Signal-mediated Nuclear Transport in Proliferating and Growth-arrested BALB/c 3T3 Cells

Carl M. Feldherr and Debra Akin
Department of Anatomy and Cell Biology, University of Florida, College of Medicine, Gainesville, Florida 32610

Abstract. Mediated transport across the nuclear envelope was investigated in proliferating and growth-arrested (confluent or serum starved) BALB/c 3T3 cells by analyzing the nuclear uptake of nucleoplasmin-coated colloidal gold after injection into the cytoplasm. Compared with proliferating cells, the nuclear uptake of large gold particles (110-270 Å in diameter, including the protein coat) decreased 5.5-, 33-, and 78-fold, respectively, in 10-, 14-17-, and 21-d-old confluent cultures; however, the relative uptake of small particles (total diameter 50-80 Å) did not decrease with increasing age of the cells. This finding suggests that essentially all pores remain functional in confluent populations, but that most pores lose their capacity to transport large particles. By injecting intermediate-sized gold particles, the functional diameters of the transport channels in the downgraded pores were estimated to be ~130 and 110 Å, in 14-17- and 21-d-old cultures, respectively. In proliferating cells, the transport channels have a functional diameter of ~230 Å. The mean diameters of the pores (membrane-to-membrane distance) in proliferating and confluent cells (728 and 712 Å, respectively) were significantly different at the 10%, but not the 5%, level. No differences in pore density (pore per unit length of membrane) were detected. Serum-deprived cells (7-8 d in 1% serum or 4 d in 0.5% serum) also showed a significant decrease in the nuclear uptake of large, but not small, gold particles. Thus, the permeability effects are not simply a function of high cell density but appear to be growth related. The possible functional significance of these findings is discussed.

Many, if not all, macromolecular exchanges between the nucleus and cytoplasm are signal mediated and occur through the nuclear pores (Feldherr et al., 1984; Breeuwer and Goldfarb, 1990; Garcia-Bustos et al., 1991). The signals required for protein transport are frequently short, basic regions consisting of five to eight amino acids; however, more complex signals containing two basic sequences separated by a spacer domain have also been described (Robbins et al., 1991). The available evidence suggests that signal containing polypeptides initially complex with cytoplasmic acceptors (Adam et al., 1990; Newmeyer and Forbes, 1990). Presumably, these complexes then interact with receptors at the cytoplasmic surfaces of the pores to initiate translocation of the targeted polypeptides, a process that may involve dilation of transport channels located within the centers of the pores.

Since exchanges across the nuclear envelope are essential for the maintenance and coordination of cellular activities, modulation of nucleocytoplasmic trafficking patterns can represent an important regulatory mechanism. Examples of proteins whose functions are dependent on the regulation of their intracellular distributions include the glucocorticoid receptor, which is localized in the cytoplasm but is transported to the nucleus in the presence of hormone (Picard and Yamamoto, 1987; Nigg, 1990); the dorsal protein, which enters the ventral, but not the dorsal nuclei in the syncytial blastoderm of Drosophila (Roth et al., 1989; Ruslow et al., 1989; Steward, 1989); and the B lymphocyte protein NF-kB, which is localized in the cytoplasm of unstimulated cells but redistributes to the nucleus in stimulated lymphocytes (Baeuerle and Baltimore, 1988). It is likely that all of the above proteins contain nuclear targeting signals, but that the signals are not always available for transport. In these instances, factors that regulate transport appear to act either by inducing conformational changes that unmask the signal, or releasing the protein from a cytoplasmic anchorage site.

It has been demonstrated, using carrier proteins conjugated with synthetic peptides containing well-characterized nuclear localization signals, that not all targeting sequences are equally effective in initiating nuclear import (Dworetzky et al., 1988; Chelsky et al., 1989; Lanford et al., 1990). In addition, transport rates are known to be dependent on the number of signals present within a given protein (Dingwall et al., 1982; Lanford et al., 1986; Roberts et al., 1987; Dworetzky et al., 1988). Thus, the nuclear import rate of a specific polypeptide can be modulated by its signal content (i.e., the effectiveness and number of targeting regions) as well as the availability of the targeting sequence.

Nucleocytoplasmic protein distributions can also be regulated by factors that have a more direct effect on the transport mechanism per se. For example, increases in the diffusion rates of macromolecules across the nuclear envelope have
been reported during the first and fourth hours after division in HeLa cells (Feldherr and Akin, 1990) and following treatment of 3T3 cells with insulin or EGF (Jiang and Schindler, 1988). Since diffusion occurs through aqueous channels located within the pores, these results reflect changes in the physical properties of the pore complexes that accompany changes in cell activity. Recently, differences in signal-mediated nuclear transport have been detected in proliferating, confluent, and differentiated 3T3-LI cells (Feldherr and Akin, 1990). Transport was studied by microinjection of various-sized nucleoplasmin-coated gold particles into the cytoplasm and determining the subsequent intracellular distribution of the tracer. Both the relative nuclear uptake rate of the particles and the functional diameter of the transport channels (~230 Å vs. 190 Å) were significantly greater in proliferating than in confluent cells. When confluent populations were induced to differentiate into adipocytes, the permeability of the nucleus increased to the level observed in proliferating cells.

The present experiments were designed to investigate further nuclear permeability during different phases of cellular activity, especially with regard to changes in the functional size of the pores. Signal-mediated transport was analyzed in proliferating, confluent, and serum-starved BALB/c 3T3 cells by monitoring the nuclear uptake of nucleoplasmin-coated gold particles after microinjection. It was found that particles with overall diameters (gold plus the protein coat) of ~230 Å were readily transported into the nuclei of proliferating cells. In confluent cells the pores remained active in signal-mediated exchange, but only a small proportion retained the ability to transport particles as large as 230 Å in diameter. In the majority of the pores, the functional diameter of the transport channel appeared to be diminished by ≥100 Å, depending on the age of the confluent cultures. Similar changes in nuclear permeability occurred when growth arrest was induced by serum starvation.

Materials and Methods

Cell Cultures

BALB/c 3T3 A31 cultures were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in DME medium containing 4 mM glutamine and 4.5 g/l glucose and was supplemented with 10% calf serum (CS), penicillin G (10,000 U/ml), streptomycin sulfate (10 mg/ml), and Fungizone (Gibco BRL, Gaithersburg, MD) (250 μg/ml). The stock cultures were maintained in T25 flasks at 37°C in 5% CO₂ and supplemented with 10% CS. After the cells had attached to the ACLAR (10-30 min) the serum concentration was reduced to 0.5 or 1%, and the cultures were maintained at this serum level for 4 or 7-8 d, respectively.

Growth arrest was monitored in confluent and serum-starved cultures by determining the percentage of cells in S phase. The cells were labeled with [3H]thymidine (1 μCi/ml) for 2 h, fixed in a mixture of 30% acetic acid and 70% ethanol, dehydrated in 95% ethanol, coated with Ilford L4 emulsion (Polysciences, Warrington, PA), exposed for 14 d, and examined by use of phase microscopy.

1. Abbreviations used in this paper: CS, calf serum; N/C, nuclear-to-cytoplasmic ratio.
Figure 2. The intracellular distribution of large nucleoplasmin-coated gold particles in a proliferating (a) and 21-d confluent cell (b). Both cells were fixed 30 min after injection. Gold has accumulated in the nucleus of the proliferating, but not the confluent cell. N, nucleus; C, cytoplasm. Bar, 0.5 μm.

the margins of the cleared regions had incorporated the label (data not shown). These results confirm that the confluent populations are growth arrested, and even the oldest experimental cultures are viable.

Nuclear Transport in Proliferating and Confluent BALB/c 3T3 Cells

In initial experiments, large gold particles (80–240 Å in diameter) were coated with nucleoplasmin, and microinjected into proliferating (1-d) and confluent (10-, 14–17-, or 21-d) cultures. The cells were fixed for EM after 30 min. Modulated contrast images of representative experimental cultures are shown in Fig. 1; micrographs showing the gold distribution in proliferating and confluent cells are seen in Fig. 2. The nuclear uptake data, expressed as nuclear-to-cytoplasmic (N/C) ratios, are given in Table I. These values were obtained directly from electron micrographs by counting gold particles in equal and adjacent areas of nucleoplasm and cytoplasm. The N/C ratios decreased progressively with increasing age of the cultures, to a maximum of 78-fold at 21 d. All decreases were statistically significant (Table I). There are two possible explanations for these results. First, the gold
particles were restricted to the site of injection in confluent cells, and, as a result, had only limited access to the nucleus; second, changes in the functional state of the cells were accompanied by changes in the transport capacity of the nuclear envelope.

To determine whether injected particles distributed differently in the different experimental populations, cytoplasmic counts were made in randomly assigned quadrants in 14 proliferating and 13 (21-d-old) confluent cells. The combined results (Fig. 3) are expressed as the percent of the total particle count in each quadrant, proceeding clockwise from the highest value. Assuming that the quadrants containing the most particles represent the injection sites, it would appear that some gold is retained in this area, perhaps because of binding; however, since the cytoplasmic particle distributions are not significantly different in proliferating and confluent cells ($P = 0.194$), it can be concluded that the differences in the N/C ratios are due to changes in nuclear permeability, rather than to the accessibility of gold to the envelope.

The size distributions of the large gold particles present in the nucleus and cytoplasm of proliferating and confluent cells are shown in Fig. 4. The data were collected directly from EM negatives. All particles present in randomly selected (but unequal) areas of nucleoplasm and cytoplasm were measured. Since the same gold fractions were used in the different experimental groups, the cytoplasmic values are presented as a single distribution in the figure. Despite the marked differences in the N/C gold ratios, the size distribution of particles in the nuclei of confluent cells (combined data from 14–17- and 21-d cultures) was not significantly different from that in proliferating cells, as determined by chi square analysis ($P = 0.234$).

Taken together, the data for the nuclear uptake rates and size distributions of the gold particles indicate that only a small number of pores remain fully active in mediated transport during growth arrest. Presumably, most pores are either lost, or have reduced functional capabilities. Two approaches were used to distinguish between these possibilities. First, the number of pores per unit length of membrane was determined by analyzing perpendicular sections through the nuclear envelope; 612 and 748 μm of envelope were examined in proliferating and 21-d confluent cells, respectively. The respective mean pore densities, 0.40 and 0.41 pores/μm, were not significantly different ($P = 0.71$). The pore diameters (membrane to membrane distance) were also measured in proliferating and confluent cells, and the mean values (728 and 712 Å, respectively) were significantly different at the 10% but not at the 5% level ($P = 0.062; n = 400$).

Second, the capacity of the pores to transport small particles was evaluated. Proliferating and 14–17 d confluent cells were microinjected with 50–80 Å (overall diameter) gold particles coated with either nucleoplasm or BSA. The cells were fixed for EM 30 min after injection. As shown in Table II, the N/C ratios for small nucleoplasm–coated particles were not significantly different in proliferating vs. confluent cells. Since it is theoretically possible for these particles to diffuse through the pores, it was necessary to demonstrate that nuclear uptake was, in fact, signal-mediated. For this purpose, BSA-gold particles, which lack targeting signals, were injected into proliferating cells. The N/C ratios for BSA-gold (Table II) were over two orders of magnitude less than those obtained for nucleoplasm-coated particles, demonstrating that the latter results were not due to simple diffusion.

The data indicate that the nuclear pores in proliferating and confluent cells are maintained at the same density (pores per μm).
per micrometer of envelope) and are equally effective in the signal-mediated transport of small particles. The major functional difference is the exclusion limit for translocation through the central pore channels. In proliferating cells, coated gold particles with an overall diameter of ~230 Å can readily enter the nucleus; however, most pores in confluent cells are unable to transport tracers of this size. To determine the overall nuclear exclusion limit in the latter cell populations, the nucleocytoplasmic distribution of intermediate-sized gold particles (overall diameter 50–150 Å) was investigated after microinjection. The gold was stabilized with nucleoplasmin, and the cells were fixed 30 min after injection. The size distributions of gold particles in the nuclei of 14- and 19-d confluent cells were analyzed in two separate experiments and compared with results obtained for proliferating cells injected with the same gold fractions (Fig. 5). The size distributions of the particles in the nucleoplasm of proliferating vs. 14- and 19-d cultures were significantly different (P < 0.001 in both cases). Particles having an overall diameter larger than 130 and 110 Å were restricted from entering the nuclei in 14- and 19-d-old cells, respectively. As expected, the cutoff points are not absolute since a small proportion of the pores can apparently transport particles with an overall diameter of ≤230 Å (Fig. 4).

### Nuclear Transport in Serum-starved BALB/c 3T3 Cells

The decrease in nuclear permeability that accompanies confluence could be caused by changes in several factors, including cell shape, cell density, and metabolic activity. In an initial effort to distinguish among these possibilities, signal-mediated transport was investigated in serum-starved cells, which are metabolically similar to confluent cells but can be maintained at lower densities. Furthermore, serum-starved cells remain extended during growth arrest.

Experimental cells were maintained in medium containing either 1% serum for 7 or 8 d or in 0.5% serum for 4 d. To verify that growth arrest had occurred, 8-d 1%, and 4-d 0.5% serum cultures were incubated in [3H]thymidine; 5.6% and 0.6% of the cells were labeled, respectively, as compared with 34.5% incorporation of label in proliferating cultures. Incorporation of [3H]thymidine returned to the proliferating level, and cell division resumed once the growth-arrested, serum-starved cultures were provided with 10% CS.

Proliferating and serum-starved cells were injected with either large or small gold preparations (overall diameters 110–270 and 50–80 Å) that were stabilized with nucleoplasmin. The cells were fixed for electron microscopy after 30 min. Table III shows that the nuclei of proliferating cells incorporated large gold particles at a significantly greater rate than either of the serum-starved populations; however, there was no significant difference in the uptake of small particles. The size distributions of the large particles that entered the nuclei in the 1% and 0.5% serum-starved populations (Fig. 6) were also significantly different from the distribution in proliferating cells (P < 0.001 in both cases). Except for the latter results, the nuclear permeability changes that occurred during serum starvation paralleled those observed for confluent cells.

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**Table II. N/C Ratio in Proliferating vs. Confluent BALB/c Cells: Fraction Containing Small Gold Particles**

| Experiment     | Coating/Agent | Cells | Total particles counted | N/C ratio ± SE | Significance* |
|----------------|---------------|-------|-------------------------|----------------|--------------|
| Proliferating  | NP            | 28    | 4,795                   | 2.33 ± 0.17    | ns P = .284  |
| 14–17-d Confluent | NP          | 25    | 4,365                   | 2.05 ± 0.19    | s P << .001  |
| Proliferating  | BSA           | 13    | 2,516                   | 0.016 ± 0.003  | s P << .001  |

* The results of each experimental group were compared with the data obtained for proliferating cells. s, significantly different; ns, not significant.

† NP, nucleoplasmin.
**Discussion**

In this study, an overall decrease in signal-mediated nuclear transport was detected when proliferating BALB/c 3T3 cells reached confluence. In general these results are similar to those reported previously for 3T3-L1 cells (Feldherr and Akin, 1990); however, there are several specific differences in the transport patterns in the two cell lines. First, there was a graded decrease in nuclear permeability in the BALB/c cells with increasing age of the confluent cultures. This effect was not observed in 3T3-L1 cells. Second, the maximal decrease in the N/C ratio for large gold particles in confluent, as compared with proliferating, BALB/c cells (78-fold), was an order of magnitude greater than in 3T3-L1 cultures. Third, in confluent 3T3-L1 cells, the decrease in the nuclear uptake of large particles was accompanied by a significant decrease in the size of the particles able to enter the nucleus. No change in size distribution was observed in confluent BALB/c fibroblasts. A significant size decrease did occur in serum-starved cells; however, large particles (overall diameter \(\leq 230 \text{ Å}\)) were present in the nucleoplasm.

These results suggest that the functional size of the entire pore population decreases when 3T3-L1 cells become confluent. In BALB/c cells it appears that a small population of pores retains the capacity to transport large particles. The fact that the nuclear uptake of small gold particles did not decrease in either confluent or starved BALB/c cells indicates that most, if not all, of the pores remain active in transport but contain functionally smaller transport channels. The diameters of the downgraded channels in 14- and 19-d-old cells were estimated to be 130 and 110 Å, respectively. The observed decrease in signal-mediated nuclear transport in confluent BALB/c cell populations was not due simply to an increase in cell density, since similar results were obtained in low-density, serum-starved cultures.

As an initial step toward establishing a possible regulatory role for the nuclear permeability changes that accompany growth arrest, it is useful to consider which essential macromolecular exchanges would be most affected by a decrease in pore size. In this regard, the functional diameter of the transport channels in 14- and 19-d BALB/c cultures (130 and 110 Å, respectively) is sufficiently large to accommodate most, if not all, proteins that normally enter the nucleus, but the export of both mRNP particles and ribosomal subunits would very likely be restricted. Limited export of these particles would occur, however, since a small population of pores appears to retain full transport capacity.

Differences in the cytoplasmic content of mRNA and rRNA have been investigated in proliferating and resting fibroblasts by Johnson et al. (1974). They reported that growing 3T3 cells contain 4.0- and 2.8-fold more poly(A)+ mRNA and rRNA, respectively, than resting cells. The higher mRNA content in proliferating cultures is apparently related to a greater efficiency of hnRNA processing (Johnson et al., 1975; Johnson et al., 1976). The regulation of rRNA is likely to be more complex, involving changes in transcription, turnover rates, and processing of precursor RNA (Johnson et al., 1976). Consistent with the idea that nuclear permeability might also be a factor in regulation, it was found that the nuclear retention time for both rRNA and mRNA was greater in resting cells (Johnson et al., 1976). Although regulation of RNA efflux at the level of the pores is a plausible mechanism for modulating overall metabolic activity, no direct supporting evidence is available.

Several factors could contribute to the variations in signal-mediated nuclear transport in proliferating and growth-arrested cells. For example, transport could be related to cell shape. Ingber and Folkman (1989) and Ingber (1990) emphasized the close correlation between the extended shape of proliferating cells and nuclear activity. They developed a model, based on the organization of cytoskeletal elements, by which tension is transmitted from the cell surface to the nucleus, and proposed that an increase in the cross-sectional area of the nucleus during growth could increase DNA syn-

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**Table III. N/C Ratio in Proliferating vs. Serum-starved BALB/c Cells**

| Experiment | Gold particle size | Cells | Total particles counted | N/C ratio ± SE | Significance* |
|------------|-------------------|-------|-------------------------|----------------|--------------|
| Proliferating | 20-50 | 23 | 3,911 | 1.98 ± 0.16 | ns P = .389 |
| 4-d, 0.5% CS | 20-50 | 8 | 1,742 | 1.74 ± 0.14 | ns P = .438 |
| 7-d, 1.0% CS | 20-50 | 13 | 3,726 | 2.19 ± 0.23 | ns P = .389 |
| Proliferating | 80-240 | 19 | 1,027 | 1.90 ± 0.20 | s P << 0.001 |
| 4-d, 0.5% CS | 80-240 | 29 | 1,274 | 0.20 ± 0.04 | s P << 0.001 |
| 7-8-d, 1% CS | 80-240 | 21 | 933 | 0.31 ± 0.05 | s P << 0.001 |

* The data from each experimental group were compared with the data obtained for proliferating cells. s, significantly different; ns, not significant.

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**Figure 6.** A comparison of the size distributions of large nucleoplasmin-coated gold particles present in the nuclei of proliferating, 4-d 0.5% serum-starved, and 7-d 1% serum-starved cells; 489, 232, and 227 particles were measured, respectively. The particle sizes do not include the protein coat. ㎡, Proliferating nuclei; ㎡, 4-d, 0.5% CS nuclei; ㎡, 7-d, 1% CS nuclei.
thesis by perturbing the nuclear matrix or, perhaps, by altering rates of nucleocytoplasmic transport. This concept is in agreement with the decreases in pore diameter detected in confluent (less extended) BALB/c cells in this study and also in confluent 3T3-L1 cultures (Feldherr and Akin, 1990). However, the fact that serum-starved cells, which also exhibit a significant decrease in nuclear transport, remain in an extended state indicates that shape alone is not sufficient to explain the observed permeability changes. It is likely, as suggested by Ingber and Folkman (1989), that shape is a "permissive regulator," but that other factors are also required. Consistent with this possibility is the report of Jiang and Schindler (1988), who found that insulin and epidermal growth factor caused a threefold increase in the diffusion of fluorescein-labeled dextrans into the nuclei of adhering, but not spherical, fibroblasts.

Variations in permeability could also be caused by alterations in the composition of the pore complex. These alterations could include changes in protein content, or the level of postsynthetic modifications, such as glycosylation or phosphorylation. Similarly, quantitative or qualitative differences in required cytoplasmic factors could affect transport. There is evidence that cytoplasmic acceptors are necessary for nucleocytoplasmic exchange (Adam et al., 1990; Newmeyer and Forbes, 1990), and it is thought that these factors function by mediating the binding of targeted molecules to the pore surfaces. Since the extent of binding to the pores can modulate both the rate of exchange and the functional size of the transport channel (Dworetzky et al., 1988), differences in the availability of acceptors could account for the growth related changes in nuclear permeability. Finally, overall differences in energy metabolism could influence the rate of ATP-dependent translocation through the pores. Experiments are currently in progress to distinguish among these possible regulatory mechanisms.

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