*EVIDENCE FOR THE MITOCHONDRIAL ORIGIN OF FROG EGG CYTOPLASMIC DNA**

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It has been shown[1] that eggs of the two amphibian species, *Rana pipiens* and *Xenopus laevis*, contain 300–500 times more DNA than somatic cells of these animals. Egg DNA is high-molecular-weight double-stranded material complementary in sequence to a small proportion of liver DNA of the same species. It has been suggested on the basis of indirect evidence that the bulk of egg DNA is of mitochondrial origin. More direct evidence for this hypothesis is presented below. In addition, the demonstration of DNA in frog egg mitochondria adds to the accumulating evidence for the general occurrence of DNA in these particles.

Materials and Methods.—Protein was determined by the Lowry method, deoxyribose-containing compounds were measured by Burton's[2] modification of the diphenylamine test. Cytochrome *c* oxidase was assayed according to Straus[3] with the modification that the final volume was 5 ml and the color was read at 550 mμ. Succinate-cytochrome *c* reductase was assayed by following the increase in absorbancy at 550 mμ in 2.4 ml of a solution containing 0.25 *M* sucrose, 0.05 *M* tris at pH 7.4, 1.5 mM KCN, 4 mM succinate, 0.08% cytochrome *c*, and 0.1% deoxycholate. Primogonyl, generously supplied by Schering AG, was used to induce ovulation in *X. laevis*. Eggs were collected, dejellied, and washed as described previously.[1]

Preparation of mitochondria: All steps were carried out at 0–4°. In addition to eggs, whole ovaries of *R. pipiens* were used as starting material for mitochondria. Ovaries were not used previously for the preparation of egg DNA[1] because of the possibility of contamination with follicle cell nuclear material. This danger was not present here, since nuclear material was removed during the purification of the mitochondria. Ovaries were gently squashed between two surfaces in 10 vol of 0.25 *M* sucrose, 0.03 *M* tris (pH 7.4), 1 mM EDTA, and filtered through gauze to remove most of the ovarian tissue.[4] Ovulated eggs were homogenized by hand in a loose Teflon-glass homogenizer in the same buffered sucrose solution. The suspension of egg or oöcyte material was centrifuged twice for 15 min at 2,000 rpm (*X. laevis*) or at 3,000 rpm (*R. pipiens*). The supernate was centrifuged for 20 min at 10,000 rpm; the resulting pellet was clearly divided into a black layer of pigment granules at the bottom and a yellow-brown layer of mitochondria above it. The mitochondria were suspended in buffered sucrose without disturbing most of the pigment granules. Centrifugation and resuspension of mitochondria were repeated once in the same way. A suspension of crude mitochondria derived from about 4,000 eggs was then layered over a 25-ml linear sucrose gradient, 0.9–2.1 *M*, which contained 1 mM EDTA. After centrifugation for 75 min at 22,000 rpm in the SW25.1 rotor of the Spinco centrifuge, 2-ml fractions were collected from the top with a syringe connected to a bent-tip glass tube. Egg mitochondria reached equilibrium in the gradient at a density of 1.215 (1.35 *M* sucrose). Some pigment granules still left in the preparation pelleted under these conditions; this material was suspended and analyzed in the same way as the other fractions of the gradient.

Preparation of DNA: DNA was extracted from mitochondrial fractions by either of two methods: lysis with 2% SDS, addition of $NaClO_4$ to 1 *M*, and deproteinization with chloroform-isoamyl alcohol;[5] or digestion with pronase (0.5 mg/ml) in the presence of 0.25% SDS for 4 hr at 37°, followed by shaking with phenol.[6] In both cases, nucleic acids were precipitated with ethanol, allowing several hours at 4° for the formation of the precipitate. Two methods were used for further purification. The material was treated with RNase at 50 μg/ml for 30 min at 37°, extracted twice with phenol, and dialyzed against 0.01 *M* tris, pH 8.0. The solution was applied to a 1 × 4-cm column of MAK, the column washed with 0.3 *M* NaCl until no UV-absorbing material appeared in the effluent, and the DNA was then eluted with 0.75 *M* NaCl. Alternatively, after reprecipitation with ethanol, the entire fraction was subjected to equilibrium density

gradient centrifugation in CsCl for 65 hr at 20° at 34,000 rpm in the SW39 rotor of the Spinco centrifuge. The DNA band was localized by measuring the absorbancy of fractions at 260 mμ, and the DNA-containing fractions were pooled.

Results.—Egg constituents were first divided into three crude fractions, each consisting of a mixture of materials.[1] The 2,000-rpm pellet consists mainly of yolk platelets and also contains most of the pigment granules (Fig. 1). This fraction contains less than 5 per cent of the egg's DNA. The 10,000-rpm pellet contains about 80 per cent of the DNA; the material in the pellet could be resolved into pigment granules, which contained no measurable DNA, and mitochondria, with which the DNA was associated (see below). Finally, there is the supernatant solution, which contained about 20 per cent of the DNA. Cytochrome *c* oxidase activity was detectable in the mitochondrial fraction only.

Isopycnic banding in sucrose gradients allowed the separation of mitochondria from other particles present in the 10,000-rpm pellet. Association of the DNA with the mitochondria in eggs and oöcytes of *R. pipiens* and *X. laevis* is demonstrated by the parallel distribution of the DNA and of two mitochondrial enzymes (Fig. 2). The constancy of the ratio of DNA to enzyme activity across the peaks emphasizes the association of DNA with the particles (*top insets*, Fig. 2).

It has been reported that DNA inside intact mitochondria is not susceptible to digestion by DNase.[7] This is also true for egg mitochondrial DNA. The mitochondrial pellet obtained from *X. laevis* eggs was incubated in 0.25 *M* sucrose, 0.02 *M* tris (pH 7.4), 5 mM $MgCl_2$, and 30 μg/ml DNase at 25° for 30 min. After subsequent purification on a sucrose gradient these particles still yielded 2 mμg DNA per egg, the same amount as preparations not treated with DNase. Furthermore, electron micrographs showed that only mitochondria were present in some preparations (Fig. 3*b*), whereas others contained a few pigment granules (Figs. 3*a* and *c*). This contamination is not considered serious because there was no apparent difference in the DNA content of preparations containing some pigment and those free of it, and since bulk pigment fractions contained no measurable DNA.

The quantity of diphenylamine-reacting material in mitochondria may be taken as a direct measure of the DNA content, since the amount of substances interfering with the assay is much smaller in the purified particles than in whole eggs. The absorption spectrum of the product of the reaction of mitochondrial extracts with diphenylamine was identical to that of pure DNA, in contrast to the spectrum

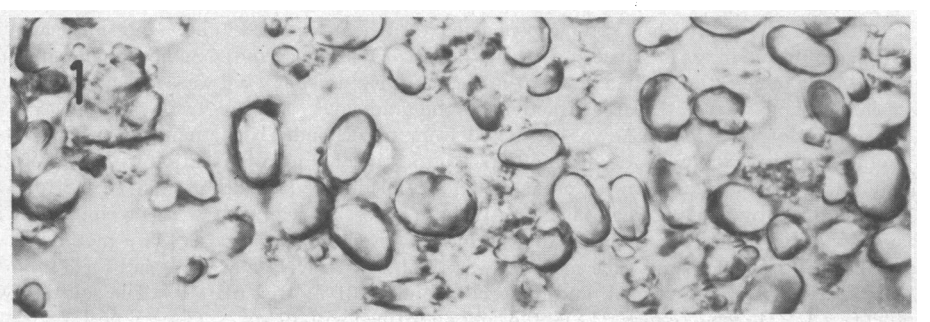


FIG. 1.—Light micrograph of unstained suspension of the low-speed pellet, ×800. The large particles are yolk granules. Micrograph by L. Arcos.

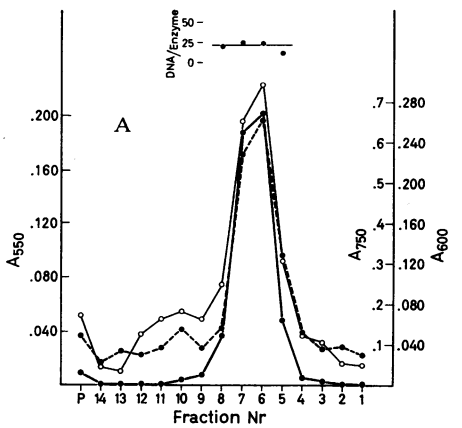
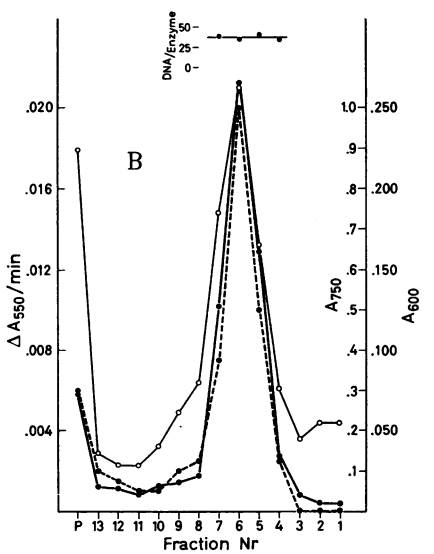
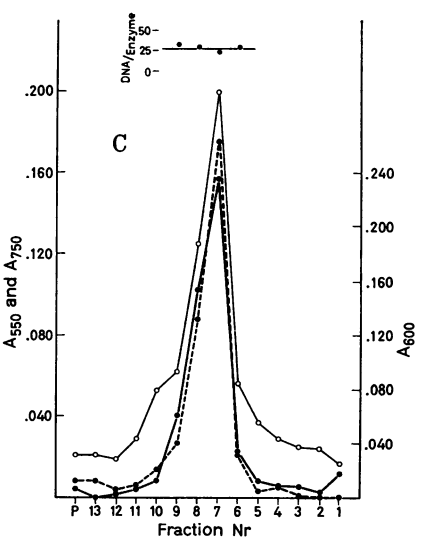


FIG. 2.—Sucrose density gradient centrifugation of egg mitochondria. (*A*) *R. pipiens* eggs; (*B*) *R. pipiens* ovarian oöcytes; (*C*) *X. laevis* eggs. Bottom of tube is to left; fraction *P* is pellet of pigment granules. Each 2-ml fraction was diluted with 1.5 ml buffer, and samples of 10–50 μl were assayed for protein by Lowry test (A_{750}, *thin line, open circles*), and for cytochrome *c* oxidase (A_{550}), or succinate-cytochrome *c* reductase (A_{550}; both *dashed line, full circles*). Remaining samples were made 10% in trichloroacetic acid, centrifuged, pellets washed twice with cold 2% trichloroacetic acid, and once with 80% ethanol containing 0.1 *M* sodium acetate, air-dried, and extracted with 0.2 ml 1 *N* perchloric acid at 75°. DNA was determined in 0.1 ml of extract by diphenylamine reaction (A_{600}, *heavy line, full circles*). Top insets show ratio of DNA content to enzyme activity in arbitrary units in the peak fractions.





shown by the product obtained with crude extracts of whole eggs.[1] Table 1 is a summary of the quantitative analyses of DNA in mitochondria and in whole eggs. The DNA associated with mitochondria accounts for the bulk of the DNA of whole eggs. Purification of DNA from mitochondria gave yields of 65–80 per cent of the total content as indicated by the diphenylamine test. The amount of DNA per mitochondrial protein averages 0.52 μg/mg and is similar to values found for liver and muscle mitochondria of several animal species.[7, 11–14] The amount of mitochondrial protein per wet weight of egg tissue is one order of magnitude lower than the amount obtained from liver by similar purification techniques.[12, 14, 15]

To allow the preparation of sufficient material, three gradients were loaded with aliquots of the same suspension of crude mitochondria and centrifuged together.

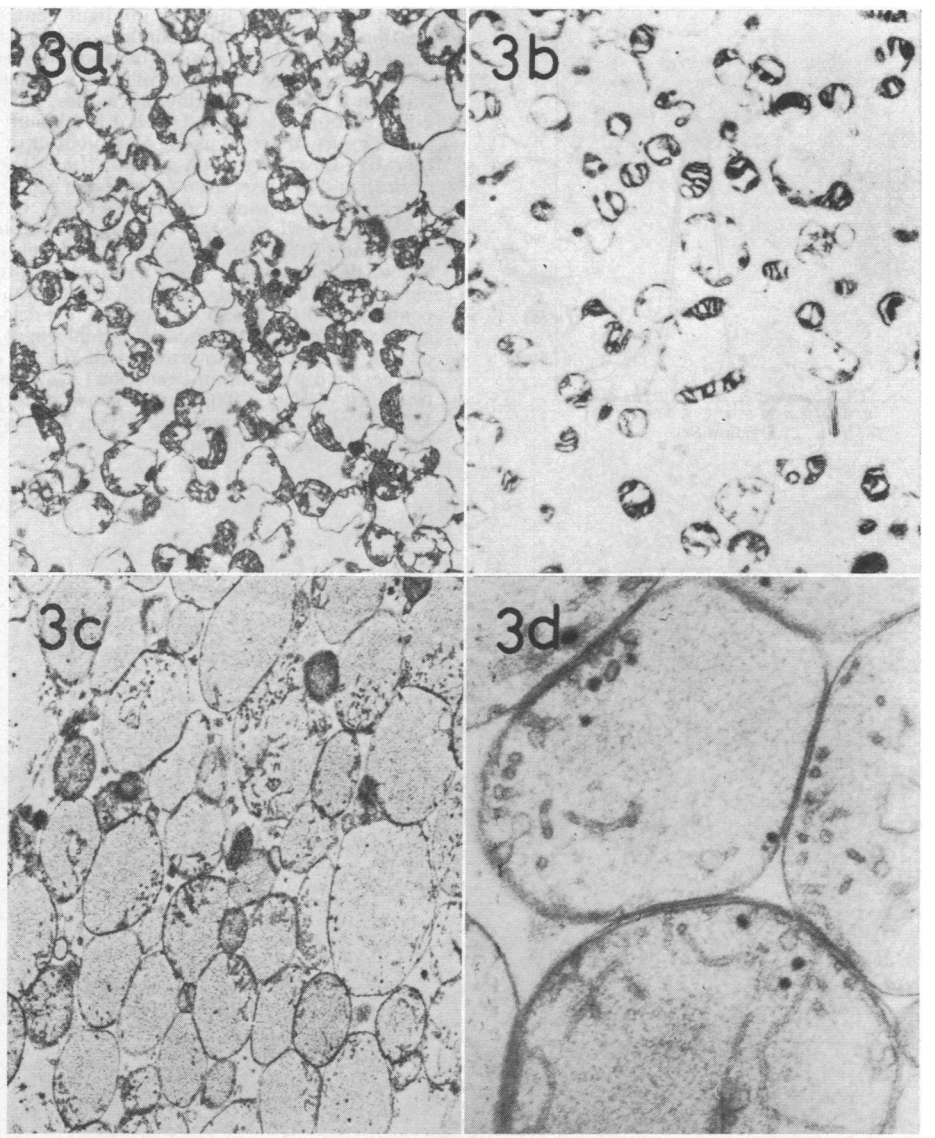


FIG. 3.—Electron micrographs of mitochondria. (*a*) *X. laevis* eggs, ×9,200; (*b*) *R. pipiens* oöcytes, ×12,000; (*c*) *R. pipiens* eggs, ×9,200; (*d*) same as (*c*), ×30,000. The electron microscopic examination was carried out by Renate Bromberg and D. R. Wolstenholme. Pellets were fixed for about 12 hr at room temperature in Kellenberger's 1% OsO_4, treated with uranyl acetate,[8] dehydrated in a graded series of ethanol, and embedded in either methacrylate-divinyl benzene[9] (*a* and *b*) or Epon[10] (*c* and *d*). Electron micrographs of thin sections were made with a Siemens-Elmiskop I.

One gradient was used for analysis (Fig. 2), and the peak tubes of the other two were used for the isolation of DNA. The purity of the DNA preparations was judged by the absence of UV-absorbing material on either side of the DNA band in CsCl gradients. Banding of egg mitochondrial DNA is shown in Figure 4. The density of the DNA's agree with those found previously for whole-egg DNA.[1]

TABLE 1

MITOCHONDRIAL DNA AND PROTEIN IN EGGS

	Content per Egg					
	Mitochondrial protein (μg)	DNA in whole egg* (mμg)	DNA in mitochondria† (mμg)	DNA from mitochondria‡ (mμg)	DNA-to-protein ratio in mitochondria (μg/mg)	DNA per diploid cell* (μμg)
X. laevis	4.7	3.1	2.4	2.0	0.51	6
R. pipiens	6.0	4.5	3.1	2.0	0.52	15

* Quoted from ref. 1; the values for whole eggs were corrected for losses in purification by an isotope dilution technique. Actual recoveries were 1.5 mμg for *X. laevis* and 3.6 mμg for *R. pipiens*.
† Assayed with the diphenylamine reaction.
‡ Purified DNA, after banding in CsCl.

Thus, in *R. pipiens*, egg mitochondrial DNA has the same density as somatic, nuclear DNA, whereas in *X. laevis*, mitochondrial DNA is more dense by 0.002 gm/cm.[3] The density of mitochondrial DNA of both species corresponds to a content of 43 per cent guanylic plus cytidylic acid.[16] Heating increased the density by 0.017 (Fig. 4*B*), as expected for double-stranded DNA. This finding is in agreement with the reported melting behavior of whole-egg DNA.[1]

The problem[7] of polysaccharide banding in the density range of 1.7 has been considered. Such material has been observed in some experiments on whole-egg DNA; it could be removed by purification of the DNA on MAK. The polysaccharide is not retarded by the column and can be washed through. Furthermore, this material can be distinguished from DNA by its ultraviolet spectrum, since it exhibits increasing absorbancy with decreasing wavelength, lacking a maximum at 260 mμ. All DNA preparations submitted to density gradient analysis had been purified on MAK and exhibited a typical nucleic acid spectrum. Also, heating caused the expected density shift. Additional criteria for the identity of egg DNA were met previously.[1]

Discussion.—Although frog eggs are quite different from liver cells in some respects, egg mitochondria could be purified by a method similar to procedures applicable to liver. It is important to use buffered sucrose during isolation; whereas ion-free sucrose solutions are quite suitable for the preparation of mitochondria from some tissues, egg homogenates are acidic and the presence of sufficient buffer is required to raise the pH to neutrality. The morphology of the isolated particles was not very well preserved (Fig. 3), probably due to the time needed for isolation and to the hypertonic conditions to which the mitochondria were exposed.

The possibility that the DNA found in the particulate fraction is only absorbed

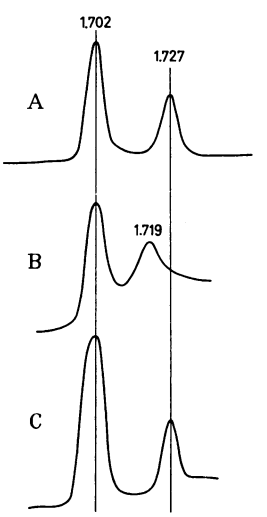


FIG. 4.—CsCl density gradient centrifugation of egg mitochondrial DNA. (*A*) *R. pipiens* oöcyte DNA; (*B*) same as (*A*), a mixture of equal amounts of native DNA and DNA denatured by heating to 95°; *X. laevis* egg DNA. The band at the density 1.727 is DNA of *Pseudomonas aeruginosa*.[16] The samples were centrifuged for 20 hr at 39,460 rpm at 20°. In order to compare with the densities given earlier for egg DNA,[1] 0.002 must be added to the present values, since a different density had been assumed for the reference DNA.

but not an actual constituent of these particles has been considered and rejected previously by following the distribution of labeled DNA added to the homogenate.[1] In other cases where mitochondrial DNA has been characterized, this DNA was mostly found to have a different density than nuclear DNA[7, 17, 18] (see *Note added in proof*). In the case of the rat,[13] though, this difference is small, similar to the situation in *X. laevis*. Clearly, mitochondrial DNA can be very different or quite similar to nuclear DNA in its density or gross base composition. Even when the density is the same, as in *R. pipiens*, the sequences of mitochondrial and nuclear DNA differ, as shown by the homology of egg DNA to only a few sequences in liver DNA.[1] The lack of homology of mitochondrial DNA to most sequences in nuclear DNA can be taken as further evidence for the supposition that the DNA found in mitochondria is an *in vivo* constituent of the organelles.

Whereas eggs contain only about 10 per cent as much mitochondrial protein as liver on a weight basis, the content per cell, or relative to the number of nuclei, is about 10^5 times higher. Since one frog egg weighs about 10^6 times as much as a liver cell, the concentration of egg nuclei per weight of tissue is only 10^{-6} that of liver nuclei. This fact causes an "inversion" of the quantitative relation of nuclear to mitochondrial DNA. In liver, the mitochondrial DNA amounts to 0.2–1.5 per cent of the total.[11–14] In contrast, eggs contain 300–500 diploid complements of DNA and the nucleus contributes less than 1 per cent to the total egg DNA. The size difference between egg and liver cells, together with the lower concentration of mitochondria in eggs, neatly accounts for this inversion.

On the basis of the evidence presented in this and a previous[1] report, the following conclusions can be reached regarding frog egg cytoplasmic DNA. At least two thirds of the total egg DNA can be found in highly purified mitochondrial preparations; there is good reason to believe that this DNA is an integral part of these organelles. The sequences of this DNA differ from the bulk of sequences present in nuclear DNA. The yolk and pigment fractions contain little, if any, DNA; results were at the limit of sensitivity of the methods. About 20 per cent of the egg DNA was found in the postmitochondrial supernatant fraction. Whether this "supernatant DNA" is derived from the breakage of mitochondria during isolation or whether a different fraction of DNA exists is not known at present. *The phenomenon of excess cytoplasmic DNA in amphibian eggs is thus caused by the large size of these cells and by the great number of mitochondria they contain.*

The situation outlined above may not be restricted to frog eggs. Recently, the isolation of fish oöcyte DNA[19] and the banding in CsCl of sea urchin DNA[20, 21] have been reported. In sea urchins, as in frogs, egg DNA has a similar density to nuclear DNA. The excess is only about 30 times the haploid value,[22] in accord with the smaller size of these eggs. Furthermore, sea urchin egg DNA is evenly distributed between nucleate and enucleate halves,[21] and so are the egg's mitochondria.[23] Furthermore, Bell and Mühlethaler[24] have shown by high-resolution autoradiography that a large part of the DNA in eggs of an aquatic fern is associated with mitochondria and proplastids. Mitochondrial DNA may then represent the bulk of excess, cytoplasmic DNA in the eggs of different animal species, while plastids will make their contribution in plants.

Mitochondrial DNA has been suggested as a basis for cytoplasmic heredity and for autonomy and self-replication of mitochondria.[7, 17, 25] These speculations may

apply to egg mitochondria in the same way as to mitochondria of other cells. The special situation in eggs and embryos may add some aspects, though. While the hypothesis suggesting that cytoplasmic DNA functions as a storage material for the assembly of chromosomes during early development has been excluded (see ref. 1), it is possible to regard the entire mitochondria of the egg as stored material. The mitochondria would be partitioned into newly forming cells during development and used as such, without degradation and resynthesis. This behavior during development would be analogous to that of egg ribosomes which are used ununchanged,[4, 26] but different from the role of the major storage material, yolk. In further analogy with the ribosomes, one might speculate that the developing embryo does not need new mitochondria for some time, although it may begin their synthesis long before the need arises.

Summary.—DNA was found in purified mitochondria from eggs and oöctyes of two amphibian species, *Rana pipiens* and *Xenopus laevis*. The association of DNA with the mitochondrial particles was demonstrated by the parallel distribution of DNA and two mitochondrial enzymes in sucrose gradients, by the DNase-stability of the DNA inside the particles, and by electron microscopic checks of the purity of the mitochondrial preparations. The DNA was isolated and its density determined; it is identical to the density of whole-egg DNA determined previously. At least two thirds of the DNA of eggs was found in the mitochondria. The bulk of egg cytoplasmic DNA is thus of mitochondrial origin and the phenomenon of excess DNA in eggs is caused by the presence of a large number of mitochondria per egg.

Note added in proof: In recent articles [Corneo, G., *et al.*, *Science*, **151**, 687 (1966), and Borst, P., and G. J. C. M. Ruttenberg, *Biochim. Biophys. Acta*, **114**, 645 (1966)], it was reported that mitochondrial DNA in several mammals had the same or a very similar density as nuclear DNA, analogous to the situation in frogs.

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Abbreviations used: SDS, sodium dodecyl sulfate; MAK, methylated albumin on kieselguhr.

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