Transportin-mediated Nuclear Import of Heterogeneous Nuclear RNP Proteins

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Abstract. Heterogeneous nuclear ribonucleoprotein (hnRNP) A1 is an abundant nuclear protein that plays an important role in pre-mRNA processing and mRNA export from the nucleus. A1 shuttles rapidly between the nucleus and the cytoplasm, and a 38-amino acid domain, M9, serves as the bidirectional transport signal of A1. Recently, a 90-kD protein, transportin, was identified as the mediator of A1 nuclear import. In this study, we show that transportin mediates the nuclear import of additional hnRNP proteins, including hnRNP F. We have also isolated and sequenced a novel transportin homolog, transportin2, which may differ from transportin1 in its substrate specificity. Immunostaining shows that transportin1 is localized both in the cytoplasm and the nucleoplasm, and nuclear rim staining is also observed. The nuclear localization of A1 is dependent on ongoing RNA polymerase II transcription. Interestingly, a pyruvate kinase–M9 fusion, which normally localizes in the nucleus, also accumulates in the cytoplasm when RNA polymerase II is inhibited. Thus, M9 itself is a specific sensor for transcription-dependent nuclear transport. Transportin1–A1 complexes can be isolated from the cytoplasm and the nucleoplasm, but transportin1 is not detectable in hnRNP complexes. RanGTP causes dissociation of A1-transportin1 complexes in vitro. Thus, it is likely that after nuclear import, A1 dissociates from transportin1 by RanGTP and becomes incorporated into hnRNP complexes, where A1 functions in pre-mRNA processing.

The heterogeneous nuclear (hn)[1] RNPs comprise a group of >20 abundant proteins, designated A through U, that associate with pre-mRNA molecules immediately upon their emergence from the transcription (RNA polymerase II [pol II]) complex (for review see Dreyfuss et al., 1993). Pre-mRNAs/mRNAs remain associated with hnRNP proteins (as hnRNP complexes) throughout their lifetime in the nucleus. Many of the human hnRNP proteins have been cloned and sequenced. Among them, hnRNP A1 is one of the best characterized. A1 binds with high affinity to RNA sequences that resemble pre-mRNA 3′ and 5′ splice sites (Swanson and Dreyfuss, 1988; Burd and Dreyfuss, 1994) and it strongly influences pre-mRNA alternative splicing in vitro and in vivo; the amount of A1 relative to that of the splicing factor SF2/ASF determines the use of alternative 5′ splice sites (Fu et al., 1992; Mayeda and Krainer, 1992; Caceres et al., 1994; Yang et al., 1994). One of the most intriguing properties of A1 is its subcellular localization and transport. A1 is a nuclear RNA-binding protein, but it is not confined to the nucleus; rather, it shuttles rapidly between the nucleus and the cytoplasm in an RNA pol II-dependent manner (Piñol-Roma and Dreyfuss, 1991, 1992). While in the cytoplasm, A1 is also bound to poly(A)$^+$ RNA, and it is therefore likely that A1 also has functions in mRNA metabolism in the cytoplasm, and that it plays an important role in the export of mRNAs from the nucleus (Piñol-Roma and Dreyfuss, 1992). Importantly, this phenomenon is not unique to A1, as many other hnRNP proteins, including A2 and K, are also shuttling proteins (Piñol-Roma and Dreyfuss, 1993; Michael et al., 1995*a*). In contrast, other hnRNP proteins, including the hnRNP C1, C2, and U proteins, are confined to the nucleus. The hnRNP C1 protein contains a nuclear retention signal that is capable of retaining in the nucleus proteins that would normally be exported (Nakielny and Dreyfuss, 1996).

The signal within hnRNP A1 that mediates its nuclear import is a 38-amino acid domain, termed M9, near the COOH terminus of A1. M9 is necessary to localize A1 to the nucleus and sufficient to localize otherwise cytoplasmic proteins to the nucleus when these proteins are fused to the M9 domain (Siomi and Dreyfuss, 1995; Weighardt

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1. *Abbreviations used in this paper*: IBB, importin β binding domain; GST, glutathione-S-transferase; hn, heterogeneous nuclear; NLS, nuclear localization signal; NPC, nuclear pore complex; pol II, polymerase II.

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et al., 1995). Interestingly, M9 does not contain any stretches of basic residues, a characteristic of the classical nuclear localization signals (NLSs); (for review see Dingwall and Laskey, 1991). M9 has also been shown to function as a nuclear export signal of A1, while, using similar assays, the classical NLSs do not have such activity (Michael et al., 1995*b*). These findings suggested that M9 mediates import of hnRNP A1 by a pathway that is different from the import pathway used by classical NLSs.

The nuclear import pathway for proteins containing classical NLSs has been studied extensively (for review see Görlich and Mattaj, 1996; Pante and Aebi, 1996). For import, the NLS-containing proteins bind in the cytoplasm to importin α (Görlich et al., 1995*a*; Imamoto et al., 1995*a*). Importin α (known also as the NLS receptor/karyopherin α) provides the NLS-binding site (Adam and Gerace, 1991; Görlich et al., 1994, 1995*a*; Weis et al., 1995) and it, in turn, interacts with importin β (known also as p97/karyopherin β; Adam and Adam, 1994; Chi et al., 1995; Görlich et al., 1995*a*; Imamoto et al., 1995*b*; Radu et al., 1995) through its importin β binding domain (IBB; Görlich et al., 1996*a*; Weis et al., 1996). The NLS–importin α/β complexes dock via importin β to nuclear pore complexes (NPCs; Görlich et al., 1995*b*; Moroianu et al., 1995) and are subsequently translocated through the NPCs. For this step, cytoplasmic RanGDP (Görlich et al., 1996*b*) and GTP hydrolysis by Ran are required (Melchior et al., 1993; Moore and Blobel, 1993). After translocation, RanGTP directly interacts with importin β in the nucleoplasm (Rexach and Blobel, 1995; Görlich et al., 1996*b*), and this causes the NLS–importin α/β complex to dissociate, and the NLS-containing proteins are then released into the nucleoplasm. At least one other protein is involved in this classical NLS nuclear import pathway, NTF2/p10 (Moore and Blobel, 1994; Paschal and Gerace, 1995), whose precise function is not yet known.

Recently, we have shown that the nuclear import of M9-containing proteins does not use the importin-mediated pathway and have identified a 90-kD protein, termed transportin, as the nuclear import mediator of M9-bearing proteins (Pollard et al., 1996). Transportin directly and specifically interacts with M9 but not with transport-defective M9 mutants (Nakielny et al., 1996; Pollard et al., 1996). Moreover, transportin mediates the nuclear import of M9-containing proteins and full length hnRNP A1 protein, but not of classical NLS-containing proteins (Nakielny et al., 1996; Pollard et al., 1996) in a digitonin-permeabilized import system (Adam et al., 1990), and inhibitors and competitors of importins α and β have no effect on M9-mediated import (Pollard et al., 1996). Thus, the transportin-mediated nuclear import pathway is distinct from the importin-mediated pathway. However, sequence comparison reveals that transportin is distantly related (24% identity) to human importin β (Pollard et al., 1996). In addition, the transportin-mediated protein import is inhibited by RanQ69L (Nakielny et al., 1996), a known inhibitor of classical NLS-bearing protein import (Melchior et al., 1995; Marshallsay et al., 1996; Palacios et al., 1996), suggesting that Ran, or a Ran-like molecule, is required for transportin-mediated protein import, as is the case for importin-mediated import. As described previously, there is a transportin homolog in *Saccharomyces cerevisiae*, yeast transportin (Pollard et al., 1996), which is the most closely related yeast protein to human transportin (35% identity; Nakielny et al., 1996). A recent report has described that a yeast protein, termed Kap104p, which is identical to yeast transportin, functions in the nuclear import of the mRNA-binding proteins, Nab2p and Nab4p, and in the reimport of exported nuclear mRNA-binding proteins (Aitchison et al., 1996).

In this study, we demonstrate that transportin is capable of interacting with hnRNP proteins other than A1 and that it mediates their nuclear import. We also describe a transportin homolog, termed transportin2, which likely has a distinct function as it has a different substrate specificity from the originally identified transportin, which hereinafter, we refer to as transportin1. By immunostaining, we show that transportin1 is localized both in the cytoplasm and the nucleoplasm and that nuclear rim staining can be observed, as is seen for importin β (Chi et al., 1995), suggesting that transportin1 interacts with NPCs during translocation. We found that nuclear localization of pyruvate kinase (PK) fused to M9, like A1, is transcription-dependent. Therefore, M9 is a transcription-dependent nuclear transport signal. We also demonstrate that transportin1-A1 complexes can be isolated from the nucleoplasm; however, no transportin1 is detectable in hnRNP complexes. Gel mobility shift assays show that addition of RanGTP causes dissociation of the transportin1-A1 complexes. Thus, we suggest that after nuclear import, A1 dissociates from transportin1 by RanGTP binding to transportin1 in the nucleoplasm and becomes incorporated into the hnRNP complexes where A1 functions in pre-mRNA metabolism (Choi et al., 1986; Mayeda and Krainer, 1992; Munroe and Dong, 1992; Caceres et al., 1994; Portman and Dreyfuss, 1994; Yang et al., 1994). We discuss the possible roles of transportins and hnRNP proteins in mRNA export.

Materials and Methods

Cell Culture, Labeling, and Cell Fractionation

HeLa S3 and HeLa monolayer-adapted clone JW36 cells were cultured at 37°C to subconfluent densities in DME supplemented with penicillin and streptomycin, and 10% calf serum. For the experiment shown in Figs. 4 *A* and 8 *C*, cells were labeled with [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) at 20 μCi/ml for 20 h in DME containing one-tenth the normal methionine level and 5% calf serum. To prepare the cytoplasmic and nucleoplasmic fractions, cells were resuspended in RSB100 (10 mM Tris-HCl, pH 7.4, and 2.5 mM $MgCl_2$ containing 100 mM NaCl) containing 35 μg/ml digitonin (Calbiochem, San Diego, CA) after washing with cold PBS. After incubation on ice for 5 min, cells were disrupted by passage through needles. Centrifugation at 1,500 *g* briefly yielded a supernatant fraction that was further centrifuged at 4,000 *g* for 15 min and designated the cytoplasmic fraction. The pellet was resuspended in RSB100, sonicated, and centrifuged on 30% sucrose cushion at 4,000 *g* for 15 min to yield a supernatant designated the nucleoplasmic fraction.

Far Western Blotting

Micrococcal nuclease-treated nucleoplasm was fractionated by single-stranded DNA (ssDNA) chromatography essentially as described previously (Piñol-Roma et al., 1988). Proteins were bound to an ssDNA-agarose column (GIBCO BRL, Gaithersburg, MD) at 0.1 M NaCl. The column was washed with 2 mg of heparin per ml in 0.1 M NaCl and eluted with 2 M NaCl. 3 μg of the ssDNA-binding proteins was analyzed by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% nonfat milk in PBS, probed with transportin1, importin β, and transportin2 (see Full Length Transportin2 Isolation) produced by

in vitro transcription–translation of plasmids His-transportin, pCRimpβ (Pollard et al., 1996), and His-transportin2, respectively, using a TnT kit (Promega Biotech, Madison, WI) in rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Amersham Corp.), and exposed to X-ray film.

The cDNA for hnRNP F (Matunis et al., 1994; containing PCR-engineered BamHI and PstI sites just outside the intiation and termination codons, respectively) was excised from pGBT9-F by digestion with PstI, mung bean nuclease, and BamHI, and subcloned into pGEX-5X-1 (Pharmacia Fine Chemicals, Piscataway, NJ) at the BamHI and SmaI sites. The plasmid was transformed and expressed in BL21(DE3) cells, and the glutathione-S-transferase (GST)-F fusion protein was purified according to the manufacturer's instructions. The BstBI/PvuII fragment of hnRNP C2 from pHCC2 (Burd and Dreyfuss, 1994) was subcloned into the same sites in hnRNP C1 in pcDNA3.1 (Nakielny and Dreyfuss, 1996). The entire hnRNP C2 in this vector was then excised with EcoRI and XhoI and subcloned into pGEX-5X-3 (Pharmacia Fine Chemicals). This plasmid was likewise transformed and expressed in BL21(DE3) cells, and the GST-C2 fusion was purified as described above.

Protein-Binding Assays

Purified wild-type GST-M9 or the import-defective GST-M9 mutant (G^{274} to A; 5 μg each) were incubated with 30 μl of glutathione-Sepharose (Pharmacia Fine Chemicals) in 500 μl of binding buffer (50 mM Tris-HCl, 400 mM NaCl, 5 mM $Mg(OAc)_2$, 2 μg/ml of leupeptin, 2 μg/ml pepstatin, and 0.5% aprotinin, pH7.5). After incubation for at least 1 h at 4°C, the resin was washed with binding buffer, and the cytoplasmic fraction of HeLa cells was added. After incubation for 3 h at 4°C, the resin was washed with binding buffer and the bound fraction was eluted by boiling in SDS-PAGE sample buffer, analyzed by SDS-PAGE, and visualized by either Coomassie staining or immunoblotting with D45 (see Preparation of Monoclonal Antibodies).

For the competition experiment, 3 μg of each bound GST-fusion protein was incubated with 15 μl of the ^{35}S-labeled transportin1 translation reaction in 1 ml of binding buffer in either the presence or absence of a 10-fold molar excess of zz-M3 peptide (Pollard et al., 1996). The samples were processed as described above, and the bound ^{35}S-transportin1 was visualized by fluorography.

Nuclear Import Assays

Nuclear import reactions were performed as described (Adam et al., 1990), except that GTP was added to 0.1 mM. HeLa S100 cytosol was prepared as described (Adam et al., 1990). The transport substrates were added at a concentration of 100 μg/ml. After the import reactions, the nuclei were fixed and processed for immunostaining (see Immunofluorescence Microscopy). Import of the GST substrates was detected with an anti-GST monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For the transportin-mediated import assays, 1 μg of transportin was added to the import substrate in transport buffer plus ATP-regenerating system.

Full Length Transportin2 Isolation

In the course of isolation of full length transportin1 cDNA described previously (Pollard et al., 1996), we obtained several partial clones (transportin2) with high similarity to transportin1. One longer clone among them was then employed as a probe to screen a ZAP II HeLa cDNA library (Stratagene Corp., La Jolla, CA) to obtain the full length cDNA of transportin2. Several positive clones were obtained and sequenced. Composite full length cDNA of transportin2 was inserted into pET28a vector (Novagen, Madison, WI) to construct the expression plasmid, His-transportin2. The DNA sequence of transportin2 is available in the EMBL/Genbank/DDBJ database under accession number AF019039.

Preparation of Monoclonal Antibodies

The anti-transportin1 monoclonal antibody D45 was obtained by immunization of a BALB/c mouse with recombinant His-tagged transportin1 (Pollard et al., 1996) purified from *E. coli.* To demonstrate the specificity of D45, immunoprecipitation was carried out in the presence of the ionic detergent EmpigenBB at 1%, 1 mM EDTA, and 0.1 mM DTT as described (Choi and Dreyfuss, 1984) from either [^{35}S]methionine-labeled HeLa cell lysate or rabbit reticulocyte lysate in which transportins1 and 2 were produced by in vitro transcription–translation using a TnT kit (Promega Biotech) in the presence of [^{35}S]methionine.

The preparation of the monoclonal antibodies 4F4 (anti-hnRNP C) and 4B10 (anti-hnRNP A1) were described previously (Choi and Dreyfuss, 1984; Piñol-Roma et al., 1988). For the experiment shown in Fig. 8 *B*, immunoprecipitations were carried out in the presence of EmpigenBB at 1% from rabbit reticulocyte lysate in which myc-PK, myc-PK-M9, and myc-A1 were produced by in vitro transcription–translation of plasmids (Michael et al., 1995*b*; Siomi and Dreyfuss, 1995) using a TnT kit (Promega) in the presence of [^{35}S]methionine.

Immunoprecipitation and Immunoblotting

Transportin1-hnRNP A1 and hnRNP complexes were immunoprecipitated from the cytoplasmic and/or nucleoplasmic fractions of HeLa cells for 10 min at 4°C with the antibodies on protein A-agarose (Pharmacia Fine Chemicals). Rabbit anti–mouse IgG antiserum was added with the D45 antibody, since D45 does not bind protein-A directly. The same secondary antiserum was included with all the SP2/0 nonimmune controls. After washing extensively, the bound fraction on protein-A beads was eluted by boiling in SDS-PAGE sample buffer, analyzed by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% nonfat milk in PBS and probed with D45, 4B10, and 4F4. The bound antibodies were detected with peroxidase-conjugated goat anti–mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and the protein bands were visualized by ECL Western blotting detection kit (Amersham Corp.).

Immunofluorescence Microscopy

Immunofluorescence microscopy was carried out essentially as described previously (Choi and Dreyfuss, 1984) with minor modifications. HeLa cells cultured on glass coverslips were fixed with 2% formaldehyde in PBS for 30 min, followed by permeabilization with 0.1% Triton X-100 for 15 min. Ascites fluids were diluted at 1:500 for D45 and at 1:1,000 for both the anti-importin β antibody 3E9 (Chi et al., 1995) and the anti-hnRNP A1 antibody 4B10 (Choi and Dreyfuss, 1984; Piñol-Roma et al., 1988). The mouse antibodies were detected with fluorescein isothiocyanate-conjugated goat anti–mouse $F(ab')^2$ (Cappel Laboratories, Durham, NC) used at 1:50 dilution in 3% BSA in PBS. Laser confocal fluorescence microscopy was performed with a confocal microscope (TCS NT; Leica, Oberkochen, Germany).

For Fig. 6, HeLa cells grown on glass coverslips in 30-mm dishes were transfected with myc-PK-M9 and myc-full length A1 plasmids (5 μg each) as described previously (Siomi and Dreyfuss, 1995). 48 h after transfection, cells were incubated in the presence or absence of actinomycin D at 5 μg/ml for 4 h before fixation for immunofluorescence microscopy.

Gel Mobility Shift Assays

Oligonucleotides encoding A1 winner sequence (Burd and Dreyfuss, 1994) were annealed and inserted in HindIII and XbaI sites of pSP64 (Promega Biotech). Oligonucleotide sequences are as follows:

A1 winner sense: 5′-AGCTTTATGATAGGGACTTAGGGTGT-3′
A1 winner antisense: 5′-CTAGACACCCTAAGTCCCTATCATAA-3′

This plasmid, termed pSPA1winner, was linearized by XbaI and used for in vitro transcription reaction. Transcription and purification of RNA were carried out as described previously (Kataoka et al., 1995).

Recombinant hnRNP A1 protein was overexpressed and purified as described (Portman and Dreyfuss, 1994). GST-transportin1 fusion protein was kindly provided by S. Nakielny and J. Zhang (University of Pennsylvania, Philadelphia, PA). The plasmids encoding Ran and RanQ69L mutant were transformed and expressed in M15[pREP4] cells. RanGTP, RanGDP, and RanQ69L (GTP form) proteins were purified as described (Bischoff and Ponstingl, 1995).

Gel mobility shift assays were essentially carried out as described previously (Kataoka et al., 1995). The binding buffer used in this study contained 10 mM Hepes (pH 7.3), 55 mM KOAc, 2.5 mM NaOAc, 2.5 mM $Mg(OAc)_2$, 0.25 mM EGTA, 1 mM DTT, 10% glycerol, 50 ng/μl of BSA, 50 ng/μl of yeast RNA (Sigma Chemical Co., St. Louis, MO), 2×10^4 cpm

of RNA (A1 winner), and 1 U/μl of RNasin (Promega Biotech). 5% native polyacrylamide gels were used to analyze the complexes.

Results

Transportin1 Mediates Import of Other hnRNP Proteins in Addition to A1

Although M9 sequence is not found in other known proteins, we considered the possibility that transportin1 may facilitate the import of other hnRNPs. To identify new nuclear import substrates, we isolated an enriched hnRNP population from HeLa cell nucleoplasm by single-stranded DNA-agarose chromatography. The partially purified hnRNP preparation was resolved by SDS-PAGE and transferred to nitrocellulose. The immobilized proteins were then probed with either ^{35}S-labeled transportin1 or importin β that were synthesized in rabbit reticulocyte lysate. Several proteins showed strong interaction with transportin1, but not with importin β (Fig. 1). In addition to A1, other candidate proteins in the profile with transportin1 included the hnRNP B, D, E, and F proteins (Dreyfuss et al., 1993). The protein band labeled "X" is an artifact that appears even after probing with an unprogrammed control reticulocyte lysate (data not shown). When the protein preparation was alternatively subjected to two-dimensional Nephge/SDS-PAGE, followed by electrotransfer and probing with labeled transportin1, one of the signals on the two-dimensional blot corresponded to a protein of molecular weight and isoelectric point coincident with hnRNP F (data not shown; Matunis et al., 1994). The possibility that hnRNP F could interact with transportin1 in vitro was investigated by expressing and purifying hnRNP F as the recombinant fusion protein GST-F and testing whether nitrocellulose-bound GST-F could interact with ^{35}S-labeled transportin1. In addition, we also tested hnRNP C2 fused to GST, since one of the strongly interacting proteins in Fig. 1 corresponds in size to hnRNP C proteins (41–43 kD). Fig. 2 *A* shows that under conditions where hnRNP C2 (Burd et al., 1989) fused to GST is incapable of binding transportin1, there is a strong interaction between transportin1 and GST-F and between transportin1 and GST-M9. To test whether the hnRNP F-transportin1 interaction could be competed by an M9-containing fragment of hnRNP A1, we carried out a protein-binding assay, in which GST-F was bound to glutathione-Sepharose beads. The complex was incubated with ^{35}S-labeled transportin1 in the presence or absence of the competitor zz-M3 (Pollard et al., 1996), a fusion protein containing the zz peptide, the interaction domain of protein A with IgG, fused to the hnRNP A1 M3 fragment, which includes the M9 domain plus an additional 32 amino acids NH_2-terminal to M9 and 15 amino acids COOH-terminal to M9 (Siomi and Dreyfuss, 1995). A 10-fold molar excess was sufficient to nearly completely block the binding of transportin1 to either GST-M9 or to GST-F (Fig. 2 *B*).

The ability of transportin1 to mediate nuclear import of hnRNP F was assessed in an in vitro import assay system (Adam et al., 1990), using digitonin-permeabilized HeLa cells. In the absence of HeLa cytosol, GST-F is unable to be imported into the nucleus, whereas the addition of HeLa S100 cytosol facilitates protein import (data not shown). In the absence of HeLa cytosol, GST-M9 can be imported with the addition of exogenous transportin1. Fig. 2 *C* shows that GST-F can also be imported in permeabilized HeLa cells upon the addition of exogenous transportin1, showing directly that transportin1 mediates hnRNP F import.

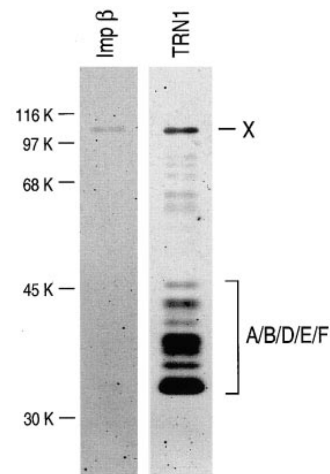


Figure 1. Transportin1 interaction with HeLa single-stranded DNA-binding proteins. Nucleoplasmic ssDNA-binding proteins (3 μg total protein each) were separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with either ^{35}S-labeled importin β (*Imp β*) or transportin1 (*TRN1*), which were synthesized in a coupled transcription–translation reticulocyte lysate system. A/B/D/E/F indicates the protein bands (likely hnRNP A, B, D, E, and F by size) specifically interacting with transportin1. X indicates an artifact band that appears even after probing with an unprogrammed control reticulocyte lysate (data not shown). The molecular weight standards are shown to the left.

Transportin2, a transportin1 Relative, Has Different Substrate Binding

In the course of isolating full length transportin1 cDNA (Pollard et al., 1996), we obtained several partial clones that had high similarity to the originally identified transportin1. One of these clones showed 84% amino acid sequence identity to transportin1. We termed this new 894-amino acid homolog transportin2. The amino acid sequence alignment of transportin2 and transportin1 is shown in Fig. 3 *A*. The two proteins are highly similar over their entire length. Two notable exceptions include differences in the acidic stretch found in the middle of transportin1 (^{350}DEDGIEEEDDDDDEIDDDD^{368}) and transportin2 (^{345}EAERPDGSEDAEDDDDDD^{362}), and most notably, an extra sequence near the COOH end of transportin2 (^{765}GRLTSPSAIP^{774}) which is not found in transportin1. Thus, while yeast contains only one transportin gene (yTRN/Kap104p; Aitchison et al., 1996; Nakielny et al., 1996) there are at least two transportin homologs in humans. Far Western blotting experiments showed that transportin2 did not bind any of the proteins in the ss-DNA-binding protein fraction, whereas transportin1, under the same conditions, bound avidly to several of them (Fig. 3 *B*). The COOH half of transportin1 (amino acids 518 to the end of the protein) is sufficient for interaction with the M9 domain of A1 (Pollard et al., 1996), and the extra sequence, located in this region of transportin2 corresponding to the M9-interacting domain of transportin1, likely modifies the interaction preference and/or strength

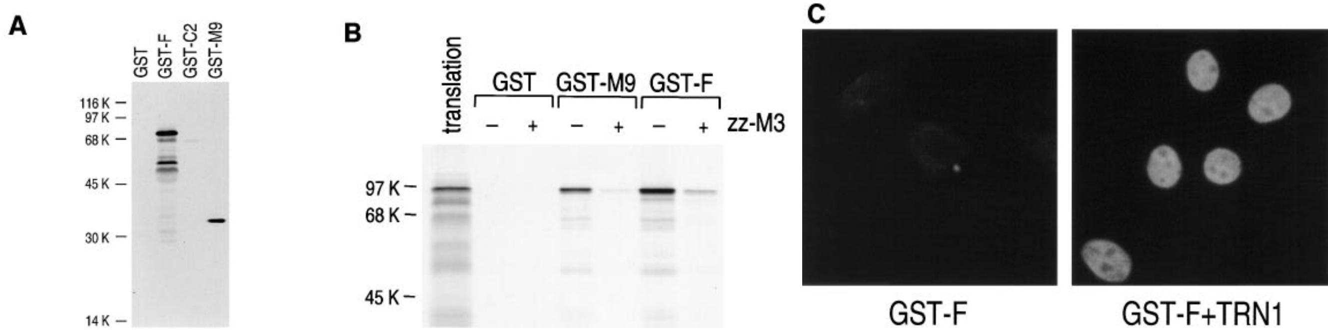


Figure 2. Transportin1 interaction with hnRNP F. (*A*) GST-hnRNP fusion proteins (*GST*, *GST-F*, *GST-C2*, and *GST-M9*; 2 μg in each lane) bound to nitrocellulose blots were probed with ^{35}S-labeled transportin1, prepared as described in Fig. 1. (*B*) Transportin1 binding to hnRNP F can be competed by the transportin1-binding domain of hnRNP A1. GST-fusion proteins (*GST*, *GST-M9*, and *GST-F*; 2 μg each) bound to glutathione-Sepharose beads were incubated with ^{35}S-transportin1 (10 μl from a 50 μl reaction) in the absence (−) or presence (+) of a 10-fold molar excess (over fusion protein) of zz-M3 peptide (Pollard et al., 1996). Transportin1 bound to the indicated fusion protein was eluted with 2× electrophoresis sample buffer and detected by SDS-PAGE and fluorography. Product of transcription–translation reaction was shown as translation (1 μl from a 50 μl reaction). (*C*) Transportin1 mediates the nuclear import of hnRNP F. Digitonin-permeabilized HeLa cells were incubated with GST-F (100 μg/ml) in the presence or absence of transportin1 (50 μg/ml). Import was detected with mouse monoclonal anti–GST-antibody, followed by indirect immunofluorescence with FITC-conjugated goat anti–mouse IgG.

of transportin2 interaction with these proteins. The identity of the nuclear import substrates of transportin2, if any, is as yet unknown.

D45, a Monoclonal Antibody Specific for Transportin1

To further characterize transportin1 and its interaction with hnRNP proteins, we generated monoclonal antibodies to it by immunizing mice with purified recombinant 6His-tag transportin1. Several monoclonal antibodies were obtained, and one of these, termed D45, was further characterized in detail. By immunoprecipitation in the presence of the ionic detergent EmpigenBB, D45 specifically immunoprecipitated transportin1 but not importin β, to which transportin1 is distantly related (Fig. 4 *A*). The specificity of D45 was further demonstrated as D45 immunoprecipitated, from total HeLa cell extract, a single 90-kD protein that comigrated by SDS-PAGE with in vitro-translated transportin1 (Fig. 4 *A*). Similar immunoprecipitation was also performed using in vitro-translated transportin2, and although the amino acid sequence of this protein is highly similar to that of transportin1, D45 did not show detectable cross-reactivity with transportin2 (Fig. 4 *B*). Deletion experiments suggest that the epitope of D45 is located within the second quarter of transportin1 (data not shown). The lack of cross-reactivity of D45 with transportin2 suggests that the acidic stretch region showing at least similarity between transportins 1 and 2 (see Fig. 3 *A*) may be the epitope of D45, and the acidic region might determine, in part, some functional difference between transportins 1 and 2.

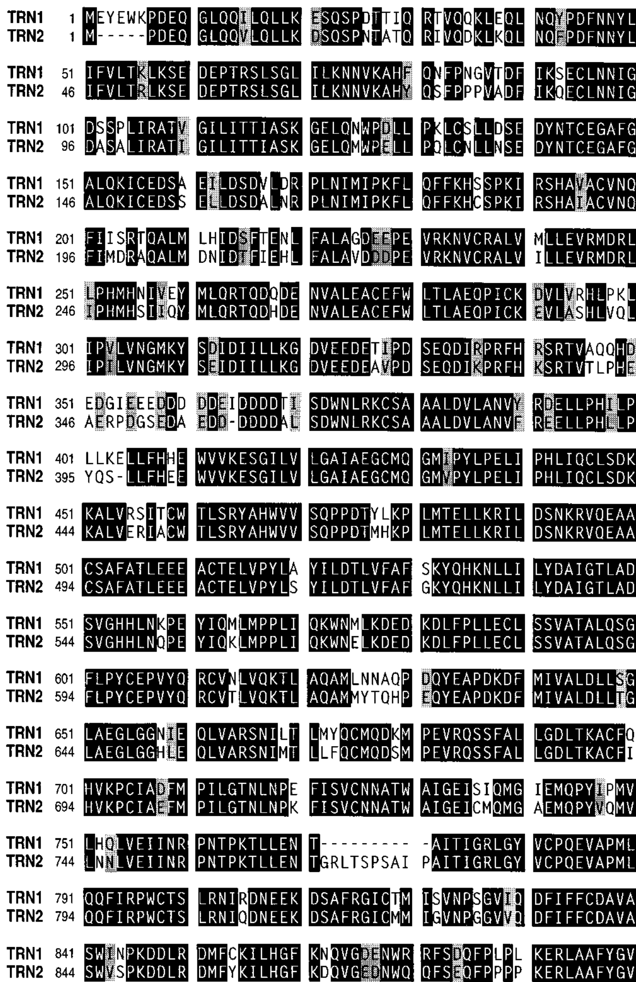
Immunoblotting using D45 on HeLa cytoplasm incubated with either wild-type GST-M9 or the import defective GST-M9 mutant (G274 to A; Michael et al., 1995*b*) at 400 mM NaCl, showed a single 90-kD protein bound to GST-M9 but not to the GST-M9 mutant (Fig. 5 *A*, *Coomassie*) that reacted with D45 (Fig. 5 *A*, *TRN1 blot*). This confirmed the specific binding of transportin1 to M9. Immunoblotting with D45 on lysates from several vertebrate organisms was carried out (Fig. 5 *B*). D45 cross-reacts with protein bands of similar mobility to human transportin1 in monkey and rabbit but not in quail and frog.

Subcellular Localization of Transportin1

Laser confocal immunofluorescense microscopy using D45 was performed to determine the subcellular localization of transportin1. For comparison, antibodies to hnRNP A1 (4B10; Choi and Dreyfuss, 1984) and importin β (3E9; Chi et al., 1995) were also used. As shown in Fig. 5 *C*, transportin1 is found not only throughout the cytoplasm, but also in the nucleoplasm. Intense nuclear rim staining was also observed, although it is not as striking as that for importin β (Chi et al., 1995). The nuclear rim staining suggests binding to the NPCs, as expected for nuclear transport factors. We also expressed transportin1 as a fusion protein with a myc epitope tag by transfection in HeLa cells and observed that the localization of the myc-tagged transportin1 in transfected cells agreed with that seen by the antibody staining with D45 (data not shown).

M9 Is a Transcription-dependent Nuclear Localization Signal

Previous studies have shown that the nuclear localization of hnRNP A1 is dependent on RNA pol II transcription (Piñol-Roma and Dreyfuss, 1991, 1992). To test whether M9 is the region in A1 that confers the transcription sensitivity to A1 nuclear localization, HeLa cells were transfected with full length A1 or PK fused to M9 and then the transfected cells were treated with a pol II inhibitor, actinomycin D for 4 h. As expected, the full length A1 overexpressed in HeLa cells behaved like the endogenous A1; in contrast, PK-M9, which was localized in the nucleus in untreated cells as full length A1, accumulated entirely in the cytoplasm and was apparently absent from the nucleus in cells treated with the pol II inhibitor (Fig. 6). In this experiment, the hnRNP C proteins were detected exclusively in the nucleus (data not shown). This indicates that M9 itself



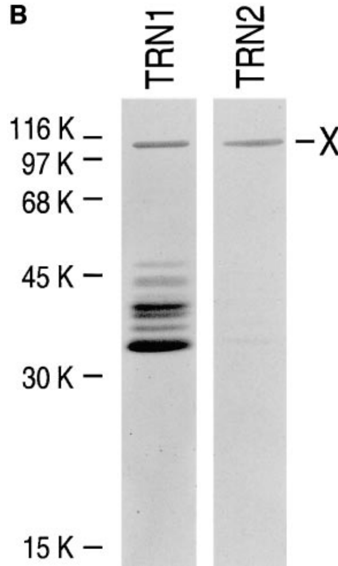


Figure 3. (*A*) Amino acid sequence alignment of transportin1 with transportin2. Identical amino acids between transportins 1 (*TRN1*; Pollard et al., 1996) and 2 (*TRN2*) are indicated by black boxes, and similar amino acids are boxed in gray. Dashed lines specify gaps in the sequences. (*B*) Far Western blotting on ssDNA-binding proteins with transportin2. The blots with ssDNA-binding proteins were prepared as described in Fig. 1 and probed with the indicated ^{35}S-labeled protein, which was synthesized in a coupled transcription–translation reticulocyte lysate system. TRN1 and TRN2 indicate transportins 1 and 2, respectively. X indicates the same artifact band observed and mentioned in Fig. 1.

is the specific sensor of A1 for transcription-dependent nuclear transport. The intracellular distribution of transportin1 was not affected with the pol II inhibitor under the same conditions (data not shown). Therefore, it is likely that the absence of pol II transcription impairs the interaction between M9 and transportin1 in the cytoplasm, resulting in the accumulation of A1 in the cytoplasm.

Transportin1 Exists as a Complex with A1 in the Nucleoplasm, But Not in hnRNP Complexes

HnRNP A1-transportin1 complexes must exist in the cytoplasm because A1 can not be imported into the nucleus without interacting with transportin1. The question we raised then, was whether A1-transportin1 complexes also exist in the nucleoplasm in living cells. To address this, we carried out immunoprecipitations from the nucleoplasm using 4B10 (anti-A1) and 4F4 (anti-C), and the presence of transportin1 in the immunoprecipitates was examined by Western blotting using D45. Under the conditions employed in this immunoprecipitation study, hnRNP complexes, consisting of >20 different hnRNP proteins on pre-mRNAs (Dreyfuss et al., 1993) can be isolated. As expected, transportin1 was in the 4B10 immunoprecipitate, demonstrating that transportin1 is still associated with A1 in the nucleoplasm after translocation through NPCs (Fig. 7 *A*). However, in the 4F4 immunoprecipitate, no transportin1 was detectable, although the immunoprecipitation of hnRNP complexes with 4F4 was efficient, as assessed by coimmunoprecipitation of A1. There are two possible explanations for this observation. First, there may be subsets of hnRNP complexes containing transportin1 that are immunoprecipitable with 4B10, but not with 4F4. Second, transportin1 may not be a component of hnRNP complexes in the nucleoplasm.

To determine which of these scenarios is more likely, similar immunoprecipitations were carried out from the nucleoplasm after treatment with RNase, which causes dissociation of all hnRNP complexes. As shown in Fig. 7 *B*, transportin1 was still in the 4B10 immunoprecipitate, whereas hnRNP C1 protein was no longer detected by Western blotting (demonstrating the efficiency of the RNase digestion). We therefore conclude that transportin1 is not associated with hnRNP complexes, or that only very small amounts are present.

M9 Is Not Accessible to Transportin1 while A1 Is in hnRNP Complexes

Several monoclonal anti-A1 antibodies have been produced in our lab (Piñol-Roma, S., and G. Dreyfuss, unpublished observations). Interestingly, when immunoprecipi-

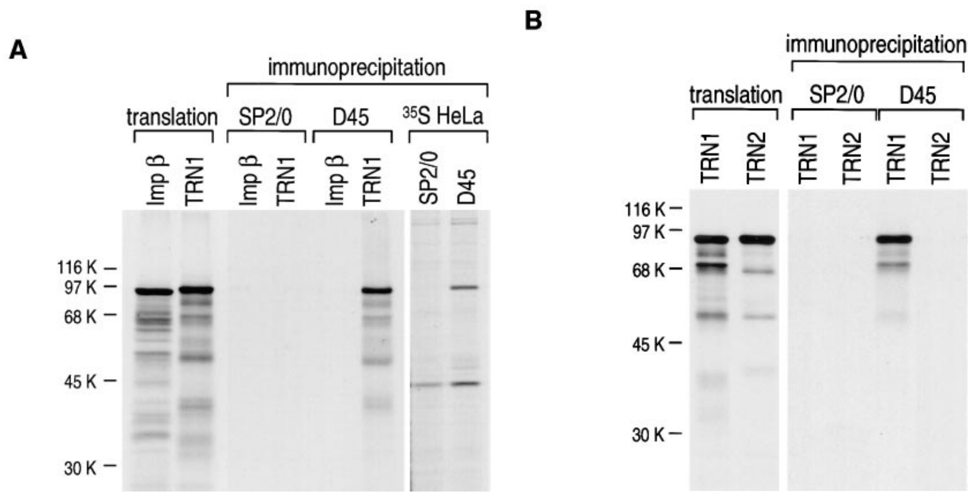


Figure 4. Specificity of the monoclonal antibody for transportin1, D45. (*A*) Transportin1 (*TRN1*) and importin β (*Impβ*) were transcribed–translated in vitro in the presence of [^{35}S]methionine (*translation*). Immunoprecipitations were carried out with D45 and SP2/0 (as a control) in the presence of the ionic detergent EmpigenBB, and the bound fraction of the translated products was analyzed by SDS-PAGE and visualized by fluorography (*immunoprecipitation*). Products of transcription–translation reaction are shown as translation. Additional immunoprecipitation was carried out from total HeLa extract labeled with [^{35}S]methionine under the same conditions. Note that D45 reacts specifically with transportin1 and does not cross-react to importin β. The positions of molecular mass markers are indicated on the left. (*B*) Immunoprecipitation of transportins 1 and 2 with D45. Immunoprecipitation was carried out with D45 using transportins 1 (*TRN1*) and 2 (*TRN2*) transcribed–translated in vitro in the presence of [^{35}S]methionine (*translation*) as described above. D45 does not cross-react to transportin2.

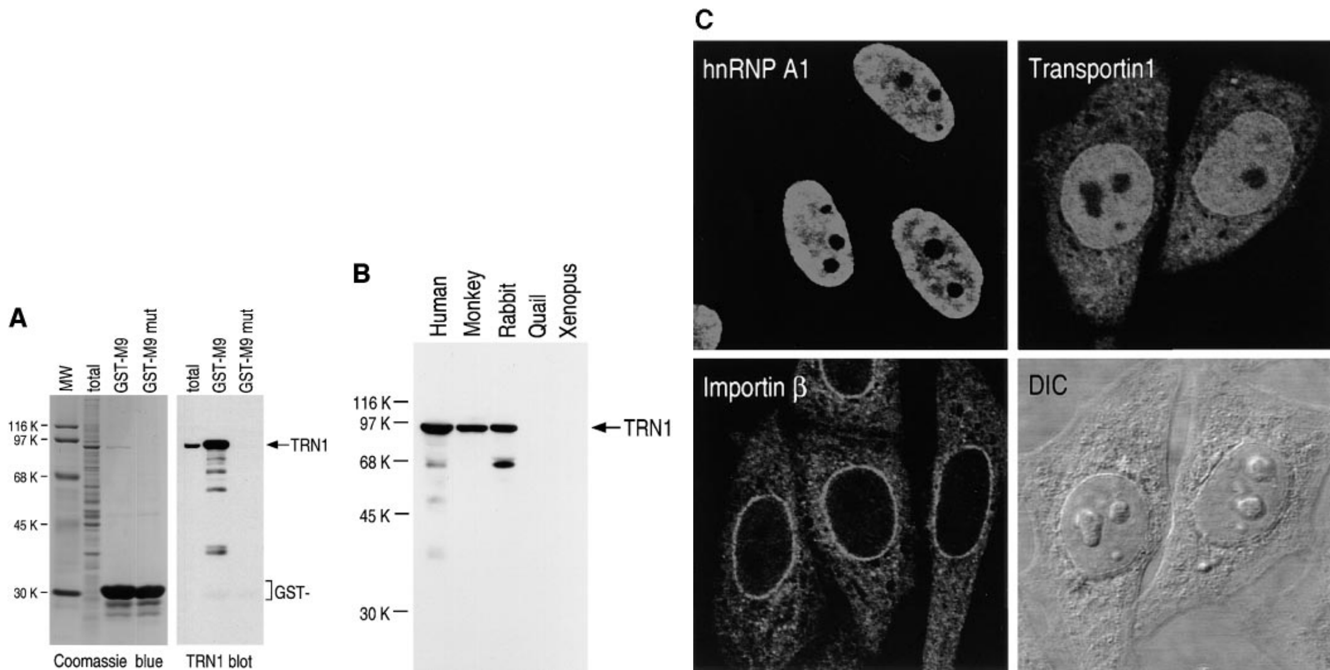


Figure 5. (*A*) M9-containing protein specifically interacts with transportin1 among all the cytoplasmic proteins from HeLa cells. GST-M9 or the import-defective GST-M9 mutant (G274 to A; Michael et al., 1995*b*) on glutathione-Sepharose (both indicated by *GST-*) was incubated with the cytoplasmic fraction from HeLa cells in the presence of 400 mM NaCl. The total HeLa cytoplasmic fraction and the bound fraction to the GST-fusion proteins were analyzed by SDS-PAGE and either visualized by Coomassie staining (*Coomassie blue*) or by immunoblotting with D45 (*TRN1 blot*). Transportin1 specifically interacting with GST-M9 but not with the mutant (*GST-M9 mut*) is indicated by TRN1 with an arrow. GST- indicates the GST-fusion proteins bound on glutathione-Sepharose beads. The positions of molecular mass markers (*MW*) are indicated on the left. (*B*) Zoo blot analysis with D45. Approximately equal amounts of total proteins from HeLa (*Human*), COS (*Monkey*), QT-6 (*Quail*), and XL177 (*Xenopus*) cells and rabbit reticulocyte lysate (*Rabbit*) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with D45. The immunoblot signals were visualized with the ECL kit (Amersham). D45 cross-reacts to protein bands of similar mobility to human transportin1 in monkey and rabbit (indicated by *TRN1* with an *arrow*), but not in quail and frog. (*C*) Subcellular localization of transportin1 in HeLa cells. HeLa cells grown on glass coverslips were fixed with 2% formaldehyde, permeabilized with 0.1% Triton X-100, and incubated with either anti-hnRNP A1 protein, 4B10 (Choi and Dreyfuss, 1984; Piñol-Roma et al., 1988), anti-importin β, 3E9 (Chi et al., 1995), or D45. The primary antibodies were recognized with FITC-conjugated goat anti–mouse antibodies, and the confocal images of the protein staining were analyzed on a Leica confocal microscope. Transportin1 is localized both in the cytoplasm and the nucleoplasm and is also accumulated in the nuclear rim as seen for importin β (Chi et al., 1995).

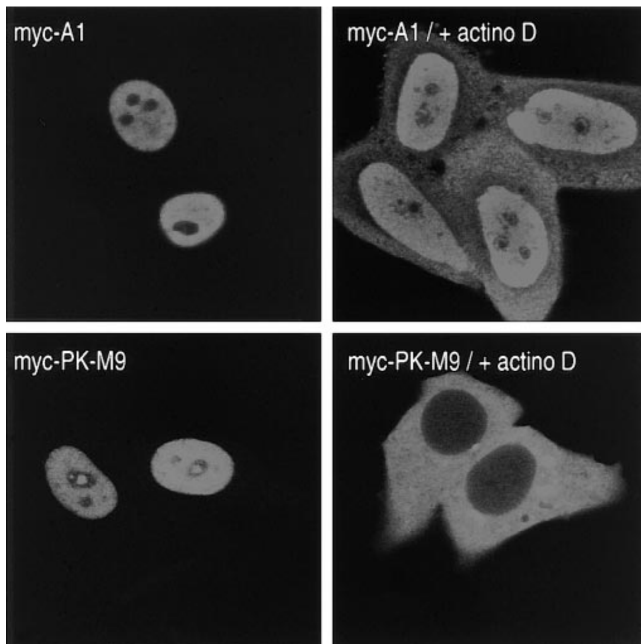


Figure 6. M9 confers the transcription sensitivity to nuclear localization of A1. Transfection of HeLa cells was carried out with either myc-full length A1 (*myc-A1*) or myc–PK-M9 (Siomi and Dreyfuss, 1995), and the transfected cells were then incubated in the presence (+ *actino D*) or absence of actinomycin D (5 μg/ml) for 4 h. Afterwards, immunofluorescence microscopy was carried out using an anti-myc antibody as described in Fig. 5 *C*.

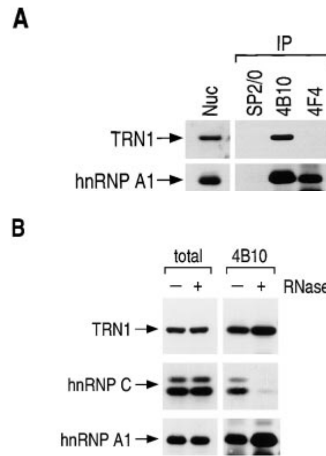


Figure 7. Transportin1 is not associated with hnRNP complexes. (*A*) Immunoprecipitations (*IP*) were carried out using anti-hnRNP A1 (*4B10*) and anti-C (*4F4*) antibodies from the nucleoplasmic fraction (*Nuc*) of HeLa cells. As a control, SP2/0 was employed in this experiment. Afterwards, immunoblotting was performed with 4B10 and D45 to show the existence of hnRNP A1 and transportin1, respectively. Transportin1 (*TRN1*) is observed in the 4B10 immunoprecipitate, but it is not detectable in the 4F4 immunoprecipitate. (*B*) Transportin1-A1 interaction is not abolished by RNase treatment. Immunoprecipitation using 4B10 was carried out from the nucleoplasmic fraction of HeLa cells pre-incubated either with (+) or without (−) RNaseA (10 μg/ml). After RNase treatment, hnRNP complexes are no longer immunoprecipitated with 4B10, since they are dissociated by RNase treatment (note that there is no detectable hnRNP C proteins in the 4B10 immunoprecipitate after RNase digestion). In contrast, transportin1 is still in the 4B10 immunoprecipitate after RNase treatment.

tations were carried out with a different anti-A1 antibody, 9H10, transportin1 was not detected in immunoprecipitates from either the cytoplasm or the nucleoplasm. However, in contrast, 4B10 coimmunoprecipitated transportin1 along with A1 from both compartments under the same conditions (Fig. 8 *A*). This difference may be due to the different epitopes recognized by 4B10 and 9H10, and we therefore performed epitope-mapping experiments. In the presence of the ionic detergent EmpigenBB, immunoprecipitations were carried out with 4B10 and 9H10 from rabbit reticulocyte lysate in which myc-tagged PK, myc-PK-M9, and myc full length A1 (Siomi and Dreyfuss, 1995; Michael et al., 1995*b*) were translated in the presence of [^{35}S]methionine. The data shown in Fig. 8 *B* clearly demonstrate that the epitope of 9H10 is located within the M9 region of A1, providing an explanation for the inability of 9H10 to immunoprecipitate A1 that is bound to transportin1. When immunoprecipitation of hnRNP complexes was carried out from HeLa nucleoplasm using 9H10, along with 4B10 and 4F4 for comparison, 9H10 did not immunoprecipitate hnRNP complexes (Fig. 8 *C*). We conclude that M9, the interaction domain of A1 with transportin1, is not accessible to transportin1 once A1 is assembled into hnRNP complexes.

RanGTP Dissociates A1-Transportin1 Complexes

RanGTP causes the NLS cargo-importin α/β complexes to dissociate at the nucleoplasmic side of the NPCs (Rexach and Blobel, 1995; Görlich et al., 1996*b*), and since transportin1 interacts with RanGTP (Nakielny, S., F.R. Bischoff, and G. Dreyfuss, manuscript in preparation), it was of interest to test whether RanGTP also dissociates transportin1-A1 complexes. To address this, we devised the following gel shift assay. A1 and an RNA probe (A1 winner; UAUGAUAGGGACUUAGGGUG, ^{32}P-labeled; Burd and Dreyfuss, 1994) were incubated in the presence of either transportin1 or bovine serum albumin (BSA), and the formation of complexes was analyzed by a gel mobility shift assay (Kataoka et al., 1995). As shown in Fig. 9 *A*, addition of BSA to A1-RNA complexes had no effect (lanes *9–11*); in contrast, addition of transportin1 resulted in the formation of a new complex of lower mobility (Fig. 9 *A*, lanes *3–5*), as expected if transportin1 could interact with A1 while it binds RNA. Transportin1 did not detectably bind to RNA on its own (lanes *6–8*).

To examine the effect of RanGTP on A1-transportin1 complexes, A1 and RNA (A1 winner) were pre-incubated on ice in the presence of transportin1, and after addition of RanGTP, the complexes were analyzed. Fig. 9 *B* shows that addition of RanGTP resulted in the disappearance of the transportin1-A1–RNA complexes, while A1-RNA complexes remained intact (lanes *5–7*). Thus, the interaction of RanGTP with the transportin1-A1–RNA complexes, most likely through transportin1, causes their dissociation. RanQ69L (GTP form) also dissociated transportin1 from A1 (Fig. 9 *B*, lanes *7–9*), whereas addition of RanGDP had no effect (Fig. 9 *B*, lanes *11–13*).

Discussion

Transportin1 interacts directly and specifically with M9, the bidirectional transport signal of the nuclear shuttling protein, hnRNP A1 (Michael et al., 1995*b*; Siomi and Dreyfuss, 1995) and mediates the nuclear import of hnRNP A1 (Nakielny et al., 1996; Pollard et al., 1996). In this study, we have shown that transportin1 is also capable of interacting with additional hnRNP proteins, such as

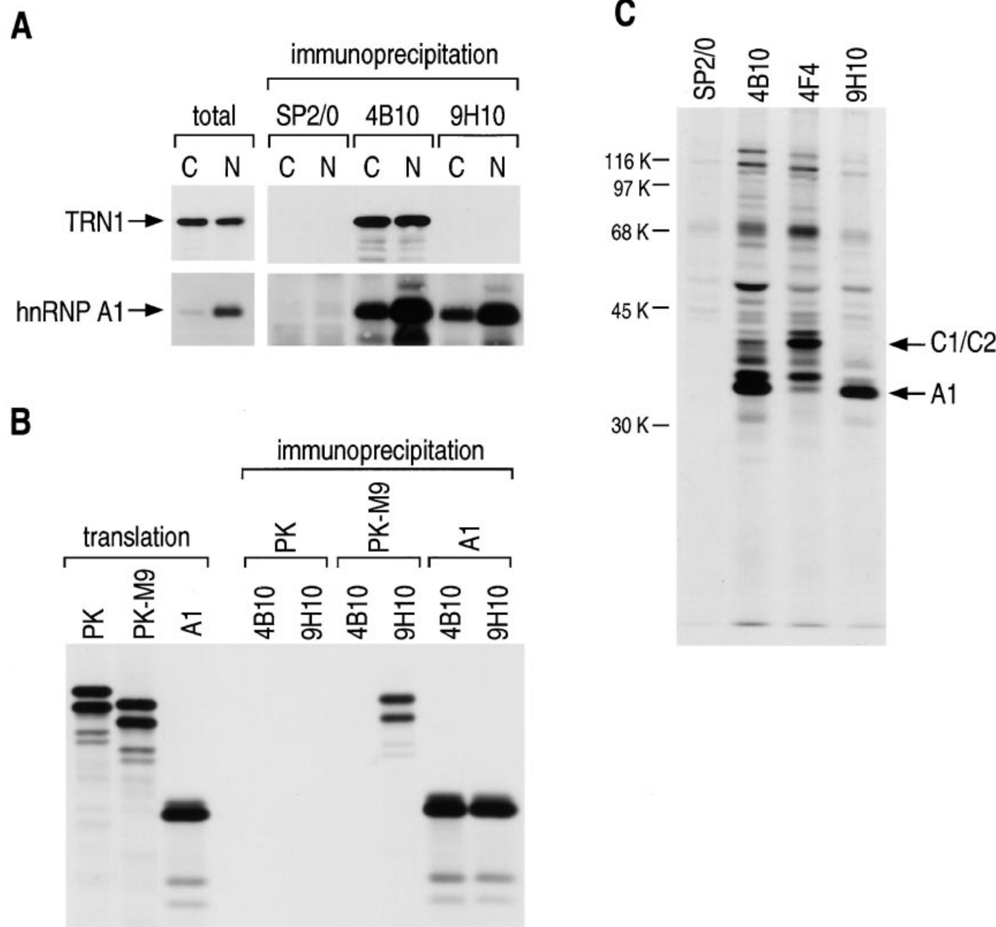


Figure 8. M9 is not accessible in hnRNP complexes. (*A*) Cytoplasmic (*C*) and nucleoplasmic (*N*) fractions were prepared from HeLa cells, and immunoprecipitations were carried out with 4B10 and 9H10 (anti-hnRNP A1 antibodies). Note that 9H10 can immunoprecipitate A1 (*hnRNP A1*); however, transportin1 (*TRN1*) is not detectable in the 9H10 immunoprecipitates from either compartment. (*B*) Epitope mapping of 4B10 and 9H10. The in vitro transcription–translation was carried out for PK, PK-M9, and full length hnRNP A1 (*A1*) in the presence of [^{35}S]methionine (*translation*), and immunoprecipitation was performed using 4B10 and 9H10 in the presence of EmpigenBB. The bound fraction was analyzed by SDS-PAGE and visualized by fluorography. Both antibodies are capable of immunoprecipitating full length A1, but only 9H10 can immunoprecipitate PK-M9, indicating that the epitope of 9H10 is within the M9 region of A1. (*C*) 9H10 does not immunoprecipitate hnRNP complexes. Immunoprecipitations were carried out from the nucleoplasmic fraction of HeLa cells labeled with [^{35}S]methionine using 4B10, 4F4, and 9H10. After immunoprecipitation, all proteins were analyzed by SDS-PAGE and visualized by fluorography. Proteins corresponding to hnRNP C1/C2 proteins are not observed in the 9H10 immunoprecipitate, indicating that 9H10 can not immunoprecipitate hnRNP complexes.

hnRNP F (Matunis et al., 1994) and mediates their nuclear import in an in vitro import assay. The interaction of hnRNP F protein with transportin1 is competed by the M3 region of A1 (Siomi and Dreyfuss, 1995; Pollard et al., 1996), suggesting that the same region of transportin1 (amino acids 518 to the end of the protein; Pollard et al., 1996) interacts with hnRNP A1 and F proteins. We have searched for an M9-like domain in the hnRNP F sequence, but no obvious sequence similarity was revealed. However, we note that hnRNP F contains a region, between the second and the third RNA-binding domains that is rich in Gly, Ser, Asn, and Tyr residues (Matunis et al., 1994), an amino acid composition similar to that of A1-M9. Therefore, transportin1 likely recognizes its import substrates by secondary and/or tertiary structural features rather than by primary sequences. Transportin1 is distantly related to human importin β (24% identity; Nakielny et al., 1996). Nevertheless, transportin1 has a few characteristics that distinguish it from importin β in terms of interacting with its import substrates: (*a*) transportin1 does not require adaptor proteins to interact with its import substrates, whereas importin β interacts with its import substrates (the classical NLS-bearing proteins) via importin α (Görlich et al., 1995*a*; Imamoto et al., 1995*a*); and, related to this, (*b*) transportin1 recognizes a wider range of sequences on its import substrates, whereas importin β binds strictly to the IBB of importin α (Görlich et al., 1996*a*; Weis et al., 1996).

Although transportin2 has very high sequence similarity to transportin1, it does not bind any of the ssDNA-binding proteins on a far Western blot under the same conditions at which transportin1 produced strong signals. One of the most obvious differences between these two protein sequences is a small peptide present near the COOH end of transportin2 located within the region corresponding to the M9-interacting domain of transportin1 (amino acids 518 to the end of the protein; Pollard et al., 1996). Therefore, it is likely that the presence or absence of this mini-exon–like sequence modifies the interaction of transportins1 and 2 with import substrates. The other notable sequence difference between transportins1 and 2 is the acidic stretches located within the second quarter of both proteins. Importin β contains such an acidic stretch, and this sequence is part of its Ran/NPC-binding domain (Chi et al., 1996; Kutay et al., 1997). In Ran also, an acidic stretch near its COOH end is required for the high-affinity binding of RanGTP to RanBP1 (Lounsbury et al., 1994; Richards et al., 1995; Bischoff et al., 1995; Ren et al., 1995) and

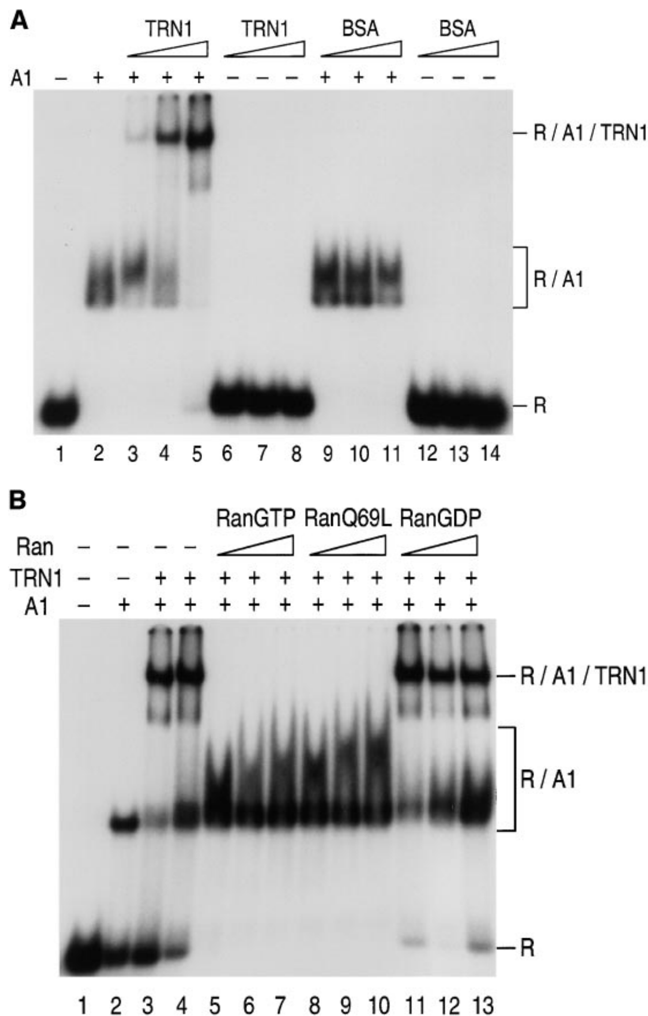


Figure 9. RanGTP dissociates transportin1–A1-RNA complexes. (*A*) Transportin1 is capable of interacting with A1-RNA complexes. A1 and ^{32}P-labeled RNA (A1 winner, Burd and Dreyfuss, 1994) were incubated in the presence of either GST-transportin1 (shown as GST-TRN1: lanes *3–5*; 0.25, 0.5, and 1 μg, respectively) or BSA (lanes *9–11*; 0.25, 0.5, and 1 μg), and the resultant complexes were subjected to 5% native polyacrylamide gel electrophoresis. Lanes *6–8* (with GST-TRN1) and *12–14* (with BSA) are showing the complexes when ^{32}P-labeled RNA was incubated in the absence of hnRNP A1. Lanes *1* and *2* show where RNA itself (*R*) and A1-RNA complex (*R*/*A1*) migrate on the gel, respectively. The formation of a new complex of lower mobility is observed when A1 and RNA are incubated with GST-transportin1 (lanes *3–5*; R/A1/TRN1). All incubations were carried out at 20°C for 10 min. (*B*) Addition of RanGTP or RanQ69L disrupts the transportin1–A1-RNA complex. After A1, ^{32}P-labeled RNA (A1 winner) and transportin1 were pre-incubated on ice for 15 min to form a complex (lane *3*), either binding buffer alone (lane *4*), RanGTP (lanes *5–7*; 0.4, 0.8, and 1.2 μg), RanQ69L (lanes *8–10*; 0.4, 0.8, and 1.2 μg), or RanGDP (lanes *11–13*; 0.4, 0.8, and 1.2 μg) was added and incubated at 20°C for another 10 min. The resultant complexes were analyzed as in Fig. 9 *A*. Lanes *1* and *2* show ^{32}P-labeled RNA itself and A1-RNA complex, respectively.

to affect the role of RanBP1 as a costimulator of RanGAP (Becker et al., 1995; Bischoff et al., 1995; Richards et al., 1995). Therefore, it is possible that transportins1 and 2 have distinct functions in protein transport through NPCs, and the acidic regions may play important roles in distinguishing their functions from each other.

Here we provide evidence that A1-transportin1 complexes dissociate by RanGTP binding to transportin1. In the nucleus, presumably after its dissociation from transportin1, A1 becomes incorporated into hnRNP complexes, where it functions in pre-mRNA processing. Together with the transportin1 import inhibition data with RanQ69L (Nakielny et al., 1996), this observation indicates that Ran and GTP hydrolysis function similarly in importin-mediated and transportin1-mediated nuclear import. However, the dissociation of A1 is not complete, since we could isolate A1-transportin1 complexes from the nucleoplasmic fraction. We also observed by immunoprecipitation experiments with D45 that not all transportin1 appears to be associated with import substrates in the nucleoplasm (data not shown), indicating that some transportin1 remains free in the nucleoplasm after dissociating from its cargo. These observations agree well with the immunostaining data with D45, which show that transportin1 is localized in the nucleoplasm to a greater extent than importin β. This suggests that transportin1 may have roles in the nucleus in addition to its role in importing hnRNP proteins from the cytoplasm. They are, however, presently not yet known. The capacity of RanGTP to completely dissociate transportin1 in vitro while transportin1-A1 complexes are found in the nucleus suggests that other factors, such as Ran-binding proteins, may stabilize these complexes in the nucleus. Alternatively, RanGTP may not be homogeneously distributed in the nucleus.

HnRNP A1 shuttles rapidly between the nucleus and the cytoplasm (Piñol-Roma and Dreyfuss, 1992). A1 is bound, at least initially, to poly(A)$^+$ RNA while in the cytoplasm, and it has been recently shown by immunoelectron microscopy that mRNA in transit through the NPC to the cytoplasm is indeed associated with hnRNP A1/A2-type proteins (Mehlin et al., 1992; Mehlin and Daneholt, 1993; Visa et al., 1996; Daneholt, 1997). Therefore, A1 is likely to play an important role in the export of mRNAs from the nucleus. Recent nuclear microinjection experiments provide additional direct evidence for this suggestion (Izaurralde et al., 1997). The M9 domain of A1 has been shown to serve as the bidirectional transport signal of A1 (Michael et al., 1995*b*; Siomi and Dreyfuss, 1995), and its NLS and nuclear export signal have not been separable so far (Michael et al., 1995*b*). The factors that interact with M9 and mediate the import and export of A1 may be the same, and in exhaustive screens, transportin1 has been the only specific M9-binding factor found. Thus, although there is no detectable transportin1 with bulk hnRNP complexes and M9 is not accessible to both transportin1 and 9H10, an anti-M9 monoclonal antibody, it is possible that transportin1 (or a close relative, such as transportin2) is involved in mRNA export. For example, if transportin1 binds to hnRNP complexes after splicing but immediately before their association with NPCs, this fraction may be too small to detect, and it would not be contained in the soluble nucleoplasmic fraction from which we can immunoprecipitate hnRNP complexes; NPCs fractionate with the insoluble “chromatin/nucleolar” pellet. It is also possible that, in contrast to the 1:1 stoichiometry (transportin1: A1) that is required for A1 nuclear import, a much smaller

amount of transportin1 (e.g., one transportin1 molecule per hundreds of A1 molecules) is sufficient to direct hnRNP complexes to the NPCs and mediate their export, and this may be below our level of detection.

Finally, the difference in the regulation of the importin- and transportin-mediated nuclear import pathways provides a framework for thinking about the need for these separable pathways. Nuclear import of some hnRNP proteins, represented by A1, is dependent on pol II transcription. In this context, it is interesting that excess free A1 microinjected into *Xenopus* oocyte nuclei specifically inhibits mRNA export (Izaurralde et al., 1997). It therefore appears likely that the reason for reducing the amount of A1 in the nucleus when pol II activity is reduced is to prevent excess A1 from competing with mRNA export. It is also possible that A1 in excess of RNA-binding sites is deleterious to the nucleus because it may be insoluble. Therefore, substrates of transportin1, such as A1, needed to evolve their own nuclear import pathway different from the importin-mediated pathway. The accumulation of PK-M9 in the cytoplasm in cells treated with a pol II inhibitor (actinomycin D) indicates that M9 is a transcription-dependent nuclear transport signal. The accumulation of M9-bearing proteins in the cytoplasm in the presence of actinomycin D is probably a result of lack of interaction of M9 with transportin1 in the absence of pol II transcription, since the intracellular distribution of transportin1 itself is transcription independent (data not shown). PK-M9 accumulates in the cytoplasm to a much greater extent than full length A1 in response to actinomycin D treatment. A1 has many functions and interactions in the nucleus while it binds pre-mRNA along with all other hnRNP proteins. Since M9 lacks the RNA-binding domains and an RGG box, which A1 contains, PK-M9 has fewer interactions with other nuclear components. This is probably why M9 accumulates in the cytoplasm to a much greater extent than A1 in the presence of actinomycin D. Transportin1 isolated from cells treated with actinomycin D is still capable of interacting with GST-M9 fusion protein on glutathione-Sepharose beads as well as that from untreated cells (data not shown). Future experiments will examine possible modifications, such as phosphorylation, that may take place on M9 and, in turn, prevent its interaction with transportin1 in transcriptionally inhibited cells.

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