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Biochemical and electron microscopic evidence that cell nucleus negatively controls mitochondrial genomic activity in early sea urchin development

([^{3}H]thymidine incorporation/DNA replication)

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ABSTRACT **Enucleated halves of sea urchin eggs obtained by centrifugation contain almost all the mitochondrial population of the egg. Removal of the nucleus followed by parthenogenetic activation stimulates the incorporation of [^{3}H]thymidine into the mitochondrial DNA, whereas no such incorporation is observed in activated whole eggs. The block is not the result of a modification in the permeability of the mitochondrial membrane. Electron microscopic observations demonstrated duplication of mitochondrial DNA molecules in activated enucleated halves. No duplication was found in the mitochondrial DNA from activated whole eggs or from nonactivated enucleated halves. We conclude that the cell nucleus exerts a negative control on the activity of the mitochondrial genome through some short-lived nuclear substance(s).**

Fertilization brings about a burst of biosynthetic activity in sea urchin eggs: the rate of protein synthesis is dramatically enhanced and so are nuclear RNA synthesis and DNA replication (see ref. 1 for a detailed review). In striking contrast to the nuclear and cytoplasmic activities is the relative quiescence of the mitochondria. Although some increase in mitochondrial RNA synthesis is observed (2–9), mitochondrial DNA replication is totally absent until the pluteus stage (ref. 10; unpublished observations). In view of the facts that replication of cell organelles is generally coupled with cell division and that the fertilized egg is actively engaged in mitosis, it is interesting to ask what mechanism prevents mitochondrial genome replication throughout the early stages of sea urchin development.

In a previous paper (11) we showed that only a modest increase in mitochondrial RNA synthesis can be observed after fertilization or parthenogenetic activation. However, when the cell nucleus was removed and the egg was parthenogenetically activated, a dramatic increase in mitochondrial RNA synthesis, mostly ribosomal, took place, suggesting that the removal of the nucleus allowed mitochondrial replication to occur immediately after parthenogenesis. This has now been verified by the direct demonstration that the removal of the egg nucleus elicits mitochondrial DNA synthesis immediately after parthenogenetic activation.

MATERIAL AND METHODS

Enucleated halves of *Paracentrotus lividus* eggs were obtained by the method of Harvey as modified by Wilt (12).

For electron microscopic analysis, the halves were fixed with 2% OsO_4 in 0.15 M phosphate, pH 7.0/2% NaCl, dehydrated in alcohol, and embedded in DER (a mixture of Dow epoxy resins). The sections were stained with Pb citrate (13) and uranyl acetate and observed in a Siemens–Elmiskop 1b (60–80 kV).

Eggs and enucleated halves (10,000/ml) were cultured in the presence of antibiotics (14). The absence of bacterial contamination was checked by optical and electron microscopy and by inoculation on plates of Difco nutrient agar.

Activation was induced with NH_4Cl at pH 9.0 as described by Epel *et al.* (15) or with butyric acid (1). Sixty minutes after fertilization or parthenogenetic activation, the cultures were incubated with [^{3}H]thymidine [10 μCi (1 Ci = 3.7×10^{10} becquerels)/ml; specific activity, 2000 μCi/mmol]. After 6 hr the samples were collected, washed, and homogenized in Dounce homogenizer at 2°C with 10 vol of 0.25 M sucrose/0.1 M Tris·HCl, pH 7.6/0.4 M KCl/and 2 μg of polyvinyl sulfate per

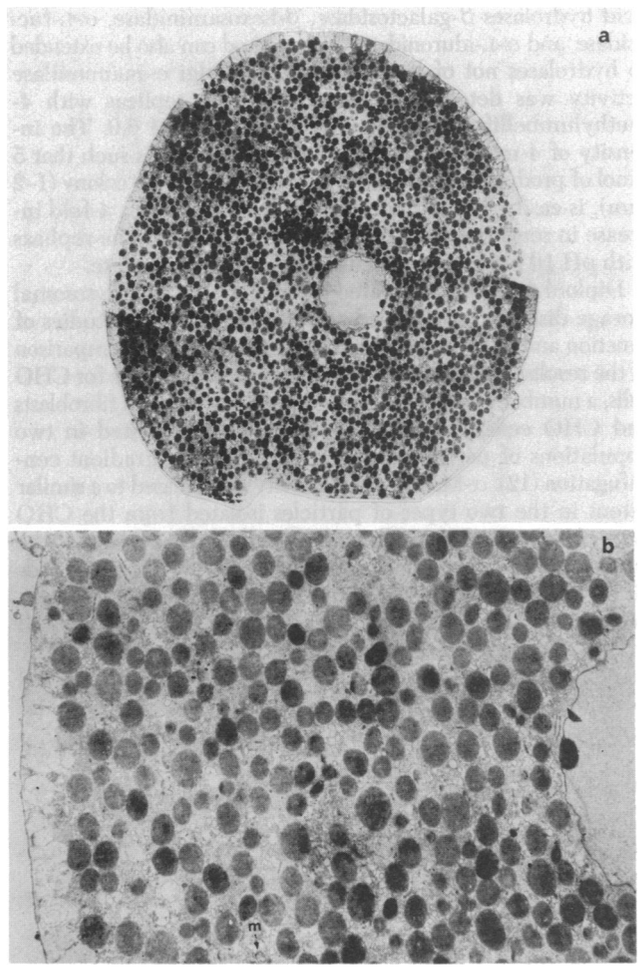

FIG. 1. Electron micrograph of nucleated half of egg. m, Mitochondrion. (*a*, ×950; *b*, ×3500.)

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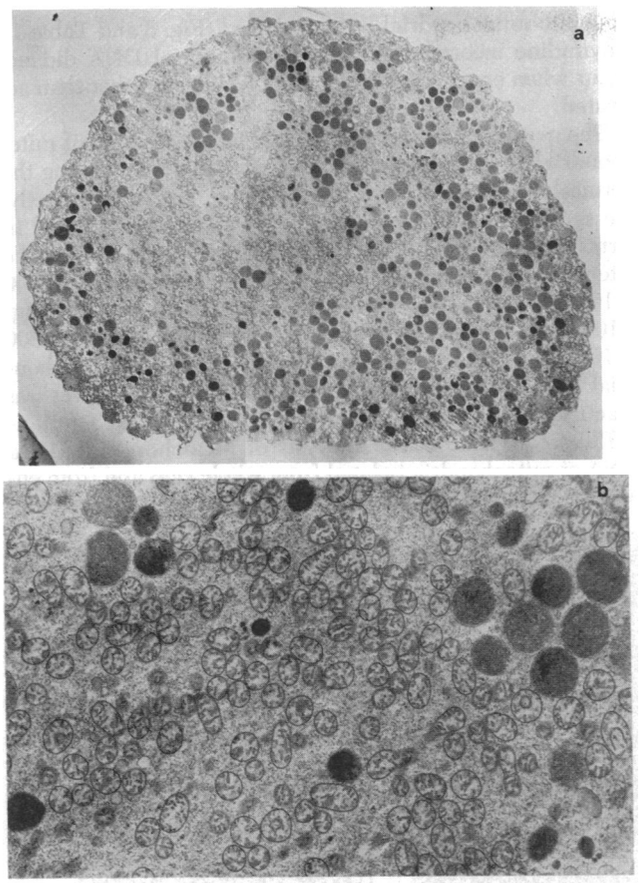

FIG. 2. Electron micrograph of enucleated half of egg. (*a*, ×2000; *b*, ×8300.)

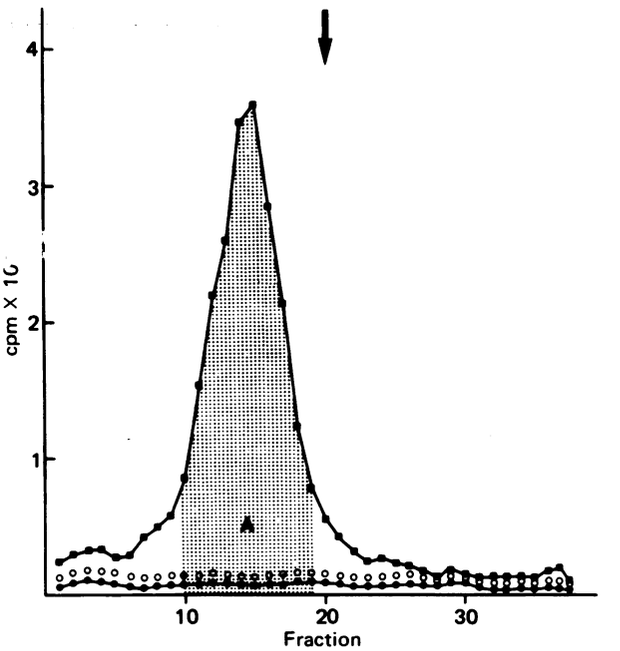

FIG. 3. CsCl/ethidium bromide gradient of mitochondrial DNA from activated eggs (●), activated enucleated halves (■), or fertilized enucleated halves (○). At 1 hr after fertilization or parthenogenetic activation, the eggs and the enucleated halves were incubated for 6 hr with [^{3}H]thymidine. The mitochondria were isolated and the DNA was purified. The gradient fractions corresponding to zone A were pooled and utilized for electron microscopic analysis. Arrow, position of purified nuclear DNA.

ml (TKS buffer). The homogenate was centrifuged at 1500 × *g* for 10 min. The pellet, washed twice with TKS, represented the nuclear fraction. The upper two-thirds of the supernatant were centrifuged at 14,000 × *g* for 10 min. The pellet, washed twice with TKS, contained essentially mitochondria. In the case of enucleated halves, the 1500 × *g* pellet was discarded. Nuclear and mitochondrial pellets were suspended in 0.5 M NaCl/0.1 M Tris·HCl/0.01 M EDTA, pH 8.0, lysed with an equal volume of prewarmed 4% sodium dodecyl sulfate, and subjected to CsCl/ethidium bromide (10) centrifugation in a Spinco 50 Ti rotor at 47,000 rpm for 18 hr at 20°C. The contents of the tubes (except for the pellets and the top layers) were transferred to a second series of tubes and centrifuged for 50 hr at 44,000 rpm.

Unlabeled nuclear DNA, used as a marker, was purified from sperm by extraction with sodium dodecyl sulfate/Pronase, deproteinization with phenol/chloroform, and purification through a 1.7 g/cm^3 CsCl gradient.

Unlabeled mitochondrial DNA, used as a marker, was obtained from mitochondria purified from unfertilized eggs in which the DNA content of the mitochondrial population is 3–6 times higher than that of nuclei (see ref. 1).

For the electron microscopic analysis of DNA molecules, the spreading solution contained 10 μg of DNA and 2 μg of cytochrome *c* per ml, 0.05 M Tris·HCl buffer (pH 8.5), 0.05 M NaCl, and 5 mM EDTA in 40% formamide. The hypophase was double-distilled water. The films were picked up on collodion-coated copper grids, stained with uranyl acetate, rotary shadowed with Pt/Pd, and examined on a Siemens–Elmiskop 1b electron microscope at an accelerating voltage of 60 kV. Replicative form I ϕ_1 was used as a marker.

Table 1. [^{3}H]Thymidine incorporation into nuclear and mitochondrial DNA fractions

	Incorporation*	
	Nuclear	Mitochondrial
Activated whole eggs	920,000	100
Activated enucleated halves	—	200,000
Fertilized enucleated halves	600,000	50

* Values are trichloroacetic acid-precipitable cpm/10^3 eggs or halves.

RESULTS

Mitochondrial Distribution in Centrifuged Eggs. When eggs of *P. lividus* are centrifuged by the method described here, each divides into a nucleated half and an enucleated half. The enucleated halves were obtained in large amounts, and the contamination by nucleated halves or whole eggs was less than 0.5% as determined by bright-field microscopy.

Both fractions were examined by electron microscopy in order to study the effect of centrifugation on the distribution of the mitochondria. Very few mitochondria appeared to be present in the nucleated halves (Fig. 1); almost all the mitochondrial population was contained in the enucleated halves (Fig. 2).

[^{3}H]Thymidine Incorporation. In order to study the replicative activity of the mitochondrial genome, at 1 hr after parthenogenetic activation or fertilization the eggs or the enucleated halves were labeled *in vivo* with [^{3}H]thymidine for 6

hr. Mitochondria were then isolated, mitochondrial DNA was purified through CsCl/ethidium bromide gradients, and the incorporation of thymidine was measured. Nucleated halves were not utilized because they contained only few mitochondria. Preliminary experiments had shown that nuclear DNA density in 1.7 g/cm^3 CsCl is 1.694 g/cm^3, whereas that of mitochondrial DNA in 1.55 g/cm^3 CsCl/ethidium bromide is 1.609 g/cm^3. A second centrifugation of mitochondrial DNA in 1.7 g/cm^3 CsCl, after removal of the dye, gave a density of 1.700 g/cm^3.

No radioactivity was incorporated in the DNA isolated from purified mitochondria of activated whole eggs (Fig. 3), whereas DNA isolated from the nuclear fraction showed active incorporation (Table 1), in agreement with the results reported by many authors (1). It is also well known that activated whole eggs can give rise to normal plutei (see ref. 1 for a detailed review). On the other hand, when the eggs were enucleated and parthenogenetically activated, an active incorporation of thymidine into mitochondrial DNA occurred (Fig. 3 and Table 1). Thymidine incorporation into mitochondrial DNA did not occur when enucleated halves were fertilized rather than activated.

The negative effect of the nucleus on the labeling of mitochondrial DNA could be the result of controls regulating the permeability of the mitochondrial membrane or, more directly, the synthesis of DNA. Our findings cannot be explained in terms of a permeability barrier because the thymidine uptake into the mitochondria of the activated enucleated halves (400 $\times 10^3$ cpm/10^3 halves) was lower than that of the activated eggs (2100 $\times 10^3$ cpm/10^3 eggs) or fertilized enucleated halves (1800 $\times 10^3$ cpm/10^3 halves). Moreover, the recovery of mitochondrial DNA from whole eggs and from enucleated halves was practically the same.

Electron Microscopic Analysis of Mitochondrial DNA. DNA of mitochondria isolated from whole eggs and from enucleated halves, regardless of whether they had been activated

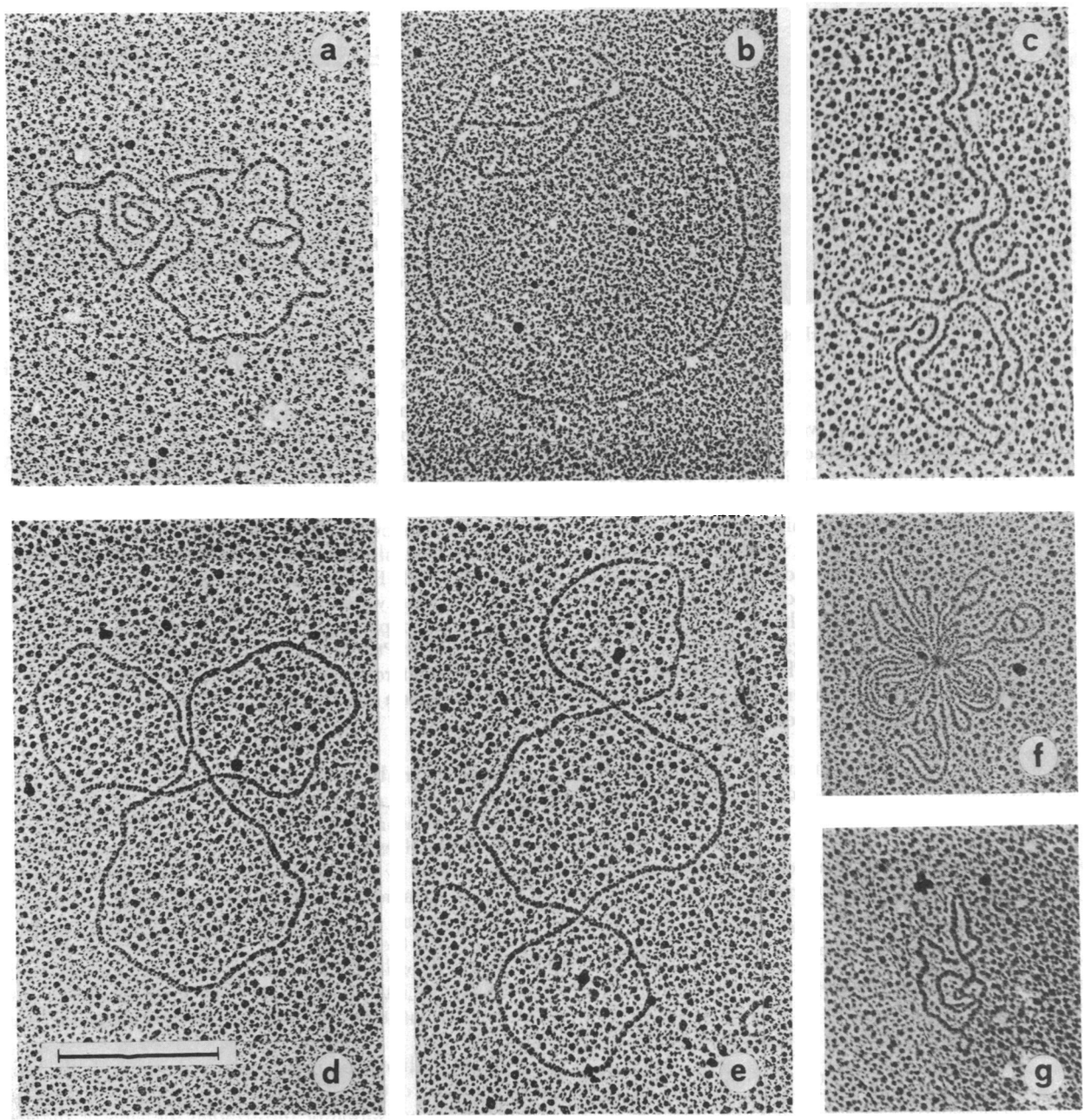

FIG. 4. (*a–f*) Mitochondrial DNA molecules obtained from activated eggs. (*g*) Replicative form I ϕ_1 as marker. Bar shows 0.5 μm.

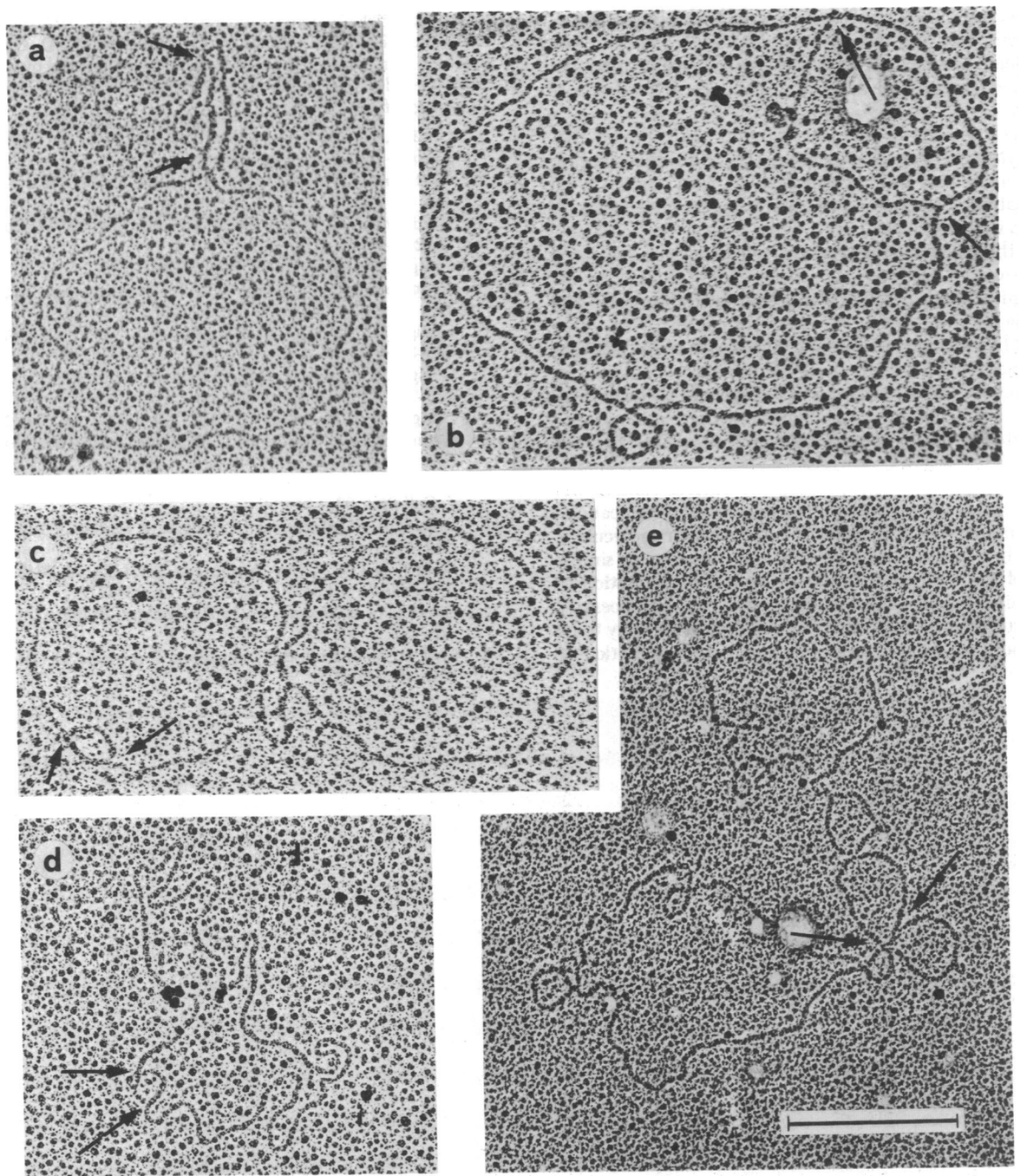

FIG. 5. (*a–d*) Mitochondrial DNA replicative molecules from activated enucleated halves. Arrows, D loops. (*e*) Concatenated dimer; the arrows indicate the genomic unit. Bar corresponds to 0.5 μm.

or not, was prepared by CsCl/ethidium bromide gradient. The fractions corresponding to zone A of Fig. 3 were pooled, the dye was removed, and the mitochondria were utilized for electron microscopic observations.

Analysis of the contour length of 56 uncoiled molecules showed a considerable uniformity of size: mean ± SEM, 4.56 ± 0.12 μm.

No replicative forms were detected among 94 molecules of mitochondrial DNA derived from parthenogenetically activated whole eggs; the DNA molecules were in the form of closed circles, some of which appeared to be supercoiled (Fig. 4). Very few molecules appeared in linear form. The same result was obtained with mitochondrial DNA derived from nonactivated whole eggs (80 molecules observed) or enucleated halves (91 molecules observed). Also, in the case of fertilized enucleated halves, no replicative forms have been found (120 molecules observed). On the other hand, when mitochondrial DNA from activated enucleated halves was analyzed, 35 replicative forms (i.e., with a D loop) and 2 dimers were detected among 156 molecules studied (Fig. 5).

DISCUSSION

The present results clearly show that, when parthenogenetic activation is preceded by the removal of the cell nucleus, active mitochondrial DNA replication is initiated in addition to the activation of mitochondrial transcription reported previously. This provides strong evidence that it is the nucleus that prevents the mitochondrial genome from replicating after fertilization or parthenogenetic activation. The mitochondrial activity is not likely to be an artifact resulting from centrifugation and cell

fission because it does not occur if not followed by parthenogenetic activation and because introduction of a nucleus into the enucleated half by fertilization fails to produce the burst of mitochondrial genome activity (11) and its replication does not occur.

Our electron microscopic investigation confirms the efficiency of the centrifugation technique for splitting sea urchin eggs into enucleated and nucleated halves, with practically no contamination of the latter with the former in this species.

What is relevant to our experiments is the lack of contamination of the enucleated halves with whole eggs or nucleated halves. However, contamination would not affect our results because mitochondrial DNA was prepared from isolated mitochondria and it cannot be mistaken for nuclear DNA when examined by electron microscopy. The results of the electron microscopic analysis exclude that thymidine incorporation can be interpreted in terms of different isotope utilization or dilution into a different internal pool and confirm that incorporation is related to DNA synthesis and not, for example, to DNA repair.

What is the mechanism by which the nucleus controls the mitochondrial genome for both transcription and replication? We have no answer at present. If some nuclear molecule is produced, however, this must be short-lived because the simple removal of the nucleus permits mitochondrial activation to occur within a few hours. Moreover, its removal is a necessary but not a sufficient condition for mitochondrial activity to be resumed because it has to be coupled to parthenogenetic egg activation.

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