder was comparable with that observed on the platinated substrate without aphidicolin (Figure 7, compare – and +). In contrast, no ladder was detected on the undamaged substrate with or without aphidicolin. These results demonstrate that the complete repair synthesis step is not required for chromatin assembly during NER. Earlier steps in the NER process involving either a specific DNA structure or a component of the NER machinery could serve as a signal of recognition and recruitment.

Discussion

Chromatin assembly and NER in *Drosophila* embryo extracts

Drosophila preblastoderm embryo extracts are shown here to perform NER efficiently and to promote a chromatin assembly pathway linked to a specific site of DNA repair. Previous studies using either preblastoderm (Becker and Wu, 1992) or postblastoderm (Kamakaka *et al.*, 1993) embryo extracts have identified a number of chromatin assembly factors. These include nucleosome assembly factor-1 (NAP-1; Ishimi *et al.*, 1984; Ito *et al.*, 1996), decondensation factor 31 (DF31; Crevel and Cotterill, 1995) and high mobility group protein (HMGP; Ner and Travers, 1994). Additional chromatin factors have been found that can mediate disrupting events associated with transcriptional activation, such as the nucleosome rearrangement factor (NURF; Tsukiyama and Wu, 1995), or that ensure nucleosome mobility (Varga-Weisz *et al.*, 1995). These factors may participate in our specific repair-coupled chromatin assembly reaction. However, the only factor shown to be associated with a specific DNA transaction is CAF1. Human CAF1 was initially identified through the analysis of an SV40 replication-coupled assembly pathway (Stillman, 1986). Analogous activities have also been found in yeast, *Xenopus* and *Drosophila* (Gaillard *et al.*, 1996; Kamakaka *et al.*, 1996; Enomoto *et al.*, 1997; Kaufman *et al.*, 1997). In addition, human CAF1 is required for chromatin assembly coupled to NER in a human cell-free system (Gaillard *et al.*, 1996). Notably, deletion of any of the three genes encoding CAF1 subunits in *Saccharomyces cerevisiae* increases sensitivity to UV irradiation, without sensitizing cells to γ-rays (Kaufman *et al.*, 1997). This is consistent with an involvement of CAF1 in NER *in vivo*. It is not yet known whether CAF1 directly interacts with components of either DNA replication or repair.

The *Drosophila* system defined here provides a new powerful approach to follow the biochemical mechanism of chromatin assembly coupled to NER. It also offers the possibility of developmental and genetic investigations, taking advantage of known UV-sensitive mutants (Dusenbery and Smith, 1996). This can be expected to open up new avenues for investigation of both chromatin assembly and NER and the interrelations between these mechanisms.

Initiation and propagation of nucleosome formation from a defined site for NER

The use of a uniquely placed lesion facilitates the study of the chromatin assembly mechanism by focusing on a

single site of NER. At early times in the reaction, mononucleosomes were formed at the repair site and contained DNA labelled during repair synthesis. The appearance of labelled polynucleosomal DNA at later times indicates that repair at a single site subsequently leads to formation of extended nucleosomal arrays (Figure 4). This demonstrates that chromatin assembly specifically operates from the repair site to generate arrays of nucleosomes. A mechanism of propagation for the assembly of nucleosomes appears particularly appropriate for the resetting of relatively large destabilized structures. Propagation may only be possible over a limited region of the genome because incomplete assembly was observed 3.5 kb away from the lesion (Figures 5B and 6). It will now be important to examine more closely the extent of nucleosomal disruption for various damaging agents *in vivo*.

Changes in the nuclease sensitivity of DNA during repair of UV lesions in mammalian cells are known to occur (Smerdon, 1989). DNA repair patches in the bulk of chromatin show an initial, transient sensitivity to nucleases. This may be accounted for if chromatin unfolds during NER (Friedberg *et al.*, 1995). The mechanism by which a native nucleosomal organization is re-established after NER is not well understood. Repair patch ligation precedes the loss of the transient nuclease sensitivity associated with unfolded or altered nucleosomal structures on DNA undergoing NER (Hunting *et al.*, 1985; Smerdon, 1986). Furthermore, most repair patches associated with isolated nucleosomes were observed to contain ligated repair patches. These results suggest that both DNA synthesis and ligation occur before the formation of a complete nucleosome structure at the repair site. However, since disruption may not be limited to a single nucleosome during NER it is also important to examine nucleosomal organization at various distances from the repair site.

We conclude that nucleosomal arrays are formed by a bidirectional propagation mechanism from the repair site, even when repair synthesis is inhibited. In the presence of aphidicolin, the region of repair was particularly sensitive to MNase (data not shown), reflecting either the presence of a sensitive intermediate in NER such as an unligated gap, or the absence of a completely formed nucleosome. Under conditions permitting the complete repair reaction to occur, labelled mononucleosomes were found as soon as synthesis was detected (Figure 4A, top panel). Thus, although DNA synthesis may be needed in order to form a stable canonical mononucleosome at the repair site in agreement with previous reports (Smerdon, 1989), it does not appear to be necessary for entry of a presumed chromatin assembly machinery during NER. Instead, we propose that the link between NER and extensive *de novo* chromatin assembly is at an earlier step in the NER process.

Comparison of repair- and replication-coupled chromatin assembly mechanisms

Although DNA synthesis is a common event in both DNA repair and DNA replication, in each of these processes it occurs in a distinct manner and it may not be surprising that the common link involves a reaction step distinct from DNA synthesis. The incision/excision step is of particular interest since it seems possible that the simple

presence of nicks can promote repair-coupled chromatin assembly (Figure 2A and B, high KCl, non-irradiated labelled DNA). Identification of proteins specifically interacting with these intermediates may reveal a factor that recruits a presumed chromatin assembly machinery.

The assembly mechanism presented here implies that a single hit initiates nucleosome assembly from one point and then further assembly proceeds in a concerted manner. Two situations can be considered. In the first scenario, the chromatin assembly machinery is recruited at the repair site and remains there to ensure that subsequent nucleosomes form at the same site, with the newly formed nucleosome being pushed toward either side to leave the repair site free for the formation of the next one. Thus, older nucleosomes would be further away from the repair site, and activities ensuring nucleosome mobility (Varga-Weisz *et al.*, 1995) would be critical in this process. A second possibility is that the chromatin assembly machinery recruits the first nucleosome over the repaired site, followed by recruitment events on both sides.

Physiological significance of nucleosome spreading

The importance of the nucleosome spreading phenomenon should be emphasized, as it allows the generation of regular arrays of nucleosomes. Although our experiments were carried out on naked DNA templates as simple models to follow an assembly process, they may also represent an open chromatin organization that may be encountered over limited regions of the genome undergoing active transcription, repair and other DNA transactions. In response to genotoxic stress, a mechanism of assembly propagating from a single site of repair in these regions would be useful to establish rapidly a nucleosomal array that could repress new DNA transactions. Such a mechanism could be used as a rapid response to genotoxic stress which arrests DNA metabolism, thus helping to establish cell cycle checkpoints.

Materials and methods

DNA templates

UV-irradiated pBluescript KS+ (pBS) plasmid DNA was prepared using a 254 nm (peak) germicidal UV lamp (Philips). The UV flux rate was measured with a Latarjet dosimeter (France). A flux of 100 J/m² induces ~1 pyrimidine dimer photoproduct in 1000 bp [~0.75 cyclobutane pyrimidine dimer and 0.25 (6-4) photoproduct]. In control experiments we treated UV-irradiated plasmid DNA with *Escherichia coli* Nth (endonuclease III) protein to cleave DNA at sites of pyrimidine hydrates (Wood *et al.*, 1995). The single lesion substrate (Pt-GTGx), containing a 1,3-intrastrand d(GpTpG)-cisplatin crosslink, and the corresponding control substrate (Con-GTGx), were constructed as described (Moggs *et al.*, 1996).

Preparation of *Xenopus* egg, *Drosophila* embryo and human cell extracts

Xenopus egg high-speed extracts were prepared as described (Almouzni *et al.*, 1991). *Drosophila* preblastoderm embryo extracts were obtained as described in Becker *et al.* (1994). Protein concentration, determined using a Bradford protein assay kit (Bio-Rad), was typically 40 mg/ml for both types of extract. The human whole cell extract used in Figure 3B was derived from SW48 cells.

Repair and supercoiling assay

Xenopus egg extracts and *Drosophila* embryo extracts were used in 25 µl reactions at 23°C containing 400 µg protein extract, 300 ng of control or UV-C irradiated pBS plasmid, 5 mM MgCl₂, 40 mM

HEPES-KOH, pH 7.8, 0.5 mM DTT, 4 mM ATP, 5 μ Ci [α - 32 P]dCTP (3000 Ci/mmol), 40 mM phosphocreatine, 2 μ g creatine phosphokinase (Type I, Sigma). The KCl equivalent in each protein extract was measured using a conductance meter (Hanna Instrument). Final equivalent KCl concentrations were obtained by the addition of KCl. Reactions were terminated by the addition of EDTA to 15 mM, SDS to 0.35% and RNase A to 200 μ g/ml. After 30 min at 37°C, proteinase K was added to 1 mg/ml and the reaction further incubated for 30 min before phenol/chloroform extraction. DNA was precipitated with 2.5 M ammonium acetate and 2 volumes of ethanol, and subjected directly to electrophoresis in a 1.2% agarose gel in TAE buffer. The total DNA loaded on the gel was visualized after ethidium bromide staining under near-UV transillumination with a CCD camera linked to an image analyser unit (Vilbert-Lourmat, France). The gel was then dried and exposed to an X-ray film to visualize the migration pattern of the plasmids labelled during the repair assay.

Quantitative analysis of repair synthesis during NER

Reactions were carried out as described above with 400 μ g of *Drosophila* embryo extract and 300 ng of either non-treated pBS, UV-C irradiated pBS, Pt-GTGx or Con-GTGx DNA substrates. pBS plasmids were linearized with *Bam*HI before electrophoresis in a 1% agarose gel. The Pt-GTGx and Con-GTGx substrates were digested by *Bsr*NI, and the digestion products were resolved in a non-denaturing 12% polyacrylamide gel. For quantitation, the bands corresponding to the DNA substrates were excised from the dried gel, and the incorporated radioactivity was directly measured by Cerenkov counting. In the case of the single lesion substrate only the 33 bp restriction fragment was excised. The incorporation of dCMP during the repair synthesis reaction was calculated taking into account the specific activity of the radiolabel and the background incorporation in the control DNA substrate. The dNTP contribution of the *Drosophila* embryo extract was determined to be ~60 μ M by isotopic dilution as described (Blow and Laskey, 1986). The repair efficiency was estimated based on the number of lesions per plasmid assuming a repair patch size of 30 nt. In the case of the single lesion substrate the number of fmol incorporated was normalized to the base composition of the damaged DNA strand.

Analysis of the dual incisions formed during NER

For dual incision reactions, 300 ng Pt-GTGx or Con-GTGx DNA substrates were incubated with *Drosophila* embryo extract at 23°C under the same conditions as the repair synthesis assay except that radiolabelled dNTPs were omitted from the reaction buffer. The analysis of dual and uncoupled 3' incisions was performed as described previously (Sijbers *et al.*, 1996). Briefly, DNA purified from incision reactions was cleaved with *Hind*III and *Xho*I before separation in a denaturing 12% polyacrylamide gel. DNA was then transferred to a nylon membrane (Hybond-N+, Amersham) by capillary transfer for 90 min. Fixed membranes were hybridized with a radiolabelled 27-mer oligonucleotide complementary to the sequence of the damaged DNA strand flanking the cisplatin-DNA adduct. The sizes of platinated oligonucleotides were determined by comparison with a 5'-phosphorylated 24-mer oligonucleotide 5'-TCTTCTTCTGTGCACTTCTTCT-3' having a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink bridging bases 10 and 12.

Analysis of micrococcal nuclease digestion products

Reactions were scaled up to a final volume of 75 μ l but using 5-fold less specific activity. When aphidicolin was added at a final concentration of 400 μ g/ml, reactions contained 6% dimethyl sulfoxide (v/v). Digestion reactions were carried out with 45 units of micrococcal nuclease (MNase) at 23°C and aliquots (90 μ l) were removed at various times and processed as described (Becker *et al.*, 1994). MNase digestion products were separated in a 1.3% agarose gel in TBE buffer. Total DNA was visualized after ethidium bromide staining of the gel as described above. For reactions carried out with pBS substrates, the gel was dried and exposed to an X-ray film to visualize the DNA labelled during the repair assay. For reactions carried out with the single-lesion substrates, the gel was processed for vacuum transfer of the DNA onto a nylon membrane (Hybond-N+, Amersham) and exposed to an X-ray film before hybridization with radiolabelled probes. The autoradiograph revealed, as for a dried gel, the migration pattern of the DNA labelled during the repair assay. Oligonucleotides (Eurogentec) specific for the damaged site ('Pt site' 5'-GAAGAGTGCACAGAAGAAGAGGCCTGG-3'), for a region diametrically opposed to the damaged site ('distal' 5'-CACCAGTAGCACCATTACCATAGCA-3'), or specific for regions ~800 bp away from either side of the lesion ('+800' 5'-CTCAGAGCATAAAGCTAATCGGT-3'; '-800' 5'-TTAATTTGCGTGATGGACAGACTCT-3'),

were end-labelled with [γ - 32 P]ATP (>5000 Ci/mmol) using T4 DNA polynucleotide kinase (New England Biolabs). Radiolabelled probes were then used successively on the same membrane to analyse the pattern of nucleosome packaging at various locations on the single-lesion substrate. Southern blot analysis was performed as described (Becker *et al.*, 1994). After hybridization with each probe the membrane was exposed to X-ray film before being stripped. The membrane was then probed with a different oligonucleotide after checking that no significant residual label remained. Because of the high specific activity of the probes (10⁷ c.p.m./pmol of oligo) the signal due to repair synthesis can be neglected.

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