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Repair of DNA Interstrand Crosslinks May Take Place at the Nuclear Matrix

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Abstract Host cell reactivation assay using Trioxsalen-crosslinked plasmid pEGFP-N1 showed that human cells were able to repair Trioxsalen interstrand crosslinks (ICL). To study the mechanism of this repair pathway, cells were transfected with the plasmids pEGFP-1, which did not contain the promoter of the *egfp* gene, and with pEGFP-G$^-$, which did not contain the *egfp* gene. Neither of these plasmids alone was able to express the green fluorescent protein. After cotransfection with the two plasmids, 1%–2% of the cells developed fluorescent signal, which showed that recombination events had taken place in these cells to create DNA constructs containing the promoter and the gene properly aligned. When one or both of the plasmids were crosslinked with Trioxsalen, the recombination rate increased several fold. To identify the nuclear compartment where recombination takes place, cells were transfected with crosslinked pEGFP-N1 and the amount of plasmid DNA in the different nuclear fractions was determined. The results showed that Trioxsalen crosslinking increased the percentage of matrix attached plasmid DNA in a dose-dependant way. Immunoblotting experiments showed that after transfection with Trioxsalen crosslinked plasmids the homologous recombination protein Rad51 also associated with the nuclear matrix fraction. These studies provide a model system for investigating the precise molecular mechanisms that appear to couple repair of DNA ICL with nuclear matrix attachment. J. Cell. Biochem. 96: 126–136, 2005.

Key words: DNA repair; interstrand crosslinks; DNA recombination; nuclear matrix; host cell reactivation

To repair lesions affecting only one DNA strand, DNA repair mechanisms such as nucleotide excision repair (NER) utilize the information in the complementary undamaged DNA strand. On the other hand, DNA lesions that affect both DNA strands, such as double strand breaks (DSB) and interstrand crosslinks (ICL) could be repaired accurately only if information from another homologous chromosome is used. Since some anticancer agents inflict DNA ICL and since the resistance to the therapy depends on the ability of the cancer cells to repair these lesions, a considerable effort has been devoted to understand how ICLs are repaired. As a result, it has been established that the repair of this type of lesion in mammalian cells is carried out with the participation of the NER proteins Ercc1 and Xpf and by proteins of the DNA homologous recombination (HR) pathway, but the details of the process are still largely unknown [Bessho et al., 1997; De Silva et al., 2000; Dronkert and Kanaar, 2001; Bessho, 2003; West, 2003; Niedernhofer et al., 2004; Rothfuss and Grompe, 2004].

Indirect immunofluorescent analyses have shown that proteins involved in NER become physically localized to sites of DNA damage and are observed as brightly staining foci in mammalian cells [Volker et al., 2001]. Immunofluorescent and Western blotting analyses reveal that some of these proteins along with the damaged DNA are recruited to the nuclear matrix soon after UV irradiation [Jackson et al.,

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1994; Balajee et al., 1998a,b; Kamiuchi et al., 2002]. These data indicate that the nuclear matrix is the site of assembly of the NER repair complexes. Concerning the HR pathway the situation is less clear. Most of the important HR proteins such as Rad51, Rad52, Rad54, etc. are reorganized into nuclear foci in response to DNA damage [Haaf et al., 1995; Tan et al., 1999; Liu and Maizels, 2000; Takata et al., 2000; Mirzoeva and Petrini, 2001; Essers et al., 2002; Tarsounas et al., 2004]. However, the relation of these foci to the underlying nuclear substructures remains to be defined [van Gent et al., 2001; Rouse and Jackson, 2002; West, 2003]. Fanconi anemia cells exhibit genomic instability and hypersensitivity to DNA crosslinking agents. A number of recent reports showed a link between the Fanconi anemia proteins and BRCA2 suggesting that these proteins and HR are part of the same network of repair pathways [D'Andrea and Grompe, 2003; West, 2003]. Immunoprecipitation data have indicated that Fanconi anemia proteins form complexes attached to the nuclear matrix [Qiao et al., 2001]. Although circumstantial, this finding suggests a role for the nuclear matrix in the process of DNA recombination. Indeed, in order for HR to occur, the homologous DNA molecules have to be brought together and precisely aligned. It could be speculated that a rigid nuclear structure should be involved in the process to serve as a platform and the nuclear matrix is the most likely candidate for this role.

To address the question of the nuclear matrix involvement in the repair of ICLs, we studied the repair of Trioxsalen crosslinked plasmid molecules transfected into K562 cells. We found out that both the recombination rate and the percentage of nuclear matrix attached plasmid DNA depended on the Trioxsalen crosslinking in a dose dependant way. After transfection of HEK293 cells with Trioxsalen crosslinked plasmids, the HR protein Rad51 was also recruited to the nuclear matrix. These results suggested that ICL repair was taking place at the nuclear matrix.

MATERIALS AND METHODS

Cells and Plasmids

Human erythroleukemic K562 cells were cultured in RPMI 1640 medium with 10% fetal calf serum and human NER deficient XP-A cells (XP25RO), were cultured in Ham's F10 medium (without hypoxanthine and thymidine) supplemented with 15% fetal calf serum. Human kidney HEK293 cells, hamster wild type V79-4 cells, and two derivative cell lines [Jones et al., 1990; Liu et al., 1998] irs1 (XRCC2 deficient) and irs3 (Rad51C deficient) were cultured in D-MEM with 10% fetal calf serum. All cells were supplemented with antibiotics and incubated in 5% CO_2 atmosphere.

Plasmids pEGFP-N1 and pEGFP-1 (promoterless) were purchased from Clontech. pEGFP-G^- (geneless) was prepared from pEGFP-N1 by excision of the sequence coding the gene for the GFP (nt 654 to 1402) with Bsp120 I and Not I. All plasmids were propagated in *E. coli* XL1-Blue [Inoue et al., 1990].

Trioxsalen Treatment

Ten micrograms of plasmid DNA was dissolved in 50 μl 10 mM Tris-HCl, 1 mM EDTA, pH 8 (TE) buffer. Ten micrograms of Trioxsalen (4′,5′, 8-trimethylpsoralen) dissolved in dimethylsulfoxide were added and irradiated with long wave UV light at 365 nm for different amounts of time [Gunz et al., 1996]. 1 M NaCl was added to a final concentration of 0.2 M NaCl and plasmid DNA was precipitated with two volumes of ethanol and dissolved in TE. The number of interstrand Trioxsalen crosslinks was determined by denaturation and electrophoresis of the linearized plasmids as described in Thompson and Mosig [1990].

Host Cell Reactivation Assay

Control and Trioxsalen treated plasmids were introduced into cells with the liposome Gene Porter 2 Transfection Reagent (Gene Therapy Systems Inc., San Diego, CA) as recommended by the manufacturer. In the case of HEK293 cells, plasmids were introduced by the calcium-phosphate procedure [Chen and Okayama, 1987]. Fluorescent cells were observed under fluorescent microscope. Six hundred cells were counted for each determination 24 h after transfection and the percentage of fluorescent cells was calculated [Atanassov et al., 2003].

Cell Fractionation

Cells were collected by centrifugation at 1,000*g* for 10 min and washed in 0.14 M NaCl, 0.01 M phosphate buffer, pH 7.0 (PBS). 10^7 cells were suspended in 10 ml of 20 mM HEPES, 5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, pH 7.5 and after 20 min on ice were homogenized by

15 strokes in Dounce homogenizer with Teflon pestle. Nuclei were pelleted down at 1,000*g* for 10 min at 4°C and the supernatant was preserved as cytosolic extract. The nuclear pellet was washed twice with the same buffer and resuspended in 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5 to make 10^7 nuclei/ml. Triton X-100 was added to final concentration 0.1% and the suspension was kept on ice for 10 min. The permeabilized nuclei were pelleted down by centrifugation in an Ependorff centrifuge. The supernatant was collected as the nucleosolic extract and the pellet resuspended in 0.2 ml 0.65 M $(NH_4)_2SO_4$, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. After 20 min on ice, the suspension was overlayered on top of a two-step sucrose gradient (0.6 ml 15% and 0.2 ml 30% sucrose in TE) and centrifuged at 10,000*g* at 4°C for 40 min. The upper layer was collected as the high salt extract and the nuclear matrix fraction with the attached chromosomal DNA and plasmid molecules was recovered from the boundary between the 15 and 30% sucrose of the gradient. This fraction was digested with BamHI and HindIII to render about 60% of chromosomal DNA soluble, which was removed by centrifugation and subsequent washes with TE buffer. DNA from all fractions was isolated by making the samples 0.5 M in NaCl and 0.5% in SDS and the proteins were digested with 300 U/ml of proteinase K at 37°C for 1 h. DNA was precipitated with ethanol and dissolved in TE.

For the immunoblotting assay, HEK293 cells were washed with PBS and collected with a cell scraper. 2×10^7 cells were spun down at 1,000*g* for 5 min, suspended in ice cold permeabilization buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.5% Triton X-100, 0.2 mM PMSF, 0.1 mM DTT) and incubated for 7 min at 4°C. After the incubation, the cells were spun down at 1,000*g* for 5 min. The supernatant was collected as the Triton X-100 soluble fraction and the pellet as the Triton X-100 insoluble fraction. The pellet was washed with 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.2 mM PMSF, 0.1 mM DTT and then resuspended in the same buffer. 40 U/ml DNase I were added and the nuclei were incubated for 30 min at 37°C. The pellet was extracted with 0.1M ammonium sulfate for 5 min on ice. After centrifugation at 1,000*g* for 5 min, the supernatant and pellet were collected as the DNase I soluble and DNase I resistant fractions, respectively. The DNase I resistant fraction was extracted with 0.65 M ammonium sulfate for 5 min on ice. After centrifugation at 1,000*g* for 7 min, the pellet and supernatant were collected as the high salt soluble fraction and the high salt insoluble nuclear matrix fraction.

Immunoblotting Analysis

Electrophoresis of the proteins was carried in 12.5% SDS polyacrylamide gels according to Laemmli [1970]. For Western blot analysis, the proteins were transferred onto 0.45 μM nitrocellulose membranes (Trans-blot transfer medium, Bio-Rad, Hertfordshire, UK) using a wet transfer. Equal loading and protein transfer was monitored by Ponceau S staining of the membrane. After the transfer the membranes were incubated in blocking buffer [5% semi dry milk in TBS-T (0.1% Tween 20, 150 mM NaCl, 25 mM Tris-HCl, pH 7.6)] for minimum 1 h at room temperature or over night at 4°C. The membranes were incubated for 1 h with the anti-Rad51 antibody (mouse, monoclonal, Abcam), washed three times for 10 min with TBS-T, and incubated for 1 h with anti-mouse IgG conjugated with horse radish peroxidase (Amersham Biosciences, Buckinghamshire, UK). Anti-β-actin (mouse, monoclonal, Sigma Aldrich Chemie, Steinheim, Germany) was used as a loading control. The antibody reactions were developed by ECL plus Western Blotting Detection System (Amersham Biosciences) as recommended by the manufacturer. The membranes were visualized by Kodak BioMAX film for chemiluminescence.

Polymerase Chain Reaction

Amplification by the polymerase chain reaction (PCR) was applied to determine the total amount of lesions in the plasmids, the recombination rates between different plasmids, and the amount of plasmids in the different cell fractions. To determine the total number of lesions (monoaducts and ICL) inflicted by the Trioxsalen treatment, a 0.853 kbp fragment (nt 438 to 1291 in pEGFP-N1) was amplified by PCR using as templates equal amounts of control and Trioxsalen treated plasmids. The primers were 5′-CCATTGACGTCAATGGGA and 5′-GGGTGCTCAGGTAGTGGTT and the thermal cycle parameters were: denaturation at 95°C for 60 s; annealing at 59°C for 45 s; extension at 72°C for 60 s; 18 PCR cycles. The ratio between the PCR products obtained from

the Trioxsalen treated samples and the control were used to calculate the average number of lesions/kbp using the Poisson formulae [Roguev and Russev, 2000].

The recombination rates between pEGFP-1 (promoterless) and pEGFP-G$^-$ (geneless) were determined in the following way. Twenty four hours after transfection total chromosomal DNA was isolated and 10 ng aliquots were used as templates to amplify a 0.853 kbp fragment (nt 438 to 1291 in pEGFP-N1) containing a 216 bp sequence from the 3′-end of the promoter region of pEGFP-G$^-$ and 637 bp from the 5′-end of the *egfp* region of pEGFP-1. The thermal cycle conditions were as above, but 28 cycles were run in this case.

To determine the plasmid distribution, all fractions were adjusted to the same volume with TE. Sonicated salmon sperm DNA was added to adjust DNA concentrations to 2 μg/ml. Five microlitres of the samples were used as templates to amplify a 0.494 kbp DNA fragment (nt 797–1291) of the pEGFP plasmid, using the primers: 5′-ACGGCAAGCTGACCCTGAA and 5′-GGGTGCTCAGGTAGTGGTT. The amplification cycles were 18.

All amplified products were run on 1% agarose gels, stained with ethidium bromide, and quantified with Gel Pro Analyzer v.3 software for Windows (Media Cybernetics, Wokingham Berkshire, UK). Alternatively, reactions were stained with SYBR Green and the amount of the generated PCR product was measured with spectrofluorimeter.

RESULTS

Repair of Trioxsalen Crosslinks

We applied the host cell reactivation (HCR) assay to measure the rate at which DNA crosslinks were repaired by different cell lines. In this assay, cells are transfected with plasmid DNA damaged in vitro, and the repair of the lesions is measured by monitoring the restoration of the expression of a reporter gene. We used the plasmid pEGFP-N1, which contains the gene for the enhanced green fluorescent protein (GFP) optimized for effective expression in mammalian cells with excitation maximum at 488 nm and emission maximum at 507 nm. It also contains the SV40 origin of replication and the SV40 early promoter. As a damaging agent, we used the compound Trioxsalen, which is a commonly used chemical agent to crosslink DNA. It represents a bifunctional furocumarin, which intercalates between the bases and upon irradiation with long wave length UV light forms chemical bonds with pyrimidine bases in a two-step reaction. In the first step monoadducts are formed, which in the second step are transformed into crosslinks provided a pyrimidine base is available at the opposite DNA strand [Chatterjee and Cantor, 1978]. Since both monoadducts and crosslinks effectively block the progress of DNA polymerases, we determined the total amount of lesions in the plasmids by PCR. To that end a 0.853 kbp fragment was amplified using as templates equal amounts of Trioxsalen treated and control plasmid DNA and the ratio between the products was used to calculate the total lesion concentration by the Poisson equation. The average number of ICLs was determined for the same plasmids after linearization, denaturation, and electrophoresis. Under these conditions plasmids without crosslinks, regardless of whether they contained monoaducts or not, moved as single strand DNA, while the plasmids containing ICLs migrated as double strand DNA. From the percentage of crosslinked DNA, using again the Poisson distribution, the average concentration of ICL was calculated. From these two sets of data, the number of monoaducts was calculated by subtracting the number of ICLs from the number of total lesions (Fig. 1).

Repair competent human K562 and HEK293 cells and hamster V79-4 cells as well as two HR deficient V79-4 derived cell lines irs1 (XRCC2-deficient) and irs3 (Rad51C-deficient) were transfected with control and Trioxsalen treated pEGFP-N1 containing 0.4 ICL/kbp and 0.6 monoadducts/kbp. At different times after transfection, fluorescent cells were counted under fluorescent microscope and expressed as percentage of the number of fluorescent cells obtained after transfection with undamaged pEGFP-N1. 44% of K562 cells, 42% of HEK293 cells, and 34% of V79-4 cells on the average were capable of repairing the damaged plasmids, while only up to 9% of the irs1 and 17% of the irs3 cells were able to restore the damaged *egfp* gene and to express the fluorescent protein (Fig. 2). The lower levels of the reporter gene restoration observed in the irs1 and irs3 cells point to the involvement of the HR pathway in the repair of the damaged reporter plasmid. These results are consistent with the interpre-

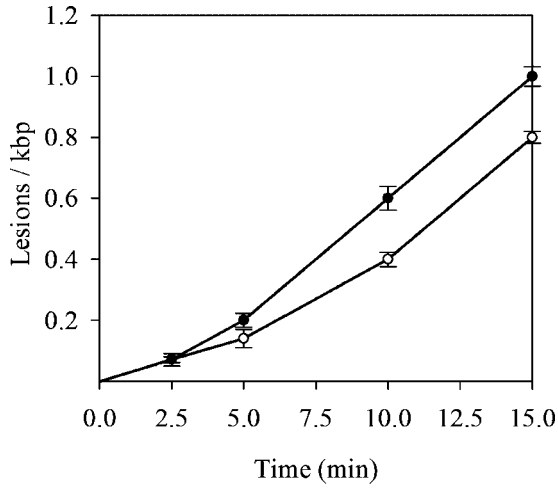


Fig. 1. Kinetics of accumulation of monoadducts and ICLs in plasmid DNA after treatment with Trioxsalen. 10 μg of pEGFP-N1 DNA were treated with 10 μg Trioxsalen and were irradiatated at 365 nm for the indicated time. Aliquots were used to amplify an 853 bp fragment and from the amount of product, the total lesion concentration was determined for each time point as described in Materials and Methods. From the same aliquots, plasmid DNAs were linearized, alkali-denatured, and subjected to agarose gel electrophoresis. Upon that treatment, plasmids with crosslinks migrated as double strand DNA fragments, while plasmids without crosslinks migrated as single strand fragments. From the percentage of the single strand DNA fraction, the number of ICLs for each time point was calculated (○–○). By subtraction the number of ICLs from the total number of lesions, the number of Trioxsalen monoadducts was determined (●–●). The results are means of three experiments and the standard deviations are shown with vertical bars.

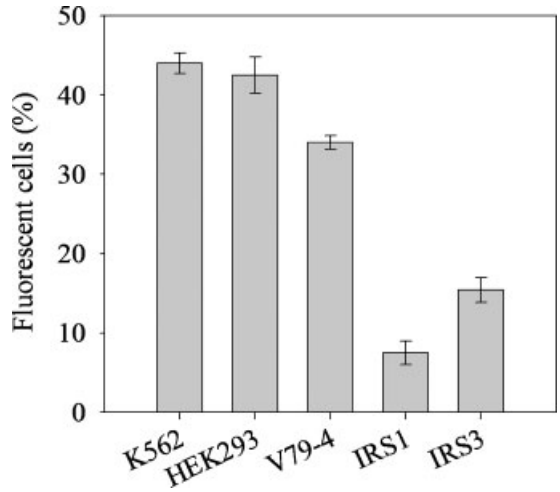
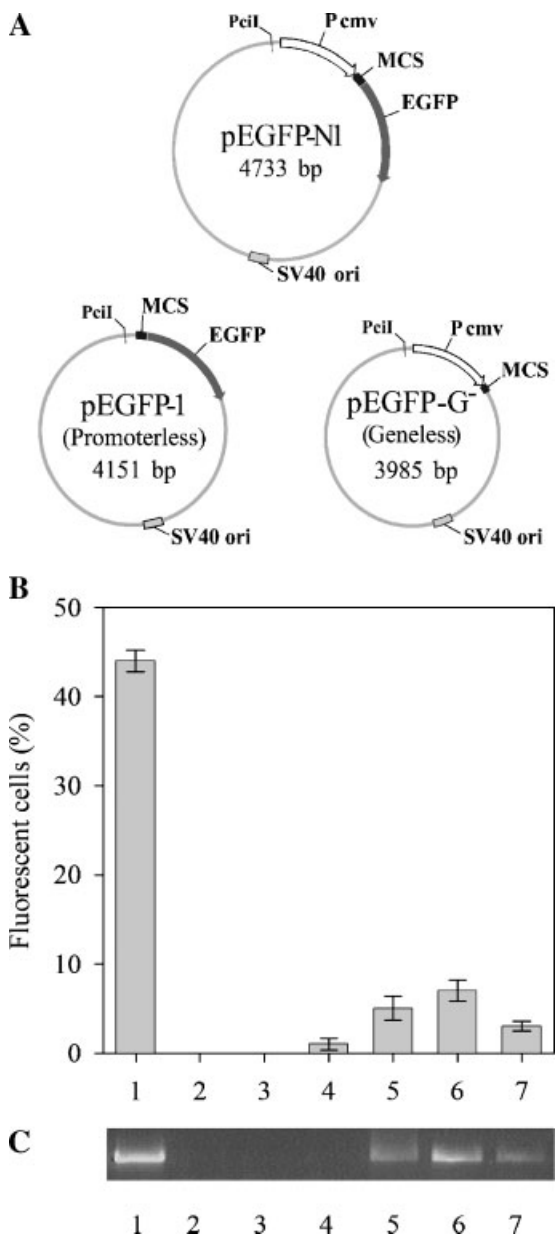


Fig. 2. HCR assay of wild type and HR deficient cells. Wild type human K562 and HEK293 cells and hamster V79-4 cells as well as two HR deficient cell lines derived from V79-4, irs1 (XRCC2 deficiency) and irs3 (Rad51C deficiency) were transfected with treated with Trioxsalen pEGFP-N1 containing 0.4 ICL/kbp and 0.6 monoadducts/kbp. Twenty four hours after transfection, the number of fluorescent cells was counted under fluorescent microscope and was expressed as percentage from the number of fluorescent cells obtained at the same time point after transfection with undamaged plasmid. 600 cells were counted for each point and the results are means of three independent experiments. Deviations from the mean are shown with vertical bars.

tation that the wild type K562, HEK293, and V79-4 cells were able to repair both the monoadducts and the ICLs, while the recombination deficient irs1 and irs3 cells repaired predominantly monoadducts by NER.

To test the suggestion that HR was taking part in the repair of Trioxsalen crosslinks in this model system, we performed the following experiment. K562 cells were transfected with the plasmids pEGFP-1 and pEGFP-G$^-$ lacking the promoter and the *egfp* gene, respectively (Fig. 3A), and the number of fluorescent cells was counted 24 h later (Fig. 3B). As a reference for comparison, K562 cells were transfected with Trioxsalen treated pEGFP-N1. When the cells were transfected with the promoterless or the geneless plasmids, no fluorescent cells were detected 24 h after transfection. On the other hand, when the cells were transfected with a 1:1 mixture of the two plasmids, some 1–2% of the cells lit up, which showed that recombination events restoring the functional integrity of the *egfp* gene had taken place in these cells. When the cells were cotransfected with 1:1 mixture of the two plasmids, one, or the other of which were crosslinked with Trioxsalen, 4%–8% of the cells developed fluorescent signal. It should be noted that in these experiments the combination of Trioxsalen treated pEGFP-1 (promoterless) and native pEGFP-G$^-$ (geneless), gave always somewhat lower numbers of fluorescent cells than the combination of native pEGFP-1 and crosslinked pEGFP-G$^-$. A possible explanation of this result could be the longer sequence of the *egfp* gene than that of the promoter sequence, making it more likely to have fewer lesions in the promoter than in the gene at the same lesion concentration. When both plasmids were crosslinked, the number of fluorescent cells decreased (Fig. 3B). These results showed that recombination between the plasmids was taking place in the cotransfected cells to give constructs with promoter and *egfp* gene properly aligned, and confirmed data that crosslinked plasmids were preferential substrate for this reaction [Hellgren, 1992]. To prove more directly that recombination events between the two plasmids were taking place, 24 h after cotransfection of K562 cells with pEGFP-1 and pEGFP-G$^-$ plasmids, total cellular DNA was

isolated and used as substrate to amplify an 853 bp fragment encompassing the 3′-end of the promoter region and the 5′-end of the *egfp* gene. This fragment was not present in either of the plasmids and could have arisen by HR between the two plasmids taking place within the common 66 bp multiple cloning site (MCS) region (Fig. 3A). Our results showed that varying amounts of this fragment were present in the DNA from the cells cotransfected with damaged plasmids, roughly corresponding to the percentage of fluorescent cells in the HCR assay (Fig. 3C).



Preferential Association of Crosslinked pEGFP to the Nuclear Matrix

Several lines of indirect evidence suggest that DNA recombination events during the repair of ICLs may take place in association with the nuclear matrix. On one hand, all other DNA transactions such as transcription, DNA replication, and NER are carried out into nuclear foci that are associated with the nuclear matrix [Berezney et al., 1995; Cook, 1999; Stein et al., 2003]. On the other hand, many proteins that are involved in HR of DNA are also relocalized into nuclear foci [Haaf et al., 1995; Tan et al., 1999; Liu and Maizels, 2000; Takata et al., 2000; Mirzoeva and Petrini, 2001; Essers et al., 2002; Tarsounas et al., 2004]. Transfection of mammalian cells is a widely used procedure, but little is known about the fate of plasmid DNA molecules after transfection other than that the plasmids are imported to the nucleus, where some of the plasmids are immobilized on the nuclear matrix [Wilson et al., 1999; Mearini et al., 2004]. To shed more light on the distribution of the plasmids in the different subcellular compartments after transfection, the cells were fractionated first into cytoplasmic and nuclear fractions. The nuclear fraction was further fractionated into nucleosolic extract, high salt extract, and nuclear matrix fraction. DNA, which is resistant to extraction with high salt buffer preceding or following digestion of nuclei with nuclease, is defined as DNA specifically bound to the nuclear matrix [Berezney et al., 1995]. In our fractionation scheme, the permeabilized nuclei were first extracted with high salt

Fig. 3. Recombination repair of Trioxsalen crosslinked plasmids containing 0.4 ICL/kbp. **A**: Physical maps of the plasmids used in this assay. **B**: K562 cells were transfected with different plasmids as specified below and 24 h later the number of fluorescent cells was determined with fluorescent microscope and expressed as percentage from the number of fluorescent cells after transfection with the undamaged pEGFP-N1. 1, Trioxsalen treated pEGFP-N1; 2, native pEGFP-1 (promoterless); 3, native pEGFP-G$^-$ (geneless); 4, 1:1 mixture of native pEGFP-1 and native pEGFP-G$^-$; 5, 1:1 mixture of Trioxsalen treated pEGFP-1 and native pEGFP-G$^-$; 6, 1:1 mixture of native pEGFP-1 and Trioxsalen treated pEGFP-G$^-$; 7, 1:1 mixture of pEGFP-1 and pEGFP-G$^-$, both treated with Trioxsalen. Experiments were made in triplicate and 600 cells were counted for each determination. Deviations from the mean are shown with vertical bars. **C**: 24 hours after transfection bulk DNA was isolated from K562 cells transfected as in (B). The isolated DNA was used as template in PCR to amplify an 853 bp DNA fragment that would have arisen from the restored sequence containing both the promoter and the *egfp* gene.

concentration. The remaining residual material contained the plasmids, which were attached to nuclear matrix and the chromosomal DNA attached to the matrix by the matrix attachment regions with the bulk of DNA looping out to form a halo. To achieve digestion of the loop chromosomal DNA without excising fragments from the plasmid DNA, the restriction nucleases BamHI and HindIII were used because they have sites in the polylinker region of pEGFP-N1 and would only linearize the plasmids. Aliquots from all fractions were used as templates in quantitative PCR reactions to amplify a fragment from the pEGFP-N1 plasmid. The results obtained showed that both the native and crosslinked plasmids rapidly entered the nucleus and no plasmid DNA was detected in the cytoplasm 4 h after transfection. In the nucleus, part of the plasmid DNA was found attached to the nuclear matrix and another part was extracted. In the case of untreated pEGFP-N1, the percentage of attached DNA increased with time to reach a maximum of about 40% 12 h after transfection and then began to decrease (Fig. 4A). The kinetics of appearance of the GFP in the cytoplasm reaches its maximum at the 24th hour [Atanassov et al., 2003, 2004]. This means that the expression of the gene followed the appearance of the tightly bound plasmid fraction and suggests that it represented plasmid molecules that were transcribed on the nuclear matrix.

When K562 cells were transfected with pEGFP-N1 treated with Trioxsalen to induce 0.4 crosslinks/kbp, the percentage of matrix attached plasmid DNA reached 60% at the 12th hour and then began to decrease. The increase of the ICL concentration to 0.8 crosslinks/kbp led to an increase in the matrix-attached plasmid DNA up to 80%, which showed that the attachment to the nuclear matrix was

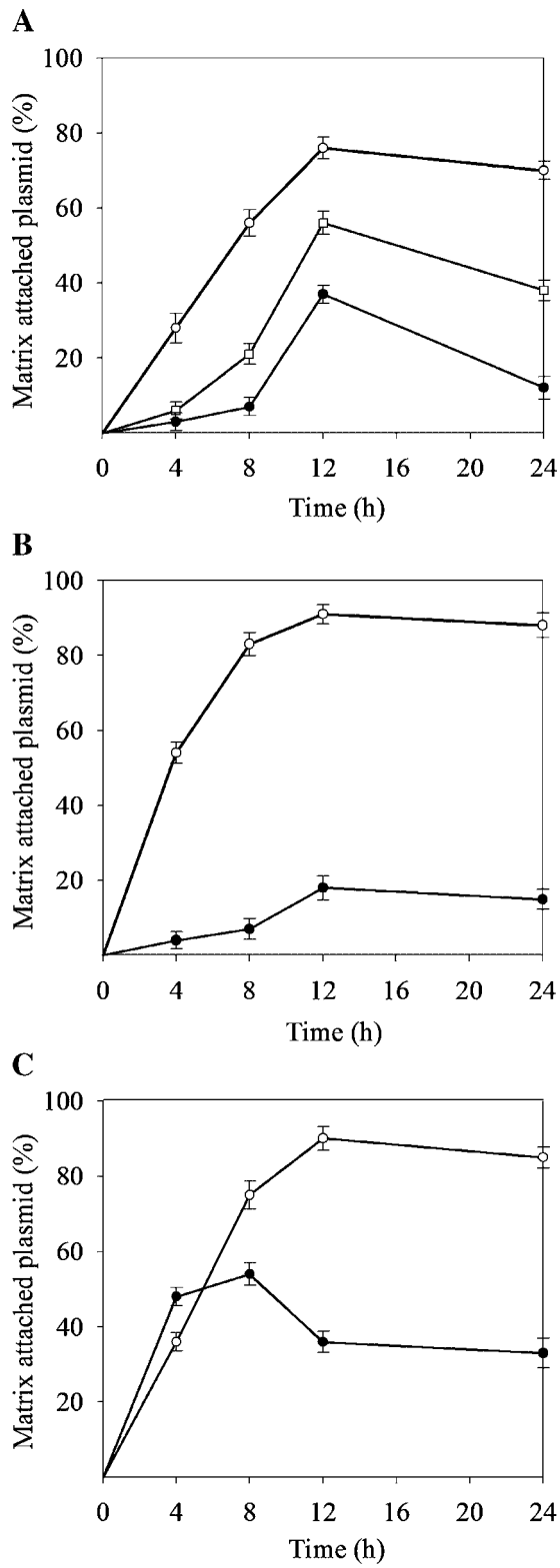


Fig. 4. Attachment of plasmids to the nuclear matrix. Cells were transfected with native or Trioxsalen treated plasmids as specified: (**A**) K562 cells were transfected with native pEGFP-N1 (●–●) and with pEGFP-N1 treated with Trioxsalen to give 0.4 ICL/kbp (□–□) and 0.8 ICL/kbp (○–○). **B**: K562 cells were transfected with either native (●–●), or Trioxsalen treated to give 0.8 ICL/kbp (○–○) pEGFP-1 (promoterless). **C**: K562 cells were transfected with pEGFP-N1 treated with UV light at 260 nm to give 1.6 UV lesions/kbp (●–●) and XP-A cells were transfected with Trioxsalen treated pEGFP-N1 containing 0.8 ICL/kbp (○–○). At different time intervals, aliquots were withdrawn and fractionated into cytosolic, nucleosolic, high salt, and nuclear matrix fractions. The plasmid distribution among the fractions was determined by quantitative PCR and the amounts of matrix attached plasmid were expressed as percentage of the total amount of plasmid in all fractions. Results are means of three independent experiments and the standard deviations are shown with vertical bars.

dynamic and dose dependant (Fig. 4A). To assess the role of transcription in the observed attachment of ICL containing DNA, native and Trioxsalen crosslinked promoterless pEGFP-1 were introduced into K562 cells and their attachment to the matrix followed. About 18% of the native promoterless plasmids were found attached to the nuclear matrix at the 12th hour, while regardless of the fact that no transcription of the *egfp* gene was taking place in this plasmid, about 90% of the crosslinked plasmids were attached to the nuclear matrix at the same time point and remained attached during the next 12 hours (Fig. 4B).

To rule out the possibility that the observed association of the crosslinked plasmids to the nuclear matrix was result of NER repairing the Trioxsalen monoadducts, two experiments were carried out. First, repair competent K562 cells were transfected with pEGFP-N1 irradiated with UV light at 260 nm to introduce 1.6 lesions/kbp (cyclobutane pyrimidine dimers and 6-4-pyrimidine-pyrimidone photoproducts, both of which are repaired by NER). This treatment brought about maximal association of pEGFP-N1 with the nuclear matrix at the 8th hour (Fig. 4C, closed circles), which is consistent with the notion that UV lesions are repaired in the first 8 h after the damage [van Hoffen et al., 1999]. However, the percentage of the attached plasmid as well as the attachment kinetics were quite different from those of Trioxsalen crosslinked pEGFP-N1 and showed that the association of the latter with the nuclear matrix was not a result of matrix associated NER. This conclusion was confirmed by the second experiment in which NER deficient XP-A cells were transfected with pEGFP-N1, crosslinked with Trioxsalen. Although no repair by NER of the Trioxsalen monoadducts was taking place, 90% of the plasmid molecules were found permanently attached to the nuclear matrix in this case (Fig. 4C, open circles).

Preferential Association of Rad51 to the Nuclear Matrix After Transfection With Trioxsalen Crosslinked Plasmids

To confirm that after transfection of the cells with Trioxsalen-treated plasmids, repair of the ICLs and the associated HR events occur on the nuclear matrix, we followed the distribution of Rad51 in different subcellular fractions by Western blotting. The mammalian Rad51 protein is a functional homolog of the bacterial RecA protein. Rad51 is a key HR protein promoting the exchange between homologous DNA strands. It is functional as a long helical polymer that contains hundreds of monomers, wrapping around DNA to form nucleoprotein filaments [West, 2003]. In untreated cells, Rad51 is predominantly localized diffusely in the nucleus, while after treatment with agents that introduce ICLs or DSBs, it relocalizes into nuclear foci [Haaf et al., 1995; Tan et al., 1999; Liu and Maizels, 2000; Essers et al., 2002; Tarsounas et al., 2004]. For this reason, it was necessary to achieve higher transfection efficiency and that is why the cell line HEK293 was transfected by the calcium-phosphate method to obtain over 70% transfected cells. At the 12th hour after transfection with native and Trioxsalen treated pEGFP-N1 (0.8 ICL/kbp), the cells were subjected to biochemical fractionation by following a routine procedure for isolation of the nuclear matrix. The cells were permeabilized by treatment with Triton X-100, which leads to a complete loss of the soluble nuclear components. DNA was digested with DNase I and the digested chromatin was extracted. The DNase I resistant nuclear fraction was extracted with high salt concentration buffer to obtain the high salt insoluble fraction representing the nuclear matrix. The proteins of all fractions were subjected to electrophoresis, transferred onto membranes, and incubated with anti-Rad51 antibodies (Fig. 5). The results show that introduction of ICLs resulted in a decrease of Rad51 in the Triton X-100 soluble fraction and its increase in the Triton X-100 insoluble fraction. After digestion and extraction of 90% of DNA, the majority of Rad51 was found in the pellet. Further extraction with high salt concentration did not release detectable amount of Rad51 in soluble form and it remained tightly associated with the nuclear matrix. These experiments suggest that the recruitment of the ICL damaged DNA to the nuclear matrix is accompanied by immobilization of the majority of the Rad51 protein on the nuclear matrix.

DISCUSSION

The HCR assay using plasmids containing well defined single ICL have been applied to show that HR independent mechanisms for removal of ICLs exist [Wang et al., 2001; Zheng et al., 2003]. However, these experiments were poorly suited to study the HR pathway since all

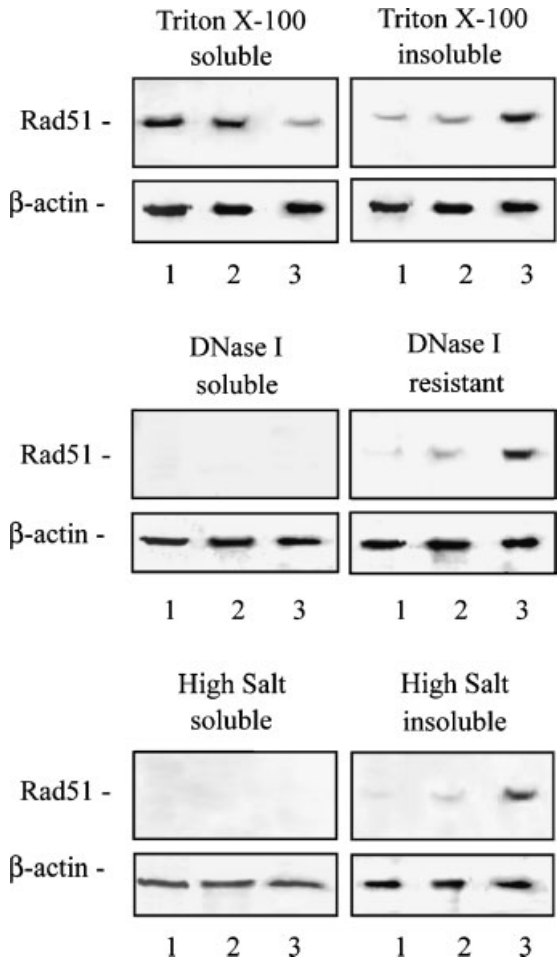


Fig. 5. Subcellular fractionation of Rad51 in HEK293 cells after transfection with pEGFP-N1 treated with Trioxsalen. The cells were subjected to biochemical fractionation into Triton X-100 soluble and insoluble fractions, DNase I soluble and DNase I resistant fractions, and high salt soluble and high salt insoluble fractions. Equal amounts of the proteins of each fraction were fractionated by SDS polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and incubated with anti-Rad51 antibodies. Anti-β-actin was used as a loading control. (1) Control not transfected cells; (2) 12 h after transfection with native pEGFP-N1; (3) 12 h after transfection with pEGFP-N1 treated with Trioxsalen to give 0.8 ICL/kbp.

plasmids carried the same ICL at the same place and undamaged homologous DNA was not available. We decided instead to carry out HCR assay using plasmids containing randomly placed ICLs as substrates. This was achieved by treating the plasmids with Trioxsalen and long wave length UV light in a controlled way to introduce well defined average amount of ICLs. Unavoidably, certain amount of monoadducts was also introduced in the process, but we believed that with proper controls it would be possible to discriminate between their repair by NER and the repair of ICLs. Our results suggest that (a) Trioxsalen ICLs introduced randomly into plasmid molecules could be repaired by HR and (b) in order for HR to take place the ICL containing plasmids should be recruited to the nuclear matrix.

Three points should be discussed in connection with the introduction of the HCR assay to study HR in higher eukaryotic cells and they are the role of transcription, the role of NER and the role of DNA replication in the ICL repair. About 40% of the native pEGFP-N1, which is actively transcribed, were found attached to the nuclear matrix and with decreasing the transcription rates of the plasmid this percentage also decreased [Fig. 4A, Atanassov et al., 2003]. It is known that transcription is taking place at the nuclear matrix and it could be argued that the observed attachment of the crosslinked plasmids to the matrix could also be a result of ongoing transcription. Against this possibility speaks the fact that the promoterless pEGFP-1, which is not transcribed is nevertheless massively attached to the nuclear matrix when crosslinked with Trioxsalen, although no such attachment was observed with the native plasmid (Fig. 4B). This conclusion is also supported by the results showing that an increase of the ICL concentration from 0.4 to 0.8 ICL/kbp brings about a twofold increase in the percentage of the attached crosslinked plasmid to the nuclear matrix (Fig. 4A). Thus, although the existence of transcription coupled recombination could not be ruled out [Islas et al., 1994; Gonzalez-Barrera et al., 2002; Garcia-Rubio et al., 2003], our data clearly showed that the observed attachment of the ICL containing plasmids to the nuclear matrix was primarily due to the presence of ICLs and not to transcription.

The second point is the role of NER in the observed matrix attachment. It is known that the repair of many lesions, including bulky monoaducts, takes place at the nuclear matrix [Jackson et al., 1994; Balajee et al., 1998a,b; Kamiuchi et al., 2002]. Since the crosslinked plasmids contain considerable amount of monoadducts, a question could arise as to whether the observed association of these plasmids with the nuclear matrix was a result of the ICLs, or rather of the repair of Trioxsalen monoadducts by NER. The fact that UV-treated plasmids were attached to the nuclear matrix in the NER

competent K562 cells by different kinetics and to different extent from the Trioxsalen crosslinked plasmids as well as the fact that crosslinked plasmids were retained to the nuclear matrix in the NER deficient but recombination proficient XP-A cells [Dronkert and Kanaar, 2001] (Fig. 4C), speaks against the latter possibility. Thus, a conclusion was drawn that the observed attachment of the crosslinked plasmids to the nuclear matrix was induced by the presence of ICLs and not by NER activity.

The third point is the potential involvement of the replication machinery in matrix attachment and recombination. ICL-induced DSB formation requires cell cycle progression into S phase, suggesting that DSBs are an intermediate of ICL repair that form during DNA replication [Akkari et al., 2000; Bessho, 2003; Niedernhofer et al., 2004; Rothfuss and Grompe, 2004]. Although the vectors used in our study are not specifically designed for episomal maintenance, they contain the SV40 replication origin and the early SV40 promoter and it was recently shown that these elements are sufficient for replication of such plasmids when attached to the nuclear matrix, even in the absence of the large T antigen [Baiker et al., 2000; Shimizu et al., 2001, 2003]. It could be suggested that after recruitment to the nuclear matrix, DNA replication is initiated on the plasmids. When replication forks reach ICLs they stall and the stalled replication forks are converted to DSBs that trigger the involvement of the HR repair pathway. This suggestion is supported by the results showing that upon transfection with the ICL containing plasmids, the HR protein Rad51 was found specifically attached to the nuclear matrix.

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