In situ photochemical crosslinking of HeLa cell mitochondrial DNA by a psoralen derivative reveals a protected region near the origin of replication

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ABSTRACT

The in vivo association with proteins of HeLa cell mitochondrial DNA (mtDNA) has been investigated by analyzing the pattern of in situ crosslinking of the DNA by 4′-hydroxymethyl-4, 5′,8-trimethylpsoralen (HMT). Either isolated mitochondria or whole cells were irradiated with long wavelength UV light in the presence of the psoralen derivative, and the mtDNA was then isolated and analyzed in the electron microscope under totally denaturing conditions. No evidence of nucleosomal structure was found. The great majority of the molecules (≥90%) had a double-stranded DNA appearance over most of their contour length, with one to several bubbles occupying the rest of the contour, while the remaining 10% of the molecules appeared to be double-stranded over their entire length. Analysis of restriction fragments indicated the presence, in ≥80% of the molecules, of a protected segment (300 to 1500 bp long) in a region which was centered asymmetrically around the origin of replication so as to overlap extensively the D-loop. Control experiments showed that at most 30% of the bubbles found near the origin could represent D-loops or expanded D-loops; furthermore, it could be excluded that some sequence peculiarity would account for the preferential location of bubbles near the origin of replication. The data have been interpreted to indicate that, in at least 55% of HeLa cell mtDNA molecules, the region around the origin is protected from in situ psoralen crosslinking by proteins or protein complexes which are associated in vivo with the DNA.

INTRODUCTION

The physical state of mitochondrial DNA (mtDNA) in vivo has been the object of considerable attention in recent years. The packaging of the 7 x 10⁶ base pairs (bp) of mtDNA of a single yeast cell¹ in a mitochondrial volume of 5 μm³, ² or of the 1.3 x 10⁸ bp of mtDNA of a HeLa cell³ in a mitochondrial volume of 130 μm³, ⁴ presumably requires some kind of compact configuration of the DNA. There is good agreement among the various investigators that mtDNA does not possess a typical nucleosomal structure; furthermore, in all cases where a careful analysis of the proteins associated with mtDNA has been done, no evidence of histones has been found (refs. 5-7; Albring and Attardi, unpublished observations). Several investigators have isolated mtDNA protein complexes on the basis of either their sedimentation behavior⁵,⁸ or their density in metrizamide gradients,⁹,¹⁰ and evidence has been presented for the presence in these
complexes of one or a few distinctive proteins. However, in these experiments, it was
difficult to exclude artifactual association of proteins with mtDNA.

In previous investigations from this laboratory, the association of a protein
structure of probable membrane derivation with HeLa cell mtDNA at or near its origin
of replication has been demonstrated by both electron microscopic and biochemical
analysis of mtDNA molecules released by Triton X-100 lysis of mitochondria in the
presence of low salt. The uniqueness and site specificity of this protein–DNA associa-
tion at the origin of replication tended to exclude a random adsorption of the protein
structure to the DNA during extraction. However, the in vivo occurrence of the mtDNA–
protein complexes could not be demonstrated in the above mentioned investigations. In
order to obtain information relevant to this question, we have analyzed, in the present
work, the pattern of photochemical inter-strand crosslinking of HeLa cell mtDNA by a
psoralen derivative. These furocoumarins are known to intercalate between adjacent
base pairs of the DNA double helix and to crosslink covalently pyrimidines on opposite
DNA strands upon irradiation with long wavelength UV light (320–380 nm). The cross-
linking sites can be identified by electron microscopic examination of the DNA under
denaturing conditions. Since the cellular membranes are permeable to the psoralens,
It has been possible to study the crosslinking effect of these compounds in vivo, and to
use them as probes for determining the association with DNA of proteins or other
molecules interfering with the crosslinkings of the DNA strands. In particular, these
drugs have been used successfully for studying chromatin structure in vivo. In the
investigations reported here, 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) has been
utilized both on intact HeLa cells and on isolated mitochondria to crosslink the mtDNA
in situ. Since this work was completed, a paper has appeared in which psoralen cross-
linking was utilized to demonstrate DNA–protein interaction in the mitochondria of
Drosophila melanogaster.

MATERIALS AND METHODS

Cell Growth and Labeling Conditions

The method of growth of HeLa cells in suspension has been previously described. Cells were labeled for 2 days with [3H]thymidine (1.25 μCi/ml) prior to the crosslinking,
in order to provide a marker to follow the mtDNA during purification.

Crosslinking of mtDNA in situ

Whole washed cells or a crude mitochondrial fraction prepared by differential
centrifugation were resuspended in either phosphate buffered saline containing 0.001 M
MgCl₂ (cells), or in a 0.25 M sucrose solution in 0.01 M Tris–HCl (pH 6.7), 0.0015 M
MgCl₂ (mitochondria). 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT), a derivative of psoralen, was added to a final concentration of 6 μg/ml from a stock solution at

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900 μg/ml in 100% ethanol. Irradiation took place in a 5 ml water-jacketed cylindrical chamber, which was placed between two General Electric Mercury-Vapor lamps (H 400 A 33–1/T16). The chamber was kept at 18°C by continuous circulation of a temperature controlled solution of cobaltous nitrate (26% cobaltous nitrate, 3% NaCl), which also acted as an UV filter allowing the passage of light between 340 and 380 nm (maximum at about 365 nm), essentially as described. Addition of a fresh equal portion of HMT solution was made every 5 min. After four such additions, the cells or mitochondria were spun out of the buffer and resuspended in fresh buffer, and the whole irradiation procedure was repeated.

**Purification of mtDNA**

Following irradiation, the intact cells were homogenized and a crude mitochondrial fraction, prepared from them; DNA was then isolated from this fraction and from the directly irradiated mitochondria by a procedure involving DNase digestion, SDS lysis and centrifugation through a 2-step CsCl-ethidium bromide gradient, as previously described. This type of gradient fractionation allows a rapid purification of mtDNA from any degraded nuclear DNA which remains near the top of the gradient. The material corresponding to the peaks of closed-and open-circular mtDNA in each gradient was pooled, the ethidium bromide was removed by extraction with isoamyl alcohol, and the DNA, collected by overnight centrifugation (SW41, Spinco rotor, 36 krpm, 16 h). Preparation from HeLa cells of closed-circular mtDNA to be used in the experiments of crosslinking in vitro of isolated DNA was carried out as described earlier.

**Preparation for Electron Microscopy**

Prior to restriction enzyme digestion, the DNA was precipitated with ethanol in order to remove the last traces of salts. The DNA was digested with Bam HI, or Pvu II or Hind III (Bio Labs) under standard conditions. Following the digestion, the sample was phenol extracted, collected by ethanol precipitation and totally denatured in 70% formamide, 0.1 M phosphate buffer, pH 7.0, 0.5 M glyoxal, at 37°C for 1 h. The denatured DNA was diluted with a solution of 40% formamide, 0.1 M Tris–HCl (pH 8.5) 0.01 M EDTA, containing 50 μg/ml cytochrome c, and spread on a hypophase containing 20 to 30% formamide, 0.01 M Tris–HCl (pH 8.5), 0.001 M EDTA. For the analysis of the D-loops in the crosslinked mtDNA preparations, undenatured DNA samples were likewise spread under formamide conditions, as described above, DNA was then picked up onto parlodion coated copper grids, stained with uranyl acetate and shadowed with Pt-Pd. Molecules were photographed on 35 mm film with a Philips 300 electron microscope, and the molecule lengths, loop sizes and distances from the ends were measured with a Hewlett-Packard 9864A Digitizer connected with a Hewlett-Packard 9820 Calculator (Hewlett-Packard Corp., McMinnville, Oregon). Only molecules whose length did not deviate more than 10% from the average of the distribution were utilized in the cross-
RESULTS
Crosslinking of mtDNA in situ

HeLa cell mtDNA was crosslinked in situ by irradiating either whole cells or a crude mitochondrial fraction with light of wavelength around 365 nm in the presence of HMT. In view of the photodestruction of the psoralen derivative, it was found necessary to add HMT to the incubation mixture repeatedly, as described in Materials and Methods.

After the photoreaction, the mtDNA was extracted from the mitochondrial fraction and separated by centrifugation in a two-step CsCl-ethidium bromide gradient. As previously described,\(^1\)\(^9\),\(^2\)\(^2\) one recognized in the lower portion of the gradient two peaks which corresponded respectively to monomeric closed-circular and open-circular mtDNA, with a partially separated peak on the faster sedimenting side of the closed-circular mtDNA, presumably representing oligomeric forms (mostly concatenated) of mtDNA.\(^1\)\(^9\),\(^2\)\(^3\) In the upper portion of the gradient, one could see a band of degraded nuclear DNA. An identical pattern was obtained for mtDNA extracted from photoreacted mitochondria and for control (uncrosslinked) mtDNA (not shown). The closed- and open-circular mtDNAs in each of the two crosslinked preparations were pooled and analyzed for the presence of crosslinks by electron microscopic examination under totally denaturing conditions. In order to prevent any possible renaturation, the molecules were denatured in the presence of glyoxal; the latter is known to react with guanylic acid residues in single-stranded DNA,\(^2\)\(^4\) thereby preventing G-C base pairing even under renaturing conditions. Under totally denaturing conditions, DNA segments containing a high density of crosslinks appears as double-stranded, while uncrosslinked regions form "bubbles."

Fig. 1 shows some typical mtDNA molecules crosslinked in isolated mitochondria. In this sample, and in mtDNA samples photoreacted in intact cells (not shown), the great majority of the mtDNA molecules (>90%) had a double-stranded appearance over 70 to 98% of their contour length (average >90%), the rest of the contour length being occupied by one or more "bubbles" of varying size, in which the two denatured uncrosslinked strands could be recognized. The remaining 10% of the mtDNA molecules had a double-stranded appearance over their entire length. We interpret this double-stranded appearance of the DNA as indicative of relatively closely spaced crosslinks, rather than as reflecting incomplete denaturation of the DNA. In fact, mtDNA which had not been submitted to crosslinking treatment, when denatured and spread under the same conditions used for the crosslinked samples, revealed only denatured single strands; furthermore, mtDNA extracted from cells or mitochondria which had been photoreacted for a shorter than the optimum overall time exhibited uncrosslinked regions with a frequency and size which increased in inverse proportion to the extent of photo-
reaction. From the average proportion of the contour length with double-stranded DNA appearance in the mtDNA molecules from extensively photoreacted cells or mitochondria (~90%), and on the basis of published data on crosslinkings of protein-free DNA, we infer that the extent of crosslinking in these molecules is probably close to one crosslink per 100 bp or less (see below).

The distribution and nature of the uncrosslinked regions in the in situ photoreacted mtDNA was further investigated, as described below.

Mapping of the Protected Regions

In order to determine whether the bubbles observed in the crosslinked mtDNA molecules under totally denaturing conditions were located in specific regions, their positions were mapped relative to several restriction enzyme cleavage sites. The enzymes used, and the positions of their cutting sites in HeLa mtDNA relative to the origin of replication, are shown in Fig. 2. The first enzyme used, Bam HI, makes a single cut in HeLa mtDNA at 2,500 bp (or 15.1% of the total genome length) from the origin of replication in the direction of heavy (H) strand synthesis (clockwise). The pattern of bubbles seen on molecules which were crosslinked in a crude mitochondrial fraction and subsequently digested with Bam HI is shown in Fig. 3. No evidence can be seen of the regular pattern of crosslinkings at 200 bp intervals which is typical of nucleosome structure. Of 94 molecules analyzed, 78% contained an uncrosslinked region, with an average size of 920 (+350) bp, at a position between approximately 15 and 20% of the total genome length from one end. The remainder of the bubbles

Figure 1. Electron micrographs of HeLa cell mtDNA crosslinked in isolated mitochondria and spread under totally denaturing conditions. (a) Circular molecule from a non-digested sample; (b) a molecule from the same sample cleaved with Pvu II.
Figure 2. Diagram showing the restriction fragments of HeLa cell mtDNA utilized in the present analysis. The positions of the origin of replication (0) and of the D-loop relative to the relevant restriction sites are indicated. The rightward arrow indicates the direction of H-strand synthesis.

appeared to be randomly distributed along the mtDNA molecule. In order to determine which of the two ends of the molecule was closer to the uncrosslinked region, and indeed whether there was a single uncrosslinked region or two uncrosslinked regions equidistant from the two ends, the same preparation of crosslinked DNA was digested with another single cutting enzyme, Pvu II; the cleaving site of this enzyme is nearly equidistant from the origin of replication as the Bam HI cutting site (2464 bp), but in the opposite direction (counterclockwise). The distribution of bubbles along Pvu II cut molecules is shown in Fig. 4. Again, in 79% of the molecules examined (84), an uncrosslinked region is seen with an average size of 930 (+ 340) bp, between approximately 12 and 18% of the total genome length from one end. The combined data from the Bam HI and Pvu II cut molecules place the most commonly seen uncrosslinked region around the origin of replication. In order to confirm that the region around the origin of replication is protected from crosslinking, the distribution of bubbles on the largest Hind III fragment, which contains the origin of replication at ~60% of the length from one end (Fig. 2), was also determined. As shown in Fig. 5, in ~75% of the molecules analyzed (17), there is an uncrosslinked region between 58 and 72% of the fragment length from one end, as expected if it were near the origin of replication. Several other preparations, including some in which crosslinking of mtDNA was performed in whole cells rather than in the
mitochondrial fraction, were analyzed in this way; in all cases, a similar proportion of molecules (~75%) was found to contain an uncrosslinked region near the origin.

The mtDNA from most animal cells investigated so far contains, in a substantial proportion of the molecules, a displacement loop (D-loop) near the origin of replication,28,29 resulting from the synthesis of a short segment of H-strand DNA (7S DNA), which displaces the parental H-strand.28 In human cells, these single-stranded DNA segments have been observed to exist as discrete size classes ranging between 550 and 680 nucleotides,30–33 in agreement with the size estimated from contour measurements
of the D-loop (~600 nucleotides). It is generally believed that H-strand synthesis takes place as a result of the extension of 7S DNA, this process producing the so-called "expanded D-loops". In all the preparations analyzed in the present work, there was a considerable variation in size and precise position of the uncrosslinked regions near the origin of replication. The majority of these uncrosslinked segments (~80%) appeared as a bubble with one end within 100–200 bp of the origin and extending in the direction of H-strand synthesis, so as to overlap the D-loop region completely, or almost so. Some of the larger uncrosslinked regions occasionally appeared as two adjacent loops. It seems unlikely that the larger uncrosslinked segments represent expanded D-loops, because of the very low frequency of the latter in the preparation (~1%, see below). An indication of the presence of the larger uncrosslinked regions can be seen in the histograms of the
bubble positions, which exhibit a peak corresponding approximately to the D-loop region, with a hint of a shoulder on both sides, more marked, however, on the side of H-strand synthesis. In all samples, the portion of mtDNA outside of the region of the origin exhibited relatively rare bubbles, which did not appear to be concentrated in any particular segment.

Frequency and Size of D-loops in the in situ Crosslinked mtDNA

Since the presence of a D-loop in a mtDNA molecule would have prevented in vivo crosslinking of the parental DNA strands in the corresponding region, it was expected that some of the bubbles observed in the D-loop region of fully denatured molecules would simply reflect the in vivo presence of the D-loop. The reported frequency of D-loops in HeLa cell mtDNA preparations (\( \approx 25\% \))\(^{35} \) (see also ref. 27) is indeed considerably lower than the frequency of bubbles detected in the origin region in the denatured samples; however, it was conceivable that the in vivo frequency of D-loop containing mtDNA molecules is much higher than in the extracted mtDNA preparations. It was reasonable to think that psoralen crosslinking would stabilize D-loops against branch migration, both by covalently linking the 7S DNA to the complementary L-strand DNA region [from the estimate of crosslinking density under the present experimental
conditions, it was anticipated that each 7S DNA molecule would have several crosslinks with the L-strand] and by crosslinking the parental strands on both sides of the D-loop. Therefore, an electron microscopic analysis was carried out on the in situ crosslinked mtDNA preparations, spread without previous denaturation, in order to identify in these preparations the molecules which contained a D-loop or an expanded D-loop. In this analysis, about 25% of the molecules showed a D-loop; this frequency agrees very well with a previous estimate of the fraction of mtDNA molecules with D-loops in HeLa cell mtDNA. In these experiments, the samples had been digested with restriction enzymes prior to spreading for electron microscopy. Since it is known that, under these conditions, there is an almost complete loss of D-loops in uncrosslinked mtDNA, the above observations strongly support the idea that psoralen crosslinking had stabilized the D-loops. These exhibited a relatively narrow distribution of contour lengths of the duplex branch, ranging between 2.9% and 3.7% (average = 3.3%) of the mtDNA length, or about 560 (+ 70) bp, as calculated by assuming a genome size of 16,569 nucleotide pairs (Figs. 3 and 4). A relatively small fraction (<5%) of the loops in the origin region exhibited a larger contour length of the duplex branch, and presumably represented expanded D-loops. The results of the analysis described above thus strongly suggested that bubbles with the structure of D-loops or expanded D-loops could only represent a minor fraction of the uncrosslinked regions observed in fully denatured samples. Confirmatory evidence for this conclusion came from an analysis of the detailed structure of the bubbles near the origin of replication in a fully denatured sample of Pvu II digested molecules. In such an analysis, D-loops were expected to be recognizable as having a double-stranded arm and a single-stranded arm. As shown in Table 1, in about 77% of the bubbles near the origin of replication, both arms appeared

<table>
<thead>
<tr>
<th>Type of bubble</th>
<th>Number of bubbles</th>
<th>Percent</th>
</tr>
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<tbody>
<tr>
<td>Single-stranded</td>
<td>44</td>
<td>77</td>
</tr>
<tr>
<td>in both arms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single-stranded in one arm and double-stranded in the other</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Unassigned</td>
<td>4</td>
<td>7</td>
</tr>
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The electron microscopic analysis was carried out on a sample of in situ crosslinked mtDNA molecules cleaved with Pvu II and spread under totally denaturing conditions.
to be clearly single-stranded (two examples are presented in Fig. 1), 7% had a double-stranded arm and a single-stranded arm, and the remainder was of uncertain assignment. Two investigators carried out the screening independently, and the positive assignments reported in Table 1 represent those on which there was agreement in the two screenings. Similar results were obtained in an analysis of a fully denatured sample of Bam HI digested mtDNA molecules (data not shown).

**Crosslinking in vitro of Isolated mtDNA**

Due to the preferential reaction of psoralens with pyrimidines, it is possible that the lack of crosslinks in a DNA segment may be related to the DNA sequence in that segment, such as an asymmetric distribution of pyrimidines, as has been found in the case of *Drosophila melanogaster* satellite DNA.\(^{36}\) An examination of the nucleotide sequence in the region around the origin of replication shows indeed that there is no portion which would not be expected to be crosslinked.\(^{26,33}\) However, in order to eliminate completely the possibility mentioned above, isolated closed-circular mtDNA from HeLa cells was crosslinked with HMT in vitro, and analyzed for the presence of uncrosslinked regions, as described above. Figure 6 shows the crosslinking pattern in a Bam HI digested mtDNA preparation having roughly the same density of crosslinks as the DNA crosslinked in situ. No evidence is observed of a preferential location of the bubbles in any position along the mtDNA molecule, even though, in the construction of the crosslinking histogram, all bubbles present in the region at 15 to 20% from one end of the molecule have been assigned to the same half (in this mtDNA preparation, the D-loops were almost absent because no special care had been taken to preserve them during the DNA isolation).

**DISCUSSION**

The present work has shown that, in the majority (<80%) of HeLa cell mtDNA

![Crosslinking pattern of Bam HI digested mtDNA photoreacted in vitro.](image)

Figure 6. Crosslinking pattern of Bam HI digested mtDNA photoreacted in vitro. See text for details.
molecules, there is in vivo a segment near the origin of replication, corresponding approximately to the D-loop region, which does not become crosslinked when the mtDNA is photoreacted in the presence of 4'-hydroxymethyl-4,5',8-trimethylpsoralen. Control experiments utilizing isolated mtDNA showed that the entire molecule, including the region around the origin, could be crosslinked in vitro to a high density, thus eliminating the possibility that some sequence peculiarity made the DNA in that region refractory to crosslinking. The bubbles observed near the origin of replication in the in situ photoreacted samples would be expected to include any D-loop and expanded D-loops which were present in the mtDNA molecules at the time of the photoreaction, and which could have become stabilized against branch migration as a result of psoralen crosslinking. However, the proportion of molecules with D-loops in the same in situ crosslinked preparations, as detected by electron microscopic examination of non-denatured samples, was found to be only about 25% (and that of the expanded D-loops, about 1%), in very good agreement with a previous estimate of the frequency of D-loops in human mtDNA preparations. Furthermore, a direct examination of the bubbles near the origin of replication in fully denatured samples of the same preparations indicated that about 80% of them were single-stranded in both arms, and therefore did not have the characteristic D-loop structure.

Excluding from consideration the fraction of mtDNA molecules which exhibited a D-loop under non-denaturing conditions, one can estimate that, in about 55% of the mtDNA molecules analyzed here, a region near the origin of replication is protected from crosslinking in situ by some structural feature other than the D-loop. This correction may actually result in an underestimate of the proportion of molecules with that particular structural feature near the origin of replication; in fact, nothing precludes the possibility that a true D-loop may also have in vivo the structural feature discussed above.

The most plausible interpretation of the nature of the structural feature which protects the HeLa cell mtDNA region near the origin of replication against in situ crosslinking by HMT is that it involves association of a site-specific protein or protein complex with the DNA. In fact, it is well established that binding of protein to DNA is apt to prevent psoralen crosslinking. The present work thus appears to give strong support to the idea of the in vivo association of a protein-containing structure with HeLa cell mtDNA near the origin of replication, which had been inferred previously on the basis of an analysis of the mtDNA molecules released by Triton X-100 lysis of mitochondria. In this earlier work, the electron microscopic observations had pointed to a probable derivation of the protein structure from the inner mitochondrial membrane to which the HeLa cell mtDNA would be attached in vivo. The present observations do not provide any evidence on this point. A membrane attachment of animal cell mtDNA
had been previously suggested on the basis of electron microscopic analysis of osmotically lysed mitochondria.\textsuperscript{37} The elucidation of the nature and functional role of the proteins bound to mtDNA near the origin of replication will have to wait for their isolation and biochemical characterization.

Observations similar to those reported here have recently been made by Potter et al. (1980)\textsuperscript{7} on Drosophila melanogaster mtDNA. However, the protected region observed in Drosophila mtDNA appeared as a series of 5 closely spaced bubbles of well-defined sizes corresponding to a total length of 2000 bp, rather than as a single bubble 300 to 1500 bp long as observed in most of the HeLa mtDNA molecules. In addition, the protected region in the Drosophila genome was located on the side of the origin of replication opposite to that in which H-strand synthesis proceeds, while in HeLa mtDNA the protected region extends from a position near the origin in the direction of H-strand synthesis. These differences may be due in part to the very different nature of the DNA sequence surrounding the origin of replication in the two organisms. In fact, in Drosophila mtDNA, the origin is embedded in an A+T-rich region, within which the protected region was mapped; no such A+T-rich region is present in the mammalian mitochondrial genomes.

In the \textit{in situ} crosslinked samples, the portion of the mtDNA contour outside the origin region had a double-stranded appearance over most of its length, exhibiting only rare bubbles, which were apparently randomly distributed. The failure to observe the 200 bp periodicity of crosslinking, which is characteristic of psoralen–crosslinked nuclear DNA, confirms the previous reports indicating absence of a nucleosomal structure and of typical histones in mtDNA.\textsuperscript{6,7}

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