Mechanism for the Selection of Nuclear Polypeptides in *Xenopus* Oocytes
II. Two-dimensional Gel Analysis

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ABSTRACT The role of the nuclear envelope in controlling intracellular protein exchanges was investigated in vivo, by determining the effect of altering nuclear permeability on (*a*) the protein composition of the nucleoplasm and (*b*) the nuclear uptake rates of specific endogenous proteins. The nuclear envelopes were disrupted by puncturing oocytes in the region of the germinal vesicle by use of glass needles. Nuclear proteins were analyzed in punctured and control cells by two-dimensional gel electrophoresis, fluorography, and double-labeling techniques. Over 300 nuclear polypeptides were identified in the fluorographs. Of this number, only ~10–15 were found to vary between punctured and control nuclei; furthermore, different polypeptides varied in each experiment. These qualitative studies indicate that specific binding within the nucleoplasm, and not selection by the envelope, is the main factor in maintaining the protein composition of the nucleus. The nuclear uptake rates of five individual polypeptides, ranging in molecular weight from 43,000 to 100,000, were analyzed by use of double-labeling procedures. Only one of the polypeptides (actin) entered the nuclei more rapidly after disruption of the envelope. That the nuclear uptake of certain endogenous proteins is unaffected by puncturing demonstrates that passage across the envelope is not a rate-limiting step in the nucleocytoplasmic exchange of these molecules.

Studies of nucleocytoplasmic exchanges in oocytes have shown that specific endogenous polypeptides accumulate in the nucleoplasm to a greater degree and at a greater rate than exogenous molecules of comparable size (1–3, 9, 10). In a previous study (10), the function of the nuclear envelope in regulating endogenous protein exchange was investigated by altering the permeability of the envelope and determining the effect of the procedure on nuclear protein uptake. In these experiments, *Xenopus* oocytes were pulse-labeled with L-[^{3}H]-leucine and then punctured, using fine glass needles, to alter the physical properties of the envelope. The nuclear uptake of labeled polypeptides from the cytoplasmic pool was compared, at various intervals, in punctured and control oocytes. At the appropriate times, isolated nuclei were analyzed on one-dimensional SDS-polyacrylamide gels. No differences were observed in either the Coomassie Blue–staining patterns or in the relative uptake rates of different size classes of endogenous nuclear polypeptides as determined by measuring radioactivity in gel slices.

It was concluded that the accumulation of specific polypeptides by the nucleus is not regulated by the envelope but rather by selective binding within the nucleoplasm. The inability to detect differences in the nuclear uptake rates of endogenous proteins in punctured cells indicates that passage across the envelope is not a limiting step in the translocation of these molecules.

It should be emphasized that the results described above were obtained by use of one-dimensional gels and refer primarily to classes of polypeptides. In this study, similar puncture experiments were performed; however, high resolution two-dimensional gels were employed in conjunction with fluorographic and double-labeling procedures. These techniques permitted a more detailed evaluation of the function of the envelope in regulating the distribution of individual molecules. The specific objectives were as follows: First, to determine whether the puncture procedure causes qualitative changes in the nucleocytoplasmic distribution of proteins. Such changes could include a loss of nuclear proteins or the acquisition of cytoplasmic proteins that are not normally present in the nucleoplasm. Second, to determine the nuclear uptake rates of individual polypeptides in punctured and control oocytes. The proteins selected for quantitative analysis included both N and

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B proteins. N proteins, as defined by Bonner (2), are localized mainly in the nucleus, whereas B proteins are present in equivalent concentrations in the nucleus and cytoplasm.

The results indicate that there are no major, reproducible, qualitative changes after disruption of the envelope. With regard to quantitative changes, the nuclear uptake of three B proteins and two N proteins were investigated. Of these substances, only one (actin) entered the nucleoplasm more rapidly in the punctured cells.

MATERIALS AND METHODS

Xenopus laevis were maintained, and the ovaries were removed, as described previously (9). Late stage-5 and stage-6 oocytes (7) were manually defolliculated in amphibian Ringer's solution at room temperature (22°–24°C). To minimize the effect of variations that occur among different animals, all of the oocytes used in a given experiment were obtained from the same ovary.

Disruption of the Nuclear Envelope

The nuclear envelopes were disrupted, in vivo, by puncturing the oocytes 25–30 times in the region occupied by the germinal vesicle. To accomplish this, glass needles were used that had tip diameters of 1–2 μm and shaft diameters of ~12 μm. 20–30 s were required to puncture each oocyte.

Evidence that this procedure is effective in altering the properties of the envelope was presented in an earlier report (10). Extensive gaps were consistently found in the envelopes of punctured cells that were examined with the electron microscope. To determine whether the morphological changes were accompanied by permeability changes, ^{125}I-labeled bovine serum albumin (BSA) was microinjected into the cytoplasm of punctured and control oocytes; after 2 h, the nuclei were isolated and analyzed for labeled protein. It was found that puncturing resulted in an 11-fold increase in the nuclear uptake of BSA. It was also shown in this earlier study that (*a*) the envelopes were not repaired during the time-course of the experiments and (*b*) puncturing does not affect protein synthesis or result in a loss of proteins from the cytoplasmic pool.

Nuclear Isolation and Gel Procedures

Nuclei were manually isolated in intracellular medium (11) and immediately transferred to 85% ethanol. Because the interval between enucleation and fixation was <30 s, it is doubtful that the in vivo composition of the nuclei was appreciably altered by this procedure. After fixation, the envelopes, along with any contaminating cytoplasm, were removed from the nuclei with watchmaker's forceps. The remaining nucleoplasm was then drained of excess alcohol and analyzed on two-dimensional gels. The procedure described by O'Farrell and O'Farrell (17) was used with the following modifications: First, the lysis buffer contained half the concentration of ampholytes, as it was added to nearly dry material. Second, dithiothreitol (0.5%) was used as a reducing agent in place of mercaptoethanol. Third, 0.5% SDS was added to the samples before isoelectric focusing (21). Fourth, a 10% SDS running gel, overlaid with a 3% SDS stacking gel was used in the second dimension (10). These modifications did not change the gel patterns, but did give more reproducible results. Other methods of processing the nucleoplasm were tried; for example, rather than fixing the nuclei in alcohol, we froze them immediately on dry ice and then subjected them to nuclease digestion. It was anticipated that this approach would optimize recovery of the nuclear proteins, but the gel patterns were no different than those obtained for alcohol-fixed nuclei without nuclease digestion.

Phosphorylase *a* (94,000 mol wt), bovine serum albumin (68,000 mol wt), ovalbumin (43,000 mol wt) and cytochrome *c* (11,700 mol wt) were used as molecular weight standards.

Qualitative Studies

Qualitative studies were performed as follows: Oocytes were incubated in Ringer's solution containing 400 μCi/ml of L-[^{3}H]leucine (sp act, ~140 Ci/mmol; obtained from New England Nuclear, Boston, Mass., or Amersham Corp., Arlington Heights, Ill.) for 30 min. The cells were then rinsed in fresh medium and divided into an experimental and a control group. The experimental cells were punctured, and both groups were incubated in Ringer's solution for 2.5 h, at which time all of the oocytes were enucleated. The control and the experimental nuclei were run on separate two-dimensional gels, stained, and fluorographed as described by Laskey and Mills (14). 25 nuclei were run on each gel.

Quantitative Studies

Double-labeling procedures were employed to compare the nuclear uptake rates of specific endogenous polypeptides in punctured and control oocytes. These studies were performed with L-[^{3}H]leucine and L-[^{35}S]methionine. A group of oocytes was labeled for 1 h in L-[^{3}H]leucine (400 μCi/ml; sp act, 140 Ci/mmol), and then rinsed in fresh Ringer's solution containing 62.5 mg of cold leucine/liter. Half of the cells were punctured immediately after rinsing, and both the punctured and nonpunctured (control) cells were incubated in Ringer's containing cold leucine for specific periods before enucleation. Under these conditions, the incorporation of tritium into precipitable counts only occurs during the labeling period (9); therefore, temporal increases in nucleoplasmic counts after labeling can be taken as a measure of ^{3}H-labeled protein uptake from the original cytoplasmic pool. A second group of oocytes was incubated for 1 h in Ringer's containing 400 μCi/ml of L-[^{35}S]methionine (sp act, 850–1,200 Ci/mmol; obtained from New England Nuclear or Amersham Corp.). After rinsing, the ^{35}S-labeled cells were immediately enucleated. These nuclei were added to each group of L-[^{3}H]leucine-labeled nuclei before running the gels, and thus served as internal standards. Equal numbers of ^{35}S- and ^{3}H-labeled nuclei were run on each gel. The total number of nuclei per gel ranged from 40 to 50.

After electrophoresis was completed, the double-labeled, two-dimensional gels were stained with Coomassie Blue (9) and the appropriate spots were cut out. The material was then extracted in 16 ml of scintillation fluid (9), and the radioactivity was measured in a model 6892 liquid scintillation counter (Tracor Analytic, Elk Grove Village, Ill.). After correcting for background and spillover, the ^{3}H:^{35}S ratios were determined for the individual polypeptides.

The time intervals used in the above quantitative experiments were derived from the normal nuclear uptake curves of the endogenous polypeptides that were selected for analysis. These data were also obtained by use of double-labeling procedures. In this case, the oocytes in each time group were labeled with L-[^{3}H]leucine and the nuclei were electrophoresed along with ^{35}S-labeled "standard" nuclei.

Considering that the present experiments were of relatively short duration, it is doubtful that reuse of label because of protein turnover was a significant factor, because the average half-life of *Xenopus* oocyte proteins is ~73 h (20).

RESULTS

Qualitative Changes

Fig. 1 shows a two-dimensional, Coomassie Blue–stained gel obtained for normal *Xenopus* oocyte nuclei. Usually, >150 polypeptides were identified in these preparations. Approximately twice as many polypeptides, including all of those observed in the stained gels, could be detected in fluorographs prepared from L-[^{3}H]leucine-labeled nuclei. For this reason, the latter technique was used to evaluate qualitative changes in punctured cells.

Fig. 2 *a* and *b* are fluorographs of control and punctured nuclei, respectively. In this experiment, nine of the polypeptides present in the punctured specimens were absent from the controls, whereas four control polypeptides could not be identified in the disrupted nuclei. These differences represent only ~4% of the total number of polypeptides; furthermore, the specific changes in the gel patterns were not reproducible. In other experiments, approximately the same number of variations were detected, but different polypeptides were involved.

Quantitative Changes

Polypeptides were selected for quantitative studies if they (*a*) were routinely present in the nucleoplasm and readily identifiable in stained gels, (*b*) consistently incorporated sufficient amounts of L-[^{3}H]leucine and L-[^{35}S]methionine for analysis, and (*c*) had molecular weights greater than ~20,000. One would expect, from studies with exogenous tracers, that molecules in this size range would be restricted by the envelope, assuming that they entered the nuclei by passive diffusion (see Discussion).

Five polypeptides were identified that fit the above criteria.

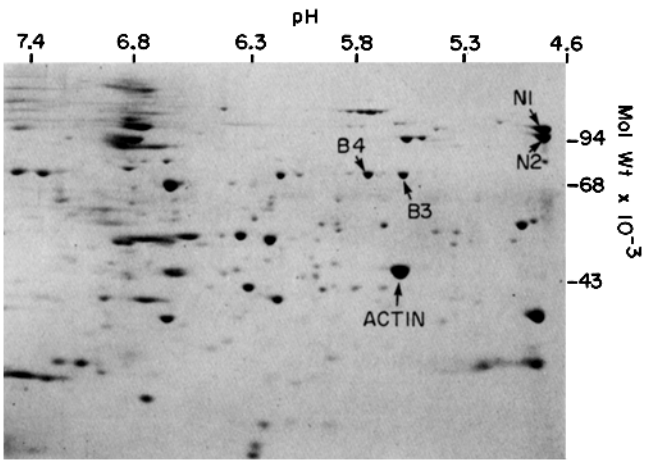

FIGURE 1 A two-dimensional gel showing the distribution of nuclear proteins in nonpunctured oocytes. The preparation was stained with Coomassie Blue. The polypeptides that were selected for quantitative studies are indicated by arrows.

They are indicated by arrows in Fig. 1, and include two N proteins (N1 and N2) and three B proteins (B3, B4, and actin). The designation of the molecules as N or B proteins is based on their similarities to polypeptides that were previously classified by DeRobertis et al. (6). This was verified in the present study by analyzing oocyte cytoplasm on two-dimensional gels. These gels (not shown) contained polypeptides that comigrated with B3, B4, and actin, but not with N1 and N2.

Actin was localized in the gels by identifying the polypeptide that coelectrophoresed with purified actin. The actin standard was prepared from oocyte homogenates by use of DNase I-affinity chromatography. The procedure was essentially the same as that described by Clark and Merriam (4).

Table I shows ^{3}H and ^{35}S counts that were obtained for individual polypeptides in a typical experiment. In this instance, the oocytes that were incubated in L-[^{3}H]leucine were not punctured and were enucleated 1 h after labeling.

Before studying the effects of the puncture procedure on nuclear protein uptake, it was necessary to determine the normal uptake rates of the five polypeptides to be analyzed. Two such experiments were performed by use of double-labeling procedures; in the first, nuclear uptake was determined 1, 3, and 6 h after labeling, and in the second, uptake was determined after 6, 9, 12, and 24 h. In addition, ^{3}H:^{35}S ratios were determined, for all of the polypeptides, immediately after labeling (0 time). The results were normalized by dividing the ratios obtained at each of the above time-points by those obtained at 0 time; thus, the starting point for the uptake determinations corresponded to the end of the labeling period. Fig. 3 *a* and *b* illustrates the relative uptake rates (i.e., adjusted ^{3}H:^{35}S ratios) for periods extending from 0–6 h and 6–24 h, respectively. The approximate equilibrium times were 1–3 h for B3 and B4, 6–9 h for actin, and 9–12 h for N1 and N2. The differences between the 6-h values in the two experiments reflect variations that normally occur among different animals.

Based on the data shown in Fig. 3, puncture experiments were performed in which the ^{3}H-labeled experimental and control oocytes were enucleated at intervals of either 1 or 2 h after labeling, that is, before the polypeptides reached equilibrium—the possible exceptions being B3 and B4, which might have equilibrated in the 2-hour studies. The results of one 1-h and three 2-h uptake experiments are given in Table II. The data are expressed as the ^{3}H:^{35}S puncture ratios divided by the ^{3}H:^{35}S control ratios. To estimate the experimental error, 2-h sham experiments were carried out in which two groups of nonpunctured oocytes were compared. It was found that an error of ±0.2 U could be expected in the final puncture:control ratios. Actin was the only protein that consistently had a puncture:control ratio that was significantly greater than one. The nuclear uptake rates of the other polypeptides were apparently unaffected by the puncture procedure.

It is possible that puncturing the oocytes has a much greater effect on nuclear uptake than is indicated by the above experiments. For example, marked increases in protein uptake could

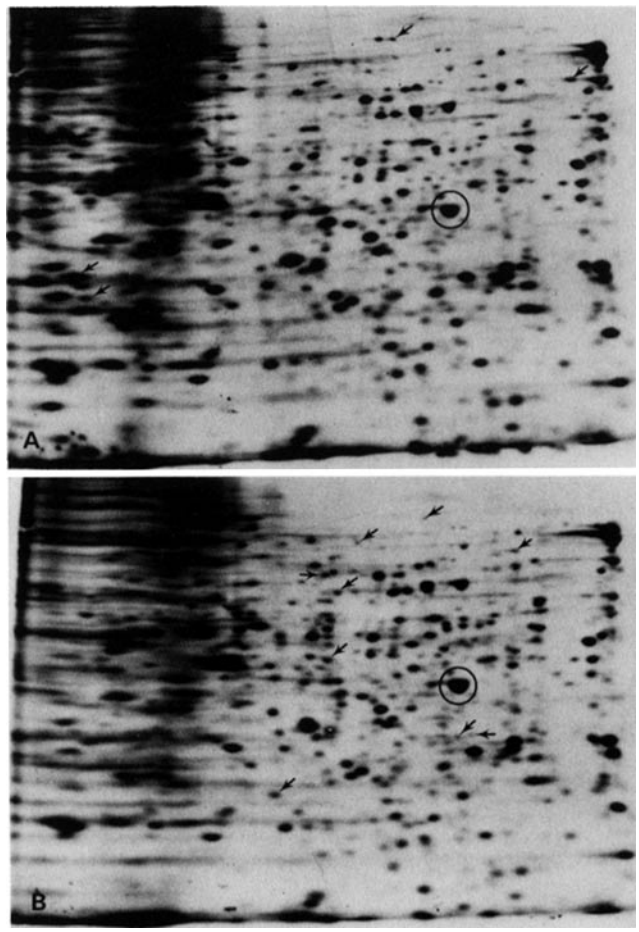

FIGURE 2 Fluorographic analysis of L-[^{3}H]leucine-labeled proteins in control (*A*) and punctured (*B*) nuclei. The differences in the polypeptide patterns are indicated by arrows. The orientation of the gels is the same as in Fig. 1. The actin spots are circled.

TABLE I

Representative ^{3}H and ^{35}S Counts for Individual Polypeptides

Protein	^{3}H	^{35}S
	cpm	*cpm*
N1	749	783
N2	779	398
B3	318	170
B4	279	160
Actin	294	388

20 ^{3}H-labeled and 20 ^{35}S-labeled nuclei were used in this study.

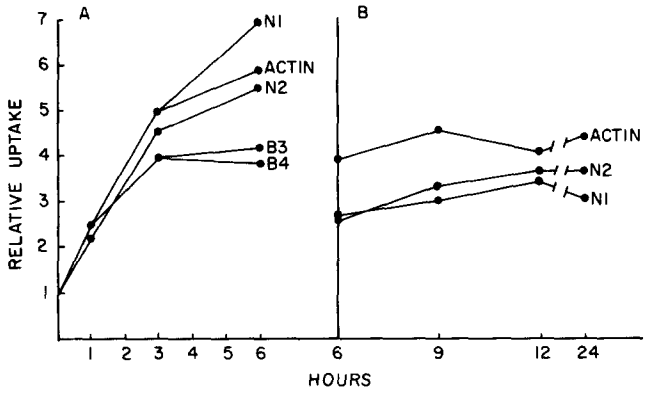

FIGURE 3 Nuclear uptake kinetics of selected polypeptides. The results of 0- to 6-h (*A*) and 6- to 24-h (*B*) experiments are shown. Relative uptake was determined by dividing the ^{3}H:^{35}S ratios that were obtained at each time-point by similar ratios obtained immediately after labeling (0 time).

be masked by accompanying increases in protein efflux, with the net change coincidently corresponding to the control values. To test this possibility, oocytes were labeled with L-[^{3}H]leucine for 1 h and then incubated for 9 h in Ringer's solution containing cold leucine. By this time the nuclear polypeptides should be at, or very near, equilibrium. After 9 h, half of the oocytes were punctured,and 2 h later, the punctured and control cells were enucleated. The nuclei were analyzed on two-dimensional gels along with ^{35}S-labeled standards. The results, which are given in Table II, show that altering nuclear permeability does not produce changes in protein efflux that would effect the interpretation of the uptake studies.

DISCUSSION

The qualitative, fluorographic studies show that disruption of the nuclear envelope does not cause major shifts in the nucleocytoplasmic distribution of proteins. The changes that were observed involved only ~4% of the polypeptides and were not reproducible. These differences could be attributable, in part, to procedural variations.

Quantitative studies were performed to evaluate the role of the envelope in regulating intracellular exchanges of specific endogenous polypeptides. The molecules that were selected for analysis covered a range of molecular weights and varied in their intracellular distribution patterns. N1 and N2 were the largest polypeptides to be analyzed. Their molecular weights, as determined in this study, were ~100,000 and 90,000, respectively; somewhat higher values were reported by DeRobertis et al. (6). It was estimated that these proteins are ~120 times more concentrated in the nucleus than in the cytoplasm (6). That they accumulate during oogenesis suggests that they might be stored for use during the early stages of development. Both B3 and B4 have molecular weights of ~70,000, and can readily be identified in the cytoplasm as well as the nucleus. The function of these proteins is not known. In *Xenopus* oocytes, actin (43,000 mol wt) makes up ~8.8% of the total oocyte protein (16) and 6% of the nuclear protein (4). It has been suggested that actin serves in maintaining the gel-like properties of the nucleoplasm (5).

Although the puncture procedure resulted in an average increase of ~72% in the rate at which actin was incorporated into the nucleoplasm, none of the other proteins were affected. The significance of this is best understood if the results are considered in relation to the nuclear uptake of exogenous tracers. Paine et al. (19) studied the nucleocytoplasmic exchange kinetics of three tritiated dextran fractions that had hydrodynamic radii of 12.0, 23.3, and 35.5 Å. The dextrans were microinjected into amphibian oocytes, and their subsequent uptake by the nuclei was followed by use of low temperature radioautographic techniques. The time required for the 12.0 and 23.3 Å dextrans to equilibrate between the nucleus and cytoplasm was 0.5 and 15 h, respectively. The nuclear: cytoplasmic ratio of the 35.5-Å fraction was only 0.1 after 23 h, at which time the experiment was terminated. These data support the view that the envelope restricts the movement of exogenous substances between the cytoplasm and nucleus, and that the degree of restriction is a function of molecular size. It was determined by Paine et al. (19) that the observed uptake rates are best explained by passive diffusion through nuclear pores having effective radii of 45 Å. A number of other exogenous tracers have been used to study nuclear permeability in oocytes. These include inulin (13), a variety of ^{125}I-labeled and fluorescein-labeled proteins (1, 18), ferritin (12, 18), and colloidal particles (8). The results are consistent with those obtained using dextrans; for example, 24 h after ^{125}I-BSA (68,000 mol wt; radius, 35 Å) was injected into oocytes, it was still five times more concentrated in the cytoplasm than in the nucleus (1).

If endogenous proteins are similarly restricted by the envelope, then puncturing the nuclei should result in a significant increase in their exchange rates, as is the case for BSA (10). This would be especially true for polypeptides with molecular weights >20,000, because diffusion of molecules in this size range is greatly restricted by the envelope (18, 19). That the nuclear uptake of certain endogenous polypeptides is not affected by puncturing demonstrates that the envelope is not a rate-limiting barrier to these substances.

The observed differences in the function of the envelope in regulating endogenous and exogenous protein exchanges can be explained in one of two ways: First, it is possible that the permeability of the envelope to both classes of molecules is basically the same, but that the rate-limiting process for certain endogenous molecules is related to the number and affinity of nucleoplasmic and cytoplasmic binding sites. Second, mechanisms might exist by which the passage of endogenous molecules across the envelope is, in some way, facilitated. Such mechanisms could include (*a*) specific transport through the pore or (*b*) passage directly across the membranous regions of the envelope, as occurs, for example, in mitochondria (15).

Overall, the qualitative data presented in this study support our earlier conclusion (10) that the accumulation of specific endogenous proteins within the nucleoplasm is attributable

TABLE II

Effect of Puncturing on the Uptake and Efflux of Nuclear Proteins

	Uptake*				
Protein	1 h	2 h	2 h	2 h	Efflux
N1	1.1	1.0	0.8	0.9	0.9
N2	1.3	1.2	0.9	0.9	1.0
B3	1.1	—	1.0	1.2	1.1
B4	1.2	—	1.1	1.2	1.0
Actin	1.8	2.2	1.4	1.5	1.0

The results are expressed as the ^{3}H:^{35}S puncture ratio divided by the control ratio. The experimental error is ±0.2.

* Three separate 2-h experiments were performed.

largely to selective binding and is not regulated by the envelope. In addition, analysis of the nuclear uptake rates of several endogenous polypeptides in punctured and control cells has demonstrated that passage across the nuclear envelope is not necessarily a rate-limiting step for these molecules.

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