ORGANIZATION OF PLASMID DNA INTO NUCLEOSOME-LIKE STRUCTURES AFTER TRANSFECTION IN EUKARYOTIC CELLS

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ABSTRACT
One of the most often used methods to monitor DNA repair in vivo is the host cell reactivation assay (HCR). It is based on the recovery of in vitro damaged plasmids after transfection in host cells. However, it is not clear to what extent plasmid molecules were degraded in the cells and whether they were packed with histones to form chromatin. Since these questions are important to evaluate the results obtained with HCR, in the present paper we studied the fate of the plasmid pEGFP-N1 after transfection in HEK 293 cells. To this end nuclei isolated from cells transfected with native and trioxsalen crosslinked pEGFP-N1 were digested with micrococcal nuclease (MNase) and DNA was subjected to electrophoresis. Southern blots were prepared and probed with digoxigenin-labeled plasmid DNA to reveal the plasmid DNA digestion pattern. Our results showed that nucleosome-like particles were formed on both native and damaged plasmid DNA after transfection. However, the nucleosome ladders were anomalous compared to the ladders generated by digestion of bulk cellular chromatin.

Keywords: host cell reactivation assay (HCR), micrococcal nuclease, enhanced green fluorescent protein

Introduction
A number of studies have demonstrated that DNA repair rates for different types of DNA damage can be measured by using the so called host cell reactivation (HCR) assay. The unique feature of the HCR assay is that it uses a non-replicating recombinant vectors containing a reporter gene pretreated with a specific DNA-damaging agent. In this assay, the transcriptional activity of the reporter gene reactivated in the host cell as a result of DNA repair is monitored at different times after transfection (1). Initially viral DNA vectors were used but later plasmid HCR assays have been described, which monitor cellular repair by measuring the transient expression of enzymatic marker genes (7, 16). The HCR assay was originally developed with the plasmid pCMVcat harboring the chloramphenicol acetyltransferase reporter gene (1), and it has also been successfully modified using a recombinant luciferase reporter plasmid pCMVLuc (17). This assay has been implemented to quantify DNA repair capacity in studies of lung cancer (21, 22), skin cancer (5, 12), head and neck cancer (2), and prostate cancer (8), after treatment with different genotoxic agents.

In our laboratory a fast and reliable variant of the assay based on the restored fluorescence of recombinant fluorescent proteins has been developed (20). A modification of this method has been used to determine the repair capacity of different cell types by counting the number of fluorescent cells under fluorescent microscope 24 hours after transfection with plasmid DNA containing the gene for enhanced green fluorescent protein (EGFP). Using this fluorescent HCR assay it has been shown that trioxsalen crosslinks in plasmid DNA were removed by homologous recombination (14).

Although transfection-based assays have been widely used to study the repair of different types of DNA damage as well as gene regulation, little is known about the fate of the plasmid molecules after their introduction into mammalian cells. There is data that upon transfection the greater part of the plasmid molecules entering the eukaryotic cells are cut and degraded in the cytoplasm (13). Because chromatin structure is essential for gene expression, recombination and repair (3, 4, 11), it is important to know when plasmid DNA enters the nucleus and whether it is packaged there with histones into chromatin. There is controversial data concerning this problem. It has been reported that micrococcal nuclease digests from transfected cell nuclei showed the presence of plasmid nucleosome ladders, whose repeat lengths were very similar to those observed for cellular chromatin (18). In contrast, other authors have shown that the transfected plasmid DNA generated anomalous repeat patterns after micrococcal nuclease digestion (10).

We have transfected human HEK 293 cells with native and crosslinked with trioxsalen pEGFP-N1 and have probed the MN-ase digests with labeled plasmid DNA fragment. Ladders with 6-7 nucleosome oligomer bands were observed, but the spacing was atypical.

Materials and Methods
Cells and plasmids
Human HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium (Applichem) containing 10 % fetal bovine serum. The cells were supplemented with antibiotics - penicillin (160 mg/l), streptomycin (100 mg/l) and kanamycin (100 mg/l) and incubated in an atmosphere of 95 % air/5 % CO₂ at 37 °C.
Plasmid pEGFP-N1 containing the gene egfp for the enhanced green fluorescent protein (EGFP) was purchased from Clontech and was propagated in E.coli strain XL1-Blue (9).

Trioxsalen treatment
Ten micrograms of plasmid DNA pEGFP-N1 were dissolved in 50 µl of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE) buffer. Trioxsalen (Sigma-Aldrich) dissolved in DMSO was added to a final concentration of 1 mM, the reactions were placed in 96-well plastic plates and irradiated in a Hanau UV illuminator box below two 15 W Sylvania black light tubes with emission maximum at 354 nm from a distance of 15 cm for 7.5 minutes to give 0.8 interstrand crosslinks (ICL) per plasmid molecule on the average (6). 1 M NaCl was added to a final concentration of 0.2 M NaCl and plasmid DNA was precipitated with 2.5 volumes of 99 % ethanol and dissolved in 1 x TE.

Introduction of pEGFP-N1 into HEK 293 cells
Control and trioxsalen-treated plasmids were introduced into cells using Lipofectamine 2000 transfection kit (Invitrogen) as recommended by the manufacturer. 24 hours later nuclei were isolated and subjected to micrococcal nuclease digestion.

γ-H2AX foci detection
Cells were seeded onto coverslips and 24 hours later were fixed with 2% paraformaldehyde in PBS (0.14 M NaCl, 0.01 M phosphate buffer, pH 7.0) for 5 minutes at room temperature, followed by permeabilization with ice cold methanol for 5 minutes at -20°C. The unspecific binding was blocked using 2% bovine serum albumin (Sigma-Aldrich) and 0.5 % gelatine from bovine skin (Sigma-Aldrich) in PBS for 60 minutes at room temperature, and the coverslips were incubated with anti-γ-H2AX mouse monoclonal antibody at 1:200 dilution (Abcam). The coverslips were washed with 0.1 % gelatine and incubated with Texas red conjugated secondary antibody (Abcam). The slides were visualized under fluorescent microscope AxioVert 200M (Carl Zeiss MicroImaging).

Micrococcal nuclease digestion and Southern blot analysis
About 10⁷ cells transfected with undamaged or trioxsalen crosslinked plasmid pEGFP-N1 were spun down by centrifugation and washed twice with 1 x PBS. The cells were resuspended in 400 µl CSK (cytoskeleton) buffer containing 0.5 % Triton X-100, 100 mM NaCl, 1 mM EGTA, 3 mM MgCl₂, 300 mM sucrose, 10 mM HEPES-KOH pH 6.9 and after 7 minutes on ice were spun down at 300 x g for 5 minutes at 4°C. The nuclear pellet was washed with 400 µl 0.25 M sucrose, 0.5 mM dithiothreitol, 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.5 (micrococcal nuclease digestion buffer) and centrifuged at 300 x g for 5 minutes at 4°C. The pellet was resuspended in 300 µl of the same buffer containing 1 mM CaCl₂ and 100 µl aliquots were digested with micrococcal nuclease (MN-ase) at room temperature. The reactions were stopped by adding 80 µl MN-ase digestion buffer and 20 µl 100 mM EDTA, 10 mM EGTA, pH 8.0 and by putting the samples on ice bath. DNA from the samples was isolated by making them 1 % in sodium dodecylsulphate and the proteins were digested with 0.5 mg/ml proteinase K at 37°C overnight. After extraction with phenol/chloroform/isooamyl alcohol (25/24/1), pH 8.0 and RN-ase digestion (50 µg/ml final concentration), DNA was precipitated with 2.5 volumes 99 % ethanol and 1/10 vol 10 M CH₃COONH₄ and dissolved in TE buffer. DNA was run on 1.5 % agarose gels, stained with ethidium bromide and transferred onto Hybond N+ membranes by capillary transfer. The membranes were then hybridized with a DIG-labeled DNA probe (0.495 kbp fragment of pEGFP-N1). The membranes were visualized using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH) as recommended by the manufacturer.

Results and Discussion

γ-H2AX foci formation
In a previous paper we have shown that a part of the trioxsalen crosslinked plasmid molecules undergo linearization after introduction into mammalian cells and that such a linearization is irrelevant to the process of ICL repair by homologous recombination (15). To investigate whether the undamaged plasmid DNA was also linearized after the introduction we transfected HEK 293 cells with either undamaged or trioxsalen crosslinked pEGFP-N1. As an indication of the DSBs formation and respectively – of the linearization of the plasmid DNA, we used the appearance of γ-H2AX foci since DSBs elicit immediate and massive phosphorylation of histone variant H2AX (19). 24 hours after transfection both in the transfected with crosslinked plasmid and in the transfected with native plasmid HEK 293 cells, γ-H2AX foci were formed (Fig. 1). The most plausible explanation was that a part of the plasmid molecules were linearized upon entering the cells regardless of whether they were crosslinked or not.

Micrococcal nuclease digestion and Southern blot analysis

![Fig. 1. γ-H2AX foci formation after transfection of HEK 293 cells with native or trioxsalen crosslinked pEGFP-N1. Exponentially growing HEK 293 cells were transfected with undamaged pEGFP-N1 or with trioxsalen crosslinked pEGFP-N1 (0.8 ICL/kb) and 24 hours later the appearance of γ-H2AX foci was monitored by indirect immunofluorescent microscopy using mouse monoclonal anti-γ-H2AX antibody. The secondary antibody was conjugated with Texas red.](image)

MN-ase digestion of bulk chromatin
Nuclei isolated from HEK 293 cells were treated with two different concentrations of the enzyme for different times. In each case the digestion rate was determined by agarose gel electrophoresis of the isolated DNA. Our results showed that with increasing MN-ase concentration nucleosome ladders
were formed from the bulk cellular chromatin (Fig. 2). The best result was observed when nuclei were digested with 0.05 U/ml MN-ase for 7.5 minutes at room temperature. Nucleosome ladders were not observed when lower concentrations of the enzyme were used, whereas the higher concentrations led to almost complete digestion of chromatin with formation of low molecular mass fragments corresponding to nucleosomal DNA.

To rule out the possibility that the MN-ase cuts the plasmid pEGFP-N1 at specific sites even when it is not packaged into chromatin, we treated naked plasmid pEGFP-N1 with increasing concentrations of MN-ase for 5 minutes at room temperature. Our results showed a lack of any nucleosome ladders in this case (Fig. 4). Thus we could speculate that the nucleosome ladders visualized after MN-ase digestion of nuclei isolated from transfected HEK 293 cells reflect the chromatin organization of the plasmid DNA in vivo.

**Plasmid DNA is packaged into chromatin after transfection into mammalian cells**

To investigate whether the exogenous plasmid DNA is complexed with histones and acquires physiologically spaced nucleosomes when introduced into mammalian cells, exponentially growing HEK 293 cells were transfected with either native or trioxsalen crosslinked pEGFP-N1. Twenty four hours after transfection nuclei were isolated and digested with 0.05 U/ml micrococcal nuclease for 7.5 minutes at room temperature. DNA was isolated, run on agarose gels and subjected to Southern blot transfer and hybridization with DIG-labeled plasmid DNA fragment. The analysis of the micrococcal digests of the native plasmid DNA showed the presence of clear although uneven nucleosomal ladder. Similar but not identical pattern was obtained from cells transfected with crosslinked plasmid. Both plasmid repeats differed from those observed for bulk cellular chromatin (Fig. 3). The reason for this difference as well as for the difference between native and crosslinked plasmid pattens is not clear at present.

![Fig. 2. MNase digestion of isolated nuclei. Nuclei isolated from exponentially growing HEK 293 cell were digested with 0.01 or 0.05 U/ml MN-ase for 5, 7.5 or 10 minutes at room temperature. DNA from each sample was isolated, purified and resolved on 1.5 % agarose gel; M – 1 kb DNA ladder (Fermentas), K – DNA from control, undigested nuclei.](image)

![Fig. 3. Southern blot patterns of pEGFP-N1 isolated from MNase digested nuclei after transfection with undamaged or crosslinked plasmid DNA. A) Nuclei isolated from HEK 293 cells 24 hours after transfection with native pEGFP-N1. B) Nuclei isolated from HEK 293 cells 24 hours after transfection with trioxsalen crosslinked pEGFP-N1. Both samples were treated with 0.05 U/ml MNase for 7.5 or 10 minutes at room temperature. DNA from each sample was isolated, purified, resolved on agarose gel and subjected to Southern blot hybridization with DIG-labeled 0.495 pEGFP-N1 fragment. Left panels – total DNA profiles after ethidium bromide staining; Right panels - plasmid DNA profiles after blotting and hybridization with DIG labeled plasmid DNA probes; Middle panels - schematic representation of total and plasmid DNA digestion patterns.](image)
Fig 4. MNase digestion of pEGFP-N1. Purified plasmid pEGFP-N1 DNA was treated with increasing concentrations of MN-ase as specified, for 5 minutes. Plasmid DNA from each sample was isolated, purified and resolved on 1.5 % agarose gel. M - 1 kb DNA ladder (Fermentas), K – control, untreated pEGFP-N1.

Conclusions
After transfection in mammalian cells, plasmid DNA enters the nucleus where it is complexed with histones to acquire nucleoprotein structure similar to that of the native chromatin. Under this form its transcription, recombination and repair are carried out by the same cellular mechanisms that carry out chromosomal transcription, recombination and repair.

REFERENCES