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Snurportin1, an m_3G-cap-specific nuclear import receptor with a novel domain structure

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The nuclear import of the spliceosomal snRNPs U1, U2, U4 and U5, is dependent on the presence of a complex nuclear localization signal (NLS). The latter is composed of the 5′-2,2,7-terminal trimethylguanosine (m_3G) cap structure of the U snRNA and the Sm core domain. Here, we describe the isolation and cDNA cloning of a 45 kDa protein, termed snurportin1, which interacts specifically with m_3G-cap but not m^7G-cap structures. Snurportin1 enhances the m_3G-cap-dependent nuclear import of U snRNPs in both *Xenopus laevis* oocytes and digitonin-permeabilized HeLa cells, demonstrating that it functions as an snRNP-specific nuclear import receptor. Interestingly, solely the m_3G-cap and not the Sm core NLS appears to be recognized by snurportin1, indicating that at least two distinct import receptors interact with the complex snRNP NLS. Snurportin1 represents a novel nuclear import receptor which contains an N-terminal importin β binding (IBB) domain, essential for function, and a C-terminal m_3G-cap-binding region with no structural similarity to the arm repeat domain of importin α.

Keywords: cap-binding protein/IBB domain/nuclear import receptor/nucleocytoplasmic transport/spliceosomal U snRNPs

Introduction

The transport of macromolecules between the cytoplasm and the nucleus occurs through nuclear pore complexes (NPC) and is generally mediated by saturable transport receptors that recognize specific nuclear localization signals (NLS) (for reviews see Görlich and Mattaj, 1996; Nigg, 1997; Izaurralde and Adam, 1998). For example, the nuclear import of proteins carrying the classical NLS, which consists of one or more clusters of basic amino acids (for review see Dingwall and Laskey, 1991), is mediated by the heterodimeric nuclear import receptor complex importin α/β (Görlich *et al.*, 1994, 1995a,b) or karyopherin α/β (Moroianu *et al.*, 1995; Radu *et al.*, 1995; for alternative nomenclatures, see Görlich and Mattaj, 1996; Nigg, 1997). Importin α contains an N-terminal importin β-binding (IBB) domain that mediates complex formation with importin β and a C-terminal domain which accounts for the NLS-binding activity and consists of eight so-called arm motif repeats (Görlich *et al.*, 1996; Moroianu *et al.*, 1996; Weis *et al.*, 1996). Importin β mediates docking of the NLS–importin complex with the NPC (Chi *et al.*, 1995; Görlich *et al.*, 1995a,b; Imamoto *et al.*, 1995; Moroianu *et al.*, 1995). Translocation of the cargo through the pore requires additional factors such as the small GTPase Ran (Melchior *et al.*, 1993; Moore and Blobel, 1993) and p10/NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995).

Recent studies, in particular those investigating the shuttling signals delineated in hnRNP A1 and K, have identified novel protein import pathways that are distinct from the basic NLS pathway (Pollard *et al.*, 1996; Michael *et al.*, 1997). Nuclear import of hnRNP A1 depends on a 38-amino acid transport signal, termed M9, which bears no sequence similarity to classical NLSs (Michael *et al.*, 1995; Siomi and Dreyfuss, 1995; Weighart *et al.*, 1995). M9 is recognized directly by transportin, which is distantly related to importin β (Pollard *et al.*, 1996; Nakielny *et al.*, 1996; Fridell *et al.*, 1997). A homologue of transportin, Kap104p, which imports a particular set of mRNA-binding proteins, has been described in yeast (Aitchison *et al.*, 1996). In contrast to importin β, transportin functions independently of an α subunit, whereas the NPC docking and Ran-dependent translocation of the hnRNP A1-transportin complex into the nucleus is mediated in a manner similar to importin β (Nakielny *et al.*, 1996; Izaurralde *et al.*, 1997a). Two other importin β-related proteins, Kap123p/Yrb4p and Pse1p, have recently been proposed to mediate nuclear import of ribosomal proteins in yeast (Rout *et al.*, 1997; Schlenstedt *et al.*, 1997). These novel importin α-independent transport receptors are all members of a large family of importin β-related transport factors (Fornerod *et al.*, 1997; Görlich *et al.*, 1997).

In contrast to protein import, the mechanism of spliceosomal U snRNP import is less well understood. Each snRNP particle consists of one (U1, U2 and U5) or two (U4/U6) snRNA molecules, a common set of eight core proteins (B, B′, D1, D2, D3, E, F and G, also denoted Sm proteins) that are bound to each of the 2,2,7-trimethylguanosine (m_3G) cap-containing snRNAs U1, U2, U4 and U5, and several proteins associated specifically with the individual U snRNPs (Will and Lührmann, 1997). With the exception of U6 snRNP, which does not leave the nucleus (Vankan *et al.*, 1990), the biogenesis of these U snRNPs requires the bidirectional transport of the snRNA across the nuclear envelope. The snRNAs U1, U2, U4 and U5 are synthesized in the nucleus with a 5′-terminal 7-monomethylguanosine (m^7G) cap structure whereas the Sm proteins are stored in the cytoplasm and

do not migrate into the nucleus in the absence of bound U snRNA. Instead, newly transcribed U snRNAs are transiently exported into the cytoplasm where the Sm proteins bind the snRNA's Sm site, to form a ribonucleoprotein complex referred to as the Sm core (Mattaj and De Robertis, 1985; Raker *et al.*, 1996). Stable association of all Sm proteins is essential for the hypermethylation of the m^7G-cap to the m_3G-cap structure (Mattaj, 1986; Plessel *et al.*, 1994). After this event and 3′-end processing of the snRNAs (Neuman de Vegvar and Dahlberg, 1990), the mature snRNP particles are transported back to the nucleus in a receptor- and energy-dependent manner.

In *Xenopus laevis* oocytes, the nuclear localization signal of U1 snRNPs is complex, with the m_3G-cap as one essential signalling component (Fischer and Lührmann, 1990; Hamm *et al.*, 1990). The second part is located within the Sm core (denoted Sm core NLS) but has not yet been defined precisely (Fischer *et al.*, 1993). Not all spliceosomal snRNAs have the same m_3G-cap requirement for nuclear transport in oocytes. Whereas U1 and U2 snRNA nuclear import absolutely requires an intact m_3G-cap, U4 and U5 snRNAs can enter the nucleus as ApppG-capped derivatives, although with significantly reduced transport kinetics (Fischer *et al.*, 1991; Michaud and Goldfarb, 1992). Although the m_3G-cap is not essential for the nuclear import of any U snRNA in somatic cells, it still accelerates their transport, indicating that it has retained a signalling role for nuclear targeting of U snRNPs (Fischer *et al.*, 1994; Marshallsay and Lührmann, 1994). The differential m_3G-cap requirements for the nuclear import of specific snRNPs in oocytes, and also between oocytes and somatic cells, results from differences in soluble cytosolic factors (Marshallsay and Lührmann, 1994).

The nature of the nuclear import receptor(s) interacting with the snRNPs and whether both parts of the complex snRNP NLS are recognized by one import receptor simultaneously or by at least two distinct receptors, is at present unclear. Recently, a general role of importin β in nuclear import of U snRNP was demonstrated in *X.laevis* oocytes (Palacios *et al.*, 1997). The inability of an excess of protein karyophiles, encompassing either a canonical basic NLS or M9–NLS, to inhibit competitively snRNP nuclear import and vice versa indicates, however, that snRNPs require snRNP-specific import receptors not shared by the other karyophile classes (Fischer *et al.*, 1991, 1993; Michaud and Goldfarb, 1991, 1992; Izaurralde *et al.*, 1997b). Here, we describe the identification and molecular characterization of a protein isolated from human cells, termed snurportin1, which specifically interacts with m_3G-cap but not m^7G-cap structures and which is involved in m_3G-cap-dependent U snRNP import *in vivo* and *in vitro*. Snurportin1 is a novel transport receptor which contains an amino-terminal IBB domain but whose C-terminal domain has no structural relationship to the arm repeat domain of importin α.

Results

Identification of a 45 kDa protein in HeLa cytoplasmic extracts with high specificity for m_3G-cap structures

For the initial identification of potential m_3G-cap-binding proteins in HeLa cell extracts, we employed a UV cross-linking assay using a chemically synthesized m_3GpppAmpUmpA-oligonucleotide (denoted hereafter m_3G-cap oligo) as a substrate whose sequence corresponds to the 5′ end of human U1 snRNA including the 2′-*O*-methylated nucleotides. After UV irradiation of HeLa cytosolic S100 extracts containing m_3G-cap oligo that had been radiolabeled at its 3′ end with [^{32}P]pCp, proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and ^{32}P-labeled cross-linked proteins were visualized by autoradiography. As shown in Figure 1A (lane 1), one major band of radiolabeled protein migrating with an apparent molecular mass of 45 kDa (arrowhead in Figure 1A) and three less intensely radiolabeled proteins with molecular masses of 25, 35 and 150 kDa were reproducibly detected. The m_3G-cap specificity of the observed cross-links, in particular that of the 45 kDa protein, was investigated by competition studies using various unlabeled cap structures. While a 10 000-fold molar excess of m^7GpppG or ApppG-cap dinucleotide had only minor inhibitory effects on the cross-linking of the radiolabeled m_3G-cap oligo to the 45 kDa protein (Figure 1A, lanes 2–4 and 5–7), a 10- to 100-fold molar excess of unlabeled m_3G-cap oligo sufficed to abolish completely the 45 kDa protein cross-link (Figure 1A, lanes 9–11). In contrast, significant inhibition of the formation of the minor cross-linked products (with the exception of the 150 kDa band) was only observed at a 1000-fold excess of m_3G-cap oligo (Figure 1A). Interestingly, a synthetic m_3GpppG-cap dinucleotide inhibited the cross-linking of the 45 kDa protein by an order of magnitude less efficiently than the unlabeled m_3GpppAmpUmpA oligonucleotide (Figure 1A, compare lanes 8–11 with lanes 12–15; see also Discussion).

The 45 kDa protein binds not only to isolated m_3G-cap structures, but also to those present in intact U1 snRNA or, most importantly, in native U1 snRNP particles. This is shown by the ability of U1 snRNA and U1 snRNP to inhibit competitively the cross-link of the m_3G-cap oligo to the 45 kDa protein in S100 cytosolic extracts (Figure 1B, lanes 2–5 and 11–14, respectively). The interaction of the 45 kDa protein with U1 snRNA and U1 snRNP is strictly dependent on the presence of the 5′-terminal m_3G-cap structure; U1 snRNA and U1 snRNP preparations whose 5′-terminal ends had been removed by DNA oligonucleotide-targeted RNase H hydrolysis, failed to compete for the cross-linking of m_3G-cap oligo to the 45 kDa protein (Figure 1B, lanes 6–9 and 15–18). It is important to note that similar concentrations of either isolated m_3G-cap oligo, U1 snRNA or U1 snRNP sufficed to inhibit completely the 45 kDa protein m_3G-cap oligo cross-link (compare Figure 1A and B). This result indicates that neither additional RNA sequences nor the Sm core proteins enhance the affinity of the 45 kDa protein for the 5′-terminal m_3G-cap structure of U1 snRNA/snRNP.

Purification of the 45 kDa m_3G-cap-binding protein, snurportin1

Based on its binding specificity and high avidity for m_3G-cap structures, the 45 kDa protein (henceforth termed snurportin1) appeared to be a promising candidate for a snRNP transport factor. In order to purify this protein, cytosolic S100 extracts from HeLa cells were initially passed over a CM-Sepharose column and the 45 kDa protein containing flow-through was then fractionated

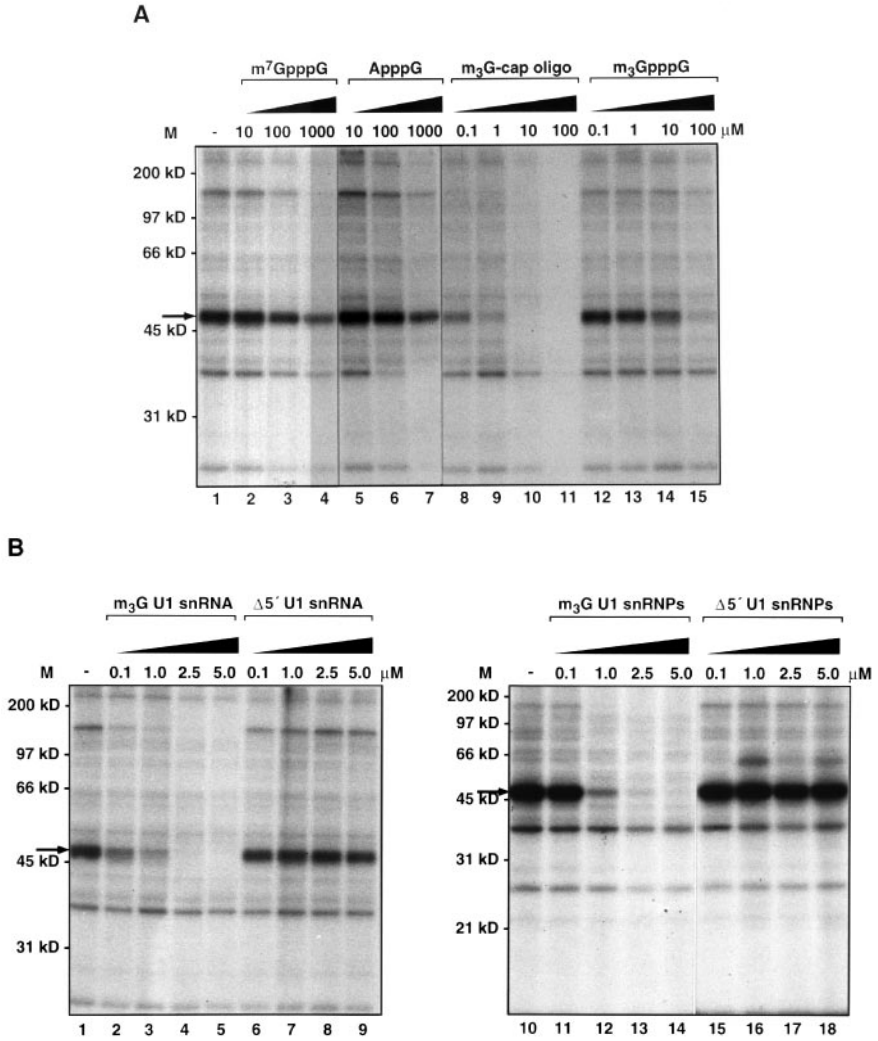

Fig. 1. Identification by UV cross-linking of a 45 kDa protein in HeLa cytosolic extracts that interacts specifically with m_3G-cap structures. (**A**) A 45 kDa protein cross-links specifically to an $m_3GpppAmpUmpA$-oligonucleotide. 1 pmol of [^{32}P]pCp 3′-end-labeled m_3G-cap oligo (~2.5×10^6 c.p.m./pmol) was incubated with 25 μg HeLa S100 cytosolic extract (in a total volume of 10 μl) and subjected to UV irradiation. Cross-linked proteins were separated by 12% SDS–PAGE and visualized by autoradiography (lane1). To assess the specificity of the cross-linking reaction, increasing amounts of unlabeled m^7GpppG- (lanes 2–4), ApppG- (lanes 5–7) and m_3GpppG- (lanes 12–15) cap dinucleotides or unlabeled m_3G-cap oligo (lanes 8–11) were added to the assay mixtures prior to UV irradiation, at the indicated concentrations. The predominant 45 kDa cross-link is indicated by an arrow. The apparent molecular masses of the proteins seen on the autoradiogram correspond to that of the cross-linked proteins plus 1.9 kDa due to the covalently bound m_3G-cap oligo. Molecular weight standards (kD) are indicated on the left. (**B**) An excess of m_3G-capped U1 snRNA and U1 snRNPs inhibit cross-link formation between the m_3G-cap oligo and the 45 kDa protein in S100 cytosolic extracts. Competitions were performed with increasing amounts (as indicated above each lane) of m_3G-capped and uncapped (Δ5′) U1 snRNA (lanes 2–9) or U1 snRNPs (lanes 11–18). Lanes 1 and 10 show the cross-links formed in the absence of competitors. In the case of U1 snRNP competitions (lanes 10–18), protein fractionation was carried out by electrophoresis on a 12.5% high-TEMED SDS–polyacrylamide gel (Lehmeier *et al.*, 1990). Molecular weight standards (kD) are indicated on the left.

by Q-Sepharose chromatography. Those fractions of the Q-Sepharose column containing the bulk of the 45 kDa m_3G-cap-binding protein (as judged by the UV cross-linking assay) were subsequently loaded onto an m_3G-cap affinity column that had been prepared by coupling a biotinylated m_3G-cap oligo ($m_3GpppAmpUmpA$-$(CH_2)_6$-biotin to streptavidin–agarose. Bound proteins were eluted stepwise with buffer containing increasing concentrations of NaCl and analysed by SDS–PAGE. A pure protein with an apparent molecular mass of 45 kDa, was eluted from the affinity column with buffer containing 0.6 to 1 M NaCl (Figure 2A, lanes 9–11). Importantly, the purified 45 kDa protein could be efficiently cross-linked to radiolabeled m_3G-cap oligo by UV irradiation (Figure 2B). These data strongly indicate that the 45 kDa protein purified from HeLa S100 extracts is both necessary and sufficient for the formation of the 45 kDa cross-link, suggesting that it alone harbours m_3G-cap-binding activity.

Snurportin1 contains an IBB domain but lacks canonical arm repeats

For the purpose of cDNA cloning, peptide sequences were obtained from the purified protein by microsequencing. All five peptide sequences identified were detected in a human expressed sequence tag (EST) present in the DDBJ/EMBL/GenBank database (Figure 3A). This full-length snurportin1 cDNA is predicted to encode a 360-amino acid protein with a molecular weight of 41 kDa (Figure 3A). A database search with the human snurportin1 sequence revealed a surprisingly high degree of homology

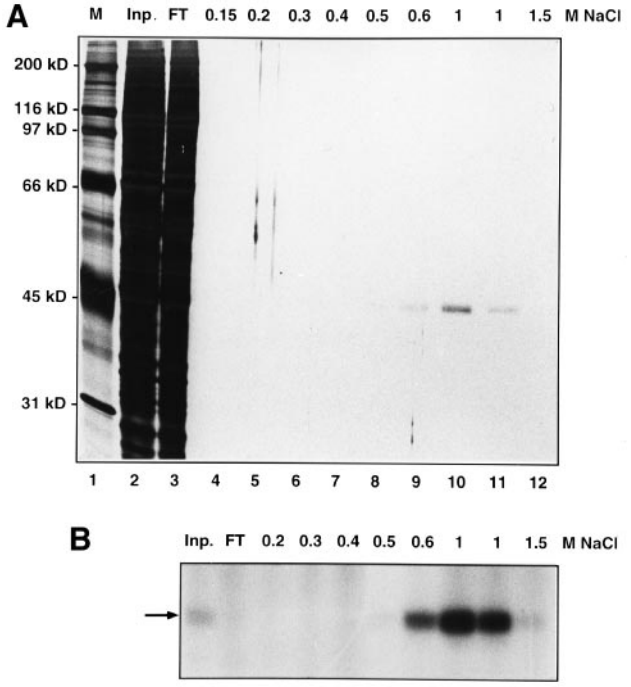

Fig. 2. Purification of the 45 kDa m_3G-cap-binding protein, snurportin1. (**A**) Pre-fractionated HeLa S100 extract (see Materials and methods) was subjected to m_3G-cap affinity chromatography. The column matrix, prepared by coupling biotinylated m_3G-cap oligo [$m_3GpppAmpUmpA$-$(CH_2)_6$-biotin] to streptavidin–agarose (see Materials and methods), was washed with 10 column volumes of buffer D and elution was performed stepwise with 2 ml of buffer D containing 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 1 and 1.5 M NaCl as indicated above each lane. For protein analysis, 10 µl of the input (lane 2), flow-through (lane 3) and each eluate (lanes 4–12) were fractionated on a 10% SDS–PAGE gel and proteins were visualized by silver staining. Molecular weight standards (kD) are shown in lane 1. (**B**) After dialysis and concentration (see Materials and methods), 5 µl of each fraction (as indicated above each lane), 1 µl of the affinity column input (Inp.) or 1 µl of the flow-through (FT) (each containing ~1.5 µg of total protein) was tested for m_3G-cap-binding activity by UV cross-linking. The cross-linked 45 kDa product (indicated by an arrow) was subjected to SDS–PAGE and visualized by autoradiography.

between its N-terminal region (residues 27–65, Figure 3B) and the IBB domain of importin α (i.e. 31% identity, 62% similarity with hSRP1, similar identities were observed with hRch1, xImpα and ySRP1; see Figure 3B). Moreover, several stretches of amino acid residues which are highly conserved among IBB domain sequences of diverse members of the importin α family (Görlich *et al.*, 1996; Weis *et al.*, 1996) are also conserved in the IBB domain of snurportin1 (indicated by black dots in Figure 3B). This suggested that snurportin1 may functionally interact with importin β (see below). In contrast to the N-terminal, extended IBB domain, the C-terminal part of snurportin1 is structurally distinct from importin α (e.g. <10% sequence identity with the C-terminus of hSRP1). In particular, no significant sequence homology was detected between snurportin1 and the arm repeat domain of importin α (data not shown), indicating that there is no evolutionary relationship between the C-terminal regions of these two proteins.

Notably, human snurportin1 sequence exhibits a high overall sequence homology with the open reading frames of several mouse ESTs (e.g. AA571557; >90% identity), a *Drosophila* EST (A541081, >40% identity), and with an open reading frame encoding a *Caenorhabditis elegans* protein of unknown function (ACC AF024493). The homology between snurportin1 and the *C.elegans* protein is not limited to the N-terminal IBB domain (43% identity, 59% similarity) but, most significantly, is also observed between the C-terminal parts of the two proteins (40% identity, 66% similarity) (see Figure 3A). It is thus likely that this protein is the functional counterpart of human snurportin1. The identification of a *C.elegans* homologue indicates that snurportin1 has been evolutionarily conserved, and therefore most likely carries out an essential function. Interestingly, we did not, however, detect an open reading frame with significant homology to human snurportin1 in the yeast database.

Snurportin1 binds importin β in vitro in an IBB-dependent manner

The presence of an IBB domain at the N-terminus of snurportin1 raised the intriguing possibility that importin β, or a variant of the importin β family, may cooperate with snurportin1 in mediating nuclear transport of snRNPs. As a first step to test this idea we investigated whether snurportin1 binds importin β *in vitro*. Histidine-tagged versions of either full-length snurportin1 or an N-terminal truncation mutant of snurportin1 (Δ1–65 snurportin1, lacking the IBB domain; see Figure 3B), as well as full-length hSRP1α and *Xenopus* importin α, were incubated with *in vitro*-translated ^{35}S-labeled importin β. Protein complexes were subsequently precipitated with Ni-NTA–agarose beads and binding of importin β was analysed by SDS–PAGE followed by autoradiography. Importin β was co-precipitated with full-length snurportin1, as well as hSRP1α and importin α, but not with Δ1–65 snurportin1 (Figure 4A, lanes 1–4). Thus, snurportin1 is capable of binding to importin β *in vitro*, and its N-terminal IBB domain is required for mediating this interaction.

The C-terminal domain of snurportin1 possesses m_3G-cap-binding activity

Importin α requires its C-terminal domain to bind the NLS of karyophilic proteins (Cortes *et al.*, 1994). To determine whether the C-terminal domain of snurportin1 is likewise involved in binding the m_3G-cap NLS of U snRNPs, cross-linking studies were performed with the m_3G-cap oligo and deletion mutants of snurportin1. Purified recombinant snurportin1, lacking the N-terminal 65 amino acid residues (including the IBB domain) could be cross-linked to radiolabeled m_3G-cap oligo as efficiently as recombinant full-length snurportin1 (Figure 4B, compare lane 7 with lane 6). Deletion of the C-terminal 32 amino acids did not compromise the cross-link formation, while deletion of additional 120 amino acids completely abolished m_3G-cap binding (data not shown). We therefore conclude that the middle part of the C-terminal region comprises the m_3G-cap-binding domain.

Snurportin1 stimulates U snRNP import in Xenopus oocytes in an IBB-dependent manner

The role of snurportin1 in the nuclear transport of U snRNPs was investigated directly by microinjection studies in *X.laevis* oocytes. Initially, m_3G-capped HeLa U1 and U5 snRNA were microinjected together with *in vitro*-

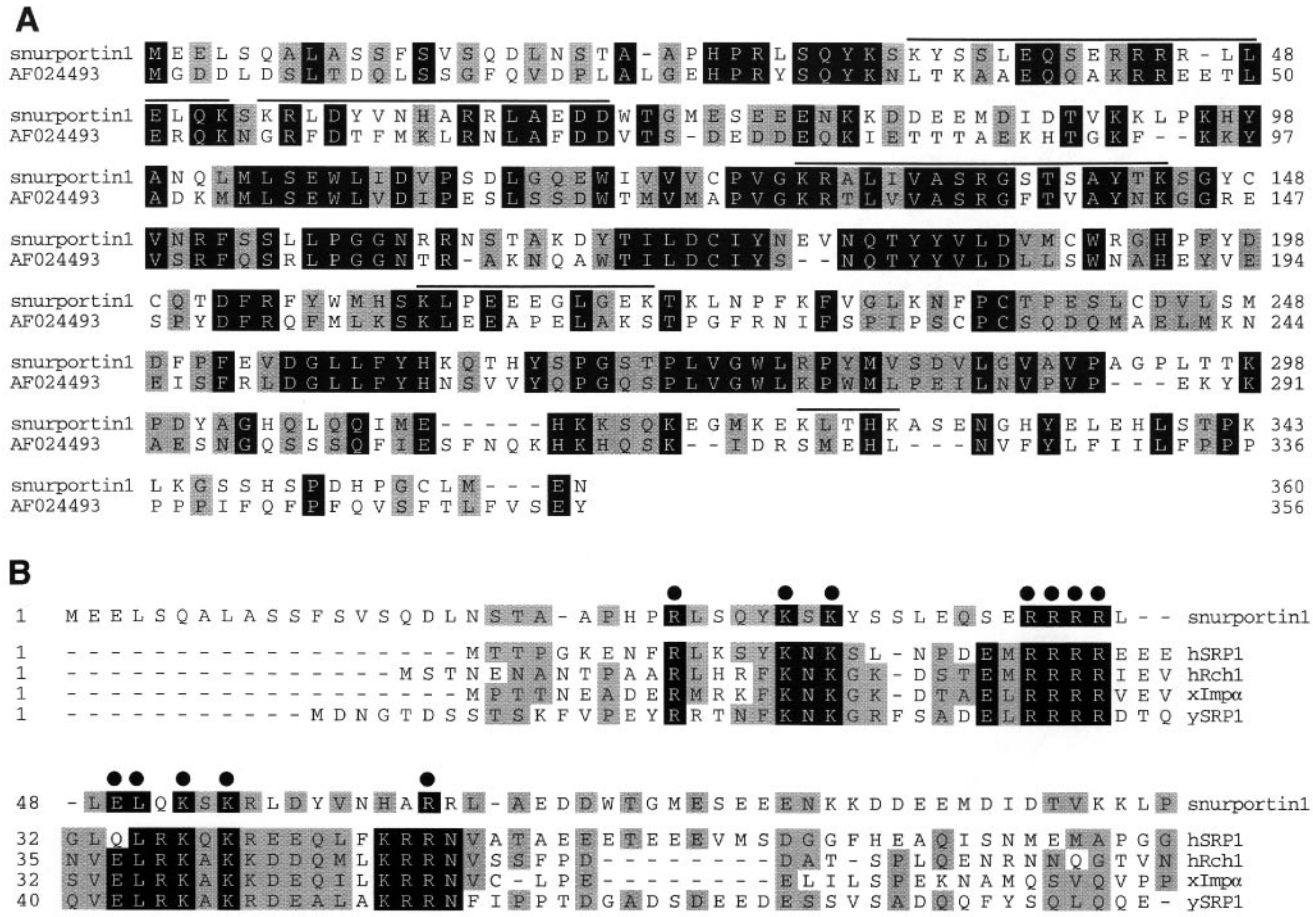

Fig. 3. Amino acid sequence of snurportin1 and its alignment with a *C.elegans* homologue and members of the SRP1/importin α protein family. (**A**) Molecular cloning of human snurportin1 and its homology with a *C.elegans* protein of unknown function (DDBJ/EMBL/GenBank accession number AF024493). The full-length cDNA sequence of snurportin1 was cloned from a human expressed sequence tag (accession number R14245; isolated from an infant brain cDNA library; WashU-Merck EST project), which was identified in the database using partial peptide sequences (indicated by the solid lines above the amino acids) obtained from the purified protein. The snurportin1 nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank data base (accession number AF039029). Human snurportin1 and *C.elegans* AF024493 were aligned using the Clustal megalign program of DNASTAR (Lasergene). Identical residues are indicated in black and related residues are shown in grey. The predicted ORF of the *C.elegans* gene encodes a protein of apparently 322 amino acids which is somewhat shorter than human snurportin1, and would thus introduce a large gap into the C-terminal half when aligned separately with snurportin1 (data not shown). Insertion of a single thymidine in front of the proposed stop codon, generates a protein of 356 amino acids with increased homology to the C-terminus of snurportin1. Therefore, we assume that this extended ORF encodes the actual putative *C.elegans* homologue of snurportin1 and have included the extended version in the alignment. (**B**) Snurportin1 contains an IBB domain. Multiple sequence alignment of the N-termini of human snurportin1 with human SRP1 (Cortes *et al.*, 1994), human Rch1 (Cuomo *et al.*, 1994), *Xenopus* importin α (Görlich *et al.*, 1994) and SRP1 from *S.cerevisiae* (Yano *et al.*, 1992) was performed as described above. The black dots above the snurportin1 sequence indicate residues conforming to the consensus of the importin α IBB domain (Görlich *et al.*, 1996; Weis *et al.*, 1996).

transcribed ApppG-capped U6 snRNA into the cytoplasm of oocytes. After 1 h, the oocytes received a second injection of either buffer or purified recombinant snurportin1, and nuclear transport was measured after 3, 5 and 8 h (Figure 5). U6 snRNA was co-injected as a control since previous data have demonstrated that this RNA is imported into the nucleus along the protein import pathway by binding to a karyophilic protein (Michaud and Goldfarb, 1991, 1992). Significantly, exogenous snurportin1 stimulated U1 and U5 snRNA nuclear import by ~50–70%, whereas no effect was seen on the transport of ApppG-capped U6 snRNA (Figure 5A, compare lanes 4–12, upper panel with lanes 13–21, middle panel; see also Figure 5B for quantitation). The same stimulatory effect of nuclear snRNP import was also observed with exogenous, affinity-purified HeLa snurportin1 (data not shown). Moreover, stimulation of U1 or U5 snRNA nuclear import by snurportin1 was m_3G-cap-dependent and not observed when snRNAs contained a 5′-terminal ApppG-cap (data not shown). Taken together, these results indicate that snurportin1 is a novel snRNP-specific nuclear import factor.

Importin α requires an intact IBB domain for function (Görlich *et al.*, 1996; Weis *et al.*, 1996). To investigate whether the IBB domain of snurportin1 is also necessary for its function, we have microinjected the N-terminal truncation mutant of snurportin1 (Δ1–65 snurportin1) together with m_3G-capped U1 and U5 snRNAs into oocytes. This mutant lacks the IBB domain but retains full m_3G-cap-binding activity (see Figure 4B, lane 7). Strikingly, Δ1–65 snurportin1 not only failed to accelerate snRNP import, but even strongly inhibited the import of m_3G-capped U1 and U5 snRNAs (Figure 5A, lower panel, lanes 22–30 and Figure 5B). The unhindered transport of

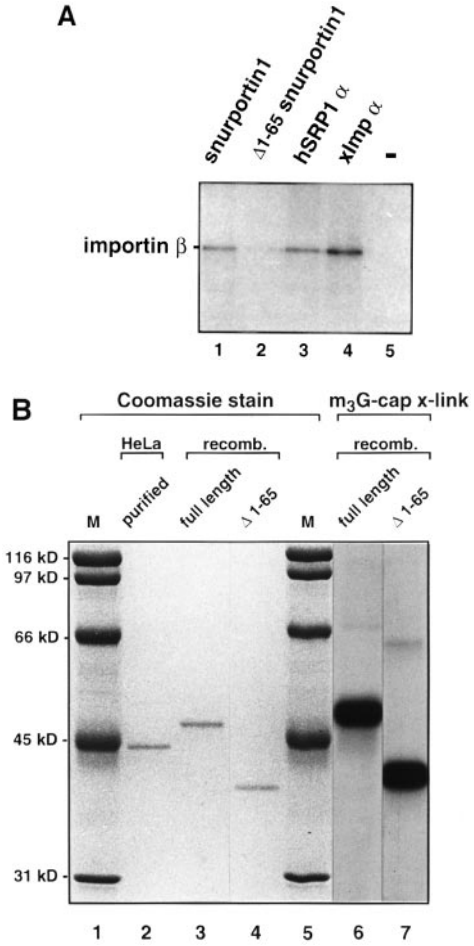

Fig. 4. The N-terminal IBB domain of snurportin1 interacts with importin β *in vitro*, whereas the C-terminal domain exhibits m_3G-cap-binding activity. (**A**) Snurportin1 interacts with importin β *in vitro*. 15 pmol of his-tagged full-length snurportin1 (lane1), the N-terminal deletion lacking the first 65 amino acids (Δ1–65 snurportin1, lane 2), hSRP1α (lane 3) or *Xenopus* importin α (lane 4) was incubated in 100 µl binding buffer with *in vitro*-translated, ^{35}S-labeled importin β. Binding was assessed by precipitation with Ni-NTA beads and bound proteins were separated by 12% SDS–PAGE and analysed by fluorography. Lane 5 shows the background binding of ^{35}S-labeled importin β to Ni-NTA beads in the absence of snurportin1/hSRP1α/importin α. (**B**) Recombinant snurportin1 and Δ1–65 snurportin1 are active in m_3G-cap binding. His-tagged full-length snurportin1 (lane 3) and Δ1–65 snurportin1 (lane 4) were overexpressed in *E.coli* and purified by sequential Ni-NTA and m_3G-cap oligo affinity chromatography steps (see Materials and methods). The purity and electrophoretic migration behaviour of the recombinant proteins were analysed and compared with that of purified HeLa snurportin1 (lane 2) by 12% SDS–PAGE followed by Coomassie staining. Note that the higher molecular weight of the full-length recombinant protein (lane 3) can be accounted for by the presence of the additional 21 amino acids derived from the his-tag. Recombinant full-length snurportin1 (lane 6) or Δ1–65 snurportin1 (lane 7) was tested for m_3G-cap binding using the UV cross-linking assay as described in Materials and methods and cross-linked proteins were visualized by autoradiography. Molecular weight standards (kD) are shown in lanes 1 and 5.

ApppG-capped U6 RNA (Figure 5A, lanes 22–30) excludes non-specific effects of Δ1–65 snurportin1 on the nuclear import machinery. This suggests that U1 and U5 snRNP import is inhibited because the Δ1–65 snurportin1 mutant competes efficiently with endogenous *Xenopus* snurportin1 for binding to the m_3G-cap of the snRNPs. These results clearly demonstrate an essential role for the IBB domain in snurportin1 function. At the same time, the strong inhibition exerted by the N-terminal deletion mutant on U1 and U5 snRNP import underscores the crucial role of snurportin1 in m_3G-cap-dependent U snRNP import in *Xenopus* oocytes.

Snurportin1 strongly accelerates the in vitro nuclear import of U1 snRNPs in digitonin-permeabilized cells

We showed previously that a 5′-terminal m_3G-cap is not essentially required for nuclear accumulation of U1 snRNPs in somatic cells (see Introduction). This indicates that U1 snRNPs can be targeted to the nucleus via a snurportin1-independent pathway. The potential role of snurportin1 in U1 snRNP nuclear import in somatic cells therefore remained unclear. To address this question, an *in vitro* transport system (Marshallsay and Lührmann, 1994) using digitonin-permeabilized HeLa cells and cytosolic extract as a source of nuclear transport factors, was employed. As nuclear import substrates, we used either purified intact HeLa U1 snRNPs or U1 snRNPs from which the 5′-terminal ~10 nucleotides of the U1 RNA including the m_3G-cap structure had been removed by DNA oligonucleotide-directed RNase H hydrolysis. The protein moiety of both forms of U1 snRNP was labeled by modification with the fluorescent dye Cy3 (henceforth referred to as U1 snRNP* or Δ5′ U1 snRNP*, respectively). We verified by SDS–PAGE and glycerol gradient centrifugation analysis that the U1 snRNP particles remained intact after the labeling procedure and that the level of Cy3 modification was similar in both forms of U1 snRNP.

As shown in Figure 6A and B, intact U1 snRNPs* are more efficiently targeted to the nucleus in the presence of HeLa cytosolic S100 extract than Δ5′ U1 snRNP* particles. In both cases, transport was energy- (Figure 6C and D) and temperature-dependent (data not shown). This result is consistent with the idea that the endogenous snurportin1 in HeLa cytosol could contribute significantly to the nuclear import of intact U1 snRNPs. To test this hypothesis, competition studies with non-fluorescently labeled U1 snRNPs or m_3GpppG-cap dinucleotide were performed. In the presence of a ~100-fold molar excess of unlabeled Δ5′ U1 snRNPs, nuclear import of intact U1 snRNPs* was reduced by 35–40 % (compare Figure 6E and 6A), while nuclear import of Δ5′ U1 snRNPs* was completely abolished (compare Figure 6F and 6B). This suggested that exogenous Δ5′ U1 snRNP particles titrated an snRNP-import receptor that is limiting in HeLa cell cytosol and distinct from snurportin1 (probably the Sm core NLS-binding receptor).

However, since a significant fraction of intact U1 snRNPs were still imported in the presence of competitor Δ5′ U1 snRNPs, the import of these particles appeared to be predominantly m_3G-cap- (i.e. snurportin1) mediated. Consistent with this notion, nuclear import of U1 snRNPs* could be inhibited by ~90%, by either an excess of intact competitor U1 snRNPs (Figure 6G) or by the simultaneous addition of competitor Δ5′ U1 snRNPs and synthetic m_3GpppG-cap dinucleotide (Figure 6H).

To provide direct evidence that snurportin1 mediates nuclear import of U1 snRNPs in somatic cells, *in vitro* import studies were performed in the presence of recom-

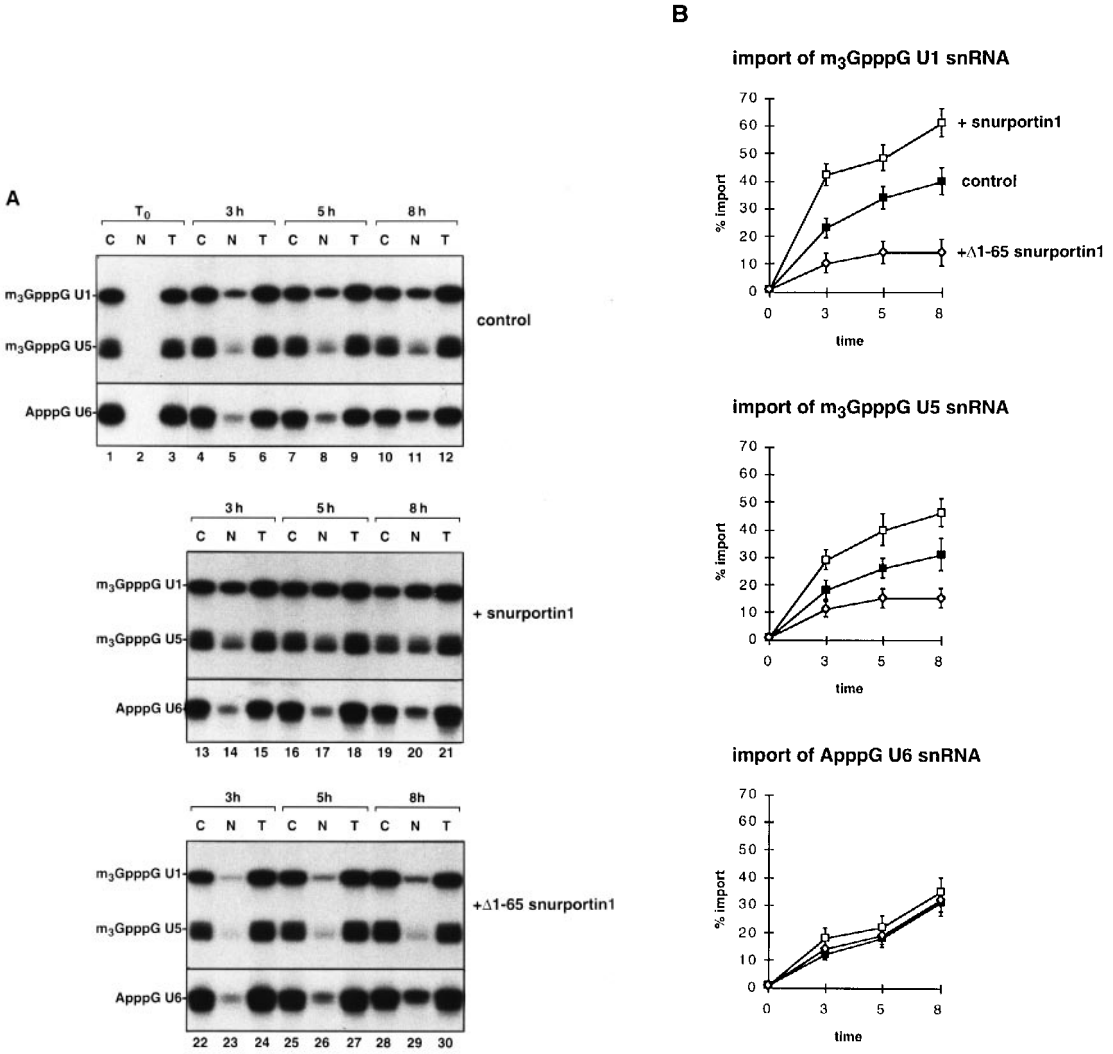

Fig. 5. Recombinant snurportin1 accelerates U snRNP import in *Xenopus* oocytes in an IBB-dependent manner. (**A**) A mixture of ^{32}P-labeled m_3G-capped HeLa U1 and U5 snRNA (~2 fmol each at 3×10^6 c.p.m./pmol), or ApppG-capped U6 snRNA (~6 fmol at 1×10^6 c.p.m./pmol) was injected into the vegetal half of *X.laevis* oocytes. Oocytes were injected 1 h later with either buffer D (control, upper panel), 20 μM recombinant full-length snurportin1 (middle panel) or 20 μM recombinant Δ1–65 snurportin1 (lower panel). RNA from cytoplasmic (C) or nuclear (N) fractions, or from total oocytes (T), was collected from four oocytes either directly (T_0, lanes 1–3) or 3 h (lanes 4–6, 13–15, 22–24), 5 h (lanes 7–9, 16–18, 25–27) and 8 h (lanes 10–12, 19–21, 28–30) after U snRNA injection. One oocyte equivalent of RNA was separated on 6% acrylamide gels containing 7.5 M urea. The identity of the U snRNAs is indicated on the left. (**B**) Quantification of the transport kinetics of m_3G-capped U1 and U5, and ApppG-capped U6 snRNA. The percent nuclear accumulation (% import) of each snRNA in control oocytes (■) and oocytes post-injected either with snurportin1 (□) or with Δ1–65 snurportin1 (◇) was determined by PhosphorImager analysis (Molecular Dynamics) and plotted against time after microinjection. The error bars indicate the standard deviation (SD) obtained from three separate experiments.

binant snurportin1. Addition of recombinant full-length snurportin1 to cytosolic S100 extract resulted in a significant increase in the nuclear accumulation of U1 snRNPs* (up to 180% in the presence of ~100 pmol exogenous snurportin1) (compare Figure 6I with 6A). This stimulation is strictly m_3G-cap-dependent, as demonstrated by the failure of exogenous snurportin1 to accelerate the transport of Δ5′ U1 snRNPs* to the nucleus (Figure 6K). Moreover, preincubation of snurportin1 with an excess of m_3GpppG-cap dinucleotide abolished snurportin1 stimulation of U1 snRNP import (data not shown). Finally, consistent with the data obtained with oocytes (see Figure 5), the enhancement of nuclear U1 snRNP* import by exogenous snurportin1 required the presence of its N-terminal IBB domain; the addition of ~100 pmol of Δ1–65 snurportin1 to cytosolic S100 extract did not accelerate, but rather inhibited, U1 snRNP import by 30–40% (compare Figure 6L with 6A). As expected, Δ1–65 snurportin1 did not inhibit the nuclear import of Δ5′ U1 snRNP* (compare Figure 6M with 6K and 6B, respectively). In summary, these data indicate that in HeLa cells at least two distinct import receptors mediate U1 snRNP nuclear import, namely snurportin1 and most likely the Sm core NLS-binding receptor, and that snurportin1 contributes significantly to the nuclear accumulation of U1 snRNPs in somatic cells *in vitro*.

Discussion

Snurportin1, an snRNP-specific nuclear import receptor

In this report we describe the structure and function of a 45 kDa protein from HeLa cytosolic extracts, termed snurportin1, which binds with high specificity the m_3G-

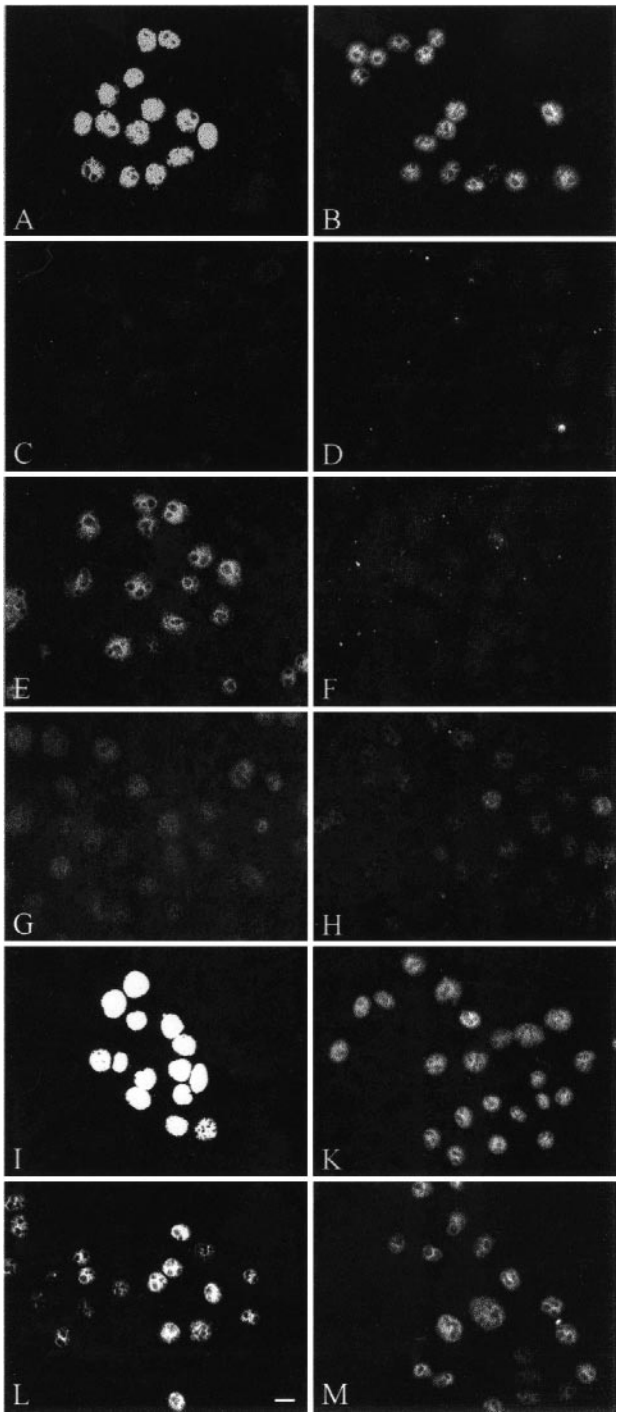

Fig. 6. Snurportin1 enhances nuclear import of m_3G-capped U1 snRNPs in digitonin-permeabilized HeLa cells supplemented with HeLa cell cytosol. Nuclear import of fluorescently labeled U1 snRNPs (**A**, **C**, **E**, **G**, **H**, **I** and **L**) or Δ5′ U1 snRNPs (**B**, **D**, **F**, **K** and **M**), in the presence of ATP (A, B, E, F, G, H, I, K, L and M) or absence of ATP (C and D), was performed as described in Materials and methods. Import reactions were supplemented with a 100-fold molar excess of unlabeled Δ5′ U1 snRNPs (E and F), a 100-fold molar excess of unlabeled U1 snRNPs (G), a 100-fold molar excess of unlabeled Δ5′ U1 snRNPs plus a 20 000-fold molar excess of m_3GpppG-cap dinucleotide (H), 100 pmol of snurportin1 (I and K) or 100 pmol of Δ1–65 snurportin1 (L and M). Scale bar, 10 μm.

cap structure of U snRNAs. Snurportin1 contains an N-terminal domain with significant sequence similarity to the importin β binding (IBB) domain of importin α (Figure 3B) and a C-terminal domain which is necessary and sufficient to bind the m_3G-cap (Figure 4B). Consistent with the fact that the m_3G-cap constitutes one part of the complex NLS of spliceosomal snRNPs (see Introduction), we demonstrate here that snurportin1 functions as an snRNP-specific nuclear import receptor. For example, recombinant snurportin1 strongly enhances the nuclear import of U1 snRNP both *in vivo*, upon microinjection of *X.laevis* oocytes (Figure 5) and *in vitro*, using digitonin-permeabilized HeLa cells (Figure 6). This enhancement is specific for m_3G-capped snRNPs and not observed for the nuclear import of ApppG-capped U6 snRNA, which is known to be imported via the protein import pathway due to its association with a karyophilic protein (Michaud and Goldfarb, 1992; Figure 5). Moreover, snurportin1 requires the N-terminal IBB domain to exert its function as a U snRNP import receptor. This is indicated by our finding that an N-terminal deletion mutant of snurportin1, which has retained the capacity to bind the m_3G-cap but lacks the IBB domain, blocks nuclear import of U snRNPs in microinjected *X.laevis* oocytes (Figure 5).

The essential role of the IBB domain for snurportin1 function further suggests that snurportin1 cooperates with importin β in mediating nuclear import of U snRNPs. This idea is supported by our finding that recombinant snurportin1 binds to *in vitro*-translated importin β in an IBB-dependent manner (Figure 4A). Moreover, our data are consistent with a recent report by Palacios *et al.* (1997), who provided evidence for a general role of importin β in the nuclear targeting of U snRNPs. In particular, they demonstrated that nuclear U snRNP import could be inhibited *in vitro*, by immunodepleting *Xenopus* egg extracts from importin β, or *in vivo* by microinjection of the importin α IBB domain into oocytes. It should be noted, however, that we have as yet failed to isolate from unfractionated HeLa cytosolic extracts a stable complex of snurportin1 and importin β using m_3G-cap affinity chromatography under low salt conditions (data not shown). This could indicate a lower affinity of snurportin1 for importin β as compared with importin α. In any case, it will be interesting to investigate whether the respective IBB domains of importin α and snurportin1 are functionally equivalent, i.e. whether they are interchangeable.

Snurportin1 is a nuclear import receptor with a novel domain structure

All members of the importin α family characterized to date share, in addition to the N-terminal IBB domain, a C-terminal domain which consists of at least eight evolutionarily conserved arm repeats. Moreover, they all mediate the nuclear import of protein karyophiles which contain classical NLS structures, and these NLSs have been shown to be bound by the importin α arm repeat domains (reviewed by Görlich and Mattaj, 1996; Nigg, 1997). If it is considered that snurportin1, in contrast to importin α, recognizes an NLS (i.e. the snRNP m_3G-cap) which is exclusively comprised of nucleic acid components (Figures 1 and 6; see also below) the presence of an IBB domain in snurportin1 is somewhat surprising. Sequence comparison of snurportin1 and importin α, however, revealed that the structural similarity between the two import receptors is confined to their N-terminal IBB domains; their C-terminal domains, which account for

NLS binding activity, are structurally distinct (Figure 3B and data not shown). Most importantly, the m_3G-cap-binding domain of snurportin1 does not contain any arm repeats. Therefore, snurportin1 represents a new type of nuclear import receptor which shares with importin α an IBB domain but lacks its canonical arm repeat region. Our results further indicate that the IBB domain can function as a nuclear import receptor module in a more versatile manner than previously expected and it will be interesting to see whether additional receptors with an snurportin1-like domain structure will be identified in the future. We note, that despite its unique structure, snurportin1, like importin α, appears to function as an adaptor molecule which bridges the cargo (i.e. the snRNP particle) to the nuclear pore docking protein (i.e. importin β).

A general property of all nuclear import receptors, including importin α, is that they shuttle between the cytoplasm and nucleus (Görlich and Mattaj, 1996; Nigg, 1997). While we presently have no direct evidence that snurportin1 shuttles, a number of observations suggest indirectly that this is the case. For example, we could isolate snurportin1 not only from HeLa cytosol, but also from nuclear extracts. Moreover, preliminary immunofluorescence microscopy data obtained with an antibody specific for snurportin1 indicate that it is localized in both compartments of HeLa cells, as well as at the nuclear membrane (data not shown).

Recently, it has been shown that the re-export of importin α from the nucleus to the cytoplasm is an active process that is mediated by a new nuclear export factor termed CAS (Kutay *et al.*, 1997). Since the IBB domain is not sufficient for nuclear export of importin α (Görlich *et al.*, 1996; Weis *et al.*, 1996), it is likely that the C-terminal arm repeat domain of importin α contributes to the direct or indirect interaction with CAS during nuclear export. Since snurportin1 lacks an arm repeat domain, it will be interesting to investigate whether a specialized export factor, distinct from CAS, mediates nuclear export of snurportin1.

At least two distinct import receptors recognize the complex NLS of spliceosomal m_3G-capped U snRNPs

Previously, we showed that nuclear U snRNP import in *Xenopus* oocytes could be inhibited by an excess of either m_3GpppG-cap dinucleotide or U1 snRNPs lacking an m_3G-cap structure (Fischer *et al.*, 1993). While these studies indicated that the two parts of the complex U snRNP NLS, namely the m_3G-cap and the Sm core NLS could be recognized by a transport receptor(s) independent of each other (Fischer *et al.*, 1993), it remained unclear whether one or more distinct import receptors would interact with the two NLS structures. The results described in this report strongly favour the idea that snurportin1 predominantly, if not exclusively, recognizes the m_3G-cap structure of U1 snRNP. For example, similar concentrations of either chemically synthesized $m_3GpppAmpUmpA$ oligonucleotide, naked HeLa U1 snRNA or purified U1 snRNP inhibited with equal efficiency the complex formation of snurportin1 with radiolabeled m_3G-cap oligo (Figure 1). Further, U1 snRNP particles lacking the 5′-terminal m_3G-cap also did not inhibit the cross-linking of m_3G-cap oligo to snurportin1 (Figure 1B). These results indicate that neither additional RNA sequences nor the Sm core proteins enhance the affinity of snurportin1 for the 5′-terminal m_3G-cap structure of U1 snRNA/snRNP. Finally, that snurportin1 does not functionally interact with the Sm core NLS of U snRNPs is strongly supported by our observations that exogenous snurportin1 enhanced significantly the *in vitro* nuclear import of intact U1 snRNPs, but not of U1 snRNPs lacking the 5′-terminal m_3G-cap structure (Figure 6). In summary, we conclude that at least two distinct nuclear import receptors recognize the complex snRNP NLS, snurportin1 binding exclusively the m_3G-cap.

What could be the function of snurportin1 in nuclear snRNP import with respect to the second, Sm core NLS-recognizing, nuclear import receptor? Considering that the m_3G-cap plays a differential role in the nuclear import of distinct snRNAs in *Xenopus* oocytes, and also when comparing oocytes with somatic cells (see Introduction), it is likely that snurportin1 also plays a differential role in a cell type-dependent manner. For example, in *Xenopus* oocytes, snurportin1 appears to be essential for mediating nuclear import of U1 snRNPs. This is indicated by previous studies demonstrating an absolute requirement for the m_3G-cap (Fischer and Lührmann, 1990; Hamm *et al.*, 1990) and by the finding described here that microinjection of a deletion mutant of snurportin1, lacking the IBB domain (Δ1–65 snurportin1) blocks U1 snRNP targeting to the nucleus (Figure 5). Since an import receptor that recognizes the Sm core NLS is also crucial for nuclear targeting of U snRNPs in oocytes (see Fischer *et al.*, 1993), it is conceivable that both import receptors interact simultaneously with a composite snRNP–NLS and mediate, in concert, snRNP nuclear import. Alternatively, the two import receptors could interact sequentially with the two parts of the NLS and possibly contribute differentially to distinct steps of the snRNP import pathway, such as the pore docking step or the actual translocation of the snRNP cargo through the pore.

In somatic cells, such as HeLa cells, the situation clearly differs from that in *Xenopus* oocytes. As shown in Figure 6, and consistent with previous observations (Fischer *et al.*, 1994; Marshallsay and Lührmann, 1994), significant nuclear transport of U1 snRNPs lacking the 5′-terminal m_3G-cap is observed in digitonin-permeabilized cells. This demonstrates that the nuclear import receptor recognizing the Sm core NLS in HeLa cell cytosol has the capacity to target U1 snRNPs to the nucleus autonomously. On the other hand, it is apparent from our data (Figure 6) that the nuclear import of U1 snRNP is significantly more efficient in the presence of a 5′-terminal m_3G-cap, indicating that in somatic cells both import receptors may also cooperate synergistically in the nuclear targeting of U snRNPs. At present we cannot exclude, however, that snurportin1, like the HeLa Sm core NLS-recognizing import receptor, may also target U1 snRNP to the nucleus via an autonomous nuclear import pathway. We are currently investigating this hypothesis in more detail.

In addition to the aforementioned differences in the activity of snRNP nuclear import receptors in *Xenopus* oocytes versus HeLa cells, the relative concentrations of snurportin1 and the Sm core NLS-recognizing factor may also vary in a tissue-specific manner. For example, we

have observed previously that the *in vitro* nuclear import of U1 snRNP is less sensitive towards inhibition by m_3GpppG-cap dinucleotide when carried out in the presence of reticulocyte lysate as opposed to HeLa cell cytosol (Marshallsay and Lührmann, 1994 and data not shown). This suggests that U1 snRNP import in the presence of reticulocyte lysate is predominantly mediated by the Sm core NLS-dependent import pathway. These apparent tissue-specific differences in the concentration and transport activities of snRNP-specific import receptors may also account for the observed differential effects that wheat germ agglutinin (Fischer *et al.*, 1991; Michaud and Goldfarb, 1992; Marshallsay and Lührmann, 1994; Powers *et al.*, 1997) or inhibitors of the Ran GTPase cycle have on nuclear snRNP import in different cellular import systems (Dickmanns *et al.*, 1996; Marshallsay *et al.*, 1996; Palacios *et al.*, 1996). Clearly, the isolation and characterization of the nuclear import receptor recognizing the Sm core NLS is the next important step which is required to clarify these questions.

Our database search revealed that snurportin1 is evolutionarily conserved between *C.elegans*, *Drosophila*, mouse and man, underscoring the important role that snurportin1 presumably plays in nuclear snRNP import in these species. Interestingly, we did not detect any obvious snurportin1 orthologue in the yeast *Saccharomyces cerevisiae*. This could indicate that the nuclear transport of m_3G-capped snRNPs in yeast occurs exclusively via the Sm core NLS receptor pathway. Alternatively, nuclear snRNP import in yeast could be mediated by an m_3G-cap-recognizing factor distinct from snurportin1. In this respect, we note that in our UV cross-linking studies with HeLa cell cytosol, additional proteins, such as the 150 kDa protein, could be cross-linked to the m_3G-cap oligo (see Figure 1). We are currently investigating whether one ore more of these cross-linked proteins also plays a role in the biogenesis of spliceosomal or other cellular m_3G-capped RNPs. On the other hand, it has yet to be demonstrated that the biogenesis of spliceosomal snRNPs in yeast involves a cytoplasmic phase, possibly obviating the requirement for snRNP import receptors.

Specificity of interaction between snurportin1 and m_3G-cap

Aside from its function as an snRNP-specific nuclear import receptor, snurportin1 is equally interesting with respect to the structural requirements which determine its specificity of interaction with the m_3G-cap. Snurportin1 binds m_3G-cap structures by approximately three orders of magnitude more avid than m^7G-caps (see Figure 1), indicating that the two additional methyl groups at the N-2 amino group of the 5′-terminal guanosine base in the m_3G-cap primarily account for the discrimination by snurportin1 between the two cap structures. We note that, compared with the m_3GpppG-cap dinucleotide, the m_3G-cap oligo (m_3GpppAmpUmpA) has an increased strength of interaction with snurportin1 (approximately by an order of magnitude; Figure 1). It remains to be seen whether this effect is due to the 3′-terminal extensions of the m_3G-cap oligo or to direct contacts between amino acid residues of the cap-binding domain of snurportin1 and the 2′-*O*-methyl groups.

Snurportin1 has to distinguish well not only between m_3G- and m^7G-caps but also between 2,2-dimethylguanosine nucleotides which are present in cellular RNA molecules such as tRNAs (Limbach *et al.*, 1994). We have experimental evidence that methylation of the N-7 position in the 5′-terminal guanosine of the m_3G-cap is of utmost importance in this respect. This is indicated by our finding that 2,2,7-trimethyl- but not 2,2-dimethylguanosine triphosphate inhibits the UV cross-link between snurportin1 and the m_3G-cap oligo (data not shown). Importantly, the alkylated N-7 group also primarily accounts for the capacity of the eukaryotic translational initiation factor 4E (eIF4E) to discriminate between m^7G-caps and unmethylated guanosine nucleotides (for review see Sonenberg, 1996). Recently, the crystal structure of murine eIF4E, complexed to 7-methyl GDP has been solved (Marcotrigiano *et al.*, 1997), which revealed that 7-methylguanosine base recognition is mediated primarily by base sandwiching between two conserved tryptophan residues. This mode of aromatic ring interaction involves enhanced π-stacking interactions between the electron-deficient 7-methylguanosine and the electron-rich tryptophan groups (see Marcotrigiano *et al.*, 1997; Ishida *et al.*, 1988, for discussion). A very similar sandwiching of the 7-methyl-guanosine base of an mRNA cap by the side chains of a phenylalanine and tyrosine residue, was observed in a co-crystal of the vaccinia m^7G-cap-specific RNA 2′-*O*-methyltransferase VP39 (Hodel *et al.*, 1997). In view of these results, it is therefore tempting to speculate that a sandwiching of the m_3G-base by aromatic side chain residues may be one important mechanism of base recognition by snurportin1. Sequence alignments of the snurportin1 m_3G-cap-binding domain (residues 87–347) with the m^7G-cap-binding regions of murine eIF4E (residues 31–209) and the vaccinia VP39 protein (residues 87–331) reveal that both are, at best, moderately homologous to snurportin1 (~20% identity and 48% similarity, data not shown). Thus, whether the m_3G-cap-binding domain exhibits significant structural similarity with either of these proteins is an open question. We note, however, that sequences similar to those surrounding the two eIF4E-conserved tryptophans (positions 107–128 and 174–212 in snurportin1) could be detected in snurportin1 which are also evolutionarily conserved (data not shown). Future biochemical and X-ray crystallography studies, should reveal how snurportin1 discriminates between mRNA and U snRNA cap structures.

Materials and methods

All enzymes used for DNA manipulations were purchased from New England Biolabs. T7 RNA polymerase and RNasin were from Promega. Pfu polymerase was obtained from Stratagene and RNase H from Boehringer Mannheim. The cap analogues ApppG and m^7GpppG were purchased from Pharmacia. m_3GpppG was synthesized and purified as described previously (Iwase *et al.*, 1989). Radiolabeled nucleotide triphosphates and [^{32}P]pCp were from Amersham. Sequences were determined with an automated DNA sequencer (Applied Biosystems) using Taq polymerase and doubled-stranded templates (PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit, Pharmacia).

Preparation of snRNPs and snRNAs

Nuclear extracts were prepared from HeLa cells (Computer Cell Culture Center, Mons) as described by Dignam *et al.* (1983). Native U1 and U5 snRNPs were isolated by affinity chromatography with monoclonal anti-cap antibody (mAb) H20, covalently attached to CnBr-activated Sepharose 4B (Bochnig *et al.*, 1987), followed by Mono Q chromato-

graphy (Bach *et al.*, 1990). For competition studies and RNase H digestion, purified U1 snRNPs were concentrated to 12 μg/μl by centrifugation at 160 000 *g* for 2.5 h at 4°C. To remove the 5′ end of U1 snRNA, U1 snRNPs (60 μg) were incubated with 10 U RNase H and a DNA oligonucleotide (5′-CAGGTAAGTAT-3′, final concentration 1.4 μg/μl) in a total volume of 50 μl as described by Lamond and Sproat (1994). Residual amounts of m_3G-capped U1 snRNPs were removed from the reaction mixture by immunoprecipitation with 25 μl mAb H20–Sepharose beads in a final volume of 100 μl phosphate-buffered saline (PBS), pH 8. After a 2 h end-over-end incubation at 4°C, the sample was briefly centrifuged and the Δ5′ U1 snRNPs in the supernatant were concentrated to 30 μg/μl using a Microcon-100 concentrator (Amicon). The purity and integrity of particles was confirmed by SDS–PAGE and sedimentation analysis on 5–20% glycerol gradients containing PBS, pH 8, in a Beckmann TLS-55 rotor as described by Marshallsay and Lührmann (1994). U1, Δ5′ U1 and U5 snRNA were isolated as described by Sumpter *et al.* (1992).

UV cross-linking studies

An m_3G-cap oligonucleotide ($m_3GpppAmpUmpA$), identical to the 5′ end of HeLa U1 snRNA, was synthesized as described previously (Sekine *et al.*, 1994, 1996). Preparative [^{32}P]pCp labeling of the m_3G-cap oligo (5 μg) was carried out as described by Fischer *et al.* (1993) except that the amount of [^{32}P]pCp was increased to 250 μCi. After phenol extraction and ethanol precipitation, radiolabeled m_3G-cap oligo was purified on 20% polyacrylamide gels containing 7.5 M urea. For the identification of m_3G-cap-binding proteins in HeLa cell cytosolic extracts by UV-cross-linking, 1 pmol of [^{32}P]pCp 3′ end-labeled m_3G-cap oligo (2.5×10^6 c.p.m./pmol) was incubated for 10 min on ice with either 25 μg S100 cytosolic extract or 1.5 μg of purified HeLa or recombinant snurportin1 (in a total volume of 10 μl). Reaction mixtures were irradiated at 254 nm with a Sylvania G8T5 germicidal UV lamp for 5 min at a distance of 2 cm. Cross-linked proteins were separated by SDS–PAGE and visualized by autoradiography.

Purification of the 45 kDa m_3G-cap-binding protein

HeLa S100 extract, prepared as described by Dignam *et al.* (1983), was pre-fractionated by a passing 960 ml (~3.5 mg/ml) over a 240 ml CM-Sepharose-FF column (Pharmacia) equilibrated in buffer D (containing 25 mM HEPES–KOH pH 7.9, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.25 mM EDTA, 8.7% glycerol, 2 mM DTT, 1 mM PMSF, 0.1 mM benzamidine and 10 μg/ml bacitracin). The flow-through, which contained the 45 kDa m_3G-cap-binding protein (as determined by UV-cross-linking), was loaded directly onto a 240 ml Q-Sepharose-FF column (Pharmacia) equilibrated in buffer D. The Q-Sepharose column was washed with 2 l of buffer D, and bound proteins were eluted with 900 ml of a linear 100 to 750 mM NaCl gradient in buffer D. Aliquots (0.5 ml) were dialysed for 4 h at 4°C against buffer D and tested for m_3G-cap-binding activity using the UV cross-link assay. Most of the activity was eluted in fractions containing 170–280 mM NaCl. These fractions were pooled (210 ml, 627 mg of protein), diluted to 100 mM NaCl in buffer D, and a 70 ml aliquot (~1.6 mg/ml) was loaded onto a 1 ml m_3G-cap affinity column (prepared as described below). The column matrix was washed with 10 column volumes of buffer D and elution was performed stepwise with 2 ml of 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 1 and 1.5 M NaCl in buffer D. A 0.5 ml aliquot of each fraction was dialysed against buffer D and concentrated to 30 μl using Microcon-10 concentrators (Amicon), and m_3G-cap-binding activity was assayed using the UV cross-link assay. The final yield of the 45 kDa protein was 0.36 mg, which corresponded to 0.01% of the total starting protein.

Preparation of m_3G-cap affinity matrix

For affinity purification of the m_3G-cap-binding protein a biotinylated m_3G-cap oligo [$m_3GpppAmpUmpAp$-$(CH_2)_6$-biotin] was chemically synthesized. A detailed description of the protocol will be presented elsewhere (M.Sekine, M.Kadokura and T.Wada, unpublished data). Coupling of biotinylated m_3G-cap oligo to streptavidin–agarose (Sigma) was performed according to Lamond and Sproat (1994). 50 nmol of biotinylated m_3G-cap oligo were coupled to 1 ml preblocked streptavidin–agarose (see Lamond and Sproat, 1994) for 18 h at 4°C in an equal volume of binding buffer (25 mM HEPES–KOH pH 7.9, 500 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol). The beads were washed with 5 vols of buffer D prior to use.

Microsequencing, cDNA cloning and expression of snurportin1

Microsequencing of snurportin1 was carried out by Toplab (Munich). In short, purified snurportin1 was first digested with endoproteinase Lys-C. Peptides were then separated by HPLC, and the amino acid sequence of several peaks was determined by microsequencing on an ABI 477A protein sequencer. The following peptide sequences, which matched to three overlapping ESTs deposited in the ATCC (DDBJ/EMBL/GenBank accession numbers H43467, H08432, R14245), were obtained: (a) KYSSLEQSERRRRLLELQK, (b) KRLDYVNHARRLAEDD, (c) KRLAIVASRGSTSAYTK, (d) KLPEEEGLGEK, (e) KLTHK. As determined by DNA sequencing, clone R14245 contained a 1.6 kb insert with an ORF containing all five snurportin1 peptide sequences. For expression of his-tagged snurportin1 and the N-terminal deletion mutant (Δ1–65 snurportin1), either the complete coding sequence of snurportin1 or a fragment coding for amino acids 66–360 was amplified by PCR from a Bluescript plasmid containing the full-length snurportin1 cDNA (pBS/spn1) and cloned into the *Nco*I–*Bam*HI sites of pET28b (Novagen). The resulting plasmids, pET28b/spn1 and pET28b/Δ1–65spn1, were transformed into *Escherichia coli* strain BL21[LysS], which was grown to an absorbance at 600 nm (A_{600}) of 0.8, and induced with isopropyl-β-D-thiogalactopyranoside for 4 h at 30°C. Cells from a 2-l culture were lysed by sonication for 1 min on ice in resuspension buffer (25 mM HEPES–KOH pH 7.9, 100 mM NaCl, 1 mM PMSF, 20 μg/ml leupeptin, 0.1 mM benzamidine and 10 μg/ml bacitracin, 5 mM imidazole and 10 mM β-mercaptoethanol). After clearing the solution by centrifugation for 45 min at 20 000 *g* and 120 min at 100 000 *g*, the supernatant was applied to a 3.5 ml nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen). Bound proteins were eluted with resuspension buffer containing 200 mM imidazole and 8.7% glycerol. For further purification, proteins were dialysed for 2 h at 4°C against buffer D and subjected to m_3G-cap affinity chromatography, essentially as described above. The following primers were used for PCR amplification: (i) pET28b/spn1-for [5′-GGGCCATGGAAGAGTTGAGTCAGGCCCTG-3′]; (ii) pET28b/Δ1–65/spn1-for [5′-GGGCCATGGCTGAAGATGACTG GACAGGGATG-3′]; (iii) pET28b/spn1-rev and pET28b/Δ1–65/spn1-rev [5′-TTTGGAT-CCCCATTCTCCATGAGGCATCCAGGGTG-3′]. All PCR-derived constructs were verified by sequencing. The expression and purification of hSRP1α and *Xenopus* importin α have been described previously (Görlich *et al.*, 1994; Weis *et al.*, 1995).

In vitro translation and protein binding assays

Importin β was produced in rabbit reticulocyte lysate by *in vitro* transcription–translation of the plasmid pKW275 (Weis *et al.*, 1996) using a TnT kit (Promega) according to the manufacturer's instructions. Binding of snurportin1, hSRP1α or importin α to importin β using Ni-NTA–agarose beads was performed exactly as described by Weis *et al.* (1996).

Procedures for labeling RNA and U snRNPs

[^{32}P]pCp labeling of gel-purified HeLa U1 and U5 snRNAs was performed as described by Fischer *et al.* (1993). *In vitro* transcription of [^{32}P]-labeled ApppG U6 snRNA was carried out exactly as described by Fischer *et al.* (1991).

For *in vitro* import assays, isolated U1 snRNPs or Δ5′ U1 snRNPs were fluorescently labeled with Cy3 monofunctional reactive dye (Amersham) according to the manufacturer's protocol. Unreacted dye was removed by repeated filtration through Microcon-100 units (Amicon) and subsequent dilution with PBS (pH 8) until the flow-through was free of Cy3 dye. Sedimentation analysis of fluorescently labeled snRNPs was performed as described above.

Oocyte injections

Microinjection was performed as described by Fischer *et al.* (1993) except OR-2 buffer (Wallace *et al.*, 1973) was used instead of MBS buffer. After incubation at 18°C for the indicated times, the oocytes were dissected manually after transferring into J-buffer (70 mM NH_4Cl, 7 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM DTT, 20 mM Tris–HCl pH 7.5, 10% glycerol). RNA was purified and analysed as previously described (Fischer *et al.*, 1993). Gels were quantified using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager system with Image Quant software, version 3.0.

Nuclear import assay

Nuclear import reactions were performed with HeLa cells grown on glass coverslips to 50–70% confluency in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco-BRL) at 37°C, 5% CO_2. After digitonin permeabilization (Adams *et al.*, 1990) cells were washed with ice-cold import buffer (25 mM HEPES pH 7.9, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.25 mM EDTA) and 25 μl of import buffer containing 0.2 mg/ml tRNA,

1 mM ATP, 1 mM creatine phosphate, 20 U/ml creatine phosphokinase (Sigma), 4 μg/ml U1 snRNP*s or Δ5′ U1 snRNP*s (labeled as described above), and 10 μl HeLa S100 cytosolic extract (5 mg/ml) was applied to the cells. Additional reagents were added as indicated in the figure legends. The import mix was depleted of ATP by omitting ATP, creatine phosphate and creatine phosphokinase, and preincubating for 30 min at 25°C in the presence of 20 U/ml apyrase (Sigma). Incubation of the import reactions was carried out at 25°C for 30 min and terminated as described by Marshallsay and Lührmann (1994). After mounting the coverslips with Fluoprep (bioMerieux), samples were visualized using the 50× objective of a Leica DM/IRB inverted fluorescence microscope and digitalized images were taken with a CCD camera. Images were processed by Adobe Photoshop version 3.0 and quantified using the NIH image software, version 1.6. For each sample, the mean fluorescence of ~100 randomly chosen nuclei from at least three independent assays was averaged.

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