MITOCHONDRIAL DNA FROM 4-CELL STAGES OF ASCARIS LUMBRICOIDES

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SUMMARY
Mitochondrial DNA (mtDNA) has been isolated from 4-cell stages of Ascaris lumbricoides. This DNA amounts to about 40% of the total quantity of 4-cell-stage DNA. Its buoyant density in neutral CsCl gradients is 1.686 g cm⁻³. Electron microscopy of mtDNA demonstrated the presence of circular molecules with an average contour length of 464 µm. About 15% of these molecules are supercoiled, covalently closed circles, whereas some 2% consist of double-forked circular molecules. The form and size of these branched molecules suggest that they are replicative intermediates.

INTRODUCTION
Mitochondrial DNA (mtDNA) of all metazoan animals isolated thus far has been shown by electron microscopy to comprise circular molecules with species-specific contour lengths ranging from 4.45 to 5.85 µm (see Borst & Kroon, 1969; Wolstenholme, Koike & Renger, 1971; Wunderlich, 1971; and Borst, 1972, for review). Circular eukaryotic DNA molecules of non-mitochondrial origin have also been found; however, these DNA molecules are not of uniform size (Hotta & Bassel, 1965; Smith & Vinograd, 1972).

A study of the process of chromatin elimination in the nematode worm Ascaris lumbricoides revealed that DNA extracted from 4-cell stages separates into 2 bands of different densities by isopycnic centrifugation (Tobler, Smith & Ursprung, 1972). The light-peak DNA was shown by electron microscopy to contain structures typical of mtDNA: circular molecules with a contour length of about 5 µm. In independent experiments, Carter, Wells & MacInnis (1972) reported that mtDNA isolated from male reproductive organs of anaerobic adult Ascaris lumbricoides worms had a buoyant density which differed somewhat from that of our own preparations. It is the purpose of the present study to analyse the structure and length of the mtDNA molecules from 4-cell stages of A. lumbricoides and to compare the results with those of Carter et al. (1972). Furthermore, since a large amount of total 4-cell-stage DNA consists of mtDNA (Tobler et al. 1972), we wondered whether we might be able to detect branched mtDNA molecules that are presumed replicative intermediates.
MATERIAL AND METHODS

Isolation of 4-cell stages from Ascaris lumbricoides

Adult females of Ascaris lumbricoides worms were collected from infected pigs in the local slaughterhouse and brought immediately to the laboratory. The isolation and incubation of eggs and the peeling of 4-cell stages were carried out as previously described (Tobler et al. 1972).

Extraction of mtDNA

Total DNA from 4-cell stages was isolated following the method of Bielka, Schultz & Böttger (1968). The separation of mtDNA from nuclear DNA was accomplished by CsCl density centrifugation. Solid CsCl was added to the DNA dissolved in 0.1 x SSC to give a final density of 1.70 g cm\(^{-3}\) and the solution was centrifuged in the No. 50 fixed angle rotor (33000 rev/min for 68 h at 20 °C) in a Beckman model L50 ultracentrifuge. (SSC = 0.15 M NaCl, 0.015 M sodium citrate.) After centrifugation, 0.15-ml fractions were collected from the bottom of the punctured centrifuge tubes. The fractions were diluted to 1 ml with 0.1 x SSC and the absorption at 260 nm determined with a Gilford model 2400 spectrophotometer. Peak fractions containing the nuclear and mtDNA were separately collected, pooled and dialysed against 0.1 x SSC for about 24 h at 4 °C.

Determination of the buoyant density of DNA

Isopycnic centrifugation was carried out in a Beckman model E analytical centrifuge with an AN-L rotor and a Kel-F centrepiece at 44000 rev/min and 25 °C to density equilibrium. Before centrifugation, the density of the CsCl solution was set to 1.70 g cm\(^{-3}\). DNA from Streptococcus mediterraneus (1.79 g cm\(^{-3}\)) was used as a density standard. Tracings of ultraviolet-absorption films were made on a Joyce-Loebel recording microdensitometer.

Spreading and shadowing

DNA was spread for electron microscopy following the method of Kleinschmidt & Zahn (1959). The hyperphase consisted of equal volumes of 1 M ammonium acetate containing 0.05 % cytochrome c and 8 μg/ml DNA dissolved in 0.1 x SSC. This solution was spread on 0.25 M ammonium acetate. The DNA-protein film was picked up on carbon-coated grids. After short immersion of the grids in distilled water and absolute ethanol, the specimens were air dried and shadowed with Pt under rotation (30 rev/min) at an angle of 5°. The distance between the source of platinum and the specimen was 5 cm.

Electron microscopy and length measurements of DNA molecules

Electron micrographs were taken with a Hitachi HS-8 electron microscope at magnifications of 10000 and 20000, respectively. The magnification of the instrument was calibrated with a diffraction grating replica (2160 lines/mm). Molecules were measured with a map ruler on positive prints at a total magnification of 194000 times.

RESULTS

Analytical and preparative CsCl buoyant density profiles of 4-cell-stage DNA

Analytical centrifugation of 4-cell-stage DNA from A. lumbricoides showed 2 bands: a heavy band with a density of 1.697 g cm\(^{-3}\) and a lighter band with a density of 1.686 g cm\(^{-3}\). Both values are in accord with our earlier results (Tobler et al. 1972). On the other hand, the fraction of the light-band DNA amounts to more than 25 % of the total mass of 4-cell-stage DNA, thus exceeding the value previously reported by Tobler et al. (1972). According to our present data, the percentage of light-band
DNA from Ascaris

DNA varies between 30 and 50% (n = 6) of the total amount of 4-cell-stage DNA in different DNA samples. The values were determined on absorption profiles of preparative CsCl density gradients.

Electron microscopy of 4-cell-stage DNA

Electron microscopy of the heavy-band DNA (\( \rho = 1.697 \text{ g cm}^{-3} \)) from preparative CsCl gradients demonstrated the presence of linear, unbranched molecules (Fig. 1). The length of the DNA molecules varies from very short fragments to molecules of up to 19 \( \mu \text{m} \). There is no detectable regularity in the size distribution of individual DNA molecules.

Light-band DNA (\( \rho = 1.686 \text{ g cm}^{-3} \)) from preparative CsCl gradients showed many circular structures in the electron micrographs (Fig. 2). The circular molecules are either open (Fig. 3a) or twisted (Fig. 3b, c). The twisted DNA molecules have been arbitrarily subdivided into loosely twisted circles carrying less than 20 points of intersection (Fig. 3b) and into highly twisted, supercoiled circles with more than 20 points of intersection (Fig. 3c, arrow). Some 300 circular DNA molecules have been examined, and on average, the frequencies of the different structural types were as follows: 10% open circles, 75% loosely twisted circles, and 15% supercoiled circles. Contour length measurements were carried out on 50 mtDNA circles with no or few twistings. Under the spreading conditions used (p. 594), a contour length of 4.64 ± 0.03 \( \mu \text{m} \) was established for \( A. \) lumbricoides mtDNA. Since supercoiled circles are too tightly twisted, it was not possible to measure accurately the contour length of these structures. However, the estimated values for supercoiled structures are in good agreement with the length determinations on open and loosely twisted circles. Linear molecules of variable lengths, but not exceeding the contour length of circular molecules, are also regularly found in the light-band fraction from preparative CsCl density gradients. These DNA molecules are interpreted to represent fragments of circular DNA molecules.

Based on the assumption that the isolated mtDNA is present in B-configuration, the length of 4.64 \( \mu \text{m} \) for circular mtDNA of \( A. \) lumbricoides corresponds to a molecular weight of 8.88 \( \times 10^6 \) Daltons (Caro, 1965). It should be noted, however, that different isolation and spreading conditions for DNAs have significant effects on contour-length determinations (Inman, 1967; Lang, Bujard, Wolff & Russel, 1967; Wellauer, Weber & Wyler, 1973).

Six out of 300 examined circular mtDNA molecules with no or few twistings showed double-forked structures (Fig. 4). For each such molecule, two of the three segments delimited by the forks were of equal length, while the third one was always longer. The sum of the lengths of one of the two shorter and the longer segment was found not to differ significantly from the length of unbranched open circular molecules in the same preparation. Moreover, all three segments of the same double-forked molecule had a rigid appearance and high contrast similar to the DNA molecules lying near to them on the same grid. Since single-stranded DNA molecules are lower in contrast relative to double-stranded DNA under the conditions used, we assume that the 2 segments of about equal length consist also of double-stranded DNA.
Thus, the double-forked circular mtDNA molecules are interpreted to represent replicative intermediates of the Cairns (1963) type. No double-forked molecules were found in which the duplicated segments exceeded 16% of the contour length of unbranched circular modules. We take this to mean that replicating molecules containing large duplicated segments are more susceptible to strand breaks in the region of the fork than mtDNA circles with only small duplicated segments.

**DISCUSSION**

Bielka et al. (1968) isolated DNA from eggs of *A. lumbricoides* and assumed that the DNA with a buoyant density of 1.685 g cm⁻³ represents mtDNA. This assumption has been directly verified by electron microscopy and renaturation kinetics (Tobler et al. 1972). The present study confirms and extends our earlier results: DNA from 4-cell stages of *A. lumbricoides* with a density of 1.686 g cm⁻³ consists of circular molecules with a contour length of 4.64 ± 0.03 μm. Electron microscopy further demonstrates the presence of either supercoiled, loosely twisted, or open circles. The supercoiled molecules are native double-stranded, covalently closed circular DNA molecules, whereas the loosely twisted and the open circles correspond to nicked double-stranded, circular molecules with at least one single-strand break (Borst & Kroon, 1969).

Assuming that mtDNA from *A. lumbricoides* does not contain uncommon bases, the average G + C content of native mtDNA may be calculated as 27%, from a buoyant density of 1.686 g cm⁻³, using the equation of Mandel, Schildkraut & Marmur (1968). Such a low G + C content has never been reported for mtDNAs from vertebrates; however, Polan, Friedman, Gall & Gehring (1973) and Bultmann & Laird (1973) demonstrated that mtDNA from *Drosophila melanogaster* has a buoyant density of 1.681 g cm⁻³, thus corresponding to an average G + C content of 21%.

Carter et al. (1972) isolated circular mtDNA molecules with a contour length of 4.79 μm from male reproductive organs of anaerobic adult *A. lumbricoides* worms. Considering the fact that different preparation and spreading conditions have large effects on contour-length determinations of spread DNA (Inman, 1967; Lang et al. 1967; Wellauer et al. 1973), the 3% lower value determined in the present experiments is in good agreement with that reported by Carter et al. (1972). Furthermore, Carter et al. (1972) reported buoyant densities of 1.690 g cm⁻³ for mtDNA and 1.698 g cm⁻³ for nuclear DNA of *A. lumbricoides* worms. The difference between their and our values cannot be due to different experimental conditions. However, since the density for nuclear DNA determined by Carter et al. (1972) is also 0.001 g cm⁻³ higher than our value, and since the buoyant density of mtDNA varied in the range of 1.688–1.690 g cm⁻³ when DNA preparations contained a large amount of nuclear DNA (Carter et al. 1972), we do not yet know whether there exists a real qualitative difference between mtDNA isolated from 4-cell stages and from adult tissues of anaerobic *Ascaris lumbricoides* worms. In yeast, Criddle & Schatz (1969) have shown that the buoyant density of mtDNA does not differ between cultures that were grown under aerobic and anaerobic conditions, respectively.

On the average, 40% of the total amount of 4-cell-stage DNA consists of mtDNA.
This value is somewhat higher than the one reported earlier (Tobler et al. 1972). Although we always used identical isolation methods in our experiments, the fraction of mtDNA varied from 30 to 50% in different DNA preparations. We do not yet know whether technical reasons alone account for this rather high variability.

Much experimental evidence indicates that mtDNA is synthesized in the mitochondria themselves (Borst & Kroon, 1969; Rabinowitz & Swift, 1970; Wunderlich, 1971; Borst, 1972). The details of the DNA replication process remain to be elucidated. Since mitochondria and bacteria share many organizational features (see e.g. Goodenough & Levine, 1970), it is possible that mtDNA follows the same replication mechanism as has been proposed by Cairns (1963) for the replication of E. coli DNA. The presence of presumed replicative intermediates of the Cairns type in mtDNA from higher organisms suggests that mtDNA may replicate like bacterial DNA. Thus far, such replicative intermediates have been described only for mtDNAs from adult rat and mouse liver tissues (Kirschner, Wolstenholme & Gross, 1968; Arnberg, Van Bruggen, TerSchegeget & Borst, 1971; Wellauer et al. 1973), 6-day-old chick embryos (Wolstenholme, Koike & Cochran-Fouts, 1973), and Chang and Novikoff rat hepatomas (Wolstenholme et al. 1973). The present communication therefore represents the first demonstration of replicative intermediates for mtDNAs in invertebrates.

The double-forked mtDNA molecules from A. lumbricoides 4-cell stages appeared in the electron microscope to be totally double-stranded. This type of presumed replicative intermediate has first been reported for mtDNA by Kirschner et al. (1968). On the other hand, double-forked circular mtDNA molecules in which either all or a considerable part of one daughter segment is single-stranded, have recently been described by several workers (Kasamatsu, Robberson & Vinograd, 1971; Arnberg et al. 1971; Robberson, Kasamatsu & Vinograd, 1972; Wolstenholme et al. 1973; Koike & Kobayashi, 1973). Since we observed only few double-forked mtDNA molecules in our preparations, it remains uncertain whether in A. lumbricoides mitochondria too, one DNA strand ordinarily replicates in advance of the complementary strand. From an evolutionary standpoint, it is rather to be expected that mtDNA of all higher organisms follows the same replication mechanism.

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REFERENCES


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Fig. 1. Electron micrograph of the heavy-band DNA (1.697 g cm⁻³) isolated from 4-cell stages of *A. lumbricoides*. Only linear, unbranched molecules of variable lengths are seen.

Fig. 2. Electron micrograph of circular mtDNA isolated from the light satellite (1.686 g cm⁻³) of *Ascaris* 4-cell-stage DNA.
Fig. 3. Electron micrographs of circular mtDNAs isolated from 4-cell stages of *A. lumbricoides*. A, open, nicked circle; B, loosely twisted circle; C, supercoiled, intact circle (arrow).
Fig. 4. Double-forked circular mtDNA molecule isolated from 4-cell stages of *A. lumbricoides*. The 2 segments between the forks (arrows) are of equal lengths.