Multiple Pathways in Nuclear Transport: The Import of U2 snRNP Occurs by a Novel Kinetic Pathway

Neil Michaud and David S. Goldfarb

Department of Biology, University of Rochester, Rochester, New York 14627

Abstract. Protein import to the nucleus is a signal-mediated process that exhibits saturation kinetics. We investigated whether signal bearing proteins compete with U2 and U6 snRNPs during import. When injected into *Xenopus* oocytes, saturating concentrations of P(Lys)-BSA, a protein bearing multiple nuclear localization signals from SV40 large T-antigen, reduce the rate of [^{125}I]P(Lys)-BSA and of [^{125}I]nucleoplasmin import, consistent with their competing for and sharing the same limiting component of the import apparatus. In contrast, saturating concentrations of P(Lys)-BSA do not reduce the rate of HeLa [^{32}P]U2 snRNP assembly or import. The import of U6 snRNP is also competed by P(Lys)-BSA. We conclude that U2 snRNP is imported into oocyte nuclei by a kinetic pathway that is distinct from the one followed by P(Lys)-BSA, nucleoplasmin, and U6 snRNP.

NUCLEAR transport is necessary not only for housekeeping cellular functions such as mRNA expression, ribosome assembly, and the biogenesis of the nucleus itself, but also for the regulation of gene expression during the cell cycle, in development, and in response to a changing environment. The bidirectional nature of nuclear transport is unique. Shuttling proteins cross the nuclear envelope repeatedly (Borer, 1989) or, in the case of ribosomal proteins, first in and then out as ribosomal subunits. Analogously, U snRNAs are exported and then, after assembly into U snRNPs, are reimported (Zieve and Sauterer, 1990).

The centerpiece of this process is the nuclear pore complex (NPC)[1] (Dingwall and Laskey, 1986; Newport and Forbes, 1987; Gerace and Burke, 1988; Goldfarb, 1989). The transporter assembly, located in the middle of the NPC, is the predominant site of karyophile binding and contains a nuclear localization signal (NLS)-triggered transport channel that can dilate to pass larger karyophiles (Akey and Goldfarb, 1989; Akey, 1990). Besides mediating NLS dependent import and RNA/RNP export, the NPC contains a ~100-Å-diameter pore, the nuclear pore, which allows the passive diffusion of microinjected inert macromolecules such as branched dextrans (Peters, 1986). It had been assumed that smaller nuclear proteins could use this pore to enter the nucleus. However, recent evidence indicates that instead they do not diffuse through the nuclear pores but are complexed by factor(s) in the cytoplasm and must, therefore, use a receptor-mediated import pathway (Breeuwer and Goldfarb, 1990). Curiously then, although the sievelike properties of the nuclear envelope are well confirmed, not a single physiologically relevant macromolecule has been shown to traverse the nuclear envelope by diffusion (Peters, 1986). Without known exception, therefore, nuclear transport is a tightly regulated process.

The export of tRNA (Zasloff, 1983) and 40S and 60S ribosomal subunits (Khanna-Gupta and Ware, 1989; Bataillé et al., 1990), and the import of karyophilic proteins (Goldfarb et al., 1986) have been shown by kinetic criteria to be receptor-mediated processes. While translocation across the nuclear envelope appears to require metabolic energy (Richardson et al., 1988; Newmeyer and Forbes, 1988), initial transport intermediates probably form in the absence of ATP (Newmeyer and Forbes, 1988; Richardson et al., 1988; Akey and Goldfarb, 1989; Newmeyer and Forbes, 1990; Breeuwer and Goldfarb, 1990). These intermediates may involve the activity of various cytoplasmic and nuclear NLS binding proteins that have been put forward as putative transport receptors (Adam et al., 1989; Yamasaki et al., 1989; Silver et al., 1989; Lee and Melese, 1989; Li and Thomas, 1989; Benditt et al., 1989).

A number of specific examples of regulated import are known. In *Tetrahymena*, where the protein composition of the micronucleus and macronucleus differ, these two nuclei exhibit distinct capabilities to import certain microinjected karyophilic proteins (White et al., 1989). Another case of nuclear discrimination occurs for the *Drosophila* protein dorsal. In the fertilized embryo, after the migration of nuclei to the periphery of the syncytium, dorsal becomes localized to ventral nuclei but remains excluded from dorsal nuclei (Rushlow et al., 1989; Steward, 1989; Roth et al., 1989). Some karyophilic proteins exhibit delayed entry into nuclei during development (Dreyer and Hausen, 1983; Borer et al.,

1. *Abbreviations used in this paper*: M_3G, trimethyl guanosine; NLS, nuclear localization signal; NPC, nuclear pore complex.

1989). For example, c-*myc* is cytoplasmic in *Xenopus laevis* oocytes but later accumulates in nuclei that form after fertilization (Taylor et al., 1986; Gusse et al., 1989).

An interesting class of proteins reside in the interphase cytoplasm until, in response to an extracellular signal, they migrate, sometimes reversibly, into the nucleus. This class includes cAMP dependent protein kinase, NF-κB enhancer binding protein, and the glucocorticoid receptor (Nigg, 1990). The mechanism of regulation for most, if not all, of these examples is probably the regulated association and dissociation of a complex between the karyophile and a karyophile-specific cytoplasmic anchor or NLS masking factor. When released from the complex, the karyophile then associates with the cell's nuclear transport apparatus. In fact, the probable anchoring or signal masking proteins have been identified for the dorsal protein (Hunt, 1989) as well as for several of the other aforementioned examples (Nigg, 1990).

U snRNPs are a unique class of macromolecular complexes that are assembled in the cytoplasm and function in the nucleus. The Ul-5 snRNPs are each composed of a small nuclear RNA, transcribed by RNA pol II, and a number of common Sm proteins (with the exception of U3) and, in certain cases, U snRNP-specific proteins (Luhrmann, 1988; Reddy and Busch, 1988; Bach et al., 1989). The Sm proteins assemble onto a consensus single stranded region of Sm-type U snRNAs that is required for both assembly, import (Mattaj and De Robertis, 1985), and cap trimethylation (Mattaj, 1986). Hamm et al. (1990) have suggested that the Sm binding site and the trimethylguanosine cap (M_3G) of Ul snRNA together comprise a bipartite nuclear targeting signal (see Fischer and Lührmann, 1990). U6 snRNP, which is transcribed by RNA pol III, contains a 5' gamma-monomethyl triphosphoguanosine cap (Singh and Reddy, 1989) and, instead of a consensus Sm protein binding site, a single-stranded region that may function analogously (Hamm and Mattaj, 1989; Hamm et al., 1990).

In the present study, we provide kinetic evidence that U2 snRNP employs a novel nuclear import pathway. These experiments were performed in *Xenopus* oocytes using P(Lys)-BSA (BSA cross-linked with synthetic peptides based on the SV40 large T-antigen nuclear localization signal), nucleoplasmin (a major oocyte nuclear protein), and U2 and U6 snRNAs isolated from Hela cells. We found that import of both nucleoplasmin and U6 snRNA is competed by saturating concentrations of P(Lys)-BSA. Importantly, however, saturating concentrations of P(Lys)-BSA did not inhibit the rate or extent of U2 snRNP import. By this criterion, therefore, U2 snRNP uses a novel kinetic pathway.

Materials and Methods

Proteins

Nucleoplasmin was purified from *Xenopus laevis* oocytes and P(Lys)-BSA was prepared using RNase-free BSA (Boehringer-Mannheim Diagnostics, Inc., Houston, TX) as described by Breeuwer and Goldfarb (1990). Synthetic peptides were provided by Dr. John Wester of Syntex Research.

Iodination of Proteins

P(Lys)-BSA and nucleoplasmin were iodinated using Chloramine T. Proteins were brought to 28 μl 125 mM Na_2HPO_4, pH 7.5, at a concentration of 2.5 μg/μl. 10 μl of 1 μg/μl Chloramine T was combined with 0.2 mCi Na[^{125}I] (Pharmacia Fine Chemicals, Piscataway, NJ), and the mixture was incubated with the protein for 15 s at ambient temperature. 5 μl of saturated L-cysteine was added to stop the labeling reaction and the sample was collected from a 5.0 ml sephadex G-25 column equilibrated with 25 mM Na_2HPO_4, pH 7.5. Labeled proteins were washed and concentrated in centricon 30 filtration units (Amicon Corp., Danvers, MA). The specific activity of labeled proteins was 500–2,000 cpm/ng.

Isolation of HeLa U2 and U6 snRNA

HeLa cells were grown in T75 flasks in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO_2. Cells were dislodged with trypsin-EDTA (Gibco Laboratories), pelleted by low speed centrifugation, and washed with phosphate-free Hank's Salts. Pelleted cells were resuspended at a density of 2–4 × 10^5 cells/ml in MEM (Gibco Laboratories) without phosphate, supplemented with 10% dialyzed FBS (Gibco Laboratories). Cells were labeled with 10 mCi [^{32}P]orthophosphate at 37°C for 16–24 h. Labeled cells were pelleted by low speed centrifugation and washed with cold PBS (0.9% NaCl, 10 mM Na_2HPO_4, pH 7.5). Cells were lysed in 5 ml 7 M urea, 2% SDS, 5 mM EDTA, 300 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mg/ml proteinase K. Protein was digested by incubation at 50°C for 1 h. After extraction with phenol/chloroform and chloroform, total nucleic acids were precipitated with 2.5 vol ethanol. DNA was removed by spooling with a closed microcapillary tube, and the remaining RNA was pelleted by centrifugation in an SS-34 rotor (Dupont, Wilmington, DE) at 8,000 rpm for 30 min at 4°C. RNA pellets were resuspended in 100 μl formamide loading buffer (85% formamide, 0.5× TBE, 0.1% SDS), heated at 95°C for 5–10 min, and then chilled on ice. RNA was electrophoresed through 8% acrylamide, 7 M urea, 1× TBE gels at 25–30 mAmps constant current for 1.5 h. RNA was located by autoradiography and regions of the gel containing U2 snRNA, U6, 5S, and tRNA were excised, crushed, and incubated overnight at ambient temperature in 300 μl 0.3 M NH_4Ac, 1 mM EDTA, 0.1% SDS. RNA was collected by low speed centrifugation through silanized glass wool and its concentration was determined by absorbance at 260 nm. The specific activity was determined by scintillation counting. After extraction with phenol/chloroform and chloroform, the RNA was precipitated in 0.3 M NaAc and 2.5 vol ethanol with 20–40 μg carrier yeast Phe-tRNA. RNA was pelleted by centrifugation at 14,000 *g* for 30 min at 4°C, washed with 70% ethanol, and resuspended in distilled water.

Microinjection of Xenopus Oocytes

Stage 6 oocytes were obtained from *Xenopus laevis* females by partial ovariectomy. Individual oocytes were defolliculated and maintained in OR-2 (Zasloff, 1983) at ambient temperature before microinjection. 50 nl of sample was injected equatorially, and the oocytes were incubated in OR-2 at ambient temperature for indicated times. The final intracellular concentrations of injected material are indicated in the figure legends. To quantitate transport of iodinated proteins, oocytes were fixed in 20% TCA. Nuclei were separated from oocytes and radioactivity in single nuclei and cytoplasms was quantified with a multi gamma counter (1261; LKB Instruments, Inc., Gaithersburg, MD). To quantitate RNA transport, oocytes were enucleated in 50 mM NaAc (pH 5.2), nuclei and cytoplasms were pooled separately, and were solubilized in 7 M urea buffer and digested with 0.1 μg/μl proteinase K at 50°C for 30–60 min. After phenol/chloroform extraction, RNA was precipitated with 2.5 vol ethanol. Nuclear and cytoplasmic RNA was pelleted at 14,000 *g* for 30 min at 4°C, washed with 70% ethanol, and resuspended in 20–40 μl formamide loading buffer. Samples were heated at 95°C for 5–10 min, chilled on ice, and electrophoresed through 8% acrylamide, 7 M urea, 1X TBE gels at 25–30 mAmps for 1.5 h. Gels were dried and bands were located by autoradiography. Bands were excised and radioactivity quantified by scintillation counting (Ecoscint; National Diagnostics, Inc., Manville, NJ).

Immunoprecipitation

Anti-Sm mouse monoclonal 7.13 antibodies were conjugated to protein A-sepharose beads with 10 μg rabbit anti-mouse IgG in LNET40 (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.1% NP-40) at 4°C. Anti-P(Lys) antiserum (Goldfarb et al., 1986) was conjugated directly to protein A-sepharose in LNET40. Oocyte nuclei, isolated in 50 mM NaAc, pH 5.2, or whole oocyte extracts were prepared in 500 μl 20 mM Tris-HCl, pH 7.5, 20 mg/ml heparin, 1 mM EDTA, and incubated with the beads with rotation at 4°C overnight. Beads were washed three times with 1.0 ml LNET40. Bound U snRNP antigen was released with 300 μl 7 M urea buffer at 95°C for 10 min. Released U snRNP proteins were digested with 0.1

mg/ml proteinase K at 50°C for 20 min and phenol/chloroform extracted. RNA was ethanol precipitated and processed for gel electrophoresis as described above. Bound P(Lys)-BSA antigen was released in 50 μl Laemmli sample buffer at 95°C for 10 min.

Results

HeLa U2 snRNA Accumulates in the Nuclei of Xenopus Oocytes

The cytosol of *Xenopus* oocytes contains large stores of uncomplexed U snRNP proteins that are normally recruited by U snRNAs transcribed and exported later in development (Mattaj, 1988). These oocyte U snRNP proteins will spontaneously assemble onto microinjected U snRNA (De Robertis et al., 1982). Uncomplexed U snRNP proteins are also present in the cytosol of somatic tissue culture cells (Sauterer et al., 1988). Initially we characterized the kinetic import properties of HeLa U2 snRNA and several other small RNAs after their microinjection into the cytoplasm of *Xenopus* oocytes. Our results are consistent with those reported by De Robertis et al. (1982). ^{32}P-labeled HeLa U2, U1, 5.8S, 5S, and tRNA were microinjected into oocyte cytoplasms and their nucleocytoplasmic distributions as a function of time analyzed by gel electrophoresis and autoradiography (Fig. 1 *A*). The extent of import was quantified by excision and scintillation counting of the U2, 5S, and tRNA bands from these gels (Fig. 1 *B*). U1 and U2 snRNA and to a lesser extent 5S RNA accumulated in nuclei while 5.8S RNA and tRNA were excluded (Fig. 1, *A* and *B*). After 20 h, ~70% of injected U2 snRNA localized to the nucleus (Fig. 1 *B*). The total counts retrieved from the U2 bands were relatively constant throughout the time course, indicating that the molecule is stable in both the cytoplasm and nucleus (data not shown).

Competition between Two Karyophilic Proteins

P(Lys)-BSA (~90 kD) is comprised of BSA conjugated with 12–17 NLS peptides (Goldfarb et al., 1986). The synthetic peptide P(Lys) is a useful tool because it contains only the

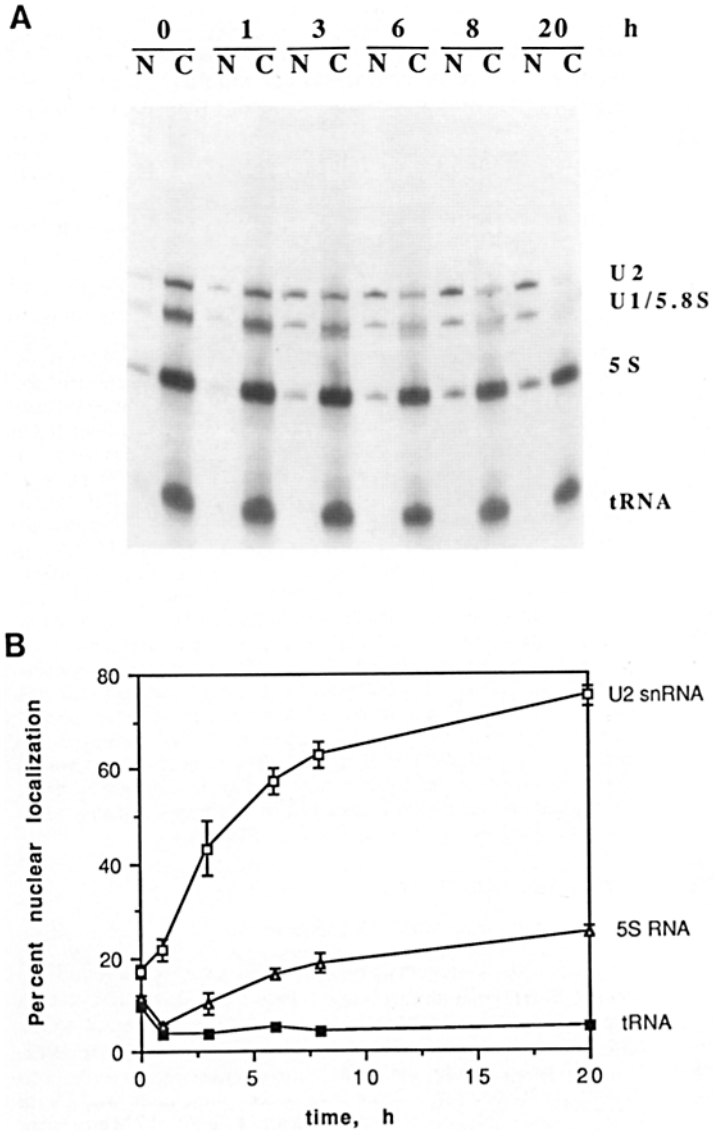

Figure 1. Accumulation of small RNAs in *Xenopus* oocyte nuclei. (*A*) ^{32}P-labeled HeLa U2 and U1 snRNA, 5S RNA, and tRNA were mixed and injected into the cytoplasm of *Xenopus* oocytes. Nuclear (*N*) and cytoplasmic (*C*) RNA isolated from groups of five oocytes incubated for the indicated time were analyzed by denaturing acrylamide gel electrophoresis and autoradiography. (*B*) Quantitation of small RNA nuclear accumulation. Bands were excised from denaturing acrylamide gels, scintillation counted, and percent nuclear localization determined. Each point is the mean of three groups of five oocytes. The error bars indicate the SEMs. Error bars for tRNA transport are included but are very small.

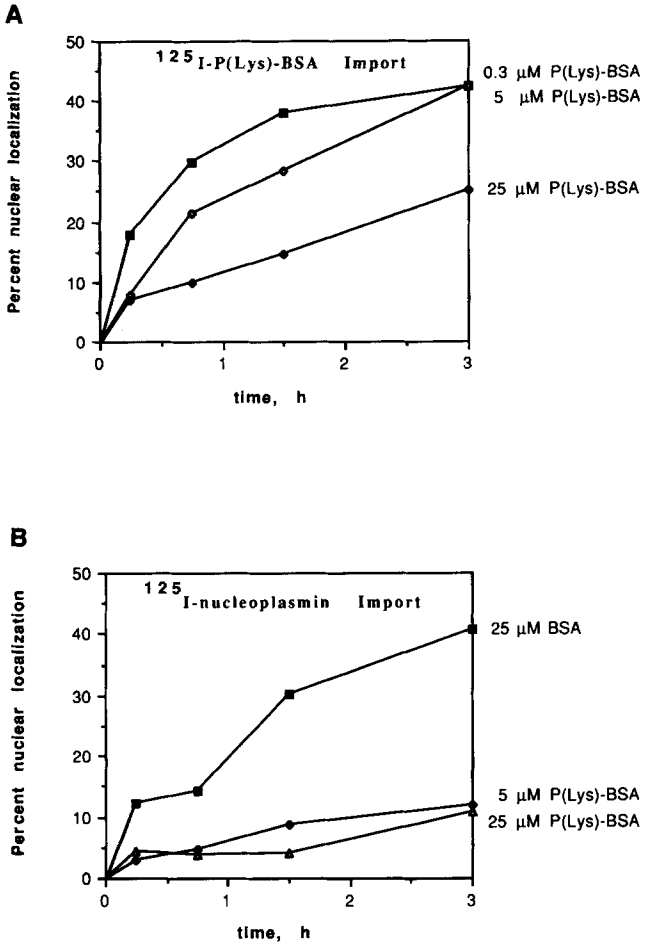

Figure 2. P(Lys)-BSA competition of [^{125}I]P(Lys)-BSA and [^{125}I]-nucleoplasmin nuclear import. (*A*) [^{125}I]P(Lys)-BSA was injected into the cytoplasm of *Xenopus* oocytes to a cellular concentration of 0.3 μM alone (*closed squares*) or with 5 μM (*open diamonds*) or 25 μM (*closed diamonds*) unlabeled P(Lys)-BSA. Nuclei and cytoplasms from TCA-fixed oocytes were separated and [^{125}I]P-(Lys)-BSA in each fraction was determined. Each point is the mean of 10–15 oocytes. *t*-tests were done on all pairs of values for each time. Differences were statistically significant between all pairs except 5 and 25 μM at 15 min and 0.3 and 5 μM at 3 h. (*B*) [^{125}I]-nucleoplasmin was injected into *Xenopus* oocytes with 5 μM P-(Lys)-BSA (*closed diamonds*), 25 μM P(Lys)-BSA (*open triangles*), or 25 μM BSA (*closed squares*) and processed as in *A*. Each point is the mean of 10–15 oocytes. T-tests indicate statistically significant differences between [^{125}I]nucleoplasmin import in the presence of BSA and import in the presence of either 5 or 25 μM P(Lys)-BSA at every data point.

minimal T-antigen NLS and would not be expected to bind cellular factors other than those specifically involved in nuclear transport. Similar peptides have been employed as affinity reagents to identify putative transport receptors (Adam et al., 1989; Yamasaki et al., 1989; Silver et al., 1989; Lee and Melese, 1989; Li and Thomas, 1989; Benditt et al., 1989). P(Lys)-BSA accumulates in the nuclei of oocytes (Goldfarb et al., 1986), a variety of vertebrate tissue culture cells (Lanford et al., 1986; Chelsky et al., 1989; Lanford et al., 1990), and *Tetrahymena* (White et al., 1989). The import of P(Lys)-BSA into oocyte nuclei occurs with a K_m apparent of ~2 μM and a V_{max} of ~200 molecules pore^{-1} min^{-1} (Goldfarb et al., 1986). Fig. 2 *A* shows that the rate, but not the extent, of [^{125}I]P(Lys)-BSA import is influenced by 5 μM P(Lys)-BSA. 25 μM P(Lys)-BSA significantly reduced its import. These kinetics are consistent with the saturation of a limiting transport component. In similar time course experiments, 5 μM and 25 μM P(Lys)-BSA reduced the rate of [^{125}I]nucleoplasmin import almost to background levels (Fig. 2 *B*). The initial rate but not the final extent of [^{125}I]nucleoplasmin import was also measurably competed by 2 μM P(Lys)-BSA (not shown). Thus increasing concentrations of P(Lys)-BSA increasingly inhibit the import of both [^{125}I]P(Lys)-BSA and [^{125}I]nucleoplasmin. By this criterion, then, both P(Lys)-BSA and nucleoplasmin share a limiting component of the transport apparatus. Although we were unable to directly saturate nucleoplasmin import, the competition studies presented here, together with results from other laboratories (Finlay et al., 1989), supports the dogma that the nuclear import of native cellular proteins is receptor mediated.

P(Lys)-BSA Does Not Compete the Import of U2 snRNA

The effect of increasing concentrations of P(Lys)-BSA on the import of [^{125}I]P(Lys)-BSA, [^{125}I]nucleoplasmin, and [^{32}P]-U2 snRNA is presented as the percent reduction in import relative to uncompeted transport at 45 min (Fig. 3). Interestingly, in this and other experiments, P(Lys)-BSA exhibits a lower K_i apparent for [^{125}I]nucleoplasmin than for [^{125}I]P-(Lys)-BSA import. Although these two proteins associate with the same limiting component, they apparently do so with different affinities (see below). Significantly, the import of [^{32}P]U2 snRNA at 45 min is unaffected by concentrations of P(Lys)-BSA that are sufficient to almost completely abolish the import of [^{125}I]P(Lys)-BSA and [^{125}I]nucleoplasmin (Fig. 3). Fig. 4 *A* shows a time course of [^{32}P]U2 snRNA import in the presence of either 20 μM BSA or 20 μM P(Lys)-BSA. In this experiment, to confirm that saturation of the P(Lys)-BSA pathway had been achieved, [^{125}I]P(Lys)-

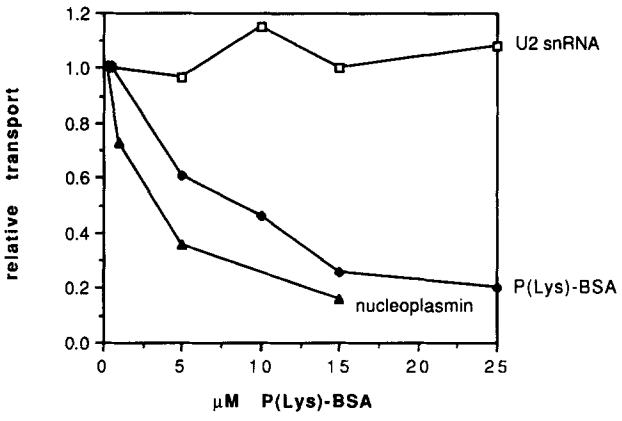

Figure 3. Dose dependence of P(Lys)-BSA competition on [^{125}I]-P(Lys)-BSA, [^{125}I]nucleoplasmin, and [^{32}P]U2 snRNA nuclear import. Nuclear import in 10–15 oocytes was assayed at 45 min after coinjection of the labeled transport substrate with increasing concentrations of P(Lys)-BSA. Normalized transport (relative transport) is expressed as the ratio of competed import, with P(Lys)-BSA, to uncompeted import, with BSA.

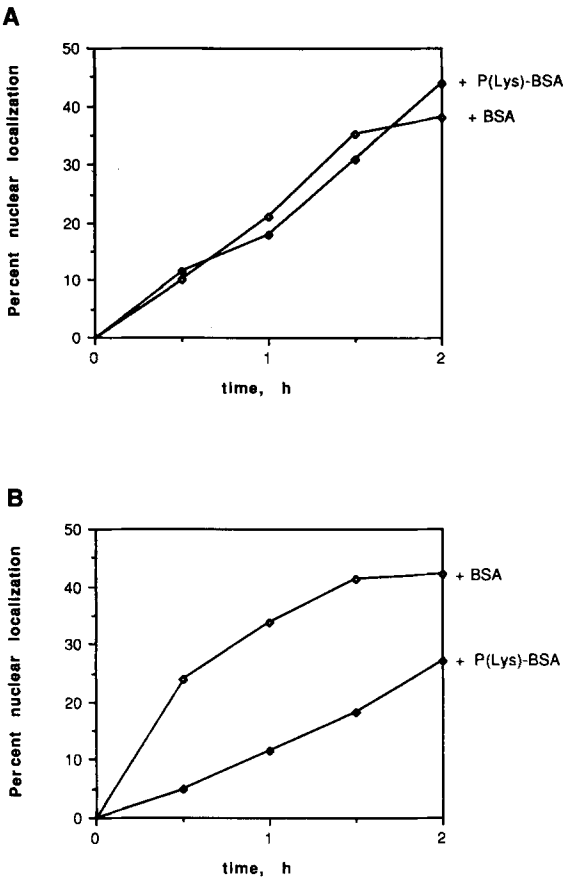

Figure 4. Time course of [^{32}P]U2 snRNP and [^{125}I]P(Lys)-BSA import in the presence of excess P(Lys)-BSA. [^{32}P]HeLa U2 snRNA (1 ng, 32 nM) and [^{125}I]P(Lys)-BSA (100 nM) were coinjected with 20 μM unlabeled P(Lys)-BSA or 20 μM BSA into *Xenopus* oocytes. Nuclei and cytoplasms from 10 to 12 oocytes at each time were separated and pooled. (*A*) [^{32}P]U2 snRNA import was determined as described in Materials and Methods. (*B*) [^{125}I]P-(Lys)-BSA import was determined by quantitating [^{125}I]P(Lys)-BSA in nuclear and cytoplasmic extracts in a gamma counter prior to extraction of RNA.

BSA import in the same cells was assessed independently by gamma counting (Fig. 4 *B*). Here it is shown that at concentrations of P(Lys)-BSA sufficient to significantly reduce the transport rate of [^{125}I]P(Lys)-BSA, the initial rate of [^{32}P]U2 snRNA is unaffected. We conclude that P(Lys)-BSA and nucleoplasmin compete for a limiting component of the nuclear transport apparatus, probably an NLS receptor, that is not required for U2 snRNA import. This conclusion is consistent with the findings of Yamasaki et al. (1989) who found that both T-antigen and nucleoplasmin synthetic signal peptides are bound by the same rat liver signal binding proteins.

U snRNP transport studies are complicated by the requirement for the labeled U2 snRNA to assemble into an RNP before import. Although unassembled U snRNAs themselves are not karyophilic, it is possible that, when mixed with P(Lys)-BSA, they might be imported artifactually as a complex with P(Lys)-BSA; that is, piggyback (Goldfarb, 1989). If this were the case, then the import of the [^{32}P]U2 snRNP-P(Lys)-BSA complex would be susceptible to the dose dependent competition characteristic of P(Lys)-BSA saturation. Thus, the fact that [^{32}P]U2 snRNA import is not competed by P(Lys)-BSA excludes the possibility that U2 snRNA import is directly mediated by P(Lys)-BSA. We confirmed that U2 snRNP assembly was occurring under the conditions of our import assays by immunoprecipitation assays using antibodies specific for either P(Lys)-BSA or the Sm-antigen component of assembled U2 snRNP. Anti-Sm IgG but not anti-P(Lys)-BSA IgG precipitated nuclear [^{32}P]U2 snRNA that had been microinjected 1 h previously together with either BSA or P(Lys)-BSA (Fig. 5). Furthermore, virtually all nuclear [^{32}P]U2 snRNA is immunoprecipitable by anti-Sm IgG and none by anti-P(Lys) antibody. [^{125}I]P(Lys)-BSA is efficiently immunoprecipitated from oocyte nuclear extracts by anti-P(Lys) IgG but not by anti-Sm IgG (not shown). Also, anti-Sm IgG can be used to immunoprecipitate assembled [^{32}P]U2 snRNP but not free [^{32}P]U2 snRNA or [^{125}I]P(Lys)-BSA (not shown). We conclude that, when microinjected together with P(Lys)-BSA. U2 snRNA assembly and import proceeds unabated and independently of P(Lys)-BSA import. The simplest explanation for this phenomenon is that the import of P(Lys)-BSA and U2 snRNP are mediated by separate transport receptors.

P(Lys)-BSA Competes the Nuclear Import of HeLa U6 snRNA

In mammalian cells, U4 and U6 snRNAs associate by base pairing to constitute a U4/U6 snRNA complex that functions in RNA splicing. However, Hamm and Mattaj (1989) reported that microinjected U6 snRNA can enter oocyte nuclei as a solitary particle, not associated with U4 snRNP. The import of U6 snRNA is particularly interesting because it lacks both the M_3G cap and Sm binding site that are essential for Ul import (Hamm et al., 1990; Fischer and Lührmann, 1990). A single stranded region of U6 snRNA, distinct from the sequence that mediates RNA–RNA interaction with U4 snRNA, is required for U6 import in oocytes (Hamm and Mattaj, 1989). The solitary U6 snRNP was also found associated with a 50-kD protein that was not detected associated with U4/U6 snRNPs (Hamm and Mattaj, 1989). Thus, the biochemical and transport properties of U6 snRNA in oocytes are significantly different from those of the RNA polymerase II transcribed U snRNAs.

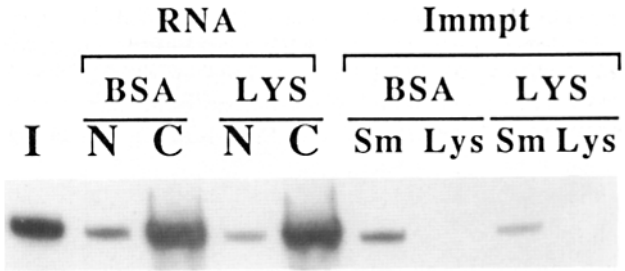

Figure 5. Immunoprecipitation of U2 snRNA from oocyte nuclei with anti-P(Lys) and anti-Sm–specific antibodies. U2 snRNA was injected into oocytes with 20 μM P(Lys)-BSA (*LYS*) or 20 μM BSA (*BSA*) and incubated for 1 h. Nuclei and cytoplasms from each treatment were separated, and RNA from each fraction isolated to indicate the level of U2 transport. Nuclear lysates from sibling oocytes in each treatment group were also immunoprecipitated with anti-P(Lys) or anti-Sm (snRNP-specific) antibodies. *I* represents the injected material. Each lane corresponds to 10 oocytes.

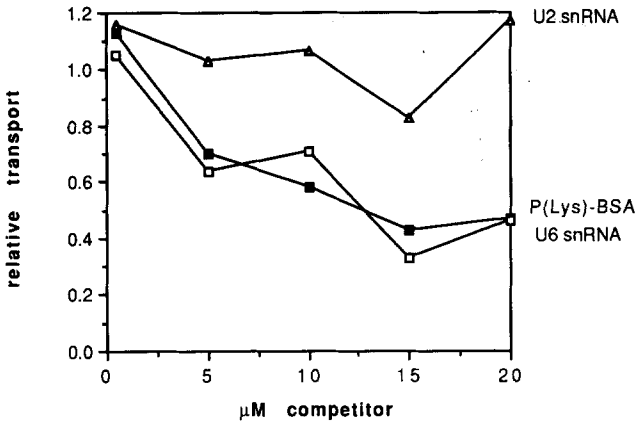

Figure 6. Dose dependent inhibition of U2 import by P(Lys)-BSA. ^{32}P-labeled HeLa U2 (*open triangles*) and U6 snRNA (*open squares*) and ^{125}I-P(Lys)-BSA (*closed squares*) were coinjected into *Xenopus* oocytes with increasing concentrations of competitor P(Lys)-BSA, and the import of each karyophile determined after 1 h incubation as described in Materials and Methods. Import of each karyophile was normalized to its import in the presence of equimolar concentrations of BSA.

In our hands, U6 snRNA import into oocyte nuclei is less efficient than is U2 import. In one trial, 18% of microinjected [^{32}P]U6 snRNA and 42% of [^{32}P]U2 snRNA accumulated in nuclei after 3 h. tRNA, for comparison, is virtually excluded from nuclei after this period (Fig. 1 *B*). Using [^{32}P]U6 snRNA, we conducted competition experiments in *Xenopus* oocytes with P(Lys)-BSA. We coinjected [^{125}I]P(Lys) BSA, HeLa [^{32}P]U2 snRNA, and HeLa [^{32}P]U6 snRNA with P-(Lys)-BSA or BSA into groups of 30 oocytes and monitored the transport of each type of molecule after 1 h. Low concentrations of P(Lys)-BSA (0.5 μM) did not significantly affect the import of [^{125}I]P(Lys)-BSA, U2 snRNA, or U6 snRNA compared to controls coinjected with 0.5 μM BSA (see below). Significantly, however, 20 μM P(Lys)-BSA decreased both U6 snRNA and P(Lys)-BSA import threefold as compared with the controls but, as expected, had no significant effect on U2 RNA import. This can be seen in the dose dependent effects of increasing concentrations of P(Lys)-BSA on the import of [^{32}P]U2 and U6 snRNA and [^{125}I]-P(Lys)-BSA (Fig. 6). Because [^{32}P]U6 snRNP is imported more slowly than [^{32}P]U2 snRNP, we incubated the oocytes for 1 h, instead of 45 min as in Fig. 3, to allow more transport to occur. Thus, because transport proceeds even in the presence of competitor, albeit more slowly, the apparent inhibition of [^{125}I]P(Lys)-BSA as a function of P(Lys)-BSA is less in Fig. 6 than in Fig. 3.

It is possible that P(Lys)-BSA acts by inhibiting the assembly of the U6 snRNP rather than as a transport competitor. Because the biochemistry of the U6 snRNP is poorly understood, U6 snRNP-specific antibodies are not yet available. Presently, therefore, we cannot directly monitor the in situ assembly of the U6 snRNP. For this reason, we restrict our conclusions to the import of the U6 snRNA and not the snRNP. We have attempted to determine whether or not P(Lys)-BSA associates strongly with the U6 snRNA before injection by gel retardation assay (Konarska, 1989). Although a small fraction of U2 snRNA can be shown to associate with P(Lys)-BSA by gel retardation, the migration of U6 snRNA in gels in unaffected by preincubating the RNA in 0.2 mM P(Lys)-BSA. Thus, it is unlikely that if P(Lys)-BSA is preventing U6 snRNP assembly it is doing so by complexing the RNA. We also asked whether the coinjection of P(Lys)-BSA with U6 snRNA has any effect on its sedimentation in glycerol gradients. In this experiment, [^{32}P]U6 snRNA was coinjected into oocyte cytoplasms with either BSA or P(Lys)-BSA, incubated for 1 h, after which time whole oocyte extracts were prepared. Glycerol gradient analysis of these extracts indicated that P(Lys)-BSA had no effect on the sedimentation of the [^{32}P]U6 snRNA (data not shown). Neither of these experiments is a good assay for U6 snRNP assembly, thus, we can not rigorously exclude the possibility that P(Lys)-BSA interferes with U6 snRNP assembly. But we think it is unlikely.

Discussion

In the first part of this study, we showed by kinetic criteria that two karyophilic proteins, P(Lys)-BSA and nucleoplasmin, compete for the same limiting component of the nuclear transport apparatus. We believe that these two proteins are representative of a much larger, general class of karyophiles that are imported by the cell's predominant import pathway. We have also observed the inhibition of [^{125}I]calf thymus histone H1 import in oocytes by P(Lys)-BSA and the inhibition of [^{125}I]nucleoplasmin import by H1 (Breeuwer and Goldfarb, unpublished observations). The ability of P(Lys)-BSA to compete the import of [^{125}I]nucleoplasmin at lower concentrations than those required to compete [^{125}I]P(Lys)-BSA import (Fig. 3) can be explained by differences in their respective affinities for a common receptor. At 45 min, the import of P(Lys)-BSA is reduced by half in the presence of $\sim$10 μM P(Lys)-BSA, whereas <3 μM P(Lys)-BSA is required to compete nucleoplasmin import to the same extent. Differences in the K_s values of these two substrates for their common import receptor may differ by as much as an order of magnitude to account for this difference. What is the molecular basis for this difference? First, the NLS of nucleoplasmin is complex and contains an element(s) that appears to be related to the T-antigen NLS motif (Burglin and De Robertis, 1987; Dingwall et al., 1988) but only insofar as they both contain clusters of lysine residues. It is difficult to envision a receptor recognizing such different sequences, present in the case of P(Lys)-BSA as a synthetic peptide, with identical affinities. Second, and perhaps more importantly, the presence of increasing numbers of NLS on the surface of a protein can increase the rate and extent of its transport (Lanford et al., 1990; Lanford et al., 1988; Roberts et al., 1987; Dworetzky et al., 1988). Although nucleoplasmin is a pentamer, the P(Lys)-BSA used for the present study has 12–17 NLS peptides/monomer. As the local concentration of signals increases on the surface of the karyophile, a previously bound receptor protein, upon dissociating, will have a statistically greater chance of reassociating with a nearby signal on the same protein resulting in higher association rate constants. The dissociation rate constant for receptor–signal complexes should not be influenced by multivalency, but the overall effect will be to increase the equilibrium binding constant. Thus, assuming each receptor has but one NLS-binding site, positive multivalency can be produced by signal proximity effects. Alternatively, as previously suggested

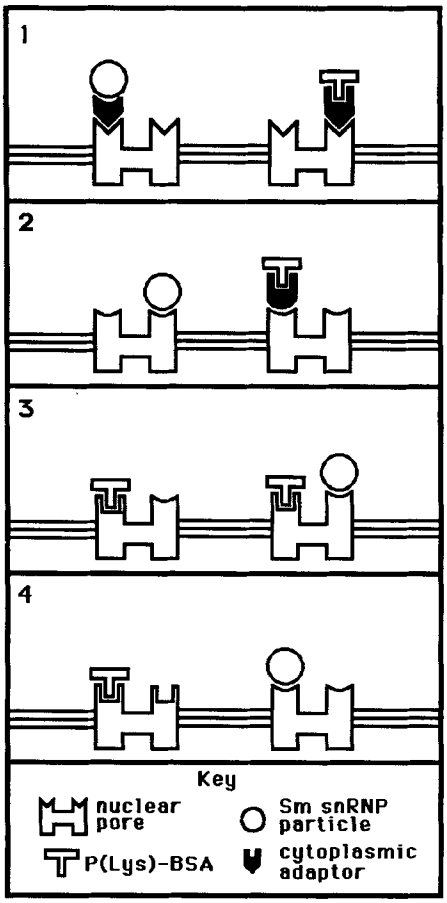

Figure 7. Diagram of import models.

(Goldfarb, 1989), positive multivalency may be achieved by the simultaneous binding of more than one NLS receptor to multivalent karyophiles. This could occur in the cytoplasm or on the central transporter assembly where multiple karyophile binding sites have been demonstrated (Akey and Goldfarb, 1990).

The key finding of this study is that U2 snRNP import occurs independently of a limiting factor that is required for P(Lys)-BSA, nucleoplasmin and U6 snRNP import. What distinguishes the import of U2 snRNP from that of U6 snRNP? Recent evidence indicates that both the M_3G cap and Sm antigen binding site of U1 are critical for nuclear import (Hamm et al., 1990; Fischer and Lührmann, 1990). U6 snRNA lacks both of these features although it is imported when artificially transcribed with a M_3G cap, but not when its single stranded region is deleted (Hamm et al., 1990). Furthermore, Fischer and Lührmann (1990) were able to inhibit U1 import with free M_3G cap. These results together with the findings of the present study suggest that the M_3G cap may have a signaling functioning distinct from the SV40 large T-antigen type of NLS. Alternatively, the M_3G cap may not be a proper signal but instead binds a factor essential for the karyophilic activation of the snRNP.

How can multiple import pathways exist in a cell whose only portal to the nucleus is the NPC? Four of the more likely models are compared in Fig. 7. Based on the lack of competition between U2 snRNP and P(Lys)-BSA import each model must provide unoccupied NPCs for U2 snRNP import in the presence of saturating P(Lys)-BSA concentrations.

In model 1, which we favor, distinct cytoplasmic adaptors mediate the targeting of the two karyophiles to the nuclear envelope. Each adapter has two domains: a karyophile-specific NLS binding domain, and a NPC binding domain. Free karyophile, in excess over its adapter, is unable to directly bind the NPC. In this model, the amount of P(Lys)-BSA receptor/adapter and not the NPC is limiting. Thus in the presence of saturating P(Lys)-BSA concentrations its adaptor, but not the NPC, becomes saturated. Cytoplasmic adaptors/signal receptors are known to function in other membrane transport pathways (Bernstein et al., 1989). SRP is a well characterized adapter/cytoplasmic receptor that mediates the targeting of all start-transfer signal-sequence-containing proteins to the ER membrane. The proposition that cytoplasmic adapters act as primary NLS receptors has received support from a number of laboratories (Yamasaki et al., 1989; Breeuwer and Goldfarb, 1990; Newmeyer and Forbes, 1990; Adam et al., 1990). What would be the function of cytoplasmic adaptors in nuclear transport? A major role in SRP in membrane transport is to maintain the translocation competence of the nascent polypeptide (Bernstein et al., 1989), which is not a requirement for nuclear import. The present study suggests that the role of putative cytoplasmic receptors in nuclear transport may be to regulate the access of multiple karyophile classes to a relatively small number of equivalent NPCs. Cytoplasmic NLS receptors may also prevent the passive diffusion of small karyophilic proteins through the nuclear pore (Breeuwer and Goldfarb, 1990).

In certain circumstances, translocation competent signal peptides can bypass the requirement for SRP and bind directly to an ER membrane-associated signal sequence receptor (Walter, 1987). A similar phenomenon could also occur in nuclear import. Thus, model 2 allows U2 snRNP, but not P(Lys)-BSA, to bypass the adaptor step and bind directly to the NPC. Model 2 predicts that P(Lys)-BSA import would be competed by saturating concentrations of U2 snRNP, but not vice versa.

In model 3, each NPC contains separate binding sites for each class of karyophile. The use of cytoplasmic NLS adaptors is not excluded by this model; however, in this case, each adaptor would bind distinct NLSs and distinct sites at the NPC. An analogous situation occurs in mitochondrial protein targeting where multiple high affinity receptors in the outer membrane mediate protein import (Hartl, 1989). The existence of multiple, spatially distinct, karyophile binding sites (peripheral binding and central docking sites) within the NPC central transporter assembly would allow for this mechanism (Akey and Goldfarb, 1989; Richardson et al., 1988; Newmeyer and Forbes, 1988).

In model 4, the nuclear envelope is studded with functionally distinct NPCs. Each karyophile class has a cognate NPC class. Although this model is consistent with our kinetic data, binding data argue strongly against this model. By electron microscopy, all the NPCs visible in extensive fields were observed to bind nucleoplasmin-colloidal gold or P(Lys)-BSA-colloidal gold (Feldherr et al., 1984; Richardson et al., 1988; Newmeyer and Forbes, 1988; Akey and Goldfarb, 1989).

In conclusion, the present data suggest that U2 snRNP and P(Lys)-BSA use kinetically distinct nuclear import pathways. If P(Lys)-BSA, nucleoplasmin, and U6 snRNP belong to one class of karyophile and U2 snRNP to another, then we may ask, how many karyophilic macromolecules belong to each class and how many total classes exist? At one extreme, snRNPs that contain either Sm antigens or M_3G caps, or both, may represent a unique and rather small family of karyophiles that are exceptional in that they do not use the cell's predominant import pathway. At the other extreme, the cell may have evolved a large number of independently regulated import pathways, each with its own characteristic NLS and receptor apparatus. The SV40 large T-antigen NLS, which can direct import in yeast and higher cells, appears to be a member of a functionally conserved class of signals. Kinetic experiments are underway to investigate exactly how large this class is and if there are many other karyophiles like U2 snRNP that fall into other classes.

We thank Drs. Sallie Hoch and Harold Smith for providing anti-Sm antibodies, Dr. William Wasserman for advice regarding oocytes and their microinjection, Dr. Robert Angerer for closely reading the manuscript, Dr. Ian Mattaj and Utz Fischer for sharing results before publication, and Roger Kornberg for helpful discussions.

This work was supported by U.S. Public Health Services FIRST and BRSG Awards, and Basil O'Connor Starter Scholar Research Award No. 5-730.

Received for publication 23 August 1990 and in revised form 9 October 1990.

References

Adam, S. A., R. S. Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807–816.

Adam, S. A., T. J. Lobl, M. A. Mitchell, and L. Gerace. 1989. Identification of specific binding proteins for a nuclear localization sequence. *Nature (Lond.).* 337:276–279.

Akey, C. W. 1990. Visualization of transport-related configurations of the nuclear pore transporter. *Biophys. J.* 58:341–355.

Akey, C. W., and D. S. Goldfarb. 1989. Protein import through the nuclear pore complex is a multistep process. *J. Cell Biol.* 109:971–982.

Bach, M., G. Winkelmann, and R. Luhrmann. 1989. 20S small nucleoprotein U5 shows a surprisingly complex protein composition. *Proc. Natl. Acad. Sci. USA.* 86:6038–6042.

Bataillé, N., T. Helser, and H. M. Fried. 1990. Cytoplasmic transport of ribosomal subunits microinjected into the *Xenopus laevis* oocyte nucleus: a generalized, facilitated process. *J. Cell Biol.* 111:1571–1582.

Benditt, J. O., C. Meyer, H. Fasold, F. C. Barnard, and N. Reidel. 1989. Interaction of a nuclear location signal with isolated nuclear envelopes and identification of signal-binding proteins by photoaffinity labeling. *Proc. Natl. Acad. Sci. USA.* 86;9327–9331.

Bernstein, H. D., T. A. Rappaport, and P. Walter. 1989. Cytosolic protein translocation factors. Is SRP still unique? *Cell.* 58:1017–1019.

Borer, R. A., C. F. Lehner, H. M. Eppenberger. and E. A. Nigg. 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell.* 56:379–390.

Breeuwer, M., and D. S. Goldfarb. 1990. Facilitated nuclear transport of histone H1 and other nucleophilic proteins. *Cell.* 60:999–1008.

Burglin, T. R., and E. M. De Robertis. 1987. The nuclear migration signal of *Xenopus laevis* nucleoplasmin. *EMBO (Eur. Mol. Biol. Organ.) J.* 6: 2617–2622.

Chelsky, D., R. Ralph, and G. Jonak. 1989. Sequence requirements for synthetic peptide-mediated translocation to the nucleus. *Mol. Cell. Biol.* 9: 2487–2492.

De Robertis, E. M., S. Lienhard, and R. F. Parisot. 1982. Intracellular transport of microinjected 5S and small nuclear RNAs. *Nature (Lond.).* 295:572–577.

Dingwall, C., and R. A. Laskey. 1986. Protein import into the cell nucleus. *Annu. Rev. Cell Biol.* 2:367–390.

Dingwall, C., J. Robbins, S. M. Dilworth, B. L. Roberts, and W. D. Richardson. 1988. The nucleoplasmin nuclear location sequence is larger and more complex than that of SV40 large T antigen. *J. Cell Biol.* 107:841–849.

Dreyer, C., and P. Hausen. 1982. Two-dimensional gel analysis of the fate of oocyte nuclear proteins in the development of *Xenopus laevis*. *Dev. Biol.* 100:412–425.

Dworetzky, S. I., R. E. Lanford, and C. M. Feldherr. 1988. The effect of variations in the number and sequence of targeting signals on nuclear uptake. *J. Cell Biol.* 107:1279–1288.

Feldherr, C. M., E. Kallenbach, and N. Schultz. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* 99:2216–2222.

Finlay, D. R., D. D. Newmeyer, P. M. Hartl, J. Horecka, and D. J. Forbes. 1989. Nuclear transport in vitro. *J. Cell Sci. Suppl.* 11:225–242.

Fischer, U., and R. Luhrmann. 1990. An essential signaling role for the m3G cap in the transport of U1 snRNP to the nucleus. *Science (Wash. DC).* 249:786–790.

Gerace, L., and B. Burke. 1988. Functional organization of the nuclear envelope. *Annu. Rev. Cell Biol.* 4:335–374.

Goldfarb, D. S. 1989. Nuclear Transport. *Curr. Opin. Cell Biol.* 1:441–446.

Goldfarb, D. S., J. Gariepy, G. Schoolnik, and R. D. Kornberg. 1986. Synthetic peptides as nuclear localization signals. *Nature (Lond.).* 322:641–644.

Gusse, M., J. Ghysdael, G. Evan, T. Soussi, and M. Mechali. 1989. Translocation of a store of maternal cytoplasmic c-*myc* protein into nuclei during early development. *Mol. Cell. Biol.* 9:5395–5403.

Hamm, J., and I. W. Mattaj. 1989. An abundant U6 snRNP found in germ cells and embryos of *Xenopus laevis*. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:4179–4187.

Hamm, J., E. Darzynkiewicz, S. M. Tahara, and I. W. Mattaj. 1990. The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite targeting signal. *Cell.* 62:569–577.

Hartl, F.-U., N. Pfanner, D. W. Nicholson, and W. Neupert. 1989. Mitochondrial protein import. *Biochem. Biophys. Acta.* 988:1–45.

Hunt, T. 1989. Cytoplasmic anchoring proteins and the control of nuclear localization. *Cell.* 59:949–951.

Khanna-Gupta, A., and V. C. Ware. 1989. Nucleocytoplasmic transport of ribosomes in a eukaryotic system: is there a facilitated transport process? *Proc. Natl. Acad. Sci. USA.* 86:1791–1795.

Konarska, M. M. 1989. Analysis of splicing complexes and small nuclear ribonucleoprotein particles by native gel electrophoresis. *Methods Enzymol.* 180:442–453.

Lanford, R. E., P. Kanda, and R. C. Kennedy. 1986. Induction of nuclear transport with a synthetic peptide homologous to the SV40 antigen transport signal. *Cell.* 46:575–582.

Lanford, R. E., R. G. White, R. G. Dunham, and P. Kanda. 1988. Effect of basic and nonbasic amino acid substitutions on transport induced by simian virus 40 T-antigen synthetic peptide nuclear transport signals. *Mol. Cell. Biol.* 8:2722–2729.

Lanford, R. E., C. M. Feldherr, R. G. White, R. G. Dunham, and P. Kanda. 1990. Comparison of diverse transport signals in synthetic peptide-induced nuclear transport. *Exp. Cell Res.* 186:32–38.

Lee, W. C., and T. Melese. 1989. Identification and characterization of a nuclear localization sequence-binding protein in yeast. *Proc. Natl. Acad. Sci. USA.* 86:8808–8812.

Li, R., and J. O. Thomas. 1989. Identification of a human protein that interacts with nuclear localization signals. *J. Cell Biol.* 109:2623–2632.

Lührmann, R. 1988. snRNP Proteins. *In* Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. M. L. Birnstiel, editor. Springer-Verlag, Berlin. 71–99.

Mattaj, I. W. 1986. Cap trimethylation of U-snRNA is cytoplasmic and dependent on U-snRNP protein binding. *Cell.* 46:905–911.

Mattaj, I. W. 1988. UsnRNP assembly and transport. *In* Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. M. L. Birnstiel, editor. Springer-Verlag, Berlin. 100-114.

Mattaj, I. W., and E. M. De Robertis. 1985. Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. *Cell.* 40:111–118.

Newmeyer, D. D., and D. J. Forbes. 1988. Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. *Cell.* 52: 641–653.

Newmeyer, D. D., and D. J. Forbes. 1990. An N-ethylmaleimide-sensitive cytosolic factor necessary for nuclear protein import: requirement in signal-mediated binding to the nuclear pore. *J. Cell Biol.* 110:547–557.

Newport, J. W., and D. J. Forbes. 1987. The nucleus: structure, function, and dynamics. *Annu. Rev. Biochem.* 56:535–563.

Nigg, E. A. 1990. Mechanisms of signal transduction to the cell nucleus. *Cancer Res.* 55:271–310.

Peters, R. 1986. Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. *Biochem. Biophys. Acta.* 864:305–359.

Reddy, R., and H. Busch. 1988. Small nuclear RNAs: RNA sequences, structure and modifications. *In* Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. M. L. Birnstiel, editor. Springer-Verlag, Berlin. 1-37.

Richardson, W. D., A. D. Mills, S. M. Dilworth, R. A. Laskey, and C. Dingwall. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope, followed by slower translocation through nuclear pores. *Cell.* 52:655–664.

Roberts, B. L., W. D. Richardson, and J. A. Smith. 1987. The effect of protein context on nuclear location signal function. *Cell.* 50:465–475.

Roth, S., D. Stein, and C. Nusslein-Vollhard. 1989. A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the Drosophila embryo. *Cell.* 59:1189–1202.

Rushlow, C. A., K. Han, J. L. Manley, and M. Levine. 1989. The graded dis-

tribution of the dorsal morphogen is initiated by selective nuclear transport in *Drosophila*. *Cell.* 59:1165–1177.

Sauterer, R. A., R. J. Feeney, and G. W. Zieve. 1988. Cytoplasmic assembly of snRNP particles from stored proteins and newly transcribed snRNAs in L929 mouse fibroblasts. *Exp. Cell Res.* 176:344–359.

Silver, P., I. Sadler, and M. A. Osborne. 1989. Yeast proteins that recognize nuclear localization sequences. *J. Cell Biol.* 109:983–989.

Singh, R., and R. Reddy. 1989. γ-Monomethyl phosphate: a cap structure in splicesomal U6 small nuclear RNA. *Proc. Natl. Acad. Sci. USA.* 86: 8280–8283.

Steward, R. 1989. Relocalization of the *dorsal* protein from the cytoplasm to the nucleus correlates with its function. *Cell.* 59:1179–1188.

Taylor, M. V., M. Gusse, G. I. Evan, N. Dathan, and M. Mechali. 1986. *Xenopus myc* proto-oncogene during development: expression as a stable maternal mRNA uncoupled from cell division. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3563–3570.

Walter, P. 1987. Two receptors act sequentially. *Nature (Lond.).* 328:763–764.

White, E. M., C. D. Allis, D. S. Goldfarb, A. Srivastva, J. Weir, and M. A. Gorovsky. 1989. Nuclear specific and temporally restricted localization of proteins in *Tetrahymena* macronuclei and micronuclei. *J. Cell Biol.* 109: 1983–1992.

Yamasaki, L., P. Kanda, and R. E. Lanford. 1989. Identification of four nuclear transport signal-binding proteins that interact with diverse transport signals. *Mol. Cell. Biol.* 9:3028–3036.

Zasloff, M. 1983. tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. *Proc. Natl. Acad. Sci. USA.* 80:6436–6440.

Zieve, G. W., and R. A. Sauterer. 1990. Cell biology of the snRNP particles. *CRC Crit. Rev.* 25:1–46.