ABSTRACT

The location of proteins on the mitochondrial DNA (mtDNA) of Drosophila virilis was investigated by Me3psoralen photoreaction of mitochondria isolated from embryos. After photoreaction the mtDNA was purified and the pattern of DNA cross-linking was determined by electron microscopy of the DNA under totally denaturing conditions. The transcribed regions of the mtDNA molecule contained some uncross-linked regions, but such regions were infrequent and randomly distributed. In contrast, the A+T-rich region around the origin of replication of the mtDNA was usually protected from psoralen cross-linking. The data were best fit by two protected sites, each approximately 400 base pairs, compared to the four 400 base pair sites observed in the equivalent region of D. melanogaster mtDNA [Potter et al. (1980) Proc. Nat. Acad. Sci. USA 77, 4118-4122]. Thus this region of the mtDNA appears to be involved in a DNA-protein structure that is highly conserved even though the DNA sequence has diverged rapidly relative to protein-coding sequences.

INTRODUCTION

The mitochondrial DNA of Drosophila is distinguished by a non-transcribed region that has a very high content of A and T bases and contains the origin of replication.1-5 This A+T-rich region shows rapid evolutionary change; from species to species it varies in size (1.0 - 5.1 kilobases) and in nucleotide sequence.3,6-8

Me3psoralen is a useful probe for the study of chromatin structure within intact cells, because it can enter cells and undergo a photochemical reaction to covalently cross-link the two polynucleotide strands of a DNA molecule. In the case of nuclear chromatin, the photoreaction is restricted to the internucleosomal linker DNA.9-12 The pattern of cross-linking on isolated DNA molecules therefore reflects some aspects of the chromatin structure in vivo. In an earlier study, we used Me3psorelen cross-linking to investigate the structure of the chromosome within the Drosophila melanogaster mitochondrion.13 We found that half of the 4.8 kilobase (kb) A+T-rich region in D. melanogaster mtDNA was protected from psoralen cross-linking in a manner that
indicated protein binding to this region of the DNA. The psoralen-protected region was adjacent to the origin of replication and appeared to be composed of four repeats each involving a stretch of approximately 400 base pairs (bp) of DNA.\textsuperscript{13}

If providing binding sites for specific proteins is an essential function for the A+T-rich segment of Drosophila mtDNA, then the pattern of psoralen protection should be conserved in Drosophila with smaller A+T-rich regions in their mtDNA. D. virilis has a 1.0 kb A+T-rich region, as small as any yet identified.\textsuperscript{3} Heteroduplex studies show no sequence homology between the A+T-rich regions of D. melanogaster and those of D. virilis, although the transcribed regions of the Drosophila mtDNAs are very similar.\textsuperscript{6,7} In the work reported here we show that, in spite of the differences in sequence and length, the A+T-rich region of D. virilis mtDNA is involved in a nucleoprotein structure that shows significant analogies to the structure in D. melanogaster DNA. It appears that the function of this A+T-rich region is conserved in spite of the extreme divergence of the nucleotide sequence, suggesting that some other features of the DNA segment, perhaps the bias in base composition, are more important in its function.

**MATERIALS AND METHODS**

Mitochondria were isolated from D. virilis embryos. Embryos were dechorionated by a 1 min treatment in 50% chlorox, washed in 0.15 M NaCl, and suspended in MSE (MSE is 0.21 M mannitol, 70 mM sucrose, 20 mM Tris, pH 7.6, 5 mM EDTA, 0.2% BSA). Cells were homogenized briefly and nuclei spun down by brief centrifugation at 1100 × g. Mitochondria were then pelleted at 14,500 × g, resuspended in MSE, and subjected to Me3psoralen photoreaction as described by Potter et al.\textsuperscript{13} Ten cycles of Me3psoralen addition and irradiation were used in an attempt to saturate all available sites on the mtDNA with the cross-linking agent. The mtDNA was then purified from the mitochondria. The mtDNA molecules, predominantly supercoiled and relaxed circular forms, were linearized by treatment with a restriction endonuclease known to cleave only once per mtDNA genome. The pattern of Me3psoralen cross-linking was analyzed by electron microscopy of the DNA under totally denaturing conditions.\textsuperscript{13}

**RESULTS**

Most of the contour length of each denatured mtDNA molecule had a predominantly double-stranded appearance (Fig. 1). With the one exception discussed below, uncross-linked "bubbles" in which the two DNA strands could
Figure 1. Electron micrographs of D. virilis mtDNA cross-linked in the mitochondrion. DNA was cleaved with Hae III and prepared for electron microscopy under totally denaturing conditions (after glyoxal treatment). Arrows indicate the two closely spaced uncross-linked bubbles regularly seen even on the most heavily cross-linked molecules, such as those shown here. (a) Full length mtDNA (last molecule of Fig. 2a). (b) End of a second mtDNA molecule (fourth molecule of Fig. 2a). d, Double-stranded φX174 form II DNA cross-linked separately and mixed with the mtDNA prior to spreading for electron microscopy. Bar = 1 kb.

be visualized were relatively infrequent (frequency = 0.1; see Fig. 2). Furthermore, they did not occur at reproducible locations relative to the ends of the molecule. The denaturing conditions used allow the reliable detection of uncross-linked regions in nuclear DNA, where Me3psoralen cross-linking occurs in linker regions between nucleosomes. Thus the apparent double-stranded regions in the mtDNA must be cross-linked significantly more often than once per 200 bp. This result indicates that the mtDNA of D. virilis, like that of D. melanogaster, is not in the nucleosomal chromatin structure typical of nuclear DNA.

On 75% of the molecules there was a significant denatured region located
Figure 2. Cross-linking maps of *D. virilis* mtDNA molecules cleaved with restriction endonucleases. a) Locations of uncross-linked bubbles on individual mtDNA molecules that were photoreacted with Me3psoralen in isolated mitochondria, then purified and cleaved with Hae III restriction endonuclease (see Fig. 1). All molecules of the correct length were included in the sample without regard to the position of the denatured bubbles. Each molecule was oriented with the half containing the largest denatured bubbles to the right. Shown here are the first 16 full-length molecules encountered on the electron microscope grid. b) Summary cross-linking histogram of 27 Hae III-cleaved molecules, including those shown in a. $\bar{X} = 15.56 \pm 0.48$ kb [mean length (relative to cross-linked φX174 DNA on the same grid) ± standard deviation]. Frequency; fraction of the molecules that had a denatured bubble in a given length increment. c) Cross-link histogram of 19 Bgl II-cleaved mtDNA molecules from the same experiment. $\bar{X} = 15.87 \pm 0.55$ kb. The histogram is offset by 47.5%, the distance between the Hae III and Bgl II restriction sites. d) Cross-link histogram of 23 mtDNA molecules that were deproteinized before being photoreacted for 80 sec with Me3psoralen. DNA was then cleaved with Hae III restriction nuclease and prepared for microscopy. $\bar{X} = 15.48 \pm 0.60$ kb. Molecules were oriented as in a. e) Shaded box; location of A+T-rich region. ▽; EcoRI restriction site.
at a fixed distance from the end. (On the remaining 25%, no such region was observed; see Discussion.) Analysis of the Hae III-cleaved mtDNA molecules (Fig. 2a, b) showed that the denatured region was located either at coordinates 83.3 - 89.2 or at coordinates 10.8 - 16.7 (all values ± 1.2; Hae III site defined as coordinate 0.0). Measurements of cross-linked mtDNA cleaved with Bgl II restriction nuclease (Fig. 2c) allowed us to place the denatured region either at 84.0 - 89.1 or at 5.9 - 11.0 (all values ± 1.5). Combining these two sets of data, it was clear that the major uncross-linked region was located entirely within the A+T-rich region, which spans coordinates 83.1 - 89.2.

The measurements allowed a more precise description of the major uncross-linked region at 83 - 89. In 63% of the cases it contained two denatured bubbles. Often these were of approximately equal size, as in Fig. 1. In 29% of the cases only one of the two bubbles was observed. In the remaining 8% there was either a single bubble or multiple small bubbles in this region. The data were fit best by two protected regions, one of 400 ± 68 bp and a second of 436 ± 144 bp. The first bubble was homogeneous in size. Its standard deviation (68 bp) was within the range of those measured for other cross-linked regions of this size, such as those occurring in the D. melanogaster mtDNA (394 ± 90 bp13) and those produced by nucleosome dimers (420 ± 60 bp12). The second bubble had a fixed left boundary but a variable right boundary, which resulted in the higher standard deviation for its measurement. The variable right-hand boundary appeared to be due to an additional, smaller protected region (270 bp) that was observed with a low frequency and, when it was seen, was usually not separated from the second bubble. Thus, a further interpretation of the Me3psoralen cross-linking pattern is that there are two protected regions of ~400 bp, the second of which is sometimes extended by an additional 270 bp. This model is summarized in Fig. 3.

Deproteinized supercoiled mtDNA was photoreacted with psoralen in vitro to give a frequency of cross-links similar to that of the DNA photoreacted in mitochondria. Molecules cleaved with Hae III restriction nuclease were measured and oriented in the same manner as the mtDNA photoreacted in mitochondria. As seen in Fig. 2d, the pattern of cross-linking in the region 83-89% from the end of the molecule was not distinguishable from that in the remainder of the molecule. Therefore we conclude that the prominent uncross-linked region observed when the mtDNA was photoreacted in mitochondria was not simply due to low Me3psoralen photoreactivity of DNA in the A+T-rich region of the genome.
DISCUSSION

The A+T-rich region of *D. melanogaster* mtDNA contains four protected regions of ~400 bp each, plus one smaller region of ~200 bp. The A+T-rich region of *D. virilis* mtDNA is too small to contain the same set of protected regions. Nevertheless, there is a clear analogy between the pattern of cross-linking in the two species. The major protected regions in the two mtDNAs have the same unit size, ~400 bp. In both species they are contained within the A+T-rich region and do not extend into the transcribed sequences (Fig. 3). The regions are both found near the origin of replication. However, the *D. melanogaster* region extends to the left on the mtDNA and covers the last DNA to be replicated in each round, while the *D. virilis* region extends to the right and covers the first DNA to be replicated. This last observation suggests that the structure is a membrane attachment site rather than a complex of replcative proteins, as the latter would not be expected to have such positional variation.

Estimates of the divergence time for *D. melanogaster* and *D. virilis* (the time since they had a common ancestor) range from 50 – 62 × 10⁶ years. The A+T-rich regions of their mtDNAs have no detectable homology in heteroduplex studies. In fact, there is already extensive sequence divergence between the mtDNA A+T-rich regions of *D. melanogaster* and *D. simulans*, and between those of *D. melanogaster* and *D. mauritiana*, pairs of species which are estimated to have diverged only 5 × 10⁶ years ago. Thus, the DNA-protein interaction in the A+T-rich region of the mtDNA has been conserved.
over a much longer time than has the nucleotide sequence of the same region.

Protection of the A+T-rich region from cross-linking was apparent on 75% of the mtDNA molecules in D. virilis and 80% in D. melanogaster. In both species, a substantial number of the molecules which were protected in this region had less than a full set of protection units (e.g., 29% of the D. virilis mtDNA molecules had only one 400 bp bubble). We previously determined that the probability of visualizing a 400 bp bubble under these electron microscopic conditions is in the range of 90 - 100%, so it is unlikely that collapse of single-stranded DNA bubbles is preventing us from seeing 400 bp regions that are protected. However, if the proteins provided less than total protection from psoralen cross-linking such that a cross-link was occasionally introduced within a 400 bp region, then the limitations of the electron microscopic technique would become evident. (The probability of visualizing a 200 bp bubble is only about 35%). In any case, we cannot exclude the possibility that a minor fraction of the mtDNA molecules has no proteins bound to the A+T-rich region.

Mitochondrial DNA molecules from species outside the melanogaster group are of the size of D. virilis mtDNA. Since the transcribed regions are evolutionarily conserved, it is likely that these species also have A+T-rich regions of 1 kilobase. The two repeats of the nucleoprotein structure seen in D. virilis mtDNA occupy -1 kilobase, and may represent the minimal functional unit of this structure. The D. melanogaster mtDNA appears to have a duplication of the minimal structure, resulting in four 400 bp stretches of psoralen-protected DNA. In earlier work we detected renaturation patterns in the D. melanogaster A+T-rich region that suggested a repetition of DNA sequences in the left half of the A+T-rich region, including the DNA protected from cross-linking.

Transcripts of the region around the origin are either absent or rare, at least in D. melanogaster. It is therefore quite possible that the only function of the A+T-rich region is the formation of this nucleoprotein structure. In that case, it is possible that evolutionary constraints on this region would be less severe than constraints on the RNA-encoding regions. For example, protein binding might be determined by the unusual base composition of the region rather than a specific nucleotide sequence. The species-specificity of the Drosophila mtDNA A+T-rich region plus its extremely biased base composition are characteristics reminiscent of the nuclear satellite DNA seen in many animals and plants. The locations of the nuclear satellite sequences around centromeres and the location of the A+T-rich region around
the origin of replication (and perhaps the site of membrane attachment) of the mtDNA suggest that both types of sequences may play structural roles.

The sequences surrounding the origin of replication in vertebrate mtDNA do not show the remarkable base composition bias seen in Drosophila mtDNA. There are, however, similarities between this region of Drosophila mtDNA and the analogous region in the mtDNA of vertebrates. Transcripts of the region around the origin are either absent or rare in all of these animals.\(^1\)\(^{18-21}\) Like the A+T-rich region of Drosophila mtDNA, the region surrounding the origin of vertebrate mtDNAs varies substantially in both length and sequence from species to species.\(^18,20-23\)

Albring et al.\(^24\) have shown that the HeLa cell mtDNA is attached near the origin of replication to a proteinaceous structure that is probably derived from the mitochondrial membrane. More recently, DeFrancesco and Attardi\(^25\) have shown that a region of the HeLa mitochondrial chromosome overlapping the site of the origin of replication is protected from psoralen cross-linking in vivo. The protected region varied in length from 300 - 1500 bp and did not have any repeated substructure as seen in Drosophila. Thus, while there may be major differences in the nature of the proteins involved, some type of stable DNA-protein interaction near the origin of replication appears to be a general feature of the mtDNA of higher eukaryotes. It is premature to dismiss the possibility that these proteins function directly in DNA replication or in transcription of the mitochondrial precursor RNA. The large size of the protected regions and their positional variability with respect to the origin of replication in the two Drosophila species, however, would seem most consistent with the possibility that the proteins have a structural role in anchoring the mtDNA to the inner mitochondrial membrane.

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