

Nuclear import of U snRNPs requires importin β

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Macromolecules that are imported into the nucleus can be divided into classes according to their nuclear import signals. The best characterized class consists of proteins which carry a basic nuclear localization signal (NLS), whose transport requires the importin α/β heterodimer. U snRNP import depends on both the trimethylguanosine cap of the snRNA and a signal formed when the Sm core proteins bind the RNA. Here, factor requirements for U snRNP nuclear import are studied using an *in vitro* system. Depletion of importin α , the importin subunit that binds the NLS, is found to stimulate rather than inhibit U snRNP import. This stimulation is shown to be due to a common requirement for importin β in both U snRNP and NLS protein import. Saturation of importin β -mediated transport with the importin β -binding domain of importin α blocks U snRNP import both *in vitro* and *in vivo*. Immunodepletion of importin β inhibits both NLS-mediated and U snRNP import. While the former requires re-addition of both importin α and importin β , re-addition of importin β alone to immunodepleted extracts was sufficient to restore efficient U snRNP import. Thus importin β is required for U snRNP import, and it functions in this process without the NLS-specific importin α .

Keywords: importin/nuclear import/nucleocytoplasmic transport/RNP transport/U snRNPs

Introduction

Macromolecular transport between the nucleus and cytoplasm is an energy- and signal-dependent process (Newmeyer, 1993; Görlich and Mattaj, 1996; Nigg, 1997). The first described and still best characterized type of nuclear import signal is the family of related short basic sequences called the nuclear localization signal or NLS (Dingwall and Laskey, 1991). Although there have been regular reports of proteins whose targeting signals do not conform to the NLS paradigm (e.g. Silver *et al.*, 1988; Mandell and Feldherr, 1990; Kambach and Mattaj, 1992), it was argued that these non-conforming signals might represent binding domains on these proteins that enabled them to interact with NLS-containing proteins and thus enter the nucleus by a piggy-back mechanism. Initially,

the only imported macromolecules for which this argument clearly did not apply were the U snRNPs.

U snRNPs are a family of nuclear ribonucleoproteins that function in diverse aspects of RNA metabolism in the nucleus. They are required mainly for various forms of RNA processing like splicing, the removal of introns from mRNA precursors (Mattaj *et al.*, 1993; Tarn and Steitz, 1997). Although not all U snRNPs assemble in the same way (Vankan *et al.*, 1990; Terns and Dahlberg, 1994), the formation of many, including the U1, U2, U4 and U5 splicing snRNPs, involves passage of the RNAs through the cytoplasm. The primary transcripts of these RNAs are made in the nucleus by RNA polymerase II, and they therefore obtain a 7-methylguanosine cap structure. The monomethyl cap, and the nuclear cap-binding complex (CBC) which binds to this cap, are requirements for efficient nuclear export of the primary U snRNA transcripts (Hamm and Mattaj, 1990; Izaurralde *et al.*, 1992, 1995; Terns *et al.*, 1993). On reaching the cytoplasm, these RNAs bind to the eight common or Sm core snRNP proteins. In a manner that depends on this RNP assembly step, the monomethyl cap is hypermethylated to form the mature U snRNP trimethylguanosine cap structure (reviewed by Mattaj, 1988; Lührmann *et al.*, 1990; see also Plessel *et al.*, 1994). Both the trimethyl cap and the core snRNP structure form the bipartite U snRNP nuclear import signal (Fischer and Lührmann, 1990; Hamm *et al.*, 1990; Fischer *et al.*, 1991, 1993).

In initial experiments to determine whether NLS-dependent protein import into the nucleus was receptor-mediated, it was found that this import could be competitively inhibited by cytoplasmic microinjection of saturating quantities of NLS peptides cross-linked to a neutral carrier protein, like serum albumin (Goldfarb *et al.*, 1986). However, the same conjugates did not affect the import of U snRNPs (Michaud and Goldfarb, 1991). Conversely, saturating quantities of either a trimethylguanosine cap dinucleotide analogue or of assembled snRNP cores competitively inhibited U snRNP import, but did not affect NLS protein import (Fischer *et al.*, 1991, 1993; Michaud and Goldfarb, 1991, 1992). These results were interpreted as evidence that at least some of the factors which mediate NLS protein and U snRNP import were different.

There has been considerable recent progress in identifying proteins required for the import of macromolecules into the nucleus. First, the NLS receptor, an NLS-binding protein with a role in NLS protein import, was characterized biochemically (Adam *et al.*, 1989; Adam and Gerace, 1991) and proposed to act as a heterodimer with a second protein, p97 (Adam and Adam, 1994). Shortly thereafter, both the NLS-binding protein (Görlich *et al.*, 1994; Imamoto *et al.*, 1995a; Moroianu *et al.*, 1995; Weis *et al.*, 1995) and the second subunit (Chi *et al.*, 1995; Görlich *et al.*, 1995a; Imamoto *et al.*, 1995b; Radu *et al.*, 1995)

were cloned from vertebrates. The subunits are now commonly called importin α and β or karyopherin α and β (for alternative names and review, see Görlich and Mattaj, 1996; Nigg, 1997). Together with the Ran GTPase and a fourth protein, NTF2/p10/pp15, the two importin subunits are sufficient for NLS-mediated protein import in an *in vitro* system (reviewed by Powers and Forbes, 1994; Adam, 1995; Görlich and Mattaj, 1996; Koepp and Silver, 1996; Nigg, 1997). Importin α binds to the NLS while importin β interacts with components of the nuclear pore complex (NPC) and with Ran (see reviews cited above). Importin α binds to β through a basic N-terminal region called the IBB (importin β -binding) domain (Görlich *et al.*, 1996a; Weis *et al.*, 1996). The IBB domain, when fused to a heterologous protein, targets the fusion to the nucleus. Thus, the only function of importin α essential for its import into the nucleus is binding to β (Görlich *et al.*, 1996a; Weis *et al.*, 1996).

Study of other proteins, particularly of the hnRNP A1 and K proteins, had meanwhile revealed additional classes of nuclear protein import signals (Michael *et al.*, 1995, 1997; Siomi and Dreyfuss, 1995; Weighardt *et al.*, 1995) which do not require importin. HnRNP A1 import depends on the M9 signal and is mediated by transportin (Aitchison *et al.*, 1996; Nakielny *et al.*, 1996; Pollard *et al.*, 1996; Fridell *et al.*, 1997). Unlike importin β , transportin does not require an importin α -like subunit, but rather binds directly to its substrate through the M9 domain (Pollard *et al.*, 1996). Saturation of the transportin import pathway blocks M9-mediated import, but has no effect on the nuclear uptake of either NLS proteins or U snRNPs (Pollard *et al.*, 1996; Izaurralde *et al.*, 1997a). Transportin exhibits some similarity to importin β , and is one of a reasonably large family of β -related proteins (Fornerod *et al.*, 1997; Görlich *et al.*, 1997). Roles for two additional members of this family in the import of ribosomal proteins have been proposed recently (Rout *et al.*, 1997). While there is also a family of importin α -related proteins, those characterized thus far can all bind to, and mediate the import of, NLS proteins (Adam *et al.*, 1995, reviewed by Görlich and Mattaj, 1996).

On the basis of the kinetic competition studies summarized above, importin α and transportin are not likely to play a role in U snRNP transport. However, little positive is known about factors that are required for snRNP import. Making use of an *in vitro* assay (Marshallsay and Lührmann, 1994), we have reported previously that U snRNP import requires Ran (Palacios *et al.*, 1996), in common with importin- and transportin-mediated transport processes (Koepp and Silver, 1996; Nakielny *et al.*, 1996; Izaurralde *et al.*, 1997b). Here we present evidence to confirm that importin α is not required for U snRNP import *in vitro*. Surprisingly however, a variety of experimental approaches led to the conclusion that importin β itself, rather than another member of the importin β family, is required to mediate U snRNP import.

Results

Importin α depletion stimulates U snRNP import

It seemed unlikely that importin α would be required for the nuclear uptake of U snRNPs since amounts of NLS peptide sufficient to saturate NLS protein import did not

affect U snRNP import *in vivo* (Michaud and Goldfarb, 1991) and only caused a moderate (34%) decrease of U snRNP import *in vitro* (Palacios *et al.*, 1996). To confirm this, we made use of an antibody raised against an N-terminal peptide from *Xenopus* importin α that binds to part of the IBB domain and disrupts binding between importin α and β (Görlich *et al.*, 1995a). As would be expected from this, the antibody efficiently blocks NLS protein import. Addition of the antibody to an import assay for fluorescently labelled NLS protein or U snRNP substrates that uses permeabilized HeLa cells and *Xenopus* egg extracts (Palacios *et al.*, 1996) abolished uptake of fluorescently labelled bovine serum albumin (BSA)-NLS peptide conjugates (Figure 1A, upper panels). In contrast, when the antibody was pre-blocked with peptide before addition, no inhibition was seen. Pre-blocked antibody also had no effect on U snRNP import but, unexpectedly, addition of unliganded antibody reproducibly increased their import to ~150% (Figure 1A, lower panels and data not shown). This effect seemed specific as it was reversed by pre-blocking the antibody with antigenic peptide.

To confirm this result, the effect of removing importin α from the extract was tested. Passing the *Xenopus* egg extract over an NLS affinity column (Görlich *et al.*, 1995a; see Materials and methods) resulted in significant, but incomplete, depletion of importin α from the extract (Figure 1C). As expected, importin β was also partially removed from the extract by this treatment, but less efficiently than importin α (Figure 1C). This depletion strongly reduced import of the BSA-NLS conjugate, and the inhibitory effect was reversed by the addition of recombinant importin α protein (Figure 1B, upper panels). In contrast, U snRNP import was stimulated by importin α depletion, and this stimulation was reversed by re-addition of recombinant importin α (Figure 1B, lower panels). These results confirm that importin α is not required for U snRNP import. The stimulation of U snRNP import observed on removal of α from the extracts mirrors the moderate inhibitory effect of NLS conjugates on U snRNP import seen *in vitro* (Palacios *et al.*, 1996) and suggests that some component of the NLS protein import pathway other than importin α was involved in U snRNP import.

The pattern of accumulation of imported U snRNPs and NLS conjugates in nuclei is different. This reflects the localization of the former in both the nucleoplasm, where they distribute reasonably uniformly, and sites of concentration which co-localize with coiled bodies (I. Palacios, unpublished data), and the tendency of NLS conjugates to bind to nucleoli and thus to be most concentrated there.

The IBB domain inhibits U snRNP import

The IBB domain of importin α effectively competes with full-length α for binding to β and thus acts as a competitive inhibitor of NLS protein import (Görlich *et al.*, 1996a; Weis *et al.*, 1996). Since importin β acts together with importin α in NLS protein import, use of the IBB domain provided a first test of whether importin β might be required for U snRNP import. Addition of a full-length IBB domain fusion protein (IBB 1-65; Weis *et al.*, 1996) to the *in vitro* import assay had a similar inhibitory effect on both NLS protein and U snRNP import, while addition

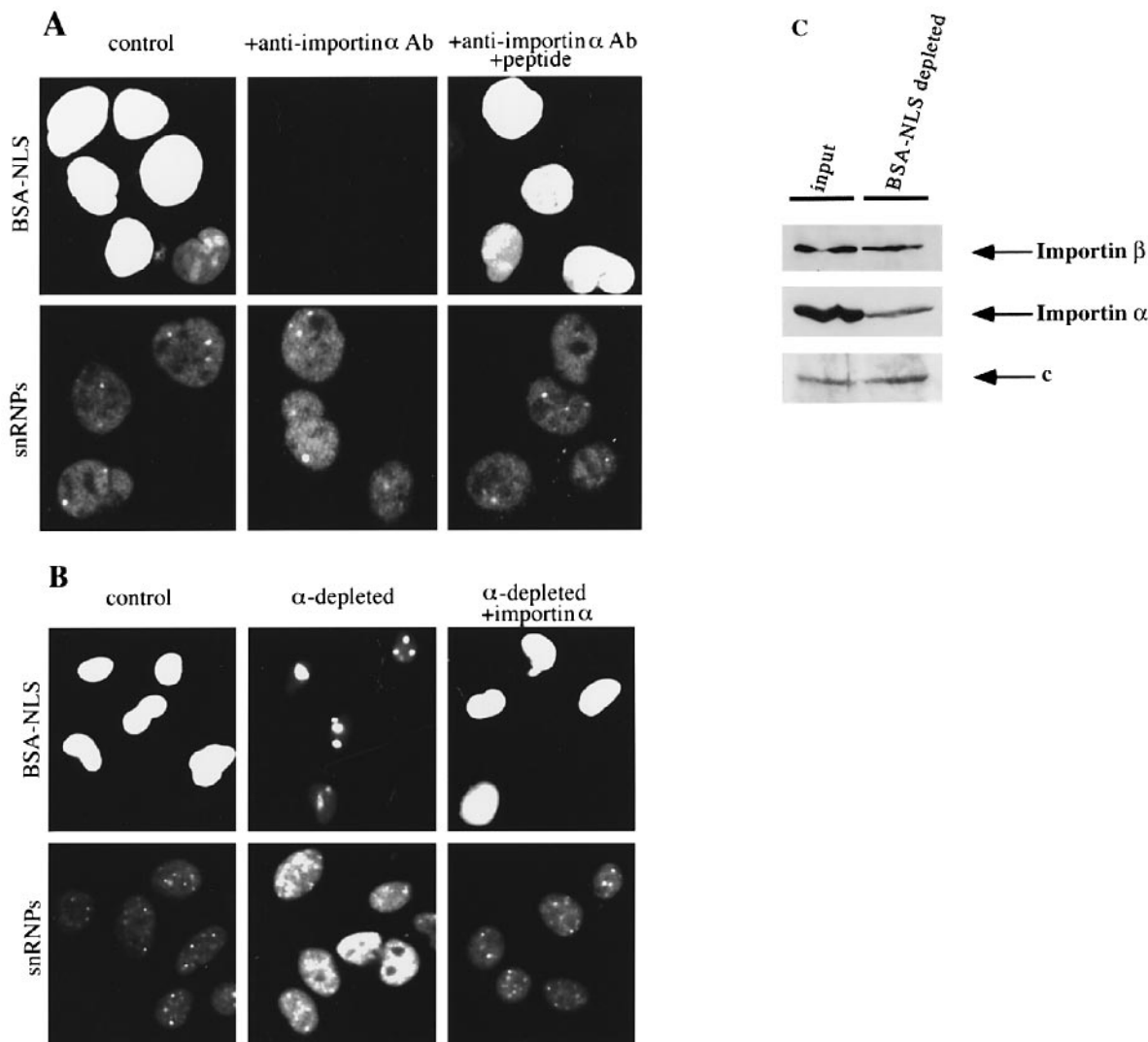


Fig. 1. Importin α is not required for U snRNP import. (A) *In vitro* import of U snRNPs is stimulated by an antibody against importin α . Digitonin-permeabilized HeLa cells were incubated with fluorescently labelled BSA-NLS (BSA-NLS) for 20 min or purified fluorescently labelled U snRNPs (snRNPs) for 120 min in the presence of *Xenopus* egg extracts, GTP, ATP and an ATP regeneration system (see Materials and methods). Transport reactions were performed in the absence (control) or presence of an antibody against the N-terminus of importin α (+anti-importin α Ab). As a control, the antigenic peptide of importin α was used to pre-block the antibody (+anti-importin α Ab + peptide). (B) Extracts depleted of importin α show increased activity in U snRNP import. *Xenopus* egg extracts were incubated with a BSA-NLS column and the import of BSA-NLS and U snRNPs was assayed in the unbound fraction. *In vitro* import of fluorescent BSA-NLS (BSA-NLS) or U snRNPs (snRNPs) was performed for 20 or 120 min respectively, in either control extract or in the unbound extracts without (α -depleted) or with addition of recombinant importin α . Importin α was added to a final concentration of 0.58 μ M. (C) The *Xenopus* egg control extract (input) or the unbound fraction from the BSA-NLS column (BSA-NLS-depleted) were separated by SDS-PAGE, transferred onto nitrocellulose and the presence of importin α and importin β was monitored by Western blotting with antibodies specific for each protein. A spurious cross-reacting band (C) was used as a loading control.

of a truncated, inactive IBB domain mutant had no inhibitory effect (Figure 2A; Table I).

This result was surprising initially because of the modest effects of NLS conjugates on U snRNP uptake discussed above. To demonstrate that the result was not an artefact of the *in vitro* system, we repeated the experiment in *Xenopus* oocytes, where IBB domain injection previously has been shown to block specifically import of NLS proteins but not of, for example, an M9-containing protein (Görlich *et al.*, 1996b; Izaurralde *et al.*, 1997a).

Oocytes were injected into the cytoplasm with either the truncated or full-length IBB domain fusion proteins. This treatment blocked NLS protein import (data not shown). A mixture of U1 and U5 snRNAs was injected into the oocyte cytoplasm and their transport was monitored by

extracting the RNAs from total oocytes (T) or from cytoplasmic (C) or nuclear (N) fractions. As expected, both RNAs accumulated in the nucleus of oocytes treated with the truncated IBB domain (Figure 2B, compare lanes 1–3 with lanes 4–6). However, in oocytes injected with the full-length IBB domain, U snRNP import was reduced drastically (Figure 2B, lanes 7–9). This suggested that either importin β or another factor that could bind the IBB domain was required for U snRNP import.

Importin β mutants and U snRNP import

Ran binds to the N-terminal region of importin β , whereas binding of the IBB domain requires roughly the C-terminal half of importin β (Chi *et al.*, 1996; Görlich *et al.*, 1996c; Chi and Adam, 1997; Kutay *et al.*, 1997). The effect of

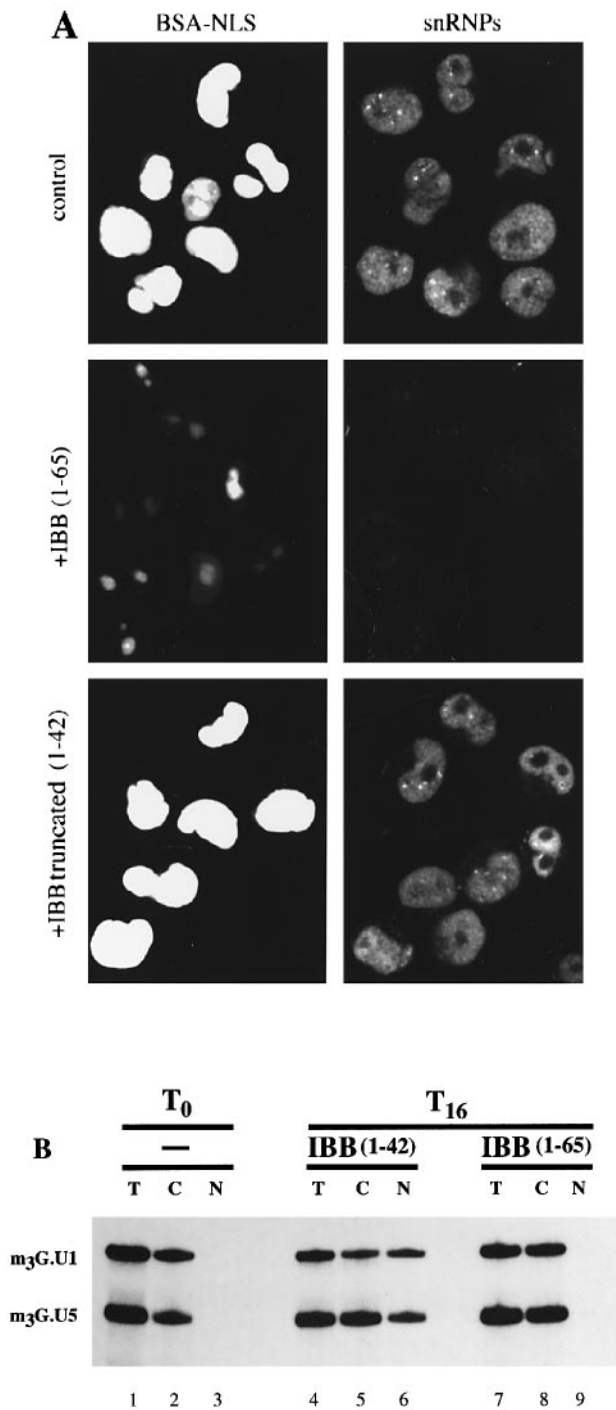


Fig. 2. *In vitro* and *in vivo* import of U snRNPs is inhibited by the importin β -binding (IBB) domain of importin α . (A) *In vitro* transport assays of fluorescent BSA-NLS (BSA-NLS) or U snRNPs (snRNPs) were performed in *Xenopus* egg extracts as described in Figure 1. Import reactions were carried out in the absence (control) or presence of 12 μ M full-length IBB (+IBB 1-65) or truncated IBB (+IBB 1-42). (B) A mixture of *in vitro* transcribed U1 and U5 snRNAs was injected into the cytoplasm of *Xenopus* oocytes that were pre-injected 1 h before with either full-length (+IBB 1-65) or truncated IBB (+IBB truncated 1-42). In lanes 1-3, RNA was extracted immediately after U snRNA injection (T₀); in lanes 4-9, RNA was extracted 16 h after the U snRNA injection (T₁₆). 'T', 'C' and 'N' indicate RNA extracted from total oocytes, or, after dissection, from cytoplasmic and nuclear fractions respectively. The final concentration of the IBB domains was 28 μ M.

Table I. Quantitation of the *in vitro* nuclear uptake of BSA-NLS and U snRNPs in the presence of full-length or truncated IBB domain

	Control	+ IBB (1-65)	+ IBB (1-42)
BSA-NLS	100 \pm 5.7	22 \pm 5.4	102 \pm 13.0
snRNPs	100 \pm 5.01	34 \pm 0.8	125 \pm 8.9

The nuclear uptake of BSA-NLS and U snRNPs in the absence (control) or presence of full-length IBB (+IBB 1-65) or truncated IBB (+IBB 1-42) was quantified. The value for both karyophiles in the 120 min control import reaction was taken as 100% import. The values obtained in the import reactions in the presence of full-length and truncated IBB (both at 12 μ M) are relative to the control. The table shows the average figures for >50 cells. The errors are standard deviations.

deletion mutants with an inactivating mutation in one or other of these binding domains on U snRNP import was tested. First, an importin β mutant (amino acids 45-876) that cannot bind RanGTP and acts as a dominant-negative inhibitor of NLS protein translocation through the NPC, but which does not prevent the docking step of import (Figure 3A, see Görlich *et al.*, 1996c; Kutay *et al.*, 1997), was tested. Since importin β mutants unable to bind Ran block all tested import and export pathways (Kutay *et al.*, 1997), and since U snRNP import in egg extracts requires RanGTPase (Palacios *et al.*, 1996), we expected this β mutant also to prevent U snRNP import, and this was indeed the case (Figure 3A, bottom panels). As previously reported (Palacios *et al.*, 1996), no stage corresponding to docking is seen with U snRNP substrates (Figure 3A).

Next, we used the least drastic C-terminal deletion mutant of importin β that prevents importin α (IBB) binding, importin β 1-771 (Kutay *et al.*, 1997). As an import substrate in this experiment, a fluorescently labelled IBB- β Gal fusion protein (Weis *et al.*, 1996) was used. Although this importin β mutant is a competitive rather than a dominant inhibitor of import than the N-terminal deletion mutant (Kutay *et al.*, 1997), it nevertheless caused strong inhibition of both protein and U snRNP import (Figure 3B). This result again suggested that importin β could be required for both NLS protein and U snRNP import, and that the requirement for importin β sequences is similar to that needed for importin α -mediated import.

Depletion of importin β inhibits U snRNP import

To obtain more direct evidence for a role for importin β in U snRNP import, we made use of monoclonal antibody (mAb) 3E9, raised against bovine importin β (Chi *et al.*, 1995). Depletion of the cytosolic extracts used in a mammalian *in vitro* nuclear import assay with mAb3E9 blocked NLS protein import (Chi *et al.*, 1995). MAb3E9 was therefore used to immunodeplete egg extract (Figure 4). Depletion of importin β was extensive but not complete, and importin α was significantly co-depleted in this experiment (Figure 4), although we found that the efficiency of importin α co-depletion varied (data not shown).

When NLS protein import was tested in these extracts, depletion with mAb3E9 had a strong inhibitory effect (Figure 5A). This effect was not reversed by addition of recombinant importin β alone, but import was restored by a mixture of importin α and β (Figure 5A), as expected. Addition of too large an excess of importin β inhibited

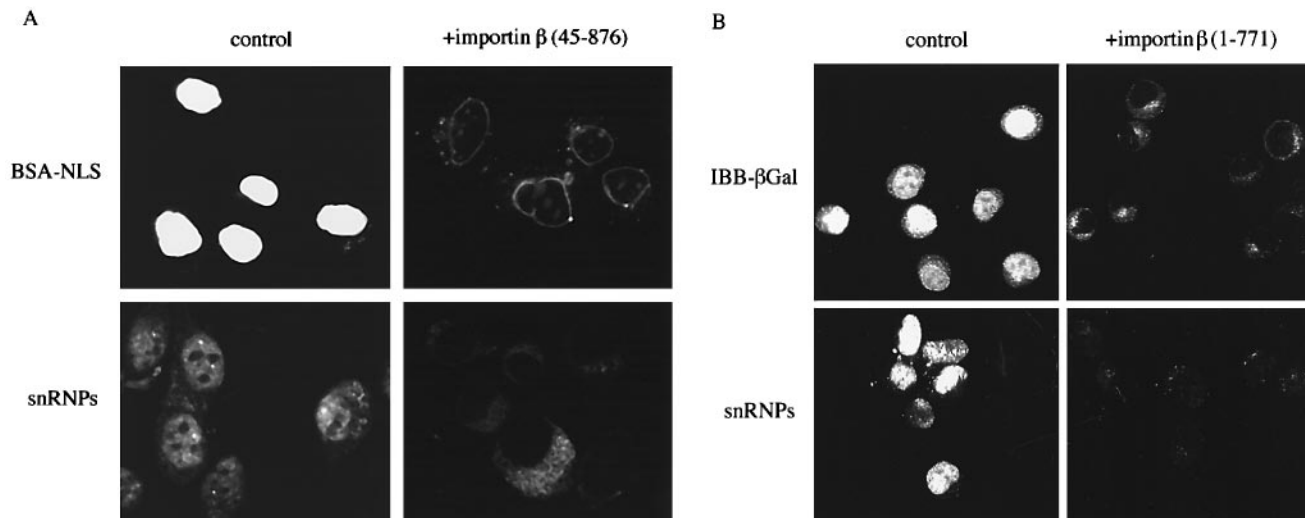


Fig. 3. Importin β function in U snRNP import requires both the N- and the C-termini of the protein. (A) *In vitro* import of U snRNPs is inhibited by a mutant of importin β that does not bind Ran, importin β (45–876). *In vitro* nuclear import reactions of fluorescently labelled BSA–NLS (BSA–NLS) or U snRNPs (snRNPs) were performed for 30 or 120 min respectively with *Xenopus* egg extracts in the absence (control) or presence of a mutant of importin β (45–876) at 1 μ M final concentration. (B) *In vitro* import of U snRNPs is inhibited by a mutant of importin β that cannot bind the IBB domain, importin β (1–771). *In vitro* nuclear import reactions of β Gal fused to the IBB domain (IBB– β Gal) or U snRNPs (snRNPs), both fluorescently labelled, were performed for 120 min with *Xenopus* egg extracts in the presence of wild-type importin β (control) or importin β (1–771), both at 1 μ M final concentration, as indicated.

both BSA–NLS and U snRNP import in both control and depleted extracts. Therefore, in all cases, an amount of importin β that resulted in maximal import levels is shown. Mock depletion of extracts and addition of recombinant importin β to complete or mock-depleted extracts did not greatly affect U snRNP import (Figure 5B). U snRNP import was, however, significantly reduced by mAb3E9 depletion (Figure 5C). In contrast to NLS protein import, addition of recombinant importin β alone could completely reverse the inhibitory effect (Figure 5C). These experiments provide direct evidence that importin β is required for the nuclear import of U snRNPs *in vitro*.

The IBB domain inhibits m_3G -independent U5 snRNP import

U snRNP import signals are bipartite, consisting of the trimethyl cap and the snRNP core structure. In *Xenopus* oocytes, the import of U1, U2, U4 and U5 snRNPs depends on the snRNP core. In contrast, while U1 and U2 snRNP import is very dependent on the trimethyl cap, U5 import is not greatly affected by its absence and U4 snRNP shows an intermediate behaviour (Fischer *et al.*, 1991; Jarmolowski and Mattaj, 1993). Since the bulk of the snRNPs present in our fluorescently labelled preparations consists of U1 snRNP, whose import is inhibited by excess trimethylguanosine cap analogue (Palacios *et al.*, 1996), it was not possible to test *in vitro* whether importin β would be required for m_3G cap-independent U snRNP import. However, by injecting a mixture of U1 and U5 snRNAs into *Xenopus* oocytes, we could compare their sensitivity to inhibition by the IBB domain.

U1 and U5 snRNAs carrying either an m_3G pppG or an ApppG cap structure were synthesized *in vitro*. Upon cytoplasmic injection into *Xenopus* oocytes, both m_3G -carrying RNAs migrate to the nucleus (Figure 6, upper panel, lanes 1–3). In contrast, when either a saturating

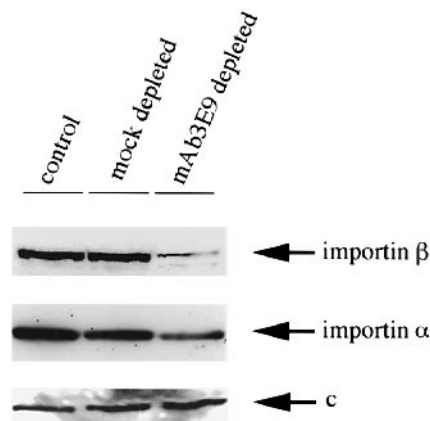


Fig. 4. Immunodepletion of importin β from *Xenopus* egg extracts. *Xenopus* egg extracts were incubated either with a column with bound mAb3E9 (mAb3E9 depleted) or with an unrelated mAb column (mock depleted). The unbound fraction of both columns and the input extract (control) were separated by SDS–PAGE, transferred to nitrocellulose and the presence of importin α and importin β was monitored by Western blotting. A spurious cross-reacting band (C) was used as a loading control.

amount of m_3G pppG is co-injected with the RNAs or when the RNAs carry an ApppG cap, only U5 snRNA can enter the nucleus (Figure 6, upper panel, lanes 4–6; lower panel, lanes 1–6). Injection of saturating amounts of the IBB fusion protein inhibits the import of m_3G -capped U1 and U5 snRNAs, and also of ApppG-capped U5 snRNA (Figure 6, both panels, lanes 7–9). Titration of the IBB domain showed that the concentration dependence of U1 and U5 snRNA import was not distinguishable (data not shown). Thus m_3G cap-independent U snRNP import also requires importin β .

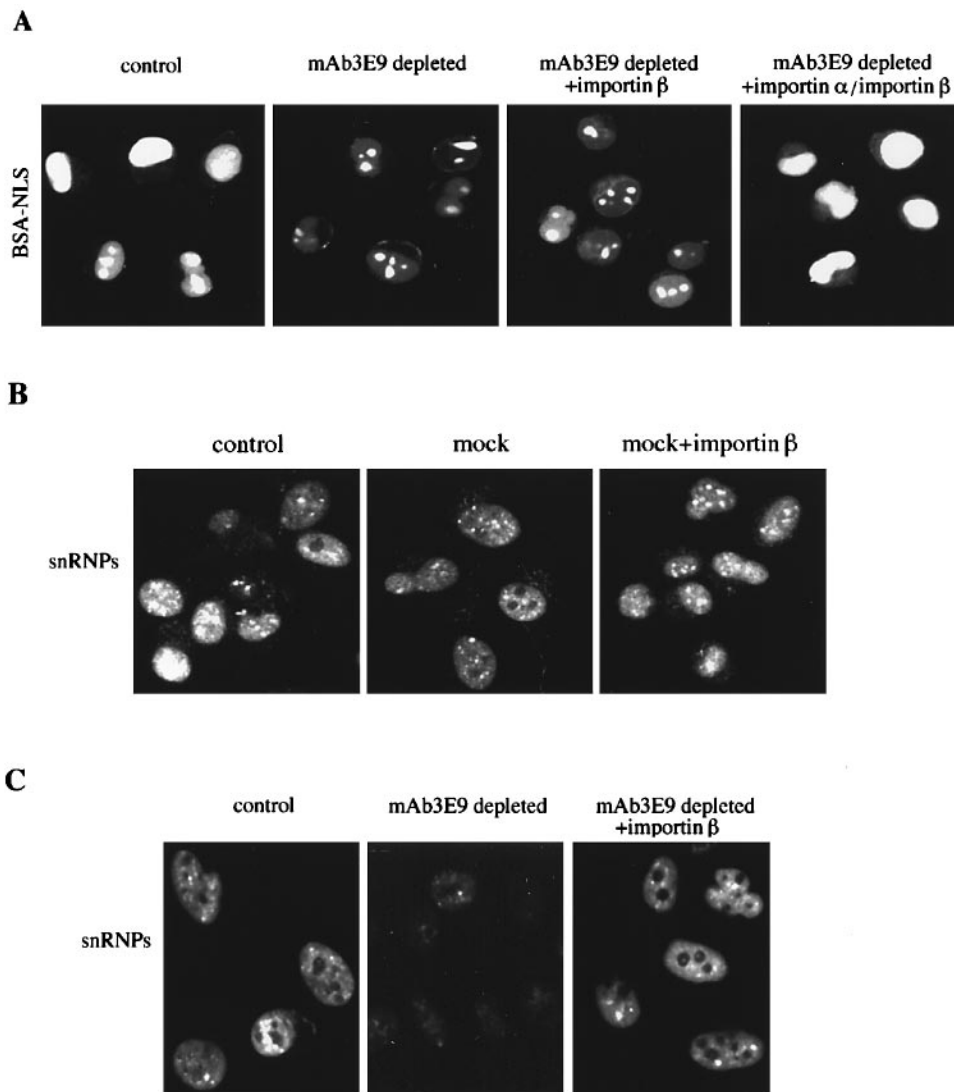


Fig. 5. The import of U snRNPs requires importin β . (A) *In vitro* import of BSA-NLS was performed for 20 min in the presence of either control *Xenopus* egg extract (control) or with mAb3E9-depleted extract. Either buffer (mAb3E9 depleted), importin β alone (at 0.45 μ M, mAb depleted + importin β) or importin β plus importin α (at 0.45 and 0.60 μ M respectively, mAb + importin α /importin β) were added to the depleted extract as indicated. (B) *In vitro* U snRNP import reactions (120 min) in control and mock-depleted *Xenopus* egg extracts with or without added recombinant importin β (0.6 μ M) as indicated. (C) The nuclear uptake of U snRNPs *in vitro* was assayed after 120 min in control and mAb3E9-depleted extracts in the absence (mAb3E9 depleted) or presence of importin β at 0.9 μ M (mAb3E9 depleted + importin β) as indicated.

Discussion

We present a series of *in vitro* experiments that lead to the conclusion that importin β , but not importin α , is required for U snRNP import into the nucleus. The IBB domain of importin α (Görlich *et al.*, 1996a; Weis *et al.*, 1996) inhibits U snRNP import *in vivo* independently of whether the import is trimethylguanosine cap dependent or independent. This requirement for importin β in the nuclear uptake of U snRNPs necessitates a re-evaluation of the interpretations of many previous experiments in this area.

The first relates to the generally accepted interpretation of previous data on the selective inhibition of specific nuclear import pathways. It has been clearly established both *in vitro* and *in vivo* that saturation of the NLS-mediated import pathway does not prevent U snRNP uptake into the nucleus and, vice versa, saturation of U snRNP import with either trimethyl cap analogues or

with U snRNP core particles does not affect NLS protein import (Fischer *et al.*, 1991, 1993; Michaud and Goldfarb, 1991, 1992; Marshallsay and Lührmann 1994; Palacios *et al.*, 1996). Since the importin heterodimer is essential for NLS protein import, these experiments led to the assumption that importin would not be involved in U snRNP uptake. However, since it is well established that importin α is the subunit which directly binds the NLS (Adam and Gerace, 1991; Görlich *et al.*, 1995a; Imamoto *et al.*, 1995a; Weis *et al.*, 1995), these data only rule out the participation of importin α in U snRNP import. Provided that importin β is present in functional excess over α in the experiments, they do not predict whether importin β will play a role.

NLS protein import can be reconstituted *in vitro* by the addition of four soluble factors, importin α and β , Ran and NTF2/p10, to digitonin-permeabilized cultured cells (see Introduction). We have been unable to reconstitute

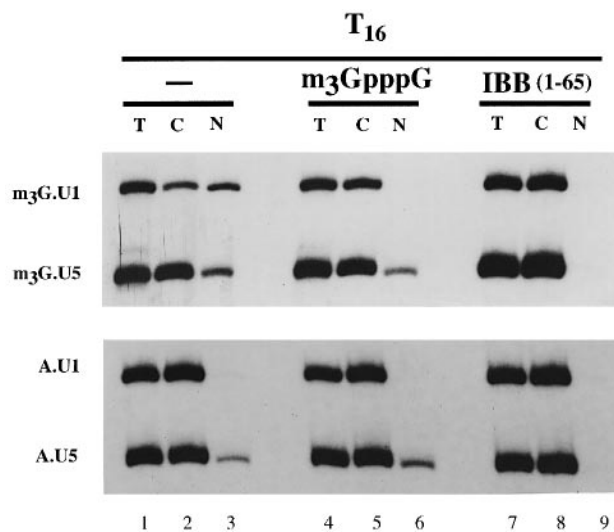


Fig. 6. Inhibition of m₃G-independent U snRNP import. Mixtures of *in vitro*-transcribed m₃GpppG- or ApppG-capped U1 and U5 snRNAs were injected into the cytoplasm of *Xenopus* oocytes. Sixteen hours later (T₁₆), their transport to the nucleus was monitored by extracting the RNAs from total oocytes (T) or cytoplasmic (C) and nuclear (N) fractions. Control import reactions for the m₃GpppG-capped (upper panel) and ApppG-capped (lower panel) RNAs are shown in lanes 1–3. The effect on import of saturating amounts (500 μM) of co-injected m₃GpppG cap dinucleotide are shown in lanes 4–6, and the effect of pre-injection of IBB at 28 μM (same protein as in Figure 2B) is shown in lanes 7–9. A truncated IBB fusion had no effect on import of these RNAs (see also Figure 2B), and the monomethyl cap dinucleotide did not affect U1 or U5 snRNA import (data not shown; Fischer *et al.*, 1991).

U snRNP import with mixtures containing importin β, Ran and NTF2/p10 (our unpublished data). This suggests that importin β does not recognize U snRNPs directly during their import, but rather collaborates with one or more adaptor proteins analogous to importin α. A corollary of this argument is that these adaptor proteins must be displaced from, or dissociate from, importin β during immunodepletion, otherwise we would not reconstitute U snRNP import with importin β re-addition alone. A further argument for the existence of such adaptors stems from the fact that U snRNP import is inhibited by the IBB domain of importin α. If U snRNPs were to bind importin β directly, one would therefore predict that they should compete with importin α binding through the IBB domain and thus competitively inhibit NLS protein import. As already discussed, this is not the case.

From the above, we can therefore deduce that when engaged in U snRNP import, importin β cooperates with an adaptor protein or proteins that is analogous to importin α but which, in contrast to α, does not bind to the NLS but rather to U snRNP import signals. One such factor does in fact exist. A protein that specifically interacts with the trimethylguanosine cap of the U snRNAs and that affects their nuclear import recently has been identified. This protein has a domain similar to the IBB domain, and binds to importin β *in vitro* (J.Huber and R.Lührmann, personal communication). However, as will be discussed in more detail below, our data show that the IBB domain also inhibits the trimethyl cap-independent import of U5 snRNP.

Importin protein families

It was quickly clear that importin α was a member of a protein family, but all the members of this family tested thus far are capable of mediating NLS protein import (for review, see Görlich and Mattaj, 1996; Nigg, 1997). In contrast, as discussed in detail in the Introduction, members of the importin β family have been implicated in the nuclear transport of a variety of proteins that do not carry an NLS but rather have other import signals (Aitchison *et al.*, 1996; Nakielny *et al.*, 1996; Pollard *et al.*, 1996; Fridell *et al.*, 1997; Rout *et al.*, 1997). This led to the expectation that the import of a different class of karyophiles, like the U snRNPs, might be mediated by one of the several importin β relatives which have no defined function as yet (Fornerod *et al.*, 1997; Görlich *et al.*, 1997). Our data instead suggest the existence of an import mediator analogous to importin α that cooperates with importin β to bring about U snRNP import. This raises the possibility that other importin β family members might act with more than one adaptor molecule. It is possible, for example, that even a protein like transportin, that interacts directly with M9-containing proteins (Pollard *et al.*, 1996; Fridell *et al.*, 1997; Nakielny *et al.*, 1997), might cooperate with as yet unrecognized adaptor molecules to transport other substrates. Of interest here is the fact that, while the importin heterodimer is conserved in yeast (for review, see Corbett and Silver, 1997), Srp1p, the importin α homologue in *Saccharomyces cerevisiae*, is the only protein in this organism with an IBB domain recognizable from its sequence (L.Englmeier, personal communication). This suggests that there may not be a separate IBB domain-containing protein that functions in U snRNP import in *S.cerevisiae*. It must, however, be kept in mind that it is as yet not clear whether U snRNP assembly in yeast involves a cytoplasmic phase.

The effect of WGA

Wheat germ agglutinin (WGA) binds to a group of glycosylated nucleoporins (NPC proteins) and inhibits NLS protein import, both *in vivo* and *in vitro* (Finlay *et al.*, 1987, 1991; Dabauvalle *et al.*, 1988, 1990; Finlay and Forbes, 1990). Since importin β was shown to be the component that directly contacts the NPC during NLS protein translocation (Adam and Adam, 1994; Görlich *et al.*, 1995b, 1996; Moroianu *et al.*, 1995; Weis *et al.*, 1996), the prediction was that interactions between importin β and one or more nucleoporins would be affected by WGA. WGA is a more effective inhibitor of NLS protein import than of U snRNP import (Fischer *et al.*, 1991; Kambach and Mattaj, 1992; Michaud and Goldfarb, 1992; Powers *et al.*, 1997). Since we now show that importin β is required for U snRNP import it is clear that the idea that WGA acts by preventing interaction between β and the NPC cannot be an adequate explanation of its effects.

A model for U snRNP import

Based on the data presented here, and on earlier information on both importin-mediated and U snRNP import, we can propose a model for the U snRNP import complex (Figure 7). Although not definitively ruled out, it seems unlikely that a single protein will mediate the U snRNP–importin β interaction for the following reasons. Both

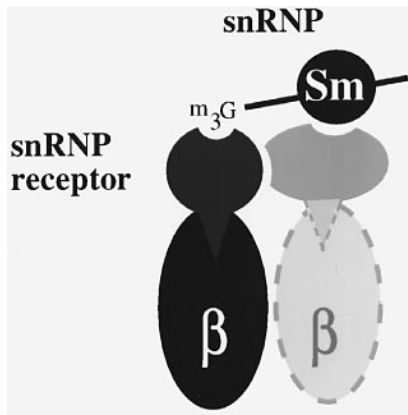


Fig. 7. A model for the U snRNP import complex. The model shows in black the known factors mediating U snRNP import, importin β and a trimethyl cap-binding protein that interacts with β through an IBB domain (see text). Also shown is the as yet hypothetical snRNP core-binding protein in grey. It is possible that this protein also has an IBB domain and that a second importin β , or importin β -like, molecule (stippled) is part of the U snRNP import complex.

trimethyl cap dinucleotide analogues and Sm snRNP core structures can saturate U snRNP import (Fischer *et al.*, 1991, 1993; Michaud and Goldfarb, 1992), suggesting that they are both recognized by essential import factors. However, a trimethyl-capped RNA that cannot bind the Sm snRNP core proteins is not imported into the nucleus (Hamm *et al.*, 1990). The factor that interacts with the trimethyl cap has been identified, and shown to carry an IBB domain (J. Huber and R. Lührmann, personal communication). Clearly, binding of this factor to importin β and to the trimethyl cap is therefore insufficient to cause formation of a functional import complex.

On the other hand, U1 snRNPs that have bound the Sm core proteins but that do not have a trimethyl cap are also either not imported or are imported inefficiently while, at least in *Xenopus* oocytes, the U5 snRNP core structure is sufficient for import (Fischer *et al.*, 1990, 1993; Hamm *et al.*, 1990; Jarmolowski and Mattaj, 1993). Thus, the Sm snRNP core can be a sufficient signal for U snRNP import in some circumstances. We show here that cap-independent import is inhibited by saturating amounts of IBB domain, i.e. that it is also importin β -mediated.

The simplest model that can be derived from these observations involves a trimethylguanosine cap-binding protein, an Sm snRNP core-binding protein and importin β bound to the IBB domain of the cap-binding component (Figure 7). A more complex variant would be necessary if trimethyl cap-independent import does not require the cap-binding protein. In this case, the Sm core-binding protein must also carry an IBB domain, and the normal import complex must contain two molecules of importin β (Figure 7). Either model requires that the interaction between the snRNP core and the core-binding factor is stronger for U5 snRNP than for U1 snRNP, since formation of the U5 snRNP import complex does not depend on the stabilization due to the interaction between the trimethyl cap and its binding protein. Although the core structures of the U1 and U5 snRNPs have identical protein compositions (Lührmann *et al.*, 1990), they have been shown previously to be non-identical in several functional respects (Jarmolowski and Mattaj, 1993), making this differential

affinity plausible. The next step in understanding U snRNP import requires characterization of the Sm core-binding factor, and we currently are pursuing this goal.

Materials and methods

T7 RNA polymerase and RNasin were from Promega. The cap analogues used to prime the synthesis of the U1 and U5 snRNAs were ApppG, from BRL, and $m^{2,2,7}$ GpppG, prepared as described by Darzynkiewicz *et al.* (1988, 1990). Labelled nucleoside triphosphates were from Amersham Corp.

Preparation of karyophiles

Fluorescein-labelled BSA-NLS was prepared as described in Palacios *et al.* (1996). IBB- β Gal was prepared as described in Weis *et al.* (1996) and it was labelled with fluorescein isothiocyanate (FLUOS, Boehringer Mannheim) following the manufacturer's instructions. U snRNPs were isolated from HeLa nuclear extracts in a one step purification as described by Bach *et al.* (1990). The anti-3mG antibody was commercial (Oncogene Science). The isolated U snRNPs were modified by 100-fold excess of FLUOS (Boehringer Mannheim) using the manufacturer's instructions. The labelled U snRNPs were re-purified by 3mG affinity chromatography and the components were analysed as described in Palacios *et al.* (1996).

Nuclear import assay

The *in vitro* transport reactions (15 μ l) contain 0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate (Sigma), 50 μ g/ml creatine phosphokinase (Sigma), 1×10^4 digitonin-permeabilized HeLa cells (Adam *et al.*, 1990) and at least 0.2 mg/ml nucleoplasm core (Görlich *et al.*, 1994). For the import reaction of U snRNPs, 4 μ l of *Xenopus* egg extract (70 mg/ml; Newmeyer *et al.*, 1986) and tRNA from *Escherichia coli*, at a final concentration of 0.2 mg/ml, were added. For the import of BSA-NLS, 2 μ l of *Xenopus* egg extracts were used. BSA-NLS, IBB- β Gal (final concentrations 70 μ g/ml) or U snRNPs (final concentration 20 μ g/ml) were used as karyophiles. The antibody and recombinant protein additions were made to the basic import reaction plus karyophile. The order of addition of the material to the nuclear import assays was as described by Dingwall and Palacios (1997). Incubations were carried out at 20°C, and the fixation, mounting and view of the reaction were as described by Palacios *et al.* (1996). Quantitation of nuclear fluorescence was also performed as described by Palacios *et al.* (1996).

Recombinant proteins

The IBB and truncated IBB domains were expressed with a his-tag and purified on nickel agarose as described by Weis *et al.* (1996), except that the lysate was spun at 45 000 r.p.m. before binding to the nickel column. His-tagged importin α was expressed as described by Weis *et al.* (1995) and purified on nickel agarose followed by MonoQ. Importin β was prepared and purified by MonoQ as described by Weis *et al.* (1996). Importin β mutant proteins were prepared as described (Kutay *et al.*, 1997) and were a kind gift from U. Kutay and D. Görlich.

Antibodies

The antibody against an N-terminal peptide from *Xenopus* importin α was prepared as described by Görlich *et al.* (1995a). The peptide used for the production of the antibody is PTTNEADERMC. The antibody used for the detection of importin β in Western blot was that of Görlich *et al.* (1995a).

The hybridoma that expressed the monoclonal antibody against importin β , mAb3E9, was produced as described by Chi *et al.* (1995). The cells were grown in culture and the supernatant, that contains the mAb, was collected, $1/20 \times$ the volume of 1 M Tris-HCl pH 8.0 was added and the mixture spun at 45 000 r.p.m. for 30 min. The supernatant (a crude IgG fraction) was then precipitated with ammonium sulfate, at 60% saturation, for 6 h at 4°C and spun as before. The pellet was resuspended in $0.1 \times$ the original volume in phosphate-buffered saline (PBS) and dialysed overnight against PBS, with at least two changes of buffer. The dialysed sample was kept at -20°C. For the preparation of the mAb3E9-protein A column, as well as for the unrelated monoclonal antibody column, the dialysed crude IgG fraction was incubated with protein A-Sepharose-CL4B (Pharmacia) for 12 h at 4°C and the bound antibody was cross-linked to the column by dimethylpimelimidate (DMP) as described (Harlow and Lane, 1988). The mAb3E9 column was kept at 4°C in PBS with 0.02% NaN_3 .

Depletion of extracts with monoclonal antibody columns

The *Xenopus* egg extracts were incubated with the mAb columns (mAb3E9 and unrelated mAb), after pre-coating them with BSA, in a ratio 4:1 (v/v) for 1 h at 4°C with mixing. The unbound fraction was recovered by spinning the Sepharose beads at 1000 g for 5 min.

Depletion of extracts with a BSA-NLS column

The BSA-NLS was prepared as described in Palacios *et al.* (1996). For the preparation of the BSA-NLS column, the BSA-NLS conjugate was incubated for 4 h at 4°C with activated CH-Sepharose 4B (Sigma), as described by the manufacturer. The *Xenopus* egg extracts were incubated with the BSA-NLS column in a 1:1 ratio (v/v) for 3 h at 4°C with mixing. The unbound fraction was recovered by spinning the Sepharose beads at 1000 g for 5 min.

Oocyte injections

In vitro transcription reactions, oocyte injections and analysis of micro-injected RNA by denaturing gel electrophoresis were performed as described by Jarmolowski *et al.* (1994).

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