Plateau distributions of DNA fragment lengths produced by extended light exposure of extranuclear photosensitizers in human cells

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ABSTRACT
We have exploited properties of photosensitizers to study an aspect of the packing of chromatin in the cell nucleus. The fluorescent photosensitizers meso-tetra(3-hydroxyphenyl) porphyrin and Photofrin II were both localized in the nuclear membrane and other membrane structures, but could not be found inside the nuclei. Light exposure of cells at 1°C in the presence of the sensitizers induced DNA double-strand breaks. The length distributions of DNA fragments were determined by pulsed field gel electrophoresis. Because DNA damage is produced mainly via singlet oxygen diffusing less than 0.1 μm from the sensitizer, DNA double-strand breaks were supposedly produced within this distance of the nuclear membrane. Consistent with this, with prolonged illumination and with increasing concentrations of sensitizer the distribution of DNA fragment lengths reached a plateau level. In contrast, with the hydrophilic, intranuclear sensitizer meso-tetra(4-sulphonatophenyl)porphyrin, no such plateau level was found. The plateau distributions of DNA fragment lengths of different cell types had the same general shape with average fragment lengths ranging from 174 to 194 kilobasepairs. Particular genes, c-myc, fos and p53, were found on broad distributions of photocleaved fragment lengths. The results indicate that on each side of the genes the locus of the chromatin fibre situated close to the nuclear membrane, varied randomly.

INTRODUCTION
The spatial organization of chromatin in the cell nucleus is believed to be important in the regulation of such nuclear functions as DNA transcription and DNA replication. There is wide agreement on some aspects of the spatial organization of the chromatin. Most of the DNA is packed in the nucleosomal 10 nm fibre and the condensed 30 nm fibre (1). The chromatin fibre is evidently partitioned in large domains by specific attachment of DNA to an insoluble protein-RNA network—the nuclear matrix—both at the periphery and in the interior of the nucleus (2—4). But the number of DNA-bases between such attachment sites is uncertain and estimates have varied significantly with the experimental procedure (5,6). Furthermore, in situ DNA hybridization shows that individual chromosomes occupy distinct domains in the nucleus (7,8).

The detailed configuration of the chromatin fibre in the chromosomes in interphase nuclei is largely unknown (4), except for special cases such as the large polytene chromosomes of Drosophila. In this case a specific set of chromosome loci frequently contacted the nuclear envelope (9). The association between membrane components and chromatin has been quantitatively determined in the Xenopus cell-free system (10). Xenopus egg extracts promote the decondensation of demembranated condensed sperm chromatin by association of membrane vesicles with chromatin. At least one vesicle binds per 100 kilobasepairs (kb) of DNA, demonstrating frequent binding between chromatin and the nuclear membrane. However, the nature of this binding, whether it is DNA sequence specific and whether mammalian cells have similar frequency of binding is unknown.

We have taken a novel approach to study an aspects of the interaction between the nuclear envelope and the chromatin. Our idea is to use lipophilic photosensitizers localized in the nuclear membrane, but not inside the nucleus to damage DNA selectively in the nuclear periphery of living cells. The photosensitizers meso-tetra(3-hydroxy-phenyl) porphyrin (3-THPP) and Photofrin II (PII) were applied. After addition to the cells' medium the fluorescence from the sensitizers is localized in membrane structures, including the nuclear membrane, but not inside nuclei. In the presence of the sensitizer light induces DNA damage, including DNA double-strand breaks. Singlet oxygen, produced by energy transfer from the sensitizer to molecular oxygen, is the dominant DNA damaging species and produces the DNA strand breaks (11,12). Because singlet oxygen diffuses less than 0.1 μm from the light excited sensitizer during its intracellular lifetime (13) the DNA double-strand breaks are expected to be produced selectively within this distance of the nuclear membrane.

We have previously used a DNA alkali unwinding method to show that extended illumination in the presence of PII induced a plateau level of DNA strand breaks in human NHK 3025 cells (14). In this work, photocleaved DNA fragments were separated by pulsed field gel electrophoresis and the distribution of fragment lengths was determined for four different cell lines and peripheral
lymphocytes. To determine whether DNA was cleaved at the same sites in all cells, the separated DNA fragments were hybridized to certain gene probes.

**MATERIALS AND METHODS**

**Cells and photosensitization**

Cells from the human, substratum attached cell lines NHIK 3025 (14) and FME (15) and the human B-lymphocyte cell lines REH (16) and U698 (17) were kept in exponential growth by subculturing two or three times a week. Less than 4% of the cells were in mitosis. The NHIK 3025 cells were grown in E2a-medium with 20% human serum and 10% horse serum. The FME cells were grown in RPMI-medium plus 10% newborn calf serum, and the REH and U698 cells were grown in RPMI-medium plus 10% fetal calf serum.

The NHIK 3025 cells contained about 100 chromosomes, the FME cells about 55, and the REH and U698 cells were nearly diploid (16,17).

Peripheral, human lymphocytes were isolated by centrifuging blood over Lymphoprep (Nycomed) and grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum. The lymphocytes were stimulated with 1 μg/ml phytohemagglutinin (Wellcome).

Cells were incubated in the dark with different, nontoxic concentrations of the photosensitizers 3-THPP, TPPS$_4$ (Porphyrin Products, Logan, U.T.) or PII (MW 600, Photomedica, Inc., Raritan, N.J.) for 18 hr. PII is a mixture of negatively charged porphyrin dimers and oligomers. The lipophilicity of 3-THPP has been measured as Triton X-114/H$_2$O partition coefficients of about 6900 at pH 7.2 (18). For the NHIK 3025 cells the serum content during incubation with the sensitizer was reduced to 2% human serum and 1% horse serum to increase the uptake of the sensitizer into the cells.

Micrographs were produced by means of a fluorescence microscope and a red-sensitive video camera (Figs. 1A and 1B) or a laser scanning microscope (Fig. 1C) operated in non-confocal mode (excitation at 488 nm and emission through a 600 nm long pass filter) (20). For 3-THPP from a known number of cells was measured in a spectrofluorimeter by exciting the dye at 400 nm and recording relative fluorescence intensity at 650 nm.

**Cell encapsulation and light exposure**

Cells attached to flasks were trypsinized after incubation with sensitizier. All cells were washed and resuspended in Dulbecco's phosphate buffered saline without CaCl$_2$. To avoid mechanical shearing of DNA during DNA isolation cells were encapsulated in 0.5% LGT-agarose beads at $5-15 \times 10^6$ cells per ml agarose, as described by Jackson and Cook (21). The cells were kept in PBS at 37°C and the agarose was cooled to 37°C immediately before mixing cells, agarose and paraffin (22°C). 250–500 μl beads were suspended in an equal volume phosphate buffered saline and placed in transparent culture tubes (Nunclon 156758) with one flat side. The tubes were kept in ice water and exposed to light at 1°C to prevent cell degradation—by means of 4 fluorescent tubes (Photophysics 3026, 24 W/m$^2$). The light source had a peak in the emission-spectrum at 405 nm, which is close to the absorption maxima of the sensitzizers (18). The relative number of excitations of sensitizer per REH cell per min, determined as previously described (18), was 6 times higher for 3-THPP (5 μM) and TPPS$_4$ (320 μM) than for PII (40 μM). During illumination the tubes were placed on the bottom of a transparent box, containing ice and water, so that the beads were in a monolayer on the flat bottom of the tubes. No DNA repair could be detected at 1°C (14).

**Pulsed field gel electrophoresis**

Immediately after illumination, the cells were lysed with 1% sarcosyl (10 mM EDTA). The beads were centrifuged and incubated overnight at 37°C in 500 μl TE-buffer (10 mM Tris and 100 mM EDTA, pH 7.5) with 1% sarcosyl and 1 mg proteinase K. After 3–4 washes, the beads were kept in TE-buffer at 4°C. Bead volumes of 10–20 μl containing less than 2 μg DNA, were pipetted into the electrophoresis wells by means of cut-off 100 μl pipette tips and covered with 1% LGT-agarose. Field inversion gel electrophoresis was run on 15 × 15 cm 1% NA-agarose gels (Pharmacia) for 12 hr at 18°C by increasing exponentially the forward pulse time from 1 to 30 or 40 sec as described by den Dunnen (22). More than 2 μg DNA applied to the wells caused a significant retardation of DNA fragments. Polymerized λ-phage DNA (> 48.5 kb) (Bethesda Research Laboratories) prepared in agarose blocks, yeast chromosomes (> 225 kb) (Pharmacia) and λ-phage DNA digested by Hind III restriction nuclease (< 23 kb) (Pharmacia) were used as size standards.

**Optical scanning**

DNA nucleic acid research was performed on 0.25 M HCl DNA for 20 min with 1 μg/ml ethidium bromide under gentle agitation and washed with water for several hours. The fluorescent gel was photographed onto 7 × 7 cm of Polaroid negative film (Polaroid 665). The relative optical densities of the DNA lanes of the film were measured with an optical scanner (Model 1650 scanning densitometer, Hoefer Scientific/Biorad) connected with a personal computer. The optical densities adjacent to the lanes were used for background correction. Absolute optical density was obtained by calibration with a photographic step tablet (Kodak 1523398). The optical density of the film was converted to relative ethidium bromide fluorescence intensity, which is proportional to DNA content, by using a calibration curve for the light sensitivity of the film (23).

The distribution of DNA density was normalized to the same total amount of DNA for all samples. The distribution of DNA fragment lengths was found by dividing the distribution of DNA density with the lengths of DNA fragments. The lengths of fragments were determined by using the DNA size standards. The mobilities of the fragments between two closely spaced size standards presumably decreased linearly with the length.

**DNA hybridization**

The gel was stained for 40 min with 1 μg/ml ethidium bromide and rinsed with water for several hours. The fluorescent gel was photographed onto X-ray film (Polaroid 665). The relative optical densities of the DNA lanes of the film were measured with an optical scanner (Model 1650 scanning densitometer, Hoefer Scientific/Biorad) connected with a personal computer. The optical densities adjacent to the lanes were used for background correction. Absolute optical density was obtained by calibration with a photographic step tablet (Kodak 1523398). The optical density of the film was converted to relative ethidium bromide fluorescence intensity, which is proportional to DNA content, by using a calibration curve for the light sensitivity of the film (23).

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**DNA hybridization**

The gel was stained for 40 min with 1 μg/ml ethidium bromide under gentle agitation, to cut DNA and facilitate blotting, and in blotting buffer (0.4 M NaOH and 0.6 M NaCl) for 60 min. DNA was transferred to Hybond-N membrane (Amersham), which was washed with neutralizing buffer (0.5 M Tris, 1 M NaCl, pH 7.0), dried and UV-fixed. The membrane was prehybridized for 1 hr at 65°C in hybridization buffer (6×SSC, 5×Denhardt solution and 0.5% SDS with 50 μg/ml denatured salmon sperm DNA). Hybridization was performed at 65°C for about 18 hr in the presence of 2 ng/ml denatured DNA probe. The probe was labelled with $^{32}$P)dCTP by the multiprime method (Amersham). Filters were washed under stringent conditions two times in
between 40 and 70%. Cells incubated with the sensitizer and kept in the dark for 18 hr. The nuclei are the dark areas inside the cells, since they could be stained by DNA specific dyes. The detection system was overexposed in part C to show the relative darkness of the nuclei. The scale bars are 7 μm.

2×SSC for 20 min, one time in 2×SSC with 0.1% SDS for 30 min and in 0.1×SSC for 10 min all at 65°C. Hyperfilm-MP (Amersham) autoradiography film was exposed to the filter with intensifying screens for 5 days and the optical density of the film was scanned. The optical density of the film increased linearly with exposure in the range of exposure used.

The plateau level of average fragment lengths for the four cell lines ranged from 174 to 194 kb (Table 1). In addition, the distribution of fragment lengths was unchanged (Fig. 3B), while the fraction of DNA moving out of the agarose gel wells varied between 40 and 70%. Cells incubated with the sensitizer and kept in the dark (Fig. 3A) or cells illuminated in the absence of sensitizer did not contain measurable amounts of DNA fragments.

It is known that photosensitizers are photodegraded (28). Hence, it was necessary to examine whether the plateau level of DNA damage reached upon extended illumination was due to such photodegradation. The fluorescence intensity of intracellular 3-THPP in REH cells was reduced to 35% after 50 min illumination (Fig. 4B). We therefore varied the concentration of sensitizer from 2 to 10 μM, which gave proportional cellular uptakes (Fig. 4C). It was found that the average DNA fragment lengths generated with these concentrations of the sensitizer were the same within the experimental error (Fig. 4A), and the rate of photo-degradation has been found to be nearly independent of the concentration of sensitizer (28). Thus, we conclude that photobleaching did not affect the plateau level amount of DNA double-strand breaks at the concentrations of sensitizer used. The distribution of photocleaved fragment lengths was determined also for a negatively charged intranuclear photosensitizer. The photosensitizer meso-tetra (4-sulphonatophenyl) porphyrin (TPPS₄) is found partly in the nuclei and partly in the lysosomes of non-illuminated cell and is further concentrated in the nucleus by light exposure (29). The chemical structure of TPPS₄ is similar to the porphyrin structure of 3-THPP except for sulphonate side groups which makes TPPS₄ hydrophilic. Its quantum yield of singlet oxygen production is high (14). With TPPS₄ no plateau level of average DNA fragment length was obtained (Figs. 4A and 3B).

The plateau levels of average fragment lengths for the four cell lines ranged from 174 to 194 kb (Table 1). In addition, the plateau fragment length distribution of newly isolated peripheral lymphocytes was determined. Lymphocytes were also stimulated with phytohemagglutinin for 48 hr prior to and during incubation with the sensitizer. A substantial fraction of the stimulated lymphocytes entered the cell cycle and the volumes of the lymphocyte nuclei increased significantly. However, the distribution of photocleaved DNA fragment lengths for...
lymphocytes was unchanged with stimulation, and the average fragment length was similar to that of the B-lymphocyte cell lines (Table 1).

The validity of the electrophoresis

Variable amounts of DNA (from 30 to 60%) were retained in the wells during electrophoresis. The retention was found to be unspecific with regard to fragment length (Fig. 3). Furthermore, the partition between the wells and the lanes of the gene probes, hybridized to the DNA of the gel, were the same as for total DNA (Fig. 2). Apparently, a variable fraction of the photocleaved DNA fragments were bound to the agarose, probably at their terminal, damaged parts. The binding to the agarose was increased if the agarose was allowed to dry, whereas the binding was slightly reduced by heating to 65°C before electrophoresis. Because more than 30% of the DNA was retained in the wells, the electrophoresis can not rule out completely the existence of photocleaved DNA fragments longer than 1000 kb. However, we previously found, by measuring DNA single- and double-strand breaks, that in NHIK 3025 cells practically all breaks occurred more frequently than every 500 kb in the treated NHIK 3025 cells (14).

The partial binding of DNA fragments to the agarose might have caused some fragments to leave the wells later than at the start of the electrophoresis. To examine this possibility, electrophoresis was run in two consecutive, perpendicular directions (Fig. 5). In the second electrophoresis, we found that the DNA fragments located in the wells after the first electrophoresis, were immobile. The same was true for the fragments located close to the wells, i.e. within a distance reached by DNA size standards of 750 to about 1000 kb (30 and 40 sec maximum forward pulse time, respectively). Hence, fragments longer than 750 kb were excluded from the determination of mean fragment length. In contrast, the high mobility part of the DNA lane from the first electrophoresis was found in a narrow lane diagonally across the gel (Fig. 5), indicating that for the fragments in this lane the electrophoretic mobility decreased monotonously with the fragment length.
DNA photocleavage at particular gene loci

The DNA fragments separated by gel electrophoresis were hybridized with three different gene probes (Figs. 2 and 6) binding to a pair of sequences of the diploid human genome. The probes hybridize with single Eco RI fragments shorter than 15 kb which is much shorter than the average photocleaved DNA fragment. The hybridization signals of different cell types were proportional to the amounts of DNA in the gels, indicating that in none of the cell types the examined genes were selectively amplified.

All of the three gene probes hybridized with DNA fragments with broad length distributions both for the diploid (Fig. 6A and 6B) and for the aneuploid cell types (Fig. 6C and 6D). DNA of a particular cell type was evidently cleaved at different lengths from the gene. The length distributions of fragments containing the genes were shifted to longer fragments when compared with the total distributions of fragment lengths (Fig. 6).

If DNA is cleaved at random with respect to a gene, the probability of finding a gene in a DNA fragment of a given length is proportional to the total DNA density of that fragment length. Figure 6 shows that the length distributions of fragments containing the genes and distributions the DNA densities are not very different for the three genes and four cell types. Furthermore, the mean lengths of fragments containing the genes are close to the predicted mean lengths by random cutting (Table 2). This indicates that on each side of the gene, the DNA locus which contacted the nuclear membrane varied randomly.

**DISCUSSION**

We have shown that light excitation of photosensitizers localized at the nuclear membrane of intact cells, induced DNA double-strand breaks and plateau distributions of DNA fragment lengths. The DNA double-strand breaks were probably caused by DNA single-strand breaks produced closer than a few base pairs from each other. The plateau distributions of DNA fragment lengths are in accordance with previous data found by measuring the number of DNA single- and double-strand breaks (14). The observation that the frequency of DNA double-strand breaks reached a plateau level indicates that DNA was cut in all parts accessible to photodamage, that is in all parts situated within the diffusion length of singlet oxygen produced at the nuclear membrane. It is unlikely that some parts of the chromatin fibre in the proximity of the nuclear membrane were completely protected from attack of singlet oxygen by histone proteins, because DNA is known to be damaged by singlet oxygen even in nucleosomes (30). The number of DNA double-strand breaks induced by possible tiny amounts of sensitizer localized inside the nucleus must be insignificant because the mean photocleaved fragment length did not vary with the amount of intracellular sensitizer (Fig. 4).

The distribution of DNA fragment lengths had the same general shape for all cell types (Fig. 6) with average fragment lengths ranging from 174 to 194 kb (Table 1). This suggests a common pattern of chromatin packing. If we assume that the chromatin is packed in 30 nm fibres, it must be required that the chromatin fibre contacting the nuclear membrane with the determined frequency, is able to fill up the volumes of the nuclei. Such a chromatin fibre of 750 kb has a length of 6.4 μm (packing ratio 40). This is approximately the diameter of the REH nuclei and the large NHK 3025 nuclei have diameters of about 8 μm (31). Furthermore, the monotonously decreasing number of fragments

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**Table 1.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Dye</th>
<th>Mean fragment length (kb)</th>
<th>S.D.</th>
<th>Number of measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>REH</td>
<td>PI</td>
<td>183</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>U698</td>
<td>PI</td>
<td>189</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>FME</td>
<td>PI</td>
<td>183</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>NHK 3025</td>
<td>PI</td>
<td>179</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>PBL</td>
<td>PI</td>
<td>174</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Stimulated</td>
<td>PI</td>
<td>181</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

* Mean fragment length at the plateau level (Fig. 4A) for fragment lengths less than 750 kb.

**Table 2.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Gene probe</th>
<th>Mean length of fragment containing the gene (kb)</th>
<th>Predicted mean length by random cutting (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>REH</td>
<td>c-myc</td>
<td>290</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>v-fos</td>
<td>270</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>p53</td>
<td>300</td>
<td>290</td>
</tr>
<tr>
<td>U698</td>
<td>c-myc</td>
<td>270</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>v-fos</td>
<td>330</td>
<td>300</td>
</tr>
<tr>
<td>FME</td>
<td>c-myc</td>
<td>300</td>
<td>330</td>
</tr>
<tr>
<td>NHK 3025</td>
<td>c-myc</td>
<td>240</td>
<td>300</td>
</tr>
</tbody>
</table>

* Determined for fragment lengths less than 750 kb. The determined mean values were not significantly different for the sensizers 3-THPP and PI.

* By random cutting, the probability of finding a small gene sequence on a DNA fragment length x is proportional to the total DNA density of that fragment length.
Figure 6. DNA hybridization of gene probes to photocleaved DNA fragments of (A) REH, (B) U698, (C) NHJK 3025 and (D) FME cells. The cells were treated with 3-THPP (A and B) or PII (C and D) and light which gave plateau distributions of DNA fragment lengths (— — —) or corresponding DNA densities (-----). The photo-cleaved fragments were hybridized with DNA probes binding to unique human gene sequences. The hybridization density curves for the c-myc (— — —), the v-fos (-----) and the p53 (— — —) gene probes represent the length distributions of fragments containing the genes. The curves are mean values for 3 to 5 measurements.

longer than 150 kb (Fig. 6) is consistent with that fact that the volume of a sphere increases with the third power of the radius. However, the very high relative numbers of fragments around 100 kb (Fig. 6) might suggest that the density of DNA close to the nuclear membrane is higher than in the interior of the nucleus. This is in accordance with the presence of peripheral heterochromatin which has a packing ratio more than 5 times higher than for euchromatin (32). The results are not inconsistent with the separation of interphase chromosomes in distinct domains provided that the chromosomes are in contact with the nuclear envelope. On the other hand, if most of the chromatin is packed in 250 nm and thicker fibers (33), the chromatin fibre could not reach into the interior of the nucleus.

It has been found that histone-depleted nuclei contain binding sites between DNA and the nuclear protein matrix, both in the interior and at the periphery of the nucleus, with a mean distance of 20–100 kb (4–6). Some of the binding sites are permanent and other binding sites appear to vary with the state of the cell (5). Because the average photocleaved fragment length was between 174 and 194 kb, it seems that DNA is bound to the intranuclear protein network at a few sites between each approach to the membrane.

DNA was photocleaved at different lengths from specific genes in different cells of a particular type. This indicates that the points of contact between DNA and the nuclear membrane were not at specific DNA loci. It is likely that the parts of the chromatin fibre which are situated close to the nuclear membrane changes on some unknown time scale. Comparison of stimulated and unstimulated lymphocytes suggests that the frequency of contact points does not change through the cell cycle. Furthermore, we do not know the effect on the chromatin of keeping cells in ice water during light exposure. There is evidence that domains of chromatin with high DAPI staining as well as nucleoli move about in living neurons (34). Chromosome domains changes their position in neurons (33), and during the cell cycle of human diploid fibroblasts (35). Moreover, the chromatin obviously loses contact with the nuclear membrane during mitosis. Taken together, it is possible that the DNA loci which are in proximity to the nuclear membrane in proliferating cells change during mitosis and/or varies through interphase. This does, however, not exclude the possibility that a few sequence specific parts of DNA are frequently found close to the nuclear membrane during interphase as observed with Drosophila polytene chromosomes (9).

The examined gene probes tended to hybridize to the longer photocleaved fragments (Fig. 6). Consequently, the parts of DNA close to the genes were less frequently cleaved than the total DNA. It is known that the non-transcribed heterochromatin form of the chromatin fibre is preferably situated in the nuclear periphery (36), perhaps within the peripheral 50% of the nuclear volume in the outer 20% of the radius. Transcribed genes contained in decondensed parts of fibers may be localized primarily in the interior of the nucleus (37–39). At least the examined c-myc gene was transcribed in the REH and NHJK 3025 cells (data not shown). Our results suggest that some genes can be found frequently in the interior of the nucleus because the chromatin fibre around the genes by chance seldom is localized close to the nuclear membrane. This rule may not apply to all parts of DNA. It is possible that random localization of genes to photocleaved fragment lengths does not apply to passive genes and that there is a relationship between chromatin fibre dynamics in general and gene activity.
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