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Nuclear transport of the U2 snRNP-specific U2B″ protein is mediated by both direct and indirect signalling mechanisms

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SUMMARY

Experiments investigating the nuclear import of the U2 snRNP-specific B″ protein (U2B″) are presented. U2B″ nuclear transport is shown to be able to occur independently of binding to U2 snRNA. The central segment of the protein (amino acids 90-146) encodes an unusual nuclear localization signal (NLS) that is related to that of the U1 snRNP-specific A protein. However, nuclear import of U2B″ does not depend on this NLS. Sequences in the N-terminal RNP motif of the protein are sufficient to direct nuclear transport, and evidence is presented that the interaction of U2B″ with the U2A′ protein mediates this effect. This suggests that U2B″ can 'piggy-back' to the nucleus in association with U2A′, and thus be imported to the nucleus by two different mechanisms. U2A′ nuclear transport, on the other hand, can occur independently of both U2B″ binding and of U2 snRNA.

Key words: U snRNP, nuclear transport, protein-protein interaction

INTRODUCTION

Nucleocytoplasmic transport of RNAs, proteins and RNPs is an energy-requiring, signal-mediated process (Feldherr et al., 1984; Dworetzky and Feldherr, 1988; Newmeyer and Forbes, 1988; Richardson et al., 1988). Nuclear accumulation of karyophilic proteins generally requires the presence of a nuclear localization signal or NLS (García-Bustos et al., 1991; Silver, 1991). NLSs are usually defined as sequences that are both necessary and sufficient for nuclear transport, although this definition is inappropriate for proteins that contain more than one independent NLS (see below). There is no strict sequence conservation between different NLSs, but most of those so far identified are short, basic sequence motifs. The best characterized example is the NLS of SV40 T antigen, which has the sequence PKKKRKV (Lanford and Butel, 1984; Kalderon et al., 1984a,b).

Several more complex types of NLS have been identified. Nucleoplasmin, for example, contains the prototype bipartite NLS that consists of two clusters of basic amino acids separated by a spacer region of roughly 10 amino acids (Dingwall et al., 1988; Robbins et al., 1991). The same kind of organization is seen in the *Xenopus* N1 protein (Kleinschmidt and Seiter, 1988) and also in the nucleolar protein NO38 (Schmidt-Zachmann et al., 1987; Robbins et al., 1991). On the basis of sequence comparisons it has been suggested that the nuclear targeting sequences of many other proteins are likely to be of the nucleoplasmin type (Robbins et al., 1991). The presence of more than one independent NLS in one protein has also been reported (Richardson et al., 1986), and sequences lying outside the minimal NLS, whose phosphorylation state influences the efficiency of nuclear accumulation, have been described (Rihs and Peters, 1989; Rihs et al., 1991; Moll et al., 1991). Some proteins contain NLSs that appear to have a fundamentally different organization. These are encoded by amino acids dispersed throughout a large protein segment that act in an additive or a cumulative way. Known cases include *Saccharomyces cerevisiae* Gal4 (Silver et al., 1988), rat hsc70 (Mandell and Feldherr, 1992), and the U1 snRNP-specific U1A protein (Kambach and Mattaj, 1992). In summary, although the involvement of short, basic NLSs in the process of nucleocytoplasmic transport is firmly established, other classes of NLS exist, and inferring the identity of an NLS from sequence data alone may be misleading.

Assembly and intracellular transport of U snRNPs has been a field of intense study for a number of years (for reviews see Mattaj, 1988; Lührmann et al., 1990; Nigg et al., 1991; Dingwall, 1992; Izaurralde and Mattaj, 1992; Newmeyer, 1993). After transcription by RNA polymerase II, U snRNAs are transported out of the cell nucleus, associate with proteins in the cytoplasm, and migrate back to the nucleus (De Robertis et al., 1982; Mattaj and De Robertis, 1985; Mattaj, 1988). In the case of spliceosomal snRNPs both binding of a group of proteins collectively called the Sm proteins and, in some cases, the subsequent cytoplasmic hypermethylation of the 7-methylguanosine cap structure have been shown to be prerequisites for the nuclear import of U snRNPs in *Xenopus* oocytes (Mattaj, 1986, 1988; Fischer et al., 1991). The movement into the nucleus of the Sm proteins is also dependent on their cytoplasmic interaction with the U snRNAs (Zeller et al., 1983; Mattaj and De Robertis, 1985; Feeney and Zieve, 1990).

In contrast, nuclear migration of several U snRNP-specific proteins has been shown to be independent of interactions with their cognate snRNAs (Feeney et al., 1989; Kambach and

Mattaj, 1992; Jantsch and Gall, 1992). For example, the U1 snRNP-specific U1A protein is transported to the nucleus by an active pathway and its import, in contrast to that of U1 snRNP, can be inhibited efficiently by wheat germ agglutinin (WGA; Kambach and Mattaj, 1992). U1A transport thus shares the characteristics of nuclear transport of other karyophilic proteins. Nevertheless, several features distinguish U1A from the majority of nuclear proteins. First, it possesses an unusual NLS (see above) and second its distribution between the nucleus and cytoplasm depends upon the number of RNA binding sites for the protein in the two compartments (Kambach and Mattaj, 1992).

One U2 snRNP-specific protein, U2B″, is highly similar to U1A (see Fig. 2; and Sillekens et al., 1987). Of particular interest from the viewpoint of nuclear transport is the fact that the similarity between the two proteins is least in the segment that, in U1A, harbours NLS activity. Furthermore, the U2B″ protein associates with another U2 snRNP-specific protein, U2A′, to form an RNA-independent complex (Scherly et al., 1990a), whereas the U1A not associated with U1 snRNP appears to be monomeric. We therefore proceeded to investigate the requirements for U2B″ nuclear import and report here that U2B″ can be targetted to the nucleus by two independent signalling mechanisms.

MATERIALS AND METHODS

Microinjection and subfractionation of oocytes

Oocytes (stages V to VI; Dumont, 1972) of *Xenopus laevis* were prepared as described (Hamm et al., 1989). For inhibition of U2 snRNA transport, a deoxyoligonucleotide complementary to a single-stranded region of U2 snRNA (U2b; Hamm et al., 1989) was injected at 300 µM final concentration together with α-amanitin at 2 µg/ml final concentration. Microinjection of in vitro translated, [^{35}S]methionine-labeled proteins, dissection of oocytes and processing of the total, cytoplasmic and nuclear fractions for protein and /or RNA analysis were carried out as described (Kambach and Mattaj, 1992). A *Xenopus* lamin L1 cDNA clone encoding a soluble form of lamin L1 (Krohne et al., 1989, mutant M8) was used as an internal control in the transport experiments. The mouse dihydrofolate reductase cDNA clone used for the construction of U2B″ fusions is described by Kambach and Mattaj (1992).

U1A, U2A′ and U2B″ mutants

A simple diagram of the sequence composition of the mutant proteins used in this study is presented in Fig. 3, below. The internal deletion mutants of U1A and U2B″ as well as the chimeric A/B″ mutants were generally constructed by making use of *Bam*HI point mutants (Scherly et al., 1989, 1990b). The double point mutation in construct 1.4 (see Fig. 2) stems from a full-length U1A parent construct (A.3) described by Scherly et al. (1990a). The *Bam*HI site at amino acids 92/93 (position 12, Figs 2, 3) was transferred with a *Stu*I restriction fragment from the appropriate point mutant (A_{12}; Scherly et al., 1989) into A.3 for construct 1.4. The internal deletion from amino acid 93 to 204 was then introduced by cutting with *Bam*HI and *Hin*dIII, and ligating with the *Bam*HI-*Hin*dIII fragment from A_{16}. A *Bgl*II site was introduced using the Amersham Corp. site-directed mutagenesis kit at amino acid positions 92/93 of $B''/A_{6/9}$. This was then cut with *Bgl*II and *Hin*dIII and ligated with the *Bam*HI-*Hin*dIII insert from $B''_{145/146}$ (Scherly et al., 1990b) to give construct 2.6. The fusion mutants with DHFR (3.1 and 3.2, Fig. 2A) were constructed in an analogous way to the fusions of internal fragments of the U1A protein to DHFR (Kambach and Mattaj, 1992). The U2A′ point mutant $A'_{44/45}$ is described by Boelens et al. (1991).

Quantification of signal strengths

Gels were quantified using the Molecular Dynamics PhosphoImager system equipped with ImageQuant™ software, v3.2.

Immunoprecipitations

Immunoprecipitations were carried out essentially as described (Kambach and Mattaj, 1992). Twelve oocytes per sample were homogenized in 250 µl oocyte extraction buffer (Vankan et al., 1990) and centrifuged twice for 15 minutes in a benchtop centrifuge. A 200 µl sample of the clear supernatant was then added to 20 µl of a suspension of antibody coupled either to Protein A-Sepharose CL4B (Pharmacia Fine Chemicals) in the case of anti-U2A′ rabbit antiserum or to anti-mouse IgG-Agarose (Promega) in the case of anti-U2B″ monoclonal antibody 4G3 (Habets et al., 1989). After rotating the samples at 4°C for 90 minutes, the supernatants were removed and precipitated by addition of 1 ml cold acetone and incubation at −80°C for 1 hour. The supernatants were centrifuged for 15 minutes at 4°C, the pellets dried and dissolved in 100 µl 1× SDS sample buffer. After incubation for 8 minutes at 95°C, 10 µl of each sample was loaded on a 12.5% SDS-PAGE gel (Lehmeier et al., 1990). The immunoprecipitated pellets were washed three times for 10 minutes with IPP150 (Mattaj and De Robertis, 1985), resuspended in 20 µl 1× protein sample buffer, incubated at 95°C as above and 20 µl of each sample loaded on the same gel.

RESULTS

Nuclear transport of the U2B″ protein can occur independently of U2 snRNA

It was recently proposed that while the U1A and U2A′ proteins migrate to the nucleus independently of U snRNA synthesis and transport, the U2B″ protein associates with U2 snRNA in the cytoplasm prior to nuclear migration (Feeney and Zieve, 1990). Since in vitro studies have shown that U2A′ and U2B″ can form a heterodimeric complex in the absence of RNA, and that U2A′-U2B″ interaction is a prerequisite for the specific binding of U2B″ to U2 snRNA (Scherly et al., 1990a,b; Bentley and Keene, 1991), the conclusions with respect to differential transport of the two U2 snRNP proteins appeared questionable.

To determine whether nuclear transport of the U2B″ protein is dependent on U2 snRNA binding, an approach identical to that previously used to study U1A nuclear import (Kambach and Mattaj, 1992) was chosen. U2 snRNA transport was blocked in *Xenopus laevis* oocytes by coinjection of a DNA oligonucleotide complementary to a single-stranded region of U2 snRNA (to mediate destruction of the accumulated endogenous U2 transcripts via RNase H) and α-amanitin to prevent new U2 snRNA transcription (Pan and Prives 1988; Hamm et al., 1989). Cleavage of the endogenous U2 snRNA was checked by northern analysis (data not shown). This cleavage removes the trimethylguanosine cap structure of U2 snRNA, and thus would prevent movement of any newly assembled U2 snRNPs to the nucleus (Fischer et al., 1991). In vitro translated U2B″ protein, which was subsequently injected into the cytoplasm of the same oocytes, accumulated in the nucleus to a level indistinguishable from that in untreated control oocytes (Fig. 1, compare lanes 1-3 and 7-9). This shows that the efficiency of U2B″ nuclear import is not influenced by removal of U2 snRNA, i.e. that U2B″ can be imported to the nucleus independently of U2 snRNA to a similar extent as in the presence of U2 snRNA. In this and other experiments, the production of

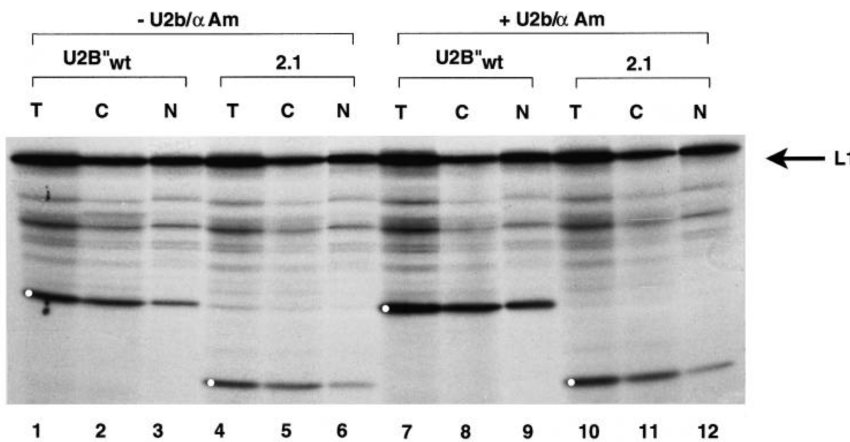

Fig. 1. Effect of U2 snRNA on U2B″ transport. *Xenopus* oocytes were injected with a mixture of α-amanitin (2 μg/ml final concn) and U2b deoxyoligonucleotide (300 μM final concn). After 2 hours incubation at room temperature, a mixture of lamin L1 and U2B″wt (lanes 1-3, 7-9) or construct 2.1 (lanes 4-6, 10-12) was injected into treated (lanes 7-12) or untreated control oocytes (lanes 1-6). After 14 hours incubation at 19°C, the oocytes were manually dissected and the fractions processed as described (Kambach and Mattaj, 1992). T, C, and N denote total, cytoplasmic and nuclear fractions, respectively. The lamin L1 derivative used is mutant M8 (Krohne et al., 1989). This derivative of lamin L1 represents an easily extractable nuclear protein, since it is soluble rather than being incorporated into the insoluble nuclear lamina. In this and other figures derivatives of U2B″ or U1A were marked with white dots to distinguish them from truncated products derived in the lamin L1 translation reaction.

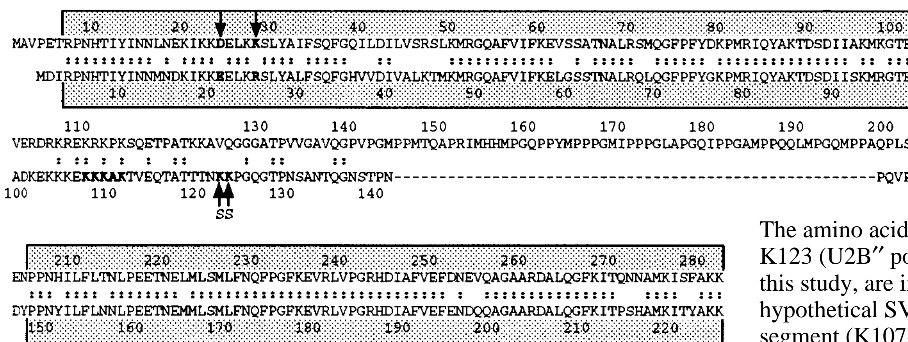

Fig. 2. Sequences of the U1A (top line) and U2B″ (bottom line) proteins (modified from Sillekens et al., 1987). The sequences are numbered with the 0 of each number positioned over the relevant amino acid. The boxed regions correspond to the amino- and carboxy-terminal RNP motifs of the proteins. The amino acid positions D24, K28 (U1A positions) and K122, K123 (U2B″ positions), which are mutated in constructs used in this study, are in boldface and are marked by arrows. The hypothetical SV40-like NLS sequence within the U2B″ central segment (K107-K111) is in boldface.

truncated versions of the internal control L1 protein sometimes made identification of the test proteins difficult. For this reason, they have where necessary been marked by a white dot.

The middle segment of U2B″ has NLS activity

As mentioned in the Introduction, homology between the closely related U1A and U2B″ proteins is least in their central segments. In the case of the U1A protein, this region was identified as the NLS (Kambach and Mattaj, 1992). The central segment of U2B″ bears a strong net positive charge, and sequences reminiscent of other defined NLSs are present between amino acids 107 and 123 (Fig. 2). We therefore wished to know whether the middle segment of U2B″ (amino acids 90 to 146) could function as an NLS when incorporated into a hybrid protein lacking other nuclear targetting signals. Initially, a U1A/U2B″ chimeric mutant containing the U2B″ middle segment flanked by the two RNP motifs of the U1A protein (construct 1.2, Fig. 3) was made. This mutant lacks the U1A sequences required for nuclear import (Kambach and Mattaj, 1992). Since this fragment of the U1A protein is completely lacking in NLS activity, and since the N- and C-terminal domains of the protein are closely related to those of the U2B″ protein, it provides an ideal background against which to test putative NLS-containing segments from U2B″ in a structural context that is likely to be very similar to that existing in the U2B″ protein itself.

A technical comment on the transport experiments must be added at this point. Since most mutants tested bear a deletion of the U1A or U2B″ middle segment (Fig. 3), they are small enough to diffuse into the nucleus. Active transport of a given mutant can thus only be inferred from the change in the cytoplasmic (C) to nuclear (N) ratio of the protein when incubations at 0 and 19°C are compared. Active transport is often more obvious from the lack of movement of a given protein to the nucleus at 0°C than from a high final nuclear:cytoplasmic ratio at 19°C. The presumed explanation for this effect is trapping of actively transported mutants in the cytoplasm through binding to elements of the transport machinery at low temperature (Breeuwer and Goldfarb, 1990). Positive (U2B″wt) and negative (construct 1.1, Fig. 3) controls were included in each transport experiment. Transport assays were quantitated, and active transport behaviour evaluated, as previously described (Kambach and Mattaj, 1992). To be considered positive, a mutant protein had to accumulate in the nucleus to at least 70% of the level of the wild-type protein, and transport had to be reduced by a factor of at least 3.5 on cooling.

Mutant 1.2 showed active nuclear transport behavior (compare lanes 13-15 in Fig. 4A,B) at a level similar to that of both U1Awt and U2B″wt (compare lanes 1-3 and 4-6 in Fig. 4A,B). The negative control, construct 1.1 (Fig. 3), did not exhibit active transport (Fig. 4A,B, lanes 7-9). Thus, the central region of U2B″ has NLS activity. In view of the lack of similarity between U1A and U2B″ in their middle segments, we wished to investigate the sequence composition of the U2B″ NLS further. First, two fusions with the cytoplasmic protein

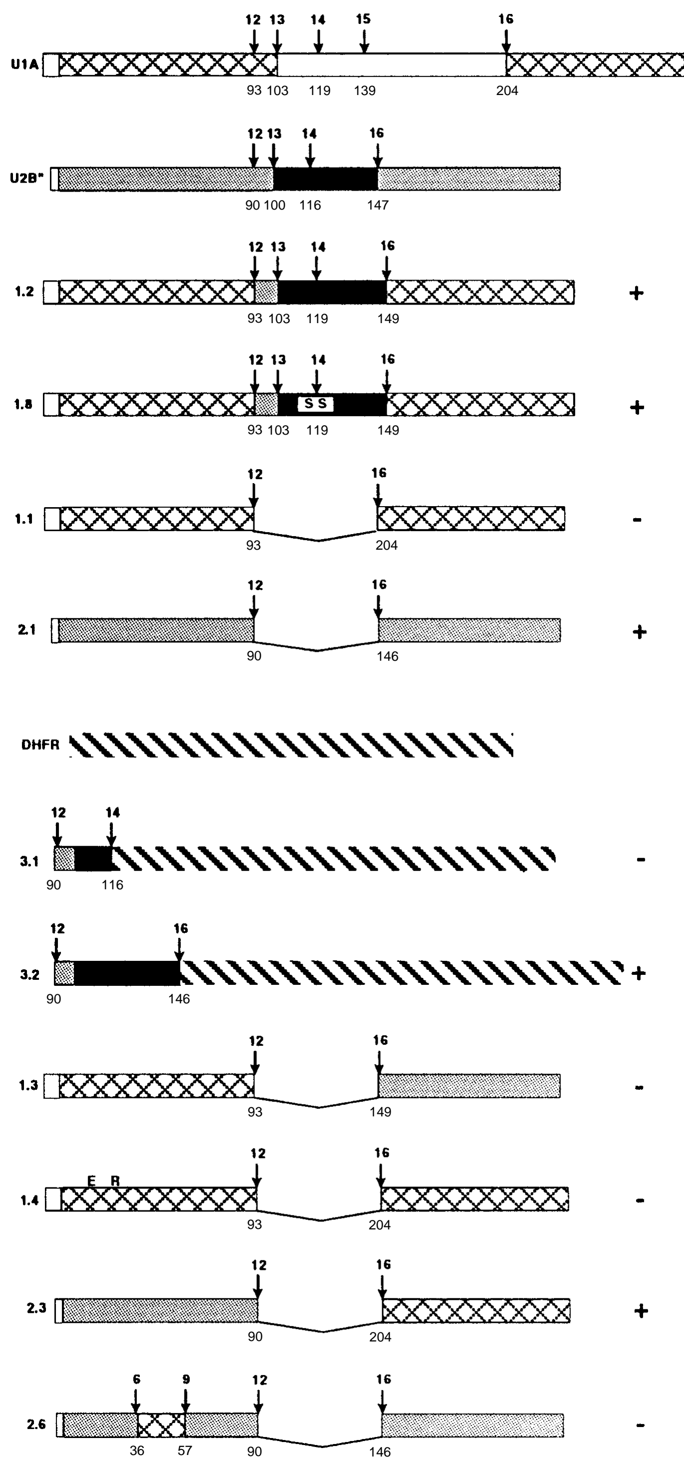

Fig. 3. Schematic representation of the mutant U1A/U2B″ constructs used in this study. The numbers beneath the bars denominate amino acid positions. The numbers above the bars refer to the numbering system of point mutants (Scherly et al., 1989, 1990b). U1A RNP motifs are depicted by cross-hatched boxes, U2B″ RNP motifs by grey boxes. The white and black boxes represent the unique segments of U1A and U2B″, respectively (Fig. 2). Deletions are represented by lines. Mouse DHFR is represented by a striped box. The relative sizes of the boxes reflect the lengths of the respective sequences. The + and – signs refer to transport activity: + designating actively transported mutants, and – transport-defective mutants.

dihydrofolate reductase (DHFR) were made (constructs 3.1 and 3.2, Fig. 3). The shorter of these (3.1) transfers the U2B″ sequence from amino acid 90 to 116 to DHFR. This region contains a putative SV40 T-type NLS (amino acids 107-111). The 3.1 fusion protein was, however, not actively transported to the nucleus (Fig. 4C, lanes 1-3 and 7-9). Transfer of the entire U2B″ middle segment led to active transport of the 3.2 fusion (Fig. 4C, lanes 4-6 and 10-12). Although constructs 3.1 and 3.2 differ in length by 30 amino acids (Fig. 3) they consistently exhibited very similar mobility on denaturing gel electrophoresis. The reason for this is unknown.

The basic stretch of amino acids between positions 107 and 111 forms part of a possible bipartite NLS together with the two lysine residues at positions 122 and 123 (Fig. 2). Since this entire putative NLS was present in 3.2, but not in 3.1, we wished to test if it was responsible for the active transport of 3.2. To this end, Lys 122 and -123 were mutated to Ser, a change that would be expected to inactivate a bipartite NLS (Robbins et al., 1991). Since other U2B″ sequences have independent NLS activity (see below) we made these changes in the context of construct 1.2. The resulting protein (1.8, Fig. 3) was transported by an active mechanism to a level indistinguishable from that of 1.2 (Fig. 5, compare lanes 1 and 2 with 8 and 9, and 5 and 6 with 14 and 15). The sequences from K_{107} to K_{123} therefore do not represent a bipartite NLS. These results also indicate that the amino acids located between positions 116 and 146, required for NLS activity (Fig. 4C, lanes 10-12), are not basic, since there are no other positively charged residues in this segment of U2B″ (Fig. 2). In summary, the results in this section show that amino acids 90-146 of U2B″ encode an NLS, and suggest that it may be organized in a similar, dispersed way to the NLS of the U1A protein.

Sequences in the N-terminal RNP motif of U2B″ can target the protein to the nucleus

Having established that the middle segment of U2B″ has NLS activity, we asked whether this part of the protein is essential for U2B″ nuclear import. We tested this by construction of a mutant bearing a deletion between amino acids 90 and 146 (construct 2.1, Fig. 3). This mutant was actively transported (Fig. 4A,B, lanes 10-12) when compared with positive and negative controls (Fig. 4A,B, lanes 1-9). In an oligonucleotide-directed RNase H experiment transport of construct 2.1 to the nucleus, like that of wild-type U2B″, was shown to occur independently of U2 snRNA (Fig. 1, compare lanes 4-6 with lanes 10-12). U2B″ must therefore contain sequences outside of amino acids 90-146 sufficient for its active nuclear import.

Constructs 1.1 as well as 2.1 (Fig. 3) consist essentially of the two RNP motifs of the U1A and U2B″ proteins, respectively. The former is not actively transported, while the latter is (Fig. 4). To establish which RNP motif in 2.1 was required for its nuclear accumulation, chimeric mutants were constructed, either containing the U1A N-terminal RNP motif connected to the C-terminal RNP motif of U2B″ or vice versa (constructs 1.3 and 2.3, Fig. 3). Construct 1.3 was not actively transported (Fig. 6A,B, lanes 8 and 9), whereas 2.3 was (Fig. 6A,B, lanes 11 and 12). The conclusion is that the N-terminal U2B″ RNP motif is sufficient to mediate active nuclear transport.

The activity of the N-terminal motif could be due to direct interaction either with some component of the transport

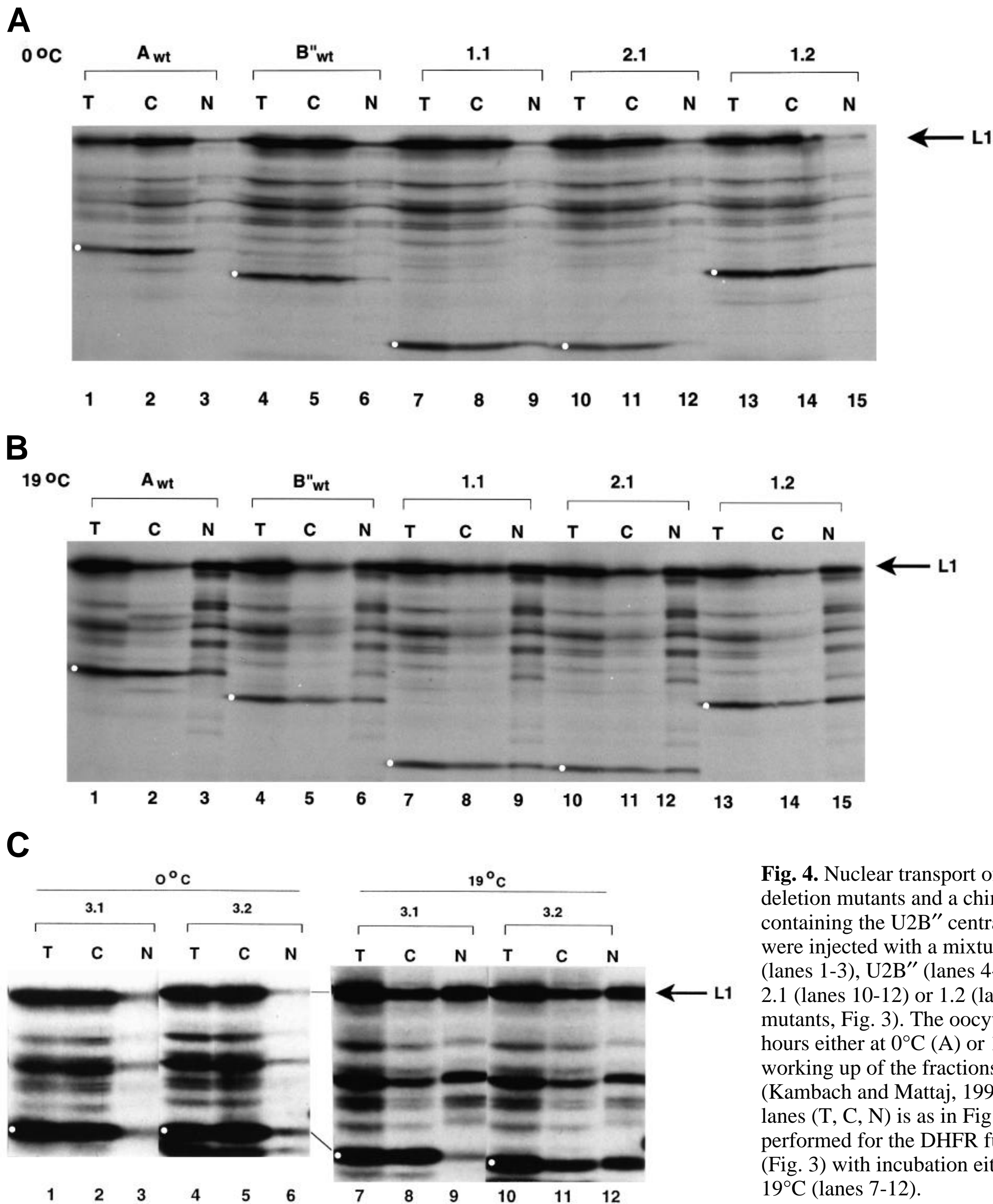

Fig. 4. Nuclear transport of U1A and U2B″ internal deletion mutants and a chimeric U1A/U2B″ mutant containing the U2B″ central segment. *Xenopus* oocytes were injected with a mixture of lamin L1 and U1Awt (lanes 1-3), U2B″ (lanes 4-6), constructs 1.1 (lanes 7-9), 2.1 (lanes 10-12) or 1.2 (lanes 13-15, for structures of the mutants, Fig. 3). The oocytes were then incubated for 14 hours either at 0°C (A) or 19°C (B). Dissection and working up of the fractions was performed as described (Kambach and Mattaj, 1992). The designation of the lanes (T, C, N) is as in Fig. 1. (C) The same experiment performed for the DHFR fusion constructs 3.1 and 3.2 (Fig. 3) with incubation either at 0°C (lanes 1-6) or at 19°C (lanes 7-12).

machinery or with some other entity (RNA or protein) that would indirectly facilitate its import (by a ‘piggy-back’ mechanism). The U2B″ N-terminal motif is known to interact both with U2 snRNA (Scherly et al., 1990b) and with the U2 snRNP-specific A′ protein (Scherly et al., 1990a; Boelens et al., 1991). Nuclear transport of construct 2.1 was shown to occur independently of U2 snRNA (see above), but the possibility that interaction with U2A′ was important remained to be tested.

To investigate this, experiments were designed based on the study of U2B″/U2A′ interaction in vitro (Scherly et al., 1990a; Boelens et al., 1991). These experiments had shown that amino acids 1-88 of U2B″ are necessary and sufficient for interaction with U2A′. Detailed analysis revealed that two amino acids in U2B″, E21 and R25, allowed strong interaction with U2A′ when introduced into the U1A protein background. These amino acid exchanges were introduced into construct 1.1 to give construct 1.4 (Fig. 3).

When tested for active nuclear transport, construct 1.4 was negative (Fig. 6A,B, lanes 13-15). This suggested that interaction with U2A′ might not be involved in targetting the N-terminal RNP motif of U2B″ to the nucleus. However, it was necessary to test whether the mutant protein actually did interact with U2A′ in vivo. To test this, the microinjected proteins were immunoprecipitated with a polyclonal anti-U2A′ antiserum (Scherly et al., 1990a). As positive and negative controls we utilized U2B″ protein and construct 1.1, respectively (Fig. 7, lanes 1-4, lanes 2 and 4 are the immunoprecip-

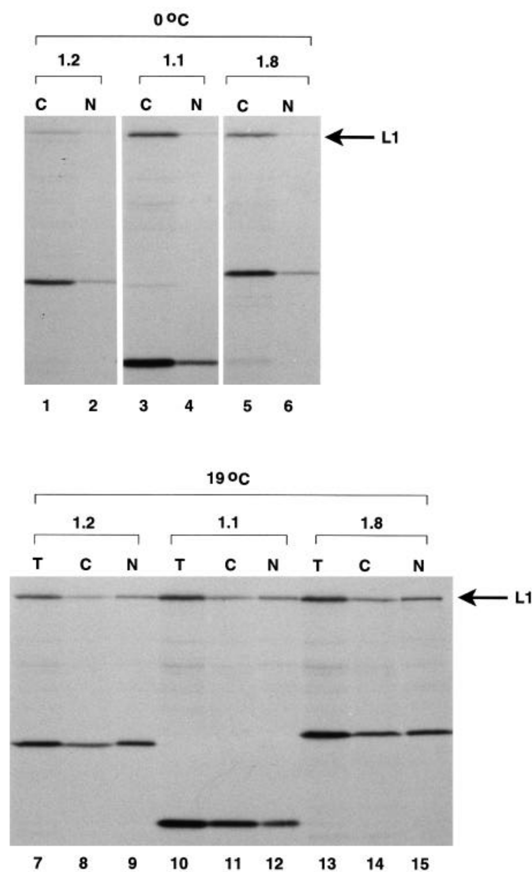

Fig. 5. Transport of constructs 1.1, 1.2 and 1.8 (Fig. 3). Lanes 1-6, incubation at 0°C. Lanes 7-15, incubation at 19°C. Only C and N fractions are shown in the 0°C experiment. The U1A mutant 1.1 (lanes 3,4 and 10-12) served as a negative control.

itated pellet fractions). Constructs 2.1 and 2.3, both of which contain the entire N-terminal U2B″ RNP motif, were found to interact with U2A′ (Fig. 7, lanes 5, 6, 9 and 10. In all supernatant lanes, 10% of the protein in the supernatant fraction was loaded), while construct 1.3, which retains only the C-terminal U2B″ RNP motif, did not (Fig. 7, lanes 7 and 8). Construct 1.4 also showed no detectable interaction with U2A′ in vivo (Fig. 7, lanes 11 and 12). This result was unexpected in the light of the association between U2A′ and A.3, a full-length version of the U1A protein containing the U2B″-specific E21 and R25 amino acids (Scherly et al., 1990a). In vitro immunoprecipitation experiments were carried out with A.3 and construct 1.4 to examine further the basis of the result obtained in vivo. U2B″ and construct 2.3 were used as positive controls. After mixing with reticulocyte lysate containing unlabelled U2A′ protein, these proteins were immunoprecipitable with anti-U2A′ antibodies (data not shown). As previously reported, A.3 interacts with U2A′. However, as in vivo (see above), construct 1.4 did not detectably associate with U2A′ in vitro (data not shown). Thus, the reason for the lack of co-immunoprecipitation with U2A′ of construct 1.4 as compared to A.3 was the deletion of amino acids 93-204 of the U1A portion of the chimeric proteins.

These results were consistent with the hypothesis that U2A′ interaction might be required for nuclear transport of the N-terminal U2B″ RNP motif, and suggested a way to test the idea more directly. A derivative of construct 2.1, containing a segment of U1A protein between amino acids 36 and 57 (construct 2.6, Fig. 3), was made. The eight amino acid changes thus introduced are known to weaken interaction with U2A′ without grossly affecting the structure of the U2B″ N-terminal RNP motif as measured by the ability of the protein to interact with RNA (Scherly et al., 1990a,b). We reasoned that, in combination with the deletion of residues 90-204 in construct 2.6, this might abolish interaction with U2A′ in vivo, and thus allow us to examine the dependence on U2A′ interaction for nuclear transport in a hybrid protein whose structure was similar to that of U2B″.

When tested by co-immunoprecipitation, construct 2.6 was found not to interact with U2A′ (Fig. 7, lanes 13 and 14). Construct 2.6 was also negative when tested for active transport to the nucleus (Fig. 6A,B, lanes 16-18). This result shows that loss of U2A′ interaction correlates with the loss of active transport, and thus implies that the active transport mediated by the N-terminal RNP motif of U2B″ is likely to depend upon interaction with U2A′.

U2A′ transport is independent of U2B″

The suggestion that U2B″ transport can occur via ‘piggy-backing’ on U2A′ is only tenable if U2A′ itself is actively transported to the nucleus. In order to examine this and to determine whether interaction with U2B″ influenced U2A′ nuclear transport we injected both wild-type U2A′ protein and a mutant derivative, $A'_{44/45}$, shown to be incapable of stable interaction with U2B″ in vitro (Boelens et al., 1991). Both were actively transported, and no significant difference between the transport activity of U2A′wt and $A'_{44/45}$ was observed (Fig. 8, compare lanes 2 and 3 with 8 and 9, and 5 and 6 with 11 and 12). To determine whether the proteins could interact with U2B″ in vivo, indirect immunoprecipitations were carried out with a monoclonal anti-U2B″ antibody (Habets et al., 1989). After microinjection of the labelled proteins, neither could be immunoprecipitated in this way (data not shown), suggesting that the U2B″ concentration in the oocyte may limit the formation of U2A′/U2B″ complexes. This possibility was tested by cytoplasmic microinjection of in vitro-transcribed U2B″ mRNA into the oocytes one day prior to injection of the two U2A′ protein derivatives. In this case U2A′wt was efficiently immunoprecipitable (Fig. 9, lanes 1 and 2) while $A'_{44/45}$ was barely detectable (Fig. 9, lanes 3 and 4). We therefore conclude that U2A′ transport to the nucleus occurs by an active mechanism that is independent of the interaction between the U2A′ and U2B″ proteins.

DISCUSSION

U2B″ nuclear transport and binding to U2 snRNA

An investigation of the nuclear import of the U2 snRNP-specific B″ protein has been presented. The U2B″ protein is highly similar in sequence and organization to the U1 snRNP-specific U1A protein (Sillekens et al., 1987; and Fig. 2) whose nuclear transport was studied previously (Kambach and Mattaj, 1992). U2B″ has several characteristics that distinguish it from U1A. First, it forms an RNA-independent complex with another U2 snRNP-specific protein, U2A′ (Scherly et al.,

Fig. 6. Transport of chimeric U1A/U2B″ constructs (Fig. 3). (A) Incubation at 0°C. (B) Incubation at 19°C. U2B″wt (lanes 1-3) served as a positive control, mutant 1.1 (lanes 4-6) as a negative control.

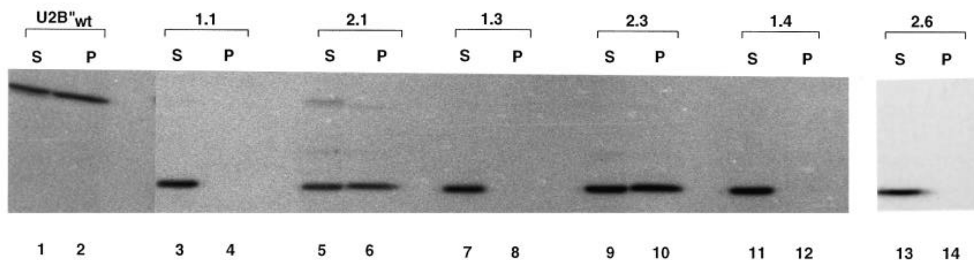

Fig. 7. Indirect immunoprecipitation of U2B″ mutants and chimeric U1A/U2B″ proteins. The mutants were injected into *Xenopus* oocytes and incubated for 14 hours. The oocytes were homogenized in oocyte extraction buffer (Vankan et al., 1990) and immunoprecipitations were carried out as described in Materials and Methods, using a polyclonal anti-U2A′ serum. 10% of the supernatants was loaded on an SDS-gel (lanes 'S'), as were the entire solubilized pellets (lanes 'P'). U2B″wt served as a positive control (lanes 1, 2).

1990a), and this interaction is required for specific binding of U2B″ to U2 snRNA (Scherly et al., 1990b; Bentley and Keene, 1991). In this study, we have elucidated which features of U2B″ are relevant for its nuclear import.

The first issue addressed was whether U2B″ required interaction with its cognate snRNA to migrate to the nucleus, as is the case for the common, or core, snRNP proteins (Zeller et al., 1983; Mattaj and De Robertis, 1985; Feeney and Zieve, 1990) but not for two U1 snRNP-specific proteins, U1A and U1C (Feeney and Zieve, 1990; Kambach and Mattaj, 1992; Jantsch and Gall, 1992). Our results showed that U2B″ nuclear import can occur independently of the presence of intact endogenous U2 snRNA, and make it likely that U2B″ joins the U2 snRNP in the nucleus.

The U2B″ central segment contains an NLS

The U1A protein contains an example of a particularly complex sequence encoding NLS activity. It extends over the

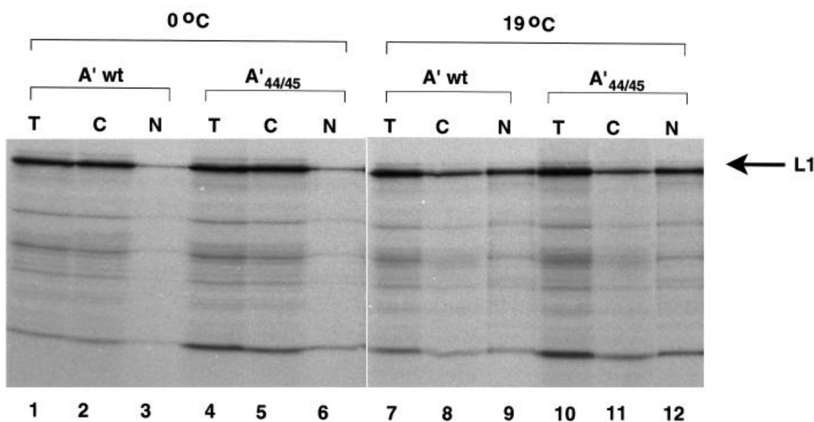

Fig. 8. Transport of U2A′wt (lanes 1-3, 7-9) and $A'_{44/45}$ (lanes 4-6, 10-12) at 0°C (lanes 1-6) and at 19°C (lanes 7-12).

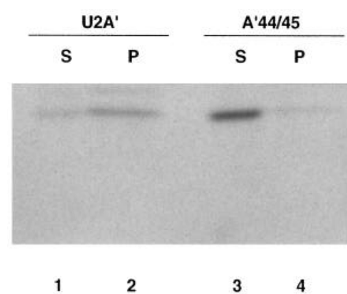

Fig. 9. Indirect immunoprecipitation of U2A′ and $A'_{44/45}$ proteins. The proteins were injected into *Xenopus* oocytes that had, 24 hours previously, been preinjected with 30 nl of a 2 mg ml^{-1} solution of in vitro-made U2B″ mRNA. After further incubation for 14 hours, the oocytes were homogenized and immunoprecipitations carried out using an anti-U2B″ monoclonal antibody. 10% of the supernatant fractions (S) and the entire pellet fractions (P) were analyzed by denaturing gel electrophoresis.

central 110 amino acids of the protein and NLS activity depends in an additive way on the presence of sequence elements dispersed throughout this segment (Kambach and Mattaj, 1992). Given the relationship between U1A and U2B″, discussed above, it was interesting to determine whether the U2B″ middle segment (amino acids 90-146, Fig. 2) would also contain an NLS. The U2B″ middle segment is considerably shorter than that of U1A (56 amino acids as compared to 112). Also, the overall sequence similarity between the two proteins is low in this region as compared to that in the flanking RNP motifs.

The results show that, in spite of this difference, the U2B″ middle region functions in nuclear import in a similar way to the U1A NLS. Although this region contains sequences reminiscent of both the SV40 T antigen (amino acids 107-112) and nucleoplasmin (amino acids 107-123) NLSs, our mutagenic analysis showed that neither of these sequence similarities was functionally important. Rather, as in the case of the better-studied U1A NLS, the activity seems to require an extended sequence, part of which (positions 101-111) is strongly basic and part of which (116-145) is not. It is possible that the NLSs of U1A and U2B″ represent the prototypes of a novel class of nuclear localization signal.

The U2B″/U2A′ interaction and active transport

Although the central segment of U2B″ is sufficient for nuclear import, removal of this region from the protein did not diminish nuclear accumulation to a significant extent. Rather, in clear contrast to the U1A protein, where both RNP motifs were dispensable for nuclear import and had no NLS activity (Kambach and Mattaj, 1992) the N-terminal U2B″ RNP motif could target fusion proteins containing the C-terminal domain of either UIA or U2B″ to the nucleus.

Two interactions of the U2B″ N-terminal RNP motif have been characterized: specific binding to U2 snRNA (Scherly et al., 1990b) and interaction with the U2 snRNP-specific A′ protein (Scherly et al., 1990a; Boelens et al., 1991). These interactions are mutually dependent to a certain degree, because specific binding of U2B″ to U2 snRNA requires U2A′ (Scherly et al., 1990a,b) and stable binding of truncation mutants of U2B″, consisting of amino acids 1-88 or 1-98, to U2A′ was enhanced considerably by the addition of U2 snRNA (Scherly et al., 1990a). Nuclear import of wt U2B″, or of the mutant derivative (2.1) lacking the NLS-encoding middle segment, did not depend on the presence of U2 snRNA.

The possibility that the interaction with U2A′ protein was responsible for the observed transport was tested with an extensive series of mutants of U1A and U2B″ in which amino acids were exchanged between the N-terminal RNP motifs of the two. Only a fraction of the results obtained are presented here. Any of the alterations made in the U2B″ RNP motif abolished transport and also prevented interaction with the U2A′ protein. There was thus a clear correlation between the ability of a given U1A/U2B″ chimera to interact with U2A′ and active nuclear transport. In conclusion, the data represent a strong indication that the U2B″/U2A′ interaction can mediate U2B″ nuclear import.

Nuclear import of U2A′ is not dependent on the U2B″/U2A′ interaction

A detailed mutagenic investigation of the interaction of U2A′ protein with U2B″ in vitro has been published (Boelens et al., 1991). The minimal segment of U2A′ that binds to U2B″ lies between amino acids 1 and 163. Of several mutants tested, amino acid substitutions at positions 44/45, 124/125 or 144/145 abolished U2A′ binding to both U2B″ and U2 snRNA (Boelens et al., 1991). The interaction-negative mutant $A'_{44/45}$ was tested for active nuclear transport. It proved to be transported to essentially the same level as the wild-type U2A′ protein and, as in vitro, not to interact detectably with U2B″. Thus, migration of U2A′ to the nucleus is independent of interaction with U2B″, and, since U2A′-U2 snRNA binding requires the prior formation of the U2A′/U2B″ heterodimeric complex, U2A′ transport to the nucleus can also occur without U2 snRNA.

In summary, our study indicates that both the U2A′ and U2B″ proteins contain NLS sequences capable of mediating independent active transport. In addition, the U2B″/U2A′ interaction can also facilitate U2B″ nuclear import by a second, piggy-back, mechanism. The relative importance of the two transport pathways in vivo is not known and, unfortunately, answering this question is currently beyond our technical capabilities.

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