THE GERMINAL VESICLE NUCLEUS OF
XENOPUS LAEVIS OOCYTES AS A SELECTIVE
STORAGE RECEPTACLE FOR PROTEINS

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ABSTRACT
The amorphous nucleoplasm of the germinal vesicle nucleus of Xenopus laevis oocytes has been selectively extracted under conditions which leave the nuclear formed elements morphologically intact. The nucleoplasm contains about 97% of the total nuclear proteins and on SDS-polyacrylamide gels some 68 polypeptides can be distinguished. On the basis of solubility differences, the nucleoplasmic proteins can be classified into two categories. The first consists of soluble or easily solubilized proteins which comprise about 34 polypeptides making up 87% of the nucleoplasm. A few of these proteins show electrophoretic mobilities similar to those of soluble proteins of the cytoplasm, but most are unique to the nucleus. The residual 13% of the nucleoplasmic proteins are tightly bound to a nucleoplasmic gel and can be extracted only by solubilizing the gel. The solubility characteristics of the proteinaceous gel suggest a complex held together by salt, nonpolar, hydrogen, and possibly disulfide bonding. Some 34 polypeptides can be distinguished in this gel fraction, including prominent and highly enriched polypeptides of about 115,000 and 46,000 daltons. The relatively soluble fraction of the nucleoplasm does not contain informofers and contains little or no nucleic acid. Evidence is presented that if histones are present in the germinal vesicle, they can comprise no more than about 8% of the total protein. The possibility is discussed that the unique polypeptides of the nucleoplasm may be sequestered there by selective adsorption to or in the nuclear gel.

Oogenesis is characterized by an unusual enlargement of the nucleus during prophase of the first meiotic division. At the completion of oogenesis in Xenopus laevis, for example, the germinal vesicle (GV) is approximately $1.7 \times 10^4$ times the volume of a somatic cell nucleus. Yet, in the meiotically duplicated chromosomes the GV contains only the tetraploid amount of DNA. The most striking quantitative difference between an oocyte GV and a somatic nucleus, therefore, is the massive accumulation of nonchromosomal material. This material has been demonstrated to contain many proteins in oocytes of Triturus cristatus (14).

In amphibians, both experimental and biochemical work have revealed that the GV of the oocyte contains many interesting biological properties. For example, when the contents of GVs are injected into blastulae, an enhancement of head structures and cement gland occurs later in development (18). In the Mexican axolotl the “o"
mutation in the homozygous state prevents development beyond gastrulation. The lesion, maternally inherited, has been shown to be due to the loss or malfunction of a macromolecular component traceable to the GV of the oocyte (5, 6).

Similarly, the ability of the Rana pipiens egg to undergo complete cleavage after activation requires material from the GV (22). The cytoplasm of X. laevis eggs can replicate injected DNA whereas the cytoplasm of the ripe oocyte has only minimal activity. The acquisition of this ability by the egg cytoplasm is correlated in time with the breakdown of the GV during the first meiotic division (2, 13). There is also an interesting change in the distribution of divergent cations within the oocyte when the GV breaks down during meiotic maturation. This change can be accomplished without ionic exchange with the cell environment (20).

Developmentally important proteins may be "stockpiled" in the GV for use during early embryogenesis. For example, the activity of DNA-dependent RNA polymerase II in the GV of R. pipiens oocytes has been reported to be as high as in an entire stage 40 tadpole containing thousands of cells (25). In addition, it has been demonstrated that the stage VI (8) oocytes of X. laevis synthesize histones (1) which then seem to be preferentially accumulated in the GV (3, 4). These observations have led to the suggestion that histones might be "stored" in the GV (1).

Thus, we see that diverse activities and factors required for normal cleavage and development reside within this giant nucleus. In this study we have carefully extracted the nucleoplasm of stage VI oocyte GVs and examined the amount and molecular heterogeneity of their polypeptides. We have compared these with the polypeptides of the GV, and with Xenopus histones. Data to be presented show that there are about 68 distinguishable polypeptides in the nucleoplasm, making up more than 97% of the proteins of the GV. They exist as (a) diffusible components, common to some of the soluble components of the cytoplasm, (b) soluble or easily solubilized components confined to the GV, and (c) components bound in a nondiffusible state to a gel matrix within the GV.

MATERIALS AND METHODS

Isolation of Germinal Vesicles

Large X. laevis females were sacrificed and the ovaries removed on the day of the experiment. Stage VI oocytes (8) were punctured near the pigmented animal pole and the GVs gently squeezed out with forceps into an isolation medium (IM) containing 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 20 mM NaCl, 3 mM MgCl₂, and 1 mM 2-mercaptoethanol, 2% (wt/vol) polyvinylpyrrolidone (PVP) of 400,000 average molecular weight. The isolated GVs were quickly cleaned by being pulled in and out of a Pasteur pipet and were immediately transferred to the appropriate medium.

Selective Extraction Procedures

The most readily soluble proteins of the GV (fraction I) were removed by incubating the freshly isolated GVs in a standard extraction medium containing 10 mM Tris-HCl (pH 8.0), 100 mM KCl, 20 mM NaCl, 3 mM MgCl₂, and 1 mM 2-mercaptoethanol. After 4-11 h of extraction at room temperature at a concentration of 100 GVs per 100 μl, the extraction fluid was withdrawn with a microsyringe and 50-75 μl of the same extraction medium again added. After 1 h most of the final extraction medium was added to the first and the combined fluid called fraction II. The residual structures were washed in several changes of the same extraction medium. The GV residues contained chromosomes, nucleoli, and nuclear granules suspended in a gelatinous matrix and surrounded by the nuclear envelope.

Substances associated with the nuclear gel were selectively solubilized by transferring the washed GV residues to a medium containing 10 mM Tris-HCl (pH 9.2), 100 mM KCl, 20 mM NaCl, 3 mM MgCl₂, and 20 mM 2-mercaptoethanol. The solubilization of the gel and extraction of its materials were accomplished during a 14-h incubation at 5°C at a concentration of 100 GVs per 40 μl. The product of a second extraction of 1 h at 21°C was added to the first and the total called fraction III. The residual structures were washed in several changes of the same extraction medium.

Electrophoretic analysis of fractions obtained by long or short incubations gave essentially the same result. Little band smearing or build-up of low molecular weight material was observed in either case, which would indicate proteolytic activity.

The structural residues were washed several times and called fraction IV. This fraction consisted of chromosomes, nucleoli, nuclear granules, a little residual nuclear gel, and the nuclear envelope.

Washed particles of yolk and other cytoplasmic particulates were prepared from enucleated oocytes. The total yolky cytoplasm was stripped from cell and follicular membranes with forceps and collected in ice-cold standard isolation medium but without PVP. After mixing with a pipet, the suspension was centrifuged for 10 min at 2,000 g at 5°C. The pellet was washed several times in the same medium and termed the cytoplasmic particle fraction (PAR). It contained yolk, pigment, and any other granules or aggregates of large size.

Fat droplets were removed from the top of the stratified homogenate and discarded. The clear, particle-free layer was removed and centrifuged for 2 h at 105,000 g at 2°C. The supernate from this spin contained the postmicrosomal material of the cytoplasm and was
terming the S-100 fraction. Histones from washed chromatin from erythrocytes of *X. laevis* were prepared from cold 0.4 N H₂SO₄ extracts after the procedure of Adamson and Woodland (1).

**Preparation of Extracts for Electrophoretic Analysis**

The S-100 and I fractions were each divided into two portions. The first was prepared for a nondissociating electrophoretic separation of proteins by dialyzing against a buffer containing 0.06 M Tris-HCl (pH 6.7), 3% sucrose, and bromphenol blue marker dye. The second portion was dialyzed by dialyzing against 10 mM Tris-HCl (pH 7.5), 120 mM NaCl to remove potassium ions, and then against a solution containing 0.5% sodium dodecyl-sulfate (SDS) and 3% 2-mercaptoethanol. To ensure complete reduction of disulfide bonds and chain dissociation, the extracts were then brought to 60°C for 2 min. This procedure gave preparations which were reproducible and showed no evidence for aggregation of molecules. After cooling, the solution was dialyzed against the electrophoresis buffer. Fractions II, III, and PAR, and total GV (GV) were prepared for SDS electrophoresis in the same way. All samples were frozen and stored at -20°C until electrophoretic analysis.

**Acrylamide Gel Electrophoresis. Acrylamide Gradient Gel Electrophoresis at pH 8.9**

5–15 μl of undissociated S-100 or fraction I extracts were layered over polyacrylamide gel slabs containing a 4-25% linear gradient of acrylamide. The gels were equilibrated and run with a 0.1 M Tris-borate buffer (pH 8.9). Electrophoresis was done at 300 V for 2 h, 5°C. Bands were visualized by staining with 0.1% Coomassie brilliant blue R in 25% methanol containing 7% acetic acid.

**SDS Gel Electrophoresis**

Dissociated proteins were separated by a procedure based on that of Laemmli (15), scaled down to accommodate tubes of 0.2 cm ID. 2–12 μl of protein solution were layered over stacking gels 0.8-cm long and containing 3% acrylamide, 0.06% N,N'bis-methylene acrylamide, and buffered with 0.06 M Tris-phosphate (pH 6.7). The running gels were 5.5 cm long and contained 10% acrylamide, 0.027% N,N'-bis-methylene acrylamide buffered with 0.38 M Tris-HCl (pH 8.9). The electrode buffer was 0.05 M Tris-0.39 M glycine, 0.1% SDS was present throughout the system. Samples in 0.06 M Tris-phosphate buffer (pH 6.7), 0.1% SDS, 1% 2-mercaptoethanol, 5% sucrose bromphenol blue were layered over the stacking gel. Electrophoresis was carried out at 65 V for 2.5 h.

A molecular weight calibration was obtained using purified soybean trypsin inhibitor, carbonic anhydrase, yeast alcohol dehydrogenase, ovalbumin, bovine serum albumin, bovine serum albumin dimer, and phosphorylase a. Densitometry of Coomassie blue-stained gels was accomplished with a Zeiss PMQ III spectrophotometer fitted with a ZK5 scanner and used at 580 nm wavelength.

**Acid Urea Gel Electrophoresis**

For the separation of histones the procedure of Panyim and Chalkley (20) was scaled down to a semimicro level. 15% polyacrylamide gels, 0.2 × 5.5 cm, were used. Electrophoresis was done at 110 V for 2 h in 2.5 M urea, acetic acid, pH 2.8.

**Sucrose Gradient Analysis of Fraction I Material**

100 freshly isolated GVs were extracted as described above to give a fraction I. 160 μl of this fraction were layered over a 10-40% (wt/vol) sucrose gradient made up in the extraction medium. In a parallel gradient, ribosomes and subunits from *Paramecium aurelia* were run as sedimentation markers. The gradients were centrifuged for 1.25 h at 40,000 rpm in a Spinco SW-41 rotor. The tubes were pumped out through a UV absorption analyzer set at 280 nm wavelength. Estimation of particle sedimentation coefficients was made by interpolation, using the ribosome and 4S RNA markers.

**RESULTS**

In an effort to establish the solubility characteristics of GV nucleoplasm and nuclear structures, fresh GVs were transferred to a drilled microscope slide in various media. Structural changes were then monitored with an inverted microscope with phase-contrast optics to establish the effect of the test media. Often, the nuclear envelope was dissected off of the isolated GV so that internal nuclear structures could be seen better.

When GVs were isolated in standard isolation medium at pH 7.6–8.0, they did not swell and retained a normal appearance for hours. By the use of the Lowry assay for proteins (16), however, it was found that proteins are slowly lost in this medium despite the retention of a normal appearance. In trials with isolated GVs from two different animals, 13% and 18% of the total protein of the GV were lost in 1 h at 21°C.

The removal of PVP from the isolation medium caused GVs to swell immediately and lose protein much faster. In standard isolation medium without PVP, the GVs lose most of their protein mass but retain chromosomes, nucleoli, nuclear envelope, nuclear granules, and nuclear gel morphologically intact.

In these solubility tests, particular attention was paid to the solubility characteristics of the nuclear
FIGURE 1 Phase-contrast photomicrographs of unfixed germinal vesicles with the nuclear envelope removed. Unextracted (A and D); after extraction of Fraction I (B and E); and the Fraction III residue (C and F). Note the fibrillar nature of the gel in B and the appearance of chromosomes in C, E, and F. Note that after solubilization of the gel, all granules have fallen to the bottom in two-dimensional array (C and F). Note the presence of chromosomes, nucleoli, and small granules (probably RNP) in the Fraction III residue (C and F). The reference mark in A is 50 μm and the magnification in A, B, and C is the same. The reference mark in D is 50 μm and the magnifications in D–F are the same.
gel in which chromosome, nucleoli, and granules are embedded. The results of many such tests are summarized in Table I. These results suggest that the gel consists of protein(s) linked together by salt, hydrogen, and/or nonpolar binding. The sensitivity to 2-mercaptoethanol at elevated pH could mean the involvement of disulfide bonding as well. The use of 20 mM 2-mercaptoethanol at pH 9.2 was adopted to solubilize the gel because the treatment leaves all the other nuclear structures intact and with a normal microscope appearance.

These observations, then, gave rise to a sequential extraction procedure which is described under Materials and Methods. Fraction I consists of amorphous material which is either in a diffusible state or solubilized very easily. Fraction II represents material either immobilized as a gel or bound to the gel and thus solubilized as the gel is solubilized. The extraction conditions are gentle but sufficiently deviant from in vivo conditions, however, to make possible the extraction of some material from the residual formed elements of the nucleus as well. Fraction III consists of the residual GV structures. Under the light microscope, these include chromosomes and attached granules, nucleoli, small granules 0.5–2 μm in diameter, the nuclear envelope, and variable-sized granules up to nucleolar size but distinguishable from nucleoli by a homogeneous texture and resistance to dissolution in high salt concentrations. The small granules attached to the chromosome loops are similar to those found suspended in the nuclear gel. They are probably ribonucleoprotein granules (23). Photomicrographs of GVs before and after these extractions are presented in Fig. 1.

It was of interest to know how much protein was represented in each of the three GV fractions. Lowry protein determinations (16) were done on a microscale on whole GVs and on the residues left after each successive extraction. The results are presented in Table II. It can be seen that proteins of the nucleoplasm, as represented by fractions I and II, make up about 97% of total GV proteins.

Work in other laboratories has shown that when isolated somatic cell nuclei are extracted under conditions virtually identical to the procedure used to obtain fraction I, a 30S particle is obtained (17, 21). We analyzed a freshly prepared fraction I on a sucrose gradient to see if the fraction contained 30S particles. The results of the analysis were very clear. There was nothing on the gradient except material of about 3–11 S, observed by its absorption at 280 nm. This result showed that fraction I contains no informosomes, only unaggregated materials of about the size of a normal population of proteins.

To check on the possible presence of nucleic acids in fraction I material, a UV absorption spectrum was obtained. In the wavelengths from 220 to 380 nm, its absorption profile was virtually identical with that of bovine serum albumin. From the extinction coefficient of RNA it could be calculated that if fraction I contained any nucleic acid at all it was less than about 0.2% of the dry mass of the fraction.

The possibility that the nuclear envelope exercises selective discrimination in allowing protein penetration has always attracted interest. To compare the most soluble proteins and polypeptides of the GV (fraction I) with those of the cytoplasm (S-100), two types of electrophoresis were utilized. First, they were analyzed on polyacrylamide gradient gels of 4–25% without reducing or dissociating agents. Second, they were treated with SDS and

Table I

| Agent      | Concentration | pH  | Liquefies gel? |
|------------|---------------|-----|----------------|
| Triton X-100 | 0.12% (vol/vol) | 7.5 | no             |
| EDTA       | 20 mM         | 7.6–9.0 | no             |
| Mercaptoethanol | 3 mM       | 9.5 | no             |
| Mercaptoethanol | 100 mM     | 7.5 | no             |
| Mercaptoethanol | 20 mM       | 9.0 | yes            |
| Urea       | 1.0 M         | 7.6 | no             |
| Urea       | 1.5–2.0 M     | 7.6 | yes            |
| NaCl       | 0.5–1.0 M     | 7.6 | yes            |
| Trypsin    | 20 μg/ml      | 8.5 | yes            |

* Each agent was included in standard isolation medium.
† Ethylenediaminetetraacetic acid.

Table II

| Exp. no. | Total protein GV | Protein/germinal vesicle % | Total protein Fraction |
|----------|------------------|---------------------------|-----------------------|
| 1        | 4.7 (100%)       | 3.9 (85%)                 | 0.55 (12%)            |
| 2        | 6.3 (100%)       | 5.5 (87%)                 | 0.69 (11%)            |

* Protein measured by the Lowry procedure (16) in duplicate in each experiment. Bovine serum albumin served as standard reference.
2-mercaptoethanol and analyzed on 10% polyacrylamide gels containing SDS. The nuclear and cytoplasmic fractions are directly compared in their electrophoretic patterns in Fig. 2.

One can immediately distinguish three classes of components in each analysis. There are components unique to the GV, unique to the cytoplasm, and common to both. On the basis of their location in the gels, there are about 11 proteins found only in the nucleus, about 13 only in the cytoplasm, and about 7 in both under non-dissociating conditions. In these gels, we also see that several of the very largest proteins are common to both nucleus and cytoplasm while some of the very smallest of these soluble proteins are confined to only one of the two compartments. All of the proteins are negatively charged, positively charged molecules having remained at the origin.

When the same two soluble fractions are analyzed under dissociating conditions, we see about 13 polypeptides only in the GV, 22 only in the cytoplasm, and about 21 in both compartments.
All of these patterns were consistently observed in many experiments.

A comparison of all of the oocyte fractions under dissociating conditions by SDS electrophoresis is presented in Fig. 3. Close inspection reveals that there is little contamination, even of fraction III, by yolk or other cytoplasmic organelles. Each pattern is substantially different from all the others. In many repetitions of these experiments the patterns obtained were always remarkably similar, despite the complexity.

The nucleoplasm, as represented by fractions I and II, shows about 68 distinguishable bands, of which about 11 have common positions in the two fractions. This can be compared with the 49 bands distinguishable in fraction III. There are about 16 bands with common positions in fractions II and III. One should note also the general skewing of the patterns towards the low molecular weight end of the gels in the more soluble S-100 and I fractions and towards the high molecular weight end in relatively insoluble fractions II and III.

Two bands of fraction II are the most prominent and enriched bands of this fraction. Their apparent molecular weights in SDS are 115,000 and 46,000 daltons. If any polypeptides are responsible for the structure of the nuclear gel, these components would be prime candidates.

The data presented by Adamson and Woodland (1) and by Bonner (3) suggested that the GV of Xenopus oocytes might be a storage receptacle for histones. To check on this possibility, an experiment was performed to give an upper limit estimation of histones present in the GV. Xenopus histones were extracted from washed chromatin of erythrocytes and analyzed both on acid urea gels and on the SDS gels used for the analysis of the GV fractions. On acid urea gels, five bands were observed. On the SDS gels, only four bands were resolved.

The positions of all four histone bands on SDS gels were determined and compared with the band patterns of GV fractions I, II, and III on SDS gels. Their locations are indicated in Fig. 3. Whenever a GV band coincided with a histone band position, it was assumed to be histone. All of the stained material at the front boundaries of all three fractions was included in this assumption. The total absorption of Coomassie blue by all bands in each fraction was then estimated by weighing the absorption scan of each which had been traced on paper. "Histone" peaks of each trace were then cut out and weighed separately. Although there are several assumptions involved, we can say that, at an absolute maximum, histones represent no more than 8% of GV protein. This would be roughly 0.44 μg per GV. It is undoubtedly an overestimate.

DISCUSSION

The most obvious result obtained in these studies is the high degree of molecular complexity observed in the polypeptides of the nucleoplasm. Assuming the involvement of one gene per polypeptide, the nucleoplasm, as seen in fractions I and II, represents an involvement of about 68 genes. It would seem that whatever the biological function of these proteins, they represent a considerable accumulation of genetic information, probably involved with several or many developmental functions.

It is difficult to compare the results reported here with those of earlier studies (14, 24) because of the differences in the species of animals and the extraction and electrophoretic techniques used. A notable difference between the GVs of the newts Triturus cristatus and T. viridescens and the GVs of Xenopus laevis oocytes lies in the differences in the solubility properties of their nuclear gels (7, 11). The KCl/NaCl concentrations which solubilize the newt gels (7, 11) are not sufficient to solubilize Xenopus gels.

The patterns of the most soluble polypeptides and proteins of the GV and cytoplasm differ substantially. This raises the interesting question of the mechanism by which the GV proteins are selectively taken up and sequestered there. Two principal mechanisms which could account for this molecular asymmetry have been advanced. The first ascribes selectivity to the nuclear envelope and its pores. The second ascribes selectivity to intranuclear and cytoplasmic binding sites. (For extensive reviews of nuclear cytoplasmic transport see references 9 and 10.)

Some illuminating studies on movement of proteins from cytoplasm to GV in Xenopus oocytes have been reported (3, 4, 12). In these investigations, radioactive proteins were injected into the cytoplasm of stage 6 oocytes and the subsequent distribution of the label was monitored. Several points emerged from this work which are of interest here. There were oocyte proteins which could be found almost exclusively in the GV or in the cytoplasm after injection and incubation, and there were some which seemed to equilibrate between both compartments. Both small and large proteins entered the GV, although at different
Comparison of electropherograms of *Xenopus* oocyte fractions. *PAR* is washed, large cytoplasmic particles; *S 100* is the cytoplasmic postmicrosomal supernate; *TGV* is the total germinal vesicle; I, II, and III are fractions I, II, and III from germinal vesicles (see Materials and Methods). All electrophoretic analyses were done on SDS-dissociated polypeptides and were stained with Coomassie blue. The absorption scans in the lower part of the figure were of the stained preparations shown in the upper part of the figure. Location of *Xenopus* histones on these gels is indicated by marks at the bottom; the location of the prominent bands of fraction II by discontinuous lines.
rates. Proteins with either net basic or net acidic charges entered the GV readily. When purified calf thymus histones were injected they accumulated in the GV but to different saturation levels. The saturation level obtained with each injected histone was not correlated with the rate of its entry into the GV (3). All of these data, especially the different intranuclear saturation levels of histones, are consistent with the concept of selective protein accumulation in the GV by intranuclear adsorption.

It is clear from many previous studies that chromosomes, nucleoli and other nuclear organelles all "bind" characteristic arrays of nuclear proteins. These proteins were associated with fraction III in the present study. In the case of the massive amorphous nucleoplasm, however, no microscopically obvious structures are present to which the complex patterns of polypeptides in fractions I and II could be bound. The only structure observable in the nucleoplasm is the nuclear gel.

The best interpretation of the findings presented here seems to the authors to be the following. The main bulk of GV proteins are probably synthesized in the cytoplasm during oogenesis. They diffuse into the GV at a speed consistent with their size, charge, and hydration. There, about 3% of the total mass of these proteins contribute to the formed nuclear structures while about 97% become associated with the nuclear gel in various degrees of tightness of adsorption. Some fraction of this latter class of proteins is either not bound at all in the cell or bound so lightly as to be in diffusible equilibrium between the nucleus and cytoplasm.

These nucleoplasmic proteins, sequestered in the GV, represent a considerable diversity of genetic information. It seems likely that, in the sequestered state, much of their functional potential is held in a nonfunctional condition. Upon meiotic maturation, when the germinal vesicle breaks down and the gel is solubilized, these proteins enter the cytoplasm in either an active or activatable state and contribute to the processes of early embryogenesis.

If the nuclear gel does indeed offer an array of binding sites capable of explaining the complex and characteristic pattern of nucleoplasmic proteins described in this study, then the nuclear gel deserves further study.

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