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Studies on the DNA of *Xenopus laevis* oocytes

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The existence of substantial amounts of deoxyribonucleic acid (DNA) in the cytoplasm of amphibian eggs is no longer a matter of discussion (review, Brachet, 1957). However, their intracellular distribution, role and origin remain controversial.

According to Dawid (1965, 1966), the bulk of the egg cytoplasmic DNA in *Xenopus* is of mitochondrial origin. His method of phenol extraction isolates only high molecular weight DNA.

On the other hand, Baltus & Brachet (1962) found that 65% of the DNA of *Pleurodeles* eggs sediments at low centrifugal speed and suggested that this nucleic acid is localized in the yolk platelets. This conclusion was based on chemical estimations of the total DNA present in the egg; they found values about 10 times higher than those presented by Dawid (1966).

Brachet & Ficq (1965) confirmed that, in ovaries of *Pleurodeles* labelled with ^{14}C-actinomycin, either *in vivo* or on histological sections, most of the radioactivity detected by autoradiography is concentrated in the yolk. For this reason, Brachet (1967) suggested the existence of at least two types of DNA, one bound to the mitochondria and the other, more labile, localized on the yolk platelets. On the basis of numerous chemical analyses, Løvtrup (1966) also concluded that the existence of two types of cytoplasmic DNA in *Rana temporaria* is highly probable.

A comparable heterogeneity in the population of DNA molecules has also been demonstrated by Agrell & Bergqvist (1962) in the nuclei of embryonic cells.

In order to obtain further information concerning the nature of the cytoplasmic DNA store, we have studied the quantitative evolution of DNA during oogenesis, and its sensitivity to several enzymic and chemical agents.

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MATERIAL AND METHODS

Removal of follicle cells

Oocytes of *Xenopus laevis* were taken from animals anaesthetized with MS 222 Sandoz, and kept in Ringer solution, to which 10 μg chloromycetin/ml was added.

Small portions of the ovaries were left for 30 s in a 0·05 % solution of pronase (Calbiochem. B grade) made up in Ringer solution. They were then washed thoroughly in Ringer solution, and the follicle cells were removed manually from each oocyte with thin forceps. The oocytes were then stored in alcohol until DNA determination (see Plate 1, A, B).

The pronase treatment does not remove the follicle epithelium cells completely; but it facilitates the manual removal of these cells.

DNA determinations and enzymic assays

DNA determinations were performed by the fluorometric technique previously described (Baltus & Brachet, 1962). The subsequent scheme briefly describes the different steps:

(*a*) The oocytes were homogenized in alcohol and the lipids were removed by treatment with several solvents (alcohol, *n*-butanol, ether).

(*b*) The dry powders so obtained were extracted with 10 % (1·80 M) NaCl for 60 min and again for 30 min.

(*c*) The saline extract was precipitated by the addition of two volumes of 94° alcohol at −20 °C.

(*d*) The pellet was dissolved in a small volume of bidistilled water and dialysed against water for 6 h at 4 °C. The dialysate was divided into several equal fractions, according to the number of enzymic assays to be performed. The enzymic assays were as follows:

(1) Control extracts were incubated with 0·005 M Tris buffer, pH 7·3.

(2) Assay with deoxyribonuclease (DNase) alone: the extract was incubated for 90 min at 37 °C in 0·005 M Tris buffer, pH 7·3, containing 10^{-3} M-$MgSO_4$ and 0·3 mg/ml DNase (Worthington, 1 × crystallized).

(3) Assay with trypsin plus DNase: the extract was incubated in 0·005 M Tris buffer, pH 8·0 containing 0·5 mg/ml trypsin (Sigma, 2 × crystallized) for 30 min at 37 °C, then with Soybean Trypsin Inhibitor (SBI) (0·3 mg/ml) (Calbiochem, 3 × crystallized, B grade) for 5 min at room temperature, and finally with DNase as described above.

(4) Assay with pronase plus DNase: the extract was incubated for 30 min at 37 °C in 0·005 M Tris buffer containing 0·5 mg/ml pronase, and then with DNase.

(5) Assay with RNase plus DNase: the extract was incubated for 30 min at 37 °C in 0·005 M. Tris buffer containing 0·1 mg/ml ribonuclease (RNase, Sigma, 5 × crystallized, type 1A, bovine pancreatic); it was then treated with DNase as described above.

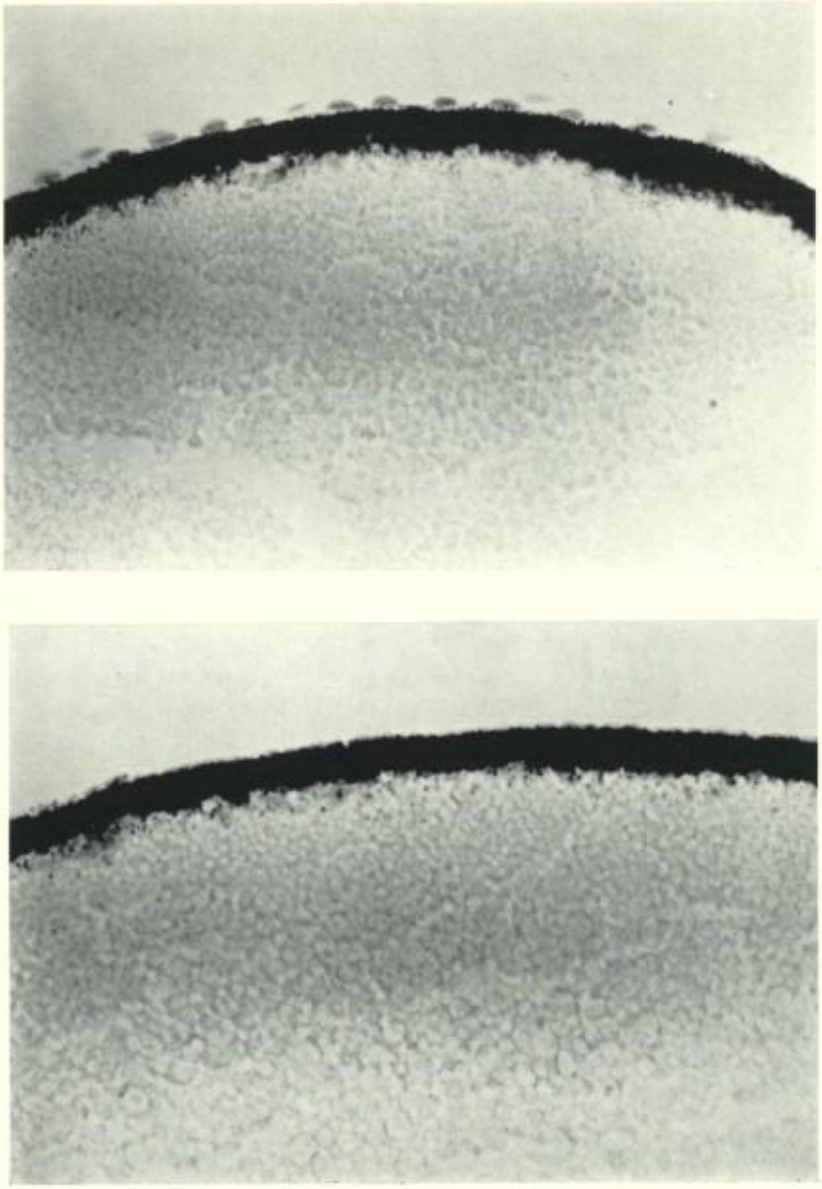

Fig. A. Partial view of an intact *Xenopus* oocyte.

Fig. B. Partial view of a *Xenopus* oocyte from which follicle cells were removed manually after a short pronase digestion.

Treated and control extracts were precipitated by the addition of 0·1 volume 10 % NaCl and two volumes of absolute alcohol at −20 °C. DNA determinations were performed using a fluorometric technique (Baltus & Brachet, 1962).

Autoradiography

Follicle cells were removed from oocytes after treatment with pronase (see above). The jelly coat of fertilized eggs was removed manually.

Oocytes (*Xenopus laevis*) and developing eggs (*Pleurodeles waltlii*) were fixed by freeze-substitution (Lison, 1960) and embedded in paraffin; 10 μ sections were treated as follows:

(1) DNase: 0·1 mg/ml dissolved in 0·1 M Tris, 0·03 M-$MgSO_4$ pH 7·0 for 90 min at 37 °C.

(2) Hydrolysis with N-HCl at 60 °C for 5, 10 and 30 min.

(3) Same hydrolysis, but followed by digestion with DNase as above.

(4) Trypsin 0·1 mg/ml. and 1 mg/ml. at 30 °C for 30 min followed by an incubation with trypsin inhibitor (0·8 mg/ml. at 30 °C for 30 min) before digestion with DNase as above.

Following the method described by Brachet & Ficq (1965), control and treated slides were dipped into a solution of ^{14}C-actinomycin (2·36 C/M, 40 μg/ml) for 30 min. After a 30 min bath in unlabelled actinomycin (5 μg/ml) and an overnight cycle with tap water, the slides were processed by the procedure of Ficq (1959) using G 5 Ilford emulsion.

RNA determination

The method described by Steinert (1951) was used.

Protein determination

We used a modification of the biuret method (Stickland, 1951).

Amino acid analysis

These determinations were carried out on a Moore & Stein automatic analyser (Beckman, model 120B).

RESULTS

Estimation of nucleic acids and proteins in oocytes of increasing size

The results are given in Table 1.

The DNA content of the oocytes is very different from one animal to another. For this reason, all experiments designed to study the evolution of DNA during vitellogenesis were performed on oocytes from the same ovary.

Table 2 shows the results obtained for different animals.

Sensitivity of the DNA of oocytes of different sizes and of developing eggs to DNase and to DNase plus trypsin

The true content of DNA has been calculated assuming that 80 % of the fluorescence is due to DNA, since this is the value which has been obtained for both very small oocytes (0·5 mm diameter) and gastrulae. In the cases where the percentage of fluorescent material removed by DNase is lower, we have supposed that the DNA is less accessible to DNase; we have considered that 80 % of the fluorescent reaction is due to DNA even in those cases.

Table 1.

	Oocyte diameter (mm)	Oocyte volume (mm^3)	DNA/ oocyte (μg)	RNA/ oocyte (μg)	Total protein (mg)
1	0·65	0·102	0·0106	2·3	0·0233
2	0·75	0·159	0·0234	3·8	0·108
3	1·130	0·550	0·0318	4·4	0·175
4	1·340	0·940	0·0435	6·0	0·240
	Increase between extreme stages				
	2 times	9·2 times	3·7 times	2·6 times	10·6 times

Table 2.

Oocyte diameter (mm)	DNA/oocyte (μg)			
	Xenopus I	Xenopus II	Xenopus III	Xenopus IV
0·65	0·011	0·029	0·015	0·008
1·35	0·044	0·089	0·035	0·020

Table 3.

Determination made on	Percentage of fluorescence removed by treatment with	
	DNase alone	DNase plus trypsin
Xenopus I		
Oocyte 0·65 mm diam.	43	65
Oocyte 0·75 mm diam.	8	44
Oocyte 1·00 mm diam.	5	60
Oocyte 1·34 mm diam.	5	75
Xenopus II		
Oocyte 1·35 mm diam.	5	46
undivided eggs	3	46
morulae	0	46
gastrulae	52	80

The action of other chemicals on the sensitivity of the oocytes DNA to DNase

(*a*) Pronase is less efficient than trypsin in hydrolysing the DNA-bound protein.

(*b*) When dry powders of oocytes are treated with N-$HClO_4$ their DNA does not become sensitive to DNase digestion.

(*c*) In the same way, treatment of dry powders of oocytes with N-HCl for 5 min at 25° or 60 °C does not change the sensitivity of their DNA toward DNase.

Behaviour of yolk platelets

Yolk platelets were isolated from oocytes of increasing size, using the method described by Wallace & Karasaki (1963). The DNA content and the sensitivity toward DNase were determined in the dry powders as already described. The DNA content of heavy particles (yolk platelets plus mitochondria) accounts for 63 % of the total egg DNA.

Table 4 shows the results, which are essentially similar to those obtained for whole oocytes.

Table 4.

Oocyte diameter (mm)	Percentage of fluorescence disappearing after treatment with: DNase alone	DNase plus trypsin
0·75	42·5	65
1·0	5·0	60
1·34	6·0	57

Analysis of the protein bound to DNA

Since the sensitivity of oocyte extracts toward DNase varies according to the size of the oocytes, and since it is increased by treatment with trypsin, we wondered about the chemical nature of the protein bound to DNA after extraction of the oocytes with 10 % NaCl.

The extracts were dialysed overnight, adjusted to 6 N-HCl and then boiled for 24 h. The amino acids resulting from the hydrolysis were chromatographed in the Moore and Stein analyser.

Table 5 gives the amino acid composition of the protein which remains bound to DNA in oocytes of different sizes after extraction with 10 % NaCl. We found that, in the four categories of oocytes studied, this bound protein amounts to about 8 % of the total proteins present in the oocytes. Table 5 also includes the amino acid compositions that we obtained from developing eggs.

One can see that the basic amino acid content of the DNA-bound protein increases slightly during the growth of the oocyte, while it decreases after fertilization. The free protein remains unchanged during development.

Although the protein which is bound to DNA contains more than 20 % basic amino acids, it is also very rich in dicarboxylic amino acids. It should be noted that its serine content is exceptionally high (31–39 %).

Table 5.

	Bound protein					Free protein				Protein extracted from frog oocytes*
Amino acid	Oocyte 0·65 mm	Oocyte 0·75 mm	Oocyte 1·34 mm	Morula	Neurula	Oocyte 0·65 mm	Oocyte 1·34 mm	Morula	Neurula	
Lysine	8·87	9·71	10·0	9·96	9·61	7·45	7·09	7·51	7·33	12·6
Histidine	3·91	4·55	4·84	4·71	4·45	2·68	3·0	2·79	2·81	7·0
Arginine	7·67	7·93	8·03	7·39	7·07	5·63	5·18	5·35	5·37	12·3
Basic amino acid (%)	20·45	22·19	22·84	22·06	21·13	15·76	15·27	15·65	15·51	31·9
Aspartic acid	7·24	6·97	7·13	6·83	6·75	8·76	8·61	8·32	8·48	8·1
Threonine	2·13	1·84	1·41	2·32	1·81	5·26	5·05	5·23	5·29	4·2
Serine	31·09	37·42	37·49	34·64	38·81	6·69	6·63	6·97	7·44	3·4
Glutamic acid	17·23	17·46	17·64	17·15	16·92	13·25	13·47	13·44	13·29	8·2
Proline	2·99	2·56	2·70	2·85	2·38	4·49	4·77	4·78	4·95	3·7
Glycine	10·18	6·17	4·83	5·98	5·24	5·64	5·08	5·23	5·21	5·1
Alanine	2·66	2·24	1·82	2·70	2·17	8·85	8·86	9·07	9·02	3·5
Valine	1·35	1·16	0·82	1·49	1·08	6·63	6·96	6·47	6·18	3·9
Isoleucine	0·97	0·70	0·51	0·90	0·62	5·41	5·71	5·43	5·21	2·9
Leucine	1·68	1·29	1·0	1·53	1·23	8·67	8·72	8·60	8·64	3·0
Tyrosine	0·62	0·59	0·85	—	0·44	3·34	3·35	3·22	3·31	2·8
Phenylalanine	1·18	1·02	0·92	0·92	0·90	4·05	4·22	4·06	4·14	2·6

*Horn (1962).

Autoradiographic study of ^{14}C-actinomycin binding in oocytes and developing eggs

Oocytes

Nuclei. The binding of actinomycin is very weak and there is no appreciable radioactivity over the nucleoli.

Cytoplasm. The number of tracks per unit area increases with the size of the oocyte. After pretreatment of the slides with DNase, there is no decrease in the amount of ^{14}C-actinomycin bound to cytoplasmic structures. Treating the slides with N-HCl at 60 °C for 5 min results in more pronounced binding of actinomycin. If the time of HCl treatment is increased (up to 60 min), this phenomenon becomes more and more obvious. When the slides are pretreated with N-HCl, and then with DNase, the binding of actinomycin is visibly decreased.

Another series of experiments was performed on ovaries of a different amphibian species, *Bufo regularis pardalis*. In this case, before treatment with DNase, some sections were submitted to digestion with trypsin, followed by incubation in trypsin inhibitor.

Table 6 summarizes the results obtained: they are expressed in number of ^{14}C-electron tracks per cell area, counted on twenty sections of each type.

Several fragments of ovaries were centrifuged (Sorvall, 2500*g* at 0 °C for 15 min). In sections of these centrifuged oocytes, it is possible to distinguish between the radioactivity bound to the lighter hyaline cytoplasm and the label bound to the yolk platelets, which accumulate at the centrifugal pole of the ripe oocytes.

Table 6.

^{14}C-actinomycin *Bufo regularis pardalis* ovaries	Cytoplasm mature oocytes	Cytoplasm young oocytes	Germinal vesicle	Follicular cells	Centrifuged oocyte hyaloplasm	Centrifuged oocyte yolk.
Controls	1·1	0·9	1·2	13·0	0·3	2·5
+RNase	1·0	1·1	1·2	12·5	0·4	1·9
+DNase	0·5	0·4	0·1	0	0·1	1·3
+DNase and RNase	0·8	0·7	0·2	0·2	0·3	1·1
+trypsin and trypsin inhibitor	0·9	1·0	0·8	7·0	0·4	1·8
+trypsin trypsin inhibitor and RNase	0·7	0·7	0·5	4·5	0·3	2·8
+trypsin trypsin inhibitor and DNase	0	0	0	0	0	0

Eggs

Nuclei. From the morula stage onwards, DNase treatment completely abolishes the binding of actinomycin to the chromatin. The same result is obtained after 5 min treatment with N-HCl.

Cytoplasm. We could not observe, at any stage, a significant decrease of ^{14}C-actinomycin binding after treatment with DNase alone.

Treating the slides with N-HCl for 5 min leads, as in the case of the oocytes, to an increased binding of ^{14}C-actinomycin. This increase is most obvious in the entomesoblastic cells.

DISCUSSION

In *Xenopus* oocytes the total DNA content increases with size. The increase in the volume of the oocytes is double that of the increase in DNA content.

The sensitivity toward DNase of the oocyte DNA decreases during vitellogenesis; it seems likely that the nucleic acid is complexed in a way which makes it inaccessible to the action of DNase.

The oocyte extracts have been treated with different agents, which could un-

mask the cytoplasmic DNA. The order of decreasing efficiency was as follows: trypsin, pronase, RNase.

Autoradiographic observations have also shown that treatment by DNase alone does not prevent the binding of ^{14}C-actinomycin to cytoplasmic DNA.

Curiously enough, N-HCl had no effect on the DNase sensitivity of DNA if applied to oocyte extracts, but it increased the binding of ^{14}C-actinomycin to cytoplasmic DNA, as shown by the autoradiographic observations. But it is still unexplained why 1 N-HCl, which probably releases proteins from a nucleoprotein complex in the case of cytoplasmic DNA, does not, at the same time, remove the purine bases of DNA; such a hydrolysis would prevent any binding of actinomycin to DNA.

Our results are in excellent agreement with those obtained by Muckenthaler & Mahowald (1966), who showed by autoradiography that incorporation of thymidine into the ooplasm of *Drosophila*, although insensitive to DNase alone, becomes labile after a pretreatment with proteolytic enzymes, especially trypsin. Our experiments also confirm, in Anurans, the observations first made on Urodeles (Brachet & Ficq, 1965): a fraction of DNA, resistant to DNase under our experimental conditions, binds actinomycin at the cytoplasmic level. A treatment with trypsin and trypsin inhibitor, followed by a DNase digestion, is evidently a drastic one; it damages the biological material and, sometimes, the photographic emulsion as well. Nevertheless, observations of the large areas remaining intact in these preparations lead to the conclusion that, after these treatments, actinomycin is no longer bound to the oocyte cytoplasm.

The analysis of the protein which remains associated with DNA during the course of extraction with 10 % (1·80 M) NaCl at 100 °C showed that it is not a pure histone, as we first thought. We might be dealing, instead, with a mixture of a histone and other proteins. Indeed, this DNA-bound protein, although it is rich in basic amino acids, has a high content of dicarboxylic acids and serine. The last point is not surprising, in view of the statement by Flickinger & Nace (1952) that hot NaCl completely dissolves the yolk platelets, in which Wallace (1963) found as much as 49 % serine.

Recently, several authors showed the existence in the nuclei of a complex between a phosphoprotein rich in serine, as reported by Langan (1967), a histone and RNA molecules. Huang & Bonner (1965) suggested that the latter could act as 'Operator–detector' substances giving specificity to the association between histone and DNA.

Several facts suggest that such a molecular association might also exist at the level of cytoplasmic DNA: the presence of histones in the yolk platelets is generally accepted (Bloch, 1962; Horn, 1962; Moore, 1963), even if it has been recently disputed by Davenport (1967). On the other hand, the resistance of some cytoplasmic particles (chloroplasts and mitochondria) to DNase could be due to the fact that their DNA is bound in a nucleoprotein complex, although

other explanations (low permeability of the membrane to the enzyme, for instance) cannot be excluded.

In the case of *Xenopus laevis*, Dawid (1966) has confirmed that mitochondrial DNA is insensitive to DNase under conditions where nuclear DNA is completely labilized. As we have seen, the same author concludes that the bulk of cytoplasmic DNA can be recovered from the mitochondria. However, our own DNA estimations, carried out on mitochondria and on yolk platelets obtained by Dawid's fractionation procedure (1966) have shown that only 5–7·5 % of the egg DNA is present in the mitochondria; the bulk of cytoplasmic DNA is concentrated in the yolk platelets. Furthermore, in autoradiograms of centrifuged oocytes, the heavy fraction (yolk) binds about 8 times more actinomycin than the light fraction of the cytoplasm. This observation confirms our previous results (Baltus & Brachet, 1962; Brachet & Ficq, 1965). Yolk DNA could be of low molecular weight, as suggested by one of us (Brachet, 1966) and by Løvtrup (1966); if so, it might be impossible to recover it by using the extraction procedure chosen by Dawid (1965). Such an interpretation is supported by Haggis's recent results (1966) showing that, in *Rana pipiens*, only nuclear DNA is entirely recovered by the classical phenol extraction procedure.

It is still difficult to understand why the DNA of the oocytes becomes more and more insensitive to DNase during the course of vitellogenesis. The amino-acid composition of the protein which is bound to DNA does not alter during oogenesis; however, the protein concentration relative to DNA steadily increases during that period. This quantitative change could perhaps explain the increasing degree of insensitivity of DNA to DNase action. More work concerning the nature and the possible repressing activity of the protein bound to yolk DNA is necessary before we can draw more precise conclusions.

SUMMARY

1. Cytoplasmic DNA increases during oogenesis in *Xenopus*.

2. This DNA is bound in a nucleoprotein complex, which becomes accessible to DNase only after treatment with proteolytic enzymes.

3. Isolated yolk platelets behave in the same way as whole oocytes towards enzymatic agents.

4. The results obtained by biochemical determinations have been confirmed by autoradiography.

RÉSUMÉ

1. Le DNA cytoplasmique des ovocytes de *Xénope* augmente en quantité au cours de l'ovogénèse.

2. Ce DNA est enfermé dans un complexe nucléoprotéique qui n'est accessible à la DNAse que s'il a été prétraité par des enzymes protéolytiques.

3. Les plaquettes vitellines isolées se comportent comme les ovocytes entiers vis-à-vis des enzymes.

4. Ces résultats ont été obtenus par des dosages biochimiques, et confirmés par autoradiographie.

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