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A Domain Distinct from Nucleoplasmin's Nuclear Localization Sequence Influences Its Transport

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We constructed mutants of the prototypical, nuclear-accumulating protein nucleoplasmin and used them in both *in vivo* and *in vitro* nuclear transport assays to search for transport-influencing domains distinct from this protein's recognized nuclear localization sequence. We identified the polyglutamic acid tract on the amino flank of the nuclear localization sequence as being involved in two stages of nuclear transport. This poly-glu tract is required for the facilitated translocation of nucleoplasmin through the nuclear pore complex, and it also enhances the subsequent binding of nucleoplasmin within the nucleus.

Specific proteins—including nuclear enzymes and structural proteins, hormone receptors, and oncogene and tumor suppressor proteins (as well as other transcription factors)—are selectively transported into the nucleus because they contain nuclear localization sequences (NLSs). Transport of NLS-containing proteins (NLS-proteins) is intricately regulated as a function of the cell-cycle and during signal transduction events (1).

NLS-protein transport is a multi-stage process involving sequential interactions of the NLS-protein with protein components of a complex and still incompletely-defined intracellular transport machinery. Recognized transport stages include: (i) directed movement within the cytoplasm to the nuclear surface (2,3), (ii) energy-independent association at the cytoplasmic side of the nuclear pore complex (NPC) and (iii) energy-dependent, facilitated translocation through the NPC (4-6), and—for many NLS-proteins—(iv) subsequent intranuclear binding (6,7). These stages operate in tandem to accomplish nuclear entry of NLS-proteins at rates far greater than possible by diffusion alone, and—for NLS-proteins whose intranuclear binding is appreciable—accumulation to nuclear concentrations which can greatly exceed their cytoplasmic concentrations.

To date, several relatively abundant, soluble components of the NLS-protein transport machinery have been isolated from the cytosol fraction of disrupted cells (8-13). In the currently accepted paradigm, two proteins, karyopherin/importin α and karyopherin/importin β, associate in a heterodimeric complex which recognizes the NLS (by specific binding to α) and targets the NLS-protein to interact (via β) with proteins of the NPC. Despite their abundance in cytosol, recent evidence suggests that importins associate with cytomatrix elements within the living cell (14); if so, they may also participate in directed transport within the cytoplasm. Facilitated translocation through the NPC requires two other transport proteins also recoverable from cytosol: the small GTPase Ran/TC4 and a Ran-interacting protein, p10. The transport roles of Ran and p10 are not fully understood, but they seem to act in concert with Ran's guanine-exchange and GTPase-activating proteins to mediate sequential association/dissociation reactions of NLS-proteins during transit through the NPC (15). Some studies have additionally implicated the family of 70 kD heat shock proteins (Hsp 70) in NLS-protein docking and/or translocation (16). Proteins responsible for the intranuclear binding of NLS-proteins have not been identified.

Nucleoplasmin (Np) was the first protein whose NLS was localized to a specific domain (17), and this NLS was eventually identified as two clusters of basic amino acids (^{147}KR and ^{159}KKKK) separated by a 'spacer' of ten nondescript amino acids (18). Similar 'bipartite' NLS motifs are present in many nuclear proteins (18-20), while other NLSs consist of only a single cluster of basic residues, and still others differ substantially from both of these motifs (21).

A primary sequence region is considered a confirmed NLS if it is sufficient to target a non-nuclear protein (to which it is covalently coupled or genetically fused) into the nucleus, and Np's bipartite seg-

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ment was validated by this criterion (18,22). This approach, of course, does not identify transport functions of NLS-protein domains distinct from the tested segment (see Discussion). To obviate this limitation, we engineered a panel of defined Np mutants, expressed them *in vivo* in *E. coli* in radiolabeled form, purified and characterized them, and measured their nuclear transport kinetics in both *in vitro* and *in vivo* assays. Our data reveal a requirement for the poly-glutamic acid (poly-glu) tract on the amino flank of Np's NLS (23) in NPC facilitated translocation. Furthermore, we constructed small molecular weight Np mutants which remain as monomers and transit the NPC by diffusion, rather than the facilitated translocation mechanism. These truncated mutants, which retain Np's bipartite NLS and poly-glu domain, permitted us to additionally resolve a contribution of the poly-glu domain to the intranuclear binding.

METHODS

Generation of Np mutants and truncated forms. We constructed a plasmid for *E. coli* expression of wild-type (wt) nucleoplasmin (Np) by adding useful restriction sites to the Np gene—BamHI in front of the initiation codon, NdeI at the initiation codon, and BamHI just after the initiation codon—utilizing PCR with the following oligonucleotide primers: NUCA (5′ end of the gene): 5′-AAAAGGATCCATATGTTCCGCAACACCAGCAAAGTT and NUCB (3′ end of the gene): 5′-AAAAGGATCCTCATTTCTTAGCAGCTGGCTT. The resulting PCR fragment was treated with Klenow polymerase, digested with NdeI and BamHI, and ligated into NdeI/BamHI-digested pET-14b vector (Novagen) to produce pET-Np. This plasmid allows expression of wt Np as a fusion molecule with six amino-terminal histidine residues (6x-His tail).

We constructed plasmids for expression of mutants Np 1, Np 3, Np 4, and Np 7 by site-directed mutagenesis according to Kunkel et al. (24), using the following oligonucleotides: 5′-GTAGCAATGGAGGAAGATTACTCTCCACCCAAAGCTGTAAAGAGA (pET-Np 1), 5′-GAATCTCCACCCAASAGCTGTAACCAAAAAGGCAGGCCAGGCA (pET-Np 3), 5′-GCAGCCACCAAAAAGGCAGGCCTTGACAAAGAGGATGAGAGC (pET-Np 4), 5′-AGCAATGGAGGAAGATTACGCCTGGGCAGAAGAGGAAGATG (pET-Np 7).

Plasmids for mutants Np 2, Np 5, and Np 6 were prepared by PCR using the following combinations of primers: Np 2 (NUCA and NUC2: 5′-AAAAGGATCCTCATTTGTCAAGTTTCTTCTTCTT), Np 5 (NUCB and NUC5: 5′-AAGGATCCATATGGAGGAAGATTACTCATGG), Np 6 (NUCB and NUC6: 5′-AAGGATCCATATGTCATGGGCAGAAGAGGAAGAT).

Plasmids for mutants Np 4/5 and Np 4/6 were prepared by PCR using pET-Np 4 as a template, with NUC5, NUCB and NUC6, NUCB as primers, respectively.

Sequences of all mutants were confirmed by restriction analysis and that of Np 1 additionally by direct sequencing of the DNA corresponding to the 71 C-terminal amino acids.

Protein expression and in vivo labeling. Wild-type Np and the mutants were expressed with 6x-His tails in *E. coli* BL 21 cells. The proteins were labeled *in vivo* with ^{35}S-Trans-Label (ICN) and purified on an affinity column of Ni-NTA resin (QIAGEN) and on Mono Q HR 5/5 FPLC (Pharmacia). This procedure yielded pure, labeled wt Np and mutants, as assayed by autoradiography of SDS-PAGE gels and western blot analyses (25). (Our antibody to wt Np recognized mutants Np 1-4 and 7, but did not react with the truncated Np 5, 6, 4/5 and 4/6).

Characterization of the Np mutants. The molecular weights of the denatured forms of the wt Np and the Np mutants were determined on SDS-PAGE gels. The molecular sizes of the proteins in solution were determined on Superose 12 FPLC (Pharmacia) equilibrated with a medium which mimicks the intracellular ionic composition of *Xenopus* oocytes (26).

Xenopus oocytes and the in vivo transport assay. *Xenopus laevis* ovaries were surgically removed into an extracellular medium (26). Full-grown (stages V-VI) (27), folliculated oocytes were manually isolated with microscissors and radiolabeled wt or mutant Np was microinjected into the cytoplasm using standard methods (28) and incubated in extracellular medium. At different times after microinjection, cells were transferred to mineral oil, and nuclei and cytoplasms were manually isolated, a procedure which obviates the loss of nuclear contents entailed in aqueous nuclear isolation methods. The amount of radioactive protein in each compartment was then measured by scintillation counting.

The in vitro transport assay. The oil-isolated nucleus (OIN) retains normal structure and functions for several hours, including its ability to selectively transport and accumulate NLS-proteins (26). This fact makes practical incorporation of an OIN into OIN:bead (OIN:B) or OIN:cytoplasm (OIN:C) 'transport pairs' (6). Briefly, while observed through a dissecting scope, an OIN is conjoined with either an aqueous, cross-linked agarose bead or a bolus of cytoplasm also isolated under oil (either approximately the size of the nucleus). Prior to forming pairs, the aqueous bead or cytoplasm is preloaded with a radiolabeled NLS-protein. Also, before some pairs are formed, one can micropuncture the OIN's envelope with the tip of a 22-gauge hypodermic needle, permitting nuclear entry of even large proteins by diffusion. At known times after pair formation, nuclei (intact or punctured) are separated from their paired bead or cytoplasm and the amount of radioactive NLS-protein within each compartment is measured. That radiolabeled NLS-transportants become localized within the intranuclear compartment, rather than only on the nuclear surface, has been established with fluorescently-labeled NLS-proteins (6).

RESULTS

Expression and purification of wt Np and Np mutants. We constructed expression vectors for wt Np and a series of Np mutants (Fig. 1) and expressed each protein in *E. coli* in radiolabeled form. We purified the proteins, assaying by SDS-PAGE and western blotting. We also characterized their *in vivo* forms by size-exclusion chromatography (Superose 12 FPLC) in an intracellular medium: mutants Np 1,2,3,4, and 7, like wt Np, form pentamers ($M_w \approx 100$ kD); the short, truncated mutants Np 5, 6, 4/5, and 4/6 remain as monomers (M_w = 7-11 kD).

Baseline transport kinetics of wt Np, in vitro and in vivo. Individual oil-isolated Xenopus oocyte nuclei (OIN) (which remain minimally disturbed with respect to *in vivo* nuclear structure and function [26]) were paired under oil with a similar size bolus of oocyte cytoplasm (C) which had also been isolated under oil and preloaded with ^{35}S-Np. This *in vitro* transport system faithfully mimicks *in vivo* NLS-protein transport (6,7). Specifically, Np is transported from the cytoplasm into the OIN and accumulates therein as a result of two sequential transport processes: facilitated transloca-

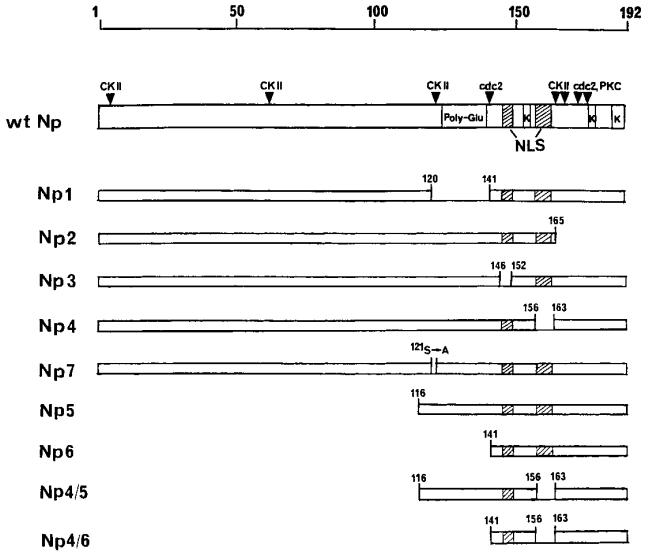

FIG. 1. Schematic of Np and Np mutants. For comparison, the structural organization of the wild-type (wt) Np is drawn to scale. Indicated are the casein kinase II (CKII) and cdc2 phosphorylation sites, the polyglutamic acid domain (Poly-Glu), and the C-terminal lysine-rich regions (K). The hatched boxes denote Np's bipartite NLS. For all deletion mutants, the boundaries are indicated, and internal deletions are marked as a single line.

tion through the NPC and subsequent intranuclear binding. Baseline transport kinetics for *E. coli*-expressed wt Np in the OIN:C 'transport pairs' are shown in Fig. 2A.

For comparison, we measured the *in vivo* transport of wt Np after microinjection into the cytoplasm of intact, living oocytes (Table I). Np's *in vivo* nuclear accumulation is somewhat slower than in the OIN assay, because in the intact cell additional time is required for Np to move from its injection site to the nuclear surface (7). (In the *in vitro* assay Np is distributed throughout the cytoplasm prior to formation of OIN:C transport pairs). Nevertheless, Np's fundamental translocation and accumulation behavior is the same *in vivo* and *in vitro*. Parallel experiments with Np 3 and Np 4, both *in vitro* (Fig. 2B) and *in vivo* (Table I), show that deletion of either half of Np's bipartite NLS abolishes nuclear entry, confirming the original report that both halves are required for Np's transport (18).

Deletion of residues on the carboxy side of the NLS does not influence transport. The sequence on the C-terminal side of Np's NLS includes basic clusters (^{177}KKGK, ^{184}RGRK, and ^{191}KK) which could potentially influence the transport function, e.g., by augmenting the local positive charge density of the NLS within the native protein conformation. Additionally, this end of the molecule contains concensus phosphorylation sites, two for CKII, one for cdc2 kinase, and one for protein kinase C (Fig. 1). To determine whether any of the basic clusters or phosphorylation sites are necessary for nuclear transport, we deleted this entire portion (residues 166-192) of the molecule (Np 2, Fig. 1). We found no significant effect on the transport kinetics, *in vitro* (Fig. 2C) or *in vivo* (Table I).

The ^{121}S CKII site is not required for transport. The amino-terminal flank of Np's NLS contains a consensus CKII site (^{121}S). A transport role for phosphorylation at this site seemed possible because its position is analogous to that of a transport-modulating CKII site (^{111}S, ^{112}S) upstream from the Ig T NLS (^{126}PKKKRKV^{132}) (29,30). To test this hypothesis, we measured the transport kinetics of Np 7, in which ^{121}S is substituted with alanine (Fig. 1). Both *in vitro* (Fig. 2D) and *in vivo* (Table I) data show that ^{121}S is not required for Np's normal transport kinetics.

A polyglutamic acid tract near the NLS is required for NPC facilitated translocation.

The amino flank of Np's NLS also contains a polyglutamic acid-rich region (residues 124-140) (Fig. 1). We reasoned that any potential transport role for phosphorylation of ^{121}S might be overwhelmed by the multiple negative charges of this polyglutamic acid (poly-glu) tract. We therefore used Np 1, with residues 121-140 deleted, to investigate whether the poly-glu tract influences Np's cytoplasm to nucleus transport. We found that this domain is absolutely required for transport, both *in vivo* (Table I) and *in vitro* (Fig. 2E). These data imply that deletion of the poly-glu tract specifically blocks NPC facilitated translocation, since Np 1 not only fails to accumulate in the nucleus, but it also fails to enter.

We examined directly the requirement for the poly-glu tract in facilitated translocation. Previously, we demonstrated that by measuring NLS-protein transport into the OIN from a conjoined agarose bead (B), rather than from cytoplasm, we could isolate the NPC facilitated translocation process, because wt Np rapidly *equilibrates* (i.e., [OIN]/[B]→1.0) in such OIN:B transport pairs (provided an ATP-regenerating system is included in the bead to power facilitated translocation), but it does not *accumulate* in the nucleus—presumably because proteins in the cytoplasm are required for Np's intranuclear binding (6). Comparative OIN:B transport data for wt Np and Np 1 (Fig. 3) confirm that the poly-glu tract is required to engage the NPC facilitated translocation.

The polyglutamic acid tract also enhances Np's intranuclear binding. We next sought to determine whether the poly-glu tract might also influence Np's intranuclear binding. It is difficult to study the intranuclear binding of wt Np or the mutants Np 1,2,3,4, and 7, in the absence of their NPC facilitated transloca-

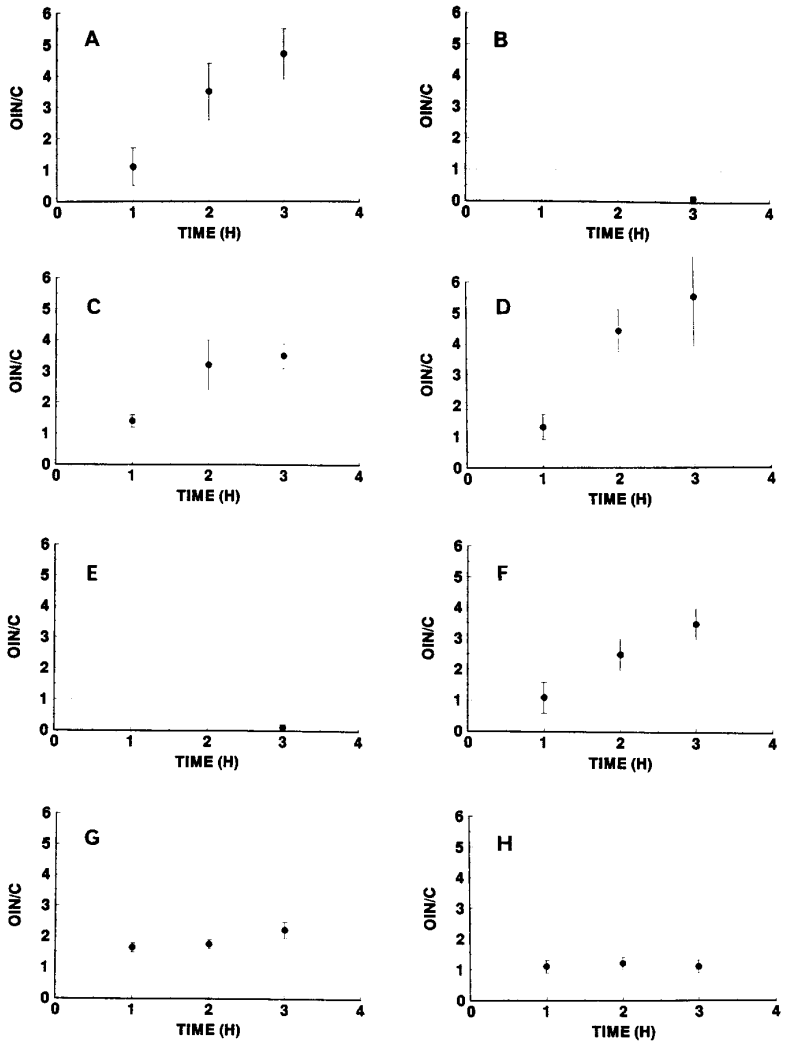

FIG. 2. *In vitro* transport kinetics in OIN:C pairs: (A) wt Np; (B) Np 3 or Np4; (C) Np 2; (D) Np 7; (E) Np 1; (F) Np 5; (G) Np 6; (H) Np 4/5.

tion, because facilitated translocation of these larger pentameric proteins into the intact nucleus obligatorily precedes their intranuclear binding. Therefore, we studied the transport of truncated Np mutants (Np 5, 6, 4/5, and 4/6, Fig. 1). Unlike wt Np and the pentameric mutants, the truncated mutants remain as monomers and pass through the NPC by diffusion, rather than by energy-dependent, NPC facilitated translocation. Thus, with the truncated forms of Np, we were able to study Np's transport into the intact nucleus *in the absence of NPC facilitated translocation* (Vancurova et al., unpublished).

In OIN:C pairs, even though the truncated mutants Np 5 and Np 6 diffuse into the nucleus, they still accumulate therein rather than simply equilibrating between the two compartments (Figs. 2F and 2G, respectively). That their accumulation is due to intranuclear binding was confirmed by experiments in which micropuncture permeabilization of the nuclear envelope prior to the formation of transport pairs did not change their accumulation behavior (data not shown), just as we originally showed for wt Np (6,7). We also measured the cytoplasm to nucleus transport of Np 5 and Np 6 microinjected into living oocytes and found that both proteins bind in the *in vivo* nucleus (Table I). Both the *in vitro* and *in vivo* data reveal that while Np's bipartite NLS alone (i.e., in the absence of the poly-glu tract, Np 6) engages intranuclear binding, the binding is significantly enhanced when the poly-glu tract is present (Np 5). Thus, in addition to being essential for NPC facilitated translocation (above), the poly-glu tract also enhances Np's intranuclear binding. As controls, we also measured the intranuclear binding of Np 4/5 and Np 4/6 *in vivo* (Table I) and Np 4/5 *in vitro* (Fig. 2H); the

TABLE I

[N]/[C] *In Vivo*

Protein transportant	[N]/[C] (no. of cells)
wt Np	7.04 ± 0.85 (n=7)
Np 1	0.26 ± 0.06 (n=6)
Np 2	7.46 ± 2.00 (n=7)
Np 3	0.37 ± 0.08 (n=5)
Np 4	0.35 ± 0.07 (n=6)
Np 5	6.43 ± 1.51 (n=4)
Np 6	2.57 ± 0.82 (n=6)
Np 7	7.29 ± 2.56 (n=3)
Np 4/6	1.76 ± 0.12 (n=5)
Np 4/5	1.67 ± 0.15 (n=3)

Note. To determine the ratio of the *in vivo* concentrations in the nucleus [N] and the cytoplasm [C], each radiolabeled protein was microinjected into the vegetal cytoplasm of living oocytes, and its nuclear and cytoplasmic concentrations were measured at 18-24 h later (as described under Methods). A minimum [N]/[C] value of ~0.3 results from residual cytoplasm on the surface of the OIN and represents an absence of nuclear entry (6).

data not only confirm that both halves of Np's bipartite NLS must be present in order to engage intranuclear binding, but they also demonstrate that the poly-glu tract is not sufficient in itself to engage intranuclear binding.

DISCUSSION

Complete understanding of NLS-protein transport probably awaits identification of additional (and likely less soluble) transport machinery proteins. Furthermore, we need more details about the interactions of NLS-proteins with the known transport proteins.

Numerous lines of evidence suggest that NLS-protein phosphorylation modulates these interactions. In the best-documented case, phosphorylation at a casein kinase II (CKII) site (^{111}S, ^{112}S) and a cdc2 kinase site (^{124}T) near the NLS of the SV40 large T antigen (Ig T) dramatically influences this protein's transport (32,33). In the case of Np, one or more of its phosphorylation sites may modulate the function of its NLS: for example, Np associates with and is phosphorylated by CKII *in vivo,* and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazol (DRB), a specific inhibitor of CKII, inhibits Np's nuclear transport (25). The results of this present study do not support a requirement for any of the phosphorylation sites on the carboxy-terminal side of Np's NLS or for the amino-terminal flanking CKII site (^{121}S). Still, it remains possible that, within the full context of native Np, the phosphorylation state(s) of one or more of these sites might regulate conformational changes and influence activity of the NLS.

Fundamental knowledge about NLS function has been obtained by joining selected, individual regions of an NLS-protein (generated from its known sequence) to a normally cytoplasmic protein. Using this approach, Robbins et al. (18) showed that pyruvate kinase is transported into the nucleus and accumulated therein if it is fused with Np's putative bipartite NLS. Importantly, however, this approach is limited, in that any one tested region of an NLS-protein may not exert its complete, *in vivo* transport function when isolated from its normal protein context. In particular, transport activities which depend on interactions of distinct regions of an NLS-protein would not be detected, and regulatory roles for one domain on the transport activity of another would go unnoticed.

To overcome this limitation, we constructed several Np mutants (Fig. 1). By measuring their nuclear transport, we systematically investigated the influence of different regions of the Np molecule on its transport. We found that, in addition to its NLS, Np's residues 124-140 (containing 12 glutamic acid residues [23,34]) are necessary for transport. More specifically, the poly-glu region is required in order for Np to engage the NPC facilitated translocation. Furthermore, experiments with novel, truncated Np mutants, which enter the nucleus by diffusion rather than facilitated translocation, reveal that the poly-glu region additionally enhances Np's intranuclear binding.

It has been previously demonstrated that for some proteins an NLS can be sufficient in itself to engage both NPC facilitated translocation and subsequent intranuclear binding (31). Nevertheless, the present results demonstrate that when Np's bipartite NLS resides within it's native context, it's ability to engage these two recognized stages of selective NLS-protein transport is influenced by the nearby poly-glu region. In the absence of detailed knowledge of Np's three-dimensional structure, the simplest explanation is that the poly-glu tract is essential to establish a conformation which makes Np's NLS sterically available for interactions with proteins of the transport machinery. One such interaction is essential to Np's NPC translocation, and another must be involved in the subsequent

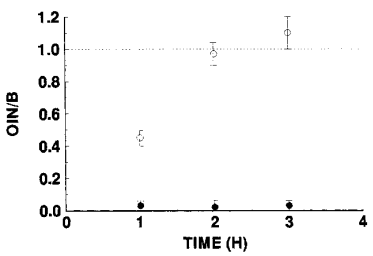

FIG. 3. *In vitro* transport kinetics in OIN:B pairs, in the presence of an energy-regenerating system: wt Np, open circles; Np 1, closed circles.

intranuclear binding of Np. Deletion of the poly-glu tract from the Np molecule may in some way mask or render the NLS cryptic for the translocation and reduce its efficiency in engaging the binding.

Additional mechanisms are conceivably involved. For example, Np's poly-glu tract may interact directly with some proteins of the transport machinery. The poly-glu tract does seem to be involved in Np's binding to basic domains of other proteins—in particular, its known interactions with histones (35,36). (Relative to such an interpretation, the transport data for the truncated Np mutants do show that Np's poly-glu tract does not—by itself—engage the intranuclear binding mechanism.) While we do not know precisely how the poly-glu tract exerts its transport influences, it is noteworthy that polyglutamic and polyaspartic acid tracts are features of many nuclear proteins (35). Indeed, it has been suggested (20), based upon analysis of protein sequence databases, that not only basic "karyophilic" clusters but also acidic clusters in NLS-proteins mediate their selective binding to proteins of the transport machinery.

The present results for the series of Np mutants show that a probe carrying an NLS isolated from its native protein context must not be assumed to display full, *in vivo* transport characteristics, and that full elucidation of the interactions of NLS-proteins with proteins of the nuclear transport machinery will require more robust approaches.

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REFERENCES

1. Feldherr, C. M., and Akin, D. (1995). Nuclear transport as a function of cellular activity. *Memb. Prot. Transport* **2,** 237–259.
2. Ambron, R. T., Schmied, R., Huang, C. C., and Smedman, M. (1992). A signal sequence mediates the retrograde transport of proteins from the axon periphery to the cell body and then into the nucleus. *J. Neurosci.* **12,** 2813–2818.
3. Schmied, R., Huang, C. C., Zhang, X. P., Ambron, D. A., and Ambron, R. T. (1993). Endogenous axoplasmic proteins and proteins containing nuclear localization signal sequences use the retrograde axonal transport/nuclear import pathway in Aplysia neurons. *J. Neurosci.* **13,** 4064–4071.
4. Richardson, W., Mills, A., Dilworth, S., Laskey, R., and Dingwall, C. (1988). Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. *Cell* **52,** 655–664.
5. Newmeyer, D., and Forbes, D. (1988). Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. *Cell* **52,** 641–653.
6. Vancurova, I., Lou, W., Paine, T. M., and Paine, P. L. (1993). Nucleoplasmin uptake by facilitated transport and intranuclear binding. *Eur. J. Cell Biol.* **62,** 22–33.
7. Paine, P. L., Yassin, R., Paine, T. M., Vancurova, I., George, C., and Sahay, N. (1995). Intranuclear binding of nucleoplasmin. *J. Cell. Biochem.* **58,** 105–114.
8. Adam, E. J. H., and Adam, S. A. (1994). Identification of cytosolic factors required for nuclear localization sequence-mediated binding to the nuclear envelope. *J. Cell Biol.* **125,** 547–555.
9. Moore, M. S., and Blobel, G. (1994). Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl. Acad. Sci. USA* **91,** 10212–10216.
10. Görlich, D., Vogel, F., Mills, A., Hartmann, E., and Laskey, R. (1995). Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377,** 246–248.
11. Imamoto, N., Tachibana, T., Matsubae, M., and Yoneda, Y. (1995). A karyophilic protein forms a complex with cytoplasmic components prior to nuclear pore binding. *J. Biol. Chem.* **270,** 8559–8565.
12. Radu, A., Blobel, G., and Moore, M. S. (1995). Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl. Acad. Sci. USA* **92,** 1769–1773.
13. Melchior, F., and Gerace, L. (1995). Mechanisms of nuclear protein import. *Curr. Opin. Cell Biol.* **7,** 310–318.
14. Hicks, G. R., Smith, H. M. S., Lobreaux, S., and Raikhel, N. V. (1996). Nuclear import in permeabilized protoplasts from higher plants has unique features. *The Plant Cell* **8,** 1337–1352.
15. Rexach, M., and Blobel, G. (1995). Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell* **83,** 683–692.
16. Okuno, Y., Imamoto, N., and Yoneda, Y. (1993). 70-kDa heat-shock cognate protein colocalizes with karyophilic proteins into the nucleus during their transport *in vitro*. *Exp. Cell Res.* **206,** 134–142.
17. Dingwall, C., Sharnick, S. V., and Laskey, R. A. (1982). A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell* **30,** 449–458.
18. Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: Identification of a class of bipartite nuclear targeting sequences. *Cell* **64,** 615–623.
19. Yamasaki, L., and Lanford, R. E. (1992). Nuclear transport receptors: specificity amid diversity. *in* Nuclear Trafficking (Feldherr, C. M., Ed.), Academic Press, San Diego.
20. Boulikas, T. (1994). Putative nuclear localization signals (NLS) in protein transcription factors. *J. Cell. Biochem.* **55,** 32–58.
21. LaCasse, E. C., and Lefebvre, Y. A. (1995). Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins. *Nucl. Acids Res.* **23,** 1647–1656.
22. Lanford, R. E., Feldherr, C. M., White, R. G., Dunham, R. G., and Kanda, P. (1990). Comparison of diverse transport signals in synthetic peptide-induced nuclear transport. *Exp. Cell Res.* **186,** 32–38.
23. Dingwall, C., Dilworth, S. M., Black, S. J., Kearsey, S. E., Cox, L. S., and Laskey, R. A. (1987). Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a cluster of sequences homologous to putative nuclear localization signals. *EMBO J.* **6,** 69–74.
24. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth. Enzymol.* **154,** 367–382.
25. Vancurova, I., Paine, T. M., Lou, W., and Paine, P. L. (1995). Nucleoplasmin associates with and is phosphorylated by casein kinase II. *J. Cell Sci.* **109,** 779–787.
26. Paine, P. L., Johnson, M. E., Lau, Y.-T., Tluczek, L. J. M., and

Miller, D. S. (1992). The oocyte nucleus isolated in oil retains in vivo structure and function. *BioTechniques* **13,** 238–246.

27. Dumont, J. N. (1972). Oogenesis in Xenopus laevis (Daudin): Stages of oocyte development in laboratory maintained animals. *J. Morph.* **136,** 153–180.
28. Gurdon, J. B., and Wakefield, L. (1986). Microinjection of amphibian oocytes and eggs for the analysis of transcription, p. 269–299. *in* Microinjection and Organelle Transplantation Techniques (Celis, J., Graessmann, A., and Loyter, A., Eds.), Academic Press, New York.
29. Rihs, H. P., and Peters, R. (1989). Nuclear transport kinetics depend on phosphorylation-site-containing sequences flanking the karyophilic signal of The Siminan Virus 40 T-antigen. *EMBO J.* **8,** 1479–1484.
30. Rihs, H. P., Jans, D. A., Fan, H., and Peters, R. (1991). The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. *EMBO J.* **10,** 633–639.
31. Vancurova, I., Jochova, J., Lou, W., and Paine, P. L. (1994). An NLS is sufficient to engage facilitated translocation by the nuclear pore complex and subsequent intranuclear binding. *BBRC* **205,** 529–536.
32. Jans, D. A., Ackermann, M. J., Vischoff, J. R., Beach, D. H., and Peters, R. (1991). $p34^{cdc2}$ mediated phosphorylation at T^{124} inhibits nuclear import of SV40 T antigen proteins. *J. Cell Biol.* **115,** 1203–1212.
33. Jans, D. A., and Jans, P. (1994). Negative charge at the casein kinase II site flanking the nuclear localization signal of the SV40 large T-antigen is mechanistically important for enhanced nuclear import. *Oncogene* **9,** 2961–2968.
34. Burglin, T. R., Mattaj, I. W., Newmeyer, D. D., Zeller, R., and DeRobertis, E. M. (1987). Cloning of nucleoplasmin from Xenopus laevis oocytes and analysis of its developmental expression. *Genes and Development* **1,** 97–107.
35. Earnshaw, W. C. (1987). Anionic regions in nuclear proteins. *J. Cell Biol.* **105,** 1479–1482.
36. Xue, Z., and Melese, T. (1994). Nucleolar proteins that bind NLSs: a role in nuclear import or ribosome biogenesis? *Trends Cell Biol.* **4,** 414–417.