Movement of a Karyophilic Protein through the Nuclear Pores of Oocytes

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ABSTRACT It has recently been shown that large karyophilic proteins are transported across the nuclear envelope in amphibian oocytes. In consideration of this, the present experiments were performed to (*a*) identify the specific sites within the envelope through which transport occurs and (*b*) determine if molecular size is a limiting factor in the transport process. The following experimental procedure was employed: Colloidal gold particles, varying in size from ~20 to 170 Å in diameter were coated with nucleoplasmin, a 165,000-mol-wt karyophilic protein, which is known to be transported through the envelope. The coated gold particles were microinjected into the cytoplasm of *Xenopus* oocytes, and the cells were fixed 15 min and 1 h later. The intracellular localization of the gold was then determined with the electron microscope. It was found that nucleoplasmin-coated particles readily enter the nucleus. On the basis of the distribution of the particles associated with the envelope, we concluded that transport occurs through the nuclear pores. Furthermore, the size distributions of the gold particles present in the nucleus and cytoplasm were not significantly different, indicating that the envelope does not discriminate among particles with diameters ranging from 50 to 200 Å (the dimensions including the nucleoplasmin coat). Colloidal gold coated with trypsin-digested nucleoplasmin (which lacks the polypeptide domain required for transport) or exogenous polyvinylpyrrolidone were largely excluded from the nucleus and showed no evidence of transport.

Exogenous macromolecules appear to enter the nucleus by diffusing through central channels located within the nuclear pores (6). In amphibian oocytes it has been estimated that the channels available for passive diffusion are ~90 Å in diameter (20). The rate of diffusion through these regions is inversely related to the size of the permeating substance. For example, in oocytes, exogenous molecules with hydrodynamic radii of ~15 Å rapidly diffuse into the nucleus, whereas substances with radii of ~45 Å diffuse at extremely slow rates, if at all (20).

Extrapolating to endogenous molecules, it is reasonable to assume that small proteins enter the nucleus by passive diffusion through the pores (although some form of facilitated uptake cannot be excluded). On the other hand, large nuclear proteins, which have a limited capacity for diffusion through 90 Å pores, are presumably transported across the envelope. Evidence for transport has been obtained in two recent studies. Feldherr et al. (9) demonstrated that RN1, a 150,000-mol-wt nuclear protein found in *Rana pipiens* oocytes, enters the nucleus approximately 20 times faster than can be accounted for by diffusion through the pores. Dingwall et al. (3) showed that nucleoplasmin, a 165,000-mol-wt pentamer, is transported across the envelope. The transport of nucleoplasmin is prevented if a 12,000-mol-wt tail region, present on each of the monomeric subunits, is removed by protease digestion.

This report is concerned mainly with two questions relating to the transport process: First, does transport occur across the membranes of the envelope or through the pores? On the basis of earlier studies, we think the pores represent the most likely pathway; however, posttranslational exchange of endogenous proteins across cellular membranes is well documented (e.g., 21, 22) and should also be considered as a possible mechanism for nucleocytoplasmic exchanges. Second, does the envelope restrict the uptake of endogenous substances on the basis of molecular size?

To resolve these problems, we coated colloidal gold particles of various sizes with nucleoplasmin and microinjected them into the cytoplasm of *Xenopus* oocytes. The intracellular localization of the particles was subsequently determined using the electron microscope. Because nucleoplasmin is known to be transported, and because the gold particles

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acquire the properties of the coating material, this approach enabled us to visualize the transport process and also to determine the effect of particle size on nuclear uptake. It was found that the nucleoplasmin-coated gold particles rapidly entered the nuclei by passing through the centers of the pores. Furthermore, the envelope showed no sieving effect for particles ranging in diameter from ~50 to 200 Å. Markedly different results were obtained using particles coated with polyvinylpyrrolidone (PVP)[1] or trypsin-digested nucleoplasmin.

MATERIALS AND METHODS

Xenopus laevis were purchased from Xenopus I (Ann Arbor, Michigan) and maintained as reported previously (7).

Nucleoplasmin Isolation: *Xenopus* oocytes were used as a source of nucleoplasmin. For each preparation, ~30 ml of ovary was digested at room temperature for 75–90 min in 340 ml of calcium-free Ringer's solution containing 200 mg of collagenase (Type 1; Sigma Chemical Co., St. Louis, MO). The dissociated oocytes were rinsed six times with calcium-free Ringer's; 1 mM of phenylmethylsulfonyl fluoride was added to the final rinse solution. The oocytes were then homogenized, cleared by centrifugation, and extracted with 1,1,2-trichlorotrifluoroethane as described by Laskey et al. (15). Nucleoplasmin was isolated using DEAE-cellulose and phenyl sepharose column chromatography and $(NH_4)_2SO_4$ extraction as outlined by Dingwall et al. (3).

It was found that our preparations occasionally contained impurities that prevented nucleoplasmin from binding to the gold particles. These impurities were removed by alcohol extraction using the following procedure: the nucleoplasmin fraction that was eluted from phenyl sepharose (see reference 3) was dialyzed against 50 mM Tris-HCl (pH 7.5) and concentrated to 1 ml. Cold EtOH was then added to a final concentration of 80% and the preparation was kept at −20°C overnight. The precipitate, which contained the nucleoplasmin, was collected by centrifugation, rinsed with fresh 80% EtOH, and dried under vacuum. Generally, 150–200 μg of nucleoplasmin was obtained from 30 ml of ovary. Gel analysis was performed as described by Laemmli (14). The samples were not boiled before electrophoresis.

Trypsin Digestion of Nucleoplasmin: Nucleoplasmin was digested after phenyl sepharose chromatography. The eluted protein was dialyzed against Tris-HCl (as above), concentrated to 4 ml, and treated with 8 μg of trypsin (180 U/mg; Worthington Diagnostic Systems, Inc., Freehold, NJ) at 33°C for 1 h. The reaction was stopped by adding 96 μg of phenylmethylsulfonyl fluoride. The trypsin-resistant core protein was purified by batch adsorption to 200 μl of DEAE-cellulose and eluted with 500 mM NaCl containing 25 mM Tris-HCl (pH 7.5). The core protein was dialyzed against 50 mM Tris-HCl (pH 7.5) and extracted with 80% EtOH as described above.

Colloidal Gold Preparations: Colloidal gold sols were prepared by reducing chlorauric acid with an ether solution of phosphorus (6). The gold sols were then stabilized with nucleoplasmin, digested nucleoplasmin, PVP (mol wt 40,000) or cytosol. Stabilization involves the adsorption of the protective agent to the surface of the gold particles.

Before stabilizing with nucleoplasmin or digested nucleoplasmin, it was necessary to determine the volume of colloid that could be protected by a given protein preparation. This was accomplished by dissolving the lyophilized protein, obtained from 30 ml of ovary, in 1.6 ml of 10 mM potassium phosphate buffer (pH 7.0) and, using small aliquots, by determining the amount required to protect 0.5 ml of colloid from precipitating when 50 μl of 10% NaCl was added. By using this criterion we found that the nucleoplasmin preparations were generally able to stabilize 12–14 ml of colloid, whereas digested nucleoplasmin could protect ~6 ml. To stabilize with PVP, 100 μg of polymer was added for each milliliter of colloid.

Cytosol was prepared by high-speed centrifugation of homogenized oocytes as described by Laskey et al. (15). Although nucleoplasm was also present in this fraction, it is estimated that >90% was of cytoplasmic origin. 5 μl of cytosol was sufficient to protect 1 ml of colloid.

After stabilization, all of the gold preparations were centrifuged at 6,000 *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 26,000 *g* for 15 min. The resulting loose pellet was removed in as concentrated a form as possible. The yields ranged from 50 to 100 μl. Finally, the preparation was dialyzed against 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).

Injection Procedures: The injection experiments were performed on late stage 5 and stage 6 oocytes (4) which had been manually defolliculated in amphibian Ringer's solution. The oocytes were placed in hemispherical depressions in a paraffin chamber and centrifuged at 650 *g* for ~7 min. This results in the migration of the nucleus to a position just below the plasma membrane at the animal pole, where it can be easily seen (13). Approximately 50 nl of colloidal gold was then injected into the cytoplasm just adjacent to the nucleus. The tip diameters of the micropipettes ranged from 15 to 20 μm.

There is evidence that after injection into oocytes, nucleoplasmin, digested nucleoplasmin, and PVP remain in solution and are able to distribute throughout the cytoplasm (3, 11). It is assumed that injected gold particles coated with these substances behave similarly and, as a result, are accessible to the nucleus.

Electron Microscopy: The fixation procedures were similar to those reported by Kalt and Tandler (12). After initial fixation in aldehydes, the oocytes were bisected at the equator, and a portion of the animal hemisphere containing the injection site (which appeared red because of the high concentration of colloid) and the adjacent region of the nucleus were removed and postfixed in OsO_4. After OsO_4 fixation the blocks were stained with *p*-phenylenediamine as described by Ledingham and Simpson (16). The material was then dehydrated, embedded in Spurr's medium, sectioned and examined with a JEOL 100S electron microscope (JEOL USA, Cranford, NJ).

The intracellular distribution of the gold particles was determined from electron micrographs taken near the injection site. Counts were made from equal, randomly selected areas of adjacent nucleoplasm and cytoplasm. The same sampling procedure was used when particle measurements were made, except that the areas of nucleoplasm and cytoplasm were not necessarily equal.

Negative staining procedures were used to estimate the overall size of the particles (gold plus adsorbed material). Colloidal suspensions were air dried on Formvar-coated grids and stained with 1% uranyl acetate. To visualize the nucleoplasmin coat, it was necessary to stain the grids twice.

RESULTS

Gel Analysis of Purified Proteins

SDS polyacrylamide gels of isolated nucleoplasmin and trypsin-digested nucleoplasmin are shown in Fig. 1. The results are similar to those published by Dingwall et al. (3). The darkly staining bands located near the top of the nucleoplasmin lane presumably represent the pentameric and tetrameric forms, whereas the lower band has an apparent molecular weight (~30,000) equivalent to that of the monomeric subunit. Digested nucleoplasmin runs as a single band with an apparent molecular weight of ~80,000.

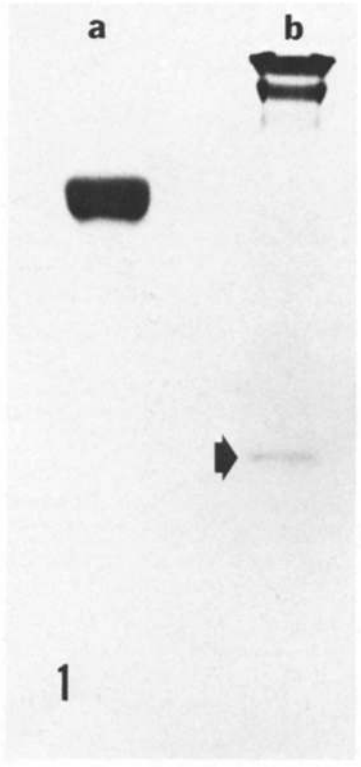
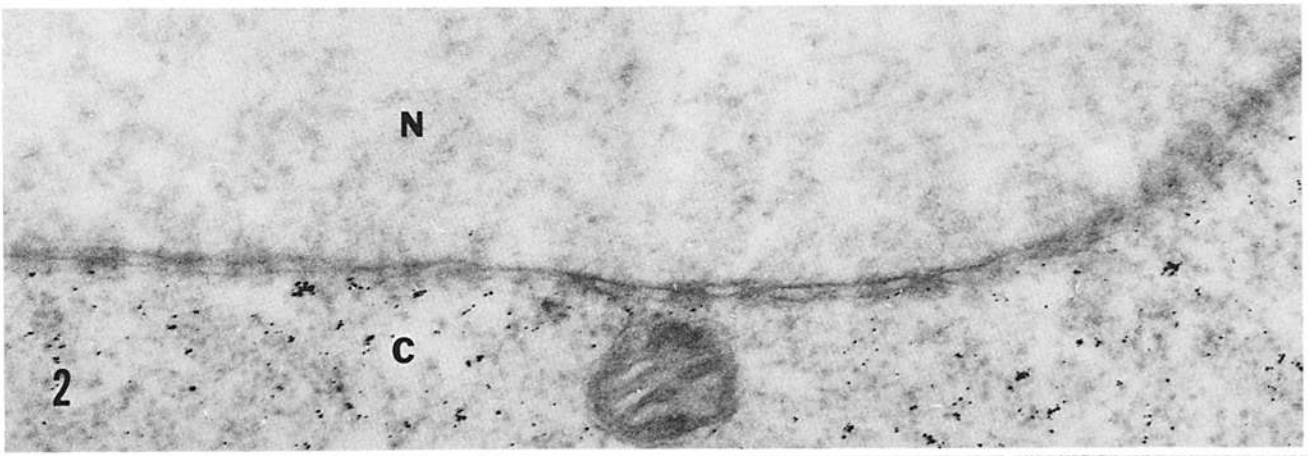
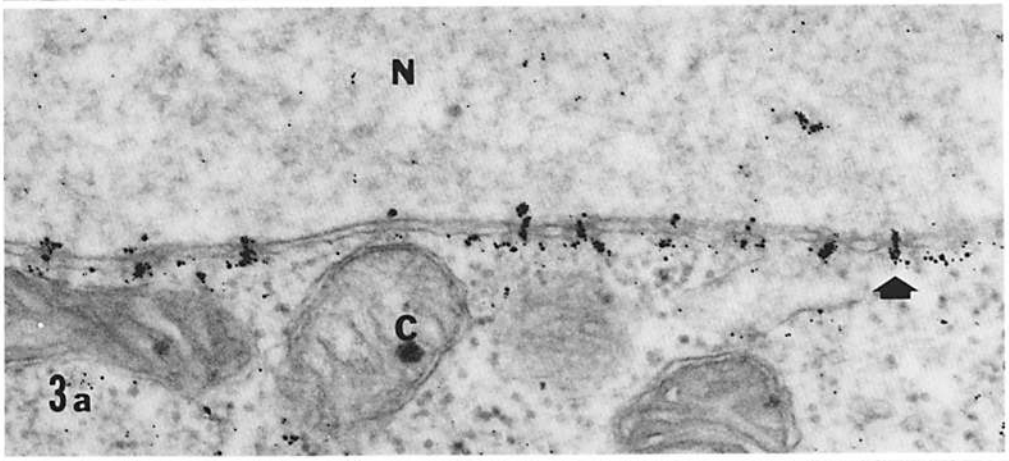
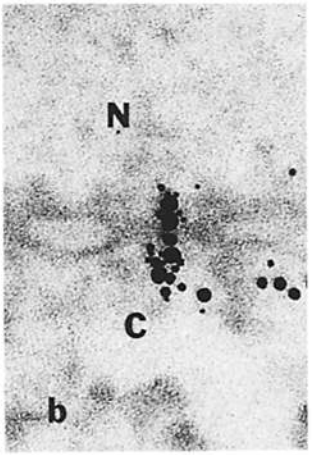


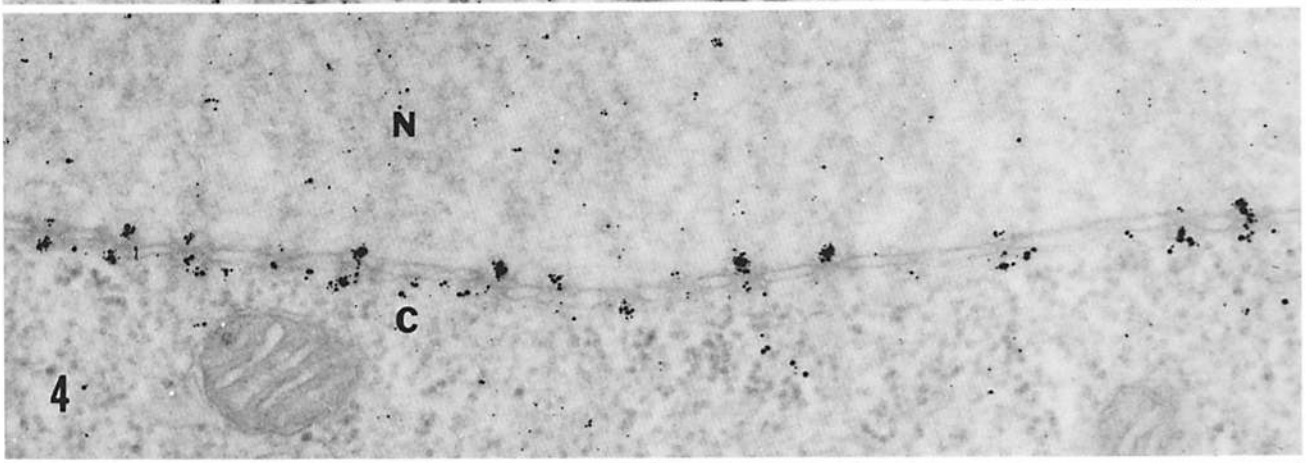
FIGURE 1 An SDS polyacrylamide gel stained with Coomassie Blue. Trypsin-digested nucleoplasmin (lane a) has an apparent molecular weight of about 80,000. Lane *b* shows the pattern obtained for native nucleoplasmin. The two major bands at the top of the gel presumably represent the pentameric and tetrameric forms. The lower band (arrow) is the monomeric form.

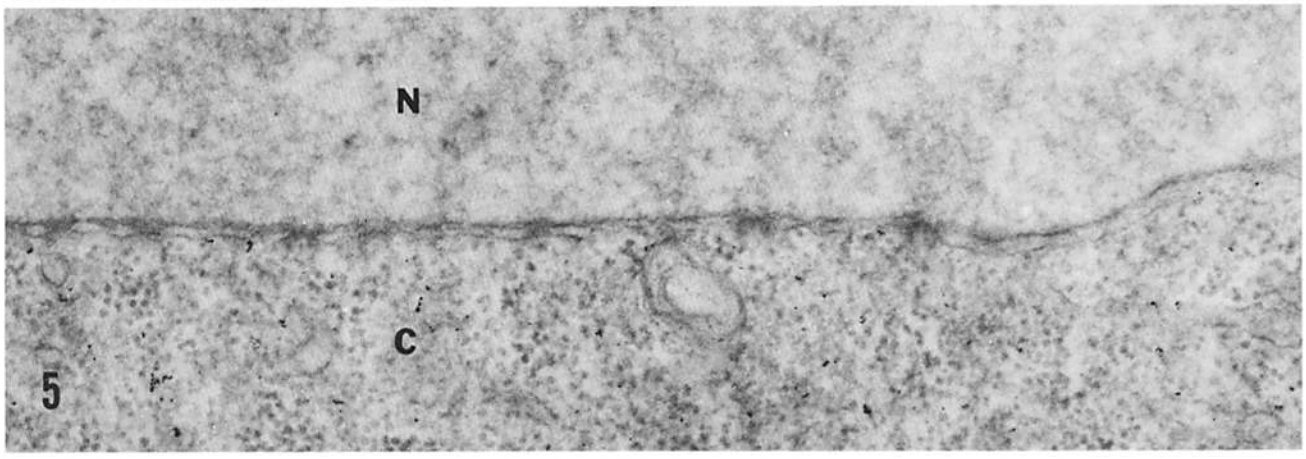
[1] *Abbreviations used in this paper:* PVP, polyvinylpyrrolidone.











When nucleoplasmin-coated gold particles were electrophoresed (data not shown), the same polypeptide pattern as seen in Fig. 1 was obtained, providing direct evidence that the protein is associated with the colloidal particles.

Injection Experiments

Oocytes were fixed either 15 min or 1 h after injection. These relatively short time intervals were selected in order to increase the probability of identifying the transport sites by fixing particles in the process of penetrating the envelope. Furthermore, any loss or redistribution of soluble cell components, resulting from the injection procedures, was minimized (17). One disadvantage of short-term studies, especially when using large cells, is that the injectate is not evenly distributed within the cytoplasm during the course of the experiments. However, it is unlikely that this was a significant source of variability inasmuch as consistent results were obtained for the oocytes within each experimental group.

Nucleoplasmin

The distribution of nucleoplasmin-coated particles 15 min after injection is illustrated in Fig. 3, *a* and *b*. Gold was present in both the nucleoplasm and cytoplasm. Within the cytoplasm the particles were excluded from organelles (i.e., mitochondria and yolk granules) but otherwise appeared to be randomly distributed in the areas examined. Within the nucleus the particle distribution also appeared random, with the exception that aggregates of gold were sometimes located at the surface of the nucleoli. Extensive accumulations of gold were found associated with the nuclear envelope, especially within the annular material at both the cytoplasmic and nuclear surfaces of the pores. In addition, particles were frequently seen extending through the centers of the pores.

Particle counts, given in Table I, show that, after 15 min, the gold concentration in the nucleus was approximately half that of the cytoplasm. The size distribution of the particles located in the nucleus and cytoplasm are shown in Table II*A*. It was determined, using Chi-square analysis, that the two populations are not significantly different ($p = 0.6$), demonstrating that particles up to ~160 Å in diameter (excluding the coat material) can readily penetrate the envelope.

The data obtained 1 h after injection with nucleoplasmin-coated gold are shown in Figs. 4 and 7, Table I, and Table II, *B*. The results are essentially the same as described above, except that by 1 h the gold concentration in the nucleus was about 2½ times greater than in the cytoplasm. There were no apparent differences in either the distribution or number of gold particles associated with the annular material in the 1-h vs. the 15-min experiments.

As a control, four oocytes were injected with nucleoplasmin-coated gold and fixed within 10 s. Gold was found in the cytoplasm of these cells but not in the nucleus or along the envelope (see Fig. 2). Thus, the presence of gold in the latter two areas after 15 min and 1 h is not an artifact caused by fixation and subsequent processing for electron microscopy.

Annulate lamellae were occasionally seen in the experimental oocytes. The distribution of gold particles in these structures (see Fig. 8) resembled that described for the nuclear envelope, that is, high concentrations of gold were associated with the annular material and particles were frequently found within the pores. Thus, the pore complexes in the envelope and lamellae have at least some common binding properties. Gold did not accumulate along the annulate lamellae when digested nucleoplasmin or PVP were used as coating agents.

Trypsin-digested Nucleoplasmin

Gold particles coated with trypsin-digested nucleoplasmin were injected into 13 oocytes. Because the same results were obtained at both experimental times (15 min and 1 h), only the 1-h data are given. Few particles were found in the nucleus (Fig. 5); the nuclear to cytoplasmic concentration ratio was only 0.009 (see Table I). Furthermore, gold did not accumulate along the envelope, and particles were rarely observed within the pores.

A comparison of the size distribution of cytoplasmic particles (see Table II) shows that the particles coated with digested nucleoplasmin were smaller than those coated with either nucleoplasmin or PVP, despite the fact that identical procedures were used to prepare the gold sols. The simplest explanation for this finding is that the larger particles aggregated during stabilization and were lost in the first centrifugation step.

Polyvinylpyrrolidone

10 oocytes were injected with PVP-coated gold. The 1-h data are presented in Fig. 6, Table I, and Table II*D*. Similar results were obtained after 15 min. In general, the PVP-coated particles distributed as expected for a large exogenous tracer. Gold was randomly distributed in the cytoplasm but was essentially excluded from the nucleus (the nuclear to cytoplasmic concentration ratio was 0.006). No accumulations of gold were observed along the envelope or in the pores.

Cytosol

The possibility exists that after injection the adsorbed coat material dissociates from the gold and is replaced by cytoplasmic components. To determine if the present results can be explained in this way, the intracellular distribution of cytosol-coated gold particles was investigated. Nine oocytes, fixed 15 min and 1 h after injection, were examined. At neither time were gold particles found in the nucleus or along the envelope. Furthermore, the gold in the cytoplasm was not randomly dispersed but localized in large masses, a distribution not observed with any other tracer. Thus, none of the results reported above could be reproduced with cytosol-coated particles.

FIGURES 2–5 Fig. 2: Nucleoplasmin gold injection, "zero time" experiment (the oocytes were fixed within 10 s after injection). Gold is present only in the cytoplasm (*C*). × 60,000. Fig. 3: (*a* and *b*) Nucleoplasmin gold injection, 15-min experiment. Colloidal particles are present in the cytoplasm and in the nucleus (*N*). Accumulations of gold can be seen adjacent to and within the pores. The pore indicated by the arrow is shown at high magnification in *b*. (*a*) × 60,000; (*b*) × 200,000. Fig. 4: Nucleoplasmin gold injection, 1-h experiment. The distribution of the gold is similar to that in Fig. 3*a* except that a higher concentration of gold is present in the nucleus. × 60,000. Fig. 5: Digested nucleoplasmin gold injection, 1-h experiment. Gold is present only in the cytoplasm. × 60,000.

Negative Staining

Fig. 9 is a negatively stained preparation of colloidal gold particles stabilized with nucleoplasmin. The thickness of the protein coat under these conditions is ~15 Å. The PVP coat was determined to be ~10 Å thick.

DISCUSSION

Gold particles coated with nucleoplasmin, an endogenous oocyte protein, readily enter and concentrate in the nucleus, whereas particles coated with trypsin-digested nucleoplasmin or PVP are largely excluded from this organelle. These findings are consistent with those reported by Dingwall et al. (3) and with earlier studies using exogenous tracers (e.g., 1, 20).

TABLE I

Intracellular Distribution of Gold Particles

Experiment	Particles in cytoplasm	Particles in nucleus	Nucleus/cytoplasm
	n		
Nucleoplasmin gold, 15 min (6)	1,721	948	0.55
Nucleoplasmin gold, 1 h (7)	722	1,751	2.43
Digested nucleoplasmin gold, 1 h (5)	1,984	17	0.009
PVP gold, 1 h (5)	1,175	7	0.006

Counts were made in equal volumes of cytoplasm and nucleoplasm. Organelles (i.e., mitochondria, yolk granules, and nucleoli) were excluded. The data were not corrected for differences in water content in the nucleus and cytoplasm. Data in parentheses are number of oocytes analyzed.

Furthermore, it was shown that the observed gold distributions were not due to either fixation procedures or alterations of the particles following injection. On the basis of this evidence, it was concluded that the intracellular localization of the gold particles is determined by the properties of the coating agent. Thus, analysis of the distribution of nucleoplasmin-coated particles should provide a model for studying the transport of endogenous proteins into the nucleus.

The results indicate that the pores are the major, if not the exclusive sites for transport. No evidence was obtained to support the hypothesis that exchanges occur directly across the membranes of the envelope. The following steps appear to be involved in the uptake process:

First, passage of the particles to the nuclear envelope. This can be explained simply by free diffusion within the cytoplasm; however, it is possible that the particles are transported to the envelope along cytoskeletal elements.

Second, binding of the particles to the annular material at the cytoplasmic surface of the pores. Binding was only observed for nucleoplasmin-coated particles and is presumably involved in selecting molecules for transport. At present there is no information concerning the specificity of the reaction or the nature of the binding sites.

Third, transport of the particles through the centers of the pores. Although the transport mechanism is not known, it is apparent that the 90 Å size limit that has been reported for exogenous molecules is not applicable to endogenous proteins. In this regard, nucleoplasmin-coated particles ranging from ~50 to 200 Å (including the thickness of the coat) were able to pass, unrestricted, through the pores. This size range

TABLE II

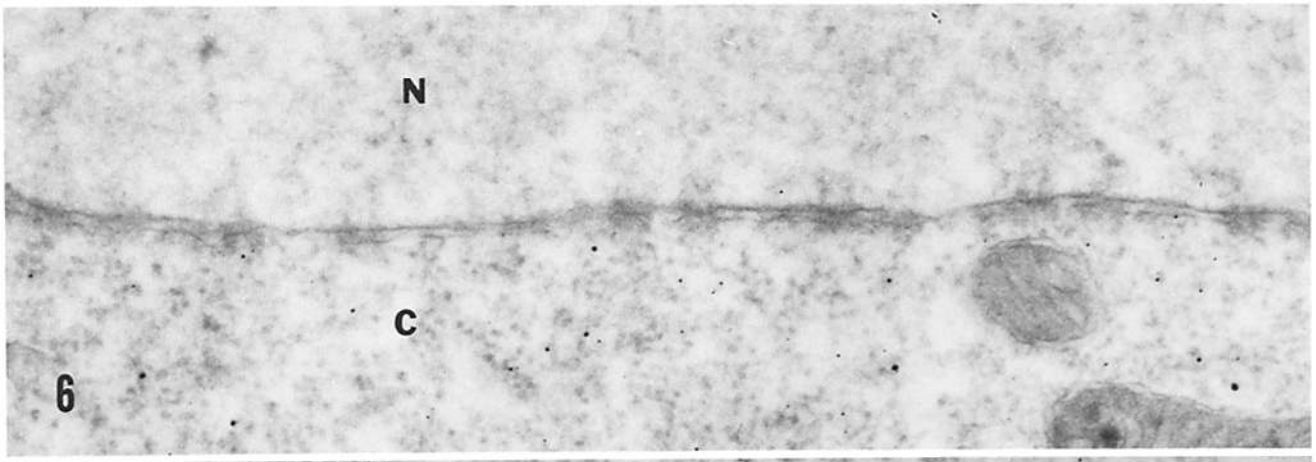
Size Distribution of Gold Particles in the Cytoplasm and Nucleus of Injected Cells

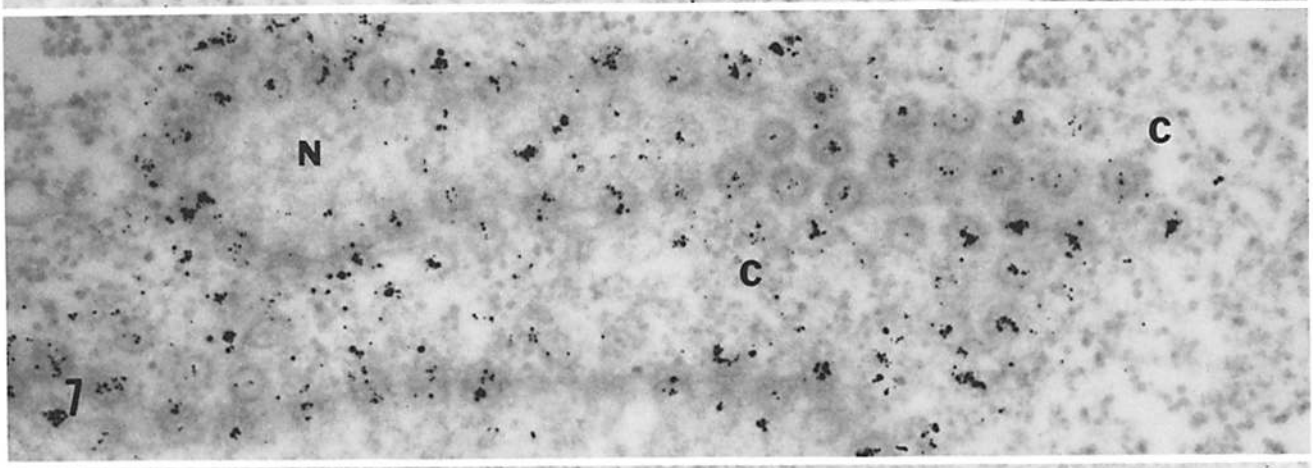
Experiment	Total number of particles measured	Particles in each size class							
		20–40 Å	40–60 Å	60–80 Å	80–100 Å	100–120 Å	120–140 Å	140–160 Å	>160 Å
	n	*n*							
A. Nucleoplasmin gold 15 min (6)									
Cytoplasm	2,254	215	1,039	543	255	122	54	20	6
Nucleus	1,645	133	771	427	177	84	38	12	3
B. Nucleoplasmin gold 1 h (5)									
Cytoplasm	933	64	452	220	115	52	18	8	3
Nucleus	1,005	56	478	269	109	54	22	11	6
C. Digested nucleoplasmin gold 1 h (4)									
Cytoplasm	876	84	592	176	20	3	1	0	0
Nucleus*	41	7	23	10	1	0	0	0	0
D. PVP gold 1 h (5)									
Cytoplasm	946	49	370	302	124	60	19	12	10
Nucleus*	26	1	11	8	5	0	1	0	0

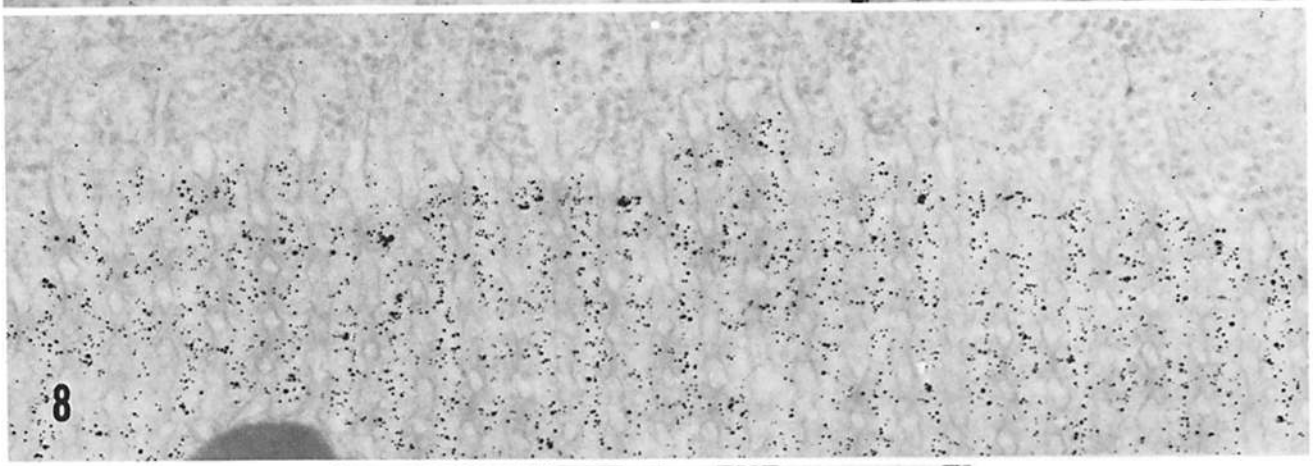
Data in parentheses are number of oocytes analyzed.

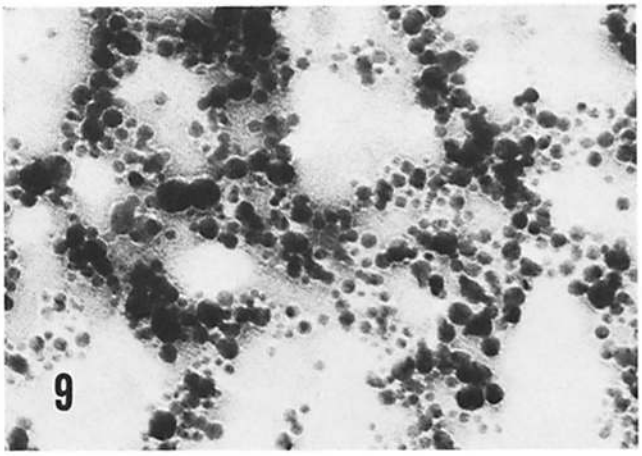
* A sufficient number of counts for statistical analysis could not be obtained.

FIGURES 6–9 Fig. 6: PVP gold injection, 1-h experiment. Gold is seen in the cytoplasm (C) but not in the nucleus (*N*) or along the envelope. × 60,000. Fig. 7: Nucleoplasmin gold injection, 1-h experiment. A tangential section through the nuclear envelope. The majority of the pores visible in the field contain gold particles. × 60,000. Fig. 8: Nucleoplasmin gold injection, 15-min experiment. A segment of stacked annulate lamellae that are continuous with cisternae of the rough endoplasmic reticulum (seen in the top half of the field). Large numbers of gold particles are located within the annular material and pores of the lamellae, but none are present in the lumens of these structures. The rough endoplasmic reticulum contains only a few gold particles. The dimensions of this stack of lamellae in the plane of the section were 7.5 × 2.3 μm. × 60,000. Fig. 9: A negatively stained preparation of nucleoplasmin-coated gold particles. The protein coat is ~15 Å thick. × 300,000.









includes all known nuclear proteins.

Fourth, dissociation from the annular material. This probably occurs after the particles have penetrated the pores. The fact that aggregates of gold particles were frequently associated with the annular material at the nuclear surface of the envelope suggests that the colloid remains bound for a short period after entering the nucleoplasm. After dissociation, the particles could either diffuse or be transported within the nucleus.

The central regions of the pores are focal points for the nuclear uptake of macromolecules and function as sites for both transport and passive diffusion. The relationship between these two processes, as well as the size restrictions that appear to be imposed by diffusion, can be explained by assuming that each pore contains a large central channel, 200 Å or more in diameter, that is available for nucleocytoplasmic exchanges. Transport of bound, endogenous molecules can be visualized as occurring along the margins of such channels; as a result, smaller, residual channels would be created through which passive diffusion could occur. Because transport is probably a continuous process, the "residual channels" would be permanent structures, although their dimensions might vary with fluctuations in transport activity. In addition, the larger "transport channels" could serve as sites for RNA efflux, which also occurs through the pores (e.g., 10, 23).

It is not known whether all of the pores have similar transport capabilities or whether there are distinct functional classes, each involved in the exchange of a specific macromolecule or group of molecules. The present results, although inconclusive, tend to support the view that all pores have similar functions. Thus, in regions adjacent to the injection sites the majority of pores appeared to be involved in the transport of nucleoplasmin-coated gold particles (see Figs. 3, 4, and 7). If transport were a highly selective process, one would expect relatively few pores to contain gold under these conditions.

The injected PVP-coated particles ranged from ~40 to 190 Å in diameter (including the coat material). As expected, very few of these particles entered the nucleus within 1 h; however, a small number of 100 to 120 Å particles (total diameter) were present in the nucleoplasm. This exceeds the 90 Å limit for exogenous substances that has been proposed in previous reports. These larger particles could have penetrated the envelope in one of two ways; first, by diffusion through a small population of "residual channels" with diameters >90 Å or, second, by binding to the annular material followed by transport through the pores. Although PVP-coated gold appears to have a low affinity for the annular material, binding will occur when the colloid is present in high concentrations, as demonstrated in previous studies utilizing amoebae (6) and isolated oocyte nuclei (5). Concentrations sufficient to initiate a limited transport reaction might have been present adjacent to the nucleus for a short period after injection.

In addition to uptake studies, the efflux of particles from the nucleus was also investigated. In preliminary experiments, nucleoplasmin-coated gold particles were injected into the nucleus, and the oocytes were fixed 15 min later. There were no indications that efflux had occurred, i.e., particles were not seen in either the pores or the cytoplasm. This suggests that transport across the envelope is irreversible; however, the experiments are not definitive. Nucleoplasmin is known to accumulate in the nucleus (e.g., 18, 19), and there is evidence that accumulation is due to binding (e.g., 2, 8). Unfortunately, we could not exclude the possibility that the injected gold particles were also bound and, as a result, inaccessible to the pores. Further experiments are planned to resolve this question.

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