Nuclear Localization Signal Receptor Affinity Correlates with in Vivo Localization in Saccharomyces cerevisiae

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Nuclear localization signals (NLSs) target proteins into the nucleus through mediating interactions with nuclear import receptors. Here, we perform a quantitative analysis of the correlation between NLS receptor affinity and the steady-state distribution of NLS-bearing cargo proteins between the cytoplasm and the nucleus of live yeast, which reflects the relative import rate for this cargo. This correlation, however, is not maintained for cargoes that bind to the NLS receptor with very weak or very strong affinity.

The segregation of the nuclear genetic material from the cytoplasmic machinery that translates it into proteins provides the eukaryotic cell with intricate mechanisms for controlling gene expression. This segregation, however, also presents the cell with a mechanistic problem. Because most intra- and extracellular signaling pathways culminate with changes in gene expression within the nucleus, signals must cross the nuclear envelope to gain access to the genetic material. This signal is almost invariably a protein, such as a transcription factor, that enters the nucleus. In addition, once a gene is transcribed, the messenger RNA must then be exported across the nuclear envelope into the cytoplasm where it is translated into protein. In fact, the nuclear envelope is a critical information barrier across which both RNA and proteins are selectively transported in a highly regulated manner to establish orderly communication and behavior within the cell (1).

The best characterized mechanism for translocation across the nuclear envelope is protein import, which depends on the

2 The abbreviations used are: NLS, nuclear localization signal; IBB, importin β-binding domain; NES, nuclear export signal; GFP, green fluorescent protein; DIC, differential interference contrast; DAPI, 4′,6-diamidino-2-phenylindole.
### NLS Affinity Dictates Localization

#### TABLE 1

| NLS variant name | NLS sequencea | \(K_D\) | \(\mu M\) |
|------------------|---------------|--------|---------|
| BPSV40          | KR–X\(_d\)–PKKKRKV | 0.03   | 0.03    |
| BPSV40A5        | KR–X\(_d\)–PKKKRKV | 0.2    | 0.2     |
| BPSV40A6        | KR–X\(_d\)–PKKKRKV | 0.9    | 0.9     |
| BPSV40A4        | KR–X\(_d\)–PKKKRKV | 1      | 1.2     |
| Myc             | PAARKVKLD      | 6      | 6.5     |
| SV40            | PKKKRKV        | 9      | 9       |
| MycA5           | PAARKVKLDE     | 13.5   | 13.5    |
| BPSV40T3        | KR–X\(_d\)–PKKKRKV | 13.5   | 13.5    |
| SV40A2          | PAARKVK       | 16.5   | 16.7    |
| MucA6           | PAARKVKLD      | 25     | 25.1    |
| SV40A5          | PEKKRKV        | 38     | 38      |
| SV40A1          | AKKKRVK       | 36     | 36.2    |
| SV40A7          | PKKKRKA        | 53     | 53      |
| MucA8           | PAARKVKAD      | 85     | 85      |
| MycA1           | AKKKRVKLD      | 120    | 120     |
| SV40A4          | SKPKARVK       | 335    | 335     |
| SV40A6          | PEKKRAVE       | 310    | 310     |
| MucA7           | PAARKVKLD      | 650    | 650     |
| SV40R3          | PEKKRKV        | 850    | 850     |
| MucA5           | PAARKVKLDE     | 1400   | 1400    |
| BP-GFP          | KR             | 2000   | 2000    |
| SV40T3          | PEKKRKV        | 3000   | 3000    |
| SV40A3          | SKPKAEVK       | 3000   | 3000    |
| MucA4           | PAARKVKLDE     | 15000  | 15000   |

*a Bold font represents amino acid changes in each variant NLS.

b Calculated values were inferred from binding affinity for full-length importin \(\alpha\).

The \(K_D\) values for full-length importin \(\alpha\) can be approximated from these numbers as follows: \(K_D\) (full-length importin \(\alpha\)) \(\approx\) 120 \(K_D\) (ΔIBB Importin \(\alpha\)).

A complete understanding of nuclear import signals requires a quantitative model for the import reaction that correlates NLS amino acid sequence, *in vitro* interaction energies, and *in vivo* functionality. We have previously attempted to decipher the energetic details of NLS recognition by importin \(\alpha\) through quantitative analysis of variant NLSs. The relative importance of each residue in two monopartite NLS sequences was determined using an alanine-scanning approach (26). This analysis was performed using the ΔIBB-importin \(\alpha\) variant, which lacks the N-terminal autoinhibitory domain, as a model for the high affinity importin \(\alpha\)-importin \(\beta\) complex. Variants of NLS sequences were generated with affinities for ΔIBB-importin \(\alpha\) that ranged between a \(K_D\) of a few micromolar to a \(K_D\) of a few picomolar (Table 1). In addition, the energy of inhibition of the importin \(\alpha\) IBB domain was measured and found to be \(\approx 3\) kcal/mol regardless of the sequence of the NLS. These data allow the generation of an energetic scale of nuclear localization sequences where a signal has a high affinity for the cytoplasmic importin \(\alpha\)-importin \(\beta\) complex and an affinity \(3\) kcal/mol weaker for the autoinhibited importin \(\alpha\) in the nucleus (26).

One goal of this quantitative analysis is to provide a thermodynamic foundation for a numerical model of the process of nuclear transport (for examples see Refs. 27–29). One would expect that the *in vivo* process of nuclear import would correlate in some manner with the energetics of the individual protein-protein interactions that drive the process. One finding from a recent modeling study is that the rates of nuclear protein import are largely governed by the level of the NLS receptor, importin \(\alpha\) (29). One implication of this finding is that the binding affinity of the NLS receptor for its cargo should be an important determinant of how efficiently that cargo is transported into the nucleus. Indeed, there is empirical evidence to indicate that there is some sort of functional threshold of affinity that an NLS must possess for importin \(\alpha\) in order for the cargo to be imported into the nucleus (3). When the SV40 NLS (PKKKRKE) is mutated to the SV40T3 sequence (PKTKKRKE), its affinity for ΔIBB-importin \(\alpha\) decreases by \(\approx 3\) kcal/mol (from \(K_D\) = 9 \(\mu M\) to \(K_D\) = 3 \(\mu M\)), and it loses its ability to function as a nuclear localization signal *in vivo* (3). Thus the energetic threshold dividing a functional NLS from a non-functional NLS exists somewhere between the binding affinity of the SV40T3 variant NLS (\(K_D\) = 3 \(\mu M\)) and the SV40 wild-type NLS (\(K_D\) = 9 \(\mu M\)).

There have been a handful of reports comparing the measured binding affinity between an NLS and its receptors with the rate of import of a cargo fused to that NLS *in vivo*. Work by Jans et al. (30–32) involved the measurement of the binding constant between several NLSs and full-length importin \(\alpha\) through an enzyme-linked immunosorbent assay followed by measurement of the import rate of these NLSs microinjected into rat hepatoma cells. They reported that the initial rate of protein import is linearly correlated with the equilibrium constant for the interaction between the NLS cargo and importin \(\alpha\). This is an interesting result, because this relationship would hold true in a simple model where the rate of protein import would depend on the equilibrium concentration of the importin \(\alpha\)-importin \(\beta\)-cargo-NLS ternary complex. Although provocative, the dynamic range of the measurements used in this study was limited: the range of \(K_D\) measurements used in this report varied only by a factor of four. Thus it would be interesting to see if this correlation suggested in these studies is general to a wider range of NLS affinities.

The quantitative data generated by our examination of the energy landscape of importin \(\alpha\) yields a numerical skeleton on which to build a comprehensive model for the complicated process of protein import. A given NLS can be situated on a linear scale that describes its affinity for the importin \(\alpha\)-importin \(\beta\) complex (using ΔIBB importin \(\alpha\) as a model) as well as its affinity for importin \(\alpha\) alone (26). For an NLS to function in nuclear import, one might hypothesize that it must have an affinity for the importin \(\alpha\)-β complex that is tight enough to stimulate the uptake of the NLS cargo in the cytoplasm, but it must also have an affinity for importin \(\alpha\) alone (26). This hypothesis is intuitive because the cargo must be transported to the nucleus. Thus, we might hypothesize that there are both upper and lower limits to the affinity of an NLS for its receptors that define a functional localization signal.

Here, we report the results of our initial experiments designed to address the correlation between *in vitro* binding energies and *in vivo* import function. We quantify the relative import rates of various NLSs in live yeast cells by measuring the steady-state distribution of the NLS-bearing cargo protein. These measurements are used to estimate the rate of nuclear import for transport cargoes containing various NLS sequences. Our results indicate that there is a complicated but monotonic quantitative relationship between the affinity of an NLS for the import receptors and the steady-state accumulation of the cargo in the nucleus.
PCR amplification and then ligated into the SV40-GFP-GFP protein in yeast. Other NLS sequences were placed in this vector by sequencing and by appropriate expression of the target protein (pAC1065). The fidelity of the resulting vector was confirmed with a second GFP molecule encoded in the vector DNA that was too large for anisotropy measurements (due to the addition of a second GFP). However, the sequences surrounding the NLS and linking the NLS to GFP were identical to those in the comparable molecules analyzed in vitro (26). Even if there is any minor change in the binding constants, we assume that all variants would be affected in the same manner and thus all our measurements, which are relevant to one another, would still provide significant insight into how binding to the NLS receptor correlates with import. NLS-reporters carrying a single copy of the GFP protein were constructed from the NLS-GFP-GFP reporter plasmids by digesting with Sall, then performing an intramolecular ligation with the resulting large fragment, which excises the second GFP open reading frame.

To construct a plasmid encoding the NLS-GFP-NES reporter, first an intermediate vector was generated encoding GFP-NES. The bacterial expression vector SV40-GFP (26) was amplified using the primers GTCCGGCGTAGAGGATCGAG, which primed 5’ to the gene in the vector, and GCCGCTCTGAGTACGAGTACGAG, which primed 3’ to the gene in the vector, and cut with the NheI site at the 3’-end of the GFP gene. The resulting DNA fragment was cut with HindIII and XhoI and ligated into pET28a. After verification by sequencing, this new vector, GFP-Not, was then amplified with primers GTCCGGCGTAGAGGATCGAG, which primed 5’ to the gene in the vector, and GCCGCTCTGAGTACGAGTACGAG, which primed 3’ to the gene in the vector, and cut with the NheI site at the 3’-end of the GFP gene. The resulting DNA fragment encoding GFP-NES was then excised using HindIII and XhoI. This fragment was then ligated into each yeast NLS reporter expression plasmid cut with the same enzymes creating NLS-GFP-NES reporters with unique NLS sequences that could be expressed in yeast.

**Microscopy**—Indirect immunofluorescence was performed as described elsewhere (34). To detect Myc-tagged importin α or the NLS-GFP reporter, Myc (EMD Bioscience) and GFP antibodies (35) were diluted 1:3000 and incubated with fixed

### Experimental Procedures

**Construction of Plasmids**—All plasmids used in this study are listed in Table 2. NLS-GFP fusion proteins were expressed in the yeast *Saccharomyces cerevisiae* under the control of the MET25 promoter using the plasmid pGFP-C-FUS (33). The NLS-GFP fusion proteins expressed in yeast were identical to the proteins expressed in *Escherichia coli* for *in vitro* binding studies (26) except that the first 17 residues of the N terminus were removed, including the N-terminal His<sub>6</sub> tag, to facilitate expression in yeast. The DNA sequences encoding the SV40-GFP fusion were amplified from the bacterial expression vector in vitro (26) using the DNA oligonucleotide GCCGCTCTGAGTACGAGTACGAG as a 5’-primer and the oligonucleotide ACTCATCTCGACGGTATCG as the 3’-primer. The DNA encoding the SV40-GFP fusion protein was then ligated into the yeast expression vector pGFP-C-Fus (33) using XbaI and ClaI restriction sites, placing the gene as an in-frame 5’ fusion with the second GFP molecule encoded in the vector DNA (pAC1065). The fidelity of the resulting vector was confirmed by sequencing and by appropriate expression of the target protein in yeast. Other NLS sequences were placed in this vector by PCR amplification and then ligated into the SV40-GFP-GFP vector using XbaI and HindIII. Removal of the His<sub>6</sub> tag, and the addition of a second GFP created a molecule that could not be directly analyzed in vitro by quantitative fluorescence anisotropy to assure that neither modification altered the binding affinity for the NLS receptor. This is due to the fact that the modified protein could no longer be easily purified (due to the removal of the His<sub>6</sub> tag) and was too large for anisotropy measurements (due to the addition of a second GFP). However, the sequences surrounding the NLS and linking the NLS to GFP were identical to those in the comparable molecules analyzed in vitro (26). Even if there is any minor change in the binding constants, we assume that all variants would be affected in the same manner and thus all our measurements, which are relevant to one another, would still provide significant insight into how binding to the NLS receptor correlates with import. NLS-reporters carrying a single copy of the GFP protein were constructed from the NLS-GFP-GFP reporter plasmids by digesting with Sall, then performing an intramolecular ligation with the resulting large fragment, which excises the second GFP open reading frame.

### Yeast Strains and Plasmids Used in Study

| Strain/plasmid | Description | References |
|---------------|-------------|------------|
|ACY192 (wild-type) | MATa ura3-52 leu2a1 try1 | 53 |
|ACY817 (Nap49-GFP) | MATa ura3-52 leu2a1 try1 yfp49-GFP::TRP1 | 44 |
|ACY339 (cmt1-3) | MATa ura3-52 his3a200 leu2a1 try1 ade2 ade3 | 46 |
|ACY642 (lep1-55) | MATa ura3-52 his3a200 leu2a1 try1lep1-55::LEU2 | 49 |
|pRS425 | 2µ, LEU2, AMPg | 24 |
|pAC253 | pSF277Y, GAL1-10, 2µ, URA3, AMPg | 39 |
|pAC958 | CSE1, 2µ, LEU2, AMPg | This study |
|pAC963 | SRP1-c-myC (3X), CEN, TRP1, AMPg | 49 |
|pAC1056 | BPSV40-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1057 | BPSV40A5-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1058 | BPSV40G6-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1059 | BPSV40T3-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1060 | MYC-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1061 | MYC-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1063 | MYC-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1065 | SV40-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1066 | SV40A5-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1067 | SV40T3-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1069 | GFP-GFP, CEN, URA3, AMPg | This study |
|pAC1350 | pGAL1-GFP, CEN, URA3, AMPg | This study |
|pAC1352 | pGAL1-SV40-NLS-GFP, CEN, URA3, AMPg | This study |
|pAC1353 | pGAL1-BP-SV40-NLS-GFP, CEN, URA3, AMPg | This study |
|pAC2046 | BPMYC-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC2047 | SV40A2-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC2048 | SV40A7-NLS-GFP-GFP, CEN, URA3, AMPg | This study |
|pAC2049 | BSVP40-NLS-GFP, CEN, URA3, AMPg | This study |
|pAC2050 | SV40-NLS-GFP, CEN, URA3, AMPg | This study |
|pAC2051 | BPSV40-NLS-GFP-NES, CEN, URA3, AMPg | This study |
cells overnight at 4 °C. The Texas Red-labeled anti-mouse secondary (Jackson ImmunoResearch) and fluorescein isothiocyanate anti-rabbit secondary antibodies (1:1000) were incubated with cells for 2 h at room temperature. Cells were also labeled with DAPI (1 μg/ml) to mark the position of the nucleus. Samples were viewed through a Texas Red-optimized filter and a GFP-optimized filter (Chroma Technology) using an Olympus BX60 epifluorescence microscope equipped with a Photometrics Quantix digital camera.

Direct fluorescence microscopy was used to localize GFP fusion proteins in live cells. For all experiments, cells were incubated with DAPI to visualize the DNA and confirm the location of the nucleus. The localization of the fusion protein was monitored by directly viewing the GFP signal in living cells through a GFP-optimized filter as described for indirect immunofluorescence microscopy.

Imaging—For imaging, yeast cells (ACY192) transformed with the NLS-GFP vectors were grown to mid-log phase in media lacking methionine (for a list of yeast strains used, see Table 2). Standard fluorescence and DIC images were collected using an Olympus BX60 epifluorescence microscope equipped with a Photometrics Quantix digital camera. Quantitative interpretation of the wide field fluorescence images were not accurate due to out of focus light and the lack of calibrated excitation sources. Thus, for quantitative analysis cells were imaged using a customized two-photon microscope with calibrated excitation and photon-counting emission detectors. The inherent three-dimensional resolution of two-photon microscopy provides an important advantage for resolving the fluorescence signal in the nucleus or the cytoplasm (36, 37). For two-photon microscopy yeast cells were harvested by gentle centrifugation and then resuspended in a small volume of media. The cells were transferred to a 35-μl plastic depression dish (Sitting Drop Bridges from Hampton Research). Vacuum grease was applied to the edges surrounding the depression, and then a glass slide was applied over the depression, sealing it through the grease. The slide assembly was then inverted, and the yeast were allowed to gently settle against the glass coverslip. The slide was then placed on the two-photon microscope for imaging.

Image Analysis—Two-photon images were sectored into nuclear and cytoplasmic compartments through a semi-automated procedure using the programs Mathematica (Wolfram Research) and Corel Photopaint (Corel). For each image, the average fluorescence per pixel was calculated for the nuclear and cytoplasmic compartments. Multiple three-dimension-resolved axial sections were imaged for each cell, and the automated sorting procedures were developed to avoid vacuolar regions as well as to ensure that compartment edges were avoided and did not play a role in the measured average signal levels.

NLS-GFP Import Assay—The NLS-GFP import assay was performed as described previously (38). Briefly, cells were grown to early mid-log phase in synthetic media containing 2% glucose (w/v) at 30 °C, pelleted, resuspended in 1 ml of glucose-free synthetic media containing 10 mM sodium azide, 10 mM 2-deoxy-D-glucose, and incubated at 30 °C for 45 min. The cells were then pelleted, washed with 1 ml of ice-cold ddH2O, repelleted, resuspended in 100 μl of glucose-containing synthetic media pre-warmed to 30 °C, and incubated at 30 °C. For scoring, 2-μl samples were removed every 2.5 min following resuspension in pre-warmed glucose-containing synthetic media. Individual cells were analyzed and counted using a GFP-optimized filter (Chroma Technology) using an Olympus BX60 epifluorescence microscope. Cells were scored as “nuclear” if the nucleus was both brighter than the surrounding cytoplasm and a nuclear-cytoplasmic boundary was visible. At least 100 cells were counted for each time point. Cells were also examined to assure the complete relocalization of nuclear proteins upon energy depletion.

Overexpression of NLS Cargo—Wild-type cells (ACY192) or srp1-55 cells (ACY642) were transformed with galactose-inducible plasmids encoding GFP (pAC1350), SV40 NLS (ESPKKKRKE)-GFP (pAC1352), bipartite SV40 NLS (KRTADGSEESPKKKRKE)-GFP (pAC1353), or as a control a known dominant negative mutant of yeast Ntf2 (N77Y Ntf2, pAC253) (14, 26, 39). Single transformants were grown in liquid culture to saturation, serially diluted (1:10), and spotted on minimal media plates containing 2% galactose or, as a control, 2% glucose. Galactose induces expression of the plasmid-encoded proteins. Plates were incubated at 25 °C for 3 days. For all experiments we employed quantitative immunoblotting to examine the level of expression of both the NLS reporter proteins and the importin α proteins. This analysis revealed that the expression levels of either the NLS reporters or the importin α proteins were comparable for all experiments (data not shown).

RESULTS

Measurements of in Vivo Import Rates—Our first goal was to determine the correlation between the affinity of an NLS for receptor and the rate of import of the NLS cargo into the nucleus. This is an important question, because this relationship would have direct implications for the mechanism of nuclear transport. We have previously generated a diverse set of NLS variants comprising a wide range of defined affinities for importin α (26). To measure the in vivo import rate associated with these variant NLSs, we constructed a reporter cargo for use in live yeast that is nearly identical to the NLS-GFP fusion proteins used in the in vitro measurements with two exceptions (26). First, the N-terminal 17 residues of the protein used in the in vitro studies were removed to facilitate expression in yeast. Although this reduces the number of residues N-terminal to the NLS (from 27 to 10 in the case of the bipartite NLS), the local context of the NLS remains identical. The second modification is the addition of a second GFP peptide to the C terminus of the reporter. This addition yields a double GFP molecule (molecular mass of ~55 kDa) with a presumably slowed rate of passive diffusion through the nuclear pore. The similarity between the in vitro and in vivo reporter molecules near the N-terminal NLS ensures that the results of one assay are directly comparable to the results of the other.

In Fig. 1A, yeast cells expressing four different NLS-GFP-GFP reporters were imaged by direct fluorescence microscopy. The level of nuclear fluorescence compared with cytoplasmic fluorescence increased with the measured strength of the affin-
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Steady-state Nuclear Transport—We can estimate the import rate of various NLS reporters in living cells under normal growth conditions by analyzing the steady-state distribution of NLS bearing protein cargoes. This approach is based on the recognition that in the steady state the relative concentrations of NLS cargo in the nucleus relative to the concentration in the cytoplasm must be related to the relative rates of nuclear import and nuclear export. This simple method does not account for the detailed molecular mechanisms of the import process nor protein synthesis and degradation but does provide a reasonable first order quantification of the relative import rate for variant NLS sequences. Nuclear import and export can occur both via signal-mediated active pathways and through passive diffusion through the nuclear pore. The average rate of protein import can thus be written,

\[
\text{Import rate} = \frac{N}{C} = C(r_{p, in} + r_{f, in}) \quad \text{(Eq. 1)}
\]

where \(C_N\) is the concentration of the molecule in the cytoplasm, \(r_{p, in}\) is a rate constant for passive diffusion from the cytoplasm to the nucleus that depends on the size and shape of the molecule, and \(r_{f, in}\) is a rate constant for the facilitated transport from the cytoplasm to the nucleus that depends on the existence of an NLS. The rates in this equation presumably reflect multiple molecular processes and are thus not directly interpretable in terms of the binding energetics of a specific protein-protein interaction, but this approach does allow us to make an initial estimate of the relative effective rates for nuclear import. Similarly, the rate of protein export from the nucleus may be written,

\[
\text{Export rate} = \frac{N}{C} = C(r_{p, out} + r_{f, out}) \quad \text{(Eq. 2)}
\]

The ratio of the nuclear to the cytoplasmic concentration can thus be computed as in Equation 4.

\[
R_{NC} = \frac{C_N}{C} = \frac{(r_{p, in} + r_{f, in})/(r_{p, out} + r_{f, out})} \quad \text{(Eq. 4)}
\]

At steady-state, the ratio of the concentration of reporter

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**FIGURE 1. Localization of NLS variants.** Steady-state fluorescence distribution in yeast expressing NLS-GFP-GFP variants as viewed through a standard fluorescence microscope. A, the top panels show GFP fluorescence (A–D). The bottom panels show the corresponding DIC image (E–H). The binding constant for each NLS cargo binding to importin \(\alpha\) is indicated below the images. B, localization of importin \(\alpha\)-Myc (pAC963) and the BPSV40-GFP-GFP reporter (pAC1056) by indirect immunofluorescence in control cells (pRS425) or cells overexpressing Cse1 (pAC958). Images of wild-type cells expressing the BPSV40-GFP-GFP reporter (A and B) and importin \(\alpha\)-Myc (C and D) are shown. Corresponding DAPI and DIC images are shown below (E–H).

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One possibility is that, because the BPSV40 reporter binds to importin \(\alpha\) with high affinity, the enhanced nuclear localization simply reflects an increase in the reporter-receptor complex rather than free reporter within the nucleus. To determine whether the BPSV40 reporter remains bound to importin \(\alpha\) in the nucleus, we localized importin \(\alpha\) and the BPSV40 reporter in yeast overexpressing the importin \(\alpha\) export receptor, Cse1. Because Cse1 relocalizes importin \(\alpha\) to the cytoplasm (40–42), if the nuclear concentration of the BPSV40 reporter were due solely to binding to importin \(\alpha\), the nuclear cargo concentration would be expected to decrease when importin \(\alpha\) is no longer concentrated in the nucleus. Thus, localization of both importin \(\alpha\) and the BPSV40 reporter was analyzed by indirect immunofluorescence in control cells or cells overexpressing Cse1. Control cells show primarily nuclear localization of both the BPSV40 reporter and importin \(\alpha\) (Fig. 1B, panels A and C). Overexpression of Cse1 caused relocalization of importin \(\alpha\) to the cytoplasm (Fig. 1B, panel D), while the BPSV40 reporter remained within the nucleus (Fig. 1B, panel B). This result shows that the nuclear localization of the BPSV40 reporter does not depend on a nuclear pool of importin \(\alpha\).
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A.

FIGURE 2. Quantitative analysis of NLS reporter localization. A, representative images taken from a two-photon fluorescence scan of yeast carrying the SV40-GFP-GFP plasmid (pAC1065). Four successive Z-scan images are shown where the spacing between each Z-scan is 1 μm. The raw data from the two-photon scan were converted into these images by taking the logarithm of the fluorescence intensity for each pixel, then scaling the entire image such that the dynamic range of the image corresponds to the dynamic range of the TIFF image file format. These images are typical of those that were used in the semi-automated sectoring of each scan into cellular compartments. B, histograms of \( R_{N/C} \) measurements from yeast cells expressing the SV40-GFP-GFP reporter. Data compiled from three independent experiments are shown as three distribution curves illustrating the reproducibility of the localization data.

TABLE 3

| NLS                        | \( R_{N/C} \) Mean | \( R_{N/C} \) 90% confidence interval |
|---------------------------|--------------------|--------------------------------------|
|                           | Low               | High                                |
| Wild type with NLS-GFP-GFP reporters |                   |                                      |
| BPMyc                     | 5.75              | 3.36                                | 7.48                              |
| BPSV40A6                  | 3.34              | 1.42                                | 5.54                              |
| Myc                       | 2.69              | 1.62                                | 4.28                              |
| SV40                      | 3.66              | 1.90                                | 6.59                              |
| BPSV40T3                  | 4.07              | 1.83                                | 6.88                              |
| MycA6                     | 3.18              | 2.39                                | 4.07                              |
| SV40A5                    | 1.91              | 1.35                                | 2.61                              |
| SV40A7                    | 2.52              | 2.02                                | 3.37                              |
| MycA1                     | 1.98              | 1.87                                | 2.09                              |
| SV40A6                    | 1.63              | 1.34                                | 1.89                              |
| SV40T3                    | 1.56              | 1.34                                | 1.90                              |
| GFP                       | 1.40              | 1.28                                | 1.57                              |
| Nup49-GFP with NLS-GFP-GFP reporters |                   |                                      |
| BPMyc                     | 4.55              | 2.80                                | 6.15                              |
| BPSV40                    | 4.95              | 2.71                                | 7.48                              |
| BPSV40A5                  | 5.24              | 3.10                                | 8.07                              |
| BPSV40A6                  | 3.59              | 2.44                                | 5.04                              |
| Myc                       | 2.12              | 1.47                                | 2.99                              |
| SV40                      | 2.32              | 1.63                                | 3.32                              |
| BPSV40T3                  | 4.61              | 2.44                                | 8.01                              |
| SV40A2                    | 2.58              | 1.71                                | 3.30                              |
| MycA6                     | 2.03              | 1.71                                | 2.43                              |
| SV40A5                    | 1.68              | 1.35                                | 2.12                              |

| Wild type with NLS-GFP-GFP reporters |                   |                                      |
| BPSV40                    | 3.20              | 1.81                                | 4.37                              |
| SV40                      | 1.66              | 1.28                                | 2.09                              |

As described above, the ratio of nuclear to cytoplasmic fluorescence \( R_{N/C} \) yields a measure of the relative import rate of the NLS-GFP reporter at steady state (Table 3). When \( R_{N/C} \) values for multiple cells carrying the same reporter are measured, the resulting values are highly variable. For example, different cells from the same population of cells expressing the SV40 NLS-GFP-GFP reporter yielded \( R_{N/C} \) values
from 1.3 to 4.9. We observe wider distributions for the tighter binding NLS cargoes. This result is presumably due to the highly efficient nuclear import of these cargoes such that the remaining cytoplasmic concentration (the denominator in the $R_{N/C}$ ratio) is less well defined. However, when different populations of cells carrying the same reporter were measured on different days, the overall distribution of $R_{N/C}$ versus the number of cells was reproducible (Fig. 2B).

Analysis of variance demonstrated the statistical significance of the observed differences in mean $R_{N/C}$ value for different NLS cargoes, as discussed below.

One potential source of the variability in our measurements of the $R_{N/C}$ values is the inherent variability in the expression level of the reporter in different cells within the population. The reporter is expressed using an extrachromosomal plasmid, and thus the total amount of NLS-GFP reporter in each cell varies. To investigate the possibility that the variability stems from changes in the amount of protein expression, the ratio $R_{N/C}$ was plotted as a function of the fluorescence per unit volume in the cell cytoplasm in Fig. 3. This analysis yields a measure of the correlation between $R_{N/C}$ and the total concentration of GFP in the cell. We found no correlation between $R_{N/C}$ and the total amount of NLS-GFP in the cell suggesting that changes in the expression level, at least over the range examined here, do not substantially alter the $R_{N/C}$ value. This finding also suggests that importin α is not rate-limiting for import of these reporter cargoes in the cell.

We found that the distribution of $R_{N/C}$ values measured for each NLS-GFP reporter varies with the strength of the NLS. Although the distributions of the $R_{N/C}$ values from different NLS-GFP reporters overlap with each other, the mean $R_{N/C}$ value for the distribution increased monotonically with the affinity of the NLS for the import receptor (Fig. 4). To confirm the statistical significance of the differences in population means, we performed one-way analysis of variance for the SV40A5, SV40, BPSV40, and BPSV40A5 samples with the null hypothesis of indistinguishable $R_{N/C}$ values firmly rejected with 99% confidence. Pairwise comparisons using the Tukey method showed that differences in mean $R_{N/C}$ for any two of these NLS sequences are significant at the 90% confidence level or better.

The relationship between the mean $R_{N/C}$ value and the affinity of the NLS is complicated. In Fig. 5, the mean $R_{N/C}$ values along with confidence limits measured for various NLS-GFP reporters are plotted as a function of the NLS affinity. Data are shown for experiments using both wild-type yeast and Nup49-GFP yeast (Fig. 5, A–C). As shown, NLSs with higher affinities for the import receptors tend to have a higher accumulation in the
nucleus at steady-state suggesting that import rate increases with the affinity of the cargo for the NLS receptor.

Although a general trend was observed in the relationship between import rate and NLS receptor affinities, the relationship exhibited a number of irregularities. One finding that impacts the dynamic range of our measurements is that the GFP-GFP fusion alone accumulated in the nucleus with a mean $R_{N/C}$ of 1.4. Because the GFP-GFP fusion has a molecular mass of ~58 kDa, we would expect that this protein is too large to pass easily through the nuclear pore complex by passive diffusion, yet it apparently possesses some intrinsic mechanism for nuclear accumulation. The $R_{N/C}$ value for GFP-GFP (1.4) was low compared with a robust NLS such as the SV40 NLS-GFP-GFP (mean $R_{N/C}$ value of 3.66). However, the modest nuclear accumulation of GFP-GFP limited the smallest affinities for which $R_{N/C}$ values could be reliably measured; furthermore, this slight nuclear localization of GFP-GFP may also slightly distort some measured $R_{N/C}$ values, particularly for low affinity NLS cargoes.

To confirm the validity of our results using steady-state measurements to estimate import rates, we carried out a kinetic NLS-GFP import assay (38) on selected reporters. This assay provided a kinetic measurement of initial import rates in live yeast cells (Fig. 6). The NLS-GFP import assay was performed using GFP-GFP, as a control, and the SV40T3, SV40, and BPSV40 NLS variants. In this assay, cells expressing NLS reporters are depleted of energy by incubation with azide and 2-deoxy-glucose, which causes the redistribution of any nuclear protein (38). Import kinetics are then measured by counting the percentage of cells showing nuclear localization of the reporter over time after azide and 2-deoxy-glucose are removed. As predicted by our steady-state analysis, the import kinetics increased as the binding affinity of the reporter for importin $\alpha$ increased (Fig. 6A). In fact, import of the BPSV40 reporter was so fast that it was virtually all nuclear at the earliest time point we could measure. To assure that the BPSV40 reporter was redistributed upon incubation of cells with azide and 2-deoxy-glucose, we examined cells expressing this reporter at each point in the assay (Fig. 6B). Cells showed diffuse localization of the BPSV40 reporter in media containing azide and 2-deoxy-glucose in comparison to cells not treated (Fig. 6B, compare A and B). After cells were washed with H$_2$O, BPSV40 remained diffusely localized throughout the cell (Fig. 6B, panel C). How-
ever, as soon as cells were placed in per-warmed glucose-contain-
ing media, BPSV40 accumulated within the nucleus (Fig. 6B, panels D and E). These results confirm that the import of rate of this NLS reporter with a very high affinity for importin α is extremely rapid as was predicted by our analysis of import rates based on the steady-state distribution of the reporter.

Relationship between in Vivo Import Rate and Cargo Size—To determine the effects of cargo size on the steady-state distribution of NLS-GFP reporters, the distributions for both an SV40 and a BPSV40 NLS fused to a single GFP were also measured. As shown in Table 3, the single GFP reporters exhibited less nuclear accumulation than the double GFP reporters. If one makes the assumption that the facilitated import rates of the NLSs, i.e. rSV40 and rBPSV40, do not change for the single or double GFP molecules, then this decreased nuclear localization must result from increased intrinsic import and export rates for the ~30-kDa single GFP molecule relative to the 58-kDa GFP-GFP. We estimate this rate increase is ~1.7 times, probably due to enhanced passive diffusion of the smaller cargo through the nuclear pore.

Addition of an NES to Increase the Dynamic Range of the Analysis—A limitation of this study was the dynamic range of R_{N/C} values available using GFP as a cargo. Due to the inherent nuclear targeting of GFP, the range was limited to values >1.4. In an attempt to increase the dynamic range of the measurements, a NES was added to the C terminus of a single GFP reporter (sequence: AAALALKLAGLNI) (45). The goal was to cause the cytoplasmic accumulation of reporters carrying weaker NLSs but still allow the nuclear accumulation of stron-
ger NLSs. Surprisingly, we found the NES, at least in this con-
text, was a much stronger targeting signal than the import signal imparted by most of the NLSs used in this study. An SV40-GFP-NES reporter showed an exclusively cytoplasmic steady-state localization (data not shown). This suggests that this particular NES dominates the trafficking of the NLS/NES combination. One possibility is that the effective nuclear concentration of the NES receptor is much higher than the effective concentration of the NLS receptor in the cytoplasm. Strikingly, when the stronger BPSV40 NLS was paired with the NES, the reporter appeared to accumulate at the nuclear rim as shown in Fig. 7. To ensure that accumulation at the nuclear rim is due to NES-dependent export, the BPSV40-GFP-NES reporter was expressed in the yeast mutant crm1-3, which contains a mutation in the yeast NES export receptor, Crm1/Xpo1 (46). In this mutant, the BPSV40-GFP-NES reporter accumulates within the nucleus (Fig. 7) consistent with the hypothesis that nuclear rim localization is a direct effect of rapid export after import. This observation suggests that the residence time of the BPSV40-GFP-NES reporter in the nuclear pore complex is large relative to the time the reporter remains in either the nucleus or the cytoplasm. One interpretation of this observation is that the initial steps of import and export are relatively fast compared with the rate of either translocation through the pore or release from the pore.

Upper Limit to Functional NLS Affinity—In our original hypothesis, we proposed that, for an NLS to be functional in vivo, it must have an adequate affinity for the import receptors in the cytoplasm to allow capture, but it must also have a weak enough affinity for the receptors in the nucleus to allow efficient release of the cargo. This admittedly simplistic model for protein import predicts that there is an upper limit to the affinity of a functional NLS for the import receptors. Surpris-
ingly, we did not see evidence for such an upper limit in our imaging studies above. There is a saturation behavior observed for the high affinity bipartite NLSs in that the relative localization of these NLSs are apparently insensitive to changes in the NLS affinity, but these high affinity NLSs efficiently localize their cargoes to the nucleus.
NLS Affinity Dictates Localization

FIGURE 8. Overexpression of NLS cargo. Wild-type (WT) yeast cells or cells that express an autoinhibitory mutant form of the NLS receptor importin α (srp1-55) as the only copy of the NLS receptor (14) were transformed with the following galactose-inducible plasmids expressing vector (pAC1350); a control protein that inhibits cell growth when it is overexpressed (pAC253); an SV40-NLS reporter (pAC1352); or a BPSV40-NLS reporter (pAC1353). Cells were grown to saturation in glucose media (no expression) and then serially diluted and spotted on glucose (left) or galactose (right) plates.

From our simple model of protein import, which assumes that no other factors are involved in the release of cargo except importin α, importin β, and Ran-GTP, we would expect that high affinity NLS cargoes would remain bound to importin α even after the dissociation of importin β from the trimeric import complex. Importin α cannot interact with its export factor, Cse1, when an NLS is bound (41, 47, 48). Thus, we would expect the high affinity NLS cargo-importin α complexes to accumulate in the nucleus and deplete cytoplasmic importin α to the detriment of cellular growth.

Because no obvious growth defects were observed in our imaging studies with the bipartite NLSs, we further examined the toxicity, or lack thereof, of high affinity NLSs by expressing the NLS-GFP-GFP cargoes at a higher level and directly analyzing cell growth. Using a galactose-inducible multicycle expression vector, we tested the effects of overexpressing both the SV40-GFP-GFP and the BPSV40-GFP-GFP reporters on growth of wild-type yeast cells. As controls, we included the empty vector and a known dominant negative variant of the protein Npt2 (39). As shown in Fig. 8 (top panel), the overexpression of either NLS cargo had no apparent effect on cell growth. This result is somewhat surprising given that the BPSV40 NLS is predicted to have nanomolar affinity for importin α in the nucleus (26). Similar results were obtained when we expressed the NLS reporters from a very strong GPD1 promoter on a multicopy plasmid (data not shown). This analysis suggests that the affinity, or lack thereof, of the NLS for importin α in the nucleus is not the limiting factor in the release of cargoes in the nucleus.

In fact, several additional factors contribute to the release of the NLS cargo from importin α within the nucleus (47, 49). These factors include the nucleoporin, Nup2 (47), the export receptor for importin α, Cse1 (48), and the autoinhibitory IBB domain of the importin receptor itself (14). To examine the role of one of these factors, the autoinhibitory IBB domain, we overexpressed the nanomolar bipartite SV40 binding cargo in cells that express a variant of yeast importin α (encoded by the SRP1 gene (50)), which has compromised autoinhibitory function. This variant, Srp1-55, has an alanine substitution in the NLS-like sequence of the IBB domain reducing its ability to compete with NLS binding through an intramolecular interaction with the NLS-binding site (14). This yeast mutant thus harbors a defect in the ability of importin α to release the NLS cargo in the nucleus. When the NLS-GFP-GFP reporters were overexpressed in srp1-55 mutant cells, a mild growth defect was observed with BPSV40-GFP-GFP expression where no defect was observed with SV40-GFP-GFP expression. This result is not due to a change in the expression level of the Srp1-55 mutant protein as compared with wild-type Srp1 as quantitative immunoblotting indicates that the proteins are expressed at levels that are indistinguishable from one another (data not shown and Ref. 14). This finding suggests that, when the NLS releasing mechanism of the protein import machinery is perturbed, cells become sensitive to high affinity NLS cargoes.

DISCUSSION

Intuitively, one would expect the function of an NLS to be related to its affinity for the NLS receptor. Jans et al. (30–32) have hypothesized that the import rate of an NLS-bearing cargo is linearly related to $1/K_{D}$ based on measurements of a small range of $K_{D}$ values. This hypothesis is consistent with a model where a number of NLS cargoes compete for a limited number of import receptors resulting in a relative rate of import that is proportional to the relative concentrations of NLS cargo/receptor complexes. In this study, we have investigated a much larger dynamic range of NLS affinities compared with that examined by Jans et al. We found that the steady-state localization and thus, indirectly, the rate at which a cargo is imported, are correlated with the binding affinity of the NLS for the import receptor complex. However, the analytical relationship between these parameters is not yet completely clear. A clearer picture of the analytical form of the relationship between affinity and import rate would provide a powerful means by which various models (29) for the complicated process of nuclear import could be tested at an experimental and quantitative level. Along with the work of Jans et al., this report provides another early step in the quantitative description of NLS-mediated nucleocytoplasmic transport.

The relationship between $R_{N/C}$ and the cytoplasmic affinity for import transporters can be described as having three regimes. NLSs with affinities weaker than $10^{-7} M$ yield $R_{N/C}$ values indistinguishable from GFP-GFP alone. NLSs with affinities between $10^{-7} M$ and $10^{-9} M$ yield $R_{N/C}$ values that generally increase with increasing affinity. NLSs with affinities greater than $10^{-9} M$ yield broad $R_{N/C}$ distributions that are invariant with the NLS affinity with a mean $R_{N/C}$ of about $5$. From these data, one can hypothesize that the import rate of an NLS is a continuous function of its affinity for the import receptors, but, at high affinities, the import rate is limited by some mechanism to a value approximately $3.6 r_{GFP-GFP}$ (assuming that the export rate constant for the NLS-GFP-GFP is identical to that of GFP-GFP alone). Thus, on average, the highest rate constant for NLS-mediated import appears to be about four times larger than the rate constant for the SV40 NLS. One interesting exception to this scheme is the BPSVT3 NLS, which has a binding affinity...
near 10 nm but exhibits the broad distribution of $R_{N/C}$ and higher mean $R_{N/C}$ value of the stronger NLSs. This may be an indication that the saturation phenomenon observed for strong NLSs may be due to a unique aspect of the fact that they are bipartite NLSs rather than just a function of their high affinity for the import receptors.

With our increased understanding of the interactions that govern the nuclear transport process, particularly the importin α/importin β-mediated import of cargoes containing a classic NLS, it has become possible to model the overall process. In recent years, two laboratories have established such models (27–29). Largely, these studies indicate that the level of nuclear Ran governs the import process. Recent work also indicates that the cellular level of the import receptor, importin α, is an important determinant of nuclear import (29). Implicit within these results is the suggestion that it is not actually the amount of importin α that governs the import of a particular cargo but rather the amount of cargo bound to the import receptor that governs import rates. If more receptor is available, then more cargo will be bound. Alternatively, if the affinity of the receptor-cargo interaction is increased, this should also lead to enhanced nuclear import. The results presented here provide the experimental data in support for this idea. Ultimately, we will need a combination of modeling studies and experimental tests of those models to fully understand the molecular mechanisms that govern the rate of nuclear protein transport.

A somewhat surprising observation in this report is that the nuclear import machinery of yeast is robust enough to handle NLS affinities that vary over several orders of magnitude. Particularly noteworthy is that exceptionally high affinity NLSs appear to be properly localized to the nucleus and released in the nucleoplasm without causing any severe disruption to cellular growth. This finding supports the suggestion that the processes of nuclear import could be regulated on a kinetic level rather than a thermodynamic level. Provocative evidence for the importance of kinetic regulation has been offered by Gilchrist et al. (47) specifically regarding the release of NLS cargo in the nucleus. Their work demonstrates that both the nucleoporin Nup2 and the export receptor for importin α, Cse1, can enhance the rate of disassembly of the importin α-NLS cargo complex in vitro (47, 51). Recent structural studies have revealed details of how both Nup2 (52) and Cse1 (48) interact with importin α. Despite the information that the three-dimensional structures of these complexes provide, it is still not clear how these factors act in a coordinated manner to facilitate NLS cargo release in vivo. Such questions highlight the importance of future experimentation defining both the energetic and kinetic behaviors of the nuclear trafficking machinery. These experiments will then provide a foundation for a more advanced model describing the nuclear transport process.

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