Visualization of DNA within mitochondria by osmium-ammine staining of mouse duodenal crypt cells

DONG FENG LIU, MOHAMED EL-ALFY and CHARLES PHILIPPE LEBLOND

Department of Anatomy, McGill University, Montreal, Quebec, Canada H3A 2B2

Summary

Previous investigators have examined mitochondrial DNA (mtDNA) in the electron microscope (EM) after extraction from mitochondria and rotary shadowing. We have observed mtDNA in situ by the osmium-ammine procedure for specific staining of DNA in the EM. The procedure was modified to improve the regularity of the staining and then applied to the rapidly dividing cells present in mouse duodenal crypts. In the stained sections of these cells, 25% of the mitochondria exhibited discrete reactive filaments. The filaments, whether observed directly or in stereopairs, appeared either irregular or arranged into distinct patterns, some of which were similar to those previously described after rotary shadowing of duplicating mtDNA: namely, simple and double circular figures, displacement loops and supercoiled forms. The filaments could be traced in serial sections of the same mitochondria and, therefore, were not artifacts. Moreover, their disappearance after DNase digestion demonstrated that they were composed of DNA. It is concluded that mtDNA can be visualized by the modified osmium-ammine technique and may show patterns that can be interpreted as phases in its replication.

Key words: mitochondria, osmium-ammine, mitochondrial DNA.

Introduction

After extraction from mitochondria and rotary shadowing, mitochondrial DNA (mtDNA) has been visualized as a closed circle formed by a 5 μm long double-stranded molecule (Sinclair and Stevens, 1966; Radloff et al., 1967; Wolstenholme et al., 1973; Piko and Matsumoto, 1977). The nucleotide sequence of the mtDNA molecule in mice consists of 16,295 base pairs, which code for two ribosomal rRNAs, 22 tRNAs and 13 proteins, five of which have been identified (cytochrome b, three cytochrome c subunits, and an ATPase subunit) (Bibb et al., 1981). Mitochondria also import many proteins encoded by nuclear DNA and synthesized in the cytoplasm (Attardi and Schatz, 1989).

In contrast to the extensive biochemical information on mtDNA, in situ morphological observations are few. Nass and Nass (1963a,b) have used sections of Epon-embedded chick embryos stained with uranyl and lead salts, and observed intramitochondrial fibers, which appear to be DNase-sensitive. Recently, Testillano et al. (1991) used sodium hydride methylation/acetylation followed by uranyl acetate to stain DNA-containing structures and depicted a stained fiber in a mitochondrion from Lowicryl-embedded onion root meristematic cells. However, neither this approach nor that of Nass and Nass (1963a,b) provided results as specific for DNA as the osmium-ammine procedure devised by Cogliati and Gautier (1973). This procedure stains nuclear DNA exclusively and no longer does so after DNase treatment, thus demonstrating its specificity. Moreover, it shows DNA in chromatin as 3 nm thick filaments that encircle electron-lucent spaces; the width of these structures, on average, is 12 nm, like that of nucleosomes (Derenzini et al., 1982). The staining procedure has been extensively used to detect not only nuclear and nucleolar DNA (Derenzini et al., 1977, 1990; Derenzini, 1979; Puvion-Dutilleul and Puvion, 1980) but also viral DNA in infected mouse hepatocytes, where it appears as 2-3 nm thick filaments (Derenzini et al., 1987).

Since mtDNA has not yet been visualized in situ by the osmium-ammine procedure, we have attempted to do so in mouse duodenal crypt cells. The procedure is carried out in two steps. First, Epon sections of formaldehyde-fixed tissue are hydrolyzed in hydrochloric acid to activate the DNA aldehyde groups; the procedure, however, yields erratic results (Derenzini et al., 1990), due in part to the sulfur dioxide being gradually released from the osmium-ammine complex during the staining period. Thus, the nuclear stain may show little contrast and precipitates may appear over the sections. We have modified the procedure in such a way as to prevent the loss of sulfur dioxide, by carrying out the reaction under pressure in a closed vessel. Reproducible results are thus obtained,
although precipitates appear occasionally; but even then the nuclear DNA is well stained.

Since the amount of mtDNA is maintained in dividing cells (Berk and Clayton, 1974), it was presumed that such cells would be likely to show figures of mtDNA duplication. In this hope, the osmium-ammine staining procedure was applied to the rapidly dividing cells of mouse duodenal crypts. Examination of mitochondria in regular and stereopaired micrographs revealed the presence of fine filaments in various forms: circles, curls, folds and supercoils. Evidence was presented that the filaments consisted of DNA.

Materials and methods

Seven adult male Swiss albino mice, approximately 3 months of age and weighing 32 to 35 g, were anesthetized with sodium pentobarbital. A piece of duodenum about 1.0 cm long was cut next to the pylorus and fixed by immersion for 4 h in 4.0% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, to which 2 mM calcium chloride was added to stabilize DNA (Schreil, 1964). In another animal, a piece of duodenum was fixed by immersion in mixed aldehydes (2.5% glutaraldehyde and 2% formaldehyde) in the same buffer. No postfixation in osmium tetroxide was used. After either method of fixation, the duodenal wall was cut into 1 mm² pieces, which were washed in buffer overnight, dehydrated in a graded acetone series and taken through increasing concentrations of Epon in acetone at room temperature. The embedding in Epon was carried out at 58°C. Blocks were oriented so that the plane of section was parallel to the mucosal surface and, therefore, duodenal crypts were cut in cross-section. Straw-colored sections were prepared at a level between three and nine cells away from the crypt bottom. In some cases, sections were cut serially and each section was placed on a different 200-mesh gold grid.

The grids with sections facing down were floated on 5 N hydrochloric acid for 30 min at room temperature, washed by dipping in two distilled water baths, floated for 1 h over distilled water, washed again and dried on filter paper. Meanwhile a 1% saturated solution was prepared of either osmium-ammine or osmium-ammine B (Olins et al., 1989), both from Polysciences, Inc; the former was preferred, since it seemed to produce better contrast. The solution was bubbled with sulfur dioxide for 30 min. After the bubbling was stopped, the solution turned brownish yellow, while a black pulverulent material settled down. The brownish yellow solution was pipetted out and deposited on small wells in a glass plate. One grid per well was floated section-face down on the solution. The plate was covered with an aluminum sheet and placed over a large waterlogged filter paper in a pressure cooker. The pressure cooker was then filled with sulfur dioxide gas under a pressure of 2 lb/in² (1 lb/in² = 6.9 kPa), and moved into a 37°C oven, where it was kept for 4-11 h. The grids were then removed, dipped in two baths of distilled water, dried and examined in a Philips EM 400.

Filaments may form distinct circles (Figs 1, 7-10). In such cases, the circular filament may branch at some point into two threads that recombine farther on, with one of the threads appearing thicker than the other (Fig. 7). In other cases, two concentric circles may be distinguished (Fig. 8). The circular structure depicted in Fig. 9 extends filaments within itself and also shows an acute angle at the lower right, suggesting continuity with a filament outside the plane of section. Similarly, in Fig. 10 the circular structure is connected to an irregular filament within the plane of section.

Stereopairs prepared for examination of osmium-ammine-stained filaments in three dimensions have confirmed direct observations and shown patterns with added clarity, such as the frequent folding of filaments upon themselves (Fig. 11), coiling of two filaments around each other (Fig. 12), and circular or elongated structures, from which filaments extend within and without (Figs 13, 14). In Fig. 13, a group of filaments seems to be limited by a smooth ovoid surface, except at the base, from which a filament emerges. In Fig. 14, a circular group at the left and an elongated group at right are composed of irregular filaments extending in three dimensions, but again limited by a smooth surface, except for narrow areas from which filaments emerge. In particular, such outside filaments appear to connect the two main groups.

Mitochondrial filaments have been analyzed in two ways. First, stained filaments have been searched for in

DNase digestion

Thin sections of formaldehyde-fixed tissue were incubated in about 0.4 ml of 0.1% DNase I (Calbiochem Corporation, CA) in 0.01 M Tris buffer, at pH 7.4, containing 1.0 mM magnesium chloride. The incubation was carried out at 37°C for 12 to 16 h. The grids were then stained with osmium-ammine as above.

Results

When sections of duodenal crypt cells fixed in formaldehyde are stained with osmium-ammine, the nucleus shows an intense reaction limited to chromatin clumps and strands (Fig. 1). In the cytoplasm, even though the density of mitochondria and endoplasmic reticulum cisternae makes their profile visible, the only structure stained by osmium-ammine consists of delicate, irregular filaments present in some mitochondria (Fig. 1). Examination of 278 mitochondrial profiles in one animal has shown stained filaments in 25% of them. The mixed aldehyde fixative used in one animal has yielded a similar pattern of intramitochondrial filaments in 24% of 188 mitochondria.

Detailed examination of stained mitochondrial filaments reveals wide variation in their size, shape and density. A filament may consist of a single straight or slightly curved strand (Fig. 2) or two filaments may run more or less parallel (Figs 3, 4). Two filaments may be associated in more distinctive pattern, in which they wind around each other in a loose (Fig. 5) or tight manner (Fig. 6).

Filaments may form distinct circles (Figs 1, 7-10). In such cases, the circular filament may branch at some point into two threads that recombine farther on, with one of the threads appearing thicker than the other (Fig. 7). In other cases, two concentric circles may be distinguished (Fig. 8). The circular structure depicted in Fig. 9 extends filaments within itself and also shows an acute angle at the lower right, suggesting continuity with a filament outside the plane of section. Similarly, in Fig. 10 the circular structure is connected to an irregular filament within the plane of section.
Fig. 1. Mouse duodenal crypt cell stained with osmium-ammine. In this and the following figures, the osmium-ammine stain has been applied to sections of formaldehyde-fixed materials. No counterstain has been used. The nucleus at the left shows stained chromatin patches (arrowheads) while the nucleoplasm (NP) is unstained; some of the stained patches follow the nuclear outline, with occasional interruption at a nuclear pore (P). In the cytoplasm (Cyt), profiles of mitochondria (Mt) and other organelles may be distinguished. Within several of the mitochondria, stained filaments (arrows) appear delicate, but the range of their electron density is comparable to that of nuclear chromatin. Several of the filaments appear to be folded. One forms a distinct circle (double arrows). The variously shaped filaments are interpreted to be mitochondrial DNA. \( \times 35,000 \). Bar, 500 nm.

two successive sections of the same mitochondrion. Thus, in Fig. 15, adjacent sections labeled A and B show parts of the same three mitochondria. Two of these include a filament traceable from section to section. Secondly, sections treated with DNase show no material stained with osmium-ammine (Fig. 16). The absence of stain in the nuclear areas normally occupied by chromatin makes them appear light, while no filaments are observed in mitochondria.

Discussion

The first question raised by the finding of mitochondrial
filaments stained by the osmium-ammine procedure in duodenal crypt cells was whether or not the stained material was an artefact. First, such filaments were never seen outside profiles of mitochondria and, therefore, they could only be components of the organelle. Secondly, the presence of stained filaments in approximately the same location in successive sections of mitochondria (Fig. 15) made it likely that they were real and not artefactual. The next question was whether the filaments consisted of DNA. When sections had been pre-incubated in DNase prior to the osmium-ammine procedure, neither intramitochondrial filaments nor intranuclear chromatin were stained (Fig. 16). It was concluded that the various types of stained intramitochondrial filaments were composed of DNA, in accord with the literature summarized in the Introduction, which indicates that every osmium-ammine-stained structure, to date, was composed of DNA.

Several precautions were taken to minimize DNA
Visualization of DNA within mitochondria

Figs 7-10. Faintly outlined mitochondrial profiles contain circularly arranged osmium-ammine-stained filaments. Under Figs 7 and 8 a hand-drawing interprets the detail of the stained filaments. x137,500. Bar, 100 nm.

Fig. 7. A darkly stained circular filament (284 nm long) branches at the left into two parallel lines that form a weakly stained loop (232 nm long) extending over nearly half of the circumference. The filaments are mainly composed of minute dots in succession.

Fig. 8. Within a darkly stained circle (337 nm long), there is a weakly stained circular filament (234 nm long).

Fig. 9. In the center, a large circular filament ends in an acute angle at the lower right, suggesting the possibility of continuity with filaments outside the plane of section, and thus perhaps joining the other two groups of filaments seen in the same mitochondrion. In addition, several filaments located within the large circle seem to be in continuity with it.

Fig. 10. A circular filament composed of successive dots is associated with a small, complex extension on its lower left side.

disruption, especially the addition of calcium to the fixative (Schreil, 1964); also acetone was used instead of ethanol for gentler dehydration, and Epon polymerization was carried out at 58°C; that is, below the temperature required for DNA denaturation. The regularity of some of the observed filaments, such as those twisted around each other, indicated that, in these cases at least, DNA disruption had been avoided.

The duodenal crypt base cells used in the present study were known to proliferate actively, since the duration of their cell cycle was 12.3 h (El-Alfy and Leblond, 1988). Presumably, the mtDNA had to be replicated at each cell cycle (Berk and Clayton, 1974) and, therefore, the patterns associated with replication were expected to be visualized by osmium-ammine staining. When the observed filaments were compared with the various stages in the model of mtDNA replication (Fig. 17) devised by Clayton (1982), on the basis of the biochemical and electron microscopic studies by his group and others (Sinclair and Stevens, 1966; Wolstenholme et al., 1973; Piko and Matsumoto, 1977), some similarities were observed. Thus the branching filaments in Fig. 7 could correspond to the D-loop occurring in the early stages of duplication (Fig. 17A,B). The association of two adjacent circles in Fig. 8 could represent parts of two newly formed circles about to separate (Fig. 17, at a stage between C and D). The two filaments coiled to different degrees in Figs 5, 6 and 12 could be part of a supercoiled mtDNA molecule (Fig. 17E).

The circumference of the observed circles (Figs 7, 8), usually between 200 and 400 nm, was considerably
smaller than that of the mtDNA molecule, which has been recorded at 5000 nm by rotary shadowing (Sinclair and Stevens, 1966; Radloff et al., 1967). Moreover, some of the circles had shapes suggesting continuity with filaments outside the plane of section (Figs 9, 10). Three-dimensional views confirmed the continuity between circles and filamentous groups (Figs 13, 14). It was concluded that the depicted circular figures were parts of, not whole, mtDNA molecules.

To account for the complexity of some filamentous groups, two factors were considered. First, Klenin et al. (1991), with the help of computer simulation, obtained
Visualization of DNA within mitochondria

Fig. 15A,B. Two successive sections, A and B, show profiles of the same three mitochondria, Mt1, Mt2 and Mt3, after osmium-ammine staining. In A stained filaments are present in all three mitochondria. In B stained filaments are absent in one of them (Mt2). In Mt1 the same filament seems to extend from one section to the adjacent one. In Mt3 a group of filaments extends in both sections. ×60,500. Bar, 200 nm.

Fig. 16. Duodenal crypt cell treated with DNase and stained with osmium-ammine. In the nucleus (N) the chromatin clumps lining the nuclear envelope and surrounding the nucleolus (Nu) appear pale (arrowheads). In the cytoplasm mitochondrial profiles can be distinguished (arrows), but intramitochondrial filaments are absent. ×17,500. Bar, 100 nm.

Figures indicating that parts of a given circular DNA molecule could take the supercoiled form at a different degree of twisting, while other parts were relaxed. This was in accord with the view that the various circular structures depicted in our figures were parts of large irregular circles, the rest of which was seen as filaments. Secondly, since mtDNA molecules were known to be enclosed by the internal mitochondrial membrane (Clayton, 1982) they were located within the matrix. They must fit within the intercristal spaces and, therefore, their three-dimensional shape was likely to involve folds and curls. The presence of a smooth edge
Fig. 17. Model for mtDNA replication (slightly modified from Clayton, 1982). (A) The circular mtDNA molecule is composed of two strands referred to as "light" (L) and "heavy (H) parental strands". At a point, O_H, known as the origin of the daughter heavy strand and defined by a distinct series of base pairs, the two strands separate and synthesis of complementary DNA begins along the parental light strand, thus giving rise to the daughter heavy strand (broken line labeled d_H). Meanwhile, the parental heavy strand, which does not synthesize DNA at this stage, extends outside the circle while remaining attached to it at both ends. Together, the separated heavy and light strands as well as the daughter heavy strand associated with the latter form a triplex structure known as the "displacement loop" or D-loop. (B) The separation of the two parental strands continues while the D-loop lengthens and the daughter heavy strand extends along the parental light strand. (C) When the duplication of this strand is 67% complete, synthesis of a daughter light strand d_L begins along the parental heavy strand at a point, O_L, known as the origin of the daughter light strand, but progresses in the opposite direction. (D) It is likely that, just before the daughter light strand is completed, the two circles separate from each other. (E) Negative (right-handed) superhelical turns are introduced into the circles. There may be 100 of these turns per molecule.

around grouped irregular filaments (Figs 13, 14) could indicate contact with the surface of cristae.

In recent unpublished work, we observed mitochondrial filaments in circular and other patterns in the cells of various tissues (hepatocytes, epithelial cells of colon and pyloric antrum).

It is concluded that the modified osmium-ammine procedure makes it possible to visualize mitochondrial DNA in situ. The results provide hope that a detailed analysis of mtDNA will be possible under various physiological and pathological conditions, particularly in mitochondrial diseases, such as the Kearnz-Sayre syndrome.

This work was supported by a grant from the Medical Research Council of Canada. Dr. Y. Clermont's critical reading of the manuscript is acknowledged.

References


(Received 5 August 1991 - Accepted, in revised form, 2 January 1992)