Nucleosome Unfolding during DNA Repair in Normal and Xeroderma Pigmentosum (Group C) Human Cells*

(Received for publication, February 24, 1998, and in revised form, April 14, 1998)

Bonnie K. Baxter‡ and Michael J. Smerdon§

From the Department of Biochemistry and Biophysics, P.O. Box 644660, Washington State University, Pullman, Washington 99164-4660

The fate of nucleosomes during nucleotide excision repair is unclear. We have used organomercurial chromatography to capture accessible thiol groups of proteins at (or near) nascent repair sites in normal and xeroderma pigmentosum (group C) human cells. The reactive groups include cysteine 110 of histone H3, which is exposed in unfolded nucleosomes. Immediately after UV irradiation and a short pulse labeling of repair patches, intact nuclei were digested with restriction enzymes to release ~18% of the chromatin into soluble fragments, which are enriched (~4-fold) in a constitu-

1 The abbreviations used are: NER, nucleotide excision repair; XPA, xeroderma pigmentosum (group A); XPC, xeroderma pigmentosum complementation group C; DTT, dithiothreitol; bp, base pair(s); PMSF, phenylmethylsulfonyl fluoride; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane.

as extreme sensitivity to UV light, often results in skin cancer. The xeroderma pigmentosum locus heterogeneity has been di-

vided into eight complementation groups (6), indicating that the molecular basis of this disease may involve one of several defects in the DNA repair pathway. Some of these defects may prevent repair enzymes from accessing damaged DNA com-

plexed with nucleosomes. Indeed, DNA damage-specific endo-

nuclease activity in extracts from human XPA and XPD cells decreases when DNA is folded into nucleosomes, while this activity is enhanced in extracts from normal human cells (8–10). Furthermore, XPC cells repair primarily the template strands of transcriptionally active chromatin and are deficient in repair of inactive (or bulk) chromatin (11–13). Since transcri-

ptionally active chromatin has a number of distinguishing features compared with bulk chromatin (reviewed in Refs. 2 and 14), factors may exist in cells that promote access to DNA lesions in condensed chromatin domains for DNA repair processing.

It is clear that NER modulates nucleosome structure (for reviews, see Refs. 3–5). During (and immediately after) repair synthesis, the association of histones with DNA appears to be locally disrupted. In early studies, nucleosome rearrangement during NER was operationally defined as (a) a change in nu-

Glease digestion kinetics of newly repaired DNA (or “nuclease sensitivity”) and (b) a change in association of newly repaired DNA with nucleosome core particles (15–17). Newly repaired DNA is almost absent from isolated nucleosome cores immedi-

ately after repair synthesis but rapidly associates with core histones (∼10 min) in intact human cells. Over longer times (1–24 h), there is a slow change in these features, resulting in the randomization of repair patches in nucleosomes, thought to reflect nucleosome migration onto newly repaired DNA (18).

Interestingly, XPC fibroblasts differ quantitatively in these features, in that nascent repair patches are less sensitive to nuclease digestion than in normal cells and completely randomize in chromatin by 1 h after labeling (i.e. are not associated with a slow rearrangement phase) (Ref. 17; also see Ref. 5). This suggests that nucleosome rearrangement, following repair, occurs more rapidly in XPC cells and that the newly repaired nucleosomes are more mobile than at least some of those repaired in normal cells.

The changes observed in both nuclease sensitivity and nu-

cleosome core association appear to be due to the same event, since the time courses of these changes are virtually identical (15). This phenomenon was referred to as “nucleosome rearrangement” to avoid implication of a mechanism for this process (15), and an unfolding-refolding model was proposed to explain these changes (19). Following this rapid rearrangement phase, newly repaired nucleosomes appear to have typical structures that contain histone H1 (20). This rapid rearrangement phase occurs regardless of the time after UV damage that repair synthesis takes place (16). Furthermore, prior to the

In eukaryotic cells, DNA is wrapped around histone octa-

mers, forming nucleosomes, which are the primary repeating units of chromatin (reviewed in Refs. 1 and 2). In processes such as DNA replication, transcription, and repair, the proteins involved must access DNA buried within this structural hier-

archy. This obstacle is formidable in the case of nucleotide
excision repair (NER),¹ as DNA lesions occur in all regions of chromatin (reviewed in Refs. 3–5). During (and immediately after) repair synthesis, the association of histones with DNA appears to be locally disrupted. In early studies, nucleosome rearrangement during NER was operationally defined as (a) a change in nuclease digestion kinetics of newly repaired DNA (or “nuclease sensitivity”) and (b) a change in association of newly repaired DNA with nucleosome core particles (15–17). Newly repaired DNA is almost absent from isolated nucleosome cores immediately after repair synthesis but rapidly associates with core histones (∼20 min) in intact human cells. Over longer times (1–24 h), there is a slow change in these features, resulting in the randomization of repair patches in nucleosomes, thought to reflect nucleosome migration onto newly repaired DNA (18).

Interestingly, XPC fibroblasts differ quantitatively in these features, in that nascent repair patches are less sensitive to nuclease digestion than in normal cells and completely randomize in chromatin by 1 h after labeling (i.e. are not associated with a slow rearrangement phase) (Ref. 17; also see Ref. 5). This suggests that nucleosome rearrangement, following repair, occurs more rapidly in XPC cells and that the newly repaired nucleosomes are more mobile than at least some of those repaired in normal cells.

The changes observed in both nuclease sensitivity and nu-

cleosome core association appear to be due to the same event, since the time courses of these changes are virtually identical (15). This phenomenon was referred to as “nucleosome rearrangement” to avoid implication of a mechanism for this process (15), and an unfolding-refolding model was proposed to explain these changes (19). Following this rapid rearrangement phase, newly repaired nucleosomes appear to have typical structures that contain histone H1 (20). This rapid rearrangement phase occurs regardless of the time after UV damage that repair synthesis takes place (16). Furthermore, prior to the
Nucleosome Unfolding during DNA Repair

Rapid rearrangement phase, nascent repair patches do not yield a DNase I footprint (16), suggesting that newly repaired DNA is not tightly bound to a surface of core histones immediately after repair synthesis. In addition, repair patch ligation precludes nucleosome formation in human cells (21, 22). Thus, completion of NER during the rapid phase of nucleosome rearrangement appears to progress from an unligated nonnucleosome structure to a ligated nonnucleosome structure and finally to a ligated nucleosome structure (3–5).

A number of studies implicate nucleosome disruption (or unfolding) during transcription (reviewed in Refs. 2 and 23). Furthermore, Alfirevic and co-workers (e.g. see Ref. 24) employed organomercurial affinity chromatography as a means to bind "open" nucleosomes at exposed thiols of disrupted (or unfolded) H3 histones to capture actively transcribing chromatin. Nucleosomes from a number of different species, including slime mold, yeast, mice, and humans, were fractionated with this method, and in each case active genes were preferentially bound to the organomercurial affinity matrix. In addition, the thiol reactivity of nucleosomes containing the genes c-fos and c-myc correlated with the expression of these genes when assayed by nuclear run-off transcription (25). Thus, when genes are actively transcribed in cells, at least some of these sequences are preferentially associated with the thiol-reactive nucleosomes. This suggests that proteins at (or near) these genes have exposed sulphydryl groups during transcription. Candidate proteins include H3 histones, which have one internal cysteine (at position 110) exposed in unfolded nucleosomes and other proteins with exposed thiols that are strongly bound to these genes (i.e. remain bound in 0.5 M salt). Perhaps the most direct evidence for the involvement of cysteine 110 of histone H3 in this assay comes from studies on Saccharomyces cerevisiae, which does not naturally possess a cysteine residue in histone H3 (26, 27). A mutant yeast strain was constructed with a cysteine substitution for alanine at position 110, and this substitution yielded binding of transcriptionally active chromatin to the organomercurial matrix (27). This binding did not occur in wild type strains. Furthermore, electron spectroscopic imaging implicates the existence of U-shaped structures in the nucleosomes collected from thiol-reactive chromatin but not in the unbound fraction of the organomercurial matrix (28).

In the present study, organomercurial chromatography has been used to fractionate labeled excision repair patches in human chromatin as an assay for nucleosome unfolding during NER. We have observed an enrichment of nascent repair patches associated with thiol-reactive nucleosomes of normal human cells, and this enrichment decreases in these cells as nucleosomes rearrange after the repair event. On the other hand, chromatin from XPC cells shows much less enrichment, suggesting that this transient change is more rapid in transcriptionally active chromatin and/or that this effect is due to NER occurring only in bulk (inactive) chromatin in normal human cells.

EXPERIMENTAL PROCEDURES

Normal human diploid fibroblasts (strain AG1518) and human XPC fibroblasts (strain GM02996) were purchased from Coriell Cell Repositories (Camden, NJ). The DHFR (dihydrofolate reductase) gene construct, pZH-15, was a gift from Drs. C. A. Smith and P. Hanawalt (Stanford University, Stanford, CA). This construct contains a 788-bp insert of genomic DNA from −315 bp to intron 2 of the human DHFR gene (17). 14C- and H-labeled thymidine was obtained from Moravek Biochemicals (Brea, CA). All restriction enzymes were obtained from Life Technologies, Inc. (Abi-Gel 50) organomercurial agarose was purchased from Bio-Rad, and agarose magnetic beads, activated by p-nitrophenyl chlorofluoromate, were synthesized by Perceptive Diagnostics (Cambridge, MA).

Repair Experiments—Cell culture conditions were as described by Smerdon et al. (17). In all experiments, to ensure a minimum amount of background replication, the cells were grown to confluence and treated with 2 mM hydroxyurea 45 min prior to UV damage. To allow comparison of data between experiments and between samples within one experiment, the "double-label" procedure described in Smerdon et al. (17) was used. Cells were pulse-labeled with 20 Ci/ml [3H]dThd during the growth phase to yield uniformly 3H-labeled DNA. At growth arrest (or confluence), cells were induced to repair by exposure to 12 J/m2 UV light in the presence of 10 μCi/ml [3H]dThd in conditioned media (or 20 μCi/ml [3H]dThd for XPC cells), which was incorporated into the nascent repair patches during specified incubation times. Therefore, the H/3H ratio accurately measures the relative repair activity in each of the organomercurial fractions. In some experiments, following this pulse label, the 3H was chased with 50 μM unlabeled thymidine (see Ref. 17).

Isolation of Nuclei and Chromatin Preparation—Nuclei were isolated as described (24) with the following modifications. Harvested cells were pelleted and resuspended in a lysis buffer containing 10 mM Tris, pH 8.0, 1.0 mM CaCl2, 8.6% sucrose, and 5.0 mM sodium butyrate (to inhibit histone deacetylation). Following centrifugation, cells were resuspended in a lysis buffer containing 10 mM Tris, pH 8.0, 1.0 mM CaCl2, 8.6% sucrose, 5.0 mM sodium butyrate, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 0.25 mM 1,2-epoxy-3-(p-nitrophenyl)propylene (EPNP), and 0.5% (w/v) Triton X-100. Nuclei, at an approximate concentration of 1 × 109 nuclei/ml, were treated with a mix of three restriction enzymes (0.5 units/μl each of HaeIII, BglI, and TaqI) for 1 h at 37 °C, with vigorous shaking to digest the chromatin. The nuclei digestion buffer contained 10 mM Tris-HCl, pH 7.4, 2.5 mM NaCl, 25 mM KCl, 3.0 mM MgCl2, 1.0 mM dithiothreitol, 5.0 mM sodium butyrate, 0.5 mM EPNP, and 0.25 mM PMSF. Chromatin fragments were released, with the addition of 50 mM EDTA, from the nuclei into the supernatant, which was collected after centrifugation at 10,000 rpm for 10 min at 4 °C. This soluble fraction will be referred to as the "S fraction."

Coupling of Phenylmercury to Activated Agarose Magnetic Beads—Magnetic agarose beads were coupled to a phenylmercury group as described by Chen-Cleland et al. (29). Alternatively, some beads were made with benzoic acid replacing mercuric benzoate, resulting in beads containing just a benzene ring (and no HgCl2). These beads were used in control experiments for nonspecific binding (see below).

Organomercurial Fractionation—The soluble chromatin (or S fraction) collected after restriction digestion of nuclei for each sample was loaded onto organomercurial columns or mixed with organomercurial magnetic beads (as described in Refs. 24 and 29) to selectively bind unfolded nucleosomes. The first column buffer, buffer 1 (10 mM Tris-HCl, pH 7.4, 2.5 mM NaCl, 25 mM KCl, 3.0 mM MgCl2, 1.0 mM dithiothreitol, 5.0 mM sodium butyrate, 0.1 mM PMSF, 0.1 mM EPNP), washed out the flow-through fraction and rinsed the column (or beads) of nonbinding material (Fig. 1). The collected fractions were called "peak 1." The organomercurial matrix was then washed with buffer 2 (buffer 1 plus 0.5 M NaCl), which releases chromatin bound by ionic interactions alone (Fig. 1). Finally, the column (or beads) was washed with buffer 3 (buffer 1 plus 0.5 M NaCl and 10 mM dithiothreitol (DTT)). The DTT reduces sulfide linkages, releasing the thiol-reactive chromatin from the matrix (Fig. 1). In the repair analysis, the three classes of nucleosomes separated by the chromatography (class 1, non-reactive; class 2, salt labile; and class 3, thiol-reactive) were stripped of protein by proteinase K digestion (500 μg/ml at 42 °C 1 h), and the level of 3H dpm and 14C dpm was determined by scintillation counting of triplicate samples (Packard 1900CA, Downer's Grove, IL).

Data Processing—To clarify the presentation of data, Table I shows scintillation counts (dpm) for a typical experiment. The ratio of 3H dpm (repaired DNA) to 14C dpm (total DNA) gives the relative amount of repair synthesis (per unit of DNA) for a given experiment. Since the levels (specific radioactivity) of 3H and 14C incorporated vary for each experiment, we normalized the ratio for the thiol-bound fraction (fraction 3) to the ratio for the flow-through fraction (fraction 1) in each experiment (Table I). Also, the average (3H+/14C) ratio in unirradiated cells was 4 ± 2.7% (mean ± 1 S.D.) that of the irradiated cells in each case.

RESULTS

Organomercurial Fractionation of Normal Human Chromatin—Normal human fibroblasts were prelabeled during the growth phase with [14C]dThd to provide a "bulk" DNA label for repair experiments and allow normalization of repair synthesis levels in each fraction from the organomercurial column (i.e. repaired DNA/total DNA in a particular fraction). A typical profile of the complete fractionation on the mercury affinity agarose column is shown in Fig. 2. Nuclei were prepared and
digested with a mixture of three restriction endonucleases (TaqI, HaeIII, and RsaI), as described under "Experimental Procedures." The released (soluble) chromatin, or S fraction, represents 18 ± 5% of the total DNA content, as measured by scintillation counting of the 14C content in the pellet and supernatant (data not shown). This value is similar to that obtained by Allfrey and co-workers for human HeLa cells (29). Thus, only a minor fraction of the total chromatin in cells is released by this procedure. This number (~18%) is higher than estimated percentages of actual transcriptionally active genes in mature mammalian cells (30); therefore, the S fraction must contain a large fraction of inactive (yet open) chromatin regions accessible to restriction endonucleases.

The soluble fraction of the digested chromatin (~18 ± 5% of total) was loaded onto the organomercurial magnetic beads. Buffer 1 (10 mM Tris-HCl, pH 7.4, 25 mM NaCl, 25 mM KCl, 2% sucrose, 5.0 mM EDTA, 5.0 mM sodium butyrate, 0.25 mM PMSF, 0.25 mM EPNP) was used to wash away unbound chromatin (fraction 1). Buffer 2 (buffer 1 plus 0.5 M NaCl) was then applied to release chromatin associated through ionic bonds (fraction 2). Buffer 3 (buffer 2 plus 10 mM DTT) reduces covalent thiol linkages and releases chromatin bound through unfolded nucleosomes (fraction 3).

The resulting soluble chromatin (S) was applied to an organomercurial agarose column (Affi-Gel 501, Bio-Rad), and the amount of DNA in each fraction was determined by liquid scintillation counting. Peak 1 represents chromatin that did not bind to the column in buffer 1 (see legend to Fig. 1). Peak 2 represents chromatin that was eluted from the column by the addition of 0.5 mM NaCl to the buffer. Peak 3 represents thiol-bound chromatin that was eluted with 10 mM DTT (i.e., the thiol-bound fraction). The inset shows magnification of peaks 2 and 3. Vertical lines indicate fractions where buffer changes were made.

The fraction of chromatin fragments expected to contain repair patches can be estimated as follows. About 18% of the total genome is liberated from the nucleus by the restriction enzyme digest (see above), and about 2% of these fragments are captured by the organomercurial matrix and released as fraction 3. Therefore, about 0.4% of the total chromatin is eluted in fraction 3. There is an average of one cyclobutane pyrimidine dimer per 6–8 kilobase pairs at 12 J/m² in these

The released chromatin, or about 0.4% of total chromatin (see below). In the transcription studies on human 3T3 cells, Allfrey and co-workers (24, 29) found that ~10% of the soluble chromatin was recovered in fraction 2 and ~7% in fraction 3. These differences from our results may reflect differences in solubility of the fragment sizes produced. These authors used different restriction enzymes (6-bp cutters) than our combination of 4-bp cutters and employed a different method for DNA quantitation (Hoescht dye) than we used (14C labeling). However, only a small percentage (<5%) of the human genome is expected to be actively transcribing (and completely disrupted) at any given time in mature human fibroblasts (30); therefore, only a small portion of the soluble fraction should be involved in repair.

To test whether the mercury affinity matrix may occlude or bind chromatin fragments nonspecifically, magnetic beads were generated containing only the phenyl group, and lacking HgCl (see "Experimental Procedures"). The 14C-labeled chromatin was then fractionated with these beads as for the repair experiments. Fraction 1, representing the nucleosomes that did not bind to the column, contained ~97% of the total soluble chromatin (14C-labeled DNA), while the wash with buffer 2 (0.5 mM NaCl) released ~3% of the chromatin. Only 0.05% of the 14C-labeled chromatin was detected in fraction 3 (10 mM DTT wash), or ~3% of the thiol-bound chromatin when the HgCl group is present. Thus, whereas the column matrix itself interacts ionically with a small fraction of the chromatin fragments, there is little chromatin retained in the presence of 0.5 mM NaCl.

The fraction of chromatin fragments expected to contain repair patches can be estimated as follows. About 18% of the total genome is liberated from the nucleus by the restriction enzyme digest (see above), and about 2% of these fragments are captured by the organomercurial matrix and released as fraction 3. Therefore, about 0.4% (2% × 0.18) of the total chromatin is eluted in fraction 3.2 There is an average of one cyclobutane pyrimidine dimer per 6–8 kilobase pairs at 12 J/m² in these

Clearly, the insoluble (or pellet) fraction, representing ~80% of total chromatin, may also contain thiol-reactive proteins at (or near) nascent repair sites, and it is expected that this value of 0.4% is less than the actual fraction of thiol-reactive fragments in total chromatin.
The Thiol-reactive Fraction—

Allfrey and co-workers have demonstrated that transcriptionally active genes are preferentially associated with the thiol-bound fraction (24). As a control for our experiments, the mercury bead fractions were tested for the presence of DHFR, which is constitutively expressed in the confluent human cells used in our experiments (35, 36). In fact, the relative rates of DHFR transcription in growing versus resting cells are similar (37), and equivalent amounts of mRNA in the two populations are detected on Northern blots (35). This gene should be in an active chromatin configuration and, especially those being actively transcribed, should be enriched in the thiol-bound fraction. Chromatin from UV-irradiated normal and XPC human fibroblasts was isolated and fractionated, as above, and equal amounts of DNA (determined by $^{14}$C dpm) were blotted onto a nylon membrane. The DNA was hybridized to a $^{32}$P-labeled riboprobe made to the human DHFR coding sequence (see “Experimental Procedures”). As shown in Fig. 4, the DHFR sequence was more prevalent in fraction 3 when compared with fraction 1 in both normal and XPC samples. When normalized to the $^{32}$P/$^{14}$C ratio in fraction 1, the average of two (XPC cells) or four (normal cells) experiments was 0.19 and 0.16 (P fraction), 1.9 and 1.8 (S fraction), 2.4 and 2.4 (fraction 2), and 3.2 and 4.0 (fraction 3) for XPC cells and normal cells, respectively. This finding is consistent with previous results (e.g. see Ref. 26) and indicates that a significant fraction of DHFR chromatin fragments are not covalently bound to the column. Thus, certain proteins associated with transcriptionally active chromatin must form strong ionic interactions with the column and retain actively transcribed sequences after the first buffer wash (e.g. see Ref. 38).

**Thiol-bound Chromatin Is Enriched in Nascent Repair Patches of Normal Human Cells**—We next analyzed the levels of repair-incorporated label ($^{3}$H)dThd in the three organomercurial bead fractions. Normal human fibroblasts were prelabeled with $^{14}$C)dThd, grown to confluence, UV-irradiated, and immediately incubated with $^{3}$H)dThd for 30 min in order to label nascent repair patches at UV photolesions. After this short pulse, some cells were harvested immediately, while others were incubated with unlabeled thymidine (chased) for 24 h. The amount of repair synthesis (per unit of DNA) was determined from the $^{3}$H/$^{14}$C ratio for each fraction, and this ratio was normalized to the ratio for the unbound sample, fraction 1, as described in Table 1. (Note that this normalization allows quantitative comparison between different experiments where different amounts of labeled dThd are incorporated.) Fractions 1–3 are the same as described earlier (Figs. 1 and 2). As shown in Fig. 5, the thiol-bound material is enriched (≈1.5-fold) in newly repaired nucleosomes from cells harvested with no chase (solid bars). This enrichment is not observed for cells that are

---

3 Since altered nucleosomes in these regions have a half-life of about 20 min (on average (15)), only a fraction should be in the thiol-accessible state (see above).
TABLE I

| Chase time (h) | Fraction | $^3$H | $^{14}$C | $^{3}$H/$^{14}$C | Fraction 3/1 |
|---------------|----------|-------|---------|----------------|--------------|
|               |          | dpm   |         |                |              |
| 0             | S        | 8182  | 2046    | 4.00           |              |
|               |          | 26327 | 12220   | 2.15           |              |
| 24            | S        | 19,820| 6127    | 3.23           |              |
|               |          | 128,765| 40,678  | 3.17           |              |
|               |          | 16,740| 5152    | 3.25           |              |
|               |          | 2690  | 939     | 2.86           | 0.91         |

Fig. 5. Repaired chromatin is enriched in the mercury-bound fraction of normal cells. Confluent human fibroblasts (prelabeled with $^{14}$C-dThd) were allowed to repair for 30 min, after 12 J/m² UV irradiation, in the presence of 10 μCi/ml $^{3}$H-dThd. Cells were harvested immediately (solid bars), or the $^{3}$H-dThd was chased with unlabeled dThd for 24 h (hatched bars). Soluble chromatin was pre-removed from restriction endonuclease digestion of nuclei and fractionated with organomercurial magnetic beads as described in Fig. 2. The ratio of repair-incorporated label ($^{3}$H) to bulk DNA label ($^{14}$C) is given for the flow-through (fraction 1), 0.5 M NaCl released (fraction 2), and the DTT released (fraction 3) fractions, all normalized to fraction 1. Error bars denote ± 1 S.E. of six (solid bars) or three (hatched bars) experiments, where values were determined in duplicate for each experiment. Relative ratios were calculated as noted in the legend to Fig. 5.

Fig. 6. Repaired DNA in the thiol-bound chromatin fraction decreases with chase time. UV-irradiated confluent human fibroblasts (prelabeled with $^{14}$Cl-dThd) were allowed to repair for 30 min in the presence of 10 μCi/ml $^{3}$H-dThd as described in the legend to Fig. 5. The medium was replaced with conditioned medium containing 50 μM unlabeled thymidine for the indicated times (chase). Chromatin was isolated, digested, and fractionated with the organomercurial magnetic beads as described in the legend to Fig. 2. The $^{3}$H/$^{14}$C ratio of the thiol-bound chromatin, relative to the ratio for fraction 1 ($R(fraction)/R(fraction 1)$), is plotted as a function of chase time. The time of UV irradiation and pulse time are indicated by arrows. Error bars denote ± 1 S.E. of three experiments, where values were determined in triplicate for each experiment. Relative ratios were calculated as noted in the legend to Fig. 5.

Repair Enrichment in Thiol-bound Fraction Decreases with Chase Time—The enrichment of repaired regions of chromatin observed in the thiol-reactive fraction may represent the nucleosome rearrangement demonstrated in prior nucleoside digestion studies (e.g., see Refs. 15–17). In these studies, it was found that the half-life of the early, rapid phase of nucleosome rearrangement is about 20 min in normal human fibroblasts. If this rapid nucleosome rearrangement does indeed reflect nucleosome refolding at nascent repair sites, then a similar time course should be observed, with a progressive loss of newly repaired nucleosomes bound to the thiol-reactive beads. Therefore, we tested the time course of preferential binding of repaired chromatin to the organomercurial beads. Once again, $^{14}$C-labeled cells were irradiated with UV light and pulse-labeled with $^{3}$H-dThd for 30 min. Following this pulse, cells were chased by incubation in 50 μM unlabeled dThd for 0–3 h. For each short chase time, chromatin was prepared, digested, and fractionated with the mercury affinity beads, as described in Fig. 1. As shown in Fig. 6, the amount of repair synthesis ($^{3}$H/$^{14}$C ratio) in the thiol-reactive fraction (relative to that of fraction 1) decreased rapidly with increasing chase time. The time course of this decrease, having a half-life of ~30 min, suggests that during the rapid maturation phase of repaired regions in chromatin they become less associated with thiol-reactive (or unfolded) nucleosomes.

The $^{3}$H/$^{14}$C ratio in fraction 2 also decreases to 1.0 ± 0.03 (relative to that of fraction 1) during the 3-h chase period (data not shown). Thus, although the bias of repair synthesis in fraction 2 is significantly less than that of the thiol-bound fraction, there appear to be tightly bound proteins transiently associated with nascent repair patches in chromatin that are ionically bound to the column and elute in high salt.

Thiol-bound Chromatin in XPC Cells Is Reduced in Nascent Repair Patches—We next analyzed the levels of repair-incorporated label in the three organomercurial-bead (or column) fractions of soluble chromatin from XPC human fibroblasts. Once again, cells were prelabeled with $^{14}$Cl-dThd, grown to confluence, UV-irradiated, and immediately incubated with $^{3}$H-dThd for 30 min. Some cells were harvested immediately,

...the3H signal is the result of repair synthesis.
and others were exposed to a chase period of 24 h. The amount of repair synthesis per unit DNA (or \( {^{3}H/^{14}C \) ratio) was determined for each fraction, and the value for fraction 3 was normalized to fraction 1 as described in Table I for each chase time. As shown in Fig. 7, the thiol-bound material is only slightly enriched in newly repaired nucleosomes from XPC cells not receiving a chase (hatched bars). This is in contrast to the enrichment seen with normal cells after no chase (solid bars). We note that the values for normal cells shown in Fig. 7 are from parallel experiments done at the same time as the XPC cell experiments and show some variation from the data presented in Fig. 5, when another batch of organomercurial agarose was used.

**DISCUSSION**

A central question concerned with DNA processing in chromatin concerns the fate of nucleosomes during the processing event (e.g., see Refs. 5, 23, 39, and 40). Indeed, it was recently shown that histones do not leave the DNA template during transcription through a single nucleosome in *vitro* by yeast RNA polymerase III, and direct internal nucleosome transfer may be involved (41). The recognition, removal, and replacement of damaged nucleotides during NER within chromatin must also require rearrangement of histones for NER proteins to gain access to (and process) the DNA. As stated in the Introduction, there is a rapid decrease in nuclease sensitivity of newly synthesized repair patches and association of these patches with nucleosome core structures. This change may represent nucleosome refolding, nucleosome sliding, or reassociation of displaced histones at nascent repair patches. Furthermore, UV-irradiated plasmids are rapidly assembled into nucleosomes following NER synthesis in *Xenopus* oocytes (or cell extracts), and this assembly is promoted by chromatin assembly factor 1 (42). Since UV photoproducts appear to be removed at similar rates from all surfaces of the DNA helix within nucleosomes during the early repair phase in human cells (33), an active process of nucleosome rearrangement may occur to allow access of DNA lesions to repair enzymes.

In the present report, we employed organomercurial affinity chromatography as an assay for the accessibility of thiol groups in newly repaired nucleosomes in human cells. We observed an enrichment of repair synthesis in the thiol-reactive fraction at early times (first 30 min) after repair incorporation in normal fibroblasts (Fig. 5). This suggests that proteins near nascent repair patches have exposed sulfhydryl groups during NER. As discussed in the Introduction, these groups include the internal cysteinal thiol at position 110 of H3 histones and support the notion that disruption of nucleosomes occurs during NER. This enrichment is short lived in pulse-chase experiments and decreases with a time course similar to the loss of enhanced nuclease sensitivity (Fig. 6; also see Ref. 15). Thus, in normal human cells, thiol-reactive groups are transiently exposed in a subset of nascent repair patches and may result from dynamic nucleosome unfolding during repair.

Altered nucleosome structure during NER is also implicated by *in vitro* studies on the salt- and temperature-induced rearrangement of nucleosomes at nascent repair sites in human cell nuclei (43). These studies demonstrated the existence of two phases of salt-induced nucleosome rearrangement in nuclei in *vitro* (44). Following the first transition, due to histone octamer sliding, newly repaired DNA remains in an altered (or nonnucleosome) state (43). This suggests that newly repaired DNA regions are resistant to canonical nucleosome formation even when nucleosome sliding is promoted in flanking regions and supports the notion of an altered nucleosome structure in nascent repair patches (19). Together with the present report, these results seem to rule out a model for nucleosome rearrangement being the result of preferential repair of linker DNA, followed by nucleosome sliding.

The transient nature associated with a mechanism such as nucleosome unfolding is implicated by the transient nature of the repair event itself. Even in normal cells where compact chromatin domains are also repaired, only a small fraction of chromatin may be in an intermediate state that has accessible cysteines. Thus, we expect that the thiol-reactive fraction from normal cells is an underrepresentation of newly repaired nucleosomes (see “Results”). However, our data from the pulse-chase experiments (Fig. 6) demonstrate a transient bias of repair synthesis for the thiol-reactive fraction. Clearly, this fraction represents a subfraction of all nascent repair patches, since some should be refolded (and not bind the mercury column), and others should co-isolate with unfolded nucleosomes of actively transcribing chromatin (although most of these have refolded). Thus, our results most likely reflect only a fraction of nascent repaired nucleosomes that are transiently unfolded *and* in transcriptionally inactive chromatin domains. Furthermore, the soluble chromatin fraction in our studies (released by restriction enzyme digestion) will also not include nuclear matrix DNA (1, 2). These regions are repaired more efficiently in chromatin (than nonmatrix regions) after low UV doses (45, 46) and may involve different structural rearrangements at nascent repair sites than are observed for the soluble chromatin fraction.

It is interesting that we observed much less enrichment of repair synthesis in the thiol-reactive fraction from XPC human cells (Fig. 7), which repair primarily the template strand of actively transcribing genes (47, 48). Since only a small fraction of chromatin is expected to be actively transcribing in mature human fibroblasts (30), some of the nascent repair patches inserted at early times after DNA damage in normal cells are located in condensed (transcriptionally inactive) chromatin (also see Ref. 5). Thus, the thiol-reactive fraction we obtain from normal cells could represent primarily nucleosomes from these regions of chromatin. Since repair sites in XPC cells are in actively transcribing chromatin, nucleosome refolding may occur more rapidly in these regions, and fewer repair sites are thiol-reactive after the 30-min labeling pulse.4 In support of

---

4 Indeed, the rate of nucleosome refolding in transcriptionally active chromatin may reflect the distinguishing features of these regions, such
this notion, nascent repair patches in XPC cells are less sensitive to exogenous nuclease after a 60-min labeling pulse than those of normal cells and are completely randomized in nucleosomes in less than an hour (Ref. 17; also see Ref. 5). These results may shed light on the repair deficiency in XPC cells.

XPC protein is found associated with HHR23B, a human homolog to yeast excision repair protein RAD23 (49–51). The XPC-HHR23B complex, together with transcription/repair factor TFIIH, appears to promote the first incision (or steps leading to the incision) by stabilizing the open complex required for the first step of NER (52). Consistent with this role, XPC has a high affinity for DNA ($K_d \sim 10^{-9} \text{M}$) in vitro (49), and extracts from cells lacking a functional XPC protein are unable to open the DNA structure in the vicinity of a cisplatin adduct (52). Stabilization of the unwound DNA would not be necessary in a transcribing strand held open by RNA polymerase, which correlates well with the phenotype of XPC cells. In addition, a partially unwound DNA construct, containing a thymine cyclobutane dimer in the single-stranded DNA region, eliminates the need for XPC-HHR23B in the incision step in vitro (53). Thus, XPC-HHR23B and TFIIH may promote nucleosome unfolding in inactive chromatin, where this process is not performed by RNA polymerase (also see Refs. 2 and 48).

It is important to note that XPC cell extracts are deficient in NER of naked plasmid DNA, and preassembly of the plasmid into chromatin is not required to observe the XPC phenotype (e.g. Ref. 54). However, the XPC protein restores NER of SV40 minichromosomes in XPC cell extracts in vitro (55), suggesting that it is required for repair of both nucleosomal and naked DNA templates. Thus, besides stabilizing an open complex in naked DNA (see above), XPC-HHR23B and TFIIH may be trans-acting factors for creating and stabilizing unfolded nucleosomes during NER (see discussion in Ref. 2). In condensed chromatin regions, where passive exchange (or migration) of histone octamers is less likely, the DNA helicase activity of TFIIH could provide the directed nucleosome disruption. A model for NER of inactive chromatin is shown in Fig. 8 and portrays a role for XPC protein in repair of inactive chromatin as helping to create and stabilize a disrupted nucleosome state. Furthermore, transient unfolding of nucleosomes may involve the reversible acetylation of core histone tails and depletion of histone H1 to relax the condensed state (e.g. see Refs. 32, 56, and 57). Thus, DNA damage may trigger acetylation of core histones in chromatin to prepare a "poised" chromatin fiber that allows binding and nucleosome unfolding by TFIIH (possibly with XPC-HHR23B) prior to NER (Fig. 8). This would form the initial structure for the series of DNA processing events by NER enzymes (e.g. see Ref. 48).

Finally, the structural hierarchy in chromatin modulates DNA damage levels (reviewed in Ref. 4), and tightly packed regions may play a role in protecting DNA from damage. Indeed, nucleosomes from HeLa S3 cells treated with N-methyl-N-nitrosoare were fractionated by organomercurial chromatography, and the nucleosomes in fraction 3 were found to be more heavily damaged by this agent (58). Thus, in normal cells the repair synthesis that is enriched in thiol-reactive chromatin at early times may partially reflect damage enriched in open regions of chromatin. Furthermore, the less prevalent UV photoprocess pyrimidine (6–4)–pyrimidine is repaired more rapidly in human cells (59, 60), and some of the patches we detect are from repair of these lesions. However, regardless of where in chromatin these nascent repair patches are located and which UV lesion is removed, our results indicate that

---

**REFERENCES**

1. van Holde, K. E. (1989) Chromatin, Springer-Verlag New York Inc., New York
2. Wolfe, A. P. (1995) Chromatin: Structure and Function, 2nd Ed., Academic Press, Inc., New York
3. Smerdon, M. J. (1989) in DNA Repair Mechanisms and Their Biological Implications in Mammalian Cells (Lambert, M. W., and Laval, J., eds) Plenum Publishing Corp., pp. 271–294, New York
4. Smerdon, M. J., and Thoma, F. A. (1998) in DNA Damage and Repair: Biochemistry, Genetics, and Cell Biology (Hoekstra, M. F., and Nickoloff, J. A., eds) Humana Press Inc., Totowa, NJ, in press
5. Smerdon, M. J., and Conconi, A. (1998) in Progress in Nucleic Acids Research and Molecular Biology (Melakawa, K., ed) Academic Press, Inc., in press
6. Cleaver, J. E., and Kraemer, K. H. (1989) in The Metabolic Basis of Inherited Disease (Scrivier, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) 6th Ed., McGraw-Hill, New York, pp. 2949–2971
7. Friedberg, E. C., Walker, G. C., and Wolfram, S. (1995) DNA Repair and Mutagenesis, American Society for Microbiology, Washington, DC
8. Parrish, D. D., and Lambert, M. W. (1990) Mutat. Res. 235, 65–80
9. Parrish, D. D., Peng, X., and Lambert, M. W. (1992) Biochem. Biophys. Res.
17524 Nucleosome Unfolding during DNA Repair

Commun. 189, 782–789
10. Parrish, D. D., Lambert, W. C., and Lambert, M. W. (1992) Mutat. Res. 273, 157–170
11. Kantor, G. J., Barsalou, L. S., and Hanawalt, P. C. (1990) Mutat. Res. 235, 171–180
12. Venema, J., van-Hoffen, A., Natarajan, A. T., van-Zeeland, A. A., and Mullenders, L. H. (1990) Nucleic Acids Res. 18, 443–448
13. Venema, J., van-Hoffen, A., Karcagi, V., van-Zeeland, A. T., van-Zeeland, A. A., and Mullenders, L. H. (1991) Mol. Cell. Biol. 11, 4128–4134
14. Elgio, S. R. C., ed. (1995) Chromatin Structure and Gene Expression, Oxford University Press, New York
15. Smerdon, M. J., and Lieberman, M. W. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4238–4244
16. Smerdon, M. J., and Lieberman, M. W. (1980) Biochemistry 19, 2992–3000
17. Smerdon, M. J., Kastan, M. B., and Lieberman, M. W. (1979) Biochemistry 18, 3732–3739
18. Nissen, K. A., Lan, S. Y., and Smerdon, M. J. (1986) J. Biol. Chem. 261, 8585–8588
19. Lieberman, M. W., Smerdon, M. J., Tlsty, T. D., and Oleson, F. B. (1979) in Environmental Carcinogenesis (Enmeleit, P., and Kriek, E., eds) Elsevier/ North-Holland and Biochemical Press, pp. 345–363, Amsterdam
20. Smerdon, M. J., Watkins, J. F., and Lieberman, M. W. (1982) Biochemistry 21, 3879–3885
21. Hunting, D. J., Drezeier, S. L., and Lieberman, M. W. (1985) Biochemistry 24, 3219–3226
22. Smerdon, M. J. (1986) J. Biol. Chem. 261, 244–252
23. Thomas, F. (1991) Trends Genet. 7, 175–177
24. Allfrey, V. G., and Chen, T. A. (1991) Methods Cell Biol. 35, 315–335
25. Chen, T. A., and Allfrey, V. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5257–5266
26. Chen, T. A., Smith, M. M., Le, S., Sternglanz, R., and Allfrey, V. G. (1991) J. Biol. Chem. 266, 6489–6498
27. Chen-Cleland, T. A., Smith, M. M., Le, S., Sternglanz, R., and Allfrey, V. G. (1992) J. Biol. Chem. 268, 1118–1124
28. Basset-Jones, D. P., Mendez, E., Czarnota, G. J., Oettnesmeyer, F. P., and Allfrey, V. G. (1996) Nucleic Acids Res. 24, 321–329
29. Chen-Cleland, T. A., Boffa, L. C., Carpaneto, E. M., Mariani, M. R., Valentin, E., Mendez, E., and Allfrey, V. G. (1993) J. Biol. Chem. 268, 23409–23416
30. Lewin, B. (1997) Genes VI, pp. 650–661, Oxford University Press, New York
31. Gale, J. M., and Smerdon, M. J. (1986) Biochemistry 27, 949–958
32. Ramanathan, B., and Smerdon, M. J. (1989) J. Biol. Chem. 264, 11026–11034
33. Jensen, K. A., and Smerdon, M. J. (1990) Biochemistry 29, 4773–4782
34. Huizier, J. C., and Smerdon, M. J. (1992) Biochemistry 31, 5077–5084
35. Fritz, L. K. (1994) Differential DNA Repair in Mammalian Ribosomal Genes, Ph.D. thesis, Washington State University, Pullman, WA
36. Fritz, L. K., Suquet, C., and Smerdon, M. J. (1996) J. Biol. Chem. 271, 12972–12976
37. Leys, E. J., and Kellems, R. E. (1981) Mol. Cell. Biol. 1, 961–971
38. Walker, J., Chen, T. A., Sterner, R., Berger, M., Winston, F., and Allfrey, V. G. (1990) J. Biol. Chem. 265, 5736–5746
39. van Holde, K. E., Lohr, D. E., and Robert, C. (1992) J. Biol. Chem. 267, 2837–2840
40. Sogo, J. M., and Laskey, R. A. (1995) in Chromatin Structure and Gene Expression (Elgin, S. C. R., ed) pp. 49–70, Oxford University Press, New York
41. Studitsky, V. M., Kassavetis, G. A., Geiduschek, E. P., and Felsenfeld, G. (1997) Science 278, 1960–1963
42. Gaillard, P. H., Martini, E. M., Kaufman, P. D., Stillman, B., Moustacchi, E., and Almouzni, G. (1996) Cell 86, 887–906
43. Watkins, J. F., and Smerdon, M. J. (1985) Biochemistry 24, 7288–7295
44. Watkins, J. F., and Smerdon, M. J. (1985) Biochemistry 24, 7279–7287
45. Harless, J., and Hewitt, R. R. (1987) Mutat. Res. 183, 177–184
46. Mullenders L. H., van Renteren van Leeuwen, A. C., van Zeeland, A. A., and Natarajan, A. T. (1988) Nucleic Acids Res. 16, 10607–10622
47. Friedberg, E. C. (1996) Annu. Rev. Biochem. 65, 15–42
48. Sancer, A. (1996) Annu. Rev. Biochem. 65, 43–81
49. Masutani, C., Sugawara, K., Yanagisawa, J., Sonoyama, T., Ui, M., Enomoto, T., Takin, K., Tanaka, K., van der Spek, P. J., Bootman, D., Hoeijmakers, J. H. J., and Hanaoka, F. (1994) EMBO J. 13, 1831–1843
50. Aboseusekhra, A., Biggerstaff, M., Shirvij, M. K., Viljo, J. A., Moncollin, V., Podust, V. N., Pretic, M., Hubscher, U., Egli, J. M., and Wood, R. D. (1995) Cell 80, 859–868
51. Mu, D., Hsu, D., and Sancer, A. (1996) J. Biol. Chem. 271, 8285–8294
52. Evans, E., Moggs, J. G., Hwang, J. R., Egli, J. M., and Wood, R. D. (1997) EMBO J. 16, 6559–6573
53. Mu, D., and Sancer, A. (1997) J. Biol. Chem. 272, 7570–7573
54. Shirvij, M. K., Eker, A. P., and Wood, R. D. (1994) J. Biol. Chem. 269, 22749–22757
55. Sugawara, K., Masutani, C., Uchida, A., Maekawa, T., van der Spek, P., Bootman, D., Hoeijmakers, J. H. J., and Hanaoka, F. (1996) Mol. Cell. Biol. 16, 4852–4861
56. Smerdon, M. J., Lan, S. Y., Calza, R. E., and Reeves, R. (1982) J. Biol. Chem. 257, 13441–13447
57. van Holde, K., and Zlatanov, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10548–10555
58. Boffa, L. C., Mariani, M. R., and Carpaneto, E. M. (1992) Mol. Carcinogenesis 5, 174–177
59. Wang, Y. C., Maher, V. M., Mitchell, D. L., and McCormick, J. J. (1993) Mol. Carcinogenesis 13, 4276–4283
60. Suquet, C., Mitchell, D. L., and Smerdon, M. J. (1995) J. Biol. Chem. 270, 16507–16509