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

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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(\text{Lys})-BSA, a protein bearing multiple nuclear localization signals from SV40 large T-antigen, reduce the rate of \text{[t}^{35}\text{P]}(\text{Lys})-BSA and of \text{[m}^{32}\text{P]}\text{nucleoplasmin import, consistent with their competing for and sharing the same limiting component of the import apparatus. In contrast, saturating concentrations of P(\text{Lys})-BSA do not reduce the rate of HeLa \text{[}^{32}\text{P}]U2 snRNP assembly or import. The import of U6 snRNP is also competed by P(\text{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(\text{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) (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 \(\sim 100\)-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.,...
A notable feature of proteins which are present in the interphase cytoplasm until, in response to an extracellular signal, they migrate, is 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 U1-5 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 and al. (1990) have suggested that the Sm binding site and the trimethylguanosine cap (m7G) of U1 snRNA together comprise a bipartite nuclear targeting signal (see Fischer and Luhrmann, 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), nucleoplasm (a major oocyte nuclear protein), and U2 and U6 snRNAs isolated from Hela cells. We found that import of both nucleoplasm 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

Nucleoplasm 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 nucleoplasm were iodinated using Chloramine T. Proteins were brought to 28 μl 125 μM Na2HPO4, pH 7.5, at a concentration of 2.5 μg/μl. 10 μl of 1 μg/μl Chloramine T was combined with 0.2 μCi Na[125I] (Pharmacia Fine Chemicals, Piscataway, NJ), and the mixture was incubated with the protein for 15 s at ambient temperature. 5 μl of saturated t-cysteine was added to stop the labeling reaction and the sample was collected from a 50 ml sephadex G-25 column equilibrated with 25 mM Na2HPO4, pH 7.5. Labeled proteins were washed and concentrated in centricrtin 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 775 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% CO2. Cells were dished 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 x 10^5 cells/ml in MEM (Gibco Laboratories) without phosphate, supplemented with 10% dialyzed FBS (Gibco Laboratories). Cells were labeled with 10 mCi [35S]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 Na2HPO4, 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, 0.5x 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, 1X 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 RNA, and U RNA were excised (gelled), and incubated and overnight at ambient temperature in 300 μl 0.3 M NH4Ac, 1 mM EDTA, 0.1% SDS. RNA was collected by low speed centrifugation through silica 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 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 intranuclear 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 cytoplasm was quantitated with a multi gamma counter (1261; LKB Instruments, Inc., Gaithersburg, MD). To quantitate RNA transport, oocytes were labeled with 10 mCi Na[35S]acetyl-5-20 μg carrier ethanol at 37°C for 16-24 h. Labeled proteins were washed and concentrated in centricrtin 30 filtration units (Amicon Corp., Danvers, MA). The specific activity of labeled proteins was 500-2,000 cpm/ng.

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% NF-40) at 4°C. Anti-P(lys) antisum (Goldfarb et al., 1988) 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 de-
scribed 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 un-
complexed U snRNPs that are normally recruited by U snRNAs transcribed and exported later in develop-
ment (Mattaj, 1988). These oocyte U snRNPs will spontane-
ously assemble onto microinjected U snRNA (De Robertis et al., 1982). Uncomplexed U snRNPs
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). 32P-labeled HeLa U2,
U1, 5.8S, 5S, and tRNA were microinjected into oocyte cytoplastms 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, indicat-
ning that the molecule is stable in both the cytoplasm and nu-
cleus (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
The import of P(Lys)-BSA into oocyte nuclei occurs with a
Lanford et al., 1990), and culture cells (Lanford et al., 1986; Chelsky et al., 1989;
cytes (Goldfarb et al., 1986), a variety of vertebrate tissue
et al., 1989). P(Lys)-BSA accumulates in the nuclei of oo-
affinity reagents to identify putative transport receptors
minimal T-antigen NLS and would not be expected to bind
cellular factors other than those specifically involved in nu-
clear 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
each fraction was separated and [125I]P(Lys)-BSA.
import of [125I]P(Lys)-BSA, [32p]U2 snRNA, and [32P]U2
snRNA is presented as the percent reduction in import
relative to uncompeted transport at 45 min (Fig. 3). Interest-
ingly, in this and other experiments, P(Lys)-BSA exhibits a
lower K_m apparent for [125I]nucleoplasmin than for [125I]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 [32P]U2 snRNA at 45 min is unaffected by concentrations
of P(Lys)-BSA that are sufficient to almost completely abol-
ish the import of [125I]P(Lys)-BSA and [125I]nucleoplasmin.
By this criterion, then, both P(Lys)-BSA and nucleoplasmin
share a limiting component of the transport apparatus. Al-
though 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 [125I]P(Lys)-BSA, [125I]nucleoplasmin, and [32P]U2
snRNA is presented as the percent reduction in import
Figure 2. P(Lys)-BSA competition of [125I]P(Lys)-BSA and [125I]-
nucleoplasmin nuclear import. (A) [125I]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 [125I]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 ex-
cept 5 and 25 μM at 15 min and 0.3 and 5 μM at 3 h. (B) [125I]-
nucleoplasmin was injected into Xenopus oocytes with 5 μM P-
(Lys)-BSA (closed diamonds), 25 μM P(Lys)-BSA (open triangles),
or 25 μM closed squares) and processed as in A. Each point
is the mean of 10–15 oocytes. T-tests indicate statistically signi-
ficant differences between [125I]nucleoplasmin import in the pres-
ence of BSA and import in the presence of either 5 or 25 μM P(Lys)-
BSA at every data point.

Figure 3. Dose dependence of P(Lys)-BSA competition on [125I]-
P(Lys)-BSA, [125I]nucleoplasmin, and [32P]U2 snRNA nuclear
import. Nuclear import in 10–15 oocytes was assayed at 45 min
after coinjection of the labeled transport substrate with increasing
centrations 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.

pore⁻¹ min⁻¹ (Goldfarb et al., 1986). Fig. 2 A shows that
the rate, but not the extent, of [125I]P(Lys)-BSA import is
influenced by 5 μM P(Lys)-BSA. 25 μM P(Lys)-BSA signi-
ficantly 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 [125I]nucleoplasmin import almost
to background levels (Fig. 2 B). The initial rate but not the
final extent of [125I]nucleoplasmin import was also measur-
bly competed by 2 μM P(Lys)-BSA (not shown). Thus
increasing concentrations of P(Lys)-BSA increasingly inhibit
the import of both [125I]P(Lys)-BSA and [125I]nucleoplasmin.

By this criterion, then, both P(Lys)-BSA and nucleoplasmin
share a limiting component of the transport apparatus. Al-
though 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.

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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 [32P]P(Lys)-BSA, the initial rate of [32P]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 artificially as a complex with P(Lys)-BSA; that is, piggyback (Goldfarb, 1989). If this were the case, then the import of the [32P]U2 snRNP-P(Lys)-BSA complex would be susceptible to the dose dependent competition characteristic of P(Lys)-BSA saturation. Thus, the fact that [32P]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 [32P]U2 snRNA that had been microinjected 1 h previously together with either BSA or P(Lys)-BSA (Fig. 5). Furthermore, virtually all nuclear [32P]U2 snRNA is immunoprecipitable by anti-Sm IgG and none by anti-P(Lys) antibody. [125I]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 [32P]U2 snRNP but not free [32P]U2 snRNA or [125I]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 M1G cap and Sm binding site that are essential for U1 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.

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**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.
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ate with P(Lys)-BSA by gel retardation, the migration of U6 snRNA though a small fraction of U2 snRNA can be shown to associate in oocyte extracts after injection by gel retardation assay (Konarska, 1989). Alternatively, the import 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 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, [32P]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 [32P]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 [3H]alaf thymus histone H1 import in oocytes by P(Lys)-BSA and the inhibition of [32P]nucleoplasmin import by H1 (Breeuwer and Goldfarb, unpublished observations). The ability of P(Lys)-BSA to compete the import of [32P]nucleoplasmin at lower concentrations than those required to compete [32P]nucleoplasmin 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 ~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, 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, 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 NLSs 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 in our hands, U6 snRNA import into oocyte nuclei is less efficient than is U2 import. In one trial, 18% of microinjected [32P]U6 snRNA and 42% of [32P]U2 snRNA accumulated in nuclei after 3 h incubation, for comparison, is virtually excluded from nuclei after this period (Fig. 1 B). Using [32P]U6 snRNA, we conducted competition experiments in Xenopus oocytes with P(Lys)-BSA. We coinjected [32P]P(Lys) BSA, HeLa [32P]U2 snRNA, and HeLa [32P]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 [32P]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 [32P]U2 and U6 snRNA and [125I]-P(Lys)-BSA (Fig. 6). Because [32P]U6 snRNP is imported more slowly than [32P]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 [32P]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, [32P]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 [32P]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 [3H]alaf thymus histone H1 import in oocytes by P(Lys)-BSA and the inhibition of [32P]nucleoplasmin import by H1 (Breeuwer and Goldfarb, unpublished observations). The ability of P(Lys)-BSA to compete the import of [32P]nucleoplasmin at lower concentrations than those required to compete [32P]nucleoplasmin 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 ~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, 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 NLSs 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 6](image-url)
the simultaneous binding of more than one NLS receptor to port (Hamm et al., 1990; Fischer and Lührmann, 1990). U6 distinguishes the import of U2 snRNP from that of U6 Goldfarb, 1990).

karyophile binding sites have been demonstrated (Akey and or on the central transporter assembly where multiple multivalent karyophiles. This could occur in the cytoplasm and Sm antigen binding site of U1 are critical for nuclear im-

cation between 102 snRNP and P(Lys)-BSA import each model are compared in Fig. 7. Based on the lack of competi-

tion between 102 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 func-

tion of cytoplasmic adaptors in nuclear transport? A major role in SRP in membrane transport is to maintain the translo-
cation 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 im-

In model 3, each NPC contains separate binding sites for each class of karyophile. The use of cytoplasmic NLS adap-
tors is not excluded by this model; however, in this case, each adapter 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 function-
ally 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 NPC visible in extensive fields were observed to bind nucleoplasmin-colloidal gold or P(Lys)-BSA-colloidal gold (Feldderr et al., 1984; Richar-
dson et al., 1988; Newmeyer and Forbes, 1988; Akey and Goldfarb, 1989).

(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 oc-

Figure 7. Diagram of import models.

3

4

Key

nuclear pore

Sm snRNP particle

P(Lys)-BSA cytoplasmic adapter

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 adapter 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 NPC visible in extensive fields were observed to bind nucleoplasmin-colloidal gold or P(Lys)-BSA-colloidal gold (Feldderr et al., 1984; Richar-
dson 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 karyophoric macromolecules belong to each class and how many total classes exist? At one extreme, snRNPs that contain either Sm antigens or M,G 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 or 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.

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