Proc. Natl. Acad. Sci. USA
Vol. 77, No. 2, pp. 1034–1038, February 1980
Cell Biology

Immunological identification and localization of the predominant nuclear protein of the amphibian oocyte nucleus

(phosphoproteins/nonhistones/nuclear sap/nucleocytoplasmic compartmentation/immunofluorescence microscopy)

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Communicated by Jean Brachet, November 27, 1979

ABSTRACT **Nuclei of vitellogenic oocytes of the frog, *Xenopus laevis*, contain a prominent protein, representing about 10% of nuclear protein, which is characterized by a polypeptide of M_r 30,000. This protein is highly phosphorylated and acidic, displays several isoelectric variants with pI values ranging from 4.7 to 5.3, shows a high thermostability, is not stably complexed with other proteins, and is readily extracted in buffer solutions. Guinea pig antibodies against this protein have allowed its specific immunoprecipitation and localization by immunofluorescence microscopy, using both frozen tissue sections and cells grown *in vitro*. The protein is located almost exclusively in the nucleus where it appears to be spread throughout the nuclear interior. It is also a major nucleus-specific protein in vitellogenic and previtellogenic oocytes of other amphibian species as well as in other cell types, including hepatocytes, follicle epithelial cells, and cultured *Xenopus* cells, but is not detected in nuclei of transcriptionally inactive cells such as erythrocytes and spermatids. An immunologically related, if not identical, protein occurs in nuclei of higher vertebrate cells, including mammals. The properties of this abundant nuclear phosphoprotein and its possible relationship to the "nucleosome assembly factor" protein are discussed. It is suggested that this soluble protein serves a general nuclear function.**

In the eukaryotic cell the nucleus and the cytoplasm have different protein compositions. According to the specific nucleocytoplasmic distribution, three major categories of proteins have been distinguished (1–6): (*i*) proteins that occur in both the nucleus and the cytoplasm; (*ii*) strictly cytoplasmic proteins; (*iii*) proteins that are not found, in detectable steady-state concentrations, in the cytoplasm but are greatly enriched in the nucleus. The specific location of proteins of the latter two categories is maintained even though the nucleocytoplasmic barrier structure, the nuclear envelope, contains pore complexes with an effective patent channel diameter of about 9 nm (4)—i.e., penetrable by most diffusible protein molecules. However, as has been shown by microinjection experiments (5), the nuclear translocation and accumulation of proteins is governed by other and more specific factors than molecular size.

An important system in studies of nucleocytoplasmic compartmentation of proteins is the amphibian germinal vesicle, which can be isolated and purified manually (1, 3, 5, 6). Its protein composition has been characterized to some degree (5–10). In experiments with isolated germinal vesicles we have observed that the most prominent nuclear protein, which is common to a broad range of species, is rapidly extracted, even from intact nuclei, in buffer solutions of various ionic strengths, physiological ones included. By using antibodies against this protein we have purified and localized it, and we show that it is present in various kinds of cells, always in an exclusively nuclear location.

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MATERIALS AND METHODS

Cells and Tissues. Late vitellogenic oocytes of the following species were used for nuclear isolation: *Xenopus laevis*, *Bufo bufo*, *Bombina variegata*, *Rana pipiens*, *R. esculenta*, *Pleurodeles waltlii*, *Triturus cristatus*, and *T. alpestris*. Oocyte proteins were labeled by incubation of ovaries for 16–20 hr at 21°C in modified Barth's solution (11) containing either 500 μCi of [^{32}P]phosphate per ml (30–100 Ci/mg; 1 Ci = 3.7×10^{10} becquerels) or 400 μCi of [^{3}H]leucine per ml (110 Ci/mmol; Radiochemical Centre, Amersham, UK). Small tissue pieces were taken from ovaries, livers, and testes of the species mentioned and were processed for freeze-sectioning (12). Cultures of kidney epithelial cells of *X. laevis* were as described (13).

Isolation of Nuclei. Nuclei and gelified nuclear contents

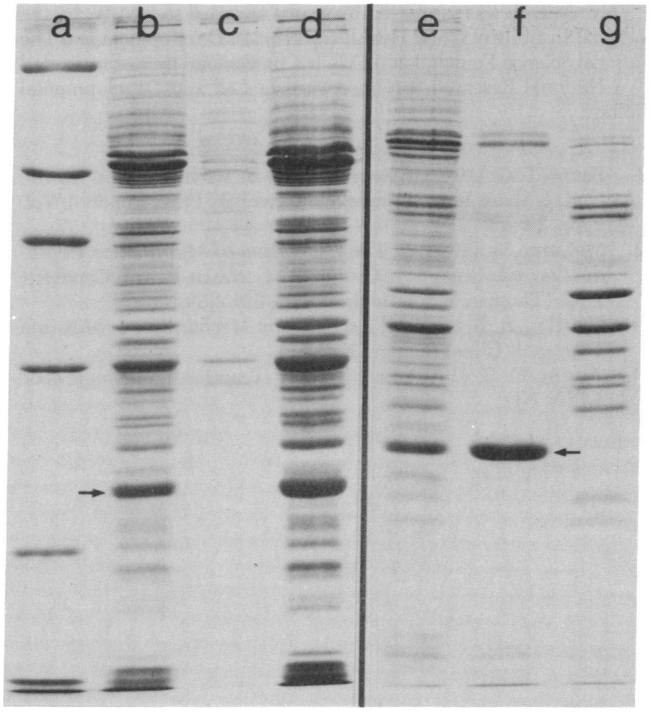

FIG. 1. One-dimensional NaDodSO$_4$/polyacrylamide (12%) gel electrophoresis of total nuclear proteins from 25 *Xenopus* oocytes (lanes b and e), nuclear proteins of pellets (lane c) and supernatants (lane d) obtained after homogenization (60 nuclei) in buffer A, and nuclear proteins soluble (lane f) and pelletable (lane g) after heat treatment. Arrows denote the protein of M_r 30,000. Reference proteins (lane a) are (from top to bottom): myosin heavy chain, phosphorylase-a, bovine serum albumin, actin, chymotrypsinogen.

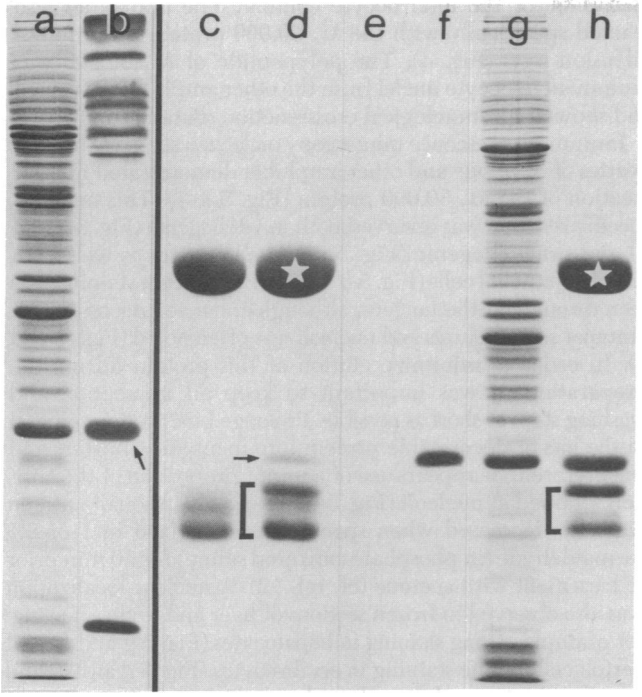

FIG. 2. Gel electrophoresis of total nuclear proteins from 25 *Xenopus* oocytes labeled with [^{32}P]phosphate seen after staining with Coomassie blue (lane a) or in autoradiofluorography (lane b, same gel). Note labeling of the protein of M_r 30,000 (lane b, arrow). Immunoprecipitate of [^{32}P]phosphate-labeled proteins obtained from nuclear material (80 nuclei) solubilized with $NaDodSO_4$/Triton X-100-containing buffer by addition of antiserum and protein A-sepharose (lane d) is compared with the result of incubation with preimmune serum (lane c, stained with Coomassie blue). The autoradiofluorogram of the same gel (lanes e and f) shows that the M_r 30,000 protein (arrow) is the only labeled protein immunoprecipitated. Immunoprecipitate from nuclear material of 60 nuclei solubilized in buffer A, treated with antiserum but not with protein A, and washed only with phosphate-buffered saline (lane h) is compared with the pattern of the proteins used for precipitation (lane g, from 25 nuclei). Stars, heavy chains of IgG; brackets, light chains of IgG.

were manually isolated from oocytes (10). They were either stored in cold (−20°C) 96% ethanol or incubated (60 nuclei per 150 μl), directly or after homogenization by sucking up and down in a fine bore pipette, for various times (10, 30, or 60 min) in one of the following solutions: (A) 83 mM KCl/17 mM NaCl/10 mM Tris·HCl (pH 7.2), with or without 10 mM $MgCl_2$ added; (B) 1.5 M KCl/17 mM NaCl/10 mM Tris·HCl, pH 7.2; (C) 10 mM Tris·HCl, pH 7.2. After incubation (21°C), the material was fractionated by centrifugation for 1.5 hr at 120,000 × *g* in a small-volume ultracentrifuge (Airfuge, Beckman Instruments, Munich). Proteins either were precipitated from the supernatants by adding 4 vol of ice-cold acetone or were briefly (12 min) heated to 80°C (cf. ref. 14). "Heat-stable" proteins not included in the sediment were precipitated with acetone.

Gel Electrophoresis and Protein Determinations. One-dimensional slab gel electrophoresis was done essentially according to Laemmli (15) in 10% and 12% gels or to Thomas and Kornberg (16) in 15% and 18% gels. In two-dimensional gel electrophoresis (17, 18), mostly ampholine buffers allowing optimal separation of proteins at pH 4.0–7.0 were used. Gels were stained with Coomassie blue or amido black and used for densitometer tracing (10) or autoradiofluorography (19). Protein was determined by using conventional methods (20, 21).

Antibody Preparation. Antibodies were raised in guinea pigs as described (22) by using the ethanol-precipitate of the contents of 1200 nuclei for the first immunization and of 600 nuclei for the booster injection. In another experiment, the polypeptide band of M_r 30,000 was excised, eluted, precipitated, and used as antigen as described for other proteins (13, 23). Sera or IgG fractions were used (13, 22).

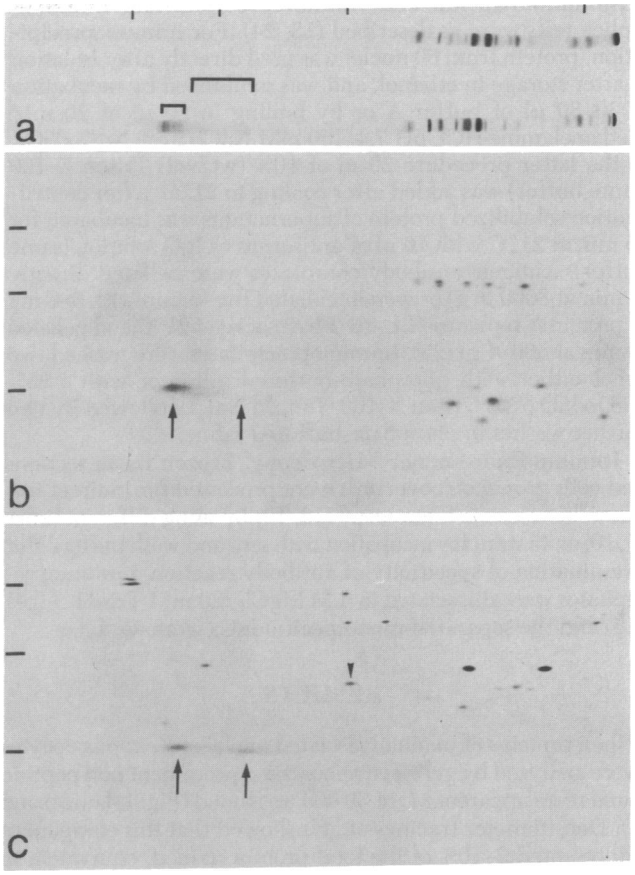

FIG. 3. Isoelectric focusing (*a*) and two-dimensional gel electrophoresis (*b*) of immunoprecipitates from solubilized nuclear proteins obtained by addition of antiserum to M_r 30,000 protein (similar to that shown in Fig. 2, lane h). The isoelectric focusing pattern (*a*, lower gel) is compared with that of the IgG from the same serum obtained by mixing with protein A-sepharose (*a*, upper gel): The pattern of the IgG is seen on the right and that of the precipitated antigen, on the left (brackets denote the two subgroups of isoelectric variants, probably representing different degrees of phosphorylation). The two-dimensional gel electrophoresis of the immunoprecipitate (*b*, heavy and light IgG chains on the right) is compared with that of the total nuclear proteins from which the immunoprecipitate has been obtained (*c*, from 30 nuclei). The precipitate contains a broad range of variants of the M_r 30,000 protein (arrows). The arrowhead (*c*) denotes the position of the nuclear actin identified by comigration with authentic β- and γ-actin. Top scale presents pI values (from left to right, 4.5, 5.0, 6.0, 7.0); horizontal bars (*b* and *c*) indicate M_r values (from top to bottom: 100,000; 50,000; 30,000).

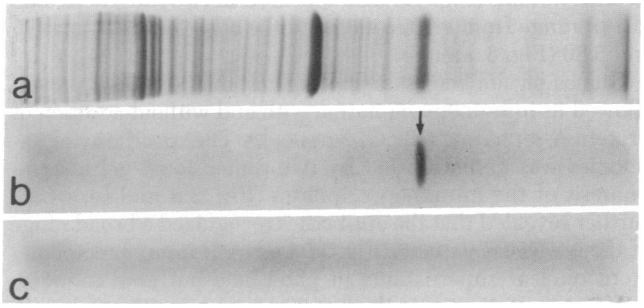

FIG. 4. Agarose immunoreplica from $NaDodSO_4$/12% polyacrylamide gel containing the separated polypeptides of 60 nuclear contents of *Xenopus* oocytes (*a*) shows that only the polypeptide of M_r 30,000 forms a precipitin with the antiserum (*b*), which is not seen in replicas containing preimmune serum (*c*). (Coomassie blue.)

Immunodiffusion and Immunoprecipitation. Immunoreplica tests were as described (13, 24). For immunoprecipitation, protein from 60 nuclei was used directly after isolation or after storage in ethanol, and was solubilized by incubation in 30–80 μl of buffer A or by boiling in 20 μl of 20 mM triethanolamine·HCl, pH 7.4/150 mM NaCl/2.5% $NaDodSO_4$. In the latter procedure 20 μl of 10% (wt/vol) Triton X-100 (same buffer) was added after cooling to 21°C. After centrifugation solubilized protein of supernatants was incubated for 45 min at 21°C with 10 μl of antiserum or IgG solution (same buffer). Antigen–antibody complexes were pelleted directly (4 min at 8000 × *g*) or were incubated for 45 min with 5–8 mg of protein A-sepharose CL-4B (Pharmacia) at 21°C and pelleted (5 min at 500 × *g*) (25). Immunoprecipitates were washed two times either with phosphate-buffered saline or with 1.25% $NaDodSO_4$/5% Triton X-100/75 mM NaCl, followed by two further washes in phosphate-buffered saline.

Immunofluorescence Microscopy. Frozen tissue sections and cells grown on coverslips were processed for indirect immunofluorescence microscopy (12, 13) by using different times (5, 10, or 45 min) for incubation with sera and wash buffers. For examination of specificity of antibody reaction, immunoprecipitates were dissociated in 4 M $MgCl_2$/20 mM Tris·HCl (pH 6.8) and the separated monospecific IgGs were used.

RESULTS

When proteins of manually isolated nuclei of *Xenopus* oocytes were analyzed by gel electrophoresis, a prominent polypeptide band of an apparent M_r of 30,000 was found (Fig. 1, lanes b and e). Densitometer tracings of gels showed that this component represented 9–10% of the total protein stained, equivalent to a nuclear content of this protein of 0.25–0.28 μg—i.e., about 4.2 mg/ml and 6×10^{12} molecules per nucleus—estimated on the basis of a mean nuclear protein content of 2.8 μg (±0.3 SD; $n = 6$; three determinations with each method; cf. refs. 8 and 9). The protein was rapidly released from isolated nuclei in buffers of low, high, and physiological strength, resulting in underestimations of this protein in nuclei not immediately analyzed. When nuclei were homogenized we found, confirming Merriam and Hill (8), that more than 90% of nuclear protein was recovered in the nonpelleted fraction, including all of the 30,000 M_r protein (Fig. 1, lanes c and d). When such nuclear supernatants were heated to 80°C, only three major polypeptides, including the prominent component of M_r 30,000, remained in solution and were absent in the bulk of nuclear protein sedimentable upon heat-denaturation (Fig. 1, lanes f and g). Incorporation of [^{3}H]leucine *in vivo* demonstrated that the 30,000 M_r protein was continuously synthesized (data not shown). The protein was intensely labeled by [^{32}P]-phosphate (Fig. 2, lanes a and b). Isoelectric focusing showed that this protein was very acidic and revealed a series of isoelectric variants (Fig. 3*a*) that appeared in two subgroups, one in a pI range from 4.70 to 4.85 and the other between pI 4.85 and 5.30 (Fig. 3 *a* and *c*).

Guinea pig antibodies directed against this 30,000 M_r protein allowed its immunoprecipitation with and without addition of protein A-sepharose (Fig. 2, lanes c–h). The specificity of antibodies was demonstrated by two-dimensional gel electrophoresis of the immunoprecipitates (Fig. 3 *a* and *b*), which further revealed that the antibodies recognized a broad range of the isoelectric variants (Fig. 3 *b* and *c*). Immunoprecipitates from nuclear supernatants in phosphate-buffered saline or buffer A contained only the 30,000 M_r protein (Fig. 2, lanes g and h). Significant amounts of immunoprecipitates were not obtained from ooplasmic supernatants of enucleated oocytes; quantitative immunopreparation experiments indicated that the cytoplasmic content of this protein, if present at all, is less than 0.4% of the total oocyte content. The antibodies also reacted specifically with the M_r 30,000 protein in immunodiffusion tests (Fig. 4). The polypeptide of M_r 30,000 was prominent in oocyte nuclei from the other amphibia examined and showed immunological crossreaction (data not shown).

Immunofluorescence microscopy on frozen sections through ovaries of *Xenopus* and other amphibia demonstrated nuclear location of the M_r 30,000 protein (Fig. 5 *a–f*). This nucleus-specific reaction was observed both in vitellogenic (Fig. 5 *a* and *b*) and previtellogenic (Fig. 5 *c* and *d*) oocytes as well as in follicle epithelial cells (Fig. 5 *b* and *f*). Fluorescent staining was seen throughout the nucleus, although enhanced decoration of granular substructures and nucleoli was often noted (Figs. 5 and 6). In order to minimize elution of this protein during the preparation, it was important to keep all incubation and washing steps as short as possible. Prolonged incubation resulted in the loss of decoratable protein into incubation buffers and the preferential appearance of a minor proportion of the antigen retained at nucleoli (Fig. 5*e*). Artificial elution of antigen was also decreased when specimens were fixed in 1 or 2% formaldehyde (in phosphate-buffered saline) for 20 min prior to treatment with acetone (cf. ref. 12). A nuclear localization was also observed in frozen sections of liver and testes, showing, for example, strong staining in hepatocytes (Fig. 5 *g* and *h*) and Sertoli cells but no staining in erythrocytes (Fig. 5 *g* and *h*) and spermatids. Accessibility of nuclear antigens to IgG was controlled by using antibodies to histones and nuclear membrane-associated nonhistones, and cytoplasmic staining was examined with antibodies to cytochrome b_5 (cf. ref. 12). Preimmune sera were negative (Fig. 6 *e* and *f*). During mitotic metaphase and anaphase the protein was found not to be associated with the chromosomes but appeared distributed over most of the cytoplasm (data not shown).

DISCUSSION

Amphibian oocyte nuclei contain an abundant, acidic, soluble phosphoprotein of M_r 30,000 that exceeds in quantity any other single nuclear protein, including the prominent actin-containing band (for actin see also refs. 5 and 8–10). Although this protein appears to be confined to the nucleus *in vivo*, it can be readily eluted from isolated nuclei (this can also be seen from the data in figure 3 of ref. 8). From gel electrophoretic studies we conclude that only minuscule amounts of this protein, if any, remain associated with isolated, sedimentable nuclear structures, such as chromosomes, nucleoli, and nuclear envelope (10).

We have also shown, by immunofluorescence microscopy and immunoprecipitation from nuclear (oocytes) or whole cell lysates, that a protein immunologically and electrophoretically related to the M_r 30,000 phosphoprotein of amphibian oocytes occurs as a major protein, again in a strictly nuclear location, in a broad range of cell types of amphibia as well as higher vertebrates (unpublished results).

The unusual properties of this protein, as well as its intranuclear location and distribution, distinguish it from other frequent nuclear proteins of similar mobility on $NaDodSO_4$/polyacrylamide gels, such as H1-histones, protein A-24 (26), and chromatin proteins of the "high mobility group" (27–29). At the moment, however, we cannot exclude the possibility that the protein described here might represent phosphorylated forms of other known nuclear proteins. On the other hand, the similarity of the properties of this nuclear protein to those of the acidic protein of M_r 29,000 described as "nucleosome assembly factor" in *Xenopus* egg homogenates (14) is obvious, and two-dimensional gel electrophoresis of the purified "assembly factor" protein (R. A. Laskey and E. M. DeRobertis, personal communication) has shown a similar mobility. Further

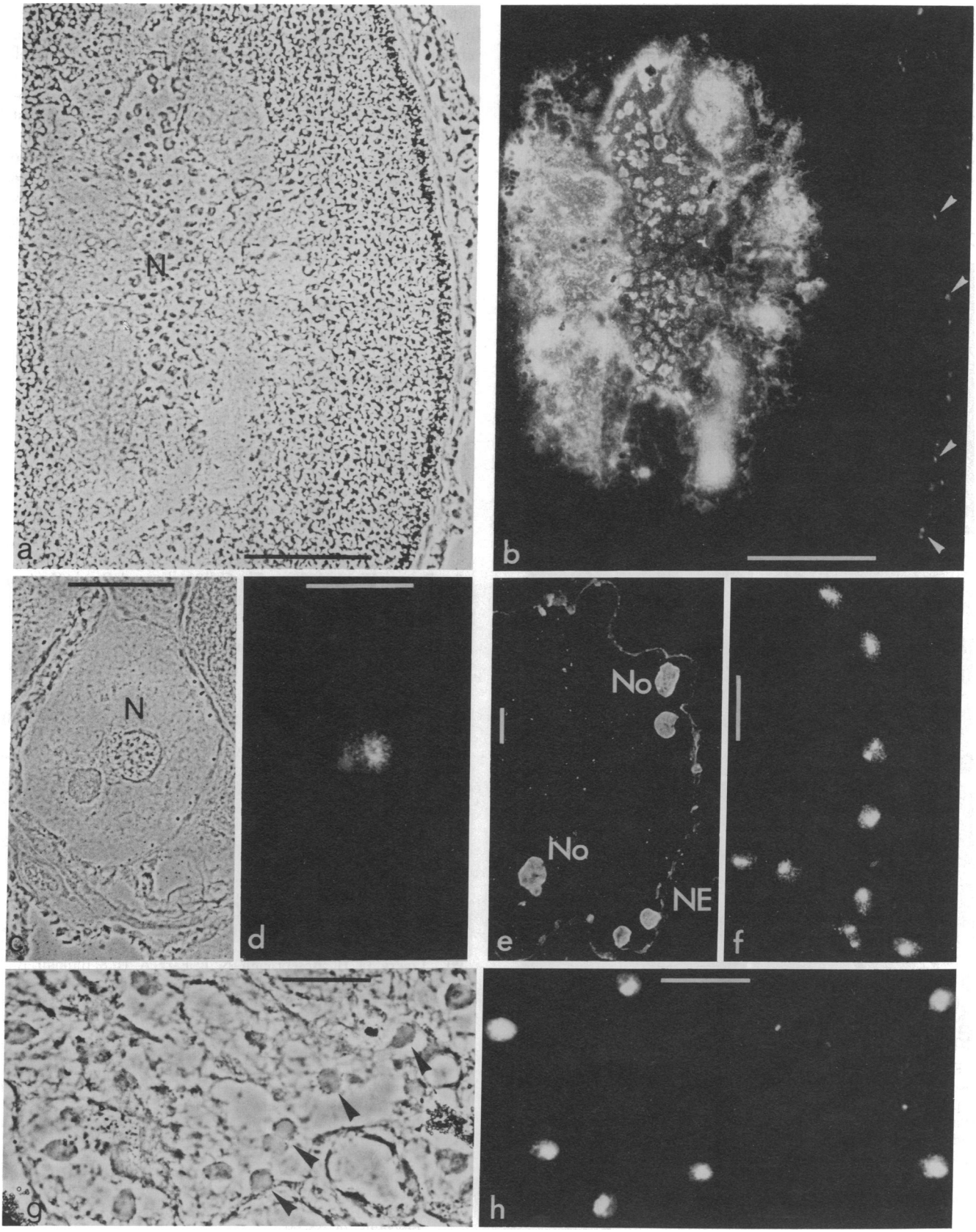

FIG. 5. Immunofluorescence microscopy of frozen sections through ovaries (*a–f*) and liver (*g* and *h*) of *Xenopus* after staining with antibodies to the M_r 30,000 protein (*a* and *b*, *c* and *d*, and *g* and *h* show the same section in phase contrast and epifluorescence optics, respectively). Staining is seen in nuclei (N) of vitellogenic (*a* and *b*) and previtellogenic (*c* and *d*) oocytes, follicle epithelial cells (arrowheads in *b*; *f*), and hepatocytes (*g* and *h*), but not erythrocytes (arrowheads in *g*). Incubation with antiserum and washes were for 10 min for the sections shown in *a–d* and *f–h*. The section shown in *e* was incubated for 45 min with antiserum, resulting in selective decoration of nucleoli (No) and nuclear envelope (NE), probably reflecting artificial elution of antigen from nuclear sap. Bars, 100 μm (*a–d*) and 20 μm (*e–h*).

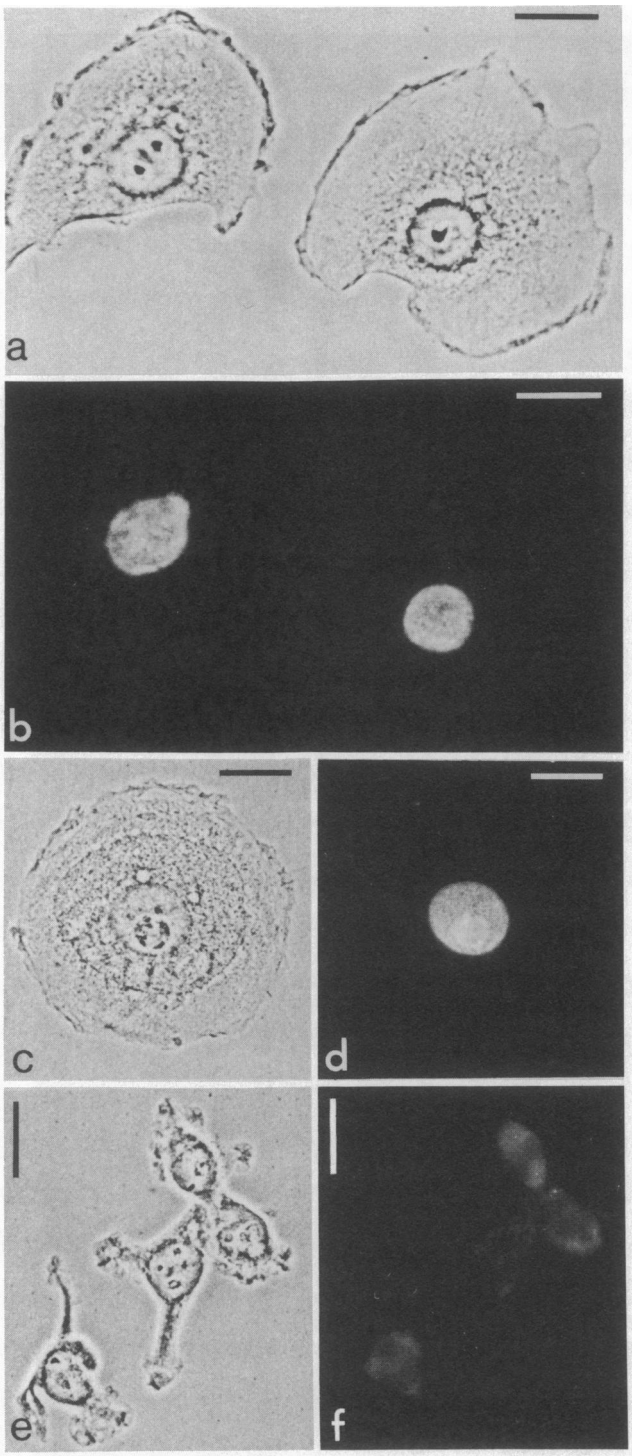

FIG. 6. Cultured cells of *Xenopus* processed for immunofluorescence microscopy with antibodies to the M_r 30,000 protein (*a–d*) or preimmune serum (*e* and *f*) are shown in phase contrast (*a*, *c*, and *e*) and epifluorescence (*b*, *d*, and *f*) optics. Note nuclear staining (*b* and *d*), sometimes revealing granular substructures (*d*), and lack of reaction in control (*f*). Bars, 20 μm.

characterization and functional tests are necessary to elucidate the relationship of this protein to other nuclear proteins.

The abundance and widespread occurrence of this protein suggest that it may serve fundamental nuclear functions and that it is involved in the general organization of nuclear activities, apparently with the exception of certain transcriptionally inactive nuclei such as in erythrocytes and spermatids. In the oocyte nucleus one could also envisage that this acidic protein might be involved in neutralization of the charges of histones abundantly present (cf. ref. 14). In this respect, however, our finding that no other proteins are coimmunoprecipitated in detectable amounts with this protein speaks against a stable association with other proteins, histones included.

The protein described here presents an example of a soluble protein that is accumulated mostly, if not exclusively, in the nucleus *in vivo* but tends to leave the otherwise structurally intact nucleus when isolated. This observation and our finding of transfer of this protein from the cytoplasm into the forming nucleus in mitotic telophase (unpublished results) then raises the question about the forces that keep this protein within the confinements of the nuclear envelope *in vivo*.

We thank Dr. E. M. DeRobertis (MRC Laboratory, Cambridge, UK) and U. Scheer (Heidelberg) for valuable discussions and the Deutsche Forschungsgemeinschaft for financial support.

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