CYTOPLASMIC DNA IN THE UNFERTILIZED SEA URCHIN EGG: PHYSICAL PROPERTIES OF CIRCULAR MITOCHONDRIAL DNA AND THE OCCURRENCE OF CATENATED FORMS*

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The mitochondrial DNA in the unfertilized egg of the sea urchin *Lytechinus pictus* is present in an amount approximately seven times that of the haploid nuclear DNA.[1] The mitochondrial DNA has a higher buoyant density than the nuclear DNA and consists of circular duplex molecules of a uniform size of about 5 μ. The circular DNA has been recovered[1] in both the intact (closed) and nicked (open) states characteristic of the circular duplex viral DNA's[2] and the mitochondrial DNA's from birds and mammals.[3, 4]

In the present study covalently closed circular duplexes were isolated by buoyant density centrifugation in ethidium bromide–CsCl density gradients.[5] This DNA has been characterized by electron microscope and sedimentation methods. Electron microscope examination revealed that a significant fraction of mitochondrial DNA molecules are in the form of interlocked dimers or catenanes such as have been recently observed in the mitochondrial DNA from HeLa[6] cells and from leucocytes of leukemic patients.[7]

Materials and Methods.—Chemicals: Optical grade CsCl was obtained from the Harshaw Chemical Co. The ethidium bromide was a gift from Boots Pure Drug Co., Ltd., Nottingham, England. Sodium dodecyl sulfate (SDS) was obtained from the Matheson Company. All other chemicals were of reagent grade.

Isolation of DNA and fractionation in an ethidium bromide–CsCl density gradient: Mature unfertilized eggs of *Lytechinus pictus* were collected, washed, and freed of the gelatinous coat as described by Tyler and Tyler.[8] The packed eggs were homogenized with 3 vol of a medium containing SDS and ethylenediaminetetraacetate (EDTA).[1] The homogenate was divided into three equal aliquots and solid CsCl added to densities of 1.55, 1.65, and 1.75 gm/ml. The solutions were layered into SW25 tubes to a volume of 28 ml. Each tube contained the homogenate from 5 to 6 ml of packed eggs. After centrifugation at 23 Krpm for 72 hr at 20°C in a Beckman preparative ultracentrifuge, the contents of the tubes were drained through a needle inserted about 1 cm above the bottom of the tube. A 5-ml vol containing the turbid polysaccharide band and the DNA[1] was collected from each tube. This material either was used directly in ethidium bromide–CsCl density gradients or was diluted with 2 vol of water and precipitated with an equal volume of ethanol. After 16 hr at 4°C, the precipitate was washed by centrifugation 2× with cold ethanol and 1× with ether, and air-dried. The precipitate was dissolved in a CsCl solution, density 1.55 gm/ml, 0.01 *M* tris, pH 7.6. Ethidium bromide in 1.55 gm/ml CsCl was added to a final dye concentration of 300 μg/ml. Thirty to 50 μg of DNA, from 3 to 5 ml of packed eggs, in 5 ml of dye–CsCl were centrifuged 48 hr in an SW65 rotor at 43 Krpm at 20°C. The tubes were fractionated in 27-μliter fractions with a Buchler drop-collecting unit into nonfluorescent plastic trays obtained from Linbro Chemical Co. The centers of the upper and lower bands were located in a darkened room with near ultraviolet light. The trays were covered and stored at −20°C. The fractions were used directly in electron microscopy. For centrifuge studies the dye was removed by chromatography through a 0.25-ml bed of Dowex 50W-X4 resin (Cs^+). The

effluent solution was diluted with 0.015 *M* NaCl, 0.0015 *M* Na citrate, pH 7 (one-tenth strength standard saline citrate) to 5 ml and concentrated by centrifugation in an SW65 rotor at 55 Krpm, 20°C, for 12 hr. The supernatant was totally decanted and the pellet redissolved in a small volume of SSC/10 and stored at −70°C.

Analytical ultracentrifugation: Band-sedimentation velocity experiments were usually performed at 20°C, in 30-mm double-sector type-III charcoal-filled Epon band centerpieces, with 7–10 μliters of lamellar solution containing 0.15–0.30 μg of DNA. The sedimentation solvents in the experiments at neutral pH contained either 1 *M* NaCl or 2.85 *M* CsCl, 1.35 gm/ml; both were buffered with 0.01 *M* tris, pH 8. The sedimentation solvent for the velocity experiments at high pH was prepared by titrating 1.35 gm/ml CsCl, 0.05 *M* K_3PO_4, with 1.35 gm/ml CsCl, 0.05 *M* K_3PO_4, 1 *M* KOH, under argon to pH 12.50.[9] The sedimentation coefficients were corrected for viscosity and solution density by the method of Bruner and Vinograd.[10] The buoyant density experiments were performed and calculated by the procedures described by Vinograd and Hearst.[11] Crab dAT from *C. antennarius* sperm was used as a density marker. The buoyant density of the marker is 1.675_6 gm/ml[9] based on a value of 1.710 gm/ml for *E. coli*. All analytical centrifugation experiments were performed in a Beckman-Spinco model E analytical ultracentrifuge equipped with a photoelectric scanner and a parallel Moseley X-Y recorder.

Electron microscopy: Specimens for electron microscopy were prepared by the method of Kleinschmidt and Zahn.[12] The 27-μliter dye-gradient fractions were diluted with 100 μliters of 4.5 *M* NH_4 acetate, pH 8. To 25-μliter aliquots were added 15 μliters of 1 mg/ml cytochrome *c* and 40 μliters 0.01 *M* EDTA, pH 8. The solution was delivered down an inclined glass slide onto a 0.15 *M* NH_4 acetate hypophase, pH 8. After 10–25 min the films were transferred to parlodion-covered 300-mesh copper specimen grids. The grids were passed through 95% ethanol containing 1×10^{-5} *M* uranyl acetate and 1×10^{-3} *M* HCl,[13] and through isopentane. The specimens were shadowed while rotating with platinum-palladium, and examined with a Philips 200 electron microscope. The magnification factor (about 5000× on film) was calibrated with a grating replica. The negatives were projected at a 20× magnification on a Nikon 6F comparator and traced onto vellum. The contour lengths were determined with a map measure.

Results.—Buoyant density fractions in ethidium bromide–CsCl gradients: The initial buoyant density centrifugation of egg homogenates in CsCl alone yields a DNA preparation which contains a considerable amount of polysaccharide and RNA. Subsequent centrifugation of this material in dye–CsCl results in a wide separation of these three components, as shown in Figure 1*a*. The RNA sediments to the bottom of the tube. The polysaccharide forms a turbid, nonfluorescent layer in the lower part of the tube. The strongly fluorescent DNA[14] is resolved into three bands well above the polysaccharide layer.

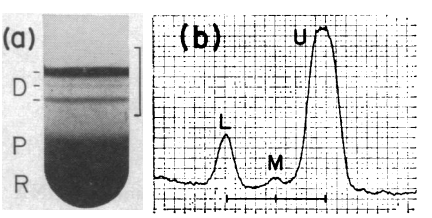

FIG. 1.—*L. pictus* egg DNA after centrifugation in 1.55 gm/ml CsCl, 300 μg/ml ethidium bromide, 48 hr at 43 Krpm, 20°C, in the SW65 rotor. The DNA was partially purified by preliminary buoyant density centrifugation in CsCl and alcohol precipitation.

(*a*) Photograph of the centrifuge tube illuminated with near-UV light. The photograph was taken through a UV filter on Polaroid type-46 film. Only the lower part of the tube containing the bands is shown (0.85×). *D* = DNA bands; *P* = polysaccharide band; *R* = RNA.

(*b*) Fluorescence scan of the same tube in the area indicated by the bracket. The tube which was moved vertically was illuminated by a narrow beam of 548-mμ light and the fluorescence at 590 mμ was recorded. The middle band (*M*) is located in the center of the lower (*L*) and upper (*U*) bands.

The upper and lower bands are separated by a distance of *ca.* 4.5 mm; the faint middle band is located midway between the two main bands. Figure 1*b* presents a scan of the fluorescence of this region and gives a measure of the relative amounts of DNA in the three layers.

The three DNA bands were analyzed in the analytical ultracentrifuge and by electron microscopy. Samples from the lower and middle bands contained a single DNA species with the buoyant density of mitochondrial DNA (Figs. 2*b* and *c*); a sample from the upper band contained mitochondrial DNA and a smaller amount of nuclear DNA (Fig. 2*a*). The dense satellite DNA reported earlier[1] to be present in *L. pictus* eggs was not detected in these preparations. Whole-egg DNA extracted by the previously described procedure[1] contained only two DNA bands, mitochondrial and nuclear, in buoyant analyses in the analytical ultracentrifuge. We estimate that a 2 per cent admixture of the dense DNA could have been observed. The source of the dense DNA and the variability of its occurrence remain at present unexplained.

Dimers and higher oligomers of circular mitochondrial DNA: Electron microscope examination of a DNA sample from the lower band showed only circular DNA molecules, the majority of which were twisted. Of 331 molecules examined at random, 95 per cent were circular monomers and 5 per cent were dimers, i.e., compound molecules consisting of two monomers. DNA samples from two separate middle band preparations also contained exclusively circular molecules. Of 745 molecules examined, 33 per cent were circular monomers, 63 per cent were dimers, 3 per cent were trimers, and 1.2 per cent were tetramers. A DNA preparation from the upper band contained long linear molecules together with open circular monomers, dimers, and a small portion of higher oligomers. The high relative concentration of dimers in the middle band indicates that circular molecules are joined to each other in such a way that one circular

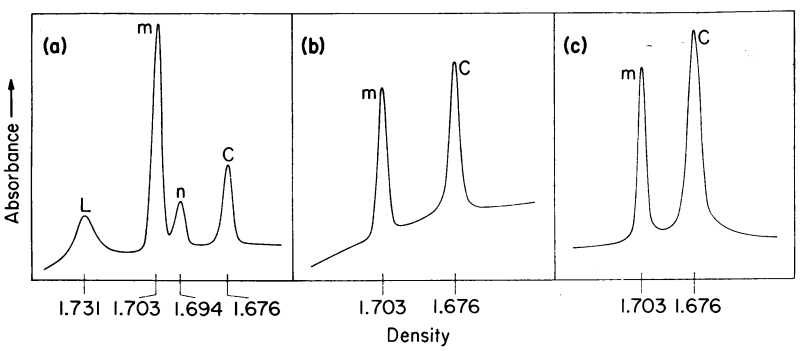

FIG. 2.—Distribution of DNA in a buoyant density experiment in CsCl, 0.01 *M* tris, pH 8. The DNA samples were obtained by dye–buoyant density centrifugation (see text): (*a*) DNA from upper band; (*b*) DNA from lower band; and (*c*) DNA from middle band.

L = DNA from *Micrococcus lysodeikticus; C* = crab dAT; *m* = mitochondrial DNA; *n* = nuclear DNA.

(*a*) and (*b*) were centrifuged in the same rotor at 39,400 rpm for 43 hr, 25°C; (*c*) was centrifuged at 44,770 rpm for 40 hr, 25°C.

unit may be nicked while the other remains intact. Such joined molecules in HeLa cell mitochondrial DNA have been shown to be interlocked circles or catenanes.[6] In the present work, in specimen grid preparations with high contrast, the open dimers could often be seen to be catenated in chainlike fashion when examined at various focal planes. The catenation could also be observed in a study of the grain pattern on negative films of electron micrographs under a dissecting microscope. Selected catenated molecules are shown in Figure 3.

The proportion of catenated molecules in sea urchin egg mitochondria was measured in an electron microscope examination of an unfractionated mitochondrial DNA preparation isolated by the method of Pikó *et al.*[1] The preparation contained less than 5 per cent linear molecules of approximately 5 μ or shorter, indicating that no significant breakdown of mitochondrial DNA occurred. Among 1295 molecules examined there were 88 per cent monomers, 11 per cent dimers, 1 per cent trimers, and 0.3 per cent tetramers.

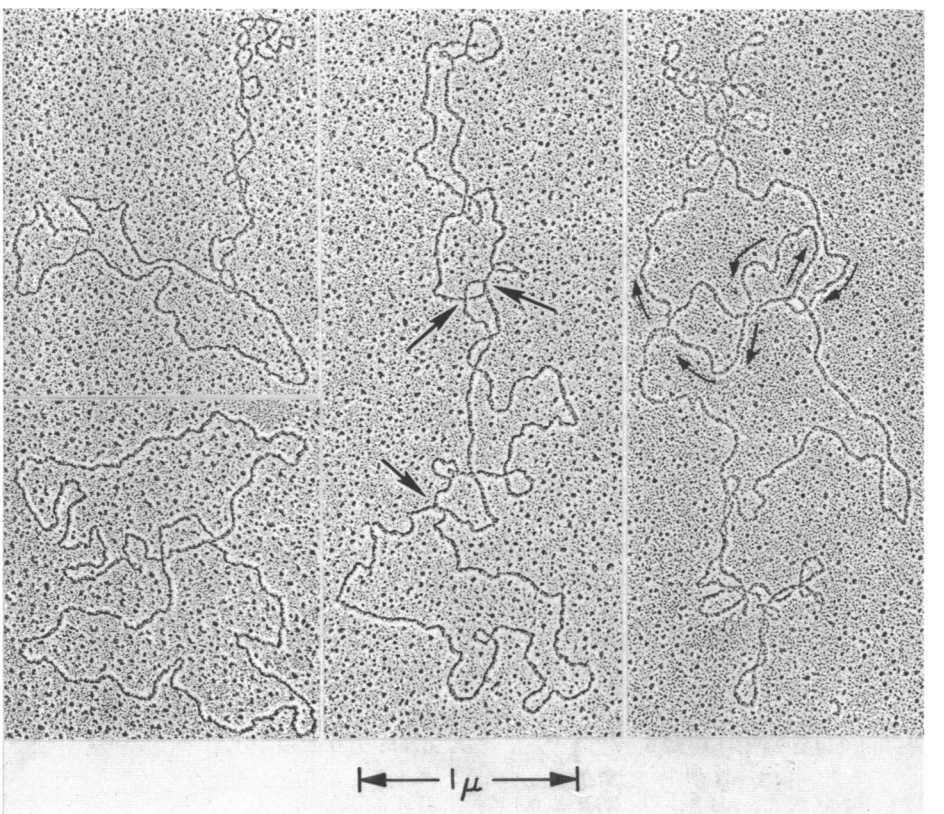

FIG. 3.—Electron micrographs of selected catenated molecules from middle band material of *L. pictus* egg DNA. *Lower left:* dimer with two relaxed circles. *Upper left:* dimer with one relaxed and one twisted circle. *Middle:* trimer with three relaxed circles; the arrows indicate the crossovers of the interlocking circles. *Right:* tetramer with two relaxed and two twisted circles; the arrows indicate the course of one of the relaxed circles. The two middle circles in the tetramer may be doubly interlocked.

TABLE 1. *Contour lengths of DNA circles in monomers and catenated oligomers in two electron microscope preparations of L. pictus mitochondrial DNA.*

Origin of circles	(a) No. of circles	(a) Average length ± SD (μ)	(b) No. of circles	(b) Average length ± SD (μ)
Monomers	43	4.65 ± 0.19	16	4.85 ± 0.21
Dimers	110	4.59 ± 0.14	18	4.90 ± 0.12
Trimers	15	4.55 ± 0.17	15	4.83 ± 0.23
Tetramers	—	—	16	4.80 ± 0.22
Total	168	4.61 ± 0.16	65	4.85 ± 0.20

Size of the circular units in monomers and oligomers: The contour lengths of the monomers and of the monomer units in the catenanes measured on the electron micrographs are presented as histograms in Figures 4*a* and *b*. Table 1 shows the average length of DNA circles in the various kinds of molecules. The tabulated results indicate that there is no significant difference in the contour length of the circular units in the monomers and the catenanes within the same electron microscope preparation. The 4 per cent difference in the mean length of the molecules in the two different preparations is within the range currently observed in our laboratories.[6]

Sedimentation properties of sea urchin egg DNA: Table 2 summarizes the data obtained for *L. pictus* egg DNA in buoyant density and sedimentation velocity experiments. In another sea urchin species, *Strongylocentrotus purpuratus*, the buoyant densities of the nuclear and mitochondrial DNA were 1.695 and 1.701 gm/ml, respectively.

Lower band material obtained by dye-buoyant density centrifugation (Fig. 1) was used for the analysis of the sedimentation properties of covalently closed mitochondrial DNA from *L. pictus*. The DNA was freed of dye by column chromatography. Sedimentation velocity experiments in neutral 2.85 *M* CsCl and 1 *M* NaCl showed the presence of two components with standard sedimentation coefficients, calculated for Na-DNA, 38*S* (I) and 27*S* (II) (Table 2). The relative amounts of the two components were 50–70 per cent I and 30–50 per cent II in various preparations. It appears likely that I and II correspond to the closed and open circular forms of mitochondrial DNA. The open circular DNA was probably formed by nicking during the dye-removal steps. Sedimentation of this DNA in alkaline 2.85 *M* CsCl again showed two components

TABLE 2. *Sedimentation properties of unfertilized sea urchin egg DNA (L. pictus).*

	Mitochondrial[a] I	Mitochondrial[a] II	Nuclear[a]
Buoyant density[b] gm/ml, CsCl, 25°C	pH 8	1.702_6 ± 0.0005 (12)	1.693_7 ± 0.0007 (10)
	pH 12.5	1.758_6 ± 0.0050[c] (6)	—
$s^\circ_{20,w}$	CsCl, pH 8[d]	37.7 ± 2.1 (5)	27.4 ± 1.5 (6)
	1 *M* NaCl, pH 8	38.2 ± 0.4 (2)	27.2 ± 1.2 (2)
s°_{20}	CsCl, pH 12.5[e]	75.8 ± 1.6 (2)	20.0 ± 0.3 (2)

[a] Mean values ± standard deviation. The number of experiments is given in parentheses.
[b] Referred to a value of 1.710 gm/ml for *E. coli*.
[c] Calculated from the center of mass of the band and referred to a value at pH 12.5 of 1.733 gm/ml for crab dAT.
[d] In 1.35 gm/ml CsCl, fully corrected by the method of Bruner and Vinograd.[9]
[e] Sedimentation coefficients at 20°C in 1.35 gm/ml CsCl. These values are not corrected.

with uncorrected sedimentation coefficients, s°_{20}, of 76*S* and 20*S*. The 76*S* component is the denatured double-stranded form of I and the 20*S* component is a mixture of the dissociated single circular and linear strands of II.[15]

In the sedimentation velocity measurements at alkaline pH, the fast-moving band appeared to decrease in amount and to broaden during centrifugation. This behavior indicates that the closed circular DNA is hydrolyzing while sedimenting. A similar effect was observed in a band-buoyancy experiment[15] in alkaline CsCl, pH 12.5. Initially, two bands were observed sedimenting from the meniscus. The fast-moving band decreased steadily in mass and disappeared completely within about $4^1/_2$ hours. At equilibrium a single broad band was observed with an average buoyant density of 1.759 gm/ml (Fig. 5). This banding pattern indicates the presence of two components, which may represent the two separated complementary strands, with an estimated density difference of about 0.005 gm/ml.

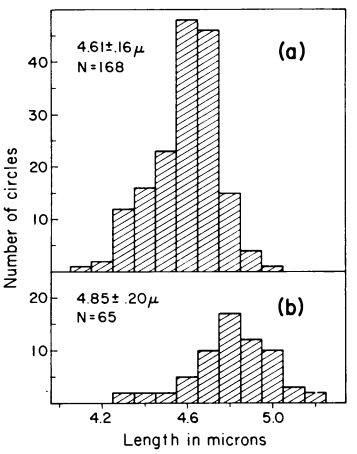

FIG. 4.—Histogram of contour length of DNA circles measured in two separate electron microscope preparations of *L. pictus* egg DNA. The mean lengths ± standard deviation are given.

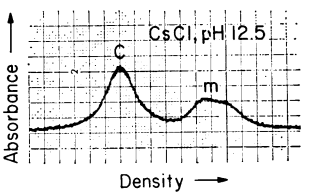

FIG. 5.—Slow-speed photoelectric scan of buoyant density bands in CsCl, 0.05 *M* K_3PO_4, pH 12.5, after 44 hr of centrifugation at 44,770 rpm, 25°C. Note the broadening of the band of mitochondrial DNA (*m*) indicating a slight difference in the buoyant densities of the two strands; the average buoyant density at the center of mass of the band is 1.759 gm/ml. *C* = crab dAT with a buoyant density of 1.733 gm/ml.

Discussion.—The DNA from unfertilized *L. pictus* eggs[1] is a particularly favorable material for analysis of the structure and properties of mitochondrial DNA which comprises 80–90 per cent of the total DNA. The ethidium bromide–CsCl method[5] has proved effective in separating the closed and open circular forms of this DNA. A third band of intermediate buoyant density was also obtained.[6, 7] Buoyant density analysis and electron microscopy showed that the middle band consists of mitochondrial DNA in which about 67 per cent of the molecules (or about 80 wt.%) are catenated dimers or higher oligomers. A study of a mitochondrial DNA preparation that was not subjected to dye-buoyant density fractionation showed that about 11 per cent of the egg mitochondrial DNA molecules are catenated dimers and 1.3 per cent are higher

oligomers. To date no circular dimer, a circular molecule twice the monomer size, was observed in sea urchin egg DNA. Circular dimers have been reported in leukemic leucocyte mitochondrial DNA,[7] colicinogenic factor,[16] and RF-ϕX DNA.[17] The finding of catenated molecules in HeLa cells,[6] leucocytes,[7] and in the sea urchin egg was facilitated by enrichment of these forms in the middle band. The interlocking nature of the joint was clearly shown[6] when grids with HeLa mitochondrial DNA were shadowed first while rotating and then from a single direction. The possibility that catenation may be the result of a recombination process has been discussed by Hudson and Vinograd.[6] The significance of catenated forms in mitochondrial structure and function is obscure at present. The occurrence of these forms in sea urchin eggs, and normal leucocytes,[7] indicates that they are not *per se* associated with abnormal cells such as the HeLa cells[6] and leukemic leucocytes.[7]

The physical properties of sea urchin mitochondrial DNA are in general agreement with those established for closed circular viral DNA[2, 9, 15] and circular mitochondrial DNA from mammals and birds[3, 4] and amphibia.[18] One difference from viral DNA appears to be the greater instability of the mitochondrial DNA at alkaline pH. The apparent difference in the buoyant densities of the denatured strands should be noted; a considerably larger difference in the densities of the two strands has been indicated recently for frog egg mitochondrial DNA.[18]

Summary.—Closed circular mitochondrial DNA, free of nuclear DNA, was isolated from *L. pictus* eggs by buoyant density centrifugation in ethidium bromide–CsCl density gradients. The closed and open circular forms of mitochondrial DNA were characterized in sedimentation velocity experiments at neutral and alkaline pH. Electron microscope examination has shown that approximately 12 per cent of the mitochondrial DNA molecules are in the form of catenanes consisting of two or more topologically interlocked circular units of the monomer size of about 5 μ.

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