The Function of the Nuclear Envelope in Nuclear Protein Accumulation

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Abstract. The mechanism by which proteins accumulate in the cell nucleus is not yet known. Two alternative mechanisms are discussed here: (*a*) selective unidirectional entry of karyophilic proteins through the nuclear pores, and (*b*) free diffusion of all proteins through the nuclear pores and specific binding of nuclear proteins to nondiffusible components of the nucleoplasm. We present experiments designed to distinguish between these alternatives. After mechanical injury of the *Xenopus* oocyte nuclear envelope, nuclear proteins were detected in the cytoplasm by immunohistochemical methods. In a second approach, nuclei from *X. borealis* oocytes were isolated under oil, the nuclear envelopes were removed, and the pure nucleoplasm was injected into the vegetal pole of *X. laevis* oocytes. With immunohistochemical methods, it was found that each of five nuclear proteins rapidly diffuses out of the injected nucleoplasm into the surrounding cytoplasm. The subsequent transport and accumulation in the intact host nucleus could be shown for the nuclear protein N1 with the aid of a species-specific mAb that reacts only with *X. borealis* N1. Purified and iodinated nucleoplasmin was injected into the cytoplasm of *Xenopus* oocytes and its uptake into the nucleus was studied by biochemical methods.

M UCH is known about the migration of proteins into or across most cellular membranes (Wickner and Lodish, 1985). Regarding the nuclear envelope, the passage of karyophilic proteins through the nuclear pores has been thoroughly studied, but the question of how these proteins concentrate in the nuclei (Paine, 1982; De Robertis, 1983) is still disputed (for review see Dingwall and Laskey, 1986). Since the initial finding by Gurdon (1970) that iodinated histones microinjected into frog oocytes migrate into the cell nucleus and accumulate more than 100-fold, two alternative models concerning migration and accumulation of proteins into nuclei have evolved. Indirect evidence supports the idea that nuclear proteins diffuse into the nucleus and accumulate by binding to a nondiffusible nucleoplasmic component (Bonner, 1978; Feldherr and Pomerantz, 1978; Feldherr and Ogburn, 1980). The second model suggests that the accumulation of nuclear proteins is due to their selective and active unidirectional transport across the nuclear envelope. The selectivity of the process (Bonner, 1975*a*, *b*; De Robertis et al., 1978; Dabauvalle and Franke, 1982) led to the proposal that the accumulation is controlled by a persisting signal within the mature molecular structure of the transported protein (De Robertis et al., 1978).

Nucleoplasmin, the most abundant protein in the nucleus of *X. laevis* oocytes (Mills et al., 1980; Krohne and Franke, 1980), can be accumulated by nuclei in vitro, and it has been shown that ATP is required for this process (Newmeyer et al., 1986*b*). Nucleoplasmin was also the first protein for

which a signal for nuclear transport was localized in a specific domain (Dingwall et al., 1982).

Kalderon et al. (1984*a*) have identified a short amino acid sequence of the SV40 large-T antigen necessary for its transport and accumulation into the nuclei of transformed Rat-1 cells. This sequence is sufficient to redirect the cytoplasmic enzyme pyruvate kinase to the nucleus (Kalderon et al., 1984*b*).

Although additional nuclear location signals have been discovered (Richardson et al., 1986; Davey et al., 1985; Hall et al., 1984), it is not clear whether these karyophilic signals are responsible for the binding of the proteins to a nondiffusible nucleoplasmic component, or whether the signals interact with the nuclear envelope to allow selective transport of the proteins into the nucleus. Davey et al. (1985) suggested that both selective transport and selective binding play a role in nuclear accumulation and that it is possible that these two activities are controlled by different regions of a protein.

Evidence supporting the first model comes from Feldherr and Pomerantz (1978) and Feldherr and Ogburn (1980), who have studied the retention and the uptake rates of endogenous nuclear proteins in nuclei of *Xenopus* oocytes punctured in vivo. We have essentially repeated these experiments, but have used more sensitive immunohistological techniques for the detection of the nuclear proteins. We found low, but significant, levels of leakage of protein from punctured nuclei and greater levels when larger lesions were introduced into the nuclear envelope. Moreover, nuclei were deprived

of their envelopes and the resulting nucleoplasm was injected into *Xenopus* oocytes with the result that nuclear proteins readily diffused from the site of the injected nucleoplasm and accumulated in the intact nucleus of the host cell.

Materials and Methods

Purification and Iodination of Nucleoplasmin

Nucleoplasmin was purified from homogenates of *Xenopus laevis* oocytes by affinity chromatography. Ovaries were incubated in 3 vol 0.2% collagenase (type I, Sigma Chemical GmbH, München, FRG) in oocyte Ringer 2 buffer without calcium (Wallace et al., 1973) for 1.5 h at 30°C. The isolated oocytes were washed extensively with cold 50 mM NaCl, 25 mM Tris-HCl (pH 7.2), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and homogenized in a final volume 1.2 times the volume of the oocytes. After centrifugation in an SW 27 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 1 h at 25,000 rpm at 4°C, the supernatant was extracted twice with 1,1,2-trichlorotrifluoroethane to remove yolk lipoprotein (Laskey et al., 1978) and the extract was stored at −80°C. For affinity chromatography, 10 ml of extract was centrifuged at 15,000 g, and the supernatant was loaded onto a 0.5 × 5-cm (1 ml) antibody column equilibrated with 50 mM NaCl, 25 mM Tris-HCl (pH 7.2), 0.1 mM EDTA (NTE buffer).[1] The column was washed with 0.8 M NaCl in NTE buffer and the nucleoplasmin was eluted with 2 M $MgCl_2$ in NTE buffer. Peak fractions were pooled and dialyzed against NTE buffer.

For iodination 20–50 µg nucleoplasmin in 10 µl of 0.1 M borate (pH 8.5), 50 mM NaCl were reacted with Bolton and Hunter reagent (1 mCi, sp act 2,000 ci/mmol; Amersham Buchler GmbH, Braunschweig, FRG) as described by Dingwall et al. (1982). Between 15 and 25% of the label was routinely incorporated.

Immobilization of Antibody to Sepharose

mAb b7-1A9, directed against nucleoplasmin, was purified from mouse ascites fluid by affinity chromatography on a protein A–Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden; Langone, 1982). The isolated antibody was coupled to Sepharose CL-4B (Pharmacia Fine Chemicals) by the procedure of Nilsson and Mosbach (1984), using tresyl chloride (Fluka AG, Neu-Ulm, FRG) as a coupling reagent. In this way 9 mg of antibody was immobilized to 1 g of wet tresyl-Sepharose.

Microinjection and Culture of X. laevis Oocytes

Ovaries were dissected from female frogs and maintained in modified Barth's saline (MBS-H) at 20°C as described by Gurdon (1976). Before microinjection, a small piece of ovary was incubated in 3 vol 0.2% collagenase (type I, Sigma Chemical GmbH) in oocyte Ringer 2 buffer without calcium for 5 min at 37°C, and for a further 15 min at room temperature. Then the oocytes were washed extensively in MBS-H and fully grown oocytes were selected for injection. Each oocyte was injected with 60 nl of protein solution into the equator region directly underneath the plasma membrane using glass needles of 15 µm diam.

Procedure for Isolation of Nucleoplasm from Oocytes

10 oocytes, manually isolated from the ovary, were washed with distilled water and transferred to a glass plate. Water was removed with absorbent paper and by rolling the oocytes over the glass plate. The oocytes were transferred into paraffin oil (E. Merck, Darmstadt, FRG, Art. 7174) when the water film on the surface of the oocytes had disappeared. Oocyte nuclei were removed manually by making a slit into the animal pole with an injection needle and squeezing the oocyte gently. The nuclear envelopes were removed with a pair of watchmaker forceps and the resulting nucleoplasms were fused into a ball. This was sucked up into a 50 µm diam glass needle filled with paraffin oil. Injection into oocytes incubated in MBS-H was performed under constant flow of nucleoplasm out of the needle.

Procedure for the Removal of Part of the Nucleus

A narrow slit was introduced with an injection needle into the animal pole

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1. *Abbreviations used in this paper*: MBS-H, modified Barth's saline; NTE buffer, 50 mM NaCl, 25 mM Tris-HCl (pH 7.2), 0.1 mM EDTA.

of an oocyte incubated in MBS-H. The oocyte was squeezed until part of the nucleus was extruded. This part was cut off by running the sharp edge of the injection needle along the surface of the oocyte. The nuclear envelope, sticking in the slit in the plasma membrane, was then gently pushed back into the interior of the oocyte with a blunt glass needle.

Processing of Oocytes after Injection of Iodinated Nucleoplasmin

After injection of iodinated nucleoplasmin, the oocytes were individually incubated in 1 ml of MBS-H. At different times, groups of five oocytes were pipetted into hot water (80°C) and 15 s later transferred into dry ethanol. 0.5 min later, the nuclei were easily removed by breaking open the oocytes with needles. Radioactivity in nuclei, enucleated oocytes, and aliquots from the incubation medium were individually determined in a gamma counter. The nuclear-to-cytoplasmic concentration ratio of the protein was computed for every oocyte and the specimen whose ratio was closest to the mean value was processed for SDS gel electrophoresis (Laemmli, 1970). The enucleated oocyte was ground in ethanol, centrifuged, and the dried sediment as well as the dried nucleus was dissolved by vortexing in 30 µl of gel sample buffer containing 1 µg of unlabeled nucleoplasmin. The samples were heated to 95°C for 10 min and loaded on a 10% SDS–polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed at −80°C with an intensifying screen. With the help of the autoradiograph, the nucleoplasmin-containing bands were identified and cut out of the dried gel, and the radioactivity was determined in a gamma counter.

Procedure for Immunohistological Studies

Oocytes were fixed in 2% TCA for 2 h and transferred to 100% ethanol for 12 h. For staining of sections with mAb b7-1A9, which is directed against nucleoplasmin, oocytes were fixed in Romeis fixative (25 ml saturated mercuric chloride, 20 ml 5% TCA, 15 ml 37% formaldehyde; Romeis, 1968) for 2 h. After dehydration in ethanol, oocytes were impregnated with a solution of polyethyleneglycol-400-distearate, 1% cetylalcohol (Koch-Light Ltd., Haverhill, Suffolk, England) in ethanol (1:1) for 1 h, and then in the same solution without ethanol for 3 h at 42°C. After imbedding in the same medium, 7-µm-thick sections were cut. For immunostaining, sections were dewaxed in 100% acetone and washed in 90, 70, 50, or 30% acetone in water and finally in PBS. The sections were incubated with ascites fluid containing the mAb (diluted 1:1,000 in PBS) for 30 min, washed three times in PBS (5 min each), and stained with affinity-purified FITC-conjugated goat anti-mouse IgG (Wedlich et al., 1985) diluted 1:100 in PBS, 0.1% Triton X-100, and 1% BSA for 30 min. After washing three times for 5 min, the slides were mounted in glycerol.

Two-dimensional Microgel Electrophoresis

The method used was that of Neukirchen et al. (1982) as modified by Y. Boxberg (unpublished observations). 0.65-mm-diam tube gels were used for the first dimension and 0.1-mm-thick slab gels were used for the second. Gels were silver stained as described in Zimmer (1987).

Results

Puncturing of Nuclei

Nuclear envelopes of intact *Xenopus laevis* oocytes were punctured in situ 30 times with 12-µm-diam glass needles as described by Feldherr and Pomerantz (1978). The control oocytes (not punctured) and the punctured oocytes were fixed 3 and 8 h later and processed for immunostaining. Sections were stained with mAbs binding to different nuclear proteins (Dreyer et al., 1985). The positions of the antigens nucleoplasmin, N1, N4, and N8 on silver-stained two-dimensional gels are indicated in Fig. 1.

Oocyte sections stained with a mAb that binds to nucleoplasmin are shown in Fig. 2. 3 h after puncturing, a significant amount of nucleoplasmin is seen in the cytoplasm, although most of the antigen is still retained in the nucleoplasm. Leakage increases with time after puncturing as
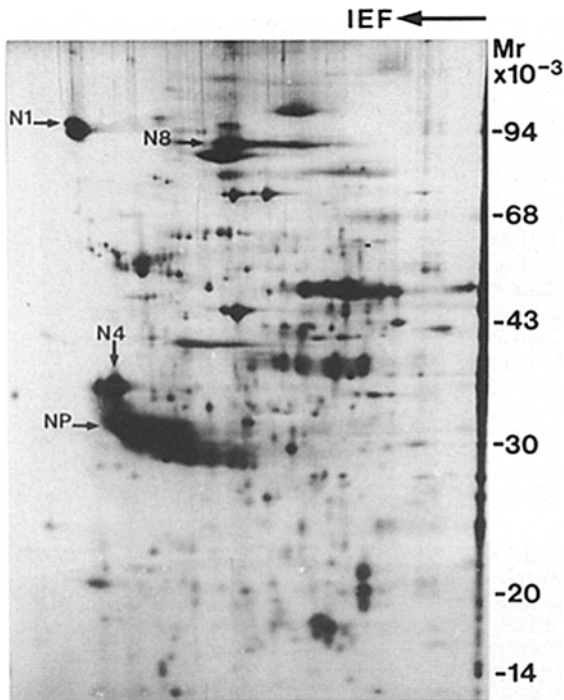
Figure 1. Nuclear proteins of *X. laevis* oocytes. Three hand-isolated nuclei were subjected to two-dimensional microgel analysis, and the polypeptides were stained with silver. Arrows indicate the nuclear proteins nucleoplasmin (*NP*), N1, N4, and N8, which are highly enriched in oocyte nuclei. Proteins N1 and N4, as described by De Robertis et al. (1978), were identified by their position in two-dimensional gels.

shown by comparison of specimens fixed 3 and 8 h after puncturing.

Leakage into the cytoplasm was only observed with nucleoplasmin, which is the most abundant oocyte nuclear protein (Mills et al., 1980; Krohne and Franke, 1980). mAbs against less abundant nuclear proteins stained the nuclei only (not shown). Either nucleoplasmin is the only nuclear protein that leaks out of the nucleus after puncturing or the less abundant nuclear proteins also leak out of the punctured nucleus but their amount remains below the limit of detection in the cytoplasm. To discriminate between these two possibilities we then tried to injure a larger area of the nuclear envelope.

Removal of Part of the Nucleus

Approximately 30% of the nuclear envelope was surgically removed after partially extruding the nucleus through a slit in the plasma membrane near the animal pole. After healing, the oocytes remained intact for at least 6 h. 10 min, 2 h, and 4.5 h after operation, the oocytes were processed for immunostaining and the sections stained with four mAbs directed against nuclear proteins.

In oocytes fixed 10 min after the operation, the cytoplasm was devoid of nuclear antigens except near the site of the operation. After longer periods of time, a considerable amount of all four nuclear antigens was distributed in the cytoplasm of the animal hemisphere as shown for nucleoplasmin and N8 in Fig. 3. Since this process is time dependent, it cannot be the immediate result of the operation itself.
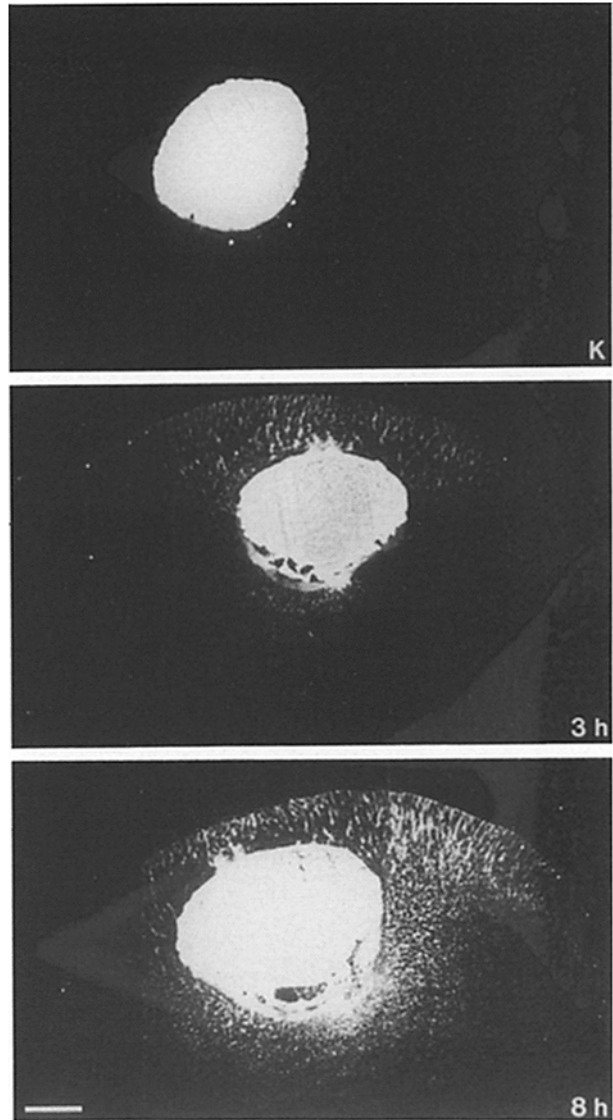

Figure 2. Immunostaining of sections of punctured *X. laevis* oocytes with mAb b7-1A9 directed against nucleoplasmin. The animal poles of oocytes were punctured 30 times with a glass needle as described in Materials and Methods. 3 and 8 h after puncturing, the oocytes were fixed in Romeis fixative and processed for immunostaining. *K,* section of nonpunctured oocyte. Bar, 0.1 mm.

The distribution patterns of the nuclear proteins N4 (De Robertis et al., 1978; antigen b7-2H4, Dreyer et al., 1985) and N1 (antigen b2-2B10, Dreyer et al., 1985) (not shown) are similar to that of N8 (Fig. 3 *b*). This demonstrates that large-scale lesion of the nuclear envelope leads to a significant loss of several nuclear proteins into the cytoplasm.

Injection of Nucleoplasm into Oocytes

A species-specific mAb (b6-3B7), directed against a nuclear protein of *X. borealis* oocytes (Dreyer et al., 1985), was used to construct the following experiment, which assessed the importance of the nuclear envelope in the process of accumulation of a protein in the nucleus.

X. borealis oocyte nuclei were isolated under oil (De Robertis, E. M., personal communication), the envelopes
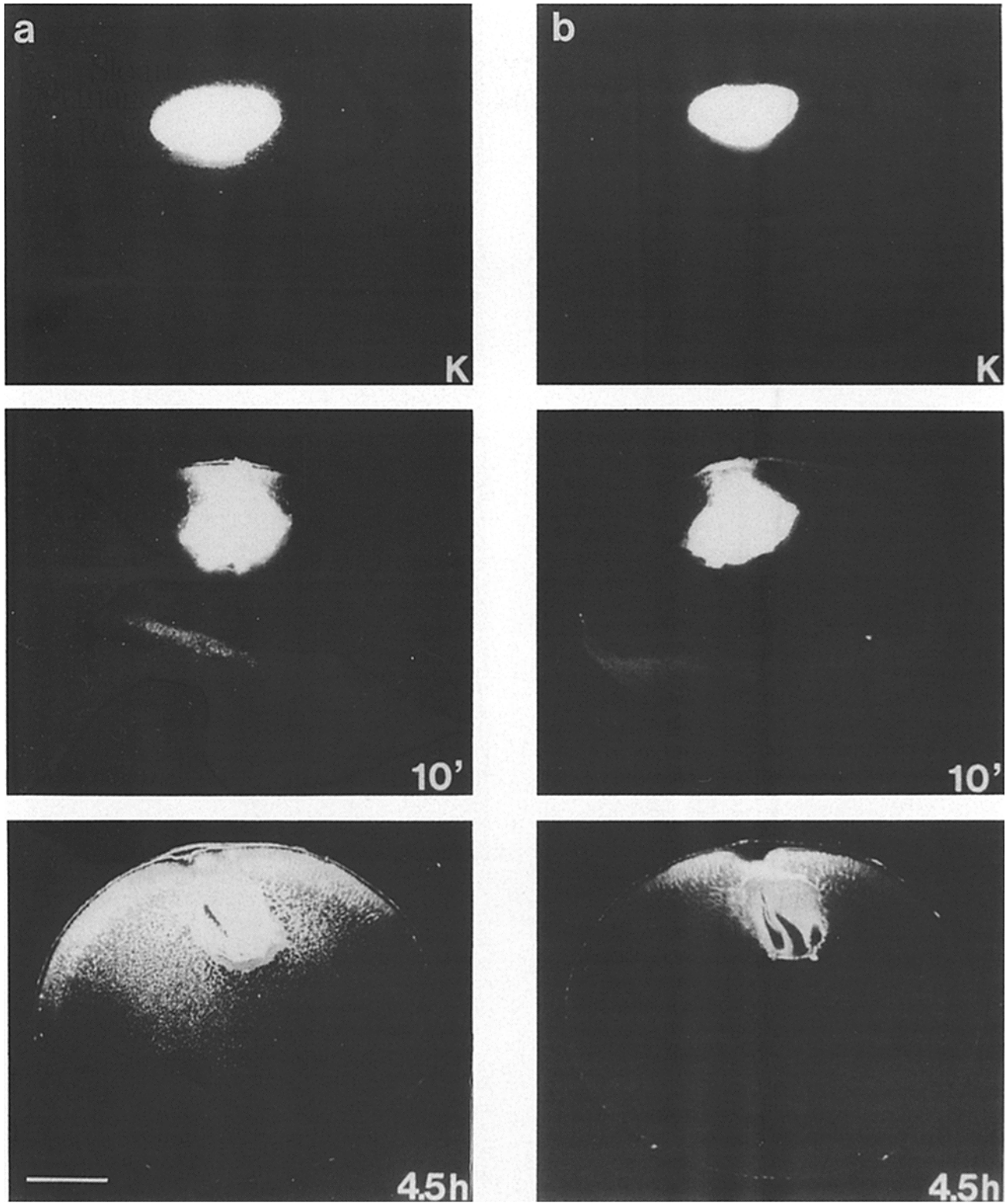
Figure 3. Immunostaining of oocyte sections after removal of part of the nucleus. Part of the nucleus of each oocyte was removed and 10 min and 4.5 h later the oocytes were fixed in Romeis fixative and processed for immunostaining with mAbs b7-1A9 (directed against nucleoplasmin) (*a*) and b6-6E7 (directed against N8) (*b*). Bar, 0.22 mm.

manually removed, and the nucleoplasm was sucked into a glass needle and injected into the vegetal pole of *Xenopus laevis* oocytes. An amount approximately equal to the volume of one nucleus was injected into each oocyte. Oocytes were fixed at different times after injection and processed for immunostaining with mAb b6-3B7. Fig. 4 shows that the *X. borealis*-specific N1 diffuses out of the injected nucleoplasm and distributes in the host oocyte.
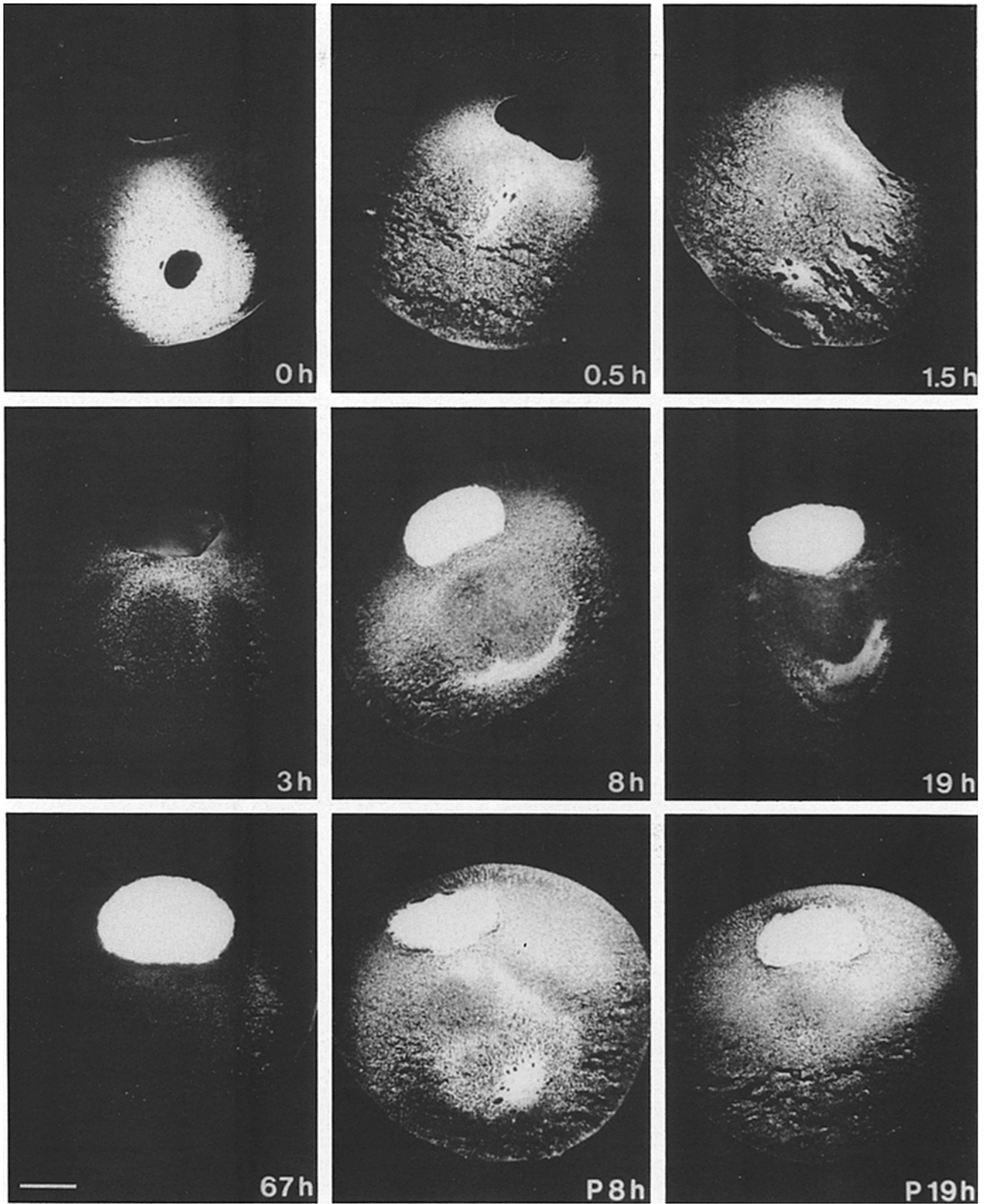
Figure 4. Immunostaining of sections of *X. laevis* oocytes injected with nucleoplasm from *X. borealis* oocytes and fixed either immediately (*0 h*) or 0.5, 1.5, 3, 8, 19, and 67 h later with 2% TCA. Two oocytes were punctured 1 h after injection 30 times with a glass needle into the animal poles. One oocyte was fixed 7 h later (*P8 h*) and the other one 18 h after puncturing (*P19 h*). Oocyte sections were stained with mAb b6-3B7, which is directed against *X. borealis* nuclear protein N1. Dark areas that are seen in the injected nucleoplasm (see *0 h*) are paraffin droplets. Bar, 0.2 mm.

b6-3B7　　　b6-6E7　　　b7-2H4　　　b2-2B10

0h

1h

1.5h

3h

8h

19h

44h
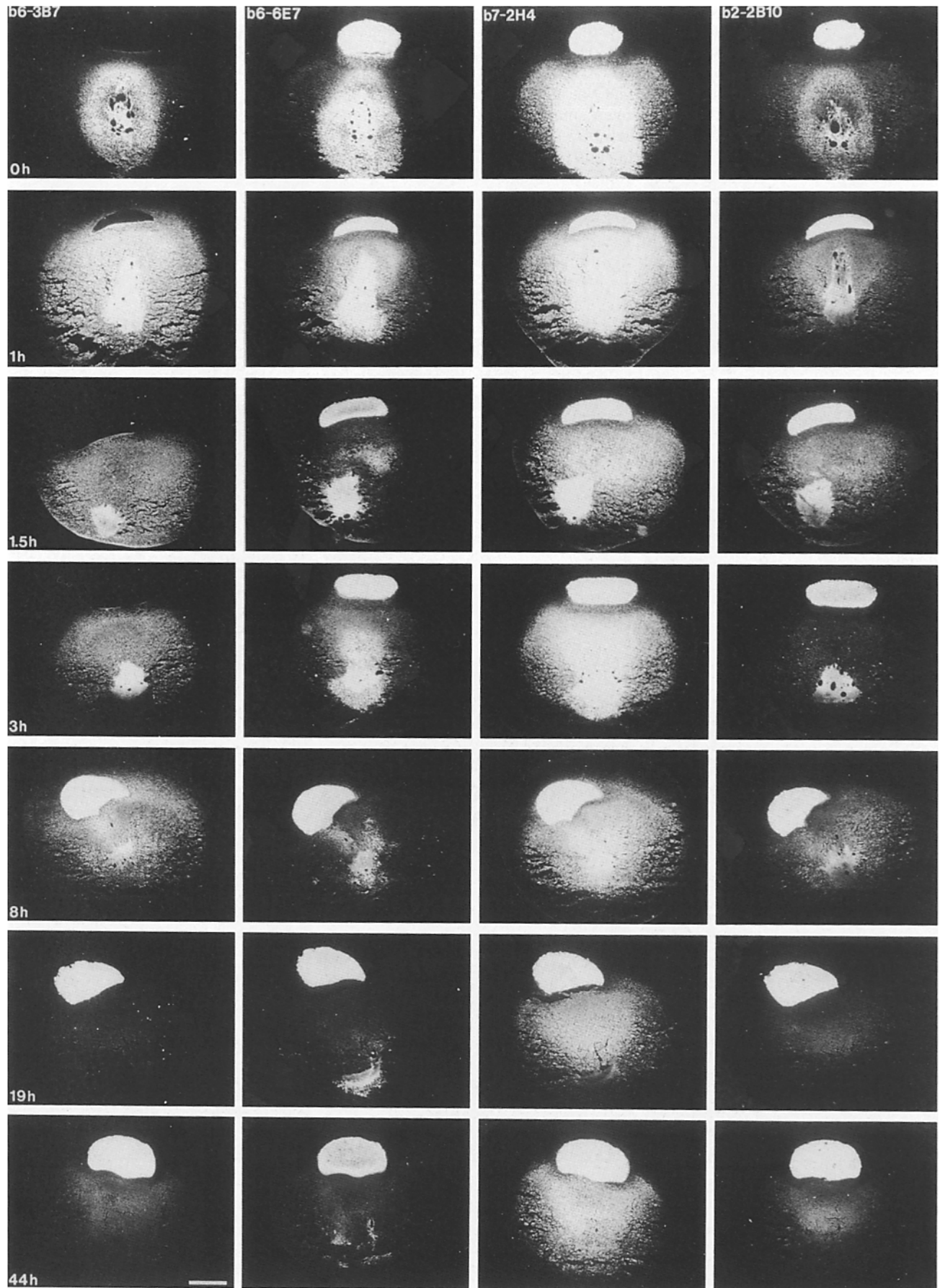
This process is so rapid that immediately after injection (*0 h* in Fig. 4) the antigen is found in an area approximately four times as large as the area of the injected nucleoplasm as visualized on sections by phase-contrast microscopy (data not shown). After 1.5 h almost all of the antigen is still in the cytoplasm, whereas after 3 h a significant amount is detected in the host nucleus, although its concentration is still higher in the cytoplasm. 8 h after injection, most of the X. *borealis*-specific N1 has accumulated in the host nucleus and after 19 h N1 is almost completely located in the nucleus. N1 remains concentrated in the nucleus for at least 2 d thereafter. The injected nucleoplasm remains compact and unaffected by the surrounding yolk initially; then it disappears between 44 and 67 h after injection. No such structure was observed after injection of an aqueous nucleoplasmin-containing solution into the vegetal pole of the oocyte (Zimmer, F. J., unpublished observations).

When the nucleus of the host oocyte is punctured 30 times with 12-μm-diam glass needles 1 h after injection of nucleoplasm, more of the X. *borealis*-specific protein N1 remains dispersed in the cytoplasm 19 h after injection as compared to a host oocyte with an intact nuclear envelope (compare *P19 h* with *19 h* in Fig. 4). These results indicate that nuclear protein N1 is not retained by the nucleoplasm but accumulates in nucleoplasm that is surrounded by a nuclear envelope. Hence, the nuclear envelope is essential for the accumulation of this protein.

The injection of nucleoplasm into oocytes was repeated and sections were stained with four different mAbs directed against different nuclear antigens (Fig. 5). The comparison of the distribution pattern of the nuclear antigens N8 (b6-6E7), N4 (b7-2H4), and N1 (b2-2B10 in X. *laevis* oocyte nuclei) with the antigen N1 from X. *borealis* (b6-3B7) in neighboring sections reveals no significantly different staining patterns. With the exception of the nuclear antigen N8 (b6-6E7), all the nuclear antigens have disappeared from the cytoplasm by 19 h after injection (cytoplasmic staining of the oocyte with mAb b7-2H4 is due to background as demonstrated on sections of uninjected oocytes; data not shown). 44 h after injection, the injected nucleoplasm has almost totally dissolved and the antigen N8 (b6-6E7) can only be detected in low amounts at the site of injection. The antibody b6-6E7 stains the nucleoli more intensely than nucleoplasm (Wedlich and Dreyer, 1988). Nucleoli persist in the injected nucleoplasm for at least 19 h, but disappear between 19 and 44 h after injection. The fact that all four nuclear antigens diffuse almost uniformly and rapidly out of the injected nucleoplasm suggests a very low affinity of these antigens for the nucleoplasm. This experiment was also done with mAb b7-1A9 directed against nucleoplasmin with the same result (not shown).

We cannot rule out the possibility that the three nuclear antigens N8, N4, and N1 (from X. *laevis*) disappear from cytoplasm due to degradation, but their simultaneous disap-

pearance from the cytoplasm and accumulation of X. *borealis* N1 in the host nucleus makes this unlikely.

Injection of Purified Nucleoplasmin into Oocytes

By injection of purified and radioactively labeled nucleoplasmin into the cytoplasm of *Xenopus* oocytes, we were able to confirm biochemically that this protein is indeed accumulated in the nucleus, as has been previously shown (Dingwall et al., 1982). Nucleoplasmin was purified from oocyte extracts by affinity chromatography and iodinated by the method of Bolton and Hunter (see Materials and Methods).

To study the accumulation of this nuclear protein, fully grown oocytes from X. *laevis* were injected with 2.5 ng of the iodinated protein (in a volume of 60 nl). The dependence of the transport into the nucleus on the injection site is shown in Fig. 6 a. Nucleoplasmin accumulates rapidly in the nucleus when injected at an equatorial or animal position. Injection was always directly underneath the plasma membrane to avoid injection into the nucleus. 5 h after injection, ∿50% of the protein is found in the nucleus. Injection into the vegetal pole, however, reduces the rate of accumulation drastically (Fig. 6 a). Therefore, all injections were performed equatorially.

The accumulation of nucleoplasmin in the nucleus during 3 d of incubation is shown in Fig. 6 b. 1 d after injection, the nuclear concentration reached an almost constant value of ∿20-fold over the cytoplasmic concentration. The accumulation in no case ever exceeded 30-fold.

Discussion

The experiments described in this paper suggest that mechanical disruption of the nuclear envelope of X. *laevis* oocytes in situ results in a loss of nuclear proteins from the nucleoplasm and leakage into the cytoplasm. These results are at variance with those of Feldherr and Pomerantz (1978) and Feldherr and Ogburn (1980). These authors incubated *Xenopus* oocytes in radioactive leucine and punctured the nucleus 30 times with 12-μm-diam glass needles. Nuclei were isolated 2.5 h later and analzyed by one- and two-dimensional gel electrophoresis. No significant differences were seen between punctured and control nuclei. They concluded that the accumulation of specific endogenous proteins within the nucleoplasm is attributable largely to selective binding. This interpretation was corroborated by their finding that the uptake of several endogenous nuclear polypeptides into the nucleus is unaffected by puncturing, suggesting that the passage across the nuclear envelope is not a rate-limiting step for these molecules.

In contrast to their results, we have observed a small but significant leakage of nuclear antigens out of the nucleus after lesion of its envelope (Figs. 2 and 3). We believe that this discrepancy is due to the more sensitive method of detection used in this study.

Figure 5. Comparison of the distribution of antigen b6-3B7 with that of three other nuclear antigens. X. *laevis* oocytes were microinjected with nucleoplasm from X. *borealis* oocytes and fixed either immediately (*0 h*) or 1, 1.5, 3, 8, 19, and 44 h later in 2% TCA. Four neighboring sections in every oocyte were stained with mAbs b6-3B7 (directed against N1 in X. *borealis* oocytes), b6-6E7 (directed against N8), b7-2H4 (directed against N4), and b2-2B10 (directed against N1). Staining with mAb b7-2H4 44 h after injection is background (see text). Bar, 0.22 mm.
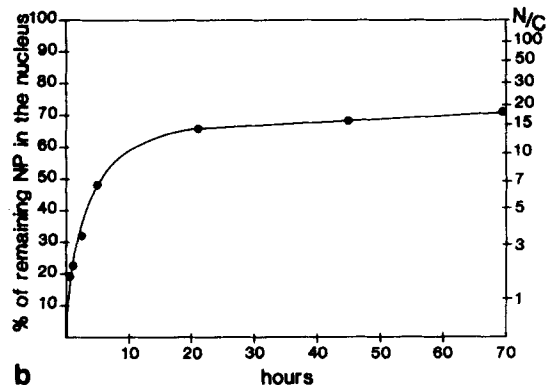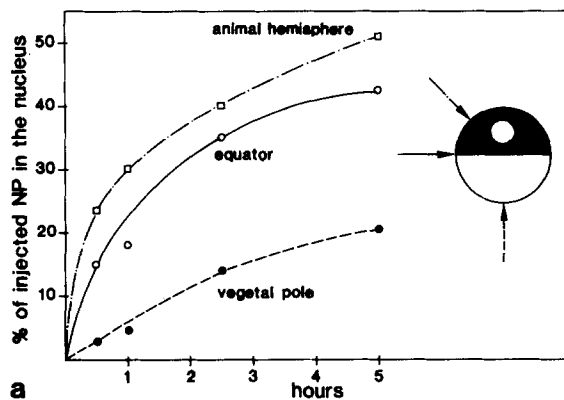
largement of the lesion in the nuclear envelope, the other nuclear proteins were detected in the cytoplasm as well.

The feasibility of preparing undiluted nucleoplasm, unaffected by aqueous medium, made possible experiments that further corroborate the result that nuclear proteins are not retained by the nucleoplasm alone. This could be shown for nucleoplasmin, N1, N4, and N8, which are all highly enriched in the nucleus in vivo.

Consequently, the mechanism of accumulation must be associated with the nuclear envelope. This is clearly illustrated in Fig. 4, where it is shown that X. borealis-specific N1 leaks out of the injected nucleoplasm and is accumulated in the host nucleoplasm surrounded by the nuclear envelope. In accordance with this idea, puncturing of the host nucleus impairs its ability to accumulate the protein (Fig. 4, P19 h). Also, the total loss of nuclear proteins from punctured nuclei (Fig. 2) must be low, since the intact parts of the envelope may counteract leakage by transport back into the nucleus.

Because the other four nuclear proteins disappear from the host cytoplasm within the same period, we assume that these proteins are accumulated in the intact nucleus like X. borealis N1 and are not simply degraded. This could be confirmed in the case of nucleoplasmin by injection of radioactively labeled protein (Fig. 6).

Dingwall et al. (1982) have previously injected purified iodinated nucleoplasmin into the cytoplasm of Xenopus oocytes and have also measured its accumulation in the nucleus. Although there is agreement that injected nucleoplasmin is accumulated in the nucleus, widely differing values on its rate of accumulation and on the concentration ratios obtained are found in the literature (Dingwall et al., 1982, 1986; Paine, 1982).

We have found that half of the amount of the injected nucleoplasmin is accumulated in the nucleus after 5 h and that the uppermost nuclear-to-cytoplasmic concentration ratio is 30. This value is at least 20-fold lower and the uptake rate 10 times slower than the values calculated by Dingwall et al. (1982). In a more recent publication Dingwall et al. (1986) obtained lower values, more in accordance with our data. Probably these discrepancies can be accounted for by differences in the methods applied and by different assumptions made. For example, we have found that the site of injection has a significant influence on the rate of accumulation observed (see Fig. 6 a). Thus different injection sites in our study and that of Dingwall et al. (1982) could explain the apparent difference in accumulation rates. Another possible explanation for discepancies is that we purified nucleoplasmin from oocytes, while Dingwall et al. (1982) used nucleoplasmin from eggs. Sealy et al. (1986) showed that nucleoplasmin from eggs is phosphorylated to a higher degree as compared to nucleoplasmin from oocytes.

We found that a substantial amount of the injected nucleoplasmin is contained in the pellet of the centrifuged homogenate of enucleated oocytes. Therefore, we isolated the nuclei from fixed oocytes and dissolved the total enucleated oocyte in sample buffer for gel electrophoresis. Furthermore, we counted the actual radioactivity of the nucleoplasmin bands in the dried polyacrylamide gels, whereas Dingwall et al. (1982) carried out quantitative autoradiography by densitometry. In addition, the above mentioned authors did not exclude the yolk volume in their calculation of the nuclear-to-cytoplasmic concentration ratio (N/C). This results in a

Figure 6. Nuclear uptake of purified nucleoplasmin after microinjection into the cytoplasm of X. laevis oocytes. (a) Dependence of nuclear accumulation of nucleoplasmin on site of injection. Oocytes were injected with ∿2.5 ng of iodinated protein either ∿45° from the animal pole (as shown in the inset), into the equator, or into the vegetal pole, and incubated in MBS-H at 20°C. The percentage of radioactivity found in the nucleus, relative to the total amount injected, is plotted against time after injection. (b) Nuclear accumulation of nucleoplasmin during 3 d of incubation. Oocytes were injected with ∿2.5 ng of iodinated protein into the equator. The nuclear-to-cytoplasmic concentration ratio (N/C) of the iodinated protein was calculated from the formula (% cpm nuclear/12)/(% cpm cytoplasmic/88), assuming that the nucleus contains 12% of the accessible aqueous volume of an oocyte (Bonner, 1975a). The percentage of the total radioactivity remaining in the oocyte, which is found in the nucleus, is plotted on the left. Note that the N/C ratio remains approximately constant after 24 h, despite degradation of injected nucleoplasmin (see text for details). NP, nucleoplasmin.

In view of the fact that the injured surface of the punctured nuclear envelope amounts to ∿1.4% (60 holes, each 12 μm in diameter, in a nucleus with a 0.4-mm diameter), the loss of proteins from the nucleus is probably too small to be detected by the method of Feldherr and Pomerantz (1978). This view is supported by our observation that only nucleoplasmin could be detected in the cytoplasm of punctured oocytes. This is probably a result of the abundance of nucleoplasmin relative to other nuclear proteins (Mills et al., 1980; Krohne and Franke, 1980; see also Fig. 1). This probably leads to cytoplasmic concentrations of these other nuclear proteins too low to be detected by immunofluorescence. After en-

threefold higher ratio compared with our calculation, which takes only the accessible cytoplasm into account (Bonner, 1975a).

Although the nucleocytoplasmic distribution ratio of injected nucleoplasmin remains constant after ~24 h after injection (Fig. 6 b), the total amount of nucleoplasmin decreases with time. The half-life of injected labeled nucleoplasmin in the oocyte was determined to be ~30 h (data not shown). This half-life is probably shorter than that of intrinsic nucleoplasmin, since nucleoplasmin is not significantly labeled after overnight incubation of oocytes with radioactive amino acids (De Robertis et al., 1978; Dreyer and Hausen, 1983). Conceivably, labeling with Bolton and Hunter reagent confers conformational changes that make the molecule more prone to degradation.

The methods we used to investigate the role of the nuclear envelope in protein accumulation aimed at either partial or total removal of the nuclear envelope. The required operations may have disturbed the structural integrity of the nucleoplasm. Mechanical injury of the nucleoplasm is unlikely to change its affinity for binding of nuclear proteins. However, we cannot rule out the possibility that contamination of the nucleoplasm with the incubation medium during puncturing or partial removal of the nucleus may change the binding properties of the nucleoplasm (Paine et al., 1983). For this reason, nuclei were isolated and demembranated under oil in order to avoid all contact of the isolated nucleoplasm with aqueous media. However, after injection the demembranated nucleoplasm will necessarily have direct contact with the cytoplasm of the oocyte. Thus, the influence of hypothetical factors which normally would be excluded from the nucleoplasm by the nuclear envelope and which could be able to change the binding properties of the nucleoplasm cannot, in principle, be ruled out. Since actin is a prevalent protein in the oocyte nucleus (Clark and Merriam, 1977), it might be possible that actin-binding factors which are uniquely located in the cytoplasm change the properties of nuclear actin, if the nucleoplasm is not protected by a nuclear envelope. An influence of ions on hypothetical interactions between nuclear proteins and nucleoplasm seems to be improbable, since the envelope of the oocyte nucleus is freely permeable to small solutes (Paine et al., 1975). Also exposure of nuclear proteins to cytoplasmic proteolytic enzymes can be neglected. This is evident from investigations by Dreyer and Hausen (1983), who found that the maternal pool of nuclear proteins persists in the developing *Xenopus* embryo, although the nuclear proteins are dispersed in the cytoplasm after the germinal vesicle breakdown.

Schulz and Peters (1986) have injected labeled nucleoplasmin into hepatoma tissue culture cells and have measured the diffusion coefficient in both the nucleus and the cytoplasm. After accumulation in the nucleus, the mobility of the protein was not significantly different from that in the cytoplasm, suggesting that accumulation of nucleoplasmin does not involve binding to nuclear structures, at least in hepatocytes. A similar conclusion was drawn by Newmeyer et al. (1986a, b), who found that accumulation of nucleoplasmin in isolated rat liver nuclei in vitro requires an intact nuclear envelope and does not involve intranuclear binding.

Since no direct evidence for the existence of nucleoplasmic binding properties leading to retention of nuclear proteins in the nucleus has been presented so far, our results lead us to conclude that the presence of an intact nuclear envelope is required for accumulation and retention of nuclear proteins.

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