MITOCHONDRIAL DNA METABOLISM IN YOUNG *PSAMMECHINUS MILIARIS* EMBRYOS

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Received 16 February 1973

1. Introduction

The sea urchin egg, as the amphibian egg, contains more cytoplasmic DNA than nuclear DNA; most of the cytoplasmic DNA, if not all, is localized in mitochondria [1]. This mitochondrial DNA (mtDNA) resembles the mtDNA of higher animals; it is circular and has a length of about 5 μm. After fertilization the nuclear DNA is synthesized to a very high degree; however, only limited information concerning the replication of mtDNA in the early cleavage stages of the sea urchin embryo and also other embryos is known.

With autoradiographic methods, labeling of mitochondria in sea urchin eggs immediately after fertilization was observed when [^{3}H]thymidine or [^{3}H]bromodeoxyuridine had been added to the incubation medium; an incorporation of the precursors into mtDNA is discussed [2, 3]. With biochemical methods, however, contradictory results were obtained. Whereas in some species no incorporation has been measured immediately after fertilization and in the early cleavage stages [4, 5], a small, but definite, incorporation of thymidine into the mtDNA of *Arbacia lixula* in the early cleavage stages has been observed [6].

The aim of these present experiments was to investigate the mtDNA synthesis in the early cleavage stages and additionally in the mesenchyme blastula- and in the gastrula stage in another species. *Psammechinus miliaris* was used because the eggs of this species are less fragile than the eggs of *Arbacia lixula* and because these eggs contain a smaller amount of pigment-granules which complicate biochemical analysis. Both in *Arbacia lixula* and in *Psammechinus miliaris* an incorporation of [^{3}H]thymidine into mtDNA was observed.

2. Materials and methods

Gametes were obtained by injecting 0.5 ml of a 0.5 M KCl solution into the animals. Eggs were washed several times in artificial sea water at 18°, sperm were kept dry. For fertilization and development the eggs were suspended to about 5×10^3 cells/ml. After fertilization the eggs were washed once. For labeling, 0.5 μCi [methyl-^{3}H]thymidine/ml (NEN 16 Ci/mmole) were added to the suspension, together with 100 units/ml penicillin G and 0.1 mg/ml streptomycin sulphate. After labeling the embryos were washed once at 4° in artificial sea water and in a solution containing 0.3 M sucrose, 0.36 M KCl, 0.001 M EDTA, 0.03 M Tris-HCl, pH 7.4 [7]. Homogenisation with glass beads (90–100 μm) was done in the same solution. The homogenate was spun twice at 200 *g* and once at 10,000 *g*. The sediment was layered atop a gradient containing 0.8–2.0 M sucrose, 0.05 M KCl, 0.005 M Tris-HCl, pH 7.4 and spun at 26,000 rpm for 3 hr. For DNAase-treatment particles were incubated in 0.4 M sucrose, 0.005 M $MgCl_2$, 0.005 M Tris-HCl pH 7.4 + 100 μg DNAase/ml for 25 min at 20° followed by sedimentation in a 10-fold volume of 0.4 M sucrose, 0.03 M EDTA, 0.005 M Tris-HCl, pH 7.4. The pellet was lysed in 0.1 ml of a solution containing 2% SDS, 0.001 M EDTA, 0.4 M CsCl, 0.01 M Tris-HCl, pH 7.4. The CsCl–ethidium bromide gradient was performed essentially according to [8]. The bands were isolated and re-

run in a second CsCl-EB gradient. The gradient was fractionated discontinuously in a special device [9]. The fluorometric evaluation was done in a Zeiss PMQ II spectrophotometer with attachment ZFM 4, excitation wavelength 346 nm measurement at 590 nm. Radioactivity was measured after TCA precipitation on 0.2 μm nitrocellulose filters in an Omnifluor scintillator (4 g/ℓ toluene) in the Packard model 3380.

DNA, free of ethidium bromide [8] and dialysed against 1/10 SSC, was hydrolyzed with calf-thymus DNA as carrier in 0.5 ml of 100% formic acid in a glass ampulla at 150° [10]. Formic acid was removed with N_2 and the bases, dissolved in 0.1 N HCl, were chromatographed on cellulose plates (Schleicher u. Schüll) in a system CH_3OH:H_2O:HCl (65:18:17) [11]. The plates were divided in 10 fields and for measurement of the radioactivity the cellulose powder was scraped from each of the fields and burned separately in a sample oxidizer (Packard Instruments, Model 305).

3. Results and discussion

After the embryos were incubated for 4 hr with [^{3}H]thymidine in artificial sea water they were homogenized, and the mitochondria were isolated and purified in a sucrose gradient where the particles banded at a density of 1.17 g/ml. Seen from the electron micrograph the particles were not very well preserved but they were free of yolk platelets which may or may not contain DNA. Before lysis of the mitochondria pellet the particles were incubated with pancreatic DNAase to destroy nuclear DNA. The mtDNA was isolated in a CsCl–ethidium bromide gradient [8]. This procedure allows the closed circular DNA to be directly isolated from the lysate.

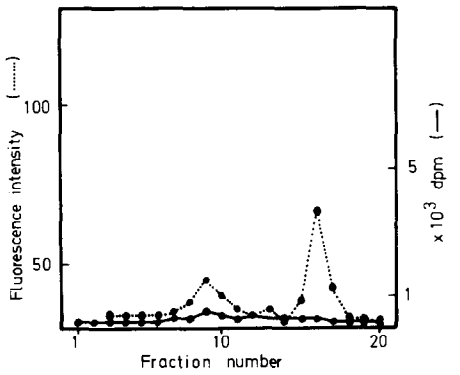

Fig. 1. Incorporation of [^{3}H]thymidine into unfertilized eggs of *Psammechinus miliaris*. FI: Fluorescence intensity in arbitrary units. Incubation: 4 hr.

Table 1
The sums of dpm and FI values of fractions constituting one band as indicated by a bracket in fig. 2 a–c.

Stage		dpm	FI	dpm/FI
Early cleavage	LB	9464	31	305
	HB	3528	27	133
Mesenchyme Blastula	LB	53236	157	339
	HB	9086	37	245
Gastrula	LB	15717	37	425
	HB	4830	16	302

LB: Light band. HB: Heavy band.

To prove that the radioactivity of the bands in fig. 2a–c was localized in the DNA, eggs were incubated after fertilization in thymidine until the 64-cell stage (4 hr); the DNA was isolated as described above. The fractions of each of the bands were combined and the DNA was hydrolyzed. The chromatogram of the bases showed that from the light band 96% and from the heavy band 81% of the radioactivity was localized only in the spot with thymine (R_f 0.67). This experiment, together with an experiment which showed that the radioactivity was neither destroyed with α-amylase nor RNAase but with DNAase [12] demonstrates that the radioactivity of the bands was linked only to DNA.

A further control experiment (fig. 1), proved that a possible incorporation of [^{3}H]thymidine in mtDNA of the oocytes, which were always present in less than 1%, had no effect on subsequent experiments. Unfertilized eggs were incubated in [^{3}H]thymidine for 4 hr. Since the heavy band, which contains the closed circular mtDNA, was not labeled, the oocytes did not influence the experiment. In the first main experiment (fig. 2a), fertilized eggs were incubated until the 64-cell stage (4 hr) after which the mtDNA was isolated. Embryos, in experiment 2 (fig. 2b), developed in sea water until the mesenchyme blastula stage before [^{3}H]thymidine and antibiotics were added. With a third experiment (fig. 2c) the precursor and the anti-

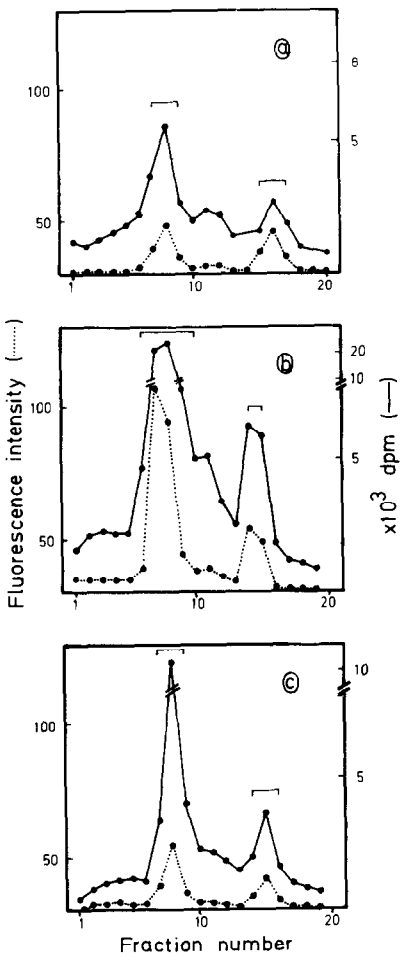

Fig. 2. Incorporation of [^{3}H]thymidine into different developmental stages of *Psammechinus miliaris*. a) Fertilized egg up to 64-cell stage; b) mesenchyme blastula stage; c) gastrula stage. Incubation: 4 hr.
The heavy band with closed circular DNA is on the right.

biotics were added during the gastrula stage. The two bands characteristic of mtDNA were observed in all gradients, as was a middle weak band containing catenanes [13]. After fractionation, the fractions were analysed fluorometrically; the middle band was no longer observed because of dilution. In all stages all bands were labeled. Table 1 contains the values for radioactive decompositions per min (dpm) and the fluorescence intensity (FI) of the fractions containing the light and the heavy bands. In the first column the sum of the dpm and in the second the sum of the FI of fractions constituting one band are listed. Fractions summed up are indicated in fig. 2 by a bracket. The third column contains the quotient dpm/FI which represents a specific activity. As seen from the table the specific activities of the light band are higher than the specific activities of the heavy bands. Since the amount of ethidium intercalated into linear DNA is higher by a factor of 1.5–2 than in closed circular DNA [14], the fluorescence intensity increases the difference in specific activity of linear and circular DNA. The light band probably still contained highly labeled nuclear DNA which clung to the mitochondria and which was not completely destroyed by DNAase. The specific activity of the middle band could not be calculated because of the small amount of DNA. Only the heavy band, which contains pure mtDNA is here discussed. When the specific activities of these heavy bands of the 64-cell stage, mesenchyme blastula stage and gastrula stage were compared, a ratio of 1:1.8:2.3 was calculated. From these data the following conclusions may be drawn: The mtDNA is metabolized also in the *Psammechinus* embryo immediately or shortly after fertilization. This fact demonstrates that the mitochondria present in a large excess in the egg are not only passively distributed to daughter cells until a concentration per cell is reached which corresponds to concentrations of these particles in adult body cells but that, in addition to mtRNA as demonstrated by Craig [15], also the mtDNA is metabolically active immediately or shortly after fertilization. The metabolism of the mtDNA is increased by a factor of 2 in the gastrula stage, provided that the rate of phosphorylation of thymidine and the equilibration between the nucleotide-pools in the cytoplasm and in the mitochondria are the same in the 64-cell stage and in the gastrula stage, This assumption is supported by the fact that the speed at which the precursor is taken up by the gastrula is the same as the speed of uptake in the early cleavage stages [16].

The values obtained by these experiments are average values of the whole embryo. It is reasonable to assume that in different cells of the embryo, different metabolic rates occur.

The question as to why no incorporation of [^{3}H]thymidine into mtDNA was observed in different sea urchin species during early development, when principally the same techniques have been applied, remains unanswered. If one assumes that in different species the basic molecular events are the same but

differ only quantitatively, it seems to be likely that in the experiments reported [4, 5] not enough mtDNA was isolated to detect a radioactivity significantly above background level. The CsCl–EB technique allows isolation and detection of amounts of DNA considerably smaller than 1 μg and if the specific activity of the DNA is low one may not be able to measure it when small amounts of DNA were isolated. In addition, different species may metabolize the mtDNA at different rates.

The specific activity of the closed circular DNA expressed in dpm/μg and calculated for an incubation time of 1 hr are in the order of magnitude of $1-3 \times 10^2$. Compared to the corresponding nuclear DNA this activity is low. Since the egg contains a large surplus of mtDNA, specific activities, smaller by one or several orders of magnitudes are to be expected. Furthermore the equilibration of the cytoplasmic and the mitochondrial pool regarding the labeled nucleotide may be slow as demonstrated for ribonucleotides in the egg of *Xenopus laevis* [17]. The results described in this publication, indicate that mtDNA metabolism may not begin as a result of a trigger mechanism in a late stage of development. The trigger mechanism for DNA metabolism may be only fertilization as mtDNA metabolism has been observed as early as the 64-cell stage. If the incorporations observed are based on a true replication mechanism, or if repair mechanisms occur to a significant degree, cannot be decided by these experiments. The increase of the specific activity from the early cleavage stages to gastrula stage favours a replication mechanism. Further investigations are planned to clarify this question.

Acknowledgements

The experiments were partially supported by the Deutsche Forschungsgemeinschaft. For the supply of the animals I kindly thank Dr. W. Greve, Biologische Bundesanstalt Helgoland. For technical assistance I thank Miss A. Warnecke and Mr. K. Hoffmann.

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