Transportins 1 and 2 are redundant nuclear import factors for hnRNP A1 and HuR

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ABSTRACT

Several mRNA-binding proteins, including hnRNP A1 and HuR, contain bidirectional transport signals that mediate both their nuclear import and export. Previously, Transportin 1 (Trn1) was identified as a mediator of hnRNP A1 import, whereas the closely related protein Transportin 2 (Trn2) was shown to interact with HuR. Here we have investigated the subfamily of transportins that consists of Trn1 (or Kap β2A) and two alternatively spliced Trn2 isoforms (Trn2a and Trn2b), also called Trn2 and Kap β2B. The sequence differences among these proteins could alter either their cargo specificity or their response to RanGTP and thus their function as import or export receptors. Using in vitro binding assays, we show that hnRNP A1 preferentially binds Trn1 and Trn2b versus Trn2a. HuR interacts with all three transportins, as well as weakly with Imp β. The hnRNP A1 and HuR shuttling domains, called M9 and HNS, respectively, are sufficient for these interactions. Despite small differences in the binding of HuR and hnRNP A1 to the three transportins, in vitro interaction studies performed in the presence and absence of RanQ69LGTP indicate that all three transportins most likely act as import factors for HuR and hnRNP A1. In digitonin-permeabilized HeLa cells, both M9 and HNS peptides compete for the import of recombinant hnRNP A1 and HuR, indicating that HuR and hnRNP A1 import pathways are at least partially overlapping. Possible nucleocytoplasmic shuttling mechanisms for hnRNP A1 and HuR are discussed.

Keywords: nuclear export; mRNA export; mRNA stability; ARE; ELAV

INTRODUCTION

The majority of characterized nucleocytoplasmic transport pathways involve importin β (Imp β)-type receptors or karyopherins (for review, see Mattaj and Englmeier 1998; Görlich and Kutay 1999; Chook and Blobel 2001). There are >20 Imp β-like transport receptors in mammalian cells that can be subclassified as importins or exportins. However, both types of Imp β-like receptors are similar in size (90–130 kD) and secondary structure, and they all interact with the small GTPase Ran (for review, see Chook and Blobel 2001). Ran appears to play a central role in both providing energy for and determining the directionality of nucleocytoplasmic transport (Izaurralde et al. 1997b; Görlich et al. 1996). Ran is predominantly GTP-bound in the nucleus, whereas it is in a GDP-bound form in the cytoplasm. Consequently, importins load their cargoes at low RanGTP concentration in the cytoplasm, translocate into the nucleus, and release them upon binding of RanGTP. Exportins bind their export substrates and RanGTP simultaneously in the nucleus; trimeric RanGTP–exportin–cargo complexes are then carried to the cytoplasm, where they dissociate upon GTP hydrolysis (for review, see Mattaj and Englmeier 1998; Görlich and Kutay 1999; Kuersten et al. 2001).

Importins and exportins recognize their cargoes via protein- or RNA-based sequence elements termed nuclear localization signals (NLS) and nuclear export signals (NES; for review, see Mattaj and Englmeier 1998; Nakielny and Dreyfuss 1999). Normally, NLS and NES signals, as well as their cognate import and export receptors, are distinct. However, several mRNA-binding proteins contain nucleocytoplasmic shuttling sequences that appear to mediate both nuclear import and export. The best characterized of these is hnRNP A1, an abundant nuclear hnRNP protein that contains a 38-amino-acid-long shuttling signal termed M9 (Fig. 1A; Michael et al. 1995; Siomi and Dreyfuss 1995).

To date, only one transport receptor, called Trn1 or karyopherin β2A (Kap β2A), has been shown to bind the M9 signal and import hnRNP A1 protein into the nucleus (Nakielny et al. 1996; Pollard et al. 1996; Bonifaci et al. 1997; Fridell et al. 1997). In addition to hnRNP A1, Trn1 is

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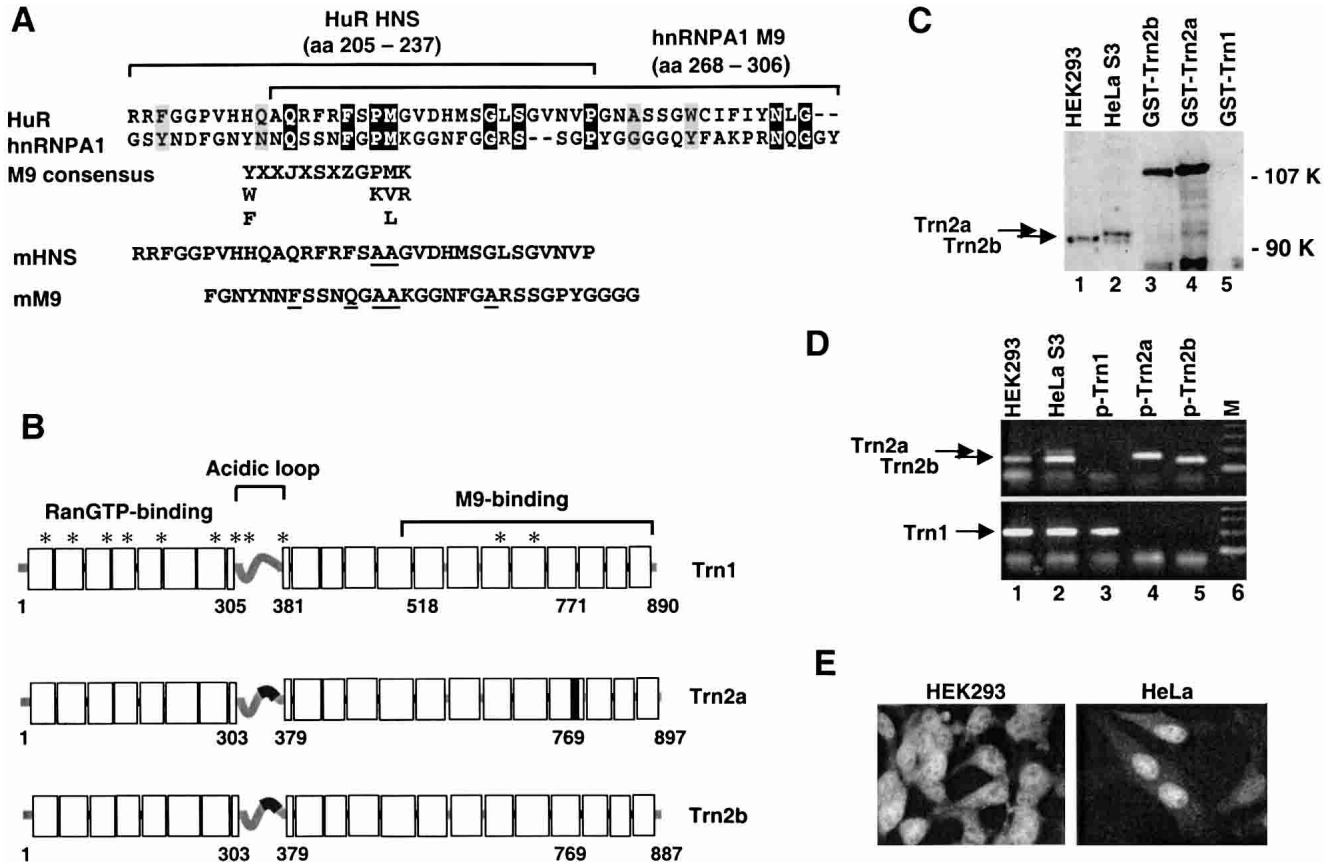

FIGURE 1. Comparison of HNS and M9 sequences, schematic representation of the domain structure of transportins, and expression of transportins in HeLa and HEK 293 cells. (*A*) Amino acid sequence alignment of HNS from human HuR (Fan and Steitz 1998a), M9 from human hnRNP A1 (Michael et al. 1995), and the M9 consensus (Bogerd et al. 1999). Amino acids identical between HNS and M9 are indicated by black boxes; similar amino acids are boxed in gray. In the consensus sequence, (J) hydrophilic amino acids; (Z) hydrophobic amino acids; (X) any residue. mHNS is the mutant HNS sequence encoded by the GST-mHNS construct (Fig. 3B), and mM9 is the sequence of the mutant M9 peptide used as a control in the competition experiments (Fig. 4B). Altered residues in mHNS and mM9 are underlined. (*B*) Schematic representation of the domain structure of Trn1 and its close homologs Trn2a and Trn2b. The Trn1 protein consists of 20 α-helical HEAT repeats (white boxes); a less-structured acidic loop (amino acids 305–381) is designated with a gray curved line, RanGTP contacts (Chook and Blobel 1999; Chook et al. 2002) with asterisks; and the C-terminal part (amino acids 518–890) of Trn1 that is sufficient for M9 binding (Pollard et al. 1996; Fridell et al. 1997) is indicated above. The most variable sequences of Trn2a and Trn2b are designated with black boxes. (*C*) Western blot analysis of Trn2a and Trn2b proteins, in which ~20 μg of HeLa S3 and HEK293 whole-cell extracts (lanes *1–2*) and 5 ng of recombinant GST-tagged Trn2b, Trn2a, and Trn1 (lanes *3–5*) were analyzed using anti-Trn2 serum. (*D*) Expression analysis of transportins by RT-PCR. (Lanes *1–5*) PCR products (243 bp) using Trn1-specific primers; (lanes *6–10*) show PCR products (194 bp for Trn2a and 164 bp for Trn2b) using Trn2-specific primers. PCR was performed on cDNA prepared from HEK293 (lane *1*) or HeLa S3 cells (lane *2*). Control PCR reactions with both primer pairs were carried out on plasmids containing Trn1 (lane *3*), Trn2a (lane *4*), or Trn2b (lanes *5*) cDNA. (Lane *6*) A 123-bp marker (Invitrogen). (*E*) Cellular localization of Trn2 in HEK293 and HeLa cells.

the import receptor for hnRNP F (Siomi et al. 1997), for the mRNA export factor TAP (Truant et al. 1999; Bachi et al. 2000), and for ribosomal proteins such as rpL23a, rpS7, and rpL5 (Jäkel and Görlich 1998). The ribosomal proteins and TAP bind Trn1 via basic lysine- and arginine-rich sequences that functionally differ from M9 because these domains exhibit only NLS but not NES activity (Jäkel and Görlich 1998; Truant et al. 1999; Bachi et al. 2000).

In the course of isolating the full-length human Trn1 cDNA, an 84% identical protein Trn2 was characterized (Siomi et al. 1997). Despite high similarity to Trn1, Trn2 did not exhibit the same high affinity in vitro for a set of test nucleic-acid-binding proteins, including hnRNP A1. Instead, HuR, another mRNA-binding protein, was shown to bind to Trn2 via its shuttling signal HNS (Gallouzi and Steitz 2001). Accordingly, the HNS sequence partially matches the M9 sequence of hnRNP A1 and the M9 consensus sequence as defined by a combination of mutational randomization followed by selection for Trn1 (Fig. 1A; Fan and Steitz 1998a; Bogerd et al. 1999). Like hnRNP A1, HuR is a nucleocytoplasmic shuttling protein (Fan and Steitz 1998b). HuR is known to stabilize unstable mRNAs that contain AU-rich sequences (AREs) within their 3′-untranslated region (3′-UTR); most likely the ability of HuR to relocalize to the cytoplasm is important for this function (for review, see Brennan and Steitz 2001). As shuttling and

mRNA-binding proteins, hnRNP A1 and HuR have been suggested also to function in mRNA export (Izaurralde et al. 1997a; Gallouzi and Steitz 2001). A more recently described variant of Trn2 termed Kap β2B has been proposed to participate in mRNA export as well, but in cooperation with the main mRNA export factor TAP (Shamsher et al. 2002).

The data presented here support the argument that Trn1 and Trn2 are redundant import factors for hnRNP A1 and HuR. First, we confirmed that the two previously reported Trn2 protein sequences (Siomi et al. 1997; Shamsher et al. 2002) represent two alternatively spliced isoforms, which we now designate Trn2a and Trn2b. Using in vitro binding assays, we show that hnRNP A1 prefers to bind to Trn1 and Trn2b versus Trn2a, whereas HuR interacts with all three transportins. The hnRNP A1 and HuR shuttling domains, M9 and HNS, respectively, are sufficient for these interactions. Further in vitro binding assays show that the presence of RanQ69LGTP decreases the affinity of hnRNP A1 and HuR for the transportins, indicating that all three transportins are most likely import factors for HuR and hnRNP A1. Competition experiments using M9 and HNS peptides in digitonin-permeabilized HeLa cells confirm that HuR and hnRNP A1 at least partially share the same import pathway(s).

RESULTS AND DISCUSSION

Transportin 2 is expressed in two spliced forms

To understand better the role of Trn1 and Trn2 in nucleocytoplasmic transport, we first compared the two separately published human Trn2 cDNA sequences, Trn2 (AF019039; Siomi et al. 1997) and Kap β2B (AF007748; Shamsher et al. 2002). They possess identical coding potential except for a 30-nt insertion in the AF019039 sequence compared with AF007748 within the 3′ part of the cDNA. A search for the two Trn2 mRNA sequences in human and mouse expressed sequence tag (EST) databases revealed two human and four mouse ESTs corresponding to AF019039 and eight human and five mouse ESTs corresponding to AF007748. Further comparison with sequences present in the EMBL/GenBank/DDBJ nonredundant database identified a putative human *Trn2* gene sequence with location *19p13.2* (http://www.ncbi.nlm.nih.gov/LocusLink). Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/maps) revealed that AF019039 and AF007748 differ only in what appears to be an alternatively spliced exon 20 that is preceded by two different 3′ splice sites (separated by 30 nt) and followed by a single 5′ splice site consensus sequence. We termed the two corresponding Trn2 proteins Trn2a (AF019039) and Trn2b (AF007748). Schematics of the domain structure of the closely related Trn1 protein, derived from its crystal structure (Chook and Blobel 1999; Chook et al. 2002), and the predicted domains of the two Trn2 proteins are presented in Figure 1B.

Next, we examined the expression of the two Trn2 isoforms in human cell lines. A rabbit polyclonal antibody recognizing both Trn2a and Trn2b was raised against a peptide (amino acids 344–361) corresponding to the sequence with the least correspondence to Trn1 located within the acidic loop (Fig. 1B; Chook et al. 2002). As shown in Figure 1C, the anti-Trn2 serum specifically recognizes and can distinguish between recombinant Trn2a and Trn2b versus Trn1 (lanes 3–5). Interestingly, only the Trn2b protein is expressed in human embryonic kidney (HEK293) cells (lane 1), whereas both Trn2a and Trn2b are detected in HeLa S3 cells (lane 2). These expression patterns were confirmed by RT-PCR analyses (Fig. 1D, top panel). However, in contrast to the Western results, RT-PCR with Trn2-specific primers indicated higher expression of Trn2b than Trn2a in HeLa S3 cells (Fig. 1D, lane 2), probably caused by preferential amplification of the shorter DNA. RT-PCR with Trn1-specific primers confirmed that Trn1 is expressed in both the studied cell lines (Fig. 1D, bottom panel).

To verify the cellular localization of Trn2 in HEK293 and HeLa cells, immunofluorescence using affinity-purified anti-Trn2 antibodies was carried out. As previously shown for Trn1 (Siomi et al. 1997) and Trn2 (Gallouzi and Steitz 2001) in HeLa cells, the Trn2 protein is localized both in the nucleus and the cytoplasm of HEK293 cells (Fig. 1E, left panel). However, Trn2 appears more abundant in the cytoplasm of HEK293 cells compared with HeLa cells (Fig. 1E, cf. left and right panels).

In conclusion, database analyses, expression studies, and previous reports (Siomi et al. 1997; Shamsher et al. 2002) demonstrate that the two proteins, Trn2a and Trn2b, are most probably the products of alternatively spliced mRNAs encoded by a single *Trn2* gene located on human Chromosome 19.

Trn1 and Trn2 interact with both hnRNP A1 and HuR in HeLa nuclear extract

Previously, it was reported that Trn1 binds a distinct set of cargoes compared with Trn2a (Siomi et al. 1997; Gallouzi and Steitz 2001). However, the lack of 10 extra amino acids within the C-terminal part of Trn2b generates a very similar sequence to Trn1 (89% identical and 94% similar between amino acids 493–890 of Trn1) in the region expected to bind cargo. This indicated that Trn1 and Trn2b might exhibit similar specificity for cargo proteins.

To test this hypothesis, we first examined HuR and hnRNP A1 interactions with transportins in in vitro pull-down assays. As shown in Figure 2, hnRNP A1 in nuclear extract binds preferentially to GST-tagged Trn1 and Trn2b (lanes 1 and 3, top panel) relative to GST-Trn2a (lane 2, top panel), and not to GST-Imp β (lane 4, top panel). HuR in nuclear extract interacts more strongly with both forms of GST-tagged Trn2 (lanes 2 and 3, middle panel) than with

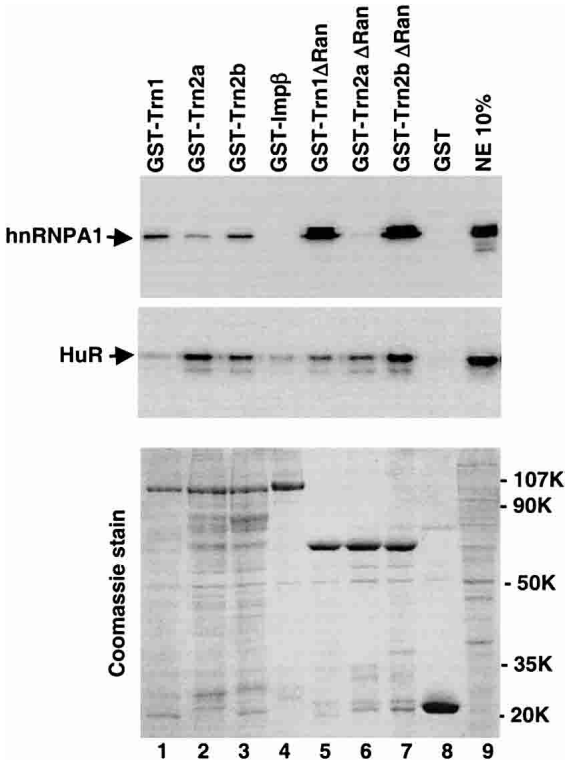

FIGURE 2. hnRNP A1 and HuR interact with transportins in HeLa nuclear extract. Glutathione Sepharose beads were precoated with GST fusions of transportins (lanes *1–3*), Imp β (lane *4*), mutant transportins lacking the RanGTP-binding domain (ΔRan, lanes *5–7*), or with GST alone (lane *8*) and incubated with HeLa nuclear extract. Bound proteins were analyzed by Western blotting using monoclonal anti-hnRNP A1 or anti-HuR antibodies. One-fifth of each binding reaction was analyzed on SDS-PAGE followed by Coomassie staining to confirm efficient binding of GST-tagged proteins to the glutathione beads (*bottom* panel). Lane *9* contains 10% of the HeLa nuclear extract used in adjacent lanes.

Trn1 and Imp β (lanes 1 and 4, middle panel). To confirm comparable binding of the GST-tagged proteins to the glutathione beads, 1/5 of each binding reaction was analyzed by SDS-PAGE followed by Coomassie staining (Fig. 2, bottom panel).

It was reported earlier that the C-terminal region of Trn1 (amino acids 518–890, Pollard et al. 1996; amino acids 547–890, Fridell et al. 1997) is sufficient for binding hnRNP A1 (see also Fig. 1B). Likewise, the GST pull-down experiments with nuclear extract showed that the C terminus of Trn1 (amino acids 494–890), as well as of Trn2b (amino acids 491–887), is sufficient for binding hnRNP A1 (Fig. 2, lanes 5–7). Thus, as reported by Siomi et al. (1997), 10 extra amino acids present in the Trn2a C-terminal region appear to hinder hnRNP A1 binding. In contrast to hnRNP A1, HuR exhibited reduced binding to GST fusions of C-terminal portions of transportins (Fig. 2, lanes 5–7, middle panel). This indicates that either HuR and hnRNP A1 interact with slightly different sites in the transportins or that the conformations of the Trn2a, Trn2b, and Trn1 C-terminal fragments are not optimal for HuR binding. Alternatively, because our in vitro binding assays were performed in nuclear extract, we cannot exclude the possibility that other proteins mediate the interactions detected between the transport receptors and hnRNP A1 or HuR.

In summary, in vitro binding experiments demonstrate that hnRNP A1 in nuclear extract interacts with GST-tagged Trn1 and Trn2b and less efficiently with Trn2a; HuR preferentially binds to Trn2a and Trn2b relative to Trn1 and Imp β. The previous data indicating that HuR binds only Trn2a and not Trn1 (Gallouzi and Steitz 2001) might have resulted if recombinant GST-Trn1 was not prepared and stored carefully, because we have observed that GST-Trn1 loses its ability to bind hnRNP A1 and HuR after a few freeze–thaw cycles, whereas GST-Trn2a and GST-Trn2b remain active in binding.

RanGTP dissociates transportin complexes with HuR or hnRNP A1

The crystal structure of the Trn1–RanGppNHp complex revealed that Trn1 consists of 20 α-helical HEAT repeats (Chook and Blobel 1999; Chook et al. 2002). Within HEAT repeat 8 (amino acids 305–381), there exists a less structured acidic loop (Fig.1B) that contacts RanGTP, as well as the C-terminal cargo-binding domain (amino acids 518–890). RanGTP hydrolysis and accompanying Ran dissociation introduce conformational changes into the protein that have been suggested to be transmitted by the acidic loop to the cargo-binding region: Trn1 with a mutated acidic loop (Fig. 1B, amino acids 341–362) can simultaneously bind both the cargo and RanGTP, but is unable to undergo Ran-mediated substrate dissociation (Chook et al. 2002). Intriguingly, the most variable sequence between Trn1 and both forms of Trn2 is within the acidic loop (amino acids 344–362 of Trn1). These observations indicated that there could be differences in the response of Trn1 and Trn2 to RanGTP binding. Indeed, Trn1 had previously been shown to function as an import factor for the TAP protein (Truant et al. 1999; Bachi et al. 2000), whereas Trn2b was suggested to be an export factor for TAP (Shamsher et al. 2002).

To ask whether hnRNP A1 or HuR interactions with transportins are influenced by RanGTP, in vitro binding experiments in the presence and absence of a GTPase deficient mutant (Bischoff et al. 1994) of Ran loaded with GTP (RanQ69LGTP) were performed. Figure 3A reveals that the strong interactions between [^{35}S]-methionine-labeled Trn1 and GST-hnRNP A1 or GST-HuR (lanes 1–4, Trn1 panel), as well as those between [^{35}S]Trn2a and GST-HuR (lanes 3–4, Trn2a panel) were significantly diminished (about sevenfold) in the presence of RanQ69LGTP. The binding of [^{35}S]Trn2b to GST-hnRNP A1 or GST-HuR was less dramatically, but reproducibly lowered (about twofold) by RanQ69LGTP (lanes 1–4, Trn2b panel). Even the very weak interactions between [^{35}S]Trn2a and GST-hnRNP A1

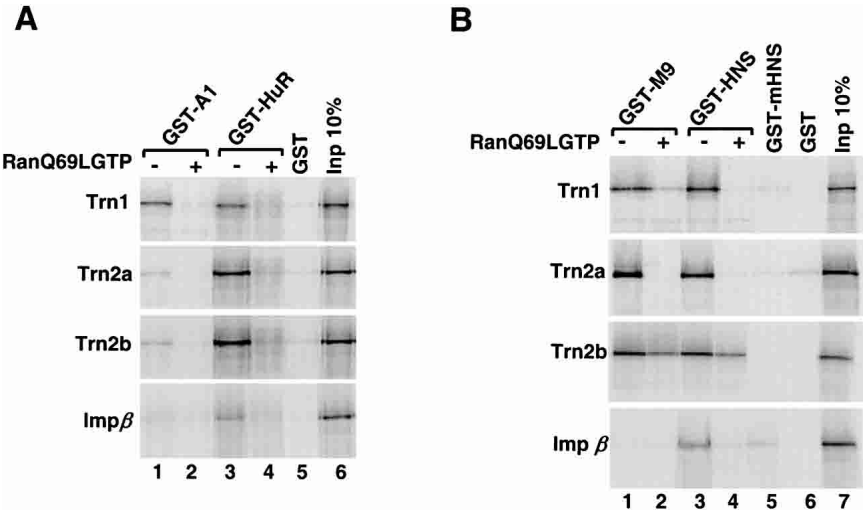

FIGURE 3. hnRNP A1 and HuR interactions with transportins are dissociated by RanGTP. Glutathione Sepharose beads were precoated with GST-hnRNP A1, GST-HuR, or GST alone (*A*) or with GST fusions of M9, HNS, mHNS (see Fig. 1A), or GST alone (*B*) and assayed for binding to [^{35}S]methionine-labeled Trn1, Trn2a, Trn2b, or Imp β in the presence (+) or absence (−) of RanQ69LGTP. Lane *6* in *A* and lane *7* in *B* contain 10% of the [^{35}S]methionine-labeled protein used in adjacent lanes. Bound proteins were analyzed by SDS-PAGE followed by autoradiography, and the efficiency of GST-tagged protein binding to the beads was confirmed as in Figure 2 (data not shown).

(lanes 1–2, Trn2a panel) and between GST-HuR and [^{35}S]Imp β (lanes 3–4, Imp β panel) were detectably disrupted by RanQ69LGTP.

Thus, despite their sequence differences, both Trn1 and Trn2a/Trn2b appear to undergo conformational changes caused by the binding of RanGTP, leading to the partial or full dissociation of hnRNP A1 and HuR. This implies that all three transportins are potential import factors for hnRNP A1 and HuR. Even Imp β might act as an import receptor for HuR because its binding to HuR is likewise sensitive to RanQ69LGTP.

HNS of HuR and M9 of hnRNP A1 are sufficient for Ran-regulated binding to transportins

The M9 shuttling sequence has been reported to be sufficient for hnRNP A1 binding to Trn1 (Pollard et al. 1996; Fridell et al. 1997) and the HNS of HuR has been observed to bind Trn2a (Gallouzi and Steitz 2001). We performed in vitro binding assays to establish whether M9 and HNS sequences are sufficient for interaction with the three transportins and whether these interactions are regulated by RanGTP, as is true of the full-length proteins. Figure 3B shows that [^{35}S]methionine-labeled transportins do bind to GST-M9 (lane 1) or GST-HNS (lane 3), whereas [^{35}S]Imp β exhibits some affinity for GST-HNS (lane 3). In contrast, GST-mHNS (see sequence in Fig. 1A) did not detectably bind any of the transportins or Imp β (lane 5). Thus, like P275 and M276 within the M9 consensus (Bogerd et al. 1999), P222 and M223 within HNS (see Fig. 1A) are essential amino acids for interaction with transportins. Note also that GST-M9 exhibits similar high affinity for [^{35}S]Trn2a and [^{35}S]Trn2b (Fig. 3B), whereas full-length hnRNP A1 favored Trn2b over Trn2a (Figs. 2, 3A). It is possible that recombinant GST-M9 does not present the M9 sequence in exactly the same way as full-length hnRNP A1.

Next, we tested the effect of RanQ69LGTP on interactions between the shuttling domains M9 or HNS and transportins. Figure 3B reveals that binding of [^{35}S]Trn1 or [^{35}S]Trn2a to GST-HNS or GST-M9 was severely diminished (sixfold to 13-fold; lanes 1–4), and interactions of [^{35}S]Trn2b with GST-HNS or GST-M9 were impaired (twofold; lanes 1–4), when RanQ69LGTP was added. Also, the weak interaction between GST-HNS and Imp β was disrupted by RanQ69LGTP (lanes 3–4).

Again, similar to the binding of the full-length proteins, GST-M9 or GST-HNS (Fig. 3, cf. A and B), interactions with Trn2b were less sensitive to RanQ69LGTP than those with Trn1 and Trn2a. This might result from either a lower affinity of Trn2b for RanGTP, a stronger interaction between Trn2b and hnRNP A1 or HuR compared with that of Trn1 or Trn2a, or a combination of both.

Previously, it was reported that Trn2b has lower affinity ($K_d \sim 300$ nM) than Trn1 ($K_d \sim 0.3$ nM) for RanGTP in a RanGAP assay, which supports the idea that Trn2b might be an export factor (Shamsher et al. 2002). However, another group (Güttinger et al. 2004) using the same assay has observed, instead, that Trn1 and Trn2b have the same affinity ($K_d \sim 1$ nM) for RanGTP. In our experiments, the ability of RanQ69LGTP to dissociate [^{35}S]Trn2b complexes with GST-M9 or GST-HNS was not increased if fourfold more RanQ69LGTP was added, if His-T7-tagged [^{35}S]Trn2b instead of Flag-tagged [^{35}S]Trn2b was used, or if reactions were performed at higher ionic strength (200 mM KCl instead of 110 mM KOAc; data not shown). However, like Trn1 and Trn2a, Trn2b binds M9 or HNS efficiently in the absence of RanQ69LGTP at relatively high ionic strength (200 mM KCl; data not shown), indicating that Trn2b is an import receptor, and probably other factors—such as competing nucleoporins or RNA—are needed to dissociate hnRNP A1 or HuR completely from Trn2b. Future experiments will be needed to test this hypothesis.

M9 and HNS peptides compete for the nuclear import of recombinant hnRNP A1 and HuR in digitonin-permeabilized HeLa cells

The results from our in vitro binding experiments indicate that transportins may act as import factors for HuR and

hnRNP A1, in turn indicating that the nuclear import pathways for hnRNP A1 and HuR might at least partially overlap. To test this possibility, we carried out in vitro import assays in digitonin-permeabilized HeLa cells (Adam et al. 1990). First, to validate the system, we asked whether the transport of GST-hnRNP A1 and GST-HuR is stimulated by components present in HeLa S3 cytoplasmic extract. As shown in Figure 4A, both GST-HuR and GST-hnRNP A1 accumulate in the nucleus in the presence of HeLa cytosol and an energy-regenerating system (see Materials and Methods) at room temperature (panels 1–4). Under the same conditions, GST alone and GST-HuR-M2, which contains only the first two RNA-binding domains (RRMs) of HuR but lacks the HNS and the third RRM, are not imported (panels 9–12). If the reaction mixture was kept on ice, both GST-HuR and GST-hnRNP A1 were retained in the cytoplasm (panels 5–8). Note that GST-HuR and GST-hnRNP A1 accumulate in the cytoplasm at 0° C (panels 5 and 7), probably because they efficiently bind mRNA.

We next tested the ability of synthetic HNS and M9 peptides to compete with GST-hnRNP A1 and GST-HuR for import into the nucleus of digitonin-permeabilized HeLa cells. Previously, it was reported that a 200-fold molar excess (over the cargo) of NLS peptide competes successfully for the import of SV40 large T antigen but not of GST-M9 in this system (Pollard et al. 1996). As shown in Figure 4B, GST-hnRNP A1 (Fig. 4B, cf. panels 2 and 3 to panel 1) and GST-HuR (Fig. 4B, cf. panels 7 and 8 to panel 6) import was significantly reduced when a 300-fold excess of M9 or HNS peptide was used. In contrast, the same amounts of the mutant M9 peptide (mM9) or of the NLS peptide (see sequences in Fig. 1A and Materials and Methods) did not affect the import of GST-hnRNP A1 (Fig. 4B, cf. panels 1, 4, 5) or GST-HuR (Fig. 4B, cf. panels 6, 9, and 10). In all our in vitro import experiments, GST-hnRNP A1 accumulation in the nucleus appeared more quantitative than that of GST-HuR because the cytoplasmic signal for GST-HuR was almost always higher than that for GST-hnRNP A1 (Fig. 4B, cf. panels 1 and 6). Consequently, the same level of M9 and HNS peptide had a greater effect on HuR than on hnRNP A1 import (Fig. 4B, cf. panels 2 and 3 to panels 7 and 8). Under these conditions, either GST-HuR import

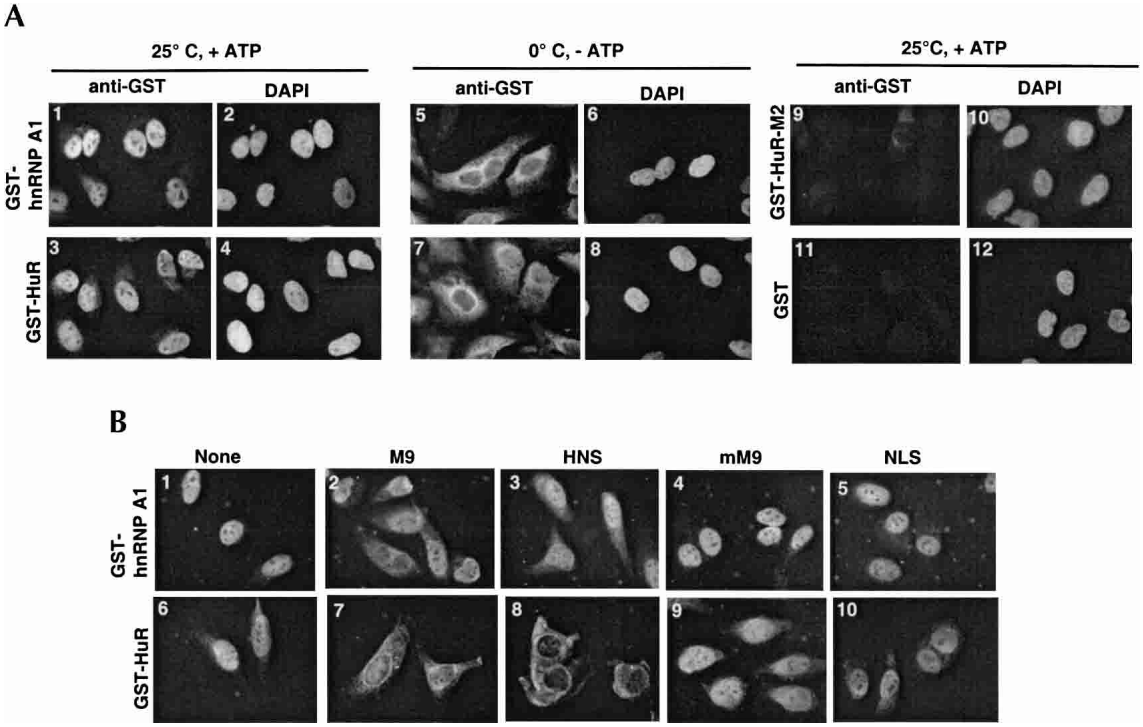

FIGURE 4. HNS and M9 peptides compete for the import of GST-hnRNP A1 and GST-HuR in digitonin-permeabilized HeLa cells. (*A*) Validation of the in vitro import assay using GST-hnRNP A1 and GST-HuR. Digitonin-permeabilized HeLa cells were incubated with HeLa cytosol along with GST-hnRNP A1 or GST-HuR, either at room temperature in the presence of ATP (anti-GST detection is shown in panels *1* and *3*; DAPI nuclear staining in panels *2* and *4*) or on ice in the absence of an energy regenerating system (anti-GST detection in panels *5* and *7*; DAPI staining in panels *6* and *8*). Panels *9* and *11* show the negative controls, GST-HuR-M2 (anti-GST detection in panel *9*; DAPI staining in panel *10*) and GST (anti-GST detection in panel *11*; DAPI staining in panel *12*), at room temperature in the presence of an energy-regenerating system. (*B*) HNS and M9 peptides compete for the import of GST-hnRNP A1 and GST-HuR. The effects of competing and control peptides (indicated on *top*) on in vitro import are presented for GST-hnRNP A1 in panels *2–5* and for GST-HuR in panels *7–10*. The controls with no peptide are shown in panels *1* and *6*. As in *A*, the negative controls GST-HuR-M2 and GST exhibited no nuclear accumulation (data not shown). The cellular localization of GST-tagged proteins was detected with anti-GST antibody (see Materials and Methods). In compiling the results from these assays, randomly picked fields containing 50–100 cells from several different experiments were evaluated by several observers.

may be slower or its affinity for cytoplasmic mRNA higher compared with that of GST-hnRNP A1. In addition, although HuR and HNS possess weak affinity for Imp β in in vitro binding assays (Figs. 2, 3), the NLS peptide does not compete for HuR import in the permeabilized cell assay (Fig. 4B, panel 10), supporting the argument that the Imp β pathway is not a major pathway for HuR import.

In summary, experiments with M9 and HNS peptides in digitonin-permeabilized HeLa cells strengthen the conclusions from our in vitro binding results. Together, these data indicate that transportins are redundant import factors for hnRNP A1 and HuR. Simultaneous with our experiments, Güttinger et al. (2004) carried out in vitro import reconstitution assays with recombinant Trn1 and Trn2b and confirmed that both function as import factors for recombinant HuR in digitonin-permeabilized HeLa cells. Similar to our results based on cross-competition in in vitro import assays using cytoplasmic extract as a source of transport factors, Güttinger et al. (2004) also conclude that M9 and HNS are imported to the nucleus along redundant pathways.

Previously, the M9 and HNS peptides fused to a cell-permeable 16-amino-acid Antennapedia peptide (AP) were reported to compete with different mRNA export pathways: specifically, AP-HNS together with a CRM1 pathway inhibitor, AP-NES, abolished c-fos mRNA export and AP-M9 inhibited DHFR mRNA export (Gallouzi and Steitz 2001). Gallouzi and Steitz (2001) therefore suggested that Trn2 and CRM1 are both export receptors for c-fos mRNA via HuR as the adapter, and that Trn1 is responsible for DHFR mRNA export, via the adapter hnRNP A1. Unfortunately, the present data neither support the concept that Trn1 and Trn2 exclusively interact with hnRNP A1 and HuR, respectively, nor do they confirm the idea that transportins function as export factors. However, because transportins might act as import factors for several hnRNP proteins and for the main mRNA export factor TAP (for review, see Izaurralde 2002), transportins could have an indirect influence on mRNA export.

What is the mechanism of hnRNP A1 and HuR shuttling?

The directionality of Ran-dependent nucleocytoplasmic transport is determined by the GTPase Ran and its regulators, and probably to a lesser extent by the asymmetric distribution of certain nucleoporins (for review, see Mattaj and Englmeier 1998; Bednenko et al. 2003). However, the directionality provided by RanGTP is not necessarily absolute. Intriguingly, Becskei and Mattaj (2003) recently showed that CRM1-mediated Ran-dependent forward export is accompanied by Ran-independent but CRM1-mediated reverse export. According to this model, reverse movements of transport receptor-binding proteins through the NPC are avoided only when those proteins are actively retained by other factors on one or the other side of the NPC. Thus, the shuttling of proteins containing the M9-type bidirectional transport signal might simply reflect the imperfection of the RanGTP-provided directionality for transportin-dependent import. Nevertheless, we cannot exclude the possibility that there exist yet uncharacterized export factors for hnRNP A1 and HuR. For instance, HuR appears to use an alternative export pathway that involves its protein ligands, pp32 or APRIL, as adapters and CRM1 as the export receptor (Brennan and Steitz 2001; Gallouzi et al. 2001).

Yet other factors might influence hnRNP A1 and HuR nucleocytoplasmic transport. Various stress conditions and activation or inactivation of certain signal-transduction pathways seem to regulate both hnRNP A1 and HuR cytoplasmic levels (van der Houven van Oordt et al. 2000; Gallouzi et al. 2001; Wang et al. 2002). In addition, inhibition of RNA polymerase II transcription by actinomycin D or DRB relocalizes both M9- and HNS-containing proteins to the cytoplasm (Piñol-Roma and Dreyfuss 1991; Fan and Steitz 1998a; Nakielny et al. 1999). This suggests that modification of hnRNP A1 and HuR or their transport factors may determine the steady-state intracellular location of hnRNP A1 and HuR. Although hnRNP A1 is known to be both methylated (Kim et al. 1997) and phosphorylated (van der Houven van Oordt et al. 2000), no modifications have been reported within the M9 region. Only one modification, methylation of R217, has been found to take place in HuR; this residue does reside within the HNS (Li et al. 2002). Whether these modifications influence the localization of hnRNP A1 and HuR remains to be studied.

MATERIALS AND METHODS

Plasmids

GST-Trn1 (Siomi et al. 1997), GST-Trn2a, myc-hnRNP A1 (Michael et al. 1995), and pCR-Impβ clones were kind gifts from G. Dreyfuss (Univ. of Pennsylvania, Philadelphia); the GST-Trn2b clone (GST-Kapβ2B; Shamsher et al. 2002) from A. Radu (Mount Sinai School of Medicine, New York); and the GST-Impβ from Y. Yoneda (Osaka Univ., Osaka, Japan). GST-HuR and GST-HuR-M2 are described in Gallouzi et al. (2000). GST-Trn1ΔRan, GST-Trn2aΔRan, and GST-Trn2bΔRan were constructed by inserting PCR fragments corresponding to the C-terminal region of transportins (amino acids 493–890 of Trn1, amino acids 491–897 of Trn2a, amino acids 491–887 of Trn2b) into the BamHI/NotI (GST-Trn1ΔRan) or EcoRI/NotI (GST-Trn2aΔRan and GST-Trn2bΔRan) sites of pGEX-4T-1 (Amersham Pharmacia Biotech). GST-hnRNP A1 was generated via insertion of PCR-generated hnRNP A1 coding sequence into the pGEX-4T-1 vector (Amersham Pharmacia Biotech). Flag-Trn2a and Flag-Trn2b clones were made by moving the BamHI/XhoI fragments from the GST clones into a Flag-pCDNA3 vector that is a derivative of pcDNA3 (Invitrogen) containing a Flag tag. pET28c-Trn1 was constructed by moving the BamHI/NotI fragment from GST-Trn1 into pET28c (Novagen). GST-M9 and GST-HNS contain PCR fragments corresponding to amino acids 263–294 of hnRNP A1 and amino acids

201–240 of HuR inserted into the EcoRI/NotI sites of pGEX-4T-1 (Amersham Pharmacia Biotech). GST-mHNS (see Fig. 1A) was made using the QuickChange site-directed mutagenesis system (Stratagen). Resequencing of the Trn2b clone revealed a point mutation, R611C, in GST-Kapβ2B (Shamsher et al. 2002). This mutation in the GST-Trn2b and Flag-Trn2b clones was reversed to correspond to the genomic sequence (NT 011295) using the QuickChange site-directed mutagenesis system (Stratagen), resulting in the GST-Trn2bC611R and Flag-Trn2bC611R clones. The GST-Trn2b protein encoded by the original GST-Kapβ2B (plasmid from A. Radu) was used for Figure 2, and Flag-Trn2bC611R for Figure 3. No significant differences in binding affinities for cargoes or RanGTP between Flag-Trn2b and Flag-Trn2bC611R were detected. pET28a-RanQ69L was made by insertion of PCR-amplified sequence corresponding to RanQ69L from pQE32-RanQ69L (gift from D. Görlich, University of Heidelberg, Germany) into the BamHI/XhoI sites of pET28a (Novagen).

RT-PCR

Total RNA from HeLa S3 and HEK293 cells was purified using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA for PCR reaction was prepared with Omniscript RT Kit (QIAGEN) according to the manufacturer's instructions; cDNA was synthesized on 2 μg of total RNA, and 1/8 of the reverse transcription reaction mix was used for the PCR reaction. Also, 10 pg of plasmid DNAs (pET28c-Trn1, Flag-Trn2a, Flag-Trn2b) was used as controls. For PCR, the following oligodeoxynucleotide primers were used: TR32, CCTTATATTCCTATGGTGTTGC ACCA; and TR33, GGGATTCACACTGATCATGGTACA; corresponding to Trn1 cDNA (NM153188) sequences 2233–2259 and 2451–2475, respectively; TR30, TTATGTGCAGATGGTCCTCAA; and TR34, TGCACCAAGGCCGGATGAA; corresponding to Trn2 cDNA (accession number AF019039) sequences 2224–2247 and 2369–2387, respectively.

Recombinant proteins

All proteins were expressed in BL21 (DE3) cells by induction with 0.2 M IPTG overnight at 25°C. GST-tagged proteins were purified essentially according to the manufacturer's instructions (Pharmacia Biotech), except that the bacterial lysate was supplemented with 1.5% *N*-laurylsarcosine and sonicated four times for 15 sec to solubilize GST-tagged proteins. To reduce protein degradation, 1 mM PMSF and 1× protease Inhibitors (Calbiochem) were included. Proteins were eluted from glutathione beads with 50 mM Tris-HCl, 400 mM NaCl, 20 mM glutathione, and 1 mM DTT; dialyzed against a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, and 10% glycerol overnight; quick-frozen on dry ice; and stored at −80°C. His-RanQ69L was purified using TALON beads (Clontech), but otherwise essentially as described by Nakielny et al. (1999). Purified His-RanQ69L was dialyzed against 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM GTP, and loaded with GTP in the presence of 1 mM GTP for 1 h at room temperature. Excess GTP was removed by a Microcon (Millipore) filter device, in which the dialysis buffer was replaced with transport buffer TB (similar to Nakielny et al. 1999) containing 20 mM HEPES (pH 7.3), 110 mM potassium acetate (KOAc), 5 mM sodium acetate (NaOAc), 2 mM magnesium acetate (MgOAc), 0.5 mM EGTA, and 1 mM DTT. RanQ69LGTP was quick-frozen on dry ice and stored at −80°C.

Protein-binding assays

To synthesize [^{35}S]methionine-labeled proteins by in vitro translation, the TnT T7 quick coupled transcription/translation system (Promega) was used according to the manufacturer's instructions. The following plasmids were used as templates: pCRImpβ (Pollard et al. 1996), pET28c-Trn1, Flag-Trn2a, and Flag-Trn2bC611R.

In all binding experiments, ~5 μg of GST fusion proteins was loaded onto 40 μL of glutathione Sepharose beads (Pharmacia Biotech) in TB buffer supplemented with 0.1% bovine serum albumine (BSA), 1 mM PMSF, and 1× protease inhibitors (Calbiochem) for 1 h at 4°C. After washing twice with 1 mL of TB, the loaded beads were incubated for 2 h in 0.4 mL of TB plus 0.05% digitonin, 1 mM PMSF, 1× protease inhibitors, 100 μg/mL of RNase A, and 0.1% BSA with either 50 μL of nuclear extract (prepared as in Dignam et al. 1983; Fig. 2) or 4 μL of [^{35}S]methionine-labeled proteins (all other figures) at 4°C, with 5 μg of RanQ69L-GTP included as indicated. The beads were then washed four times with 0.5 mL of TB supplemented with 0.05% digitonin, boiled in SDS-PAGE sample buffer, and analyzed on SDS-PAGE followed by Western blot or autoradiography. The results presented in Figure 3 were quantified using PhosphorImager (Molecular Dynamics). For Western blots, monoclonal anti-hnRNP A1 4B10 (Piñol-Roma et al. 1988) or anti-HuR 3A2 (Gallouzi et al. 2000) was used diluted 1:5000 or 1:50,000, respectively (Fig. 2). For Figure 1C, the rabbit polyclonal anti-Trn2 serum was used at 1:2,000 dilution. HRP-conjugated goat anti-mouse IgG (Fig. 2) or goat anti-rabbit IgG (Fig. 1C; Pierce) was used at 1:5000 dilution as the secondary antibodies. The blots were developed using Western Lightning Reagent (PerkinElmer Life Sciences) and Chemidoc Imaging equipment (Bio-Rad).

Rabbit anti-Trn2 antibodies were raised by the Yale University Immunization Service against the Trn2-specific peptide (amino acids 344–357) CTLPHEAERPDGSED (synthesized by the Keck Facility at Yale University) conjugated to carrier using the Imject Maleimide Activated mcKLH kit (Pierce) and affinity-purified with SulphoLink Kit (Pierce).

In vitro import assay and immunofluorescence

HeLa S3 cytosolic extract (S100T), as the source of transport factors, was prepared by combining the Dignam et al. (1983) and Adam et al. (1990) protocols. Briefly, HeLa S3 spinner cells were resuspended in five packed cell volumes of cold buffer A (10 mM HEPES at pH 7.9, 1.5 mM $MgCl_2$, 1 mM DTT, 10 mM KCl, 1 mM PMSF). Cells were allowed to swell for 10 min on ice and lysed with eight strokes of a Kontes B pestle. Nuclei were removed by spinning at 1000*g*. To the supernatant, 0.11 volume of buffer B-100T (0.3 M HEPES at pH 7.3, 1.1 M KOAc, 30 mM MgOAc) was added, followed by ultracentrifugation at 39,400 rpm for 1 h in a type 70Ti rotor. The clear fraction was then dialyzed against the TB buffer containing 10 % glycerol, frozen in liquid nitrogen, and stored at −80°C.

In vitro import assays were performed essentially as in Adam et al. (1990). The HeLa cells grown 24 h on slides were washed twice with ice-cold TB buffer and permeabilized with 20 μg/mL digito-

nin (Calbiochem). Permeabilized cells were washed three times with cold TB buffer and incubated 10 min on ice with TB plus 1× Protease Inhibitors (Calbiochem). Then, 25 μL of transport mix (50% of S100T in TB, supplemented with the energy regeneration system [0.5 mM GTP, 0.5 mM ATP, 10 mM creatine phosphate, 20 U/mL creatine kinase]) and 1 μM GST-tagged recombinant import substrate were added, covered with parafilm, and incubated for 30 min at room temperature in a humidified box. As indicated, the following peptides (synthesized by the Keck Facility at Yale University) at 300 μM were added to the import assay: NLS, CGGGPKKKRKVED; M9, NQSSNFGPMKGGNFGGRSSG PYGGGGQYFAKPRNQGGY (corresponding to amino acids 268–306 of hnRNP A1); HNS, RRFGGPVHHQAQRFRFSPQ MGVDHMSGISGVNVPG (corresponding to amino acids 205–238 of HuR), and mM9, FGNYNNFSSNQGAAKGGNFGARSSG PYGGGG (corresponding to amino acids 263–294, with the mutated amino acids underlined). After the import mixture was removed, cells were washed twice with TB, and imported proteins were detected by immunofluorescence according to Fan and Steitz (1998a). Rabbit polyclonal anti-GST Z-5 (Santa Cruz Biotechnology) diluted 1:200 as primary and Texas red conjugated goat anti-rabbit IgG diluted 1:800 as secondary antibodies were used. For Figure 1E, affinity-purified rabbit polyclonal Trn2 antibody was used at dilution 1:10.

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