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Expression and segregation of nucleoplasmin during development in *Xenopus*

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Summary

The spatial segregation of informational molecules in the unfertilized egg and embryo has been hypothesized to be a necessary phenomenon for the normal progression of development leading to the determination of cellular phenotypes. This study describes the selection of a monoclonal antibody (Mab: 2G6) that identifies an antigen (Ag: 2G6) which is localized in the germinal vesicle of oocytes and has a discrete pattern of inheritance during embryogenesis. The antigen displayed biochemical and physical characteristics very similar to nucleoplasmin, which is the histone-binding and nucleosome-assembly protein previously described. Immunoblot analysis with purified oocyte nucleoplasmin confirmed this relationship.

Indirect immunofluorescence was used to study the temporal expression and spatial distribution of nucleoplasmin. From early cleavage stages through gastrulation, it is preferentially localized in nuclei of blastomeres at the animal pole. By tadpole stages, it was detected only in nuclei of postmitotic cells of the central nervous system and in nuclei of striated muscle. It was not detected in adult tissues. Western blot analysis during embryogenesis revealed at least five immunologically related polypeptides that displayed distinct patterns of expression during development. The different species observed most likely represent different levels of phosphorylation of nucleoplasmin. The more acidic forms, known to be more active in nucleosome assembly, were present during cleavage stages.

Analysis of labelled oocyte proteins by two-dimensional immunoblots and autoradiography revealed that synthesis of nucleoplasmin was first detected in stage-2 oocytes, reached 60 % maximum levels at stage 3, peaked at stage 4 and was undetectable in stage-6 oocytes. The amount of nucleoplasmin message present does not follow a similar pattern during oogenesis. These results suggest that the message undergoes pronounced changes in translational efficiency during oogenesis.

A comparative immunoblot analysis using proteins from a variety of adult tissues revealed that, whereas the polyclonal antisera against amphibian vitellogenic oocyte nucleoplasmin recognized several different, tissue-specific polypeptides, two different monoclonal antibodies (Mab: b7-1D1, Mab: 2G6) failed to recognize any of the adult tissues tested. We conclude that nucleoplasmin is a family of closely related proteins with distinct embryonic and adult members.

Key words: nucleoplasmin, *Xenopus*, monoclonal antibody, oocyte.

Introduction

The nucleus (germinal vesicle) in *Xenopus* oocytes is approximately 2×10^5 times the volume of a somatic nucleus, and evidence indicates that it is a receptacle for the stockpiling of components required during embryogenesis (Merriam & Hill, 1976; DeRobertis, 1983). The embryonic genome does not become transcriptionally active until the midblastula stages (MBT) (Newport & Kirschner, 1982), requiring that proteins necessary for cleavage events are either preformed and stored in the oocyte or translated from maternal messenger RNA. For example, DNA polymerase (Ford *et al.* 1975), RNA polymerase (Roeder, 1974) and histones (Woodland & Adamson, 1977) are all sequestered within the germinal vesicle (GV) in quantities sufficient to supply the embryo's needs until gastrulation (reviewed in Davidson,

1976). The most abundant protein in GVs is a nucleosome assembly factor called nucleoplasmin, which has been studied extensively (Mills *et al.* 1980; Krohne & Franke 1980*a*,*b*; Sealy *et al.* 1986; Kleinschmidt *et al.* 1985; Dingwall *et al.* 1982).

In addition to the 'household' proteins stored in the GV that are required by all cells, there are those that may play a role in cellular diversification. One such category of proteins may be those required for gastrulation. A classic example is the 'O' mutant factor which is concentrated in germinal vesicles of Mexican axolotl oocytes and is required for normal development past gastrulation (Humphery, 1966; Briggs & Cassens, 1966; Briggs, 1972). Another such category of proteins might be those that affect dorsal axial structures. GV extracts from mature *Rana pipiens* oocytes have been found to enhance head structures and cement glands after injection into midblastula embryos (Malacinski, 1974).

The embryonic fate of proteins stored in the GV and released into the ooplasm at GV breakdown has been studied by a number of workers (Hausen *et al.* 1985; Dreyer & Hausen, 1983; Dreyer *et al.* 1981, 1982; Merriam & Hill, 1976; Dabauvalle & Franke, 1984). Whereas the majority of GV proteins are found in all embryonic nuclei, there are those few that become enriched in nuclei of specific embryonic cell types (Dreyer *et al.* 1983). These proteins are candidates for playing a role in the process of cellular differentiation.

The present communication describes the temporal and spatial expression of a germinal vesicle-specific antigen (Ag:2G6) that appears to be inherited by a subset of blastomeres at the animal pole. In swimming tadpoles, Ag:2G6 is detected only in postmitotic nuclei of a restricted set of cell types. A monoclonal antibody (Mab:2G6) that recognizes Ag:2G6 was also found to recognize purified nucleoplasmin, suggesting that Ag:2G6 is identical or closely related to nucleoplasmin.

Materials and methods

Preparation of antigen for immunization

Stage-12 *Xenopus laevis* gastrulae were obtained from natural matings of adult frogs injected with 1000 i.u. of human chorionic gonadotrophin (Sigma). Embryos were chemically dejellied as previously described (Newport & Kirschner, 1982) and transferred to 100% Steinberg's solution. Vitelline membranes surrounding the embryos were removed manually and the embryos were dissociated for 45 min in calcium-/magnesium-free, modified Stern's solution (Nakatsuji & Johnson, 1982). The dissociated cells were washed in 0·01 M-phosphate-buffered saline solution (PBS) and fixed in 4% paraformaldehyde for 30 min at room temperature. Whole fixed cells were washed in 0·01 M-PBS and cells from one embryo (approximately 50 000 cells) were injected into 6-week-old female BALB/C mice.

Immunization of mice

Mice were injected subcutaneously and intraperitoneally with cells from one embryo on days 1 and 30. Approximately 50% lysis of cells was detected upon passage through a 21-gauge needle as monitored under a light microscope. Approximately 10–15 days following the second immunization, mice were tail bled and the titre of the serum was determined using an enzyme-linked-immunosorbent assay (ELISA) (Voller *et al.* 1980). For ELISA, each well of a 96-well microtitre plate was coated with dissociated whole fixed gastrula cells (equivalent to one gastrula per well). Spleen cells from the mouse with the highest circulating antibody titre were fused to Sp2/0-Ag/4 myeloma cells (Shulman *et al.* 1978) as described by Chapman *et al.* (1984). The supernatants were tested for antibodies (Abs) using ELISA. The Abs were isotyped using an isotyping kit from Hyclone (Logan, Utah). Mab:2G6 was isotyped as IgG2B.

Hybridoma-secreting specific Abs were cloned by limiting dilution into 96-well microtitre plates. Cloned lines were retested using ELISA and by indirect immunofluorescence. Cloned hybrids were frozen and stored in liquid nitrogen cell banks.

Indirect immunofluorescent staining of sections

Oocytes were surgically removed from adult females, manually dissected apart and staged according to Dumont (1972). Embryos were staged according to Nieuwkoop & Faber (1967). Oocytes (stages 1–6) and embryos (4 cell to stage 40) were fixed in Romeis fixative (20 ml of 10% paraformaldehyde, 2 ml 50% TCA and 30 ml saturated mercuric chloride) (Hausen *et al.* 1985). Oocytes were fixed for 1 h and embryos for 2 h at room temperature. Embryos older than stage 40 were fixed overnight at 4°C in 4% paraformaldehyde. They were dehydrated in a graded series of ethanol (30–100%) and transferred to 50% and then 100% polyester wax polyethylene glycol diesterate (Hausen *et al.* 1985). Sections (5 μm) were blocked by treatment with 0·1 M-glycine followed by 2% BSA/3% normal goat serum for 45 min. Sections were washed in PBS, incubated in primary antibody (1° Ab) (1:2 dilution in PBS) for 2 h, washed three times and stained with FITC-conjugated goat anti-mouse IgG (Hyclone, Utah) (1:100 in PBS) for 1 h. After washing three times, sections were counterstained for 10 min with DAPI (diamidino-2-phenylindoldihydrochloride) (Boehringer Mannheim) and mounted in glycerol. Photographs were taken with a Leitz Orthoplan microscope equipped with epifluorescence optics.

Detection of antigen by protein blotting

Oocytes or embryos were extracted in 0·5% NP-40, 100 mM-KCl, 10 mM-Pipes and 1 mM-phenylmethylsulphonylfluoride (PMSF), and the protein content was determined according to the procedure of Smith *et al.* (1985). Proteins were separated on a 10% SDS–polyacrylamide gel (PAG) (Laemmli, 1970) or by two-dimensional gel analysis

(O'Farrell, 1975). Following electrophoresis, the proteins were electroblotted as described by Towbin *et al.* (1979). Following blotting, molecular weight standards were stained with 0·1 % amido black. To monitor the amount of transfer, gels were silver stained (Switzer *et al.* 1979). Blots were blocked with 5 % nonfat dried milk in 0·01 M-PBS for 1 h at 37 °C. Following incubation in 1 °Ab at 4 °C overnight, strips were washed and incubated in HRP-conjugated goat anti-mouse IgG for 1 h. 4-chloro-1-naphthol with 0·03 % H_2O_2 was used as substrate for the HRP reaction.

[^{35}S]methionine labelling

Oocytes and early gastrulae (stage 10) were labelled continuously for 5 h at 18 °C in OR2 medium (Wallace *et al.* 1973) containing 1 mCi ml^{-1} [^{35}S]methionine (Amersham, NJ). Labelled GVs were obtained by hand isolation (Paine *et al.* 1983). GVs were transferred directly into either Laemmli sample buffer (Laemmli, 1970) for 1-D gel analysis or lysis buffer for 2-D gel analysis (O'Farrell, 1975). In the latter case, the second dimension was a 7–15 % linear SDS–PAG gradient. Gels were either silver stained or electroblotted onto nitrocellulose and then probed with Ab followed by exposure to XAR-5 X-ray film (Chesapeake X-Ray, MA).

Quantification of Ag:2G6 synthesis and/or accumulation

Accumulation of Ag:2G6 during oogenesis was determined by two methods. First, equal numbers of GVs were loaded per lane and fractionated on a one-dimensional 10 % SDS–PAG, electroblotted to nitrocellulose and probed using Mab:2G6. Each lane of the Western blot was scanned using an LKB laser densitometer and the area under each peak determined using the LKB 2400 Gel-Scan XL software. The second method employed was an ELISA. Proteins extracted in 0·5 % NP-40 from oocytes at stages 1, 3 and 6 were applied to the microtitre plate at various concentrations (varying from 0·2–10 μg). Mab:2G6 was used at a dilution of 1:100 and the relative amounts of Ag:2G6 bound to the wells was determined using a standard colorimetric ELISA. The relative rate of synthesis was determined by scanning autoradiographs of two-dimensional gels using the LKB Laser densitometer and LKB 2400 Gel-Scan XL software as previously described (King & Barklis, 1985).

Results

In order to study the processes of cellular diversification that occur during gastrulation, specific probes recognizing subpopulations of cells within the gastrula would be highly desirable. To this end, monoclonal antibodies (Mabs) were raised against dissociated whole fixed cells of embryos at this stage. The resulting Mab's were screened on tissue sections of gastrulae by indirect immunofluorescence and Mabs that recognized a subset of cells within the embryo were chosen for further study. Among the Mabs initially selected by this procedure was Mab:2G6, which preferentially stained nuclei of blastomeres at the animal pole. Mabs to internal antigens appear to have resulted from lysis of whole fixed cells during the immunization procedure. As a first step towards understanding how such a pattern of expression might originate, we examined the expression of the antigen (Ag:2G6) recognized by Mab:2G6 at various developmental stages during oogenesis and embryogenesis.

Temporal and spatial expression of Ag:2G6 during oogenesis

Indirect immunofluorescence of frozen ovarian tissue sections revealed that Ag:2G6 is localized in the germinal vesicle of oocytes at all stages of oogenesis (Fig. 1). In contrast, note that the nuclei of follicle cells and fibrocytes in the cellular layers surrounding each oocyte did not stain (Fig. 1E,F). Antibody staining was essentially uniform within the germinal vesicle with the exception of the many nucleoli that appeared unstained. However, any staining at the periphery of nucleoli would not be detected. A series of control sections treated with either preimmune serum or secondary antibody alone showed no background fluorescence (data not shown).

Interestingly, the ooplasm failed to stain with Mab:2G6 at any stage of oocyte development. To confirm the strict nuclear localization of Ag:2G6, NP-40-solubilized proteins from hand-isolated GVs, enucleated oocytes and whole stage-6 oocytes were fractionated on an SDS–PAG, immunoblotted and probed with Mab:2G6. Equal oocyte equivalents were loaded per lane. A typical immunoblot is shown in Fig. 2 and indicates that Ag:2G6 was not detected in enucleated oocyte samples, but was prominent in the GV material and migrated as a doublet at 29·5 and 28$\times 10^3$ M_r (29·5K and 28K, respectively). These results are most consistent with the antigenic determinant being associated with a protein which is rapidly translocated into the GV after its synthesis in the cytoplasm.

Is Ag:2G6 nucleoplasmin?

A number of similarities between *Xenopus* nucleoplasmin (Laskey *et al.* 1978) and Ag:2G6 became obvious in the course of this study. For example, both are abundant GV proteins, both appear to be rapidly translocated into the nucleus (Dingwall *et al.* 1982; Figs 1, 2), their electrophoretic mobilities on one- and two-dimensional gels are identical (Dingwall *et al.* 1982; Dreyer & Hausen, 1983; Figs 2, 8), both have the same sedimentation coefficient (8S) (Laskey *et al.* 1978) (data not shown) and both display an apparent shift in pI between the oocyte (Sealy *et al.* 1986) (Figs 8, 9) and embryo species. To determine if Ag:2G6 was indeed nucleoplasmin, we obtained pure

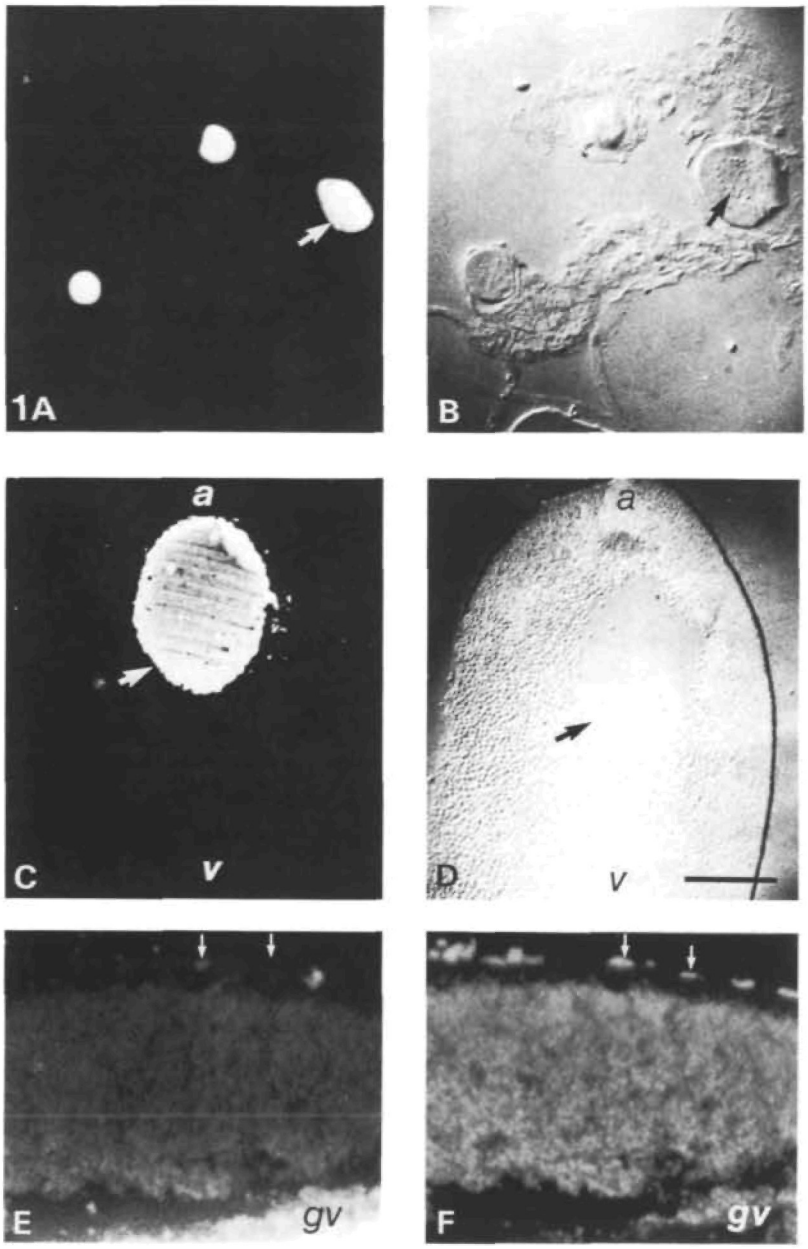

Fig. 1. Immunofluorescent staining of transverse sections through stage-1 and -2 and stage-6 oocytes using Mab:2G6. Arrows denote germinal vesicles (GV) in A–D and nuclei of follicle cells in E,F. *a*, animal pole, *v*, vegetal pole. Bar 143 μm. (A) FITC staining of stage-1 and -2 oocytes; (B) corresponding DIC image to A; (C) FITC staining of stage-6 oocytes; (D) corresponding DIC image to C; (E) FITC staining showing negative response in follicle cells; (F) corresponding DAPI image to E.

nucleoplasmin from Dr L. Sealy and used it as antigen on a protein blot probed with Mab:2G6. Mab:2G6 recognized nucleoplasmin and the pattern of response was identical to that observed for Ag:2G6, i.e. as a doublet at 29·5/28K (Fig. 3B). Furthermore, an anti-*Xenopus* nucleoplasmin monoclonal antibody from Dr P. Hausen (Mab: b7-1D1) gave an identical response on two-dimensional immunoblots to Mab:2G6 indicating that they both recognized proteins of the same molecular weight and pI (Fig. 8; Dreyer & Hausen, 1983).

However, nucleoplasmin has been detected in most adult somatic nuclei by immunofluorescence microscopy with a polyclonal antibody (Krohne & Franke, 1980*b*). This is in contrast to our findings with Ag:2G6 (Fig. 1E,F). To determine if the epitope recognized by Mab:2G6 was accessible to Mab:2G6 in our tissue sections of adult material, identical immunoblots of adult tissues (testes, heart, blood) extracted with NP-40 were probed with either polyclonal antiserum (gift of Dr Franke) or Mab:2G6. This polyclonal antiserum stains nuclei of hepatocytes and Sertoli cells but not erythrocytes and spermatids (Krohne & Franke, 1980*b*). The results presented in Fig. 4B are in agreement with our indirect immunofluorescence data and suggest that Ag:2G6 is absent from the adult tissues tested. In contrast, the polyclonal antisera recognized a 30K protein in heart tissue (mostly cardiac muscle) and gastrula, a 40K and a very weakly staining 30K protein in testes, and nothing in whole blood, as expected (Fig. 4A). From these results, we concluded that Ag:2G6 is identical

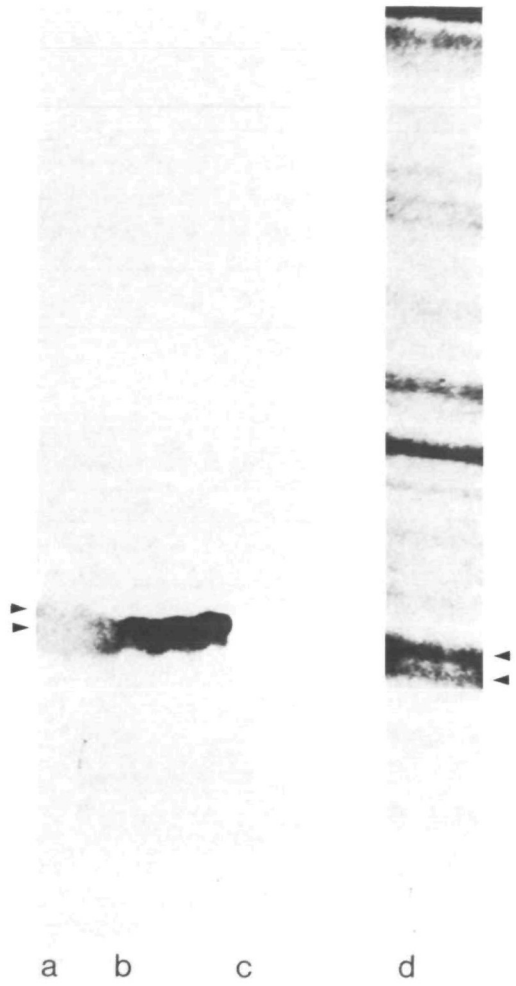

Fig. 2. Gel electrophoresis of NP-40-soluble stage-6 oocyte proteins using 10 % SDS–PAGE. Equal oocyte equivalents loaded per lane. Lanes a–c electroblotted and probed with Mab:2G6. (a) Whole oocytes, (b) GVs and (c) enucleated oocytes. Lane d represents Commassie-blue-stained GV proteins. Arrow denotes the 29·5/28K doublet.

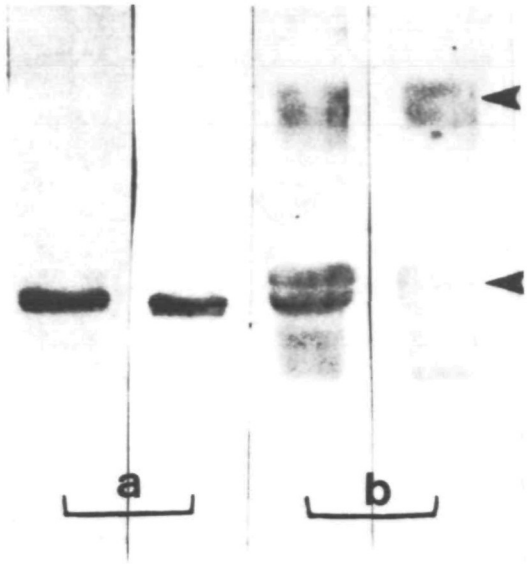

Fig. 3. Mab:2G6 cross-reacts with purified nucleoplasmin. Duplicate immunoblots of (a) NP-40-extracted stage-6 oocyte protein probed with mouse anti-*Xenopus* nucleoplasm (Mab:b7-1D1 – from P. Hausen), and (b) purified oocyte nucleoplasmin (1 μg) probed with Mab:2G6. Arrows denote nucleoplasmin running as monomer and partly as dimer.

or very closely related to nucleoplasmin and, therefore, Ag:2G6 will hereafter be referred to as nucleoplasmin. Furthermore, we suggest that Mab:2G6 is recognizing an immunologically distinct nucleoplasmin in embryos not present in adult tissue.

Nucleoplasmin expression during embryogenesis

Immunostaining of transverse sections through early cleavage stage embryos revealed a specific pattern of accumulation (Fig. 5). Serial sections from a minimum of five embryos at each stage were analysed to reconstruct and provide information on the whole embryo. Because fluorescent staining patterns can be altered by the type of fixative, length of time in the fixative and the temperature at which tissue was embedded, several different fixatives (including 4 % paraformaldehyde and Romeis fixative) were used. Tissues were either embedded in low-melting-point polyester wax or frozen in cryostat freezing medium.

At the 4-cell stage, nucleoplasmin was detected predominantly in the peripheral cytoplasm of blastomeres at the animal pole (Fig. 5A). During these early cleavage stages, blastomeres are dividing synchronously every 30 min. G_1 and G_2 are very much shortened (Newport & Kirschner, 1982) and the appearance of an interphase nucleus is rare. At the 32-cell stage, cytoplasmic staining of the dividing animal pole cells was more diffuse than it was at the 4-cell stage, but still showed a concentration of nucleoplasmin at the apical surface with an unstained area at the basal end of cells (Fig. 5B,C). Interphase nuclei are actually more common towards the vegetal pole, where the division of cells is slightly retarded, yet nucleoplasmin was not detected in blastomeres in this region. The few interphase nuclei in the animal hemisphere that were observed were stained while their cytoplasm was unstained. By the 512-cell stage, nucleoplasmin is highly enriched in nuclei of animal pole cells (Fig. 5D,E). The pattern of distribution observed implies that the protein cycles from cytoplasm to nucleus during each mitotic cycle.

At two later points in development, stage-21 (late neurulae) and stage-32 (tail-bud stage) Mab:2G6 was found to stain only nuclei, but was distributed throughout the embryo (data not shown). This universal nuclear distribution of nucleoplasmin does

not persist and 10 days later at stage 49 (swimming tadpole) a very restricted and reproducible pattern of staining is observed. Nucleoplasmin is found in nuclei of postmitotic cells in the brain (Fig. 6A,B), retina (Fig. 6C,D), spinal cord and striated muscle (Fig. 6E,F). Interestingly, nucleoplasmin was not detected in smooth muscle as sections that included gut tissue were unstained. Nucleoplasmin was never detected in adult tissues (brain, spinal cord, eye, skin, lung, liver and gut) (data not shown).

Immunoblot analysis

From the above analysis, it is clear that the presence of nucleoplasmin is not restricted to any given period of development but is present at all embryonic stages examined. The questions which remain are whether the antigenic determinant recognized by Mab:2G6 is (1) expressed on just one protein or many and (2) expressed on the same protein or different ones throughout development. To answer these questions NP-40 extracts of oocytes and embryos at various stages (4–8 cell, 32–64 cell, 512 cell, gastrula, neurula, late neurula and tadpole) were fractionated on SDS–PAG, electroblotted and probed with Mab:2G6 (Fig. 7). Western blot analysis showed that in oocytes Mab:2G6 binds to a doublet with an apparent relative molecular mass of 29·5 and 28×10^3. After fertilization, there is a slight but very reproducible increase in molecular weight to a 31/30K doublet (Fig. 7). This pattern persists until late-swimming-tadpole stages, when there is an apparent shift in M_r back to the 29·5/28K doublet. In addition, between the 4- to 8-cell stage and stage 12, a slower migrating species is also observed at 33K (brackets, Fig. 7). This third species is apparent only during cleavage stages when cell division is occurring rapidly. Sometimes higher molecular weight species, always observed as two components, could be identified which migrated with an apparent M_r of 60/66 and 150×10^3 as has been previously described for nucleoplasmin (Dingwall *et al.* 1982). Treatment of the protein sample with 5% β-mercaptoethanol before electrophoresis reduced these higher molecular weight doublets to the lower molecular weight species, indicating that they were related. In summary, Mab:2G6 recognized a protein epitope expressed on at least five immunologically related polypeptides with distinct patterns of expression during development.

Synthesis and accumulation of nucleoplasmin during oogenesis

The volume of the GV increases by some 500-fold during oogenesis, yet the intensity of immunofluorescent staining did not diminish in the larger oocytes. Therefore, it seemed likely that nucleoplasmin was synthesized throughout oogenesis. To examine this point, oogenic stages 1, 2, 3, 4 and 6 were metabolically labelled with [^{35}S]methionine. Nucleoplasmin was identified on two-dimensional gels by probing immunoelectroblots with Mab:2G6. An example of such an experiment with stage-2, -3, and -6 oocytes is shown in Fig. 8. To facilitate the correct superposition of the autoradiogram over the blot, a series of discrete spots of radioactive dye were placed on the nitrocellulose. Furthermore, some blots were probed with a mixture of our Mab:2G6 and Mab:b7-1D1 from Dr Hausen (Dreyer & Hausen, 1983), which also cross-reacts with several other polypeptides including actin, thus allowing a very accurate match with the autoradiogram. Actin, at a pI of 5·6, also served as a marker for any shift in charge for nucleoplasmin.

The relative amount of newly synthesized Ag was quantified from autoradiograms at each stage using a

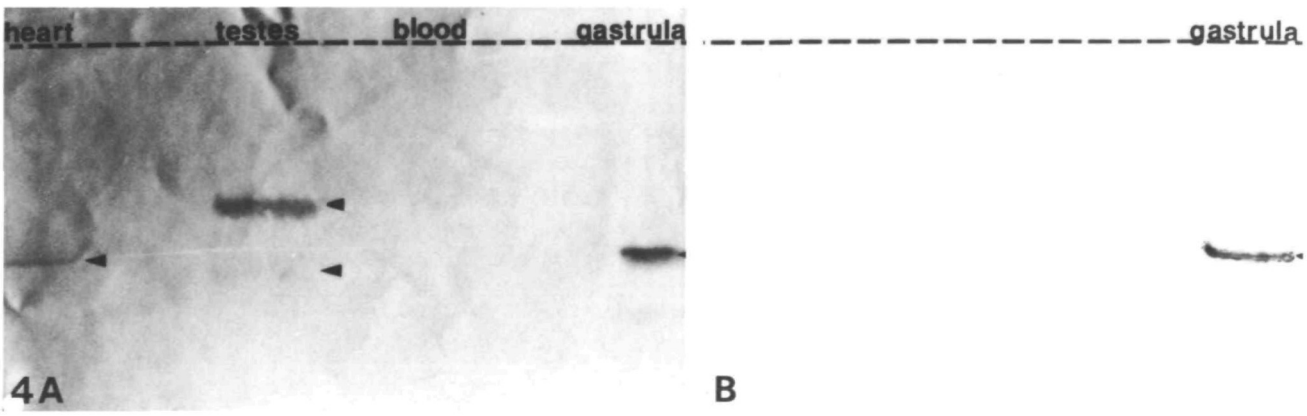

Fig. 4. Immunoblot of NP-40-soluble proteins from adult *Xenopus* tissue. Equal amounts of protein from heart, testes and blood were separated on a 10% SDS–PAG. NP-40-soluble protein from gastrula embryos were used as controls probed with (A) guinea pig anti-*Xenopus* nucleoplasmin polyclonal antibody and (B) Mab:2G6. The arrows indicate the position at which a positive reaction was noted.

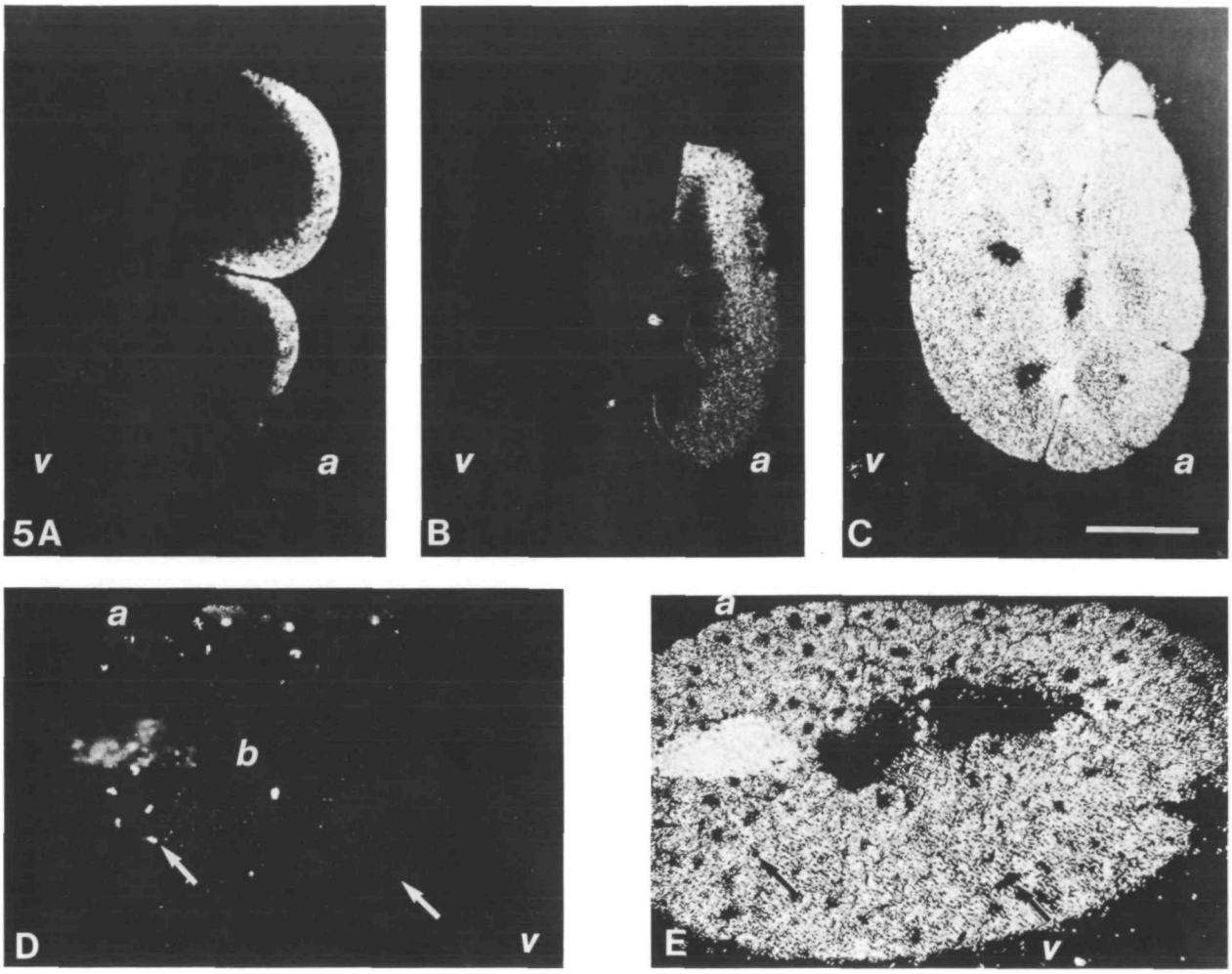

Fig. 5. Immunofluorescent staining of transverse sections through early cleavage-stage embryos using Mab:2G6. Bar 143 μm. *a*, animal pole; *v*, vegetal pole; *b*, blastocoel. (A) FITC-stained 4-cell embryo; (B) FITC-stained 32-cell embryo; (C) corresponding DIC image to B; (D) FITC-stained 512-cell embryo (Arrows denote examples of either stained or unstained nuclei); (E) corresponding DIC image to D.

computer-assisted laser densitometer as previously described (King & Barklis, 1985). Synthesis of nucleoplasmin was undetectable in stage-1 oocytes, barely detectable at stage 2, reached 60 % maximum levels at stage 3, peaked at stage 4 and was undetectable in stage-6 oocytes (Figs 8, 10),

To determine if nucleoplasmin was synthesized during embryogenesis at a time when the embryo's own genome had become transcriptionally active, we labelled early gastrula (stage 10) with [^{35}S]methionine and analysed two-dimensional gels of the resulting protein products as described for oocytes. Fig. 9 shows that nucleoplasmin is synthesized during gastrulation, at a time when message levels are below those detectable by Northern blot analysis (Burglin *et al.* 1987). Furthermore, the two-dimensional gel analysis and immunoblot data (Figs 8, 9) and the results of Sealy *et al.* (1986) with ovulated eggs revealed an apparent shift in pI values for nucleoplasmin to a more acidic form in the embryo than that found in oocytes.

The pattern of nucleoplasmin accumulation during oogenesis differs from the pattern of synthesis as described above. Accumulation was determined in two ways and similar results were obtained with both methods. As a first estimate, equivalent numbers of GVs from stage-1, -3 and -6 oocytes were electroblotted and probed with Mab:2G6. At each stage, four different protein amounts were quantified using the LKB laser densitometer and values taken within the linear range of response (Fig. 10). The second method employed was an ELISA (see Methods and materials). The results indicated that maximum accumulation of nucleoplasmin occurs in stage-6 oocytes. If the stage-6 value is taken as 100 %, then 10 % of this value has accumulated by stage 1, and

37 % by stage 3. The synthesis and accumulation results are summarized in Fig. 10.

Discussion

The most compelling evidence that Ag:2G6 is identical to nucleoplasmin is the demonstrated cross-reactivity of Mab:2G6 with purified nucleoplasmin by immunoblot analysis (Fig. 3). Additional support for this conclusion comes from a comparison of their biochemical and physical characteristics. The most striking similarity is that they are both abundant nuclear proteins (Figs 1, 2) and display identical electrophoretic mobilities on one- and two-dimen-

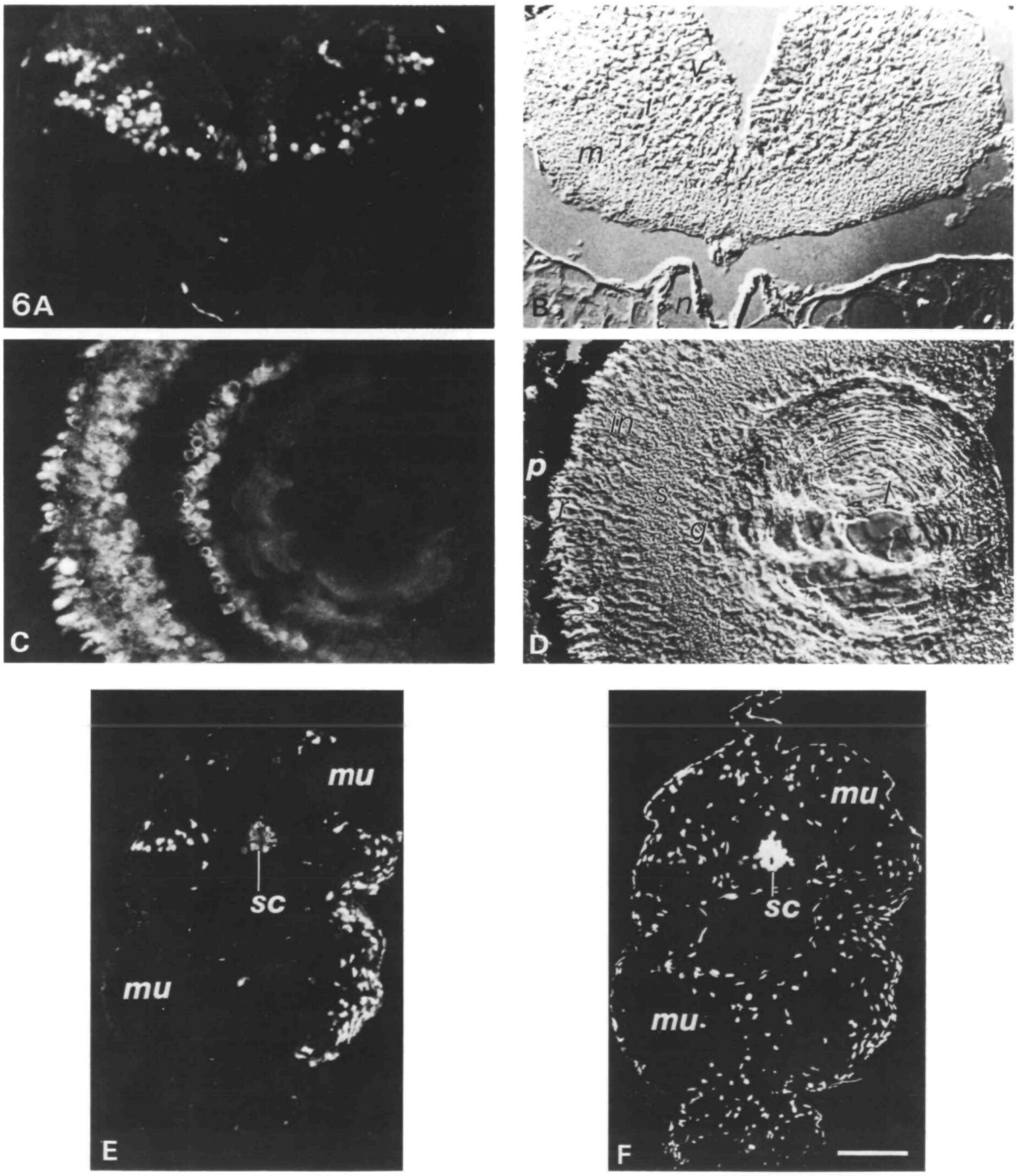

Fig. 6. Immunofluorescent staining of transverse sections through stage-49 embryos using Mab:2G6. (A) FITC-stained embryonic brain; (B) corresponding DIC image to A; (C) FITC-stained retina; (D) corresponding DIC image to C; (E) FITC-stained caudal section; (F) corresponding DAPI stain to E. *v*, ventricular zone; *i*, intermediate (postmitotic) zone; *m*, marginal zone; *n*, notochord; *p*, pigment layer; *r*, receptor layer; *in*, intrinsic neurone layer; *s*, synaptic layer; *g*, ganglion layer; *l*, lens; *sc*, spinal cord; *mu*, muscle.

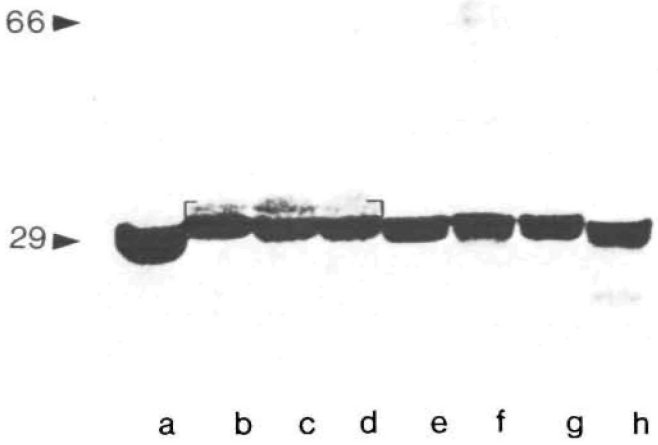

Fig. 7. Immunoblot of proteins from (a) stage-6 oocytes, (b) 4- to 8-cell embryos, (c) 32- to 64-cell embryos, (d) 512-cell embryos, (e) stage-12 embryos (f) stage-16 embryos, (g) stage-20 embryos and (h) stage-40 embryos. The bracket ([ ]) denotes the shift in molecular weight detected during cleavage.

sional gels (Dingwall *et al.* 1982; Dreyer & Hausen, 1983; Figs 7, 8). A structural heterogeneity between egg and oocyte nucleoplasmin has been described that is due to extensive additional phosphorylation of the egg protein which is apparent as an increase in molecular weight and a shift in pI to more acidic forms (Sealy *et al.* 1986; Cotton *et al.* 1986). We have observed an identical increase in molecular weight and shift in pI between the oocyte and the embryo (Figs 7, 8, 9), although we have not directly shown that this is due to phosphorylation. Finally, our results with immunofluorescence microscopy indicating Ag:2G6's predominant, if not exclusive, nuclear location (Figs 1, 2) are consistent with the observation that nucleoplasmin rapidly translocates into the nucleus after injection into the ooplasm (Dingwall *et al.* 1982). From these observations we conclude that Ag:2G6 is, most likely, nucleoplasmin.

There are conflicting reports in the literature as to whether nucleoplasmin is present in nuclei of adult somatic cells or not. Krohne & Franke (1980*a*,*b*) examined several tissues (ovary, testes, kidney cell line, liver) by indirect immunofluorescence microscopy using a polyclonal antiserum and found immunologically related nuclear proteins in all cell types except two characterized by low levels of transcription (erythrocytes and spermatids). However, the actual identity and relationship of the proteins in the different cells were not determined. In contrast, Dreyer & Hausen (1983) found no such cross-reactivity with adult material using their monoclonal antibodies. Our results with Mab:2G6 indicate no cross-reactivity with extracts of adult somatic cells on Western blots (Fig. 4) or on tissue sections by indirect immunofluorescence microscopy (data not shown). In an attempt to clarify these discrepancies, we tested the polyclonal antiserum of Krohne & Franke (1980*a*) against adult tissue extracts on Western blots and found cross-reactivity with a very weakly staining 30K species in heart and testes, which comigrated with the species in gastrula as well as a major 40K species in testes (Fig. 4). One interpretation of these results is that nucleoplasmin is really a family of closely related proteins and that immunologically distinct members of this family are present in nuclei of embryonic cells and adult somatic cells. This would explain why monovalent antibodies raised against embryonic material do not recognize any determinants in adult tissue, whereas polyvalent antibodies recognize epitopes in embryos and adult organs. It is well known that nucleoplasmin exhibits protein heterogeneity on SDS gels and migrates as a characteristic smear or, more rarely, as a series of 5–7 spots on two-dimensional gels (Figs 8, 9; Dreyer *et al.* 1985). More recently, the message for nucleoplasmin has been cloned and slightly variant cDNAs have been reported (Burglin *et al.* 1987). Taken together, these observations provide strong evidence for the conclusion that nucleoplasmin is a family of closely related proteins with distinct embryonic and adult members. The functional significance of different molecular forms of nucleoplasmin remains to be elucidated.

Our observations on the immunolocalization of nucleoplasmin confirm previously published work on oocytes (Krohne & Franke, 1980*a*,*b*; Dreyer & Hausen, 1983; Dreyer *et al.* 1985) and provide new information on its localization during embryogenesis. Nucleoplasmin was detected exclusively within the GV at each oogenic stage (Krohne & Franke, 1980*a*,*b*; Dreyer & Hausen, 1983; Fig. 1). The youngest oocytes contained 10% of the maximum accumulated amount of nucleoplasmin present in stage-6 oocytes as analysed by Western blot analysis or by an ELISA (Fig. 10). However, its synthesis, as analysed from two-dimensional autoradiograms, was not detected until stage 2, suggesting that nucleoplasmin present in stage-1 oocytes had been synthesized at an earlier point in germ cell development. From stage 2 to 3, there was a sharp increase in the rate of synthesis which peaked at stage 4. These high levels of synthesis correlate with a phase of rapid growth during oogenesis which includes a dramatic increase in the size of the GV (Callen *et al.* 1980). By stage 6,

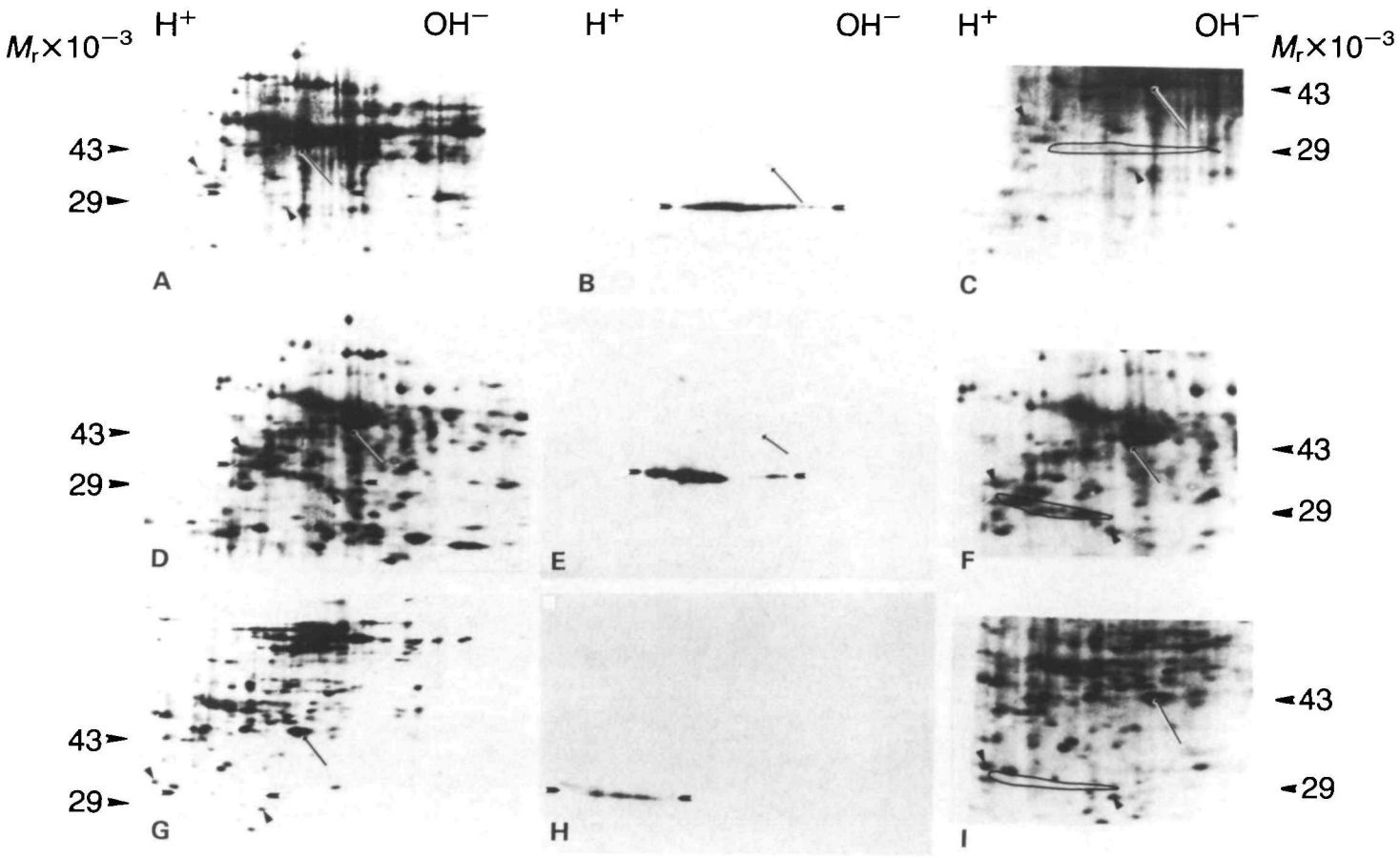

Fig. 8. Two-dimensional gel analysis of Ag:2G6. 1×10^6 cts min^{-1} of TCA-precipitable protein plus 80 μg of unlabelled protein were loaded on each gel. The (→) arrow denotes the position of actin and the (▶) wedge indicates the two proteins used as position markers. Ag:2G6 is indicated by the (➧) arrow. In each case, in panels C, F and I the area of the autoradiograph that includes Ag:2G6 has been enlarged and Ag:2G6 encircled to show the exact position as determined by superimposing the immunoblot on the autoradiograph. (A–C) stage-2 oocyte proteins probed with Mab:2G6 and Mab:b7-1D1 (from P. Hausen); (D–F) stage-3 oocyte proteins probed with Mab:2G6 and Mab:b7-1D1, and (G–I) stage-6 oocyte proteins probed with Mab:2G6.

synthesis of nucleoplasmin was undetectable and accumulation had reached maximum levels (Fig. 10). Dreyer & Hausen (1983) report no synthesis in stage-1 and -2 oocytes and minimal synthesis at stage 6 although these findings were not quantified. However, nucleoplasmin mRNA is present at the same abundant levels as it is in stage-1 oocytes (Burglin *et al.* 1987), suggesting that its translational efficiency declines significantly some time between stage 4 and 6 during oogenesis.

The embryo appears to inherit a large maternal stockpile of nucleoplasmin which does not appear to decline in amount at least until tadpole stages (Fig. 7). Upon GV breakdown, nucleoplasmin is extruded upwards towards the animal pole, eventually spreading over the whole animal half with a small amount extending into the marginal zone towards the vegetal pole (Hausen *et al.* 1985). Our results show that nucleoplasmin is distributed almost exclusively to the future animal pole blastomeres during subsequent cleavage, most likely cycling between the cytoplasm and the nucleus with each mitotic division (Figs 1, 5). By the 512-cell stage, most, but not all, nuclei at the animal pole were positive, suggesting different levels of nucleoplasmin exist in different blastomeres. Western blot analysis revealed a third molecular weight species (33K) present after fertilization but missing by gastrulation (stage 12). This species correlates with a higher phosphorylated state for nucleoplasmin and a protein more active in nucleosome assembly (Sealy *et al.* 1986). This species occurs precisely at the time of maximum DNA synthesis in development as has also been observed by Burglin *et al.* (1987).

The mechanism for restricted parcelling is unknown, but Hausen *et al.* (1985) showed that three different nucleoplasmic proteins all display different

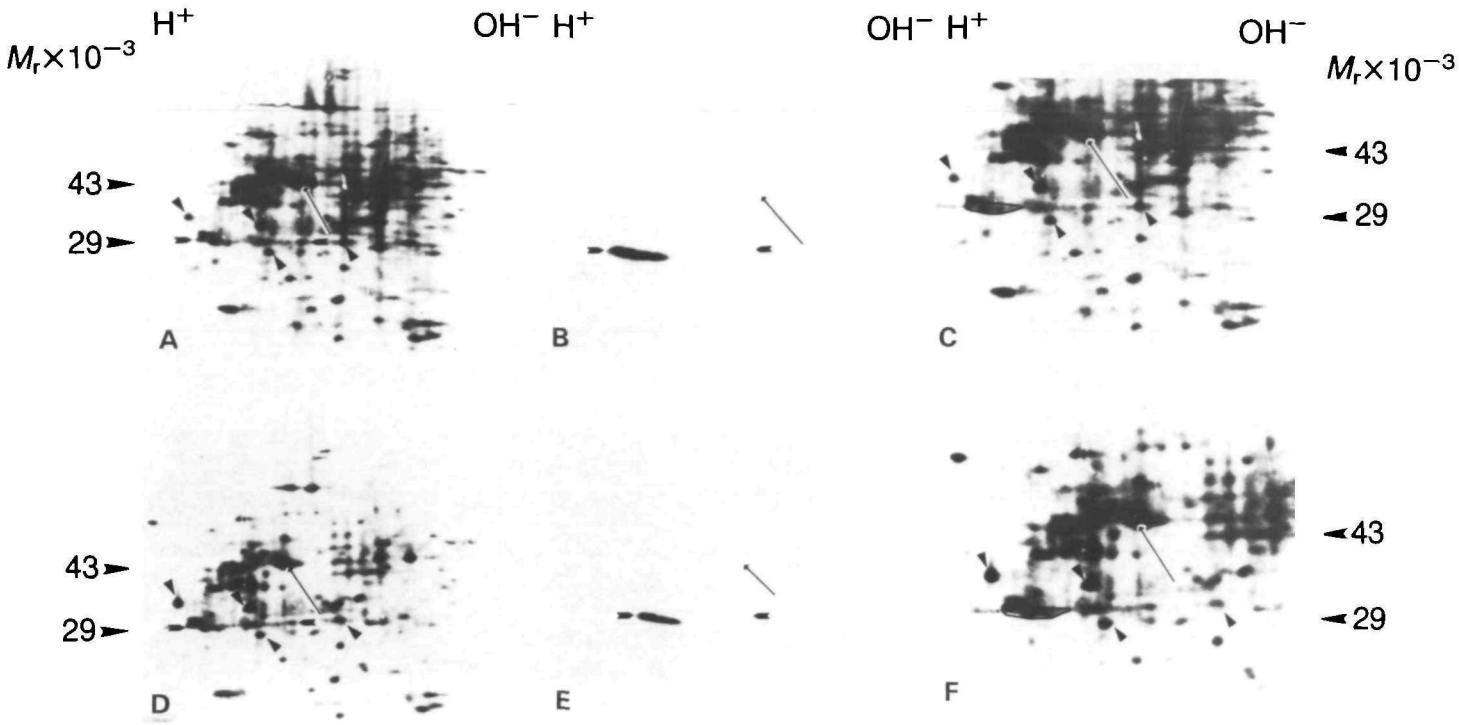

Fig. 9. Two-dimensional gel analysis of Ag:2G6 in gastrulae. 1×10^6 cts min^{-1} of TCA precipitable proteins plus 80 μg of unlabelled protein was loaded on each gel. The (→) arrow denotes the position of actin and the (►) wedge indicates four proteins used as position markers. Ag:2G6 is indicated by the (◗) arrow. In each case in panels C and F, the area of the autoradiograph that includes Ag:2G6 has been magnified and Ag:2G6 encircled to show the exact position as determined by superimposing the immunoblot on the autoradiograph. (A–C) probed with Mab: b7-1D1 and (D–F) probed with Mab:2G6.

patterns of behaviour after germinal vesicle breakdown. It seems likely that inheritance depends on a quality inherent to the protein, either chemical or structural, rather than to egg structural rearrangements that occur at oocyte maturation.

As development proceeds, nucleoplasmin is eventually detected in nuclei of all embryonic cells. It is possible that nucleoplasmin is actually present in all nuclei at all stages of development, but at sharply different concentrations. One can imagine that as nuclei get progressively smaller, as a result of mitotic division, the concentration of nucleoplasmin increases to detectable levels in the cellular descendants of the vegetal blastomeres. Interestingly, Northern blot analysis fails to detect any nucleoplasmin mRNA from cleavage through tadpole stages (Burglin *et al.* 1987) suggesting that new synthesis of nucleoplasmin after oogenesis is negligible or non-existent. Our results indicate that, in fact, a low level of synthesis is occurring during gastrulation, perhaps maintaining the observed steady-state levels of the protein through development (Fig. 9).

By stage 40 (swimming tadpole), nucleoplasmin was detected only in nuclei of specific postmitotic cells of the CNS and in striated, but not smooth, muscle cells. The shift from a universal nuclear distribution to a highly restricted one in postmitotic, but transcriptionally active, nuclei is correlated with the return of the 28·5/29K oocyte species, presumably reflecting a change to a less-phosphorylated state, and a protein less active in nucleosome assembly (Sealy *et al.* 1986). This restricted pattern is most likely explained by nucleoplasmin being diluted to levels below detection in mitotically active cells whereas cells that have left the mitotic cycle earlier in development (i.e. striated muscle) have retained nucleoplasmin at levels sufficient for detection. Such a pattern of expression is entirely consistent with the hypothesized role of nucleoplasmin in nucleosome assembly. An alternative explanation is that nucleoplasmin functions in ways not yet described, such as in stabilizing chromatin in cells that are no longer dividing.

Existing evidence strongly supports a role in nucleosome assembly and histone binding for nucleoplasmin (Earnshaw *et al.* 1980). However, only a minor fraction of the 0·25–0·28 μg present in germinal vesicles (representing 8–10 % of total nuclear protein) is found in histone complexes. At the ultrastructural level, nucleoplasmin is associated with active chromatin and transcription products, suggesting a

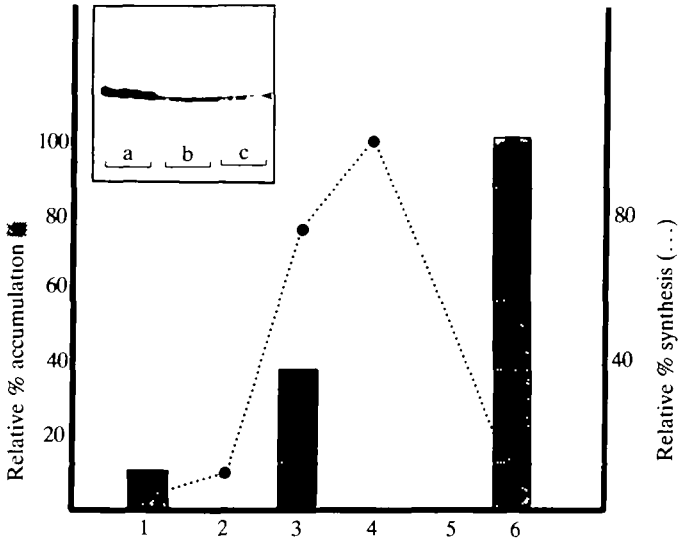

Fig. 10. Synthesis and accumulation of nucleoplasmin during oogenesis. Levels of accumulation determined by scanning one-dimensional immunoblots or by the ELISA (■). Levels of synthesis determined by computer-assisted integration of two-dimensional autoradiographs (·····). Accumulation represented as arbitrary units with stage 6 assigned a value of 100. The amounts accumulated at the other stages are represented relative to stage 6. Synthesis represented in arbitrary units with stage 4 assigned a value of 100. The amounts synthesized at the other stages are represented relative to stage 4. The data have been corrected for background. The inset represents an example of the immunoblot used to determine accumulation. Equal oocyte equivalents loaded on each lane of (a) stage 6, (b) stage 3 and (c) stage 1 and probed with Mab:2G6.

role in transcription (Moreau *et al.* 1986). Nucleoplasmin does appear to be absent from transcriptionally inactive cells (Krohne & Franke, 1980*a*,*b*; Moreau *et al.* 1986). Yet, again, it is unclear that the association with RNP particles accounts for a significant portion of the large accumulation of nucleoplasmin. It may be that nucleoplasmin provides an ionic environment within the nucleus facilitating a number of nuclear events such as histone accumulation during oogenesis, as has been suggested (Kleinschmidt *et al.* 1985; Burglin *et al.* 1987).

Why is the large maternal stockpile of nucleoplasmin specifically shunted into the animal pole region? Perhaps the distribution reflects the final number of nuclei that will form along the A/V axis as suggested by Hausen *et al.* (1985). Further studies on nucleoplasmin should shed light on the mechanisms by which proteins are distributed to a subset of blastomeres and, by analogy, how cellular determinants might also be segregated.

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