

Enzymatic activities involved in the DNA resynthesis step of nucleotide excision repair are firmly attached to chromatin

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ABSTRACT

In this study the role of nuclear architecture in nucleotide excision repair (NER) was investigated by gentle dismantling of the cell and probing the capability of chromatin to carry out repair *in vitro*. The rationale behind this approach is that compartmentalization of NER at nuclear structures would make the enzymatic activities refractory to extraction by buffers that solubilize cellular membranes. In order to obtain intact chromatin primary human fibroblasts were encapsulated in agarose microbeads and lysed in isotonic buffers containing the non-ionic detergent Triton X-100. Under these conditions the majority of cellular proteins diffuse out of the beads, but the remaining chromatin is able to replicate and to transcribe DNA in the presence of triphosphates and Mg²⁺. UV irradiation of confluent repair-proficient human fibroblasts prior to lysis stimulated the incorporation of deoxynucleotide triphosphates in Triton X-100-isolated chromatin, even under stringent lysis conditions. In addition, experiments with UV-sensitive xeroderma pigmentosum (complementation groups A and C) and Cockayne's syndrome fibroblasts (complementation group A) revealed that this repair synthesis was due to global genome repair activity. Transcription-coupled repair was only detectable in cells permeabilized by streptolysin O (SLO). Repair synthesis in Triton X-100-isolated chromatin amounted to 15% of the total repair synthesis as measured in SLO-permeabilized cells. To allow the detection of these activities *in vitro*, presynthesis complexes have to be formed in intact cells, indicating that chromatin from Triton X-100-lysed cells is unable to initiate NER *in vitro*. Our data indicate that the components involved in the resynthesis step of NER are tightly associated with

chromatin. A substantial fraction of total proliferating cell nuclear antigen (PCNA), which is required for the resynthesis step in NER, has been reported to become Triton X-100 non-extractable and tightly associated with nuclear structures after UV irradiation of cells. We propose that Triton X-100-resistant repair synthesis might be mediated by this chromatin-bound fraction of total PCNA.

INTRODUCTION

There is firm evidence that repair of UV-induced photolesions is heterogeneous across the genome and that several hierarchies of DNA repair in mammalian cells exist (1,2). Based on the efficiency of repair of UV-induced cyclobutane pyrimidine dimers in various genomic regions, different levels of nucleotide excision repair (NER) can be distinguished: (i) slow repair of ribosomal genes and transcriptionally inactive tissue-specific genes; (ii) fast and efficient repair of (potentially) active genes; (iii) accelerated repair of the transcribed strand of active genes which involves a close coupling of repair to RNA polymerase II-driven transcription (transcription-coupled repair). It is obvious that transcription is not the sole determinant governing the accelerated repair of active genes. This is indicated by the fact that in the absence of transcription, (potentially) active genes are still more efficiently repaired than inactive genes (3). This suggests that other factors, such as chromatin structure, play a role in determining the efficiencies of NER in various parts of the mammalian genome.

It has become clear that nuclear functions such as DNA replication and transcription are compartmentalized within the nucleus and that components involved in these processes are associated with a nuclear skeleton termed the nuclear matrix (4–6). One way to accomplish efficient repair of active genes is to concentrate repair enzymes at the nuclear matrix in proximity to the attached transcription machinery and active genes (7).

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Indeed, investigations into the localization of repair of UV-induced DNA damage have revealed evidence for the association of NER with the nuclear matrix. Analysis of the distribution of repair sites in UV-irradiated human cells has indicated that DNA sequences at the attachment sites of DNA loops to the nuclear matrix are the primary target for initial repair activity (8). Additionally, in cells expressing only transcription-coupled repair (i.e. xeroderma pigmentosum group C cells), repair is confined to DNA sequences located proximal to the nuclear matrix and is virtually absent in loop DNA (9). This preferential localization of UV-induced repair sites at the nuclear matrix in human cells correlates well with the ability to perform preferential repair of cyclobutane pyrimidine dimers (CPD) in (potentially) transcriptionally active DNA. Recently it was shown by Park *et al.* (10) that the XPG endonuclease, which is an enzyme essential for NER, is tightly associated with nuclear structures identical to the nuclear matrix.

The finding that the products of several repair genes (xeroderma pigmentosum groups B and D, trichothiodystrophy group A) are components of the human transcription factor TFIIH (required for the initiation of class II gene transcription; 11) may be additional evidence for nuclear architecture playing a role in DNA repair. Recent studies have indicated that activities involved in transcription are localized in discrete domains rather than diffusely distributed (12–14). Biochemical studies have provided evidence that RNA polymerase II activity as well as nascent RNA are attached to the nuclear matrix (15,16) and that transcriptionally active genes are attached via specific nuclear matrix attachment regions (MARs) (17,18). Thus the compartmentalization of transcription activity at the nuclear matrix could have a major impact on the distribution of repair activity within the nucleus and could influence repair rates across the genome.

One step forward to elucidate the role of the nuclear matrix in DNA repair is to demonstrate that enzymatic activities involved in NER are associated with chromatin. In order to address this question experimentally, it is essential to measure repair in chromatin isolated under conditions which preserve structure and function. Cook and co-workers have developed a method of chromatin preparation that fulfils these requirements as much as possible (19). In their approach cells encapsulated in agarose microbeads are permeabilized under ionic conditions that mimic the cytoplasm. Chromatin prepared in this way contains intact DNA loops associated with the nuclear skeleton and is able to replicate (in an S phase-dependent manner) and to transcribe.

In this study we have investigated whether NER activity is associated with chromatin prepared from UV-irradiated normal human fibroblasts and UV-sensitive XP and CS fibroblasts. Our results indicate that enzyme activities involved in the DNA resynthesis step of NER are tightly attached to chromatin even when cellular and nuclear membranes are solubilized and the majority of cellular proteins are removed. The Triton X-100 (Triton)-resistant repair synthesis amounted to 15% of total repair synthesis when compared with cells mildly permeabilized by the bacterial cytotoxin streptolysin O (SLO). This fraction of total repair activity closely parallels the fraction of total proliferating cell nuclear antigen (PCNA), a protein required for the resynthesis step in NER (20), that has been reported to become Triton resistant and tightly associated with nuclear structures after UV irradiation of cells (21,22). We propose that Triton-resistant repair synthesis might be mediated by this fraction of total PCNA.

MATERIALS AND METHODS

Cell culture and radioactive labelling

Primary human fibroblasts were grown in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics in a 2.5% CO₂ atmosphere. To prelabel the DNA the cells were incubated for 3 days in the presence of 0.1 μCi/ml [³H]thymidine (82 Ci/mmol). After 3 days the medium was replaced by fresh medium and the cells were grown to confluence. To label proteins, cells were grown for 24 h in the presence of 1 μCi/ml [³⁵S]methionine (1000 Ci/mmol) in methionine-depleted medium.

The following primary fibroblast cell lines were used: VH16 and VH25, derived from two normal individuals; XP1TE and XP21RO, derived from xeroderma pigmentosum complementation group C patients (XP-C); XP25RO and XP30RO derived from xeroderma pigmentosum group A (XP-A) and variant (XP-variant) patients respectively; CS3BE derived from a Cockayne's syndrome patient belonging to complementation group A. In some of the experiments α-amanitin (final concentration 20 μg/ml) was added to confluent fibroblasts and the cells were kept in this medium for 16 h. SV40-transformed normal human fibroblasts (MRC-5) were grown in Ham's F10 medium supplemented with 10% newborn calf serum and antibiotics in a 5% CO₂ atmosphere and prelabelled under the same conditions as primary cells.

Encapsulation and permeabilization of cells

Exponentially growing MRC-5 cells were encapsulated in agarose microbeads to a final concentration of 3–5 × 10⁶ cells/ml beads and subsequently lysed essentially as described by Jackson *et al.* (19). In each experiment a part of the cells was not encapsulated, but lysed in 0.5% SDS and the specific activity of the cells (³H counts/10⁶ cells) was determined by scintillation counting. Encapsulated cells were incubated in complete medium for 1 h, washed with PBS and lysed in a physiological buffer (PB) (19) containing 100 mM KH₂PO₄, 130 mM KCl, 10 mM Na₂HPO₄, 1 mM MgCl₂, 1 mM Na₂ATP (Sigma type II), 1 mM DTT, pH 7.4, supplemented with 0.5% Triton X-100. Lysis was performed by keeping the microbeads at 4°C for 15 min. In some experiments the lysis step was repeated twice. Subsequently the beads were washed four times at 4°C with large volumes of PB without Triton.

Primary fibroblasts were grown to confluence and encapsulated at a concentration of 3–5 × 10⁶ cells/ml beads. The cells were grown for 1 h in medium and lysed in PB supplemented with 0.5% Triton as described above. In several experiments SLO (Wellcome) was used instead of Triton to mildly permeabilize the cells. The cells were incubated in PB supplemented with 0.045 U/ml SLO for 30 min on ice. Unbound SLO was removed by washing twice with large volumes of PB (4°C). Subsequently, the beads were incubated for 2 min at 37°C to permeabilize the cellular membranes and again washed twice with PB (4°C).

To determine the amount of protein removed by cell lysis and subsequent washing and centrifugation, aliquots of supernatant and beads (lysed completely by incubation with 1% SDS for 90 min at 37°C) were taken and TCA was added to 10%. Precipitates were collected, dissolved in 0.2 N NaOH and processed for liquid scintillation counting.

UV irradiation

Prior to UV irradiation, encapsulated cells were kept in medium for 1 h, washed twice with PBS and resuspended in 3 vol PBS. Irradiation was performed with a Philips TUV lamp (predominantly 254 nm) at a dose rate of 0.2 J/m²/s; the effective dose of irradiation of encapsulated cells was ~50% as deduced from frequencies of cyclobutane pyrimidine dimers induced in cells encapsulated in microbeads and in monolayer cells in Petri dishes. The cells were post-UV incubated in PBS or in complete medium at 37°C for 30 min, washed with PB and lysed as described above. In every experiment part of the cells were mock irradiated and processed like the irradiated cells.

Replication, transcription and repair assays

For repair and replication experiments a 10× concentrated nucleotide mix was prepared, which contained 2.5 mM dGTP, dCTP, dTTP, 1.0 mM GTP, CTP, UTP, 10 mM ATP, 50 mM KPO₄, pH 7.4, 25 mM MgCl₂ and 400 μCi/ml [³²P]dATP (3000 Ci/mmol). For transcription experiments the 10× concentrated nucleotide mix (transcription mix) contained 2.5 mM GTP, 2.5 mM CTP, 20 μM UTP, 10 mM ATP, 50 mM KPO₄, pH 7.4, 25 mM MgCl₂ and 400 μCi/ml [³²P]UTP (3000 Ci/mmol). In several of the experiments the transcription mix was supplemented with dNTPs to a final concentration of 25 or 250 μM for each nucleotide in the transcription reaction, thus resembling the conditions for measurement of repair replication. The effect of α-amanitin (final concentration 20 μg/ml) was investigated either by treatment of intact cells with the inhibitor or by addition of α-amanitin to the transcription mix only. Aliquots of 0.9 vol lysed and washed cells in microbeads and 0.1 vol nucleotide mix were kept at 37°C for 2 min and subsequently mixed. At various time intervals aliquots were taken, washed four times with excess ice-cold physiological buffer and lysed completely by incubation with 1% SDS for 90 min at 37°C. TCA was added to 10% and precipitates were collected on glass fibre filters and processed for liquid scintillation counting.

CsCl density gradient centrifugation

Prior to encapsulation, exponentially growing ³H-labelled MRC-5 cells were incubated for 1 h in medium containing 10 μM bromodeoxyuridine (BrdU) and 1 μM fluorodeoxyuridine (FdU). Addition of BrdU and FdU to the medium enables the replicated DNA to be separated from the parental DNA in CsCl density gradients. The cells were encapsulated, lysed and incubated with nucleotides as described above, but with 2.5 μM BrdUTP instead of dTTP. After the reaction the beads were incubated with *Hae*III (500 U/ml) for 1 h at 37°C in the appropriate buffer, followed by complete lysis of the chromatin with 0.1% sarcosyl and 100 μg/ml proteinase K at 37°C and isolation of DNA by electroelution. The purified DNA was centrifuged to equilibrium in CsCl density gradients. The gradients were fractionated, TCA precipitated and radioactivity of acid-insoluble material present in each fraction was determined.

For size analysis of newly synthesized DNA, permeabilized encapsulated cells were incubated with nucleotides, lysed with 1% SDS and subjected to electrophoresis in 0.6% agarose gels equilibrated with a buffer containing 0.03 N NaOH and 1 mM EDTA. The lanes containing DNA were sliced, the gel slices were

melted in 1 N HCl at 90°C, mixed with scintillation solution and the radioactivity measured.

Treatment of chromatin with T4 endonuclease V

Encapsulated cells were UV irradiated (15 J/m²), permeabilized in Triton-containing PB as described above and resuspended in PB supplemented with 5 mM EDTA. The chromatin was treated or mock treated with the CPD-specific enzyme T4 endonuclease V for 15 min at 37°C (23) and the beads were extensively washed with cold PB containing 5 mM EDTA. Subsequently, the treated and mock-treated samples were incubated with nucleotide mix at 37°C and incorporation of labelled nucleotides was measured in samples taken at various incubation times up to 30 min. Aliquots were taken to confirm the presence of T4 endonuclease V-induced incisions by alkaline gel electrophoresis.

RESULTS

Isolation and characterization of chromatin

In order to isolate intact and functionally active chromatin we employed cells encapsulated in agarose microbeads as described by Jackson and Cook (19,24). Basically the cells are mixed with low gelling agarose to achieve encapsulation and lysed under isotonic conditions to obtain chromatin. The chromatin is protected by the agarose coat from shearing and aggregation, but remains fully accessible to macromolecules up to 1.5 × 10² kDa. Moreover, the chromatin has been reported to be able to perform DNA metabolic activities, such as replication and transcription.

The aim of our study was to address the question of whether this chromatin is capable of performing NER and which of the NER repair subpathways, i.e. transcription-coupled repair or/and global genome repair, are involved. Therefore, we utilized confluent primary normal human fibroblasts as well as fibroblasts from patients suffering from xeroderma pigmentosum (XP) and Cockayne's syndrome (CS). First we established lysis conditions and replication activities in exponentially growing immortalized cells as previously described (24) and subsequently applied the methodology to confluent or exponentially growing primary cells. After encapsulation in agarose microbeads, immortalized as well as primary cells remained viable and were able to grow in the presence of culture medium. The latter was tested by growing the cells in medium containing BrdU for 24 h and immunostaining with antibodies against BrdU-containing DNA. Lysis of immortalized or primary cells in PB supplemented with 0.5% Triton resulted in release of 80% of the [³⁵S]methionine-labelled cellular proteins. Repeated incubations with 0.5% Triton-containing buffer did not lead to further release of proteins. Based on trypan blue staining, all cells became permeable. Permeabilization of encapsulated cells by SLO (25) resulted in release of ~5% of the cellular proteins leaving the cellular membrane fully intact.

Replication and transcription activities

The capability of performing DNA replication was determined in chromatin isolated from exponentially growing MRC-5 and primary cells by incubation in PB supplemented with nucleotides (including [³²P]dATP), Mg²⁺ and ATP at 37°C. The replication activity, measured as the incorporation of [³²P]dATP into acid-precipitable counts, was fully dependent on the presence of Mg²⁺ and ATP in the reaction mix and was inhibited by

aphidicolin. Both primary and immortalized cells exhibited increased incorporation of [^{32}P]dATP with time. Figure 1A shows the results obtained with immortalized cells. However, the efficiency of the reaction was strongly dependent on the dATP concentration of the reaction mixture: the incorporation of nucleotides was strongly enhanced by an increase in the dATP concentration in the reaction mix, indicating that submicromolar concentrations of dATP are rate limiting for the replication reaction (data not shown). To prove the validity of the system with regard to replicative synthesis, chromatin derived from exponentially growing primary fibroblasts was incubated with replication mix containing BrdUTP, cut with restriction enzymes and lysed completely with sarcosyl and proteinase K. Subsequently, the newly synthesized DNA was analysed by neutral CsCl density gradient centrifugation. As shown in Figure 1B, the ^{32}P -labelled newly synthesized DNA strands were shifted to higher density in the gradient compared with the non-replicated DNA fraction, suggesting that efficient replication takes place in chromatin. To investigate the efficiency of DNA synthesis in more detail, samples were taken at various times after addition of the replication mix to the chromatin and run in an alkaline agarose gel; lanes containing DNA were sliced and the amount of radioactivity in each slice was determined by liquid scintillation counting. The results indicated that the newly synthesized Okazaki fragments are very rapidly ligated into large DNA molecules during the course of the reaction (data not shown). When encapsulated, immortalized cells were irradiated with UV (effective dose 15 J/m^2) prior to permeabilization, a clear inhibition of replication activity was observed (Fig. 1A).

Lysis and washing of the cells with Triton-containing PB results in release of the majority of the cellular proteins and both cellular and nuclear membranes. Although the chromatin from Triton-extracted cells is able to replicate, we examined whether the alterations induced by Triton affected the efficiency of replication. Therefore, we lysed exponentially growing fibroblasts in SLO, which punches holes in the cellular membranes but maintains the morphology of cells to such an extent that clonal survival is only moderately affected at the concentrations used in this study. When encapsulated cells were permeabilized by SLO or Triton, incorporation of [^{32}P]dATP (employing $1.25 \mu\text{M}$ cold dATP) in SLO-treated cells was ~ 5 -fold more efficient than in Triton-treated cells (Fig. 2).

Since transcription is required for selective repair of the transcribed strand of active genes, experiments were performed with either exponentially growing MRC5 cells or confluent human fibroblasts to determine transcription activity in encapsulated chromatin (Triton lysis) or SLO-permeabilized cells. Figure 3 shows the results of *in vitro* transcription in SLO-permeabilized XP-C cells. Incubation with ribonucleotide triphosphates, Mg^{2+} and ATP (transcription mix) resulted in increased incorporation of [^{32}P]UTP into acid-insoluble material with time. This incorporation was inhibited by UV irradiation of intact cells prior to the reaction and by addition of α -amanitin (final concentration $20 \mu\text{g/ml}$) to the transcription mix. Several nucleotide combinations (NTPs and dNTPs) and concentrations were tested and it appeared that under the conditions used for replication and repair, transcription was equally efficient compared with transcription mix.

The effect of solubilization of membranes by Triton on the efficiency of transcription was comparable with that observed for replication. When encapsulated cells were permeabilized and

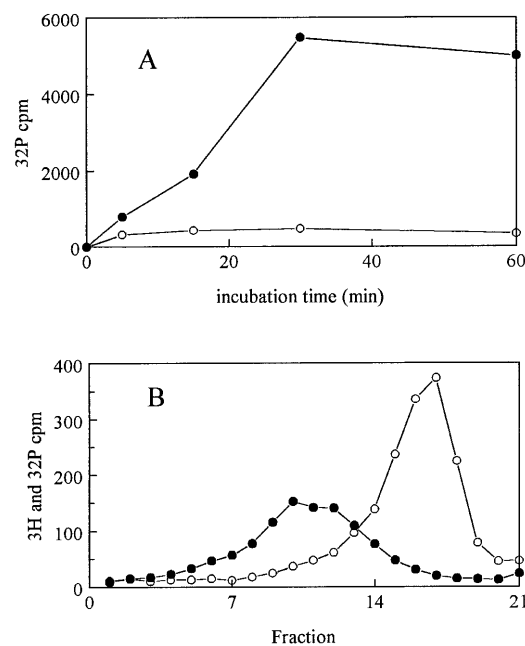


Figure 1. *In vitro* replication of chromatin. (A) Exponentially growing MRC5 cells were encapsulated in agarose microbeads and cultured for 1 h prior to UV irradiation (15 J/m^2). Chromatin was prepared in Triton-containing PB and assayed for *in vitro* replication activity. ● -UV; ○ +UV. (B) CsCl density gradient centrifugation of ^3H -labelled parental DNA and nascent DNA labelled *in vitro* for 60 min with [^{32}P]dATP in the presence of BrdUTP. Chromatin was prepared from exponentially growing VH25 cells extracted with Triton-containing PB. Fractions 1 and 21 are respectively bottom and top of the gradient. ● ^{32}P ; ○ ^3H . The results shown in these graphs are representative of a series of experiments.

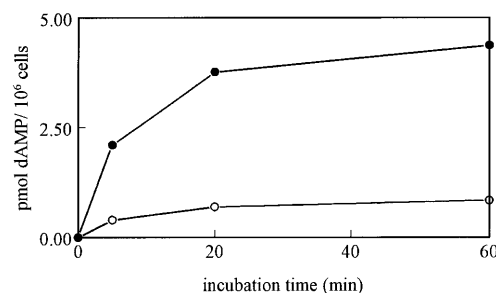


Figure 2. Effect of Triton on efficiency of *in vitro* DNA replication. Exponentially growing VH25 fibroblasts were encapsulated in agarose microbeads and cultured for 1 h. Cells were either permeabilized by SLO or extracted with Triton X-100 containing PB and assayed for replication activity. ● SLO; ○ Triton. The results shown in this graph are representative of a series of experiments.

lysed in SLO or Triton, incorporation of labelled precursors into SLO-lysed cells employing $20 \mu\text{M}$ cold UTP was ~ 5 -fold more efficient than in Triton-lysed cells; this difference was abolished when reactions were performed in the absence of cold UTP (data not shown).

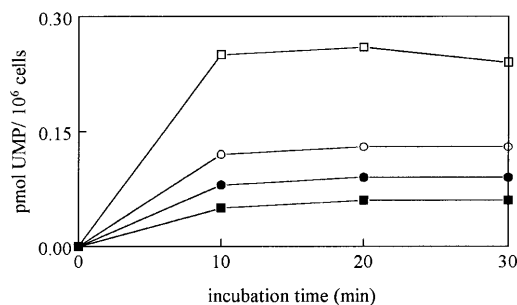


Figure 3. *In vitro* transcription of chromatin. Confluent XP21RO cells pretreated or not with α -amanitin were encapsulated in agarose microbeads, UV irradiated (15 J/m^2) or mock irradiated and kept for 30 min in buffer. Cells were permeabilized in SLO-containing PB and assayed for transcription activity in the presence or absence of α -amanitin. \square -UV; \circ +UV; \blacksquare -UV + α -amanitin; \bullet +UV + α -amanitin. The results shown in this graph are representative of a series of experiments.

DNA repair activities in normal human fibroblasts

To study repair of DNA damage by NER in intact chromatin, stationary primary fibroblasts prelabelled with [^3H]thymidine were encapsulated and irradiated with an effective dose of 15 J/m^2 UV in PBS or mock irradiated. The cells were left at room temperature for variable time periods, permeabilized with either Triton or SLO, washed with PB and incubated with nucleotides (including [^{32}P]dATP), Mg^{2+} and ATP. At various times after the start of the incubation, aliquots were taken and the amount of incorporated label per 10^6 cells was determined.

When stationary primary normal fibroblasts were encapsulated, UV irradiated and lysed immediately in Triton-containing PB, no effect of UV on incorporation of ^{32}P -labelled nucleotides was observed. However, when the cells were irradiated and incubated either at room temperature or at 37°C in PBS prior to lysis, incorporation of [^{32}P]dATP was clearly enhanced in irradiated cells compared with non-irradiated cells. This stimulation of incorporation reached a maximum when chromatin was prepared from encapsulated cells 30 min after UV irradiation (Fig. 4). Incubations of encapsulated and UV-irradiated cells for longer periods than 30 min did not lead to a further stimulation of incorporation. Based on these observations, all subsequent experiments were performed with UV-irradiated cells kept at 37°C for 30 min prior to lysis to prime the repair reaction. Under these conditions, chromatin from UV-irradiated and Triton-lysed cells is able to incorporate [^{32}P]dATP up to 60 min following the start of incubation. However, the majority of incorporation takes place during the first 20 min of the reaction. After the initial 20 min incorporation in chromatin from UV-irradiated cells continues, albeit at a slower rate, but the rate of incorporation clearly exceeds that in chromatin prepared from unirradiated cells. To confirm that UV-stimulated incorporation of [^{32}P]dATP was due to repair synthesis, we performed *in vitro* repair reactions in the presence of BrdU and analysed the DNA by CsCl density gradient centrifugation. All label incorporated in chromatin from UV-irradiated cells appeared to be present in the parental DNA fraction (Fig. 4B). Since the absolute levels of incorporation varied in a series of experiments, the extent of repair incorporation in the various experiments was quantified by expressing the incorporated label in UV-irradiated cells relative to that in

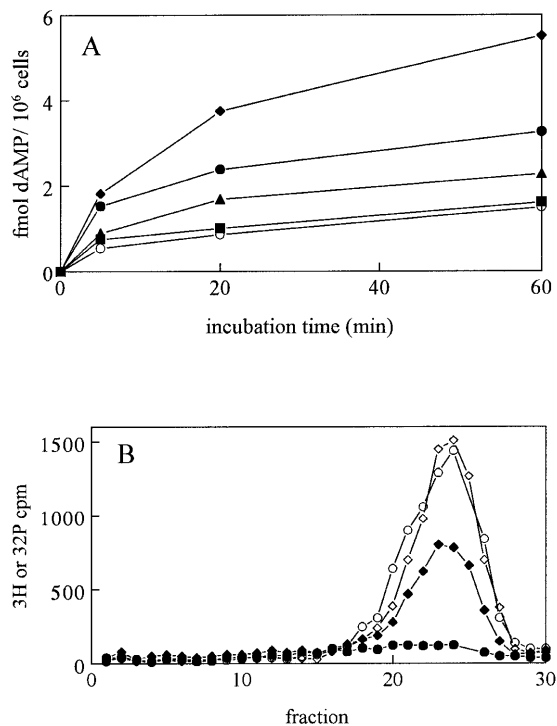


Figure 4. *In vitro* repair in primary fibroblasts. Confluent VH25 cells were encapsulated in agarose microbeads, UV irradiated (15 J/m^2) or mock irradiated and kept for various time periods (A) or for 30 min (B) in buffer. Cells were extracted with Triton-containing PB and assayed for repair activity. (A) \circ -UV; \blacksquare +UV, 0 min; \blacktriangle +UV, 5 min; \bullet +UV, 15 min; \blacklozenge +UV, 30 min. (B) CsCl density gradient centrifugation of ^3H -labelled parental DNA and DNA labelled *in vitro* for 60 min with [^{32}P]dATP in the presence of BrdUTP. \bullet ^{32}P , -UV; \circ ^3H , -UV; \blacklozenge ^{32}P , +UV; \diamond ^3H , +UV. The results shown in these graphs are representative of series of experiments.

non-irradiated cells. This ratio is termed the UV stimulation factor (UVst).

We addressed the question of whether soluble proteins remaining after incomplete lysis or extraction by Triton might account for the repair incorporation in chromatin from Triton-lysed cells. The results of experiments summarized in Figure 5A indicate that this repair activity cannot be attributed to incomplete lysis of the cells. In these experiments *in vitro* repair synthesis was measured in chromatin from UV-irradiated cells either lysed with increasing concentrations of Triton or subjected to repeating washing with 0.5% Triton. Mild permeabilization of encapsulated and UV-irradiated cells (SLO or 0.001% Triton) was taken as a standard for maximal repair synthesis and under these conditions a 10-fold stimulation of incorporation was observed when compared with unirradiated cells. When the Triton concentration was increased to 0.05%, the UVst dropped from >10 to ~ 3 –4. However, an increase in the Triton concentration up to 0.5% or three times repeated washing with 0.5% Triton (each step incubated for 15 min on ice) did not lead to a further reduction in the capacity of chromatin to incorporate [^{32}P]dATP. These results suggest that enzymatic activities involved in the resynthesis step of NER are firmly associated with chromatin.

From our results it is evident that chromatin in this *in vitro* system, although it is able to perform repair synthesis, lacks essential factors to carry out complete NER reactions from

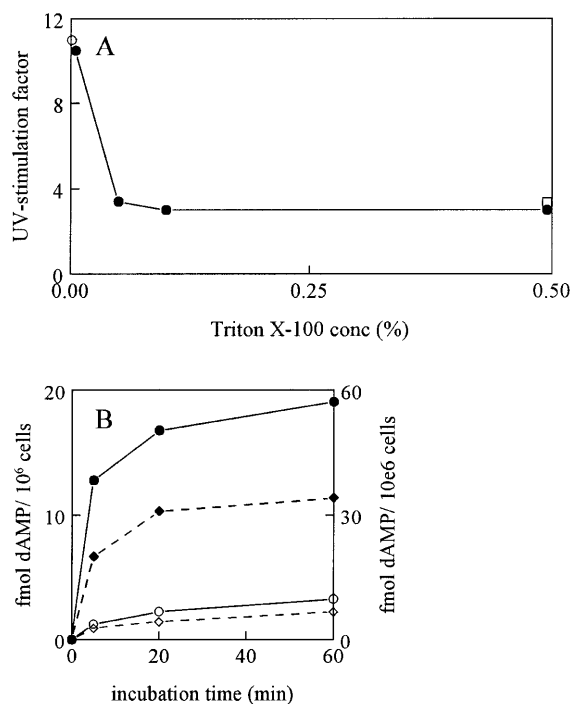


Figure 5. *In vitro* repair in VH25 fibroblasts: effect of Triton extraction. Confluent VH25 cells were encapsulated in agarose microbeads, UV irradiated (15 J/m^2) or mock treated and kept for 30 min in buffer. (A) Cells were permeabilized in SLO or extracted with PB containing increasing concentrations of Triton X-100 ranging from 0.001 to 0.5% and assayed for repair activity (60 min labelling). ○ SLO; ● Triton; □ Triton, repeated extractions. Repair incorporation was expressed relative to the incorporation in mock treated cells. (B) Repair replication in Triton-extracted cells (left y-axis) and SLO permeabilized cells (right y-axis). ● SLO, +UV; ○ SLO, -UV; ◆ Triton, +UV; ◇ Triton, -UV. The results shown in these graphs are representative of a series of experiments.

incision to the final ligation step. Since lysis of cells in Triton extracts >80% of the cellular proteins, we investigated the possibility that mild permeabilization of the cells would allow us to study repair synthesis *in vitro* without the necessity to prime intact cells for repair. However, even under conditions of mild SLO permeabilization repair incorporation could not be detected unless intact cells were UV irradiated and post-UV incubated prior to permeabilization. Nevertheless, distinct differences in repair incorporation exist between Triton- and SLO-lysed cells. As indicated in Figure 5B, UV-stimulated incorporation of [^{32}P]dATP in Triton-lysed cells is relatively low, on average 15% of that observed in SLO-permeabilized cells. Since incorporation in unirradiated cells is less affected by Triton lysis than in UV-irradiated cells, the UVst drops from an average value of 8.0 to 4.5 after SLO and Triton lysis of VH25D cells respectively. Basically, the loss of repair activity by Triton lysis mimics the effect of Triton on replicative synthesis, as Triton-lysed cells retain only 20–25% of their replication activity when compared with SLO-permeabilized cells (see Fig. 2).

In several permeable cell systems (27,28) repair replication is strongly enhanced by treatment with the enzyme T4 endonuclease V, which specifically nicks DNA containing CPD. We investigated the effect of T4 endonuclease V by pretreatment of chromatin with the enzyme prior to the *in vitro* repair incubation. We found that repair synthesis in Triton-isolated chromatin was

not stimulated by preincubation with T4 endonuclease V. Alkaline agarose electrophoresis revealed that T4 endonuclease V specifically nicked the chromatin of UV-irradiated cells, demonstrating that the lack of stimulation of incorporation by T4 endonuclease V cannot be attributed to insufficient incision at the sites of lesions.

DNA repair activities in UV-sensitive human fibroblasts

In mammalian cells UV-induced photolesions are processed by two NER subpathways, i.e. the global genome repair pathway and the transcription-coupled repair pathway. The latter removes DNA photolesions from the transcribed strand of active genes and depends on active transcription. We addressed the question whether *in vitro* repair synthesis was due to global genome repair or to transcription-coupled repair by investigating repair synthesis in XP-C and CS fibroblast strains that can only carry out transcription-coupled repair or global genome repair respectively (7,26).

As shown in Figure 6A, in normal human cell lines (VH16 and VH25) the UVst after Triton lysis amounted to ~4.0–4.5 (mean of five and six experiments respectively). UV irradiation did not have any effect on incorporation of label in XP-A cells (completely defective in NER), providing additional proof that incorporation of [^{32}P]dATP into chromatin is due to DNA repair synthesis. Also, chromatin from Triton-lysed XP-C cells did not exhibit any stimulation of repair by UV, suggesting that the repair activity detected in chromatin from normal cells cannot be attributed to transcription-coupled repair. This conclusion is in line with the observation that the extent of repair synthesis in chromatin derived from CS-A cells was very similar to normal human cells. Taken together, the results indicate that the vast majority of repair synthesis in chromatin prepared from UV-irradiated human fibroblasts is due to global genome repair.

Different results were obtained by mild permeabilization of cells by SLO (Fig. 6B). Whereas in XP-C cells after Triton lysis no repair activity could be detected, a 2-fold stimulation of incorporation of [^{32}P]dATP was observed in SLO-permeabilized XP-C cells (Figs 6 and 7). Moreover, repair synthesis in SLO-permeabilized XP-C cells appeared to be sensitive to the RNA polymerase II inhibitor α -amanitin, but only when the inhibitor was added to intact cells prior to UV irradiation: after incubation with 20 $\mu\text{g/ml}$ α -amanitin the repair activity in these cells was completely abolished (Figs 6B and 7), whereas in normal cells no effect was seen (data not shown). When α -amanitin was added to the transcription mix only, no inhibition of repair was found (Fig. 7A), indicating that repair synthesis occurs only at sites which were under repair at the time of lysis. SLO-permeabilized XP-A cells did not reveal significant stimulation of repair synthesis, whereas in SLO-permeabilized CS-A cells the UVst was comparable with that in normal human cells.

In control experiments we investigated repair synthesis in chromatin prepared from UV-irradiated XP variant cells that were actually NER proficient but had a defect in post-replication repair. The results showed that the level of repair synthesis in chromatin prepared either by SLO or Triton treatment of XP variant cells was similar to normal cells.

DISCUSSION

In this study we approached the role of nuclear architecture in NER by gently dismantling the cell and probing the capability of

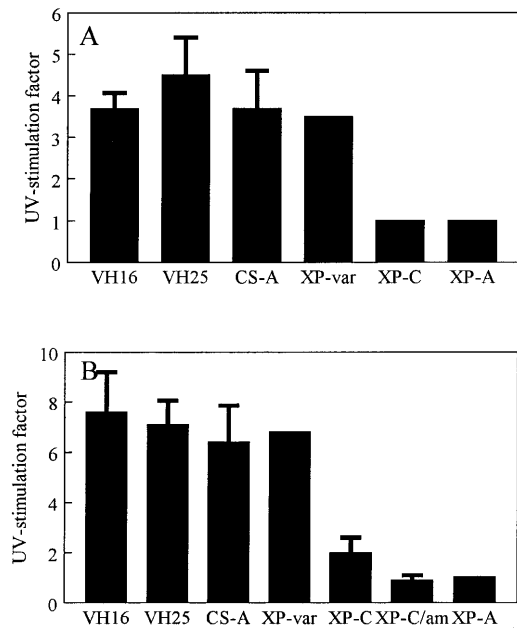


Figure 6. Repair replication in normal and UV-sensitive human fibroblasts. Confluent cells were encapsulated in agarose microbeads, UV irradiated (15 J/m^2) or mock treated and kept for 30 min in buffer prior to Triton-extraction (A) or SLO permeabilization (B). Incorporation of labelled triphosphates (after 60 min incubation) was determined in UV-irradiated cells and in mock-treated cells. The relative level of repair synthesis was expressed as the UV stimulation factor (+UV/-UV). The error bars represent standard errors of the mean (five independent experiments). UVst values without error bars represent the mean of two independent experiments.

chromatin to carry out repair *in vitro*. The rationale behind this approach is that compartmentalization of NER at the nuclear skeleton would, by analogy with replication and transcription, make the enzymatic activities refractory to extraction by buffers that solubilize cellular membranes. In order to obtain chromatin with preserved cellular functions, we adapted the cell-free system developed by Jackson *et al.* (6), which has previously been used to study replication and transcription in HeLa cells (19). Chromatin from these cells isolated with Triton in a physiological buffer exhibited efficient replication and transcription approaching, under optimal conditions, the rate *in vivo*; however, UV-induced nucleotide excision repair could not be measured (25).

Primary human cells resemble HeLa cells with regard to replication and transcription capacities of chromatin. After Triton extraction 80% of the cellular proteins diffuse out of the beads but chromatin prepared from exponentially growing fibroblasts still synthesizes DNA and RNA in the presence of magnesium, triphosphates and ATP. To allow a direct comparison between replication and repair synthesis, replication assays were performed under suboptimal conditions, i.e. in the presence of micromolar concentrations of labelled deoxynucleotide triphosphate. Obviously, at this low triphosphate concentration replicative synthesis in Triton-lysed cells is much less efficient than in more intact cells, as demonstrated by the 5-fold difference in incorporation between the SLO- and Triton-permeabilized cells. This difference in replication efficiency has also been observed for HeLa cells when replication was compared in cells either lysed by Triton or permeabilized by complement and assayed

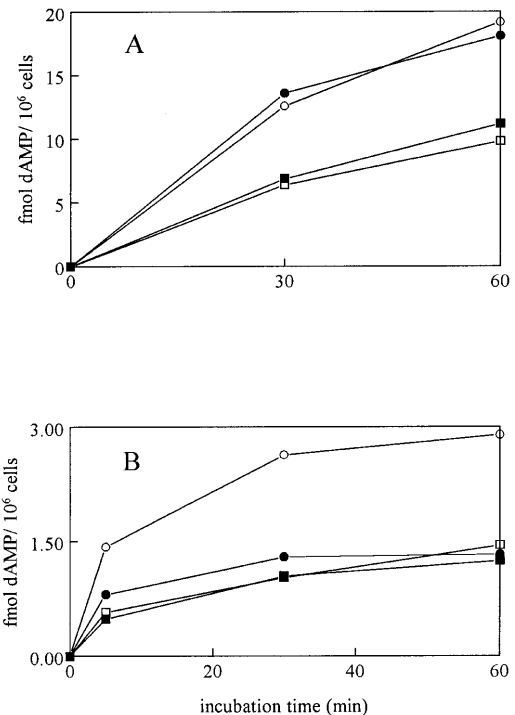


Figure 7. Transcription-coupled repair in SLO-permeabilized XP21RO fibroblasts. Confluent XP21RO cells were pretreated (B) or not with α -amanitin (A), encapsulated in agarose microbeads, UV irradiated (15 J/m^2) or mock irradiated and kept for 30 min in buffer. Cells were extracted with SLO-containing PB and assayed for repair activity. (A) *In vitro* incubations were performed in the presence or absence of α -amanitin. □ -UV; ○ +UV; ■ -UV + α -amanitin; ● +UV + α -amanitin. (B) Cells were exposed to α -amanitin for 16 h prior to encapsulation and UV irradiation. □ -UV; ○ +UV; ■ -UV + α -amanitin; ● +UV + α -amanitin. The results shown in these graphs are representative of series of experiments.

under conditions of suboptimal deoxyribonucleotide triphosphate concentration (19).

When exponentially growing cells were UV irradiated prior to Triton lysis, replicative synthesis in chromatin was almost completely inhibited, reflecting the situation in intact cells. However, the remaining replication activity was still too high to reveal any repair synthesis. In contrast, UV irradiation of confluent fibroblasts clearly stimulated incorporation of deoxynucleotide triphosphates. Similarly to replicative synthesis, this UV-stimulated incorporation depended on magnesium and triphosphates, including ATP. Like replicative synthesis, UV-stimulated incorporation was reduced in Triton-isolated chromatin when compared with SLO-permeabilized cells, but clearly detectable. This is in contrast to HeLa cells, in which UV-stimulated DNA synthesis could be detected in SLO-permeabilized cells, but not in Triton-isolated chromatin (25). By several criteria the UV-stimulated incorporation in primary fibroblasts was shown to be DNA repair synthesis: firstly, XP-A cells, which are completely deficient in NER, do not show this stimulation; secondly, repair label is recovered in parental DNA in CsCl density gradients in contrast to label incorporated by replicative synthesis. A substantial fraction of the repair synthesis ($\sim 15\%$ of the repair synthesis in SLO-lysed cells) is associated with chromatin even under stringent lysis conditions. Repair synthesis

in Triton-isolated chromatin cannot be stimulated by incubation with the CPD-specific enzyme T4 endonuclease V, in contrast to several permeable cell systems in which a clear stimulation was found (27). This suggests that repair components retained in chromatin are tightly associated with sites of repair halted at lysis and are incapable of starting DNA repair synthesis by diffusion to sites incised *in vitro*. Interestingly, other studies have shown that proteins involved in the resynthesis step of NER are tightly bound to chromatin. PCNA is the auxiliary protein of polymerases δ and ϵ and required for DNA replication and NER (20,29–31). UV irradiation of quiescent human fibroblasts triggers the appearance of PCNA in chromatin, visualized by immunofluorescent staining (21,30–32) and part of the total PCNA becomes resistant to extraction by Triton (22) or high salt (21). Consistent with these observations we found, by Western blotting analysis, that a major fraction of PCNA associates with Triton-isolated chromatin in UV-irradiated normal fibroblasts, whereas PCNA is virtually absent in chromatin prepared from unirradiated cells. In contrast to this, in HeLa cells UV-induced binding of PCNA to chromatin could not be demonstrated (unpublished results), which might underlie the absence of Triton-resistant repair synthesis in these cells (25). Thus, the possibility emerges that Triton-resistant repair synthesis is mediated by a fraction of total PCNA that becomes tightly associated with the nucleus after UV irradiation. In this respect the requisite to leave the irradiated cells intact for a period of time before permeabilization in order to detect *in vitro* repair synthesis might be related to damage-dependent relocation of PCNA to the nucleus. Since different types of PCNA complexes might exist (31), a specific form of PCNA may associate with a subfraction of chromatin or with nuclear structures, possibly the nuclear matrix (33).

By employing repair replication as a measure of repair we were unable to detect initiation of repair in Triton-lysed chromatin. The most plausible explanation is that factors involved in the initiation of NER are free in the nucleoplasm and extracted by Triton. However, even in cells mildly permeabilized by SLO, initiation of repair was virtually absent, suggesting that the damage recognition and incision steps of NER are very sensitive to small perturbations of the cellular membrane. In contrast to Triton-lysed XP-C cells, SLO-permeabilized XP-C cells showed a 2-fold increase in incorporation following UV irradiation. However, it is evident that this repair depends also on halted repair events *in vivo*, as inhibition of *in vitro* transcription by α -amanitin did not affect repair synthesis. If Triton affects the efficiency of transcription-coupled repair to a similar extent as global genome repair (i.e. 80% loss of activity after lysis with 0.5% Triton), repair synthesis mediated by the transcription-coupled repair pathway would be below the detection level.

Taken together, our results suggest that a substantial fraction of the enzymatic activities involved in the resynthesis step of NER is tightly attached to chromatin. However, presynthesis complexes have to be formed in intact cells to allow detection of these activities *in vitro*. So far, repair of UV-induced lesions in chromatin under cell-free conditions has only been achieved with SV40 chromosomes (34), but in this system repair was only efficient in the presence of naked plasmid DNA. Combining nuclear extracts with the chromatin template described in this study provides the opportunity at the biochemical and immunochemical level to characterize the role of chromatin structure in NER and to isolate proteins essential for processing DNA damage in chromatin.

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