MECHANISM FOR THE SELECTION OF NUCLEAR POLYPEPTIDES IN *XENOPUS* OOCYTES

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ABSTRACT

The function of the nuclear envelope in regulating the cellular distribution of proteins was studied by experimentally altering nuclear permeability and determining the effect of the procedure on the incorporation of exogenous and endogenous polypeptides into the nucleoplasm. Using fine glass needles, nuclear envelopes were disrupted by puncturing oocytes in that region of the animal pole occupied by the germinal vesicle. This resulted in a highly significant increase in the nuclear uptake of cytoplasmically injected [^{125}I]-bovine serum albumin ([^{125}I]BSA), demonstrating that the envelopes had lost their capacity to act as effective barriers to the diffusion of macromolecules.

Endogenous proteins were labeled by incubating oocytes in L-[^{3}H]leucine. After appropriate intervals, nuclei were isolated from punctured and control cells and analyzed for tritiated polypeptides. Both total precipitable counts and the proportion of label in different size classes of polypeptides were compared. The results showed that puncturing the oocytes had no apparent quantitative or qualitative effects on the uptake of endogenous polypeptides by the nuclei. It can be concluded that the accumulation of specific nuclear proteins is not controlled by the envelope but rather by selective binding within the nucleoplasm.

KEY WORDS nuclear envelope · permeability · nuclear proteins · nucleoplasm · oocytes

A number of investigators have studied the permeability of the nuclear envelope to macromolecules by injecting exogenous substances into the cytoplasm of intact cells and following their subsequent uptake into the nucleoplasm. Experiments of this type have been performed on amoebae and oocytes using ferritin (4, 19), colloidal gold (9), fluorescein, or ^{125}I-labeled proteins (1, 14, 19) and tritiated dextrans (20) as tracers. The results show that molecular size is a major factor in determining whether a given substance will enter the nucleus. The maximum size particle capable of penetrating the nuclear envelope is ~90 Å in amphibian oocytes (20) and 125 Å in *A. proteus* (5); however, these values may vary during the cell cycle (6). There is evidence that the channels through which macromolecular exchanges occur are located in the central regions of the nuclear pores (5). The fact that a variety of chemically different exogenous materials can enter the nucleus, provided that they do not exceed a certain size, suggests that the nuclear envelope is not a highly selective barrier and may not play a major role in regulating nucleocytoplasmic exchanges.

In two recent investigations, the nuclear

uptake of endogenous proteins was studied using *Xenopus* oocytes. Bonner (2) identified three classes of oocyte proteins based on their nucleocytoplasmic distribution. One class, designated N proteins, accumulates in the nucleus and contains polypeptides with mol wt up to 130,000. The second class, C proteins, are located predominantly in the cytoplasm. B proteins, which make up the third class, are equally distributed between the nucleus and cytoplasm. Feldherr (7) injected tritiated amino acids into the cytoplasm of oocytes and followed the nuclear uptake of labeled polypeptides, as a function of size, using sodium dodecyl sulfate (SDS)-polyacrylamide gels. It was found that large endogenous polypeptides, which are synthesized in the cytoplasm, enter the nuclei at considerably greater rates than exogenous tracers of comparable size. For example, on a volume basis, polypeptides with mol wt of 94,000 and above are approximately twice as concentrated in the nucleus than in the cytoplasm within 3 h after labeling. This is assuming that the nucleus occupies 4% of the total cell volume. On the other hand, Bonner (1) has shown that 24 h after injecting bovine serum albumin (68,000 mol wt) into oocytes, the nuclear concentration was <20% that of the cytoplasm. There are two likely explanations for the differences in uptake (7). First, it is possible that both classes of substances simply diffuse across the nuclear envelope, but endogenous polypeptides are bound within the nucleoplasm and thus accumulate more rapidly. It has been calculated that the observed results can be explained in this way if, at equilibrium, the endogenous polypeptides are about 10–30 times more concentrated in the nucleus than in the cytoplasm. Concentrations of nuclear proteins in this range have been reported previously for amoebae (17) and amphibian blastula (21). Furthermore, Bonner (2) demonstrated that *Xenopus* germinal vesicles accumulate specific N proteins up to 50 times the cytoplasmic level. The second possible explanation is that the nuclear envelope possesses specific transport systems which select and accumulate nuclear polypeptides.

The objective of this study was to determine the function of the envelope in regulating the cellular distribution of endogenous polypeptides in *Xenopus* oocytes. To accomplish this, nuclear envelopes were mechanically disrupted, thereby altering their capacity to restrict macromolecular exchanges. The effect of this procedure on the nuclear uptake of both nonspecific and endogenous proteins was then evaluated. Nonspecific uptake was studied by injecting oocytes with ^{125}I-labeled bovine serum albumin (BSA). The incorporation of endogenous polypeptides into the nuclei was investigated by incubating the cells in L-[^{3}H]leucine and subsequently determining the amount of precipitable label, as well as the size range of labeled polypeptides present in the nucleoplasm. The results indicate that the nuclear envelope does not have a major role in controlling the influx of nuclear proteins.

MATERIALS AND METHODS

Xenopus laevis were maintained in dechlorinated tap water and fed beef heart. Ovaries were removed surgically (12) and stored in sealed watch glasses at 4°C for no longer than 2 h. Small pieces of ovary were transferred to amphibian Ringer's solution (at 22°–24°C) as needed. Except where indicated, all experiments were performed in this solution. Late stage 5 and stage 6 oocytes (3) were dissected from their follicles using watchmaker's forceps. In each individual study, control and experimental cells were randomly selected from a pool of oocytes obtained from a single ovary. In this way the quantitative and qualitative variations which normally occur among different animals could be avoided.

Disruption of the Nuclear Envelope

Nuclear envelopes were disrupted by puncturing the cells with glass needles which had shaft diameters of ~12 μm and tip diameters of 1–2 μm. In most experiments the oocytes were punctured 25–30 times in that region of the animal pole occupied by the nucleus. The tips of the needles were inserted at least halfway through the cells, i.e., into the vegetal pole.

Nuclear Isolation

Oocyte nuclei were isolated by hand (11) in an intracellular medium consisting of 102 mM KCl, 11.1 mM NaCl, 7.2 mM K_2HPO_4, and 4.8 mM KH_2PO_4 (pH 7.0 ± 0.1), and immediately transferred to absolute ethanol. The entire procedure required <30 s, and it is unlikely that there was an appreciable loss of nuclear protein during this period. After fixation the nuclear envelopes and any contaminating cytoplasm were dissected off with watchmaker's forceps; thus, only substances localized in the nucleoplasm were analyzed (7, 11).

Electron Microscopy

Punctured and control oocytes were fixed in a trialdehyde solution, postfixed in OsO_4, dehydrated, and embedded according to the method of Kalt and Tandler (15). Thin sections were cut with a Porter Blum MT-1 ultramicrotome (DuPont Instruments–Sorvall, DuPont Co., Wilmington, Del.) and examined with a Siemens Elmiskop 101.

Preparation and Injection of [^{125}I]BSA

BSA (Calbiochem, San Diego, Calif.) was labeled with ^{125}I ($Na^{125}I$, 17 Ci/mg; New England Nuclear, Boston, Mass.) using a modification of the chloramine-T procedures described by Greenwood et al. (13). After labeling, the protein was (*a*) passed through a Sephadex G-15 column previously equilibrated with intracellular medium (see above), (*b*) dialyzed against intracellular medium, (*c*) concentrated with polyethylene glycol, and (*d*) redialyzed. The final BSA concentration was 0.05–0.1%, and over 99% of the ^{125}I could be recovered in an alcohol precipitable fraction. Both labeled and unlabeled BSA showed similar banding patterns when run on SDS-polyacrylamide gels. Measurements of radioactivity in gel slices indicated that ~99% of the ^{125}I was associated with polypeptides having mol wt of 68,000 or above.

Oocytes were injected with micropipettes calibrated to deliver 20 nl. The number of cpm contained in this volume of [^{125}I]BSA solution varied in different preparations but ranged from 25,000 to 30,000. After injection, radioactivity was measured in whole cells or isolated nuclei using a Beckman Radioimmuno Analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

Labeling and Counting Procedures for Endogenous Proteins

Endogenous proteins were labeled by incubating the oocytes in amphibian Ringer's solution containing 150 μCi/ml of L-[4,5-^3H]leucine (sp. act 60 Ci/mmol; New England Nuclear) for 25 min. Then the cells were rinsed several times in fresh Ringer's solution. There was no appreciable increase in precipitable counts beyond this point.

To determine the amount of L-[^3H]leucine incorporated into precipitable material, whole oocytes or isolated nuclei were extracted in 10% TCA for 20 h and 90 min, respectively, and then dissolved in 10 ml of scintillation fluid, consisting of 3.5% Protosol (New England Nuclear, Pilot Chemicals Div., Watertown, Mass.) and 0.4% Omnifluor (New England Nuclear) in toluene. Radioactivity was measured in either a Packard Tri-carb model 2425 (Packard Instrument Co., Inc., Downers Grove, Ill.) or a Beckman LS-100C liquid scintillation counter.

SDS-Polyacrylamide Gel Electrophoresis

Discontinuous SDS-polyacrylamide gels (3% stacking and 10% running gels) were employed to determine the amount of label in different size classes of polypeptides. Alcohol-fixed nuclei or whole cells (not extracted with TCA) were dissolved in sample buffer and run on a Bio-Rad vertical slab apparatus (model 220; Bio-Rad Laboratories, Richmond, Calif.) using a modification of the method outlined by Laemmli (16). After fixation and staining, the gels were sectioned transversely at 2-mm intervals. The slices were extracted in scintillation fluid (see above) and counted. A detailed description of the procedures, including solubilization of the samples, preparation of the gels, staining, and extraction are given in references 7 and 8. Phosphorylase *a* (mol wt 94,000), BSA (mol wt 68,000), ovalbumin (mol wt 43,000), and cytochrome *c* (mol wt 11,700) were used as molecular weight standards.

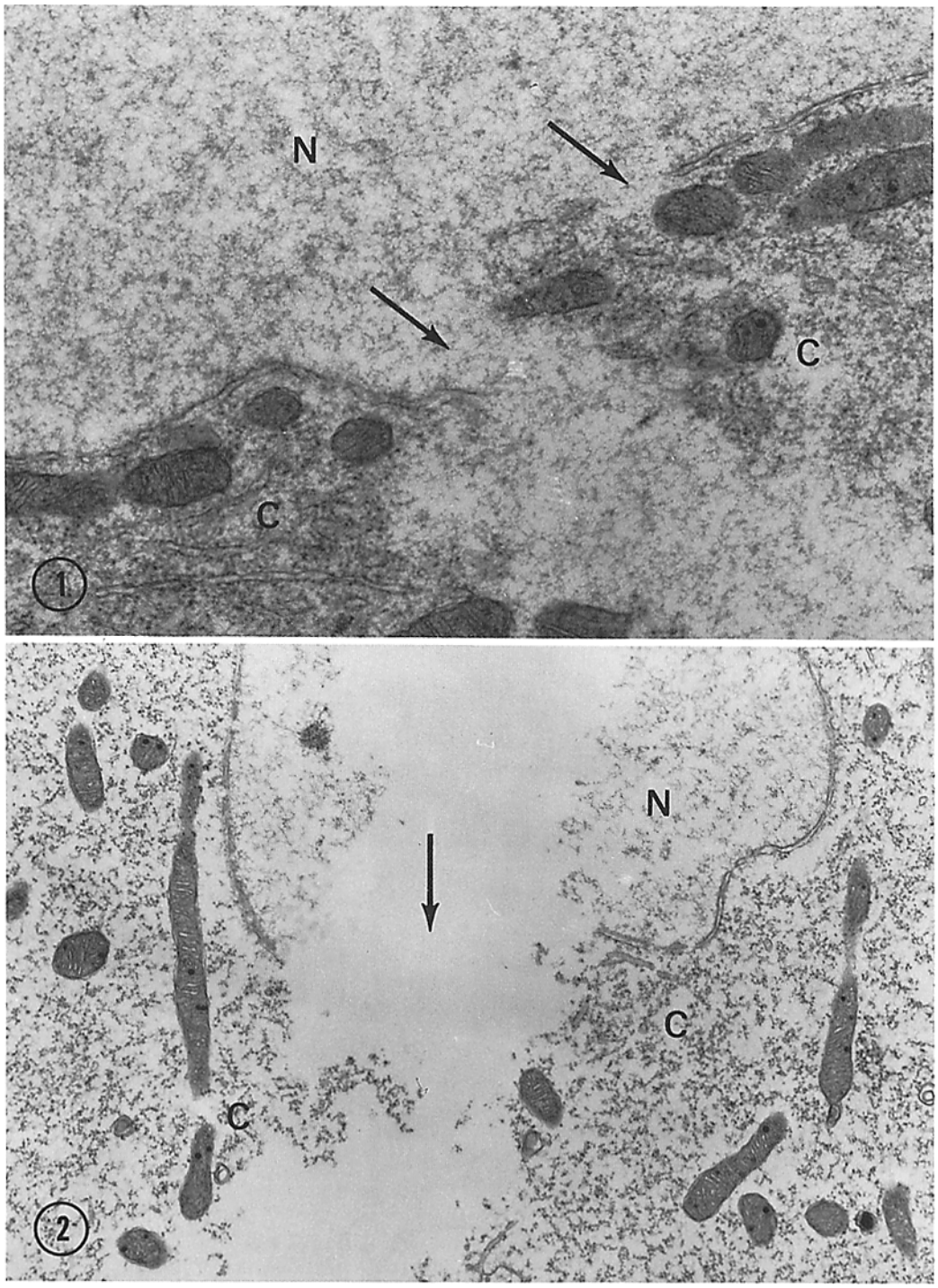
RESULTS

Electron Microscopy

Four oocytes were punctured 25–30 times and fixed for electron microscopy 2 1/2 h later. In all instances numerous breaks, up to several microns in diameter, were seen in the nuclear envelopes (Figs. 1 and 2). Similar gaps were not detected in the envelopes of control cells.

Nuclear Uptake of [^{125}I]BSA

Two experiments were designed to evaluate changes in the nuclear uptake of exogenous proteins resulting from mechanical disruption of the envelope. In one study the oocytes were injected with [^{125}I]BSA, punctured 20–30 min later, and enucleated 2 h after being punctured. In the second study the oocytes were punctured, injected with tracer after 2 h, and left in Ringer's solution for an additional 2 h before enucleation. The control cells for both experiments were injected with [^{125}I]BSA and enucleated after 2–2$^1/_2$ h. Table I gives the data obtained when oocytes were punctured 25–30 times. Nuclear uptake in both experimental groups was significantly greater than in the controls ($P << 0.001$); however, the experimental groups were not significantly different from each other ($P >> 0.2$), demonstrating that recovery does not occur within 2 h. Differences in uptake were also observed if the oocytes were punctured 5 times, but the increases were not as great and the results were more variable.



FIGURES 1 and 2 Electron micrographs of punctured cells showing disruptions (arrows) in the nuclear envelopes. *N*, nucleus; *C*, cytoplasm. Fig. 1, × 33,000; Fig. 2, × 18,000.

TABLE I

Nuclear Uptake of [^{125}I]BSA

	Control	Injected before puncture	Injected after puncture
Number of nuclei	32	25	9
Mean (SD) cpm	52 (±60)	578 (±148)	686 (±312)
% of Cytoplasmic concentration*	4.7	52.5	62.3

* Calculated on the basis that the nucleus occupies 4% of the total cell volume and that 27,500 cpm were injected.

Nuclear Uptake of Endogenous Polypeptides

Oocytes were incubated in L-[^{3}H]leucine, rinsed, and separated into an experimental and a control group. The experimental oocytes were punctured 25–30 times, and both groups of cells were enucleated either 30 min or 2½ h later. The differences in total precipitable counts incorporated by the nuclei of punctured and control cells were not significant. In 2½-h experiments, the means were 8,400 (± 2,730) and 9,100 (± 1,370), respectively. 10 nuclei were counted in each group.

SDS-polyacrylamide gel patterns for nuclei isolated from 2½-h punctured and control oocytes are shown in Fig. 3. There are no apparent differences in the stained bands. By analyzing SDS gel slices, the size distribution of the labeled polypeptides which were present in the nuclei 30 min. (Fig. 4) and 2½ h (Fig. 5*a* and *b*) after puncture were determined. The quantitative and qualitative differences seen in Fig. 5*a* and *b* can be explained by the fact that the two experiments were performed on different animals. Such variations are not unusual, and it was for this reason that oocytes used in any given experiment were taken from a single ovary. Variations between the patterns obtained for punctured and control cells in the individual experiments could easily have resulted from minor deviations in migration rates or slicing procedures. The overall differences between nuclear uptake in the 30-min and 2½-h studies, i.e., an increase in total counts and a change in the proportion of large molecular weight polypeptides, are consistent with earlier results (7). It has also been demonstrated previously that the polypeptides which are localized in the nucleoplasm are synthesized in the

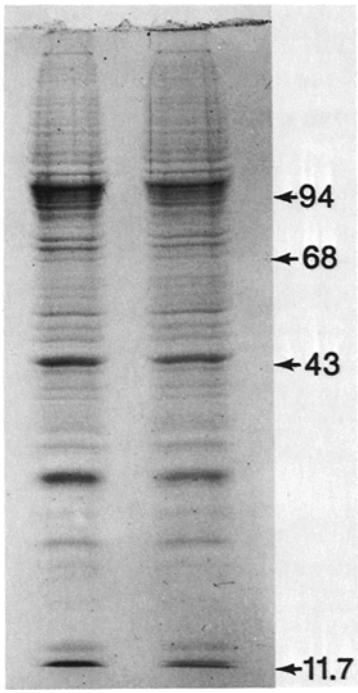


FIGURE 3 SDS-polyacrylamide gels stained with Coomassie Blue. The polypeptide-banding patterns for control (left) and punctured nuclei (right) are shown. The molecular weights (× 10^{-3}) are indicated by arrows.

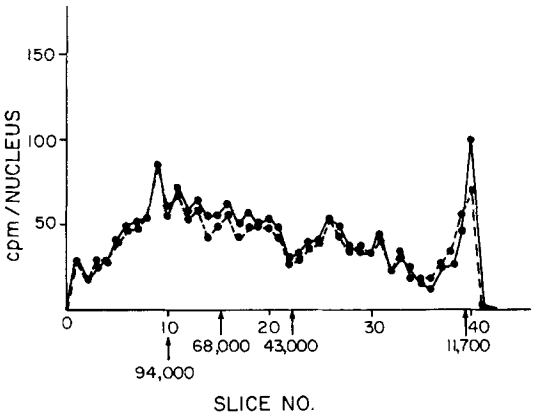


FIGURE 4 SDS-polyacrylamide gel patterns of L-[^{3}H]leucine-labeled polypeptides from 30-min punctured (—) and control (- - -) nuclei. The gels were loaded with 13 and 11 nuclei, respectively. The arrows indicate molecular weights.

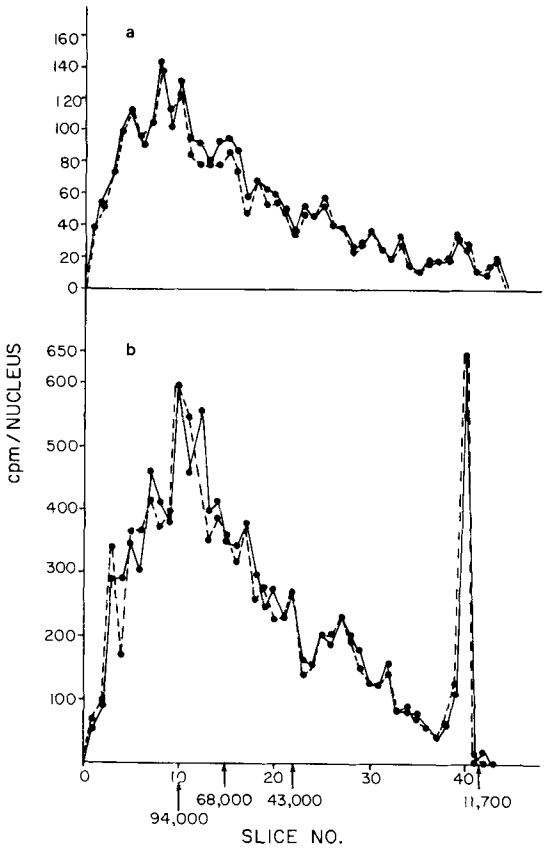


FIGURE 5*a* and *b* SDS gel patterns of labeled nuclear polypeptides from two separate 2 1/2-h experiments. 13 punctured (—) and 12 control (- - -) nuclei were used in Fig. 5*a*. 13 and 11 nuclei, respectively, were used in Fig. 5*b*. The arrows indicate molecular weights.

cytoplasm and must, in some way, penetrate the nuclear envelope (7, 10).

Whole Cell Incorporation of L-[^{3}H]leucine

Since the number of labeled molecules which will enter the nucleus during a given interval will depend on the cytoplasmic concentration of similar unlabeled molecules, experiments were undertaken to evaluate the effect of the puncturing procedure on cellular protein synthesis. 10 oocytes were punctured 25-30 times and 2 1/2 h later they were incubated in L-[^{3}H]leucine, rinsed, and immediately fixed in 10% TCA. The mean for total precipitable counts was 182,000 (± 52,000) cpm; a value of 181,400 (± 72,500) cpm was obtained for 10 control cells. A comparison of the size distribution of labeled polypeptides, as determined from slices of SDS-polyacrylamide gels, is shown in Fig. 6. There are no obvious qualitative differences in the polypeptide patterns. It should be noted that the number of counts recovered from the gels is less than the total TCA precipitable counts. This is most probably due, at least in part, to the fact that the oocytes were not completely solubilized by the sample buffer.

These results show that puncturing the cells does not alter protein synthesis during a time period equivalent to the duration of the nuclear uptake studies.

DISCUSSION

It is apparent that puncturing oocytes in the central region of the animal pole produces visible gaps in the nuclear envelope, which significantly alter its physical properties. Specifically, the envelope no longer acts as an effective barrier to the diffusion of exogenous proteins. This effect is not reversible, at least within the time-course of the experiments.

If the envelope functions as a selective barrier, involved in the accumulation and exclu-

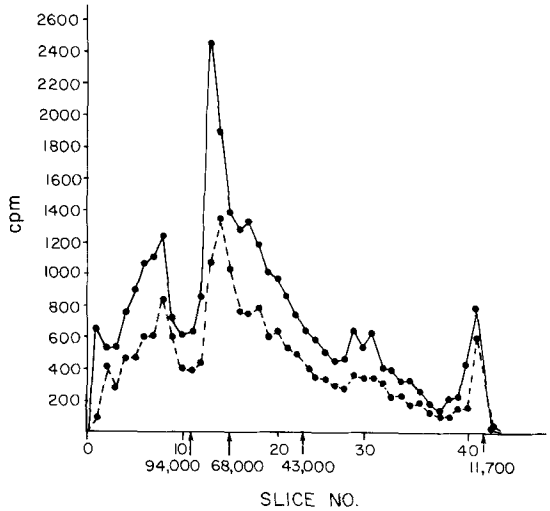


FIGURE 6 SDS gel patterns of labeled polypeptides present in whole oocytes. The experimental cells (—) were labeled 2 1/2 h after being punctured. (- - -), Controls. Five cells in each group were fixed in alcohol and dissolved in 250 μl of sample buffer; 30 μl of each sample were then run on SDS gels. The arrows indicate molecular weights.

sion of specific substances, mechanical disruption as extensive as that produced by puncturing the cells should have a detectable effect on the nuclear uptake of endogenous polypeptides. This was not the case; both the amount of precipitable label and the proportion of different size polypeptides incorporated into the nucleoplasm were the same in both experimental and control cells. Furthermore, the banding patterns of stained gels remain unchanged.

Despite the fact that the envelopes and associated material were removed before analysis, it is necessary to consider the possibility that the nucleoplasmic fractions may have been contaminated with cytoplasmic proteins. Evidence arguing against such contamination has been provided in an earlier report on *Xenopus* oocytes (7). It was demonstrated in that study that incorporation of cytoplasmically injected L-[^{3}H]leucine into TCA precipitable material is complete within 30 min. Furthermore, the SDS-polyacrylamide gel patterns obtained for labeled polypeptides were the same for whole cells examined either 30 min or 3 h after injection. Since protein synthesis does not occur in the nucleus, it can be concluded that the cytoplasmic pool of tritiated polypeptides is established within 30 min. If the observed nuclear counts and gel patterns were due primarily to contaminating cytoplasmic proteins, one would expect similar results in 30-min and 3-h experiments. This was not the case; there were fewer total nuclear counts after 30 min as compared to 3 h (767 vs. 5,560 cpm/nucleus), and there was a lower proportion of high molecular weight polypeptides in the nuclear fraction at the earlier time. These marked quantitative and qualitative differences cannot be explained simply in terms of cytoplasmic contaminants, but are most likely due to a time-dependent uptake of protein into the nucleoplasm.

It can be concluded from the present data that the observed accumulation of specific polypeptides within the nucleus is not regulated by the envelope but is dependent on the binding characteristics of the nucleoplasm. Merriam and Hill (18) have previously suggested that selective absorption to nuclear components may be responsible for the sequestration of unique nuclear polypeptides.

The data fit the hypothesis that endogenous polypeptides diffuse across the envelope and accumulate as a result of binding. As discussed in the introduction, nuclear concentrations of 10- to 30-fold, at equilibrium, would be necessary to account for the observed rates of incorporation of these substances. This calculation is based on the assumption that endogenous and exogenous materials having similar dimensions, permeate the envelope at comparable rates. If this explanation is correct, one would expect the nucleoplasmic-binding sites to be occupied more rapidly if the envelope is not able to function as an efficient diffusion barrier. There was no indication from the results of the 30-min or $2^1/_2$-h experiments that the uptake rates of nuclear polypeptides were greater in punctured cells. This suggests that passage across the envelope is not a limiting step in the nuclear incorporation of endogenous substances.

The proposal that the nuclear envelope is not primarily responsible for controlling the specificity of nuclear protein uptake in oocytes is based on an analysis of those polypeptides which can be detected using the above procedures. It is conceivable that some of the nuclear proteins were either lost during isolation or insoluble in the sample buffer. Until it can be established that the gels contain a complete spectrum of nuclear proteins, one cannot exclude the possibility that at least some polypeptides are selected by the envelope. It should also be emphasized that the present study is only concerned with the distribution of proteins; the envelope may have a more important role in the regulation of nucleic acid efflux in oocytes. In fact, future experiments are planned in which the experimental approaches described above will be used to study the nucleocytoplasmic translocation of RNA.

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