Definition of a Consensus Transportin-specific Nucleocytoplasmic Transport Signal*

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Recent progress in understanding the mechanisms that drive the nucleocytoplasmic transport of proteins and RNAs suggests that both nuclear import and export are mediated by a family of proteins related to the prototypic import factor importin β/karyopherin β1 (Imp-β1) (reviewed in Refs. 1–3). Directionality of movement appears, at least in part, to be controlled by the low cytoplasmic and high nuclear concentration of the GTP-bound form of Ran (4, 5). Thus, import factors and export receptors bind RanGTP directly, yet this interaction produces opposite effects; in the former case, RanGTP binding induces nuclear cargo release, whereas in the latter, RanGTP binding induces nuclear cargo assembly. Therefore, nuclear import and export receptors and their protein recognition sites are predicted to be distinct. Nevertheless, the ~38-amino acid M9 sequence present in heterogeneous nuclear ribonucleoprotein A1 has been reported to serve as both a nuclear localization signal and a nuclear export signal, even though only one protein, the nuclear import factor transportin, has been shown to bind M9 directly. We have used a combination of mutational randomization followed by selection for transportin binding to exhaustively define amino acids in M9 that are critical for transportin binding in vivo. As expected, the resultant ~12-amino acid transportin-binding consensus sequence is also predictive of nuclear localization signal activity. Surprisingly, however, this extensive mutational analysis failed to dissect M9 nuclear localization signal and nuclear export signal function. Nevertheless, transportin appears unlikely to be the M9 export receptor, as RanGTP can be shown to block M9 binding by transportin not only in vitro, but also in the nucleus in vivo. This analysis therefore predicts the existence of a nuclear export receptor distinct from transportin that nevertheless shares a common protein-binding site on heterogeneous nuclear ribonucleoprotein A1.

Recent progress in understanding the mechanisms that drive the nucleocytoplasmic transport of proteins and RNAs suggests that both nuclear import and export are mediated by a family of proteins related to the prototypic import factor importin β/karyopherin β1 (Imp-β1) (reviewed in Refs. 1–3). Directionality of movement appears, at least in part, to be controlled by the low cytoplasmic and high nuclear concentration of the GTP-bound form of Ran (4, 5). Thus, import factors, such as Imp-β, are believed to bind to nuclear localization signals (NLSs) or adaptor molecules, such as importin/karyopherin α, in the cytoplasm, where RanGTP is found at very low concentrations. Once the resultant import receptor-substrate complex reaches the nucleus, where RanGTP exists at high concentrations, the direct interaction of Imp-β with RanGTP induces the release of the protein cargo (5–7). Conversely, Imp-β-related nuclear export factors such as Cas and Crm1 are believed to bind proteins containing a cognate nuclear export signal (NES) in the nucleus only in the form of a ternary complex involving RanGTP (8, 9). Once the resultant export receptor-substrate complex reaches the cytoplasm, RanGTP is hydrolyzed to RanGDP by the cytoplasmic RanGAP and RanBP1 or RanBP2 proteins, thereby inducing the release of the export receptor from both Ran and the export substrate.

A prediction of the hypothesis that RanGTP is critical for both NES substrate binding and NLS substrate release in the cell nucleus is that Imp-β-like transport factors should be dedicated to either import or export, depending on whether RanGTP induces substrate release or binding (1–3). A corollary of this prediction is that protein NES and NLS sequences should be recognized by different transport factors and should therefore be distinct. In general, this is indeed the case. Thus, leucine-rich NES sequences of the type first identified in human immunodeficiency virus type 1 Rev function only in nuclear export, whereas basic NLS sequences of the type found in SV40 T-antigen and nucleoplasmin function only to mediate nuclear import (1–3). Yet, one clear exception to this prediction exists, i.e. a sequence termed M9 that is present in heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) and related mRNA-binding proteins and that has been reported to function as both an NLS and an NES (10–12).

One group has defined the M9 NLS/NES as a 38-amino acid sequence located between residues 268 and 305 of hnRNP A1 (10), whereas a second group, which primarily analyzed nuclear import, has localized the hnRNP A1 NLS to a largely overlapping sequence extending from residues 260 to 289 (12), thus suggesting that the core of the M9 NLS is likely to be located between hnRNP A1 residues 268 and 289. Little additional information on the identity of the M9 NLS/NES has been published, although it is known that mutation of either glycine 274 or proline 275 to alanine blocks both NLS and NES function (10). Attempts to identify a protein that might mediate M9 function resulted in the cloning of an Imp-β-related transport factor termed transportin (Trn) or karyopherin b2 (13–15). Trn not only binds the M9 NLS/NES specifically both in vitro and in vivo, but also can mediate the specific import of substrate proteins bearing the M9 NLS into isolated nuclei in vitro.

Because of the expectation that the M9 NLS and NES functions should reflect the interaction of M9 with distinct nuclear import and export receptors (1–3) and given that the M9 sequence remains both large and ill defined, we have attempted to dissect the M9 NLS/NES by using a previously reported

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‡ The abbreviations used are: Imp-β, importin β/karyopherin β1; NLS, nuclear localization signal; NES, nuclear export signal; hnRNP, heterogeneous nuclear ribonucleoprotein; Trn, transportin; GST, glutathione S-transferase.
strategy (16) of sequence randomization followed by in vivo selection for M9 sequences that retain their ability to bind Trn. Using this approach, we have defined the sequence requirements for M9 NLS function in detail and report that these are indistinguishable from those required for M9 NES function. Possible interpretations of this surprising result are discussed.

**EXPERIMENTAL PROCEDURES**

**Construction of Molecular Clones—**Alanine-scanning mutants of the M9 NLS/NES were constructed in the previously described pGBT9/M9 yeast expression plasmid, which expresses the Gal4 DNA-binding domain fused to the UAS1 promoter (15). Mutated sequences were generated using the Quick Change site-directed mutagenesis kit (Stratagene) using complementary oligonucleotide primers that introduced the sequence 5'-GCCGCGCGTCA-3' in place of wild-type M9 sequences. This mutation substitutes four alanines for each set of four targeted M9 residues, as shown in Table I, and also introduces a unique NcoI restriction enzyme site that introduced a translation termination codon after the first two alanine codons. Sequences encoding M9 residues 256–263 were deleted by polymerase chain reaction mutagenesis and substituted by two glycine codons. All scanning mutants were verified by DNA sequence analysis, and their expression was quantitated by Western blot analysis as described previously (16).

Libraries of randomized M9 sequences were constructed in the pGBT9/M9 sequence context as described previously (17) using two flanking primers and two overlapping primers that introduced either 9 or 12 random bases into the M9 sequence context. Amplification products were cleaved with EcoRI and XhoI and then ligated into pGBT9 digested with EcoRI and SfiI. The ligated randomized libraries were then phenol/chloroform-extracted, ethanol-precipitated, and introduced into XL1-Blue bacteria (Stratagene) by electroporation. Each library contained $5 \times 10^8$ independent clones. Yeast transformants expressing M9 variants that retained the ability to interact with Trn in the two-hybrid assay were then identified as described previously (17).

The two synthetic M9 derivatives 8.4.10 and 11.4.11, which express M9 variants mutated between residues 265 and 280, were each generated by insertion of the relevant EcoRI-XhoI DNA fragment encoding M9, as described above, into pGEX4T-1 (Amersham Pharmacia Biotech). The parental two-hybrid vectors pGBT9 (CLONTECH) and pVP16 and their derivatives, pGBT9/M9 and pVP16/Tn5 (541–890), have been described (15). Full-length cDNA forms of human Trn and Tm2 were excised from the previously described pGEM3-Trn and pBS-Trap2 expression plasmids (15, 18) by cleavage with NotI and XhoI and then cloned into pGEX4T-1 to generate pGEX4T-1 (1–890) and pGEX4T2 (1–894), respectively. Similarly, a plasmid encoding a fusion protein consisting of the Gal4 DNA-binding domain linked to full-length human Ran was generated by excision of the Ran cDNA from a previously described pGEX4T-1/Ran expression plasmid (19) by cleavage with BamHI and XhoI followed by ligation into pGEX4T-1.

A plasmid (pGEM3-Trn) that permits efficient expression of full-length human Trn in a coupled in vitro transcription/translation system has been described (15). Also described is a prokaryotic expression plasmid encoding GST linked to wild-type human Trn (19). This latter plasmid was modified to encode GST linked to the Ran mutant Q69L (20), which is GTPase-deficient.

**Protein Purification—**Wild-type and mutant GST/M9 proteins were expressed in bacteria and purified by standard protocols in the absence of detergents as described previously (15). Purified proteins were dialyzed against phosphate-buffered saline and then concentrated by centrifugation using Centricon 10 concentrators (Amicon, Inc.). Recombinant non-fusion Ran Q69L mutant protein (20) was expressed and purified as described previously for wild-type Ran protein (19).

**Cell Culture and Microinjection—**HeLa cells were cultured in Dulbecco's modified Eagle medium containing 5% fetal bovine serum, gentamycin, and Fungizone. Two days prior to injection, 2 x 10^5 cells were plated on glass coverslips in 35-mm culture plates. To increase the fusion of binuclear cells, eight pieces of culture medium was replaced with serum-free Dulbecco's modified Eagle medium on the evening prior to microinjection. At ~16 h later, the cells were refed with Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and cultured for a further 4–6 h prior to use in microinjection assays.

Prior to microinjection, each GST/M9 fusion protein was diluted to ~2 mg/ml in phosphate-buffered saline and supplemented with 1 mg/ml rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). After microinjection, which took from 8 to 10 min/sample at room temperature, each coverslip was incubated with fresh Dulbecco's modified Eagle's medium containing 5% fetal bovine serum at 37 °C for between 1 and 30 min (cytoplasmic injections) or for 20 min (nuclear injections) prior to fixation using 3% paraformaldehyde. The subcellular localization of each injected protein was then determined by double-label immunofluorescence as described previously (15).

**In Vitro Protein Binding Assays—**GST/M9-G274A-Trn complexes were formed in the presence of binding buffer (20 mM Tris, pH 7.5, 200 mM NaCl, and 10 mM magnesium acetate) for 30 min at 25 °C and then collected by addition of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Beads containing M9-Trn complexes were washed once with binding buffer and then incubated for 30 min at 25 °C in Ran buffer alone (phosphate-buffered saline and 1 mM magnesium acetate) or with 10 μg of Ran Q69L in the presence of 5 mM GTP or GDP. After washing with Ran buffer, proteins still bound to the Sepharose beads were eluted with 1 M MgCl2 and analyzed by SDS-polyacrylamide gel electrophoresis (10% gel). In vitro translated (TNT, Promega), 35S-labeled Trn was visualized by fluorography (ENHANCE, NEN Life Science Products).

**RESULTS**

As noted above, Michael et al. (10) have localized the M9 NLS/ NES by deletion mutagenesis to between residues 268 and 305 of hnRNP A1, whereas Weighardt et al. (12), using a comparable approach, localized the hnRNP A1 NLS to between hnRNP A1 residues 260 and 289 (Fig. 1). To more clearly identify which residues within this region might contribute to NLS and/or NES function and to Trn binding, we performed alanine-scanning mutagenesis by sequential insertion of clusters of four alanines between residues 265 and 292 of the M9 sequence in the context of the previously described yeast two-hybrid plasmid pGBT9/M9 (Fig. 1) (15). The pGBT9/M9 plasmid encodes the Gal4 DNA-binding domain linked to residues 256–320 of hnRNP A1. These M9 mutants were then analyzed for their ability to interact with the M9-binding domain of human Trn using the yeast two-hybrid assay as described previously (15). The G274A mutant of M9, which fails to interact with Trn (15), served as a negative control.

As shown in Fig. 1, mutant M9-D2, which bears alanines at residues 273–276, was not able to detectably interact with Trn, a result that was expected in light of the inactivity of the G274A missense mutant. In contrast, all other mutants retained at least weak Trn-binding activity. The least active of these latter mutants was M9-D1, which retained only ~2% of the activity of the wild type. However, mutants M9-D0 and M9-D3 were also significantly inhibited in their ability to bind Trn, retaining ~26 and ~7% of wild-type activity, respectively. In contrast, mutants M9-D4, M9-D5, and M9-D6 retained wild-type levels of Trn-binding activity. Western blot analysis of these yeast transformants showed that the wild-type and mutant forms of these Gal4/M9 fusion proteins were all expressed at comparable levels (Fig. 2). Otherwise, these data therefore suggest that the Trn-binding domain of hnRNP A1 is localized to between residues 265 and 280, with the most critical residues located between positions 269 and 276. To more fully confirm this hypothesis, we generated several M9 deletion mutants in the context of the Gal4/M9-(256–320) fusion protein and investigated whether these retained Trn-binding activity.
in the two-hybrid assay. As shown in Table I, hnRNP A1 residues 256–284 or 256–280 retained high levels of Trn binding, whereas the 256–272 deletion mutant was, as expected, inactive. Although the M9 deletion mutant retaining hnRNP A1 residues 264–284, a total of only 21 amino acids, also retained strong Trn-binding activity, the 264–280 mutant did show a significant drop in Trn binding, retaining only 8% of the activity of sequence 256–320 (Table I). All M9 deletion mutants were again expressed at comparable levels in yeast cells, as determined by Western blot analysis using an anti-Gal4 antiserum (Fig. 2). These deletion data therefore confirm the alanine-scanning analysis shown in Fig. 1 and map the core Trn-binding sequence of hnRNP A1 to between residues 264 and 280.

### Nuclear Import Activity of M9 Mutants—

As noted above, Trn has been shown to mediate the nuclear import of substrates bearing the M9 NLS/NES, and M9 mutants that fail to bind Trn, such as G274A, also fail to import (13, 15). To examine the NLS activity of the M9 mutants given in Fig. 1, we purified recombinant proteins consisting of GST linked to this same region of hnRNP A1. The sequences at the bottom give the M9 NLS as defined by Weighardt et al. (12) (upper row) and the M9 NLS/NES as defined by Michael et al. (10) (lower row). The indicated clustered alanine-scanning mutants were constructed and tested for Trn binding by yeast two-hybrid assay, with activity given on the right as percent of wild-type M9 binding as measured by lacZ induction in the Y190 yeast indicator strain averaged over several experiments. M9 NLS and NES function was determined as described under “Results” and is given as ++, partially active and −−, inactive.

### Table I

| Transportin binding activity of M9 deletion mutants |
|-----------------------------------------------------|
| The Y190 yeast indicator strain was transformed with plasmids encoding the VP16/Trn-(582–890) fusion protein and Gal4 DNA-binding domain fusion proteins that included the indicated hnRNP A1 residues. Induced β-galactosidase activities were determined as described in the legend to Fig. 1 and are given as a percentage of the level seen with the Gal4 fusion protein containing hnRNP A1 residues 256–320. |
|-----------------------------------------------------|
| hnRNP A1 residues tested | Trn binding |
|---------------------------|-------------|
| 256–320 (wild-type M9) | 100 |
| 256–284 | 61 ± 22 |
| 256–280 | 45 ± 16 |
| 256–272 | <0.1 |
| 264–284 | 56 ± 13 |
| 264–280 | 8 ± 3 |
| 264–272 | <0.1 |

Fig. 1. Structure and biological activity of M9 mutants. Alanine-scanning mutants were constructed in the context of pGBT9/M9, which expresses the Gal4 DNA-binding domain linked to residues 256–320 of hnRNP A1. The resultant M9 mutants were then tested for biological activity in the yeast two-hybrid assay and as recombinant fusion proteins consisting of GST linked to this same region of hnRNP A1. The sequences at the bottom give the M9 NLS as defined by Weighardt et al. (12) (upper row) and the M9 NLS/NES as defined by Michael et al. (10) (lower row). The indicated clustered alanine-scanning mutants were constructed and tested for Trn binding by yeast two-hybrid assay, with activity given on the right as percent of wild-type M9 binding as measured by lacZ induction in the Y190 yeast indicator strain averaged over several experiments. M9 NLS and NES function was determined as described under “Results” and is given as ++, partially active and −−, inactive.

Fig. 2. Quantitation of the expression level of Gal4/M9 fusion proteins in yeast. The yeast strain Y190 was transformed with plasmids expressing the indicated wild-type (WT) or mutant forms of the Gal4/M9-(256–320) fusion protein. Western blot analysis of selected transformants was performed using an anti-Gal4 polyclonal antiserum as described previously (16).

The indicated clustered alanine-scanning mutants were constructed and tested for Trn binding by yeast two-hybrid assay, with activity given on the right as percent of wild-type M9 binding as measured by lacZ induction in the Y190 yeast indicator strain averaged over several experiments. M9 NLS and NES function was determined as described under “Results” and is given as ++, partially active and −−, inactive.
the GST/D2 fusion failed to detectably accumulate in the nucleus even by 30 min after microinjection (Fig. 3, M), whereas the GST/D2 fusion failed to detectably accumulate in the nucleus even by 30 min after microinjection (Fig. 3, M), the GST/D2 fusion failed to detectably accumulate in the nucleus even by 30 min after microinjection (Fig. 3, M), itants both showed significant nuclear accumulation by 30 min after microinjection (Fig. 3, E, F, I, and J). However, these mutant M9 sequences are clearly a substantially less effective NLS than the wild-type M9 sequence, as judged by the weak nuclear accumulation seen at 1 min after injection (Fig. 3, G, H, K, and L). It is therefore apparent that the markedly reduced affinity for Trn displayed by the M9-D1 and M9-D3 mutants (Fig. 1) is nevertheless sufficient to mediate nuclear import, albeit with reduced efficiency. The other M9 mutants, i.e. M9-D0, M9-D4, M9-D5, and M9-D6 (Fig. 1), were all found to mediate nuclear entry with an efficiency comparable to that seen with the wild-type GST/M9 fusion protein, consistent with their ability to bind Trn effectively in vivo (Fig. 1 and data not shown).

**Nuclear Export Activity of Mutant M9 Sequences**—We next wished to investigate whether the mutations described in Fig. 1 would affect the NES activity of the M9 sequence. Because M9 is a more effective NLS than NES, protein substrates bearing the M9 sequence appear to localize to the cell nucleus at steady state even though they have been shown to continuously shuttle between the nucleus and cytoplasm (22). To measure NES activity, we have therefore developed an assay for protein nucleocytoplasmic shuttling that depends upon the microinjection of recombinant proteins into a single nucleus in a cell containing two or more nuclei. Such cells normally exist at a low level in a culture of HeLa cells, but their prevalence can be increased by overnight culture in serum-free medium followed by 4–6 h of culture in medium containing serum.

Such an experiment, in which binuclear cells were injected with wild-type or mutant GST/M9 proteins together with an IgG tracer, is shown in Fig. 4. After 20 min of incubation at 37 °C, much of the microinjected wild-type GST/M9 protein migrated from the injected nucleus, identified by the IgG tracer, to the un.injected nucleus (Fig. 4, A and B). Similarly, both the GST/D1 and GST/D3 proteins were also able to migrate to the other nucleus, although this process appeared less efficient and also led to a low but detectable level of cytoplasmic protein accumulation (Fig. 4, E and K). Finally, the GST/D2 mutant, which failed to import into the cell nucleus (Fig. 3), also failed to exit the injected nucleus (Fig. 4, HI). All the other M9 mutants described in Fig. 1, i.e. M9-D0, M9-D4, M9-D5, and M9-D6, all demonstrated efficient nucleocytoplasmic shuttling and therefore clearly retain NES activity (data not shown).

**Selection of Novel Functional M9 Sequences**—Although the M9 mutants delineated in Fig. 1 failed to segregate M9 NLS and NES activity, these mutants are clearly too crude to prove this point clearly. As an alternative approach, we therefore decided to totally randomize each of the four amino acid segments defined by mutants M9-D0, M9-D4, M9-D5, and M9-D6 and then select for retention of the ability to bind Trn effectively using the two-hybrid assay in yeast. This approach, which we have previously used to define the sequence requirements for leucine-rich NES function (17), should fully define the sequence requirements for Trn binding in vivo and also generate a large pool of variant M9 sequences that should retain NLS activity. However, if M9 NES function is dependent on an interaction with an unknown nuclear export receptor distinct from Trn, then this extensive pool of M9 sequence variants should allow segregation of M9 NLS function from M9 NES activity.

The M9 sequence present in pGBT9/M9 was therefore randomized in four 4-amino acid segments, and each library was analyzed for Trn binding in the yeast two-hybrid context by screening for expression of the *his3* selectable marker. M9 variants that permitted yeast colony growth under selective conditions were then recovered and retransformed to confirm that the observed *his3* expression was indeed mediated by an interaction with Trn. These secondary transformants were also analyzed for levels of expression of a second indicator gene, *lacZ*, as a measure of the affinity of the interaction of Trn with the various recovered M9 derivatives.

Based on a comparison of the number of yeast transformants observed on *his3*-selective versus nonselective plates, we observed that ≥20% of the R0 library transformants, ≥25% of the R1 library transformants, and ≥40% of the R3 library trans-
The M9 sequence was randomized in four clusters of 4 amino acids coincident with hnRNP A1 residues 265–268, 269–272, 273–276, and 274–275, respectively. The sequences of the recovered M9 variants from these two libraries, for the R2 region, we recovered several sequences with amino acid coincident with hnRNP A1 residues 265–268, 269–272, 273–276, and 274–275, respectively. The sequences of the recovered M9 variants from these two smaller libraries are given in the R2 column, with the invariant residues indicated in lowercase letters. Sequences recovered more than once are indicated by numbers in parentheses. All sequences gave levels of Trn interaction comparable to the wild type (WT) as determined by the level of lacZ induction observed in yeast indicator cells, with the following exceptions: *20–50% of wild-type M9; and +, 5–20% of wild-type M9. Residues that displayed evident selection are indicated by arrows, whereas sequences subsequently tested for NLS and NES activity are indicated in boldface. All such M9 variants displayed levels of NES and NLS function comparable to the wild type (see Fig. 7 for representative data).

Unlike the R0, R1, and R3 libraries, the R2 library, which randomized residues (positions 273–276) that blocked Trn binding when mutated to alanine (Fig. 1), resulted in only a very small number of yeast colonies upon selection for his3 expression. Although several of these were picked, only one (R2-1) proved to be a real Trn interactor, whereas the remaining positives all contained frameshift mutations that generated autonomous transcription activation domains (data not shown). To generate additional information as to sequence requirements in this critical region of M9, we therefore generated two additional libraries in which only 3 amino acids at a time were randomized, i.e. \(2^{7}FP\) or \(2^{7}GP\)B. These two libraries allowed –1 and –2%, respectively, of the yeast transformants to generate colonies under his3-selective conditions. The amino acid sequences of 17 of these transformants are given in Fig. 5. Four transformants were predicted to encode the wild-type M9 amino acid sequence, but had different underlying nucleotide sequences. Unlike the R0 and R3 libraries, for the R2 region, we recovered several sequences multiple times upon randomization, and also noted that the number of such recovered sequences appeared to have a lower affinity for Trn, as suggested by lower levels of induced lacZ indicator gene expression in the yeast indicator cells. Analysis of the recovered protein sequences showed complete conservation at only one position, i.e. serine 271. However, this serine is clearly not absolutely essential for either Trn binding or M9 function, as mutation to alanine, in M9-D1, permitted a low level of both Trn binding and NLS/NES activity (Figs. 1, 3, and 4). Other residues that appeared non-random in the selected sequences included position 266, which was always Tyr, Trp, or Phe; position 269, which was invariably a small hydrophilic residue; position 273, which was generally hydrophilic; position 274, which was glycine in all but two transformants; position 275, which was always either proline or lysine; position 276, which always contained a large hydrophilic residue; and position 277, which was generally either lysine or arginine. No other positions in M9 displayed a clear sequence requirement for Trn binding (Fig. 5). The consensus sequence for efficient Trn binding by M9 suggested by these data is given in Fig. 6.

Having identified a range of M9 sequences that permitted Trn binding in vivo, we next investigated whether these sequences would also permit M9 NLS and NES function. The M9 sequences highlighted in Fig. 5 were therefore prepared as GST fusion proteins, and their NLS and NES activity was assayed as shown in Figs. 3 and 4. To briefly summarize this work, all these randomized sequences were found to mediate nuclear protein import and export with an efficiency comparable to the wild type. Results from representative assays showing nuclear shuttling by the R1-9 mutant of M9 (i.e. M9 bearing sequence 9 from the R1 pool shown in Fig. 5) as well as by the R2-11, R2-12, and R3-10 mutants of M9 are given in Fig. 7.

To take this analysis a step further, we next generated two M9 variants containing combinations of the selected randomized sequences given in Fig. 5. In particular, these two mutants, termed 11.4.11 and 8.4.10, respectively, substituted the R1-11, R2-4, and R3-11 sequences (i.e. \(2^{60}ESAFGKM-FK786\)) or the R1-8, R2-4, and R3-10 sequences (i.e. \(2^{60}RTYFFGKMTSYR\)) in place of wild-type sequence 269–280 present in M9. Both of these very extensive M9 variants displayed close to wild-type Trn binding in yeast cells, and both also gave good NLS and NES activity when assayed by microinjection in HeLa cells (Fig. 7, I and J; and data not shown).

Effect of RanGTP on the M9-Trn Interaction—Although the mutational analysis presented in Fig. 1 and the randomization/selection for Trn binding given in Fig. 5 between them varied every single amino acid between residues 265 and 292 in the hnRNP A1 M9 NLS/NES, they never the less failed to identify any mutants that segregated the NLS activity of M9 from its NES activity. This analysis therefore raises the possibility that Trn binding is, in fact, not only the import but also the export factor for M9. If this were the case, one would predict that the M9-Trn interaction should be stable in the presence of RanGTP, which is found at high levels in the cell nucleus (1, 4). However, as shown in Fig. 8, addition of RanGTP resulted in the release of Trn from an M9 column when tested in vitro, although RanGDP had no such effect. Nevertheless, it remains possible that this release is an in vitro artifact that would not occur under in vivo conditions.

To test whether Ran binding and M9 binding to Trn are indeed mutually incompatible in the nuclear environment, we

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**Fig. 5. Identity of random M9 sequence variants selected for Trn binding.** The M9 sequence was randomized in four clusters of 4 amino acids coincident with hnRNP A1 residues 265–268, 269–272, 273–276, and 274–275, respectively. The sequences of the recovered M9 variants from these two libraries are given in the R2 column, with the invariant residues indicated in lowercase letters. Sequences recovered more than once are indicated by numbers in parentheses. All sequences gave levels of Trn interaction comparable to the wild type (WT) as determined by the level of lacZ induction observed in yeast indicator cells, with the following exceptions: *, 20–50% of wild-type M9; and +, 5–20% of wild-type M9. Residues that displayed evident selection are indicated by arrows, whereas sequences subsequently tested for NLS and NES activity are indicated in boldface. All such M9 variants displayed levels of NES and NLS function comparable to the wild type (see Fig. 7 for representative data).

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**Fig. 6. Consensus Trn interaction motif.** A consensus Trn protein-binding motif, derived from the data shown in Fig. 4, is given and aligned with the wild-type M9 Trn-binding sequence (residues 266–277 of hnRNP A1). J, hydrophilic amino acid; Z, hydrophobic amino acid; X, any residue.

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| R0 | R1 | R2 | R3 |
|----|----|----|----|
| **WT** | **NYNN** | **GSSN** | **FGPM** | **KGGM** |
| **1** | SWRF | SSSL | AGPM | RT1 |
| **2** | RWLA | SHER | fGPM | RT1A |
| **3** | QWCF | SPSL | fGPMV2 | RTGQ |
| **4** | WWGF | SPSG | fGKM | RTRA |
| **5** | GYLS | SRRR | AGPM3 | RYAP |
| **6** | IYEL | SPSA | fGPM2 | RHNH |
| **7** | TYFG | DPSS | fAPL3 | RQTI |
| **8** | RYTF | DISM | VGPM | R1HT |
| **9** | LFPF | DMSG | *IKGm | KNVN |
| **10** | VFAF | EQSR | *TGKm | TSYR |
| **11** | IFVH | EESA | *TTKm | FKQY |
| **12** | SEFE | NESN | *VCKm | YPSL |

**Consensus** | **Y** | **X** | **J** | **Z** | **M** | **W** |

**Wild Type 266–277** | **YNQNSNF** | **G** | **P** | **L** | **K** | **V** | **R**
11.4.11 mutant substitutes residues 269–280 of M9, as described under Trn Randomized M9 sequence variants that had been selected for binding to Fig. 4, active. R1-9 is the ninth sequence variant in the R1 library, as given in Fig. 4, i.e. substitution of DMSG for residues 369QSSN372 in M9. The 11.4.11 mutant substitutes residues 269–280 of M9, as described under “Results.”

used the yeast two-hybrid assay, which measures nuclear protein-protein interactions, i.e. interactions that occur in the presence of endogenous RanGTP. As noted above, human Trn residues 582–890 interact strongly with Gal4/M9 in the two-hybrid setting (15), and this result is reproduced in Table II. However, VP16/Trn-(582–890) does not interact with Gal4/Ran, a result that is expected given that the Trn Ran-binding domain, as for other Imp-β-related proteins, is expected to map to the amino-terminal region (23). More important, expression of a VP16 fusion protein containing full-length Trn (amino acids 1–890) gave rise to a readily detectable interaction with Gal4/Ran (which is presumably in the form of RanGTP), although no interaction with Gal4/M9 was detected. Therefore, as in the in vitro situation (Fig. 8), binding of full-length Trn to RanGTP and to M9 represent mutually exclusive interactions in the yeast nucleus in vivo.

A final possibility that we considered is that the export factor for the M9 NES would be a variant form of Trn. Such a protein has, in fact, been reported and termed transportin 2 (Trn2) (18). Although little is known about the role of Trn2 in vivo, Trn2 was reported to be unable to interact with hnRNP A1 and other hnRNPs in vitro. However, if Trn2 is an M9-specific NES receptor, then hnRNP A1 binding would be expected to also require RanGTP binding to Trn2, a possibility that was not tested. In Table II, we have investigated whether full-length Trn2 (amino acids 1–894) would be able to bind M9 in the yeast cell nucleus. In fact, even though the VP16/Trn2-(1–894) fusion protein gave readily detectable levels of binding to Gal4/Ran, no M9 binding was detected. We therefore conclude that Trn2 is unlikely to be the export receptor for the M9 NES.

**DISCUSSION**

The goal of this work was 2-fold. First, we wished to define the sequences in M9 required for interaction with the Trn nuclear import receptor and hence for M9 NLS function. Second, we wished to test whether the reported NES activity of M9 could be segregated from its NLS activity. As described in detail in the Introduction, such a segregation is predicted by the hypothesis that nuclear RanGTP serves to dissociate NLS-import factor complexes, yet to stabilize NES-export factor complexes (1–3). These dual aims were addressed by first delineating the sequences in the M9 domain important for Trn binding by alanine-scanning and deletion mutagenesis (Fig. 1 and Table I) followed by randomization of these sequences, together with selection for retention of Trn binding, in order to exhaustively identify residues that contribute to the M9-Trn interaction (Figs. 5 and 6).

The mutational data presented in Fig. 1 and Table I demonstrate that the M9 residues that contribute to Trn binding are located between positions 264 and 280 of hnRNP A1, with the most critical residues located between positions 269 and 276. The randomization/selection data presented in Fig. 5 demonstrate that no single residue in M9, with the possible exception of serine 271, is absolutely critical for efficient Trn binding. However, serine 271 is dispensable for at least modest Trn binding based on the mutational data presented in Fig. 1. Taken together, these data demonstrate that no single residue located in the hnRNP A1 M9 domain is essential for Trn binding in vivo and instead suggest that this protein-protein interaction results from the combined activity of multiple residues in M9, as defined by the consensus Trn-binding site given in Fig. 6. Nevertheless, it is also apparent that certain residues make particularly important contributions to this interaction. In addition to serine 271, these appear to include glycine 274 and the slightly more variable residues located at positions 275 and 276. Although residues 266, 269, 273, and 277 also make contributions to the M9-Trn interaction, as documented by the selection shown in Fig. 5, these residues appear to be less tightly conserved.

As predicted, M9 mutants and variants that retained Trn-binding activity, including all the M9 variants given in Fig. 5 that were tested, retained NLS activity. The Trn-binding consensus sequence given in Fig. 6 therefore also defines the consensus sequence for M9 NES function. Mutants of M9 that were significantly attenuated for Trn binding, such as M9-D1 and M9-D3 (Fig. 1), also retained detectable NES activity, although this was clearly reduced with respect to the wild-type M9 sequence (Fig. 3). Unexpectedly, all M9 mutants and variants that retained Trn-binding activity also displayed readily detectable NES function, including the “composite” M9 mu-

![Diagram](image-url)
tants 11.4.11 and 8.4.10, which were extensively mutated between hnRNP A1 residues 269 and 280 (Figs. 5 and 7). In contrast, mutants that had lost all Trn-binding activity, such as M9-D2 and G274A, also had lost all NES activity (Figs. 1 and 4). Therefore, these data provide strong evidence in favor of the hypothesis that Trn binding is predictive of not only M9 NLS but also NES activity in vivo.

Could transportin be not only the import but also the export receptor for hnRNP A1? Based on several findings, this appears unlikely. As shown in Fig. 8 and also reported recently by others (5, 18), preformed M9-Trn complexes are efficiently dissociated in vitro by RanGTP, but not by RanGDP. As RanGTP is present at high levels in the cell nucleus (1–4), this finding is most consistent with the hypothesis that M9-Trn complexes are dissociated in the nucleus after import from the cytoplasm. These in vitro data were further validated in vivo by the finding that full-length Trn is unable to interact with M9 in the yeast two-hybrid assay, which measures nuclear protein-protein interactions, even though it efficiently interacts with Ran (which is presumably in the RanGTP form), whereas deletion of the amino-terminal Ran-binding domain of Trn blocks Ran binding, but activates in vivo M9 binding (Table II). Therefore, both in vitro and in the nucleus in vivo, RanGTP binding and M9 binding by Trn are mutually exclusive. Recent immunoprecipitation studies also suggest that Trn is unlikely to bind to hnRNP A1 assembled into nuclear hnRNP complexes in vivo in that precipitation of such complexes using an hnRNPC-specific antibody failed to coprecipitate Trn (18). Interestingly, these studies also demonstrated that the M9 domain of hnRNP A1 was not accessible to an M9-specific monoclonal antibody when hnRNP A1 was assembled into nuclear hnRNP complexes, even though M9 appears not to be bound to Trn. These studies therefore raise the possibility that M9 is bound in the nucleus by a nuclear export factor distinct from Trn.

The data presently available therefore provide us with a paradox. On the one hand, the extensive mutational analysis presented in this report demonstrates that Trn binding is predictive of M9 NES function. On the other hand, several lines of data suggest that Trn is not the M9 NES receptor. Could an isoform of Trn or a closely related protein be the M9 NES receptor? Only one such closely related protein is known, i.e. the Trn2 protein (18). However, at least by two-hybrid analysis, full-length Trn2 does not appear able to interact with M9 in the yeast cell nucleus, although Trn2 does bind Ran effectively (Table II). This result therefore appears inconsistent with the hypothesis that Trn2 mediates M9 NES function. Alternately, it is possible that the sequence determinants for Trn binding by M9 actually define not so much a linear protein recognition sequence as a particular protein structure. If so, this might explain why a similar consensus sequence could mediate an interaction with both Trn and a perhaps only distantly related M9 NES receptor. As the identity and target sequences for nuclear export and import factors are rapidly being defined, it should only be a matter of time before this conundrum is resolved.

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