The human Tap protein mediates the sequence-specific nuclear export of RNAs containing the constitutive transport element and is likely also critical for general mRNA export. Here, we demonstrate that a previously defined arginine-rich nuclear localization signal (NLS) present in Tap acts exclusively via the transportin import factor. Previously, transportin has been shown to mediate the nuclear import of several heterogeneous nuclear ribonucleoproteins, including heterogeneous nuclear ribonucleoprotein (hnRNP) A1, by binding to a sequence element termed M9. Although the Tap NLS and the hnRNP A1 M9 element are shown to compete for transportin binding, they show no sequence homology, and the Tap NLS does not conform to the recently defined M9 consensus. The Tap NLS also differs from M9 in that only the latter is able to act as a nuclear export signal. The Tap NLS is therefore the first member of a novel class of transportin-specific NLSs that lack nuclear export signal function.

Although the mechanisms governing the nuclear export of noncoding RNAs are becoming increasingly well understood, the pathway(s) utilized for nuclear export of mRNA molecules has yet to be defined and appears likely to be subject to complex regulation (reviewed in Ref. 1). At least two possible protein mediators of mRNA export exist. The first is actually a class of highly expressed proteins, termed heterogeneous nuclear ribonucleoproteins (hnRNPs),1 of which the prototype is hnRNP A1 (2). The hnRNPs associate with nuclear pre-mRNAs and mRNAs and a subset of the hnRNPs including hnRNP A1, then accompany mature mRNAs from the nucleus to the cytoplasm, where they are released (3, 4). This nucleocytoplasmic shuttling is not passive, in that many hnRNPs contain not only a nuclear localization signal (NLS) but also a nuclear export signal (NES) (5, 6). In the case of hnRNP A1, these functions are both encoded within a short sequence element termed M9 (see Fig. 1), and exhaustive analysis has failed to separate NLS from NES function (6, 7). Efforts to define the cellular proteins that mediate M9 NLS function led to the identification of the transportin (Trn) import factor, a member of the importin β (Imp β) family of nucleocytoplasmic transport factors (8–10). Surprisingly, however, several lines of evidence suggest that M9 NES function is not mediated by Trn, and the nuclear receptor for this NES therefore remains to be defined (7, 11, 12).

A second candidate nuclear mRNA export factor is the Tap protein. Tap has been shown to bind the retroviral constitutive transport element (CTE) RNA target sequence and to mediate the nuclear export of mRNAs bearing the CTE (13–16). Several lines of evidence suggest that Tap may also be a critical component of general mRNA export. Thus, microinjection of high levels of CTE RNA into Xenopus oocyte nuclei selectively inhibits all mRNA export, and this inhibition can be rescued by microinjection of recombinant human Tap (14, 17, 18). Genetic analysis in yeast has demonstrated that Mex67p, the yeast homolog of Tap, is critical for the nuclear export of poly(A)+ RNA (19). Expression in yeast of human Tap together with a second human protein termed p15, which may be a Tap cofactor, rescues both nuclear poly(A)+ RNA export and cell viability in yeast cells lacking Mex67p (20). It has therefore been proposed that Tap is a critical component of an evolutionarily conserved nuclear mRNA export pathway (20).

Experimental analysis has led to the identification of several functional domains in Tap (14, 16, 20). An RNA binding domain, which is necessary and sufficient for specific binding to the CTE RNA target, extends from approximately residues 80 to 372 in the 619-amino acid Tap protein (see Fig. 1) (14, 16, 20). At the C terminus of Tap is a nucleocytoplasmic shuttle domain that is critical for Tap-dependent CTE RNA export (16). This NLS/NES element, which has been recently shown to directly interact with the FG-repeat domain of nucleoporin Can/Nup214 (20), may serve to target Tap, together with any bound RNA cargo, to the nuclear pore. Importantly, the Tap NLS/NES has no homology to the hnRNP A1 M9 sequence and does not interact with Trn in our hands (data not shown). Last, Tap also contains an NLS, located between residues 61 and 102 (Fig. 1), that contains 10 arginine residues yet lacks any lysine residues (16, 20). Because lysine is critical for binding to the Importin α (Imp α) nuclear import receptor (21), the Tap NLS, despite its overall positive charge, nevertheless appears unlikely to be functionally similar to Imp α-dependent basic NLSs.

In this manuscript, we demonstrate that nuclear import of substrates bearing the Tap NLS is independent of both Imp α and Imp β and is instead exclusively mediated by Trn. Although the Tap NLS binds Trn specifically in vitro, this interaction is disrupted by Ran-GTP, as is expected for a function-
a 50-μl total volume of ACB buffer (50 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM dithiothreitol). Column washes were carried out with 50 μl of ACB buffer. Columns were finally eluted (bound fractions) with 50 μl of 500 mM magnesium chloride in ACB buffer. The entire flow-through and bound fractions from each column were mixed with sample-loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis on 10–20% gradient gels (ReadyGel, Bio-Rad) and then Coomassie-stained (R-250, Life Technologies, Inc.). Ran-GTP release assays were carried out using Recombinant RanGDS, GST-Tap-NLS protein, GTP or GDP, and glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), essentially as described elsewhere (7, 23).

**Mammalian Expression Plasmids**—Mammalian expression plasmids encoding β-galactosidase (β-Gal) fused to the C-terminal M9 domain of human hnRNP A1 (amino acids 288–318) or to the N-NLS of Tap (amino acids 61–102) were constructed using polymerase chain reaction primers that introduced BamHI and Xhol restriction sites at the N and C terminus, respectively, of the M9 or Tap-N-NLS sequence. The resultant PCR DNA fragments were digested with BamHI and Xhol restriction enzymes and ligated into BamHI/Xhol-digested β-gal/Nab2 (22). The resultant plasmids express the M9 or Tap-N-NLS fused to the C terminus of β-Gal.

**Nucleocytoplasmic Shuttling Assays**—The heterokaryon assay was carried out essentially as described previously (6). Human HeLa and mouse NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. HeLa cells were transfected with expression plasmids encoding β-Gal fused to M9 or the Tap-N-NLS using the FuGene reagent (Roche Molecular Biochemicals). At 36 h post-transfection, HeLa cells were mixed with mouse 3T3 cells in a 2:3 ratio, seeded onto glass coverslips at 5 × 10^3 cells/coverslip, and further incubated for 6 h at 37 °C. Cells were then treated with cycloheximide (100 μg/ml) for 3 h, fused with 50% polyethylene glycol (PEG 3350, Sigma) for 2.5 min, then incubated for another 2 h at 37 °C in medium containing cycloheximide. Cells were then fixed and permeabilized as described previously (25). The β-Gal fusion proteins were visualized by indirect immunofluorescence using a primary mouse monoclonal anti-β-Gal antibody (Promega) and a secondary rhodamine-conjugated goat anti-mouse antibody (Cappel). Hoechst dye 33258 (Sigma) was included at 1 μg/ml with the secondary antibody incubation. Images were digitally captured with a Leica DMRB fluorescence microscope under the 100× objective and visualized using Adobe Photoshop 4.0 software.

HeLa cells were maintained and micoinjected as described previously (7, 16). Briefly, 2 days before microinjection, HeLa cells were seeded onto CELLocate microgrid coverslips (Eppendorf Scientific) at a density of 2 × 10^5/35-mm dish. To increase the prevalence of multinucleated cells, cultures were serum-starved overnight and then re-fed with serum-containing media 4–6 h before injection. A recombinant fusion protein consisting of MBP fused to the M9 NLS/NES was expressed and purified as described previously (22). GST-Tap-N-NLS (final concentration in PBS, ~2 μg/μl) was mixed with tetramethylrhodamine isothiocyanate-conjugated MBP-M9 (final concentration in PBS, ~1.5 μg/μl) and then co-injected into either one nucleus of a multinucleated cell or into the cell cytoplasm. After injection, cells were incubated at 37 °C for 40 min and then fixed with 3% paraformaldehyde.
in PBS. The GST-Tap-N-NLS fusion protein was detected by indirect immunofluorescence using a polyclonal affinity-purified rabbit anti-GST antibody and fluorescein isothiocyanate-conjugated donkey antirabbit antiserum (Jackson Immunoresearch). The subcellular localization of the injected proteins was visualized as described above.

RESULTS AND DISCUSSION

Transportin Is Necessary and Sufficient to Mediate Tap-N-NLS-dependent Nuclear Import—Previously, we and others demonstrated that the human Tap nuclear RNA export factor contains an NLS, located between residues 61 and 102 of the 619-amino acid Tap protein, that is able to induce the nuclear localization of both a GST and a green fluorescent protein fusion in human cells (16, 20). In Fig. 2, we examined whether the Tap N-NLS can also mediate the nuclear uptake of recombinant protein substrates in vitro and also whether known peptide binding targets for selected nuclear import factors would be able to competitively inhibit this nuclear uptake when present in excess. The inhibitors chosen were the IBB, which represents the site on Imp α that binds to Imp β (26, 27), the SV40 T-NLS, which binds to Imp α directly (28), and the M9 NLS/NES, which binds to Trn (8–10). An M9 mutant peptide that differs from M9 at only one residue (Gly-274 to Ala) yet lacks both NLS function and Trn binding ability served as a negative control.

The substrate proteins tested, all of which are FITC-labeled GST fusion proteins, contain either the Tap N-NLS, the SV40 T-NLS, the hnRNP A1 M9 NLS, or the HIV-1 Tat NLS. As shown in Fig. 2, panels A through D, all four substrates were imported into isolated HeLa cell nuclei in vitro upon addition of reticulocyte lysate as a source of native import factors. The addition of the IBB peptide blocked both SV40 T-NLS function and HIV-1 Tat NLS function but did not affect M9 or Tap N-NLS uptake (panels E to H). The IBB peptide competes the binding of Imp α by Imp β (26, 27) and, therefore, is expected to block uptake of Imp α-dependent NLSs such as the SV40 T-NLS (28). Although the Tat NLS directly interacts with Imp β, this is also competed by IBB (23).

As predicted, an excess of the SV40 T-NLS peptide blocked SV40 T-NLS-dependent nuclear import, presumably by competing for Imp α binding, but did not affect the Tat NLS, which binds Imp β directly (22), or the M9 NLS, which binds Trn (8–10). The Tap N-NLS was also unaffected by this competitor (Fig. 2, panel I). Finally, the wild-type M9 peptide, which directly binds to Trn (8–10), blocked not only M9 NLS function but also Tap N-NLS-dependent nuclear import (panels M and O). This inhibition was specific in that the M9 peptide did not, as predicted, affect SV40 T-NLS- or HIV-1 Tat NLS-dependent

![Fig. 3. Trn is sufficient for Tap-N-NLS-dependent nuclear import in vitro.](image)

A, suspension-grown HeLa cells were permeabilized using digitonin and then pelleted through a sucrose cushion (22). Reconstituted nuclear import assays were performed in the presence of a buffer containing Ran, an ATP regeneration system, 10 mM ATP, and 0.1 mM GTP. The recombinant transport factors listed at the top were added at a ~1 mM concentration, whereas the FITC-labeled GST fusion proteins listed at the left were added at an ~2 mM concentration. After incubation at 25 °C for 20 min, the permeabilized cells were fixed and analyzed for nuclear import by fluorescence microscopy. B, panel a is identical to panel j in Fig. 3A. In panel b, no Ran has been added. In panel c, 5 mM Ran Q69L mutant has been added. In panel d, the 0.1 mM GTP has been substituted with 1 mM GMP-PNP. In panel e, the 10 mM ATP has additionally been substituted with 1 mM AMP-PNP, whereas 20 units/ml hexokinase, 1 mM glucose, and 20 μM ADP have been added to deplete any endogenous nucleotide triphosphates (29). The bar in panels l and e ~ 20 μm.

![Fig. 4. Nuclear uptake of the Tap-N-NLS is sequence-specific.](image)

Panels A and C were derived as described in Fig. 3, whereas panels B and D were obtained as described in Fig. 2. The substrate proteins consisted of FITC-labeled fusions of GST to the wild-type Tap-N-NLS or to the nonfunctional A1 mutant shown in Fig. 1. The bar in panel D ~ 20 μm. Retic, reticulocyte.
Trn is both necessary and sufficient for the specific nuclear import assessed in Figs. 2 through 4, demonstrating that when a functional Tap-N-NLS is tested. Nuclear uptake visualized in Figs. 2 and 3 is therefore only seen in the presence of cytoplasm (panel D) and recombinant Trn (panels A and C) but failed to induce the nuclear localization of either Trn-dependent NLS chimeras.

We next asked whether Trn was not only necessary but also sufficient for Tap N-NLS function. As shown in Fig. 3A, recombinant Trn, when added to a transport buffer containing Ran, ATP, and GTP, was indeed able to mediate the in vitro nuclear uptake of not only a GST-M9 fusion protein but also a similar GST-Tap-N-NLS fusion protein. In contrast, a combination of Imp α and Imp β could mediate the nuclear uptake of a GST-T-NLS fusion but failed to induce the nuclear localization of either Trn-dependent NLS chimeras.

Recently, it has been proposed that nuclear import in general and Trn-dependent M9 nuclear import in particular is independent of energy or Ran and that the sole role of the GTP-bound form of Ran is, in fact, to mediate cargo release at the nuclear pore (29, 30). As shown in Fig. 3B, we continued to see nuclear import of the GST-Tap-N-NLS fusion in the absence of added Ran (panel b), in the presence of a GTP analog (panel d), and even in the presence of not only ATP and GTP nucleotide analogs but also of an agent (hexokinase) that should effectively hydrolyze any residual endogenous nucleotide triphosphates (panel e). Although nuclear import in this latter case did appear less efficient (compare panels b and e), this result is nevertheless consistent with the hypothesis (29, 30) that transport through the nuclear pore is not energy-dependent. To test whether the interaction of Trn with the Tap-N-NLS substrate would be subject to release by Ran-GTP (11), we next added the Q69L mutant of Ran, which binds GTP but resists hydrolysis to GDP (31), to the import reaction. As shown in Fig. 3B, panel c, the added RanGTP indeed prevented the nuclear uptake of the GST-Tap-N-NLS substrate.

Previously, we showed that the A1 mutation of the Tap-N-NLS, which consists of three alanines introduced in place of Arg-Val-Arg at residues 69 to 71 of the Tap protein (Fig. 1), entirely blocks Tap-N-NLS function in vivo (16). As shown in Fig. 4, this same mutation also blocks nuclear uptake in vitro in both the presence of recombinant Trn (panel C) and in the presence of cytoplasm (panel D). The Trn-dependent in vitro nuclear uptake visualized in Figs. 2 and 3 is therefore only seen when a functional Tap-N-NLS is tested.

**Fig. 5. In vitro binding of Trn by the Tap-N-NLS.** A, protein microaffinity chromatography followed by SDS-polyacrylamide gel electrophoresis was used to show a direct interaction of a recombinant GST-Tap-N-NLS protein with recombinant Trn but not with Imp α or Imp β. Ft, flow-through fraction; B, bound fraction. B, the GST-Tap-N-NLS protein was mixed with recombinant Trn in the presence of buffer alone or buffer containing RanQ69L-GTP or RanQ69L-GDP. Bound proteins were collected using glutathione-Sepharose beads and visualized by SDS-polyacrylamide gel electrophoresis. C, identical to panel A, except that the wild-type GST-Tap-N-NLS fusion protein is here contrasted with the A1 mutant in terms of Trn binding ability. Wt, wild type.

**Fig. 6. Heterokaryon fusion nuclear shuttling assay.** Human cells expressing a β-Gal-M9 fusion protein (panels D through F) or a β-Gal-Tap-N-NLS fusion protein (panels A through C) were fused to murine cells in the presence of a protein synthesis inhibitor. Nucleocytoplasmic shuttling is indicated by movement of the β-Gal fusion protein from the human to the murine nuclei. Murine nuclei give a punctate staining pattern when treated with Hoechst 33258 dye (panels B and E) and are also indicated by a stippled edge in all panels. As may be observed, only the β-Gal-M9 fusion (panel D) and not the β-Gal-Tap-N-NLS fusion (panel A) showed nucleocytoplasmic shuttling, although both proteins are clearly nuclear at steady state.

**Fig. 7. HeLa cell microinjection assays.** Binuclear HeLa cells were selectively microinjected into one nucleus with a mixture of recombinant GST-Tap-N-NLS and MBP-M9 fusion proteins (panels A through C). Alternately, this same protein mixture was microinjected into the cytoplasm of normal HeLa cells (panels D through F). After 40 min of incubation at 37 °C, the cells were fixed, and the localization of the injected proteins was determined by fluorescence microscopy.

**Functional Comparison of M9 and the Tap-N-NLS—**As noted above, the M9 sequence is not only a Trn-dependent NLS but also an NES, and these properties have not been mutationally segregated despite a considerable, even exhaustive, effort (6, 7). In this manuscript, we report that the Tap-N-NLS is not only Trn-dependent but that M9 competes with Tap-N-NLS for binding to Trn (Figs. 2 and 3). We therefore asked whether the Tap-N-NLS, like the hnRNP A1 M9 sequence, would demonstrate NES function using the assay first used to show M9 NES function, i.e. a heterokaryon fusion nuclear-shuttling assay (6). In this assay, human cells are first transfected with expression plasmids encoding the relevant proteins (in this case M9 and Tap-N-NLS fusions to β-Gal). At 36 h after transfection, mouse 3T3 cells are
mixed with the transfected HeLa cells, and after attachment, the culture is treated with cycloheximide to block further protein synthesis. The cells are then fused using polyethylene glycol, incubated for a further 2 h in the presence of cycloheximide, and finally analyzed for the subcellular localization of the fusion protein. Staining with Hoechst 33258 dye allows the human and murine nuclei to be readily distinguished, in that only the latter give a marked punctate staining pattern (6).

As shown in Fig. 6, we were indeed able to confirm that M9 can mediate the nucleocytoplasmic shuttling of the M9 β-Gal fusion protein from a human nucleus to an introduced murine nucleus (panels D and E). In contrast, we failed to see any evidence of shuttling by the Tap-N-NLS β-Gal fusion protein, although this protein clearly remained able to localize to the human nucleus (Fig. 6, panels A and B). Analysis of several heterokaryons failed to reveal any evidence of nucleocytoplasmic shuttling by the β-Gal-Tap-N-NLS, whereas shuttling by the β-Gal-M9 fusion protein was routinely detected.

To further confirm that M9 and the Tap-N-NLS indeed differ in terms of their ability to mediate nuclear protein export, we prepared recombinant fusion proteins consisting of GST fused to the Tap-N-NLS and MBP fused to M9. These were then co-injected into one nucleus of binuclear HeLa cells. As previously shown, nuclear injection of a protein bearing both an NES and an NLS results in the shuttling of the microinjected protein from the injected nucleus to the un.injected nucleus, whereas proteins bearing an NLS but lacking an NES remain trapped in the injected nucleus.

As shown in Fig. 7, the MBP-M9 fusion protein indeed proved able to shuttle from one nucleus to the other (panel A), while the co-injected GST-Tap-N-NLS fusion remained in the injected nucleus (panel B). To confirm that the GST-Tap-N-NLS remains fully active as an NLS, we also co-injected these two proteins into the cytoplasm of HeLa cells. As shown in Fig. 7, panels D and E, both fusion proteins were able to localize to the nucleus of the injected cells. We note that the cells visualized in Fig. 7 were in fact incubated for ~40 min after microinjection, whereas, as previously shown (7), equilibration of a microinjected protein bearing the M9 NES/NLS between the two resident nuclei actually is complete within ~20 min. Therefore, this experiment clearly demonstrates that the Tap-N-NLS is unable to mediate any detectable nuclear export in a cell that can effectively support the nuclear export of a fusion protein bearing the M9 NES/NLS.

A Novel Class of Trn-dependent NLSs—Two types of protein NLS sequences have previously been shown to use Trn to mediate their nuclear import. The prototype of the first of these is, as noted above, termed M9 and was initially defined in human nucleus (Fig. 6, 5–10). Although this protein clearly remained able to localize to the human nucleus (Fig. 6, panels A and B). As shown in Fig. 7, panels D and E, both fusion proteins were able to localize to the nucleus of the injected cells. We note that the cells visualized in Fig. 7 were in fact incubated for ~40 min after microinjection, whereas, as previously shown (7), equilibration of a microinjected protein bearing the M9 NES/NLS between the two resident nuclei actually is complete within ~20 min. Therefore, this experiment clearly demonstrates that the Tap-N-NLS is unable to mediate any detectable nuclear export in a cell that can effectively support the nuclear export of a fusion protein bearing the M9 NES/NLS.

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