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Nuclear matrix and nucleotide excision repair: damage-recognition proteins are not constitutive components of the nuclear matrix

Research Article

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Summary

The nuclear matrix is a structure involved in organization of DNA structure and regulation of replication and transcription. It is generally believed that some enzymatic activities of nucleotide excision repair are localized in this nuclear subfraction and that the nuclear matrix anchorage affects the preferential repair of (potentially) active genes. Thus answering the question what is the role of nuclear matrix is very important to fully understand the DNA repair mechanisms. We have studied the *in vitro* interactions between nuclear matrices from rat liver cells and damaged DNA. A specific 36-bp DNA sequence was either UV-irradiated or damaged by benzo(a)pyrene diol epoxide and N-acetoxy-acetylaminofluorene. The data presented in this communication show that damaged DNA did not preferentially bind to nuclear matrices; damage-recognition proteins were loosely attached to the nucleoskeleton and were easily extracted from chromatin.

I. Introduction

The genetic material of eukaryotic cells is organized into structural and functional domains. In the interphase chromatin such domains are frequently termed "DNA loops". This loop organization seems to be maintained by anchorage of specific DNA sequences (MAR/SAR) to a protein network of the nucleoskeleton. The skeletal structures can be purified after removal of majority of DNA and chromatin proteins (nuclei are treated with nucleases and extracted with high salt buffers). Such a residual structure is called the "nuclear matrix". The nucleoskeleton/nuclear matrix is thought to be involved not only in nuclear morphology but also in regulation of DNA replication and gene expression (reviewed in Bodnar 1988; Garrard 1990; Jackson et al, 1992; Boulikas 1995; Iborra et al, 1998; Davie et al, 1998).

Genomes of all organisms are under permanent pressure of genotoxic agents that can introduce damage into DNA structure. Many laboratories showed that genotoxic carcinogens preferentially damage DNA attached to skeletal structures of the nucleus. The levels of DNA lesion induced by simple alkylating agents (Ryan et al, 1986), polycyclic aromatic hydrocarbons (e.g. benzo(a)pyrene) (Ueyama et al, 1981; Mironov et al, 1983; Obi et al, 1986; Perin-Roussel et al, 1988; Widlak & Rzeszowska-Wolny, 1993), aromatic amines (e.g. 2-acetylaminofluorene) (Gupta et al, 1985; Widlak & Rzeszowska-Wolny, 1994), UV-radiation (Widlak et al, 1996) and metal ions (Xu et al, 1994) were found to be higher in the matrix-attached DNA as compared to non-matrix chromatin fractions. It is suggested that preferential damage of the matrix-attached DNA reflects a specific active state of this chromatin fraction (Boulikas, 1992; MacLeod, 1995).

It has been postulated that carcinogen-induced damage of the nucleoskeleton might be an important causative factor of functional and genetic instability of cancer cells (Pienta et al, 1989; Pienta & Ward, 1994). Thus, efficient repair of DNA fraction attached to this nuclear structure may be a very important task for cellular defense mechanisms. In fact, some DNA lesions can be removed faster from the matrix-bound DNA fractions than from non-matrix DNA.

Such faster repair of the matrix-attached DNA fraction was shown for lesion induced by benzo(a)pyrene (Widlak & Rzeszowska-Wolny, 1993), chromium (Xu et al, 1994) and ionizing radiation (oxidative base damages like thymine glycol or 8-hydroxyguanine) (Zastawny et al, 1997). In contrary, damages induced by dimethylnitrosamine (Ryan et al, 1986) and 2-acetylaminofluorene (Gupta et al, 1985; Poirier et al, 1990) were not preferentially removed from the matrix-attached DNA.

The nucleotide excision repair (NER) is an important and universal pathway which removes a broad spectrum of DNA damage. NER can recognize and repair not only bulky lesion induced by UV-irradiation, polycyclic hydrocarbons or aromatic amines but also oxidative base damages. The process starts from recognition of a lesion followed by incision of DNA chain on both sides of the damage. Then, removal of the lesion-containing oligonucleotide takes place along with DNA repair synthesis and ligation to fill and close the resulting gap. In mammalian cells NER is tremendously complex and involves about 30 polypeptides. The first step of repair complex formation seems to be binding of a heterodimer XPA/RPA to damaged fragment, then other protein complexes can bind to target DNA. Binding of transcription factor TFIIH leads to unwinding of DNA helix around the damage and enables the action of endonucleases XPG and XPF/ERCC1 to incise the damaged strand on both sides of the damage. Some components of NER pathways are specific for global genome repair (XPC protein) or transcription-coupled repair (CSA and CSB proteins) (rev. in: Sancar, 1996; Friedberg, 1996; Lindahl et al,1997; DNA Wood, 1997). The XPA protein recognizes a broad spectrum of DNA damage and seems to be a general recognition factor, however other damage-recognition proteins are probably also involved in NER. Among them is XPE factor (UV-DDB protein), which shows the highest affinity to UV-induced 6-4 photoproducts (Naegeli, 1995). Different levels of NER efficiency can be distinguished: (i) slow repair of inactive genes, (ii) fast repair of transcriptionaly active (or potentially active) genes and (iii) accelerated repair of the transcribed strands, which involves transcription-coupled repair (TCR). Preferential repair of (potentially) active genes results not only from TCR involvement but is also determined by other factors (e.g. chromatin structure) (reviewed in Boulikas, 1992). Efficient removal of DNA damage from the matrix-bound fraction might simply reflect the association of preferentially-repaired transcriptionally active genes with the nuclear matrix. On the other hand, preferential repair of (potentially) active genes could result from their close proximity to the nuclear matrix where the repair machinery is localized (rev. in: Mullenders et al, 1990, Boulikas, 1995).

The data which suggested that nucleotide excision repair might be localized in specific nuclear compartment originally concerned DNA repair synthesis. It was shown that UV-induced unscheduled DNA synthesis occurred in association with the nucleoskeleton (McCready & Cook 1984; Harless & Hewitt 1987). Mullenders et al (1984, 1988, 1990) found that DNA repair patches were enriched in the nuclear matrix of human cells irradiated with low doses of UV. This effect was enhanced in cells deficient in global genome repair (xeroderma pigmentosum group C cells), but in cells deficient in transcription-coupled repair (Cockayne's syndrome cells) the effect was lost. In contrary, we have found that unlike replicative DNA synthesis, the nuclear matrix from liver cells of rats injected with 2-aminofluorene was not enriched in newly synthesized DNA (Widlak & Rzeszowska-Wolny, 1994). This observation probably reflects the fact that 2-aminoflurene-induced damage is not preferentially removed from the matrix-attached DNA and (unlike UV-induced damage) may not be preferentially removed from transcriptionally active genes in rodent cells (Tang et al, 1989). On the other hand, if the matrix localization of DNA synthesis is a general feature of NER, some differences in the experimental model used may also cause this discrepancy. As the repair patches are short (~30 nucleotides) and the rate of synthesis is rapid there is enough time during experiment for damage to be repaired at a skeleton and then for the repaired DNA to detach from it. Secondly, repaired DNA which was originally attached to the nucleoskeleton (probably through polymerases) might be subsequently detached from it during the matrix purification procedure. In fact, there is evidence showing that even in UV-irradiated cells newly synthesized repair patches of DNA can be easily released from the nucleoskeleton (Jackson et al, 1994).

The data showing that DNA repair synthesis process is localized in the nucleoskeleton suggests the model in which damage in genomic DNA is recognized and brought into association with the nuclear matrix, where NER "machinery" is localized. Such a model has been confirmed by different lines of evidence. Park et al, (1996) showed that repair endonuclease XPG was firmly (but reversibly) associated with the nuclear matrix. Koehler & Hanawalt (1996) detected transient binding of damaged DNA to the nuclear matrix in UV-irradiated cells. Some activities involved in NER (unlike resynthesis step) are very loosely attached to chromatin (Bouayadi et al, 1997). This may suggest that the process is initiated by soluble proteins which scan the genome then mediate binding of damaged DNA to the matrix-localized repair machinery. The damaged-DNA binding (DDB) proteins are naturally best candidates for such a role. In fact, UV-irradiation of mammalian cells induced translocation of UV-DDB, XPA and RP-A proteins (which all are involved in damage recognition) from a chromatin fraction loosely associated with the nuclear matrix to the tightly associated fraction (Otrin et al, 1997).

Previously we had shown that UV-damaged MAR sequences bound preferentially to the nuclear matrices from rats which were not treated with any DNA damaging

factors (Widlak et al, 1996). It might suggest that some damage-recognition proteins are constitutive components of the nuclear matrix. It has been postulated that proximity to the nuclear matrix is one of the factors involved in fast repair of (potentially) active genes (Boulikas, 1996). Thus, the nuclear matrix localization of damage-recognition proteins could partially explain the mechanisms of preferential repair of this fraction of a genome. The aim of this work was to elucidate whether damage-recognition proteins are residual components of the nuclear matrix.

II. Results and Discussion

We studied the *in vitro* interactions between the nuclear matrices from rat liver cells and damaged DNA. A 36-bp duplex oligonucleotide was UV-irradiated or adducted by benzo(a)pyrene diol epoxide (BPDE) and N-acetoxy-acetylaminofluorene (AAAF). DNA lesions induced by the first two factors, but not the last one, are known to be removed with high efficiency from the matrix-bound DNA and they may differ as a substrate for strand- and gene-specific preferential repair.

To evaluate the effects of DNA lesions upon protein-DNA interactions, the *in vitro* binding of DNA to nuclear matrices was studied according to Cockerill & Garrard (1986) method. The nuclear matrices contained about 15% of total nuclear proteins, mainly lamins and other high molecular weight proteins but not histones. The fraction of matrix-bound DNA was separated from unbound DNA after centrifugation of matrices. The data from experiment in which matrix-DNA complexes were formed in the presence of non-damaged non-specific competitor (*E. coli* DNA) are shown in **Figure 1A**. We have found that none of the tested lesions affected the affinity of probed DNA to nuclear matrices. The binding efficiency of DNA carrying a lesion induced by AAAF, BPDE or UV was similar to that of undamaged DNA control. To confirm data obtained with a non-specific competitor, we studied formation of complexes in the presence of excess undamaged/damaged homologous competitor. DNA damaged by AAAF or BPDE was used as a radioactive probe while the same oligonucleotide or the undamaged oligonucleotide were used as non-radioactive competitors. The data which are shown in **Figure 1B** and **1C** confirmed that both damaged and undamaged DNA have the same affinities for the nuclear matrices.

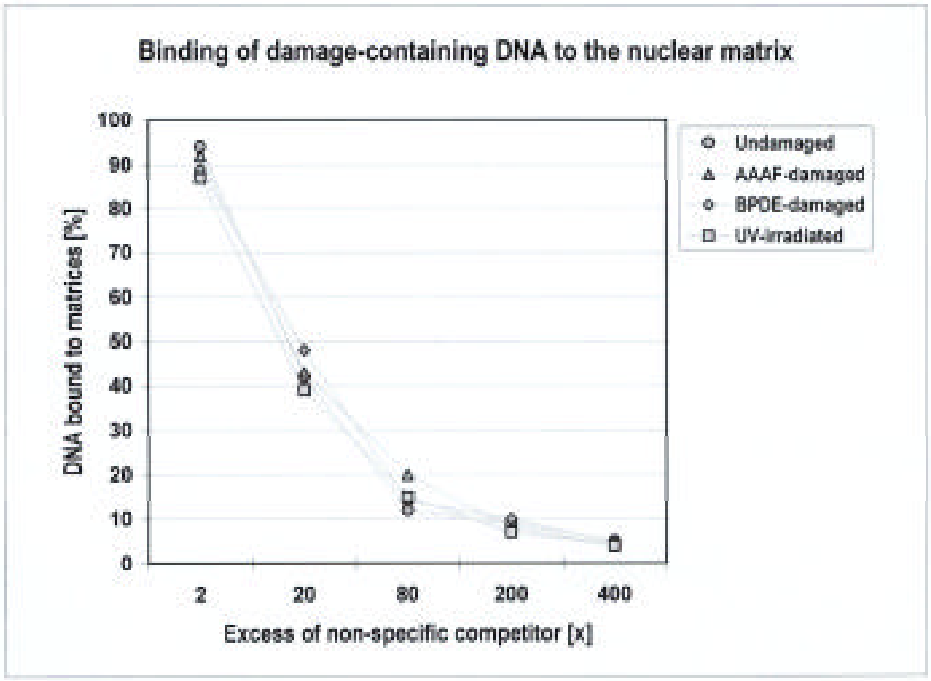

Figure 1 A. The *in vitro* binding of damaged DNA to the nuclear matrices. The complexes between nuclear matrix proteins and DNA probes (either undamaged or damaged) were formed in the presence of excess undamaged non-specific competitor. The matrix-binding efficiency is presented as a relative amount of the probe bound to matrices. Values are means from 3 assays.

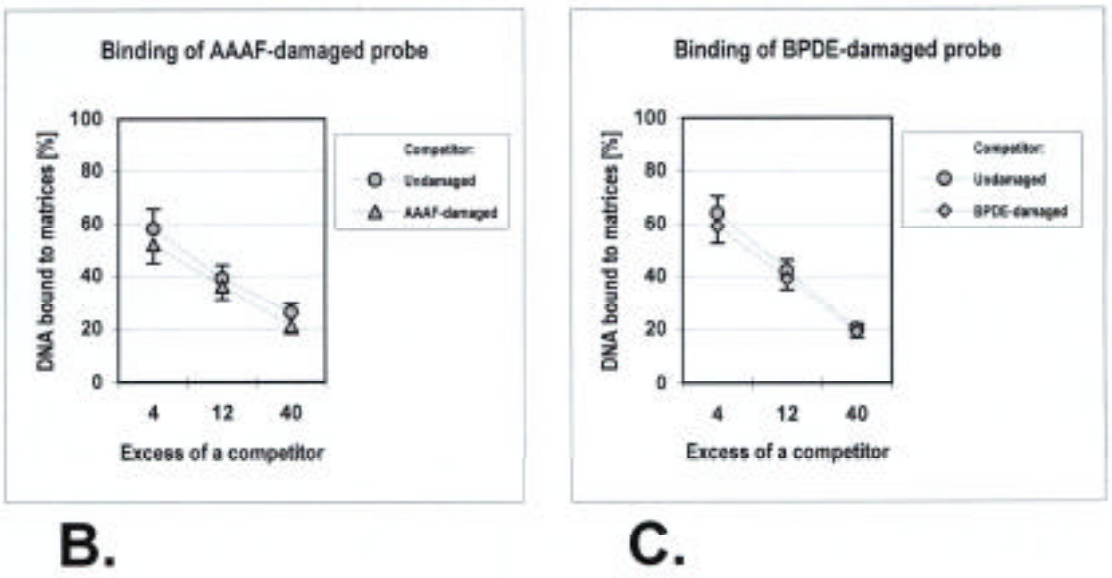

Figure 1 B and C. The *in vitro* binding of damaged DNA to the nuclear matrices. The complexes between nuclear matrix proteins and AAAF-damaged or BPDE-damaged probes were formed in the presence of excess of undamaged or damaged homologous competitor. Values are means from 3 assays (± S.D.).

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We also compared the distribution of a damage between fractions of DNA that did or did not bind to nuclear matrices. In this experiment about 50% of the probed DNA (in which one lesion per about 10 molecules was present) became bound to the matrices. The patterns of lesions in matrix-bound and unbound DNA fractions are shown in **Figure 2**. The adducts patterns in both fractions were similar for all three DNA-damaging factors. We calculated the relative levels of damage (the amount of adducts per the amount of total DNA) and compared these values between fractions. The ratio between adduct levels in the matrix-bound and the unbound DNA fractions was 1.4, 1.1 and 1.3 for AAAF-damaged, BPDE-damaged and UV-irradiated DNA, respectively (the values are means from three experiments). The data show that the nuclear matrices bound damage-carrying DNA molecules with very weak (yet detectable) preferences over undamaged DNA. We showed previously that the presence of UV-induced damage increased the efficiency of MAR DNA binding to the nuclear matrix *in vitro* (Widlak et al, 1996). In an experiment in which we studied binding of UV-irradiated kappa Ig MAR DNA to rat liver nuclear matrices the level of lesion in matrix-bound DNA was 5-fold higher as compared to unbound fraction. The data presented in this communication suggest that the observed phenomenon was specific for MAR sequence but not for the DNA sequence studied here (the MAR-binding proteins recognize some specific structural features of DNA which are probably affected by the UV-induced photoproducts).

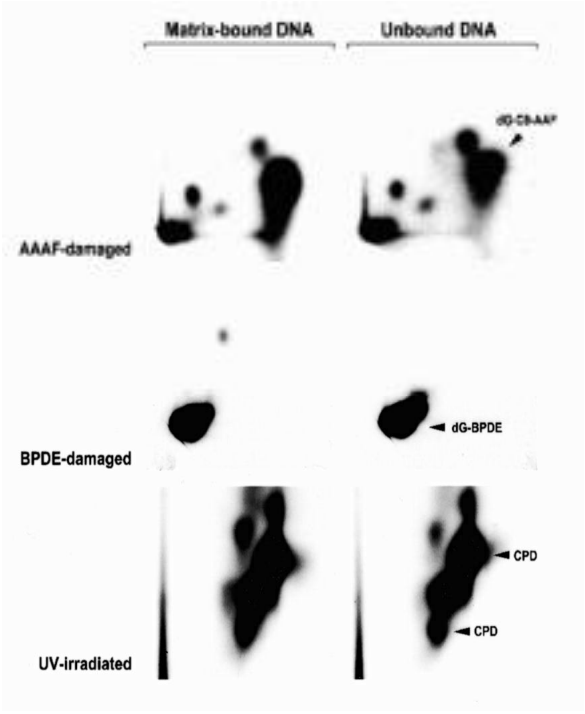

Figure 2. The chromatograms of damaged DNA complexed *in vitro* with the nuclear matrix proteins. Shown are fractions bound to the matrices as well as unbound material. About 100 ng of each DNA was used for the assay. Denoted are major forms of lesions (according to synthetic standards). CPD - cyclobutane pyrimidine dimers.

The data presented above suggest that most of damage-recognition proteins were extracted out from nuclei during the preparation of matrices. To study the presence of damage-recognition proteins in nuclear extracts we used a method based on electrophoretic mobility shift assay (EMSA). In this method, damaged DNA is complexed *in vitro* with soluble protein extracts in the presence of non-damaged competitor DNA. The presence of proteins that have high affinity to damaged DNA is detected by gel electrophoresis in the form of retarded bands containing protein-DNA complexes (Protic & Levine, 1993). In our experiments we have used DNA damaged by AAAF, BPDE or UV-radiation as radioactive molecular probes. The damage-recognition proteins were detected in protein fractions extracted from nuclei with increasing concentrations of NaCl, thus differing in stringency of their binding to the chromatin and nuclear matrix. The data showing the presence of proteins that preferentially bound to either UV-irradiated or AAAF-damaged DNA are shown on **Figure 3**. The proteins that specifically recognized UV-irradiated DNA (UV-DDB) were loosely attached to chromatin and could be eluted from the nucleus with low-salt buffers (0.15 M NaCl). The proportion of UV-DDB proteins to total extracted proteins markedly decreased in fractions extracted with 0.8 M (and higher) NaCl. The proteins which specifically recognized AAAF-damaged DNA (AAAF-DDB) were moderately attached to nuclear structures. They were present in fractions removed from nuclei with 0.4 M (and higher) NaCl (the highest proportion of AAAF-DDB proteins to total extracted proteins was detected in 0.6 M NaCl extracts). When BPDE-damaged DNA was used as a radioactive probe any complexes specific for damaged DNA could not be detected (data not shown). However, if BPDE-damaged DNA was used as a specific competitor we found that the same proteins which recognized AAAF-induced lesions also bound to DNA damaged by BPDE, yet with much lower efficiency (see panel **B** in **Figure 3**). The amount of proteins used for reactions was adjusted according to the total protein amount in extracts. However, different amounts of proteins (in proportion to total nuclear content) were extracted with increased salt concentration. Extracts used in this experiment contained about 12, 34, 51, 86, 92 and 90 percent of the total nuclear proteins (in 0.15, 0.4, 0.6, 0.8, 1.0 and 2.0 M extracts, respectively). Thus, the observed decrease in DDB protein level in the fractions extracted with higher salt concentration was due to their "dilution" with other nuclear proteins (e.g. histones). As treatment of the nuclei with high salt buffers (up to 2 M NaCl) neither increased the amount nor released any new species of DDB-proteins one can conclude that damage-recognition proteins are weakly bound to chromatin or the nuclear skeleton.

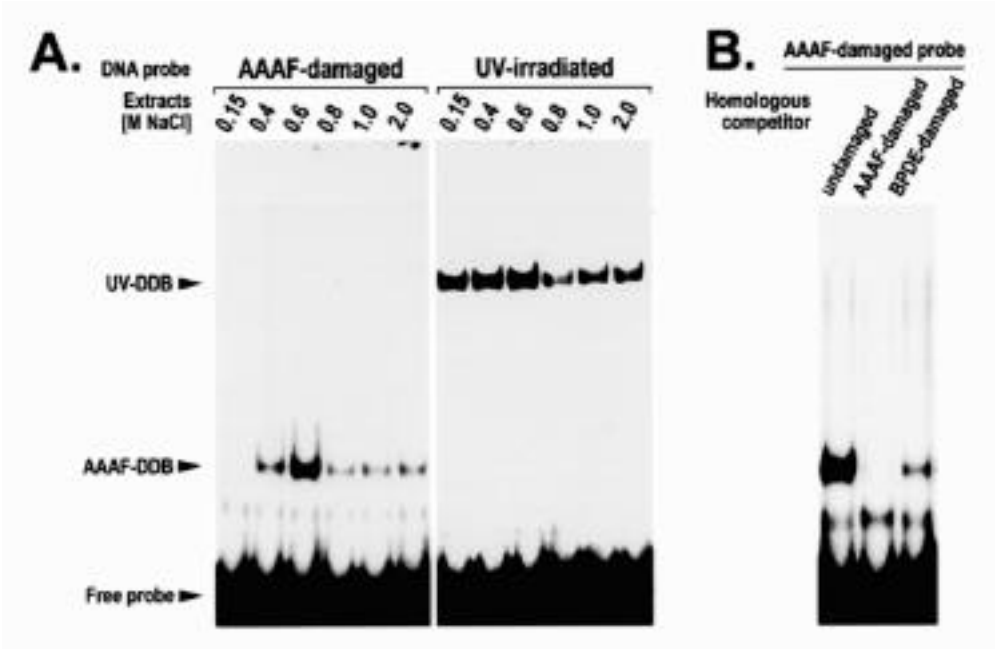

Figure 3. The EMSA analysis of DNA damage-recognition proteins from rat liver nuclei. Panel **A.** The *in vitro* complexes were formed between radioactive DNA probes (UV-irradiated or damaged by AAAF) and nuclear proteins extracted with the indicated NaCl concentrations (from 0.15 to 2.0 M), in the presence of non-specific undamaged competitor. The complexes were analyzed by polyacrylamide gel electrophoresis. Denoted are positions of free probe and retarded complexes containing UV-DDB and AAAF-DDB proteins. Panel **B**. The complexes were formed between radioactive AAAF-damaged probe and proteins extracted with 0.6 M NaCl, in the presence of excess non-radioactive oligonucleotide (undamaged and damaged by AAAF or BPDE) as a specific competitor.

Another *in vitro* method that can be used as a supplementary analytical tool to detect DDB-proteins is the Southwestern blot (Protic & Levine, 1993). In this method the probed DNA forms complexes with membrane-bound proteins after their electrophoretic resolution (usually on SDS/polyacrylamide gels). This assay depends on the ability of tested proteins to recover their structure (thus being limited to proteins which do not form oligomers). However, it can be successfully used to study nuclear matrix proteins (Widlak et al, 1995). **Figure 4** shows the binding of DNA probes to specific proteins that were either extracted from nuclei with 0.2, 0.4 and 1.0 M NaCl (low-salt extract, medium-salt extract and high-salt extract, respectively) or present in the nuclear matrices. Under the conditions of the experiment (40-fold excess of the competitor over the radioactive probe) the undamaged control DNA bound exclusively to histone proteins (both histone H1 and core histones). A similar binding pattern was seen with UV-irradiated DNA as a probe and no specific UV-DDB proteins were detected. This is in agreement with the fact that major UV-DDB protein is a heterodimer (Otrin et al, 1997). Some additional protein bands were seen on the filter tested with DNA damaged by AAAF. Such non-histone proteins which bound AAAF-damaged DNA were detected mostly in nuclear extract fractions. Some AAAF-DDB protein bands detected in the nuclear matrix probes were not matrix-specific as they were seen also in nuclear extracts.

The data presented above show that damage-recognition proteins interact loosely with the nuclear matrix and can be easily extracted from nuclei. As the nuclear matrices used in our experiments were purified from cells not pretreated with DNA damaging factors this left open the possibility that such a treatment would affect the nuclear distribution of damage-recognition proteins. Such an effect was observed in cultured cells, where UV-irradiation caused transient translocation of proteins participating in damage recognition to chromatin fractions more tightly attached to the nucleoskeleton (Otrin et al, 1997). To determine whether carcinogen treatment induces translocation of damage-recognition proteins we purified nuclear proteins from animals treated with 2-acetylaminofluorene or benzo(a)pyrene. Rats were i.p. injected with the carcinogens (50 mg/kg of body weight) then liver samples were collected at different time intervals (from 6 hours to 7 days after the treatment). The levels of adducts in hepatic DNA (assayed by the ^{32}P-postlabeling method) were highest 48 hours after 2-AAF treatment (about 16 fmol/μg DNA) and 24 hours after B(a)P treatment (about 13 fmol/μg DNA). We found that the nuclear matrices prepared from liver cells of animals treated with 2-AAF or B(a)P (48 and 24 hours after treatment, respectively) did not show more effective binding of DNA damaged by AAAF or BPDE (as compared to non-treated control animals, data not shown).

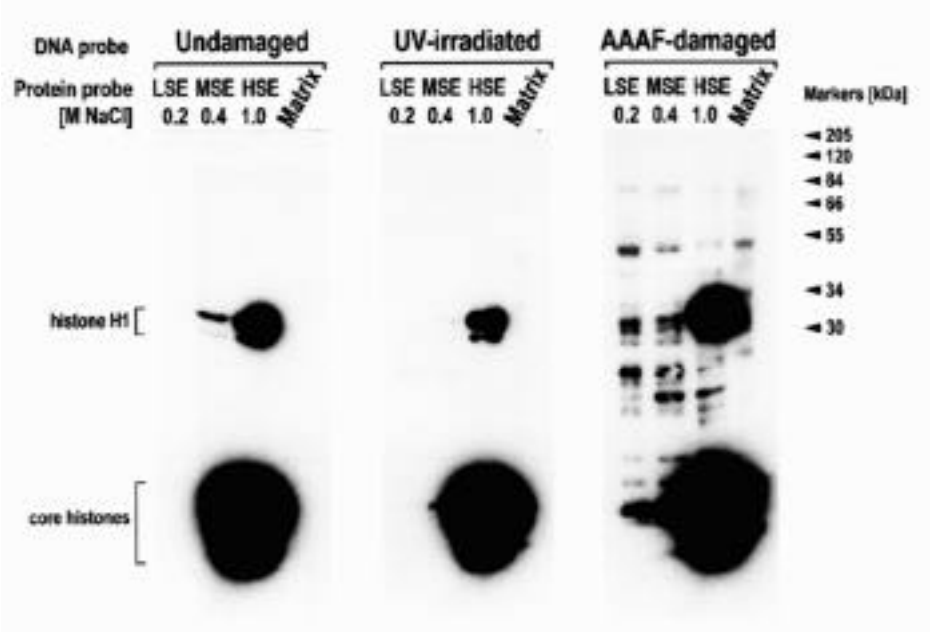

Figure 4. The Southwestern blot analysis of DNA damage-recognition proteins from rat liver nuclei. The *in vitro* complexes were formed between radioactive DNA probes (undamaged or damaged by AAAF or UV-irradiated) and nuclear proteins extracted with 0.2 M (low-salt extract - LSE), 0.4 M (medium-salt extract - MSE) and 1.0 M NaCl (high-salt extract - HSE) or the nuclear matrix proteins (Matrix). Nuclear proteins were resolved by electrophoresis on 13% polyacrylamide/SDS gel. The complexes were formed in the presence of excess undamaged non-specific competitor. Denoted are positions of histones and molecular size markers.

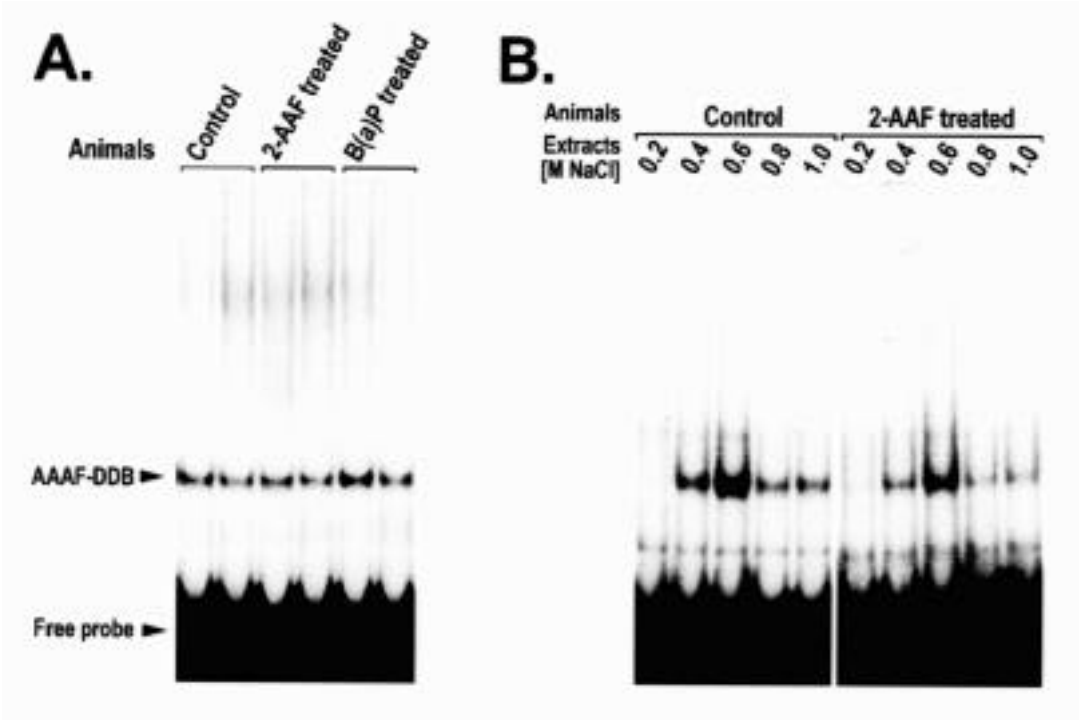

Figure 5. The analysis of AAAF-DDB proteins in liver cells of carcinogen-treated animals. **Panel A.** The complexes were formed *in vitro* between AAAF-damaged DNA probe and nuclear proteins extracted with 1.0 M NaCl from liver cells of control animals and animals injected with 2-AAF or B(a)P (24 hours after the treatment). Two animals in each group are shown. The complexes were formed in the presence of non-specific competitor. **Panel B.** The complexes were formed between AAAF-damaged DNA probe and nuclear proteins extracted with buffers of increasing NaCl concentration (0.2-1.0 M) from liver cells of untreated control rats and animals injected with 2-AAF (48 hours after the treatment).

In another experiment we assayed the level of AAAF-damaged DNA binding proteins in 0.4 and 1.0 M NaCl nuclear extracts purified from liver cells of animals injected with 2-AAF and B(a)P. We found that treatment with neither 2-AAF nor B(a)P (at any time tested) changed the levels of AAAF-DDB proteins (see **Figure 5A**). To clarify whether the carcinogen treatment affected the association with nuclear components the relative levels of these proteins were assayed in nuclear hepatic fractions extracted using increasing salt concentrations from control and 2-AAF-treated (48 hours after injection) animals. **Figure 5B** shows that the treatment of animals with 2-AAF did not change the association of AAAF-DDB protein to the hepatic chromatin. As the treatment with the 2-AAF did not affect the nuclear distribution of AAAF-DDB proteins this may reflect the fact that repair pathways specific for these lesions and UV-induced lesions are somehow different.

It is generally believed that nuclear matrix localization of certain activities involved in NER contributes to preferential repair of the DNA fraction associated with this nuclear structure. One can assume that the nuclear matrix localization of damage recognition factors may affect the repair of this DNA fraction. The data presented in this communication show that damage-recognition proteins are not constitutive components of the nuclear matrix. Thus, other factors which account for preferential repair of the matrix-attached DNA have to be searched for.

Experimental Procedures

A. DNA probes

Synthetic double-strand 36bp-long oligonucleotides (5'-AATTCGTAGG CCTAAGAGCA ATCGCACCTG TGCGCG-3', with blunt ends) were used as molecular probes. Oligonucleotides were UV-irradiated (5 kJ/m^2) using a 254 nm UV-crosslinker (Stratagene). Alternatively, oligonucleotides (at 10 μM concentration) were incubated for 4 hours at 37^{o}C with 40 μM AAAF or BPDE (Midwest Research Institute), then purified by phenol/chloroform extraction and ethanol precipitated. In all three cases, used procedures introduced lesion into about 15% of DNA molecules (on the average), which was checked by the ^{32}P-postlabeling method. Oligonucleotides were end-labeled with γ^{32}P ATP using T4 polynucleotide kinase and purified by polyacrylamide gel electrophoresis.

B. Preparation of nuclear proteins

Nuclei were purified from homogenized liver tissue from adult male WAG rats. To obtain extracts of nuclear proteins, nuclei were incubated for 30 minutes at 4°C with buffer consisting of: 10 mM Hepes-NaOH pH 7.9; 1.5 mM $MgCl_2$; 0.1 mM EGTA; 0.5 mM DTT; 5% glycerol; protease inhibitors and NaCl at different molarity (ranging from 0.15 to 2.0 M). Nuclei extracted at salt concentration higher than 0.5 M were briefly (2-3 sec.) sonicated. Insoluble remnants of the nuclei were pelleted by centrifugation for 30 minutes at 16,000 rpm at 4°C. Nuclear matrices were prepared by the DNaseI/"high salt" method as in previous experiments (Widlak & Rzeszowska-Wolny, 1994; Widlak et al, 1995), without copper stabilization. Briefly, nuclei were purified by centrifugation in 2.2 M sucrose and washed with 1% Triton X-100. The nuclei were then treated with DNaseI (10 µg/mg of nuclear protein) for 1 hour at 20°C in 0.1 M NaCl and next extracted with 0.5 M NaCl followed by 2 M NaCl to obtain residual matrix fraction.

C. Assay of DNA adducts

DNA was assayed for the presence of adducts according to ^{32}P-postlabeling method (Gupta et al, 1982). UV-induced adducts were analyzed according to Bykov et al (1995). Adducts induced by 2-AAF derivatives were enriched by butanol extraction, while adducts induced by B(a)P derivatives were enriched by nuclease P1 treatment (Widlak et al, 1996b). ^{32}P-labeled nucleotides were resolved by multi-dimensional thin layer chromatography. Adduct spots were visualized by autoradiography and quantitated by scintillation counting. To calculate the level of adducts in damaged oligonucleotides the number of total nucleotides was assayed according to Gupta et al (1982).

D. Complex formation between DNA and nuclear matrices

About 25 µg of the matrix proteins were suspended in 0.1 ml of the binding buffer comprising 50 mM NaCl, 2 mM EDTA, 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 25 µg BSA, 25 ng of ^{32}P-end-labeled DNA probe and different amounts of non-radioactive non-specific (sonicated *E. coli* DNA) or specific (oligonucleotides) competitor. After 1 hour incubation at 25°C matrices were recovered by centrifugation. To determine the amount of DNA bound to the matrices the radioactivity of pellets (matrix-bound fraction) and supernatants (unbound fraction) was quantitated in scintillation counter. In separate experiments, 25 µg of the matrix proteins were incubated with 250 ng of the oligonucleotides in the absence of non-specific competitor. Under these conditions about 50% of added DNA remained in the matrix-bound fraction. Both the pellet and the supernatant fractions were treated with proteinase K/SDS and extracted by phenol/chloroform. DNA was then recovered by ethanol precipitation and assayed for the presence of the adducts.

E. Electrophoretic mobility shift assay (EMSA)

Radioactive DNA probes (25 ng) were incubated with nuclear proteins (5µg) for 30 minutes at 4°C. The binding buffer consists of: 20 mM Tris-HCl pH 7.6; 5 mM $MgCl_2$; 0.5 mM EDTA; 1 mM DTT; 5% glycerol and 150 mM NaCl (final concentration). Complexes (in final volume 20 µl) were formed in the presence of non-specific (sonicated *E. coli* DNA, 2µg) or specific (1µg) competitors. Complexes were resolved by electrophoresis on 6% polyacrylamide gel (in 0.5 x TBE running buffer) and detected by autoradiography.

F. Southwestern blot analysis

The nuclear proteins (60 µg per slot) were fractionated on 13% polyacrylamide/SDS gel and electrotransferred onto PVDF membrane (Hybond-P; Amersham) in 25 mM Tris, 190 mM glycine and 20% methanol. Filter-bound proteins were renatured by incubation in the hybridization oven for 5 hours at 25°C with 25 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$ and 2.5% BSA. After washing with the binding buffer (same composition as above except for 0.25 % BSA added) filters were incubated for 5 hours at 25°C it the binding buffer supplemented with 500 ng of ^{32}P-end-labeled DNA probe and 20 µg of non-radioactive *E. coli* DNA (final volume 10 ml). Filters were then washed and autoradiographed.

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