

Translocation of RNA-Coated Gold Particles Through the Nuclear Pores of Oocytes

Steven I. Dworetzky and Carl M. Feldherr

Department of Anatomy and Cell Biology, University of Florida, College of Medicine, Gainesville, Florida 32610

Abstract. In the present study, various sized gold particles coated with tRNA, 5S RNA, or poly(A) were used to localize and characterize the pathways for RNA translocation to the cytoplasm. RNA-coated gold particles were microinjected into the nucleus of *Xenopus* oocytes. The cells were fixed after 15, 60 min, or 6 h, and the particle distribution was later observed by electron microscopy. Similar results were obtained with all classes of RNA used. After nuclear injection, particles ranging from 20–230 Å in diameter were observed within central channels of the nuclear pores and in the cytoplasm immediately adjacent to the pores. Particles of this size would not be expected to diffuse through the pores, suggesting that some form of mediated transport occurred. In addition, it was found that the translocation process is saturable. At least 97% of the pores analyzed appeared to be involved in the translocation process. Gold coated with nonphysiological polynucleotides (poly[I] or poly[dA])

were also translocated. When nuclei were injected with either BSA-, ovalbumin-, polyglutamic acid-, or PVP-coated gold, the particles were essentially excluded from the pores. These results indicate that the accumulation of RNA-gold within the pores and adjacent cytoplasm was not due to non-specific effects. We conclude that the translocation sites for gold particles coated with different classes of RNA are located in the centers of the nuclear pores and that particles at least 230 Å in diameter can cross the envelope. Tracer particles injected into the cytoplasm were observed within the nuclear pores in areas near the site of injection. However, only a small percentage of the particles actually entered the nucleus.

It was also determined, by performing double injection experiments, that individual pores are bifunctional, that is, capable of transporting both proteins and RNA.

THE nuclear envelope in eukaryotic cells is the site of continual macromolecular exchange between the nucleoplasm and the cytoplasm. Such exchanges can occur either by passive diffusion or mediated transport through the nuclear pores (13). Microinjection of various sized exogenous molecules has been used in previous studies to determine the dimensions of the diffusion channels within the pores. The channels were estimated to be ~90 Å in diameter in amphibian oocytes (6, 17), and are available for diffusion both into and out of the nucleus (28). Evidence for protein transport across the envelope was initially obtained for RN1 (19) and nucleoplasmin (14), both of which are major karyophilic proteins found in amphibian oocytes. The specific sites of transport for nucleoplasmin have been identified by microinjecting nucleoplasmin-coated gold particles into the cytoplasm of the oocytes (20). It was found, by electron microscopy, that transport of the colloidal particles occurred through channels, at least 200 Å wide, located in the centers of the nuclear pores. Thus, the regions of the pores available for transport are considerably larger than those available for diffusion.

The exit of mRNA from isolated nuclei has been studied in a number of laboratories (reviewed by Clawson et al., 7).

These investigations have shown that efflux is a temperature-dependent, energy-requiring process, indicating that some form of transport is involved (3, 10). In support of this view, isolated nuclear envelope preparations were found to contain nucleoside triphosphatase activity which is affected by the same factors (ionic composition, pH, etc.) that regulate RNA efflux (3, 8, 9). It has also been suggested, on the basis of results obtained in several laboratories, that the poly(A) tail of mRNA is involved in transport across the envelope (2, 5).

The efflux of tRNA from the nucleus has been investigated by Zasloff (35) using *in vivo* procedures. He obtained evidence for a saturable, carrier-mediated transport mechanism for tRNA^{met} in the amphibian oocyte. De Robertis et al. (11) have demonstrated that microinjected 5S RNA can migrate either into or out of the nucleus in *Xenopus* oocytes, however, the mechanism of exchange was not determined.

It is generally believed that RNA transport occurs through the nuclear pores. Indirect evidence supporting this view has been reviewed by Franke and Scheer (22); direct evidence was obtained by Stevens and Swift (32). These latter investigators observed that 400 Å RNP granules synthesized at the Balbiani rings in *Chironomus* salivary gland cells exit through the pores. As the particles enter the pores they are

Table I. Amounts of Coating Agent Required to Stabilize Gold Sols*

| Coating agent | Coating agent/ml of stabilizing solution | Size range of colloidal particles stabilized | Solution needed to stabilize 1 ml of colloid |
|---------------------|--|--|--|
| | mg | Å | μl |
| tRNA ^{met} | 0.2 | 20–160 | 200 |
| | | 20–50 | 50 |
| tRNA ^{phe} | 0.5 | 20–160 | 300 |
| 5S RNA | 0.4 | 20–160 | 400 |
| Poly(A) | 0.5 | 20–160 | 70 |
| | | 120–220 | 70 |
| Poly(dA) | 0.25 | 20–160 | 350 |
| Poly(I) | 0.5 | 20–160 | 400 |
| Nucleoplasmin | 0.1 | 120–220 | 60 |
| PVP | 0.1 | 20–50 | 40 |
| BSA | 1.0 | 20–50 | 60 |
| Ovalbumin | 1.0 | 20–160 | 250 |
| Polyglutamic acid | 10 | 20–50 | 200 |

* These are average values, intended to serve as a guide. The exact amounts of coating agents required for stabilization should be determined for each individual gold preparation.

transformed into rod-like structures with diameters of ~150–200 Å.

There is evidence that RNA is not freely diffusible within the nucleus and cytoplasm but is associated with structural elements of the nuclear matrix and cytoskeleton (1, 18, 21, 34). Based partly on these findings, Agutter (1) postulated that the efflux of RNA occurs in three stages by a solid-phase mechanism; first, transport to the nuclear envelope along the nuclear matrix, second, translocation through the pores, and third, transport along the cytoskeletal matrix.

The focus of the present investigation is on the second step of the efflux process, that is, translocation across the envelope. The main objectives are, first, to localize and characterize the specific regions of the nuclear pores involved in the translocation of different classes of RNA-coated gold to the cytoplasm and, second, to determine whether individual pores can function in both RNA efflux and protein uptake, or whether separate classes of nuclear pores exist. To obtain the resolution necessary to examine individual exchange sites, coated colloidal gold particles were used as tracers. The particles were microinjected into oocytes, and subsequently localized by electron microscopy.

The results can be summarized as follows: first, gold particles coated with tRNA (met or phe), 5S RNA, or poly(A), are all translocated to the cytoplasm through central channels, at least 230 Å in diameter, located within the nuclear pores. Particles stabilized with nonphysiological polynucleotides are also translocated, however, the number and distribution of the particles associated with the pores varied for different coating agents. Second, ~97% of the pores in *Xenopus* oocytes can function in the translocation of RNA-gold. Third, the accumulation of particles in the pores is a saturable process. Fourth, individual pores can function in both RNA efflux and protein uptake. Control experiments ruled out the possibilities of fixation artifacts and non-specific exchange processes.

Materials and Methods

Xenopus laevis were purchased from Xenopus 1 (Ann Arbor, MI) and maintained as reported previously (17).

Nucleoplasmin Isolation

Nucleoplasmin was isolated from a starting volume of 30 ml of *Xenopus* ovaries. The isolation procedure was similar to that described by Dingwall et al. (14), with the exception that an anti-nucleoplasmin IgG affinity column was substituted for the DEAE-cellulose and phenyl sepharose columns. Polyclonal antibodies against nucleoplasmin were generated in rabbits and affinity purified. A cleared cell homogenate, obtained from the lysed oocytes (20), was passed over an anti-nucleoplasmin affinity column, and the column was washed free of nonbound proteins. Nucleoplasmin was eluted in 1 ml fractions with 50 mM glycine-HCl buffer (pH 2.5) into microcentrifuge tubes containing sufficient 1 M Tris (pH 9.0) to increase the pH to 7.5. The protein was monitored at 280 nm, and the fractions containing nucleoplasmin were pooled and treated with (NH₄)₂SO₄ (55% saturated) overnight in the cold. The soluble (NH₄)₂SO₄ fraction was then dialyzed against a solution containing 0.05 M Tris and 0.05 M NaCl (pH 7.2), after which the nucleoplasmin was precipitated in 80% alcohol and lyophilized as described previously (20). Gel analysis was performed, as described by Laemmli (26), to determine the purity of the preparation.

Gold Preparation and Stabilization

All glassware and solutions used in experiments involving RNA were treated with 0.01% (vol/vol) diethyl pyrocarbonate and then autoclaved. Colloidal gold particles were prepared by reducing chloroauric acid with either trisodium citrate (23) or a saturated solution of white phosphorus in ether (16). In this study the trisodium citrate method gave a particle distribution of 120–220 Å in diameter, whereas the phosphorus ether preparations ranged from either 20–50 Å or 20–160 Å, depending on the initial concentration of gold chloride used.

The gold sols were stabilized with tRNA (met or phe), 5S RNA, poly(A) (3,500 bases), poly(I) (500 bases), poly(dA) (500 bases), nucleoplasmin, polyvinylpyrrolidone (PVP¹; 40 kD), polyglutamic acid, ovalbumin, or BSA. The RNAs were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) or Sigma Chemical Co. (St. Louis, MO). The purity of the RNAs was tested by running the preparations on 10% polyacrylamide gels containing 8.3 M ultrapure urea; ethidium bromide was used as a stain. Polyglutamic acid, BSA, ovalbumin, and PVP were purchased from Sigma Chemical Co.

In each instance, the minimum amount of coating agent required to stabilize the particles, that is, prevent precipitation in 1% NaCl, was determined as described earlier (20). Before stabilization, tRNA, poly(A), poly(I), and poly(dA), were dissolved in 10 mM KCl, 7.2 mM K₂HPO₄ and 4.8 mM KH₂PO₄ (pH 7.0). 5S RNA was rehydrated in 2 ml of sterile ion-free water resulting in a salt concentration of 1 mM Tris-HCl, 10 mM NaCl and 0.1 mM MgCl₂ (pH 7.5). Nucleoplasmin, polyglutamic acid, PVP, BSA, and ovalbumin were solubilized in a low ionic strength buffer containing 7.2 mM

1. Abbreviation used in this paper: PVP, polyvinylpyrrolidone.

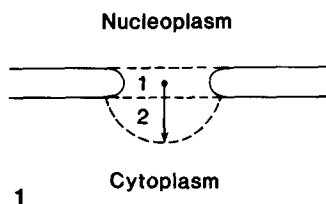


Figure 1. A schematic representation of the regions used for electron microscope analysis of the particle distribution within the pores. It is assumed that the particles in region 1 are undergoing translocation and that particles in region 2 have completed the process. The arrow which defines the outer limits of region 2 represents a distance of ~ 500 Å.

K_2HPO_4 and 4.8 mM KH_2PO_4 (pH 7.0). Concentrations of coating agents and amounts used to stabilize the gold preparations are given in Table I.

After stabilization, the 20–160 Å preparations were centrifuged at 6,000 *g* (at the bottom of the tube) at 4°C for 10 min to remove any large aggregates of gold. This step was not necessary for the 20–50 Å and 120–220 Å fractions. 5 to 7 ml of stabilized colloid were then concentrated to 70–100 μ l in Minicon concentrators (Amicon Corp., Danvers, MA). Finally, the samples were 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) for 3 h at 4°C.

Injection

Frogs were anesthetized on ice for 1 h and the ovaries removed. Late stage 5 and stage 6 oocytes (15) were manually defolliculated with watchmakers forceps and maintained in Ringer's solution (12) at 22°C.

The defolliculated cells were centrifuged at ~ 650 *g* for 8–10 min as described previously (18, 25). During centrifugation, the nucleus migrates to a position just underneath the plasma membrane at the animal pole and its outline can be visualized due to the displacement of pigment granules in the cortex. Nuclear injections could then be accomplished. Calcium-free Ringer's was used as an extracellular medium during injection to prevent possible precipitations of the colloid as the micropipettes were introduced into the cells. Immediately after injection the cells were returned to complete Ringer's solution until subsequent fixation. The total exposure to calcium-free Ringer's was less than 30 min. The tip diameters of the micropipettes were 10–15 μ m.

Electron Microscopy and Analysis

The cells were fixed using a procedure similar to that described by Kalt and Tandler (24). The oocytes were initially fixed overnight at 4°C in 100 mM Hepes containing 3% glutaraldehyde (wt/vol), 2% paraformaldehyde (wt/vol), 2.5% DMSO (vol/vol) and 1 mM $CaCl_2$ (pH 7.2). The nuclei were then dissected out with their surrounding cytoplasm, post-fixed in 2% OsO_4 (wt/vol) for 1 h, and stained with 0.5% diphenylhydramine (wt/vol) in 70% acetone for 30 min. Finally, the samples were dehydrated in a graded series of acetone and embedded in Spurr's medium. Thin sections were cut on a Reichert microtome and analyzed with a JEOL 100S electron microscope (JEOL USA, Cranford, NJ).

In the RNA efflux studies it was presumed that gold particles located within the pores were in the process of translocation, and that particles present in the cytoplasm just adjacent to the pores had completed the translocation process. These areas will be referred to as regions 1 and 2, respectively (see Fig. 1). Pores that contained one or more particles in either or both of these regions were considered to be actively involved in efflux. To determine the functional diameter of the transport channels, the size distribution of the particles in regions 1 and 2 were obtained and compared with the size of the injected particles (i.e., particles within the nucleus). To standardize the results, pores were analyzed in regions where the gold concentration in the adjacent nucleoplasm was ~ 100 particles/ 0.36 μ m.²

Negative staining procedures were used to estimate the overall diameters of the particles, that is, the gold plus the adsorbed coat material.

Results

Purity of Stabilizing Agents

Approximately 600 μ g of nucleoplasmin was isolated from 30 ml of ovary using affinity chromatography. SDS-polyacrylamide gels of the isolated protein showed major bands with apparent molecular masses of 165 and 145 kD, and a minor band with a molecular mass of ~ 33 kD. The gel pattern (not shown) was identical to that obtained for nucleoplasmin isolated using DEAE and phenyl sepharose columns (20).

On a 10% polyacrylamide gel containing urea, tRNA ran as a single distinct band corresponding to ~ 70 bp, whereas the pattern obtained for 5S RNA contained one major band corresponding to ~ 130 bp and minor bands of smaller sizes, probably representing breakdown products. Gel scans of the

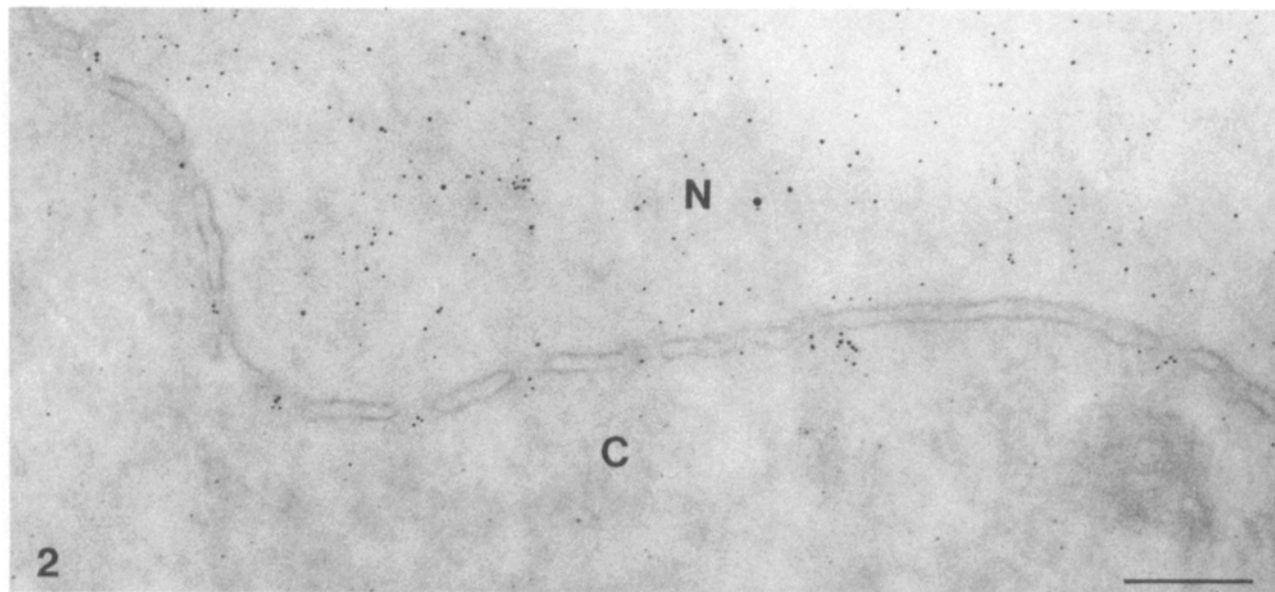


Figure 2. tRNA-gold, nuclear injection - 15-min experiment. Gold particles (20–160 Å) are observed evenly distributed throughout the nucleus (N). Particles are seen within the centers of the majority of the pores and also in the adjacent cytoplasm (C). Bar, 0.2 μ m.

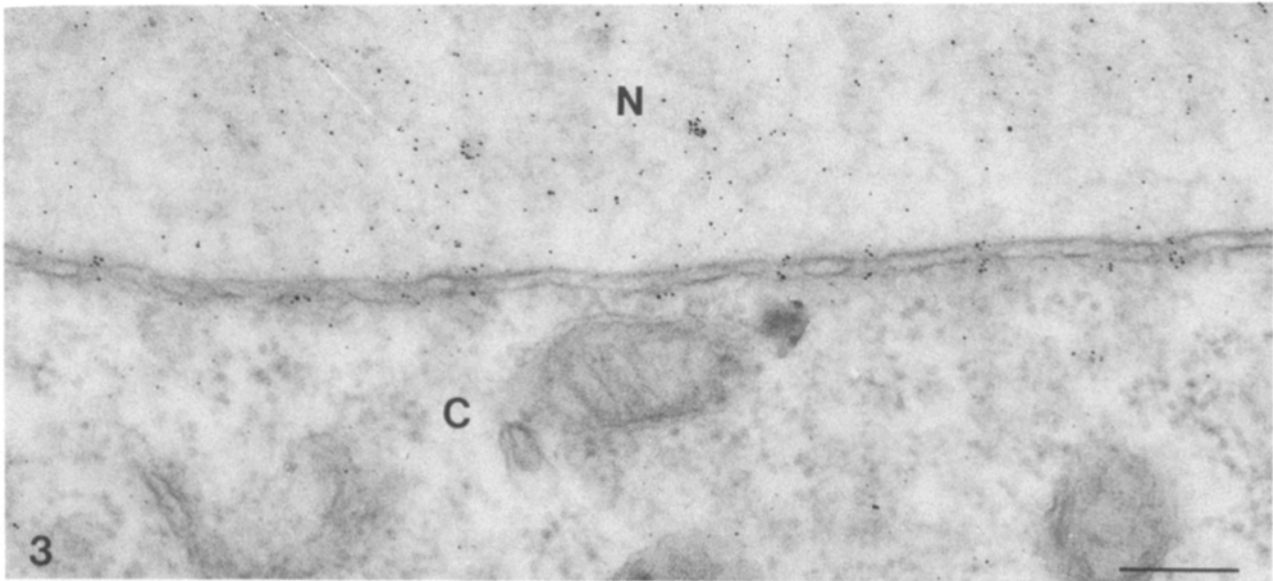


Figure 3. 5S RNA-gold, nuclear injection - 60-min experiment. The tracer particles (20–160 Å) show a similar distribution as described in Fig. 2. Particles are present within the pores and the adjacent cytoplasm (C). N, nucleus. Bar, 0.2 μm.

tRNA and 5S RNA showed the major band in each sample to be >80% of the total RNA.

Nuclear Injection of tRNA-, 5S RNA- and Poly(A)-coated Gold

It was determined initially that varying the amount of the injectate from 8–20 nl (the RNA content ranged from 30–100 ng) had no effect on the results. Routinely, 8 nl of colloid were injected, this volume contained a sufficient number of particles for electron microscopic analysis and minimized possible damage to the nucleus. The oocytes were fixed 15 min, 1 h, or 6 h after injection. 18–20 cells were examined for each type of RNA at each time interval.

The results were essentially the same for 20–160 Å particles coated with tRNA (met or phe), 5S RNA, or poly(A). At all time intervals, particles were randomly distributed throughout the nucleoplasm, except for aggregates of gold that were occasionally observed at the surface of the nucleoli. After 15 min and 1 h, particles were associated with almost all of the nuclear pores. Representative results for

tRNA and 5S RNA are shown in Figs. 2 and 3, respectively. Based on the assumption that the presence of gold particles within the pores or in the adjacent cytoplasm (regions 1 and 2 in Fig. 1) is indicative of nucleocytoplasmic exchange, it was concluded that over 97% of the pores were involved in translocation 15 min and 1 h after injection (see Table II). There was an obvious decrease in the percentage of pores that contained particles in the 6-h experiments, but these results were not quantitated. In all instances, particles were observed in the cytoplasm beyond the immediate vicinity of the pores (i.e., beyond region 2). However, even after 6-h the cytoplasmic to nuclear concentration ratio was only 1:14.

The size distributions of tRNA- and 5S RNA-coated particles present within the nucleoplasm and also associated with the nuclear pores (regions 1 and 2) are given in Table III. Since the thickness of the RNA coat, in both cases, was estimated to be 15–20 Å, the results indicate that particles with an overall diameter of at least 170 Å can pass through the centers of the pores. Since larger particles could not be stabilized with tRNA or 5S RNA, it was not possible to determine

Table II. Translocation of Gold Particles as a Function of the Coating Agent

| Experiment (1 h) | No. of particles translocated* | | | Particles/Pore | Percentage of pores active in translocation |
|------------------|--------------------------------|----------|-------|----------------|---|
| | Region 1 | Region 2 | Total | | |
| tRNA-gold | 437 | 414 | 851 | 4.3 | 97 |
| 5S RNA-gold | 458 | 425 | 883 | 4.4 | 98 |
| Poly (A)-gold | 309 | 479 | 788 | 3.9 | 98 |
| Poly (dA)-gold | 334 | 144 | 478 | 2.4 | 98 |
| Poly (I)-gold | 397 | 543 | 940 | 4.7 | 96 |
| VVP-gold‡ | 3 | 5 | 8 | 0.04 | 4 |
| BSA-gold‡ | 5 | 3 | 8 | 0.04 | 4 |
| Ovalbumin-gold | 2 | 9 | 11 | 0.05 | 6 |

* Data based on the analysis of 200 pores all within equivalent concentrations of gold particles.

‡ The size of the gold particles in these experiments ranged from 20–50 Å. In all other experiments the particle sizes ranged from 20–160 Å.

Table III. Size Distribution of Gold Particles Present in the Nuclei and Pores

| Experiment (1 h) | Total no. of particles measured | Percentage of particles in each size class* | | | | | | | | | |
|---------------------|---------------------------------------|---|-------|-------|--------|---------|---------|---------|---------|---------|------|
| | | 20-40 | 40-60 | 60-80 | 80-100 | 100-120 | 120-140 | 140-160 | 160-180 | 180-200 | >200 |
| | | Å | Å | Å | Å | Å | Å | Å | Å | Å | Å |
| tRNA ^{met} | | | | | | | | | | | |
| Nuclei [‡] | 486 | 0.3 | 27.9 | 51.5 | 12.8 | 4.2 | 1.5 | 1.7 | — | — | — |
| Pores [§] | 462 | 0.4 | 22.1 | 52.6 | 15.4 | 6.1 | 1.3 | 2.1 | — | — | — |
| 5S RNA | | | | | | | | | | | |
| Nuclei | 1,110 | 3.2 | 35.8 | 48.4 | 10.0 | 1.8 | 0.45 | 0.36 | — | — | — |
| Pores | 773 | 2.6 | 37.0 | 48.4 | 10.3 | 1.3 | 0.25 | 0.15 | — | — | — |
| Poly A | | | | | | | | | | | |
| Nuclei | 906 | — | — | — | — | — | 6.6 | 35.5 | 43.8 | 9.8 | 4.2 |
| Pores | 593 | — | — | — | — | — | 5.2 | 37.3 | 42.0 | 13.6 | 1.9 |

* Particle dimensions do not include the thickness of the coat. Negative staining indicated that the coat thickness adds 30–40 Å to the overall diameters of the particles.

[‡] Particles present within the nuclei.

[§] Total number of particles present within the pores and in the cytoplasm immediately adjacent to the pores.

an upper size limit for translocation using these coating agents. In contrast, 120–220 Å particles could be stabilized with poly(A). The results obtained with poly(A)-gold are illustrated in Fig. 4. Despite the fact that relatively few particles were injected into the nuclei, gold was found in over 70% of the pores after 15 min and 1 h. The size distribution of poly(A)-coated particles associated with the pores is shown in Table III. It is apparent from the data, which is based on the examination of 12 cells, that particles at least 230 Å in diameter (including the coat) are translocated through the pores.

Nuclear Injection of Poly(I)- and Poly(dA)-coated Gold

In addition to the studies performed with tRNA, 5S RNA and poly(A), nuclei were also injected with poly(I)- or poly(dA)-coated particles. The volumes injected, and the procedure used for analysis of these nonphysiological tracers were the same as described above. In these experiments, the oocytes were fixed 1 h after injection, and 5 cells were examined in each group. The results are shown in Table II. Particles coated with either poly(I) or poly(dA) were translocated through the centers of the pores; however, differences were observed in the numbers and distribution of the particles as-

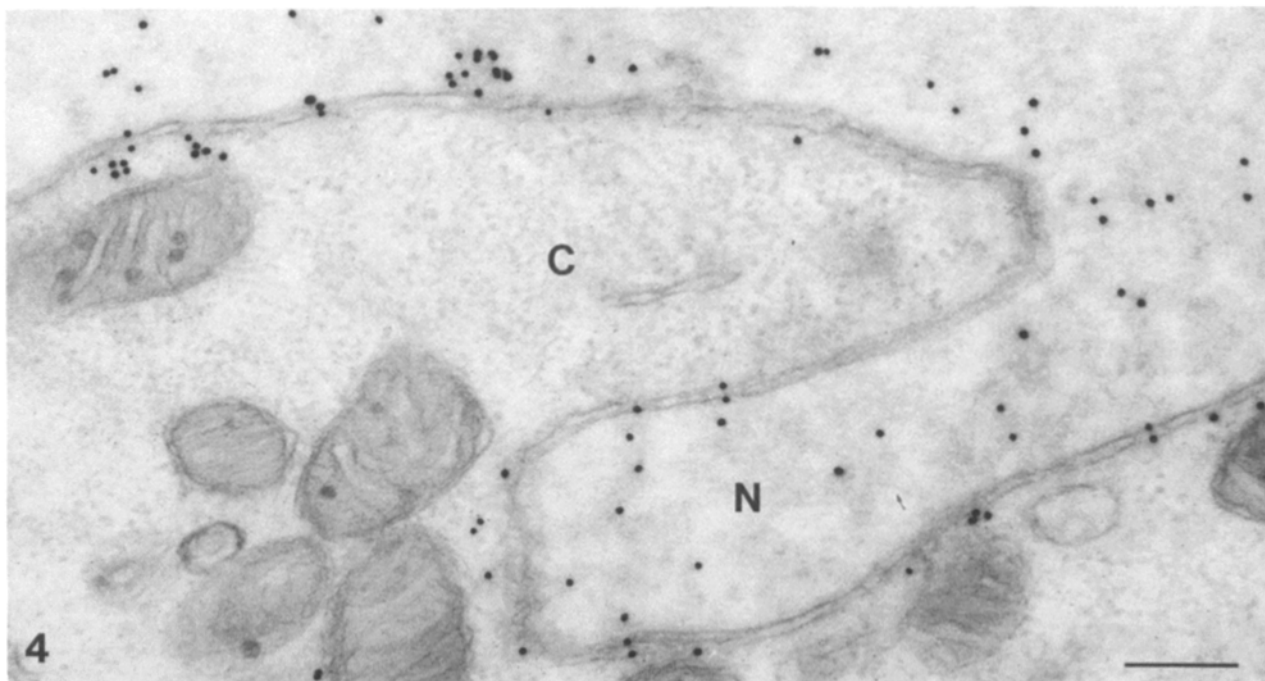


Figure 4. Poly(A)-gold, nuclear injection - 60-min experiment. Large gold particles (120–220 Å) are observed extending through the nuclear pores and are present in the adjacent cytoplasm (C). N, nucleus. Bar, 0.2 µm.

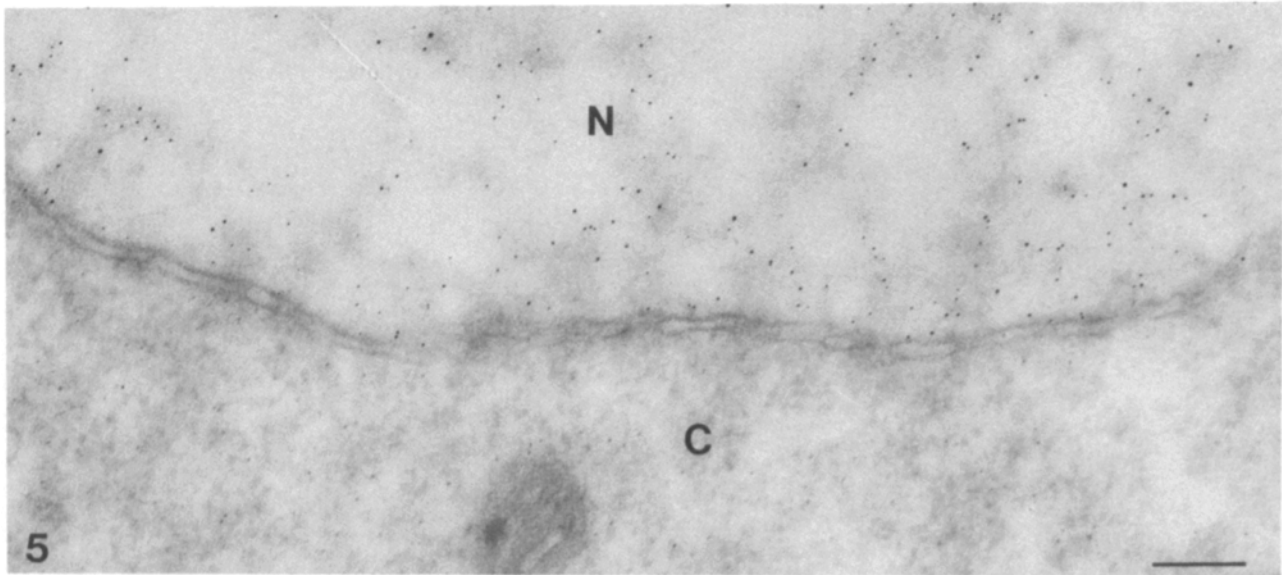


Figure 5. PVP-gold, nuclear injection - 60-min experiment. Gold particles are retained within the nucleoplasm and are rarely seen associated with the envelope or within the nuclear pores. C, cytoplasm; N, nucleus. Bar, 0.2 μm .

sociated with these structures. Compared with poly(dA)-coated gold, almost twice as many poly(I)-coated particles were present in the pore areas and, in addition, a higher proportion of these particles were located in region 2. These results suggest that some polynucleotides are translocated more efficiently than others.

Nuclear Injection of BSA-, Ovalbumin-, Polyglutamic Acid-, and PVP-coated Gold

To determine if the translocation of RNA-gold is a selective process, nuclei were injected with gold fractions that had been stabilized with the exogenous molecules PVP, BSA, or ovalbumin. The volumes injected, and the experimental times (15 min, 1 and 6 h), were the same as those used in the RNA studies. The data are based on the examination of 6–9 cells per time interval for each coating agent that was used. The result of a 1 h nuclear PVP-gold injection is illustrated in Fig. 5. Similar distributions were observed when particles stabilized with BSA or ovalbumin were injected. It was found, at all time intervals, that essentially all of the particles coated with exogenous substances were retained within the nuclei and less than 6% of the pores contained gold particles (Table II). It can also be seen in Table II that the total number of control particles translocated per 200 pores was only $\sim 1\%$ of the total number of translocated RNA-coated particles. These results demonstrate that the translocation of

RNA-gold was due to the specific properties of the adsorbed coat material.

To determine if the translocation of polynucleotide-coated particles is due simply to a high negative charge density, gold particles were coated with polyglutamic acid, which, like RNA, is a polyanion. Since polyglutamic acid is not a highly effective stabilizing agent, the colloid preparations that were injected were relatively dilute. To compensate for this factor, tRNA-gold preparations having an equivalent particle concentration were injected in parallel experiments. The results, which are based on an analysis of 5 cells, are shown in Table IV. Tracer particles coated with polyglutamic acid were observed in only 8% of the pores, compared with a value of 83% obtained for tRNA-gold. These findings demonstrate that the translocation of the RNA-coated particles is not simply a charge effect.

Controls

Control experiments were performed on 6 oocytes to establish if the results obtained for RNA-gold could be due to a redistribution of the particles during fixation. In this study, the nuclei were injected with tRNA-coated gold and the oocytes fixed within 10 s. The particles near the site of injection were rarely observed within the pores or in the adjacent cytoplasm. These data indicate that the presence of RNA-

Table IV. Translocation of Gold Particles Coated with Polyglutamic Acid

| Experiment (1 h) | No. of particles translocated* | | | Particles/Pore | Percentage of pores active in translocation |
|------------------------|--------------------------------|----------|-------|----------------|---|
| | Region 1 | Region 2 | Total | | |
| Polyglutamic acid-gold | 21 | 2 | 23 | 0.1 | 8 |
| tRNA-gold | 175 | 164 | 339 | 1.7 | 83 |

* Data based on the analysis of 200 pores. In both instances the concentration of gold particles (20–50 \AA) in the adjacent nucleoplasm was ~ 60 particles/ $0.36 \mu\text{m}^2$.

Table V. Concentration Dependence of tRNA-gold Translocation*

| Particle no./ 0.36 μm^2 | No. of particles translocated | | | Particles/ Pore | Percentage of pores active in translocation |
|---------------------------------------|-------------------------------|----------|-------|--------------------|---|
| | Region 1 | Region 2 | Total | | |
| 30 | 34 | 84 | 118 | 1.2 | 61 |
| 60 | 77 | 94 | 171 | 1.7 | 82 |
| 90 | 159 | 197 | 356 | 3.6 | 98 |
| 120 | 154 | 205 | 359 | 3.6 | 98 |
| 190 | 188 | 174 | 362 | 3.6 | 98 |

* Data based on the analysis of 100 pores for each gold particle concentration. The size of the gold particles ranged from 20–50 Å, and the cells were fixed 1 h after injection.

gold within the pores at 15-min and 1-h intervals was not a fixation artifact, but reflected an *in vivo* exchange process.

To ascertain whether the presence of excess RNA itself could alter the properties of the pores, nuclei of 6 oocytes were simultaneously injected with PVP-coated gold and 100 ng of soluble tRNA. Fewer than 4% of the 200 pores analyzed were involved in translocation. Thus, the injection of RNA in amounts greater than those normally used in this investigation, have no apparent effect on the physical properties of the pores.

Concentration Dependence of RNA Translocation

To determine the concentration dependence of the translocation process, the gold distribution in the pores was analyzed in areas in which different concentrations of colloid were present in the adjacent nucleoplasm. These experiments

were performed with tRNA-coated gold particles, and the cells were fixed after 1 h. To ensure that an appropriate concentration range was obtained, different dilutions of colloid were injected. The amount of tRNA in the injectate varied from 6–30 ng. The results are shown in Table V, and are based on the examination of 6 oocytes. It can be seen that a maximum number of particles per pore is obtained at a concentration of 90 particles/0.36 μm^2 . Above this concentration, no differences were observed either in the numbers or distribution of the particles, suggesting that the translocation process is saturable.

Double-label Experiment

The high percentage of pores involved in RNA translocation suggested that each pore may be a bidirectional channel, capable of both protein and RNA transport. To address this possibility directly, large gold fractions (120–220 Å) coated with nucleoplasmin and small gold fractions (20–50 Å) stabilized with tRNA were used. The tRNA particles were injected into the nucleus and nucleoplasmin-coated gold was injected into the cytoplasm of the same cell. The sequence of the injections varied in different experiments, however, in all instances the interval between the first and second injection was 15 min, and the oocytes were fixed after a total of 45 min. In all, 12 cells were examined. The results, which were the same regardless of the injection sequence, are illustrated in Fig. 6, *a–d*. Small RNA particles can be observed immediately adjacent to the cytoplasmic surface of the pores and large nucleoplasmin particles are present on the nuclear side of the same pores. These distributions clearly demonstrate that individual pores can function in both protein uptake and RNA efflux.

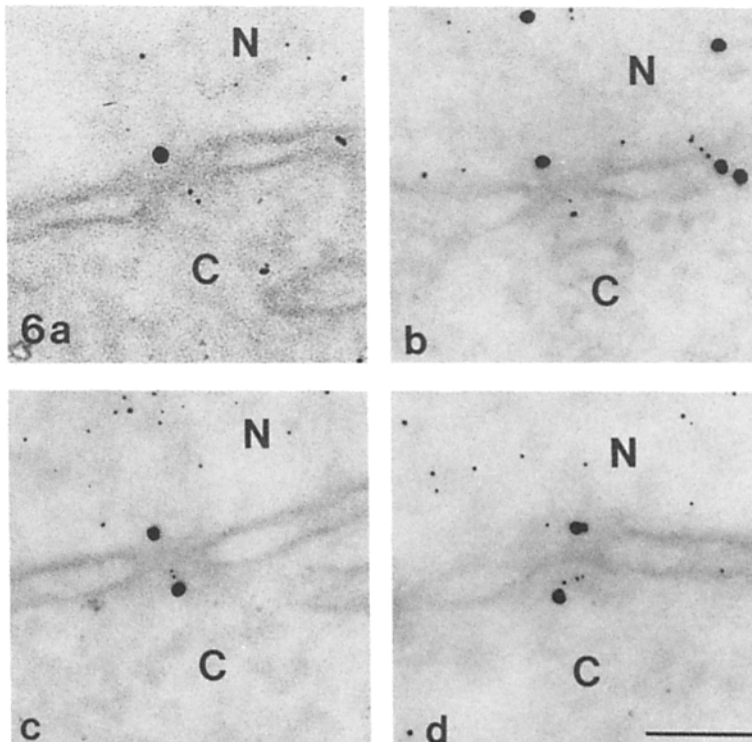


Figure 6. (*a–d*) Double injection experiment—45 min. tRNA-gold (20–50 Å) was injected into the nucleus (*N*), and the adjacent cytoplasm (*C*) was injected with nucleoplasmin-gold (120–220 Å). The large nucleoplasmin-coated particles can be seen just on the nuclear sides of the pores and small RNA particles are present on the cytoplasmic side. Bar, 0.1 μm .

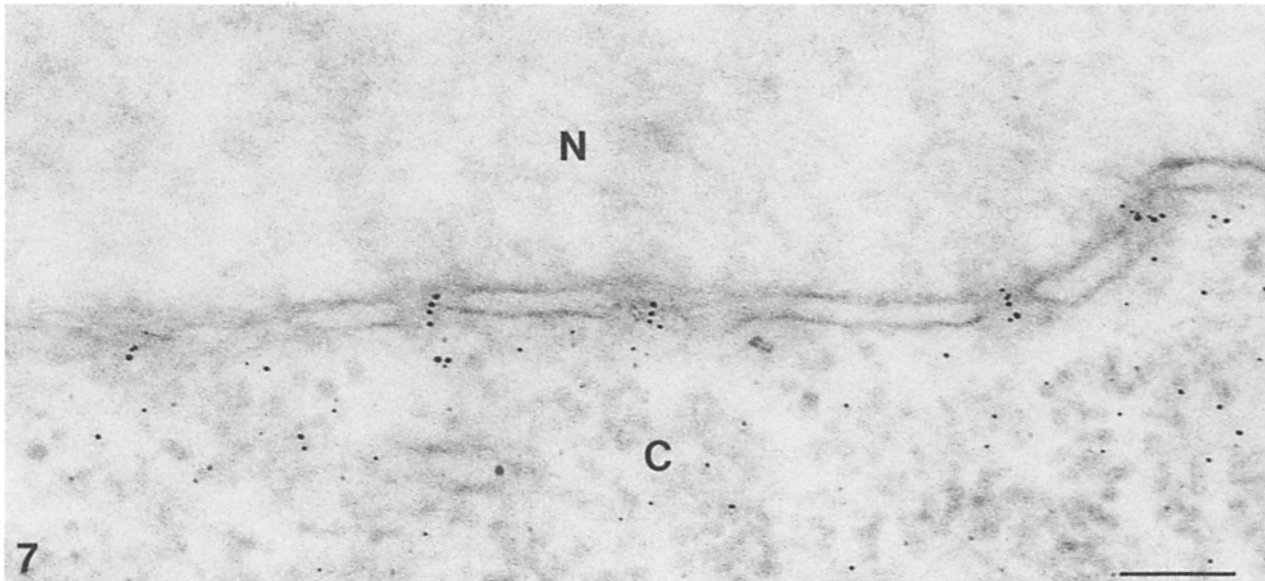


Figure 7. 5S RNA-gold, cytoplasmic injection: 15-min experiment. Gold particles are present in the cytoplasm (C) and can be seen extending through the nuclear pores. Only a small percentage of particles enter the nucleus (N). Bar, 0.1 μm .

Cytoplasmic Injections

The RNA-gold preparations [tRNA, 5S RNA, and poly(A)] were injected into the cytoplasm to determine if transport is reversible. Approximately 40 nl of colloid (containing ~ 200 ng of RNA) were introduced adjacent to the nuclear envelope in a total of 20 centrifuged cells. Similar distributions were obtained for all RNA's used. In both 1- and 6-h experiments, gold was seen within the pores near the site of injection, but relatively few particles were found in the nucleoplasm. The nuclear to cytoplasmic concentration ratios were $\sim .01$ after 1 h. The 5S RNA-gold results are illustrated in Fig. 7. It should be pointed out that the number of pores that contained gold varied considerably from cell to cell, therefore, no effort was made to quantitate these results.

Discussion

Colloidal gold procedures have been used previously to identify and characterize the regions of the pores that are involved in the transport of karyophilic proteins across the nuclear envelope (20). It was found that the transport channels are located in the centers of the pores and have functional diameters of at least 200 \AA . In this study, the same experimental approach was used to characterize the pathways for RNA efflux and, in addition, to determine whether individual pores have the capability of transporting both RNA and protein.

The presence of RNA-coated gold particles within and along the cytoplasmic surface of the nuclear pores provides direct evidence that these tracers are translocated across the envelope at these sites. Furthermore, almost all of the pores (97% or more) can function in the translocation process. The biological significance of these findings, however, is dependent on demonstrating that the pathways visualized for the colloidal gold tracers are the same as those normally used for the exchange of endogenous RNA. In this regard, we have attempted to demonstrate that the translocation of RNA-gold

through the nuclear pores is both a selective and active process, and that the observed gold distributions were not due to technical artifacts.

The fact that gold was not present in the pores of cells fixed 10 s after injection shows that accumulation in these structures is not simply a result of the injection procedures per se or a redistribution of the particles during fixation and processing for electron microscopy. Control studies also demonstrated that the injection of excess RNA does not alter the overall properties of the pores.

Gold particles coated with synthetic polymers (PVP or polyglutamic acid), exogenous proteins (BSA or ovalbumin) and even endogenous karyophilic proteins (nucleoplasmin, 20), are essentially excluded from the pores after nuclear injection, demonstrating that the translocation of RNA-gold is a selective process. The data obtained with poly(I) and poly(dA) indicate that the capacity for translocation is not necessarily restricted to physiologically active RNAs but may be a general property of polynucleotides. The chemical and/or physical characteristics of RNA that facilitate translocation are not known although a comparison of the results obtained with poly(A) and poly(dA) suggests that the sugar moieties could be involved. The high negative charge density of RNA does not appear to be a major contributing factor since particles coated with polyglutamic acid are largely excluded from the pores.

In view of the results obtained by Zasloff (35), which showed that the transport of labeled tRNA is markedly reduced by a single substitution of G to U at position 57, one might expect a greater degree of specificity for the translocation of polynucleotides. It should be kept in mind, however, that Zasloff measured the overall efflux of RNA, whereas our results relate specifically to the translocation step across the envelope. Thus, the decreased rate of transport of the variant is not necessarily due to an affect on translocation through the pores, but could be due to increased binding within the nucleoplasm or changes in the rate of migration in the cytoplasm.

As discussed in the introduction, there is evidence that macromolecules larger than 90-Å in diameter are unable to diffuse across the nuclear envelope, whereas, particles as large as 200-Å in diameter, which contain nuclear targeting signals, can be transported through central channels in the pores. It was found in this study, that RNA-coated gold particles as large as 230 Å in diameter (including the coat) readily penetrate the pores. Since this far exceeds the upper limit for diffusion, these results suggest that a transport process is involved. Consistent with this interpretation is the finding that the accumulation of RNA-gold in the pores is a saturable process. Saturation indicates the presence of a carrier-mediated transport process. However, saturation would also occur if the particles simply occupied all of the available space within and adjacent to the pore channels. The latter explanation, which would have little bearing on the mechanism of exchange, is unlikely for two reasons. First, direct electron microscopic examination of region 1 shows that these areas are not fully occupied at saturation levels. Second, particles coated with nucleoplasmin can reach more than twice the concentration in region 2, than tRNA-coated gold (data not shown), demonstrating that the nature of the coat material rather than the total number of tracer particles is the determining factor.

Colloidal tracers, although well suited for localizing and characterizing exchange sites, are not appropriate for detailed kinetic studies, which require numerous time points and involve the analysis of large samples. For this reason a comprehensive examination of the temperature dependence of translocation was not attempted. However, a cursory study was performed at 4°C and at 21°C, comparing the accumulation and distribution of tRNA-coated gold particles in the pores. After 15 min, the average number of particles per pore (based on the analysis of 100 pores) at 21°C and 4°C was 3.8 and 1.5, respectively. Furthermore, at 4°C only 25% of the particles were located in region 2, compared to 50% in cells kept at 21°C. The temperature effects are very likely influenced by two separate processes, binding to receptors and movement through the pores; thus, the results are difficult to interpret. Despite this problem, however, the overall effect of temperature is greater than would be expected for a physical process, such as diffusion, and is consistent with the view that an energy requiring step is involved.

Since the translocation of gold particles through the pores is (a) dependent on the properties of the adsorbed RNA-coat, (b) appears to involve a transport process and (c) is not an artifact of the technique, it is concluded that the pathways visualized for the translocation of RNA-coated colloidal tracers are the same as those normally used for endogenous RNA. These pathways are located in the centers of the nuclear pores and have apparent functional diameters of ~230 Å. These results are consistent with those obtained previously by Stevens and Swift (32) for mRNA efflux in *Chironomus* salivary gland cells.

It is not known whether tRNA and 5S RNA complex with specific proteins prior to exiting the nucleus. There is evidence that the poly(A) tails of mRNA bind different polypeptides in the nucleus and cytoplasm (4, 30, 31); however, it has not been determined if these proteins are involved in transport.

The fact that over 97% of the pores are involved in RNA efflux, combined with the earlier observation that the

majority of the pores contained nucleoplasmin-coated particles following cytoplasmic injections (20), suggests that these pathways are bifunctional. The double injection experiments provided direct evidence supporting this view. Thus, it appears that the central channels within the pores can function in the translocation of both RNA and protein. However, this does not necessarily mean that the same molecular mechanisms are employed.

6 h after injection, the nuclear to cytoplasmic concentration ratio of RNA-coated gold was found to be 14:1. Correcting for the difference in the volumes of the two compartments, it is estimated that no more than 36% of the particles entered the cytoplasm. Based on data presented by Zasloff (Table I in reference 35) an equivalent amount of radiolabeled tRNA would be expected to leave the nucleus in ~3 h. There are several factors that could account for this difference: first, the conformation of tRNA could be modified after adsorption to the gold particle. In this regard, Tobian et al. (33) found that point mutations, which alter the conformation of tRNA, also decrease its rate of transport to the cytoplasm. Second, since several tRNA molecules are adsorbed to the gold particles (the exact number has not been determined) the binding avidity to components within the nucleus, pores or even the adjacent cytoplasm could be affected. Third, the size of the tracers could influence the transport rates. Although RNA-coated particles leave the nucleus at a reduced rate, it should be emphasized that the properties of RNA required for translocation through the pores are retained.

Zasloff (35) and De Robertis et al. (11) reported that radiolabeled tRNA is unable to enter the nucleus following injection into the cytoplasm; however, the exchange of 5S RNA across the envelope does appear to be bidirectional (11). Gold particles coated with tRNA, 5S RNA, or poly(A) were able to pass from the cytoplasm into the nucleus, but exchange across the envelope was greatly restricted. Even after 1 h the nuclear to cytoplasmic concentration ratio was only ~0.01. Considering these low rates of uptake, it was surprising to find that RNA-coated particles were observed extending through the centers of the pores. These results could be interpreted to mean that the translocation of RNA through the pores is a reversible process, but that release into the nucleoplasm, which could require a separate mechanism, might be a limiting factor. However, before any definitive conclusion can be drawn it will be necessary to obtain reliable quantitative data concerning the percentage of pores that contain gold and additional information relating to the nuclear uptake rates. Furthermore, it is not known whether translocation is initiated by the adsorbed RNA itself or whether the particles fortuitously bind karyophilic proteins which, in turn, induce transport (27). Hopefully it will be possible to resolve some of these questions using isolated nuclei.

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