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The location of the transport gate in the nuclear pore complex

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SUMMARY

Signal-mediated nuclear transport is a gated process that occurs through a central transporter element located within the pore complex. The purpose of this investigation was to identify the region of the transporter that functions as the gate; i.e. the region that restricts passive diffusion of macromolecules through the pores. To accomplish this, small gold particles coated with polyethylene glycol (PEG; total particle diameter 40-70 Å) or large PEG-particles (total diameter 110-270 Å) were microinjected into the cytoplasm or nucleoplasm of *Xenopus* oocytes. Since PEG does not contain either nuclear import or export signals, it is assumed that the particles distribute by simple diffusion. The cells were fixed after 5 or 30 minutes and subsequently examined using TEM. The distribution of the particles located adjacent to and within the pore complexes was then mapped. The results obtained at both 5 and 30 minutes after cytoplasmic injections of small gold were basically the same. The particles readily entered the transporter but, on the average, were approximately 11 times more concentrated in the cytoplasmic half of this structure. The opposite distribution was observed following nuclear injections, i.e. the particles that were located in the transporter were approximately 7 times more numerous in the nuclear half. Our data indicate that there is a single transport gate located in the central domain of the transporter that restricts passive diffusion. The large particles that were injected into the cytoplasm migrated to the surface of the pore complex, but entered the transporter less frequently than small gold. Interestingly, the diffusion of large PEG-particles to the surface of the pores following nuclear injection was greatly restricted; however, this was not the case for similar size particles that were coated with protein containing nuclear export signals (NES). The latter results suggest that the NES is not only required for translocation, but also for migration within the nucleoplasm.

Key words: Transporter element, Pore complex, Nuclear transport

INTRODUCTION

The nuclear pore complexes represent the sites of macromolecular exchange across the nuclear envelope and, as such, can play a significant role in regulating cellular activity. Each of these structures has a molecular mass of approximately 125×10^3 kDa and is thought to be composed of at least 100 different proteins (nucleoporins; see reviews by Bastos et al., 1995; Doye and Hurt, 1997). The pore complexes exhibit eightfold radial symmetry, and are composed of several distinct structural elements (see reviews by Goldberg and Allen, 1995; Pante and Aebi, 1996). The major element is the spoke-ring assembly (Hinshaw et al., 1992; Akey and Radermacher, 1993), which occupies most of the pore area (the space created by the fusion of the outer and inner nuclear membranes). Extending from the outer and inner surfaces of this assembly are, respectively, the cytoplasmic filaments and the nuclear basket. Signal-mediated macromolecular transport occurs through a cylindrical tubular element (Feldherr et al., 1984; Akey and Goldfarb, 1989; Rutherford et al., 1997), referred to as the transporter, which is located along the central axis of the pore complex, within the spoke-ring assembly. Although the transporter element is a labile structure, and often difficult to preserve for detailed EM studies, a 3-D structural model has been proposed by Akey and Radermacher (1993) based on cryo-electron microscopic analysis. According to this model, the transporter is approximately 625 Å in length, and is made up of two hollow globular domains, each having a diameter of about 420 Å at the widest point. The diameter at the waist of the transporter, where the globular domains join, is approximately 320 Å. The transporter has also been visualized using field emission in-lens scanning electron microscopy (Goldberg and Allen, 1996).

Signal-mediated transport, especially nuclear import, has been studied extensively, and involves the following steps (see reviews by Gorlich and Mattaj, 1996; Corbett and Silver, 1997; Gorlich, 1997; Nigg, 1997): (1) initial binding of the transport substrate, which contains a nuclear localization signal (NLS), to cytoplasmic receptors; (2) association of the substrate-receptor complex with elements of the cytoplasmic fibrils of the pore complex; (3) translocation through the central transporter. The mechanism of translocation through the pores is not well understood; however, there is evidence that several additional cytoplasmic factors, including Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993) and p10/NTF2 (Moore and Blobel, 1994; Paschel and Gerace, 1995) are required, and it has been proposed that the process involves sequential steps of adsorption and desorption of the substrate/receptor complex to nucleoporins that line the transporter element. Signal-mediated nuclear export differs from import in several respects. Different signal sequences have been identified, and, presumably,

different signal receptors are required (see reviews by Fischer et al., 1996; Nakielny and Dreyfuss, 1997). Furthermore, since the pore complexes are asymmetrical, it is likely that different nucleoporins are involved in the initial (binding) stages of export. Aside from these variations, there are also similarities, most notable is the fact that both import and export utilize a common pathway, i.e. the central transporter element (Dworetzky and Feldherr, 1988; Daneholt, 1997).

In addition to signal-mediated transport channels, the pore complexes are known to contain open channels that are approximately 90-100 Å in diameter (Paine et al., 1975; Peters, 1984). Macromolecules below this size are able to cross the envelope by passive diffusion; however, due to steric hindrance and other factors, the rates of passive exchange decrease dramatically as the diameter of the permeant approaches the dimensions of the channel. For example, in *Xenopus* oocytes, dextran with a diameter of 24 Å reaches diffusion equilibrium between the nucleus and cytoplasm in 30 minutes, whereas 46 Å dextran, well below the exclusion limit, has a 15 hours equilibration time (Paine et al., 1975). There are two possible sites for diffusion across the pores. First, it has been suggested that the central transporter element functions in both diffusion and signal-mediated transport (Feldherr et al., 1984). Second, 3-D modeling of the pore architecture indicates that there should be 8 peripheral channels located within the spoke-ring assembly that could also act as diffusion sites (Hinshaw et al., 1992).

Whereas only relatively small molecules are able to diffuse through the nuclear pores, particles with diameters as large as 230-250 Å can enter the nucleoplasm by signal-mediated transport (Dworetzky et al., 1988; Feldherr and Akin, 1990). Clearly, transport is accompanied by dilation of the transporter element. Although this gating process is essential for regulating the nucleocytoplasmic exchange of large targeted substances, essentially nothing is known about the mechanism of dilation, or even the location of the gate. Akey (1990) and Akey and Radermacher (1993) have suggested that both of the globular domains of the transporter act as gates. A dual gating system could have the advantage of blocking non-specific diffusion as large targeted substances are being transported; however, there is no data either supporting or contradicting this hypothesis.

The objective of this investigation is to determine the location of the gating mechanism that functions in signal-mediated transport. The experimental approach is based on the assumption that the gates correspond to the sites, within the transporter elements, that restrict passive diffusion of macromolecules. To locate the diffusion barrier, gold particles coated with PEG, which lacks a nuclear targeting signal, were microinjected into either the nucleus or cytoplasm of stage 6 *Xenopus* oocytes. The subsequent distribution of the particles within and adjacent to the pores was then mapped using TEM, and the data was compared to the 3-D models of the pore complex. The results indicate that each pore contains a single gate that transverses the central, constricted region of the transporter element.

MATERIALS AND METHODS

Xenopus laevis were purchased from Xenopus I (Ann Arbor, MI), and maintained as described previously (Lamian et al., 1996). The frogs were anesthetized by hypothermia, and portions of the ovaries were excised. Late stage 5 and stage 6 oocytes, which were used for microinjection, were manually defolliculated and stored in OR-2 solution (Wallace et al., 1973).

Preparation of BSA-NES conjugates

A peptide, CGGGLQLPPLERLTLDGG, which contains the Rev NES (underlined; Fischer et al., 1995), was synthesized by the University of Florida protein core facility. The peptide was conjugated with bovine serum albumin (BSA; Sigma, St Louis, MO) by using the bifunctional cross-linker *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce, Rockford, IL), as described by Lanford et al. (1986). The NES to BSA ratio was approximately 10, as determined by SDS-PAGE.

Preparation of colloidal gold

Small colloidal gold fractions containing particles ranging from 10 to 40 Å in diameter were prepared by reducing chloroauric acid with a saturated solution of phosphorus in ether. The procedure was identical to that used previously to prepare 's-fraction' gold (Feldherr, 1965) except that the amount of reducing agent was increased 3-fold. Large gold particles, 80 to 240 Å in diameter, were obtained by using sodium citrate as a reducing agent (Frens, 1973). The colloidal particles were coated with either PEG (15-20 kDa; Fisher Scientific, Fair Lawn, NJ) or BSA-NES conjugates, according to the procedure described by Dworetzky et al. (1988). It is estimated that the coating process increases the total particle diameter by about 30 Å. The coated particles were concentrated approximately 200-fold using Centriprep and Microcon concentrators (Amicon, Inc., Beverly, MA), and, finally, dialyzed against intracellular medium (Feldherr and Pomerantz, 1978).

Microinjection procedures

Prior to microinjection, the oocytes were centrifuged at approximately 650 ***g*** for 8 minutes. This procedure, originally employed by Kressmann and Birnstiel (1980), results in the migration of the nucleus to the surface of the animal pole, where it can be easily visualized. Not only does centrifugation facilitate nuclear injections, it also makes it possible to reproducibly inject just adjacent to the cytoplasmic surface of the nuclear envelope: see Feldherr et al. (1984) for details. Cytoplasm and nucleoplasm were injected with approximately 40 and 20 nl of colloid, respectively.

Electron microscopy and sample analysis

The cells were fixed for TEM either 5 or 30 minutes after injection. The details of the procedures used for fixation (glutaraldehyde followed by OsO_4), dehydration, and embedding have been reported previously (Dworetzky and Feldherr, 1988). Sections were examined using a JEOL 100 CX electron microscope.

The relative rates of nuclear import (following cytoplasmic injections) and export (following nuclear injections) of the gold were determined by counting particles in equal and adjacent areas of nucleoplasm (N) and cytoplasm (C). The results are expressed as N/C or C/N ratios, respectively. The specific distribution of gold in the region of the pores was analyzed as follows. Random areas of the nuclear envelopes were first photographed at a magnification of 20 K. All pores that were sectioned transversely through their central region were then analyzed by projecting the images with an enlarger to a final magnification of 530 K. These images were superimposed over outlines of the pores drawn to the same magnification, which also included the components of the pore complex as described by Hinshaw et al. (1992) and Akey and Radermacher (1993); see Fig. 1. The position of the individual gold particles was then marked on the drawings. All particles within radii that extended from the pore center to approximately 1,800 Å into the cytoplasm and 1,500-2,000 Å into the nucleoplasm were recorded.

RESULTS

Cytoplasmic injections

Fig. 2A,B shows electron micrographs of oocytes that were

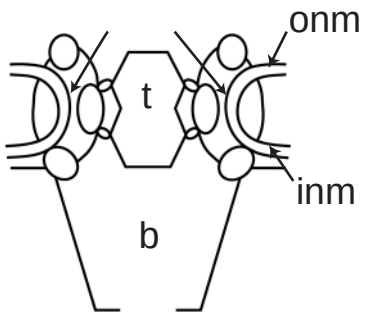

Fig. 1. A diagram of a *Xenopus* oocyte nuclear pore, based on 3-D models proposed by Hinshaw et al. (1992) and Akey and Radermacher (1993). inm, inner nuclear membrane; onm, outer nuclear membrane; t, transporter element; arrows, sites of putative peripheral channels; b, nuclear basket. The remaining unlabeled structures are components of the spoke-ring assembly.

injected near the cytoplasmic surface of the nucleus with either small (Fig. 2A) or large (Fig. 2B) PEG-gold. The N/C gold ratios obtained 5 and 30 minutes after the cytoplasmic injection of small particles were 0.004 and 0.02, respectively, demonstrating that these particles (40 to 70 Å overall diameter) can diffuse through the pores, but at a low rate. In general, these results are consistent with previous diffusion studies using similar size tracers (e.g. Paine et al., 1975; Peters, 1986). As expected, the large PEG-particles were essentially excluded from the nucleus (N/C ratio, 0.0004 after 30 minutes).

The distribution of the small particles within and just adjacent to the pores after 5 and 30 minutes is shown in Fig. 3A and B, respectively. Fig. 3A is a composite of 161 pores in which 1,016 gold particles were analyzed. 7.2% of the particles were located in the region that corresponds to the cytoplasmic half of the transporter element (this region represents approximately 6.5% of the total area examined), but only 0.4% of the particles were found in the nuclear half of the transporter. Basically, the same results were obtained in 30 minute experiments (Fig. 3B). At this time point, 281 pores and a total of 1,083 particles were analyzed; 4.8% were present in the cytoplasmic half of the transporter and 0.7% in the nuclear half. At both time points a relatively small number of particles (approximately 0.3% of the total) were found near the margins of the pore complex, that is, in the area of the proposed peripheral diffusion channels (Hinshaw et al., 1992).

The results obtained 5 and 30 minutes after the injection of large gold are shown in Fig. 3C and D, respectively. Gold was located along the surface of the pores; however, only 1.1% of the particles (average for both times) were present in the transporter, 1/5 the average value obtained for small gold. Furthermore, no particles were detected at the pore margins. A combined total of 369 pores and 809 particles were analyzed.

The above results demonstrate that small PEG-gold has access to the cytoplasmic half of the proposed transporter element, but that migration into the nuclear half is quite restricted. This is consistent with the interpretation that there is a single barrier to diffusion that is located in the central region of the transporter. Since there is limited nuclear uptake of the tracer, the gold in the nuclear half of the transporter element would then represent particles that have diffused across the gate and are in the process of entering the nucleoplasm.

Nuclear injections

If there is a single diffusion barrier at the center of the transporter, as suggested by the above results, it would be expected that small PEG-gold particles entering the pore complex from the nucleoplasm would readily migrate into the nuclear, but not the cytoplasmic domain of the transporter. To test this hypothesis, nuclear injections were performed with both small and large PEG-gold particles.

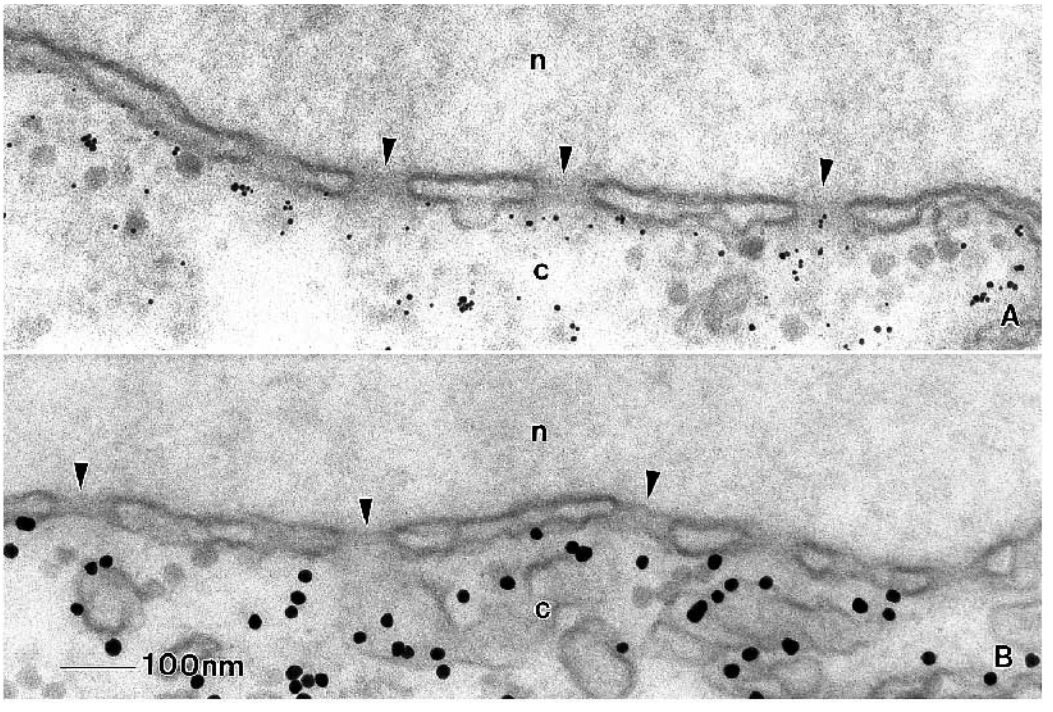

Fig. 2. (A,B) Electron micrographs illustrating the intracellular distribution of small (A) and large (B) PEG-gold particles 5 minutes after cytoplasmic injections. c, cytoplasm; n, nucleus; arrows, nuclear pores.

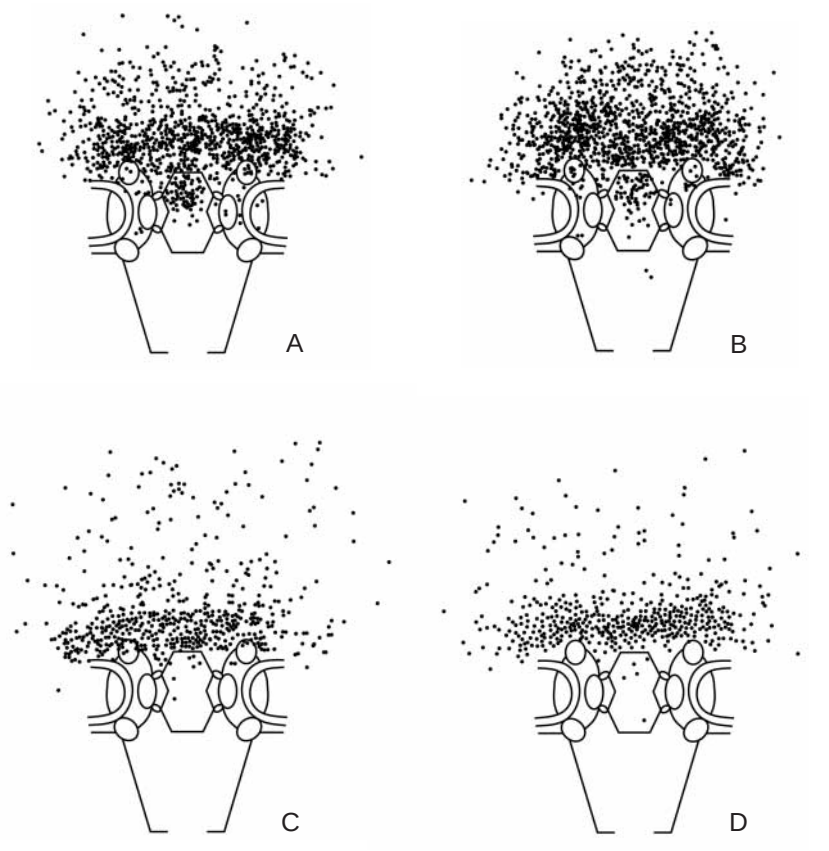

Fig. 3. (A-D) The distribution of small and large PEG-gold particles adjacent to and within the pores following injection into the cytoplasm. The data were compiled as described in Materials and Methods. Each dot represents an individual gold particle. The number of pores and gold particles analyzed is given in parenthesis. (A) Small PEG-gold, 5 minutes after injection (161 pores; 1,061 particles). (B) Small PEG-gold, 30 minutes after injection (281 pores; 1,083 particles). (C) Large PEG-gold, 5 minutes after injection (215 pores; 399 particles). (D) Large PEG-gold, 30 minutes after injection (154 pores; 410 particles).

Oocytes injected with small gold were fixed after 5 minutes. The C/N gold ratio at that time was 0.019, demonstrating that limited export had occurred. The distribution of the particles in the area of the pores is show in Fig. 4A. A total of 120 pores and 1,014 particles were analyzed. 12.7% of the particles were located in the transporter; 11% in the nuclear half and 1.7% in the cytoplasmic half. These results are consistent with the proposal that there is a single, centrally located gate within the transporter element. The greater proportion of particles within the transporter following nuclear, as compared to cytoplasmic injections, is likely due to a higher concentration of active water (water available for diffusion) in the adjacent cytoplasm. This expanation is consistent with the fact that there is limited diffusion of large gold in the nucleoplasm (see below).

Large PEG-gold showed a very different distribution when injected into the nucleus. With the exception of several particles located in an area thought to be occupied by the distal end of the basket, gold was not found along the nuclear face of the pores or within the transporter, even after 30 minutes (see Fig. 4B). These results demonstrate that there is

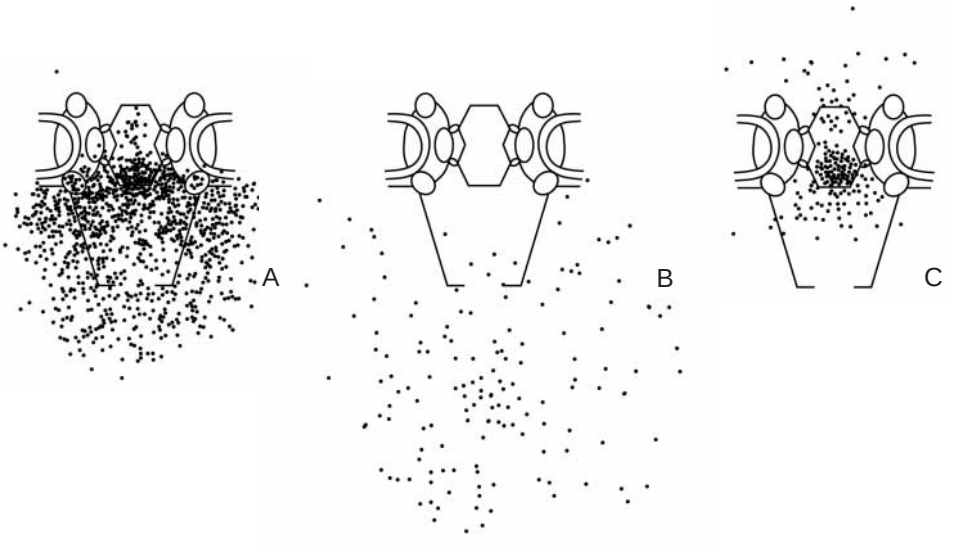

Fig. 4. (A-C) The distribution of pore associated gold particles following nuclear injections. The location of the individual particles is indicated. The number of pores and gold particles analyzed is given in parenthesis. (A) Small PEG-gold, 5 minutes after injection (120 pores; 1,014 particles). (B) Large PEG-gold, 30 minutes after injection (160 pores; 152 particles). (C) Large BSA-NES-gold, 30 minutes after injection (85 pores; 198 particles).

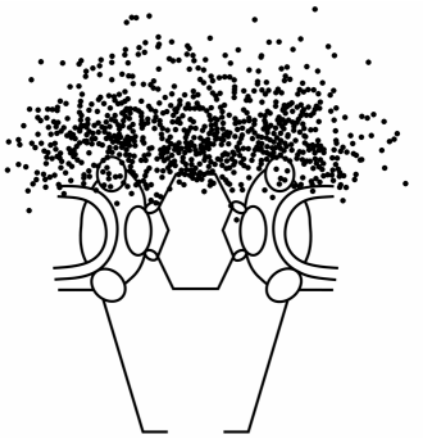

Fig. 5. The gold distribution obtained for pre-fixed nuclei treated with small PEG-gold. 147 pores and 755 particles were analyzed.

restricted diffusion of 110 to 270 Å (total diameter) exogenous particles in the region of the nucleus adjacent to the pores.

Since endogenous materials (specifically RNP particles) that are within the 110 to 270 Å size range rapidly exit the nucleus through the pores (Bataille et al., 1990; Daneholt, 1997), experiments were performed to determine if the intranuclear migration of large particles is facilitated by the presence of export signals. For this purpose, large gold was coated with BSA-Rev NES conjugates and subsequently injected into oocyte nuclei. It has been demonstrated that the Rev export signal is active in *Xenopus* oocytes (Fischer et al., 1995). The location of the BSA-NES-coated gold at the pores after 30 minutes is shown in Fig. 4C. Unlike the PEG-gold, the large particles containing NESs not only had access to the surface of the pore complex, but were found throughout the transporter element and within the cytoplasm (N/C gold ratio 0.65).

Fixation controls

It is necessary to consider the possibility that the gold localizations reported above do not reflect the in vivo migration of the particles, but, instead, are due to a redistribution of the gold and/or alterations in pore structure following fixation. In this regard, experiments were carried out in which stage 6 oocytes were manually enucleated directly into the same glutaraldehyde fixative used in the analysis of microinjection cells. The isolated nuclei were then treated for 5 minutes with small PEG-gold diluted with fixative to approximately the same concentration present in injected cells (PEG does not cross react with glutaraldehyde). The nuclei were then rinsed in fixative, incubated for an additional 30 minutes, at which time they were post fixed in 2% OsO_4 for 30 minutes. The concentration of gold particles associated with the pores was similar to that observed in in vivo injections; however, as seen in Fig. 5, there were significant differences in the distribution. Particles were located along the cytoplasmic face of the pores, and at the outer margin of the transporter, but gold was unable to enter the interior of the transporter, and no particles were found within the nucleoplasm. It was concluded from these findings that the gold localizations observed in the injection experiments were not artifacts of fixation.

DISCUSSION

Analysis of the distribution of small PEG-gold particles within the nuclear pores following microinjection into either the cytoplasm or nucleoplasm demonstrated that the particles readily migrated to the central region of the transporter element. Some migration beyond that point also occurred, but the concentration of particles in the distal half of the transporter (away from the injection site) was, on average, approximately 10-fold lower than that in the proximal half. In control experiments, it was demonstrated that the observed gold distributions were established in vivo, and were not fixation artifacts.

On the basis of the above results we propose that there is a single diffusion barrier located at the center of the transporter, and that this barrier represents the gate for signal-mediated exchange. Since there was limited, passive exchange of the small gold across the envelope, we further suggest that the particles located beyond the central domain of the transporter had diffused through the gate and were in the process of entering the nucleoplasm or cytoplasm (depending on the site of injection). Thus, the transporter element appears to function not only in signal-mediated transport, but also as a site for passive exchange through the pores. The presence of gold at the margins of the pores indicate that, in addition to the transporter, diffusion might also occur through peripheral channels located within the structural framework of the pore complex, as suggested by Hinshaw et al. (1992).

The distribution of large PEG-gold in the pore area following cytoplasmic injection was similar to that found for small particles; however, there were interesting differences. First, the large particles were not detected at the margins of the pore complexes. This is consistent with the proposed dimensions of the peripheral channels, approximately 100 Å in diameter (Hinshaw et al., 1992), which would be too small to accommodate the large gold. Second, compared to small gold, there were proportionately fewer large particles located within the transporter element (an average of 1.1% versus 6.3%, for the small particles). This suggests that passive migration to the transport gate is related to particle size. Since large endogenous materials that are targeted to the nucleus would be subject to the same physical restrictions, it is possible that the early steps in translocation, which include initial binding to cytoplasmic pore filaments followed by directed movement to the transporter element, counteract the effects of reduced diffusion. The early translocation steps, which enhance the accumulation of transport substrates in the vicinity of the pores, would also be expected to facilitate the import of small targeted molecules, even though these substances should be able to diffuse into the pores.

The inability of 110 to 270 Å PEG-gold to reach the transporter element when injected into the nucleus demonstrates that the diffusion of large particles in the vicinity of the pores is greatly restricted. Whether the nuclear basket or the density of the adjacent nuclear material functions as the diffusion barrier could not be determined. It is of interest, however, that similar particles coated with an NES containing protein readily entered the transporter. It would appear, at least for large particles, that the NES is not only required for passage through the pores, but is also necessary for transport to the translocation sites. Structures that could be involved in the latter process include the pore complex basket or a cable system that

connects the pores with the interior of the nucleus. There is evidence, for example, that the basket is involved in mediating the efflux of large messenger RNP particles in *Chironomus* salivary gland cells (Kiseleva et al., 1996). Directed movement along, or within an intranuclear network of cables has also been implicated in the export of endogenous materials through the pores (Ris, 1997).

One advantage of the dual gating system originally proposed by Akey (1990) is that it would prevent diffusion of non-targeted substances from occurring while targeted molecules are being transported. To a large extent this could also be accomplished by a single gate provided that dilation is variable, and only occurs to a degree sufficient to accommodate the substrate-receptor complex. In fact, evidence for variable dilation of the transporter has been obtained by Dworetzky et al. (1988).

The major problems that remain to be resolved regarding the gating process include the characterization of the nucleoporins that make up the gate, and the mechanism of dilation that permits the translocation of large targeted molecules. At this time no nucleoporins have been identified that are localized specifically to the center of the pore complex; however, p62 appears to be present throughout the region occupied by the transporter element (Grote et al., 1995), and could represent at least one component of the gate. Considering the lack of data regarding the molecular composition and organization of the gate, it is difficult at this time to develop models that could explain the dilation process.

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