Intracellular Distribution of the U1A Protein Depends on Active Transport and Nuclear Binding to U1 snRNA

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Abstract. Nuclear transport of the U1 snRNP-specific protein U1A has been examined. U1A moves to the nucleus by an active process which is independent of interaction with U1 snRNA. Nuclear localization requires an unusually large sequence element situated between amino acids 94 and 204 of the protein. U1A transport is not unidirectional. The protein shuttles between nucleus and cytoplasm. At equilibrium, the concentration of the protein in the nucleus and cytoplasm is not, however, determined solely by transport rates, but can be perturbed by introducing RNA sequences that can specifically bind U1A in either the nuclear or cytoplasmic compartment. Thus, U1A represents a novel class of protein which shuttles between cytoplasm and nucleus and whose intracellular distribution can be altered by the number of free binding sites for the protein present in the cytoplasm or the nucleus.

THE U1A protein is a component of the U1 small nuclear ribonucleoprotein particle (U1 snRNP). This particle belongs to a family of snRNPs involved in the removal of introns from pre-mRNA (splicing) and the vertebrate snRNP consists of a U1 snRNA molecule, a set of at least seven proteins common to the major spliceosomal snRNPs, and three specific proteins named U1A, U1-70K, and U1C (for a recent review see Lührmann et al., 1990).

The major U snRNAs (with the exception of U6) are transcribed by RNA polymerase II, and obtain a 7-methylguanosine cap cotranscriptionally. After transcription, they move to the cytoplasm, where they assemble with at least a subset of the common U snRNP proteins and undergo further processing, including cap trimethylation and 3'-end trimming. Subsequently, they return to the interphase nucleus (Eliceiri, 1974; Zieve et al., 1988; Mattaj, 1988). Both the trimethylguanosine cap and the binding of the core snRNP proteins have been shown to be essential for the nuclear migration of some snRNAs in *Xenopus* oocytes, though there are marked differences in the requirement of different snRNAs for the presence of the cap (Fischer et al., 1991; Lamond, 1990; and references therein).

Vertebrate U1-specific proteins appear to be essential for the function of U1 snRNP in splicing, at least in part because of their role in interaction of the snRNP with 5'-splice sites (Heinrichs et al., 1990; Mount et al., 1983; Hamm et al., 1990*a*). However, the binding of U snRNP-specific proteins does not seem to be required for nuclear migration of their snRNAs, as was demonstrated for the U2 snRNP-specific proteins U2A' and U2B'' (Mattaj and de Robertis, 1985) as well as for the U1 proteins U1A, 70K, and C (Hamm et al., 1990*b*). It is therefore possible that independent pathways exist for the nuclear migration of U1 snRNA and the U1-specific proteins, and evidence has been presented that the U1A and U1C proteins may enter the nucleus independently of U1 snRNA synthesis (Feeney et al., 1989).

Nuclear transport of most karyophilic proteins is mediated by nuclear localization signals (NLSs)[1]. Although there is no strict consensus between the signals identified so far, most are rich in basic amino acids. The prototype NLS is present in the SV-40 T antigen (Kalderon et al., 1984*a*,*b*; Lanford and Butel, 1984), and has the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val. However, several NLSs are not of the SV-40 T antigen type (Hall et al., 1984; Silver et al., 1988). Though the sequence of the SV-40 T antigen NLS, in the form of a peptide, is sufficient to direct a non-nuclear protein to the nucleus (Kalderon et al., 1984*b*), additional sequences NH_2-terminal of the minimal peptide are required for full functional activity of the NLS (Rihs and Peters, 1989). Together with the finding that some NLSs are multipartite (Dingwall et al., 1982; Richardson et al., 1988), this indicates that NLSs may in general be more complex than originally thought. The U1A sequence element conferring nuclear localization examined here is uncommonly large, encompassing many, or all, of the central 110 amino acids of the protein. NLS activity appears to depend on the presence of amino acids dispersed throughout this segment. Several proteins have been found to shuttle back and forth across the nuclear envelope (Borer et al., 1989; for a recent review see Goldfarb, 1991), and U1A is shown to belong to this class. At equilibrium, the distribution of the protein between nucleus and cytoplasm does not only depend on the transport rates, however, but on the interaction of U1A with its specific binding sites.

1. *Abbreviations used in this paper*: NLS, nuclear localization signal; Wt, wild-type; WGA, wheat germ agglutinin.

Materials and Methods

Microinjection and Subfractionation of Oocytes

Growing oocytes (Stages V-VI; Dumont, 1972) of *Xenopus laevis* were prepared as described (Hamm et al., 1989). For inhibition of U1 snRNA transport, a deoxyoligonucleotide complementary to the 5'-end of U1 snRNA (U1-5'; Pan and Prives, 1989) was injected at 300 μM final concentration together with α-amanitin at 2 μg/ml final concentration. In vitro translated proteins (30–50 nl/oocyte) were injected into the cytoplasm. Oocytes were incubated in Barth's medium (Gurdon, 1976) containing 200 μg/ml cycloheximide, to inhibit incorporation of [^{35}S] into endogenous proteins, for 14 to 16 h at 19 or 0°C, as indicated. Cycloheximide did not influence the nuclear transport of either U1A protein or lamin L1 (see also Krohne et al., 1989). Dissection of oocytes was performed manually in J-Buffer under a dissecting microscope. Dissected cytoplasms and nuclei were transferred immediately to TNE (50 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA). For experiments not involving RNA analysis, the fractions of eight oocytes per sample were pooled and homogenized in 200 μl TNE through repeated pipetting. For experiments with RNA analysis, nine oocytes per sample were fractionated and homogenized in 250 μl TNE. Total oocytes were analyzed in parallel as recovery controls. The homogenized oocytes were spun twice for 15 min at 13,000 rpm to remove yolk, and 150 μl of the clear supernatant precipitated with 5 vols acetone for 1 h at −80°C. After spinning for 15 min, the samples were dried, dissolved in 20 μl 1× SDS-PAGE sample buffer by 5 min vigorous shaking, incubated for 10 min at 95°C, and loaded on a 12.5% SDS-PAGE gel (Lehmeier et al., 1990). The gels were fixed, subjected to signal enhancement by Entensify (New England Nuclear, Boston, MA), dried, and exposed on XOMAT film (Eastman Kodak Co., Rochester, NY) for 14 h to 3 d.

For RNA analyses, 50-μl aliquots were taken after the second centrifugation (see above), 150 μl of water was added and the samples were extracted twice with phenol/chloroform. 1/10 vol of 3 M Na-acetate and 6 μl of linear polyacrylamide as carrier (Gaillard and Strauss, 1990) was added and the samples were precipitated with ethanol. After dissolving in 20 μl RNA loading dye, 4 μl (~1 oocyte equivalent) was run on an 8% denaturing acrylamide gel and analyzed by hybridization with antisense probes against U1, U2, U5, and U6 snRNAs (Vankan et al., 1990).

The U1ΔDsnRNA mutant, the RNA polymerase III-transcribed U1 snRNA (Pol III U1), and the U2 snRNA gene used for transcription in oocytes have been described (Hamm and Mattaj, 1990; Mattaj and Zeller, 1983).

T7 U snRNA Synthesis

The U1, U5, and U6 gene constructs for transcription by T7 RNA polymerase and the T7 transcription are described in Fischer et al., 1991.

WGA Injections

Wheat germ agglutinin (WGA) (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS to a concentration of 20 mg/ml and coinjected with the proteins. As a control, WGA was mixed with βD-*N*-Acetylglucosamine at a concentration of 0.25 M (Sigma Chemical Co.) and coinjected as above.

Expression of Cloned cDNA In Vitro

The *Xenopus* lamin L1 clone contains a point mutation that alters the COOH-terminal Cys codon into a stop codon. The mutated lamin L1 is soluble and does not associate with the nuclear envelope (Krohne et al., 1989) (mutant M8). The mouse dihydrofolate reductase clone used for the construction of U1A fusion mutants is a BamHI/HindIII subclone of pDS 5 (described in Bujard et al., 1987) in pBluescribe(−). This clone and all U1A fusion mutants were linearized with HindIII and transcribed with T7 RNA polymerase, essentially as described (Scherly et al., 1989). In vitro translation was either in wheat germ extract (Promega Corp., Madison, WI) according to the manufacturer's protocol, or in rabbit reticulocyte lysate, as follows: 2–5 μl of mRNA (1 mg/ml) was incubated for 90 min at 30°C together with 2.5 μl amino acid mix lacking methionine (Promega Corp.), 7.5 μl [^{35}S]methionine (Amersham Corp., Arlington Heights, IL), 25 μl rabbit reticulocyte lysate (Promega Corp.) and 35–38 μl water.

U1A Mutants

The point mutant A52/53 has been described (Scherly et al., 1989). Most of the internal deletion mutants are described in Boelens et al. (1991). The remaining mutants were constructed in the same way, making use of point mutants (Scherly et al., 1989). Position 15 in this numbering system corresponds to amino acids 138/139.

The fusion mutants with mDHFR were constructed by subcloning a BamHI/HindIII fragment of the corresponding U1A point mutant (Scherly et al., 1989) into the BglII/HindIII-cut mDHFR clone (see above) for COOH-terminal U1A fragments, or by subcloning a BamHI/HindIII fragment of mDHFR into the BamHI/HindIII-cut corresponding U1A point mutant for NH_2-terminal U1A fragments. The fusion mutants containing internal U1A fragments were constructed by subcloning a BamHI/BamHI fragment of double point mutants (at positions 12/14 and 12/16, see Scherly et al., 1989) into the BamHI site ($N_tA_{12/14,16}f$) or the BglII site ($C_tA_{12/14,16}f$) of mDHFR. The double point mutants were obtained either by site-directed mutagenesis of A14 at position 12 with the Amersham Corp. site-directed mutagenesis kit ($A_{12/14}$), or by subcloning the NarI/HindIII fragment of A16 into the NarI/HindIII-cut A12 mutant.

Quantification of Signal Strengths

Most gels were quantified using the Molecular Dynamics (Sunnyvale, CA) PhosphorImager system equipped with ImageQuant™ software, version 3.0. Where films were quantified instead of gels, the program Image 1.37 for the Apple Macintosh was used in conjunction with a video camera. Only bands from the same experiment and measured by the same technique were compared with one another.

Immunoprecipitations

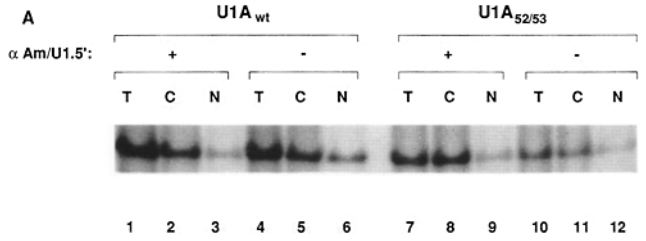
Immunoprecipitations were carried out as follows: 10 oocytes were homogenized in 1 ml oocyte extraction buffer (Vankan et al., 1990) and spun twice for 15 min to remove yolk. To the clear supernatant, 10 μl of a 10% NP-40 solution and 20 μl of a suspension of the antibody coupled to Protein A–Sepharose CL4B (Pharmacia Fine Chemicals, Piscataway, NJ) were added. The samples were rotated for 90 min at 4°C, washed three times with IPP150, incubated 4 min with 30 μl of 2× SDS-PAGE sample buffer at 95°C, and loaded on a denaturing protein gel (see "Microinjection and Subfractionation of Oocytes").

Anti-Sm precipitations were carried out with Y12 mAB (Lerner et al., 1981). Anti-U1A precipitations used the polyclonal rabbit antiserum #856, directed against a section of U1A spanning amino acids Ile 93 to Ser 202, kindly provided by J. Hamm (Columbia University, New York).

Results

The Nuclear Migration of the U1A Protein Does Not Depend on its Prior Binding to U1 snRNA

To address the issue of whether nuclear transport of the U1A protein shows any dependence on U1 snRNA binding, a direct approach was chosen. First, U1 snRNA transcription was inhibited in *Xenopus laevis* oocytes by injection of α-amanitin into the cytoplasm. A DNA oligonucleotide complementary to the 5'-end of U1 snRNA (U1-5') was coinjected with the α-amanitin. This results in the removal of the 5'-end of the endogenous U1 snRNA by RNase H cleavage (Pan and Prives, 1988), and thereby also the 5'-3mG cap of U1 snRNA, which is essential for its nuclear migration (Hamm et al., 1990*b*, Fischer and Lührmann, 1990). This ensures that U1 snRNA transport is effectively inhibited. The removal of the U1 snRNA 5'-end was checked by Northern analysis (data not shown). In vitro–translated wild-type (wt) U1A protein which was subsequently injected into the cytoplasm of the same oocytes accumulated in the nucleus to a certain level. Compared with untreated control oocytes (Fig. 1 *A*, compare lanes *3* and *6*), this level was somewhat lower, however. There are several possible explanations of this: (*a*) some U1A protein moves to the nucleus together with U1 snRNA; or (*b*) the presence of newly transcribed U1 snRNA molecules in the nuclei of the control oocytes stimu-



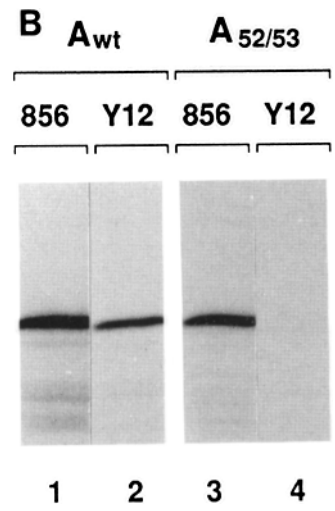


Figure 1. Influence of U1A binding to U1 snRNA on its nuclear transport. (*A*) *Xenopus laevis* oocytes were injected with a mixture of a DNA oligonucleotide complementary to the 5′ end of U1 snRNA (U1-5′, 300 μM final concentration) and α-amanitin (lanes *1–3*, *7–9*, 2 μg/ml final concentration), and incubated for 2 h at room temperature. In vitro–translated U1A wt (lanes *1–6*) or A52/53 (lanes *7–12*) protein was then injected into the cytoplasm of treated, or untreated oocytes. *T*, *C*, and *N* denote protein extracted 14 h after injection from total oocytes, cytoplasmic or nuclear fractions, respectively. Oocytes in lanes *4–6* and *10–12* were not treated with α-amanitin or the U1-5′ deoxyoligonucleotide. (*B*) Immunoprecipitation of A wt and A52/53 with anti-U1A and anti-Sm antibodies. *Xenopus laevis* oocytes were injected with either in vitro–translated A wt protein (lanes *1* and *2*) or A52/53 (lanes *3* and *4*). After incubation for 14 h at 19°C, the oocytes were homogenized and immunoprecipitated with either a polyclonal anti-U1A rabbit antiserum (*856*, lanes *1* and *3*) or a monoclonal anti-Sm antibody (*Y12*, lanes *2* and *4*).

lates nuclear accumulation of U1A (see below). Nevertheless, the fact that nuclear accumulation of U1A is observed under conditions where U1 snRNA transport is inhibited indicates that U1A can be transported to the nucleus by a mechanism which is independent of binding to U1 snRNA. Further evidence comes from a variant of the experiment described above (Fig. 1 *A*, lanes *7–12*). Here, the protein injected after the treatment with α-amanitin/U1-5′ was A52/53. This point mutant of U1A is unable to bind U1 snRNA in vitro (Scherly et al., 1989). To determine whether this is also the case in vivo, A52/53 as well as wt U1A were immunoprecipitated from oocytes with an antibody against the common U snRNP proteins present in U1 snRNP (Y12, Lerner et al., 1981). This antibody can precipitate U1A only if it is associated with U1 snRNA in the assembled U1 snRNP. The result (Fig. 1 *B*, lanes *2* and *4*) shows that wt U1A is precipitated by this antibody, whereas A52/53 is not. Both forms of the protein can be immunoprecipitated by an anti–U1A-antibody (#856, Fig. 1 *B*, lanes *1* and *3*). Thus, in vivo as well as in vitro, A52/53 cannot bind to U1 snRNA. In spite of this, it migrates to the nucleus to a certain level (Fig. 1 *A*, lane *12*), a process which is not markedly influenced by the inhibition of U1 snRNA transport (Fig. 1 *A*, compare lanes *9* and *12*).

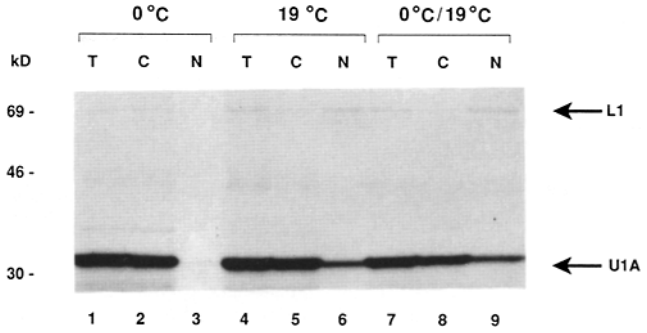


Figure 2. Inhibition of U1A transport by incubation at 0°C. A mixture of in vitro–translated U1A wt protein and lamin L1 was injected into the cytoplasm of *Xenopus laevis* oocytes. The oocytes were then incubated at 0°C (lanes *1–3*, *7–9*) or 19°C (lanes *4–6*) for 14 h. Half of the oocytes kept at 0°C were then incubated for additional 12 h at 19°C (lanes *7–9*).

U1A Enters the Nucleus by an Active Transport Mechanism

Having established that the nuclear transport of U1A and U1 snRNA can be independent of each other, we investigated whether U1A reaches the nucleus by simple diffusion, or whether there is an active transport mechanism. For a variety of nuclear proteins it has been possible to discriminate between these two possibilities by incubating the experimental system at 0°C (Newmeyer et al., 1986; Breeuwer and Goldfarb, 1990). This reduces active transport considerably,

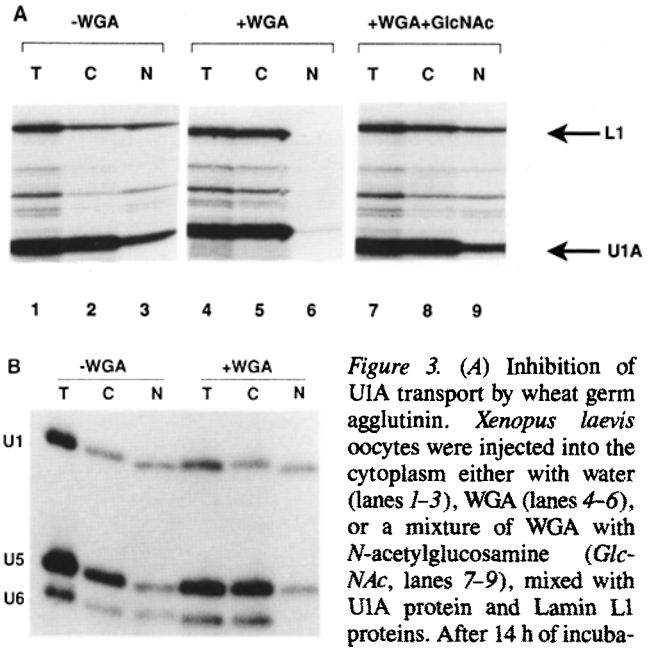


Figure 3. (*A*) Inhibition of U1A transport by wheat germ agglutinin. *Xenopus laevis* oocytes were injected into the cytoplasm either with water (lanes *1–3*), WGA (lanes *4–6*), or a mixture of WGA with *N*-acetylglucosamine (*GlcNAc*, lanes *7–9*), mixed with U1A protein and Lamin L1 proteins. After 14 h of incubation, oocytes were processed as described in Materials and Methods. (*B*) Nuclear migration of U1 snRNA is not inhibited by WGA. T7 U1, U5, and U6 snRNA were injected either alone (*left*) or with a solution of 20 mg/ml WGA (*right*) into the cytoplasm of oocytes. The oocytes were dissected after 14 h incubation into nuclear and cytoplasmic fractions, the RNAs were extracted and analyzed on an 8% polyacrylamide denaturing gel.

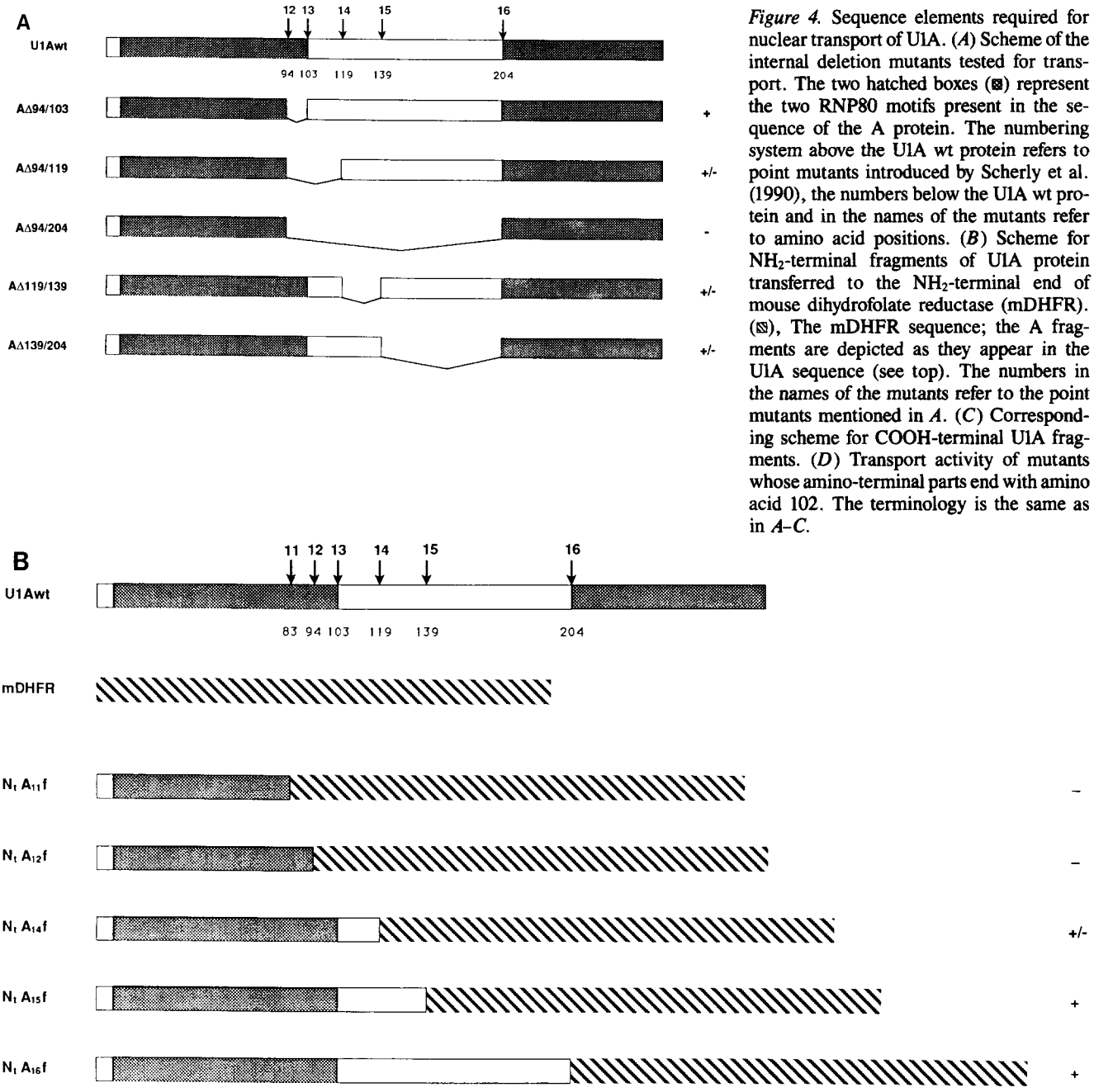
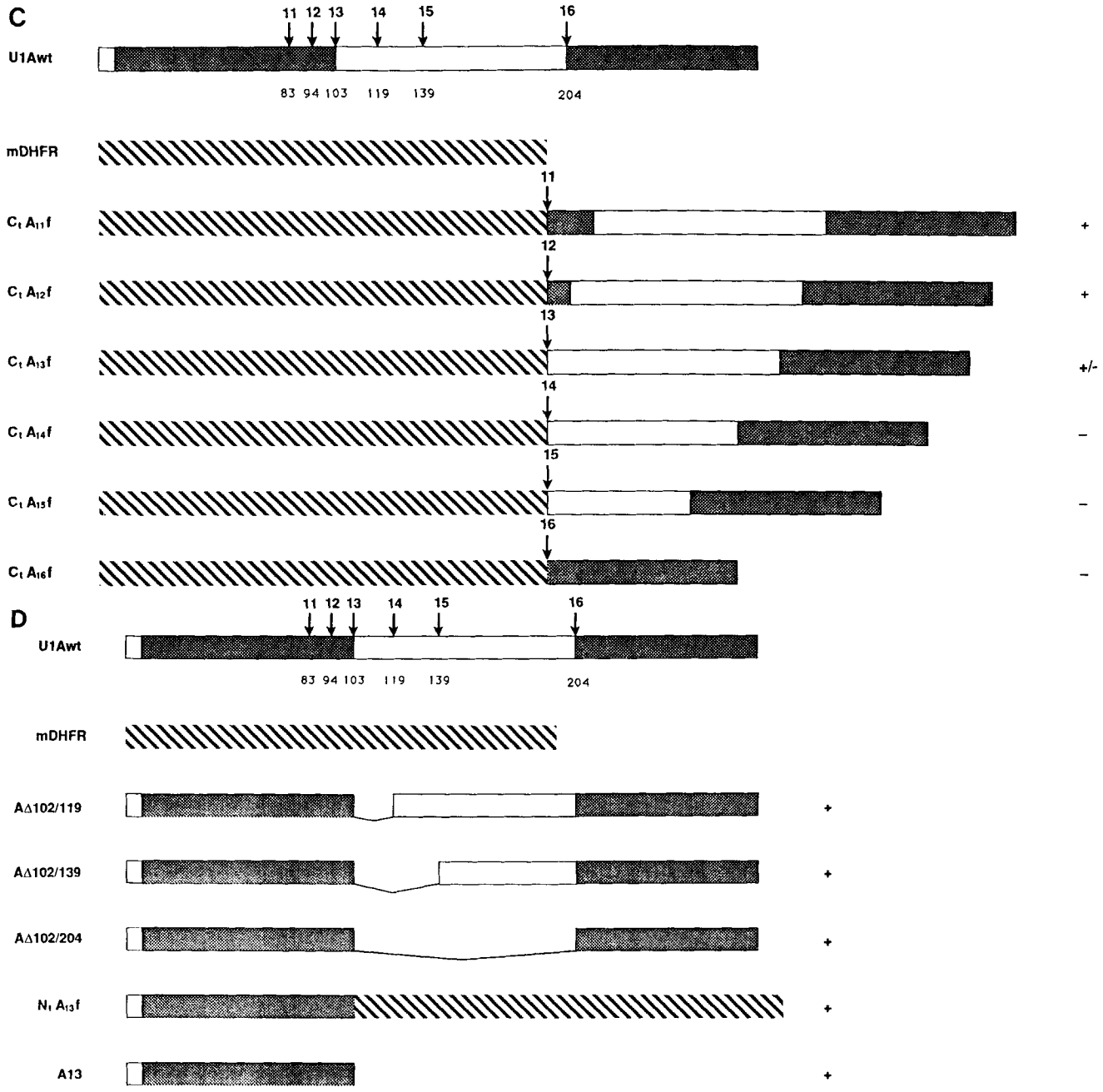


Figure 4. Sequence elements required for nuclear transport of U1A. (*A*) Scheme of the internal deletion mutants tested for transport. The two hatched boxes (▩) represent the two RNP80 motifs present in the sequence of the A protein. The numbering system above the U1A wt protein refers to point mutants introduced by Scherly et al. (1990), the numbers below the U1A wt protein and in the names of the mutants refer to amino acid positions. (*B*) Scheme for NH_2-terminal fragments of U1A protein transferred to the NH_2-terminal end of mouse dihydrofolate reductase (mDHFR). (▨), The mDHFR sequence; the A fragments are depicted as they appear in the U1A sequence (see top). The numbers in the names of the mutants refer to the point mutants mentioned in *A*. (*C*) Corresponding scheme for COOH-terminal U1A fragments. (*D*) Transport activity of mutants whose amino-terminal parts end with amino acid 102. The terminology is the same as in *A–C*.

whereas diffusion is only reduced to ~90% of the level observed at ambient temperature. To assay U1A transport, U1A wt was injected together with the actively transported nuclear protein Lamin L1 (Krohne et al., 1989) into the cytoplasm of oocytes and incubated at 0 or 19°C, respectively (Fig. 2, lanes *1–3* and *4–6*). Subsequently, the cytoplasmic and nuclear fractions were analyzed. Nuclear accumulation of U1A is drastically reduced in the oocytes incubated at 0°C (lane *3*) when compared with oocytes incubated at 19°C (lane *6*). The same is true for L1. Most importantly, this effect is reversible. Oocytes which have been incubated at 0°C for 14 h and then at 19°C for an additional 12 h accumulate U1A and L1 in the nucleus to essentially the same level as control oocytes kept at 19°C (Fig. 2, compare lanes *9* and *6*). As a control, the mouse dihydrofolate reductase (mDHFR) protein was injected into the cytoplasm of *Xenopus* oocytes. After 14 h incubation at 0 or 19°C, respectively, the oocytes were dissected into nucleus and cytoplasm and analyzed on a denaturing protein gel. Nuclear accumulation was almost identical at 0 or 19°C, indicating free diffusion (data not shown).

The existence of an active transport mechanism for U1A led us to investigate the effect of a general inhibitor of the transport of a variety of nuclear proteins, the plant lectin WGA (Finlay et al., 1987; Dabauvalle et al., 1988). *Xenopus laevis* oocytes were coinjected with a solution of 20 mg/ml



WGA (see Materials and Methods) and U1A with Lamin L1 as internal control (Fig. 3 *A*, lanes *4–6*). This treatment inhibits U1A and L1 transport to a marked extent (compare lane *6* with the nuclear signal from untreated control oocytes, lane *3*). The effect is specific, since it can be overcome by the coinjection of the competing sugar *N*-acetylglucosamine (Fig. 3 *A*, compare lanes *9* and *6*). *N*-acetylglucosamine is known to relieve the inhibitory effect of WGA (Finlay et al., 1987). The transport of the pol III-transcribed U6 snRNA is similarly inhibited by WGA, while neither U1 nor U5 snRNA transport is markedly affected by WGA at these concentrations in our experiments (Fischer et al., 1991; Fig. 3 *B*). This again suggests that transport of U1 snRNA and U1A protein are mechanistically dissimilar. The lack of significant amounts of nuclear U1A protein in the presence of WGA (Fig. 3 *A*, lane *6*) further suggests that most, if not all, of the U1A transport observed in these experiments occurs independently of U1 snRNA.

The Region of U1A Which Is Necessary and Sufficient for Nuclear Transport Consists of the Central 110 Amino Acids of the Protein

Given the evidence for active, U1-independent transport, we next investigated the requirements in terms of primary structure in U1A for this process. Of particular interest was the

observation of a sequence in U1A (arg107 glu lys arg lys pro lys; Feeney and Zieve, 1990) which bears a resemblance to the SV-40 T antigen class of nuclear localization signals (see introduction). To identify the regions of U1A important for transport competence, we performed an extensive mutagenic analysis. Since the experimental system showed a high degree of variability in terms of transport activity, an internal control (usually lamin L1) had to be coinjected with each mutant, and the transport competence of a given mutant was always compared to the nuclear accumulation of wt U1A protein in the same batch of oocytes. As a further means to correctly assay the active transport of a mutant, each experiment was conducted at 0 and 19°C, and the nuclear accumulation was compared. Gel bands were quantified either by direct scanning with an analytical imaging system, or by densitometry of exposed films. Mutants were classified as positive, intermediate, or negative. To be scheduled as positive, a mutant had to accumulate in the nucleus to a level >70% of the wt, and the transport had to be reduced upon cooling to 0°C by at least a factor of 3.5. Intermediate phenotype mutants accumulated in the nucleus to within 40–70% of the wt and showed a reduction of transport by a factor of 1.5 to 3.5 upon cooling to 0°C. Any activity below these values was scheduled as transport negative. Each mutant was classified according to the outcome of several independent experiments. Because of the large number of mutants tested and the complexity of the results obtained, most of the data is presented in summary form in Fig. 4.

The first series of constructs tested consisted of internal deletion mutants (Fig. 4 *A*). Deletion of the region containing the putative NLS (mutant $A_{\Delta 94/119}$) decreased, but did not abolish, nuclear transport which already indicated that although this sequence element may contribute to transport competence, it is not alone sufficient for a transport-positive phenotype. Non-overlapping internal deletions spanning various lengths of the central 110 amino acids of U1A ($A_{\Delta 119/139}$, $A_{\Delta 139/204}$) also decreased nuclear transport. Only the removal of the whole middle segment of the U1A protein led to complete loss of active transport (mutant $A_{\Delta 94/204}$).

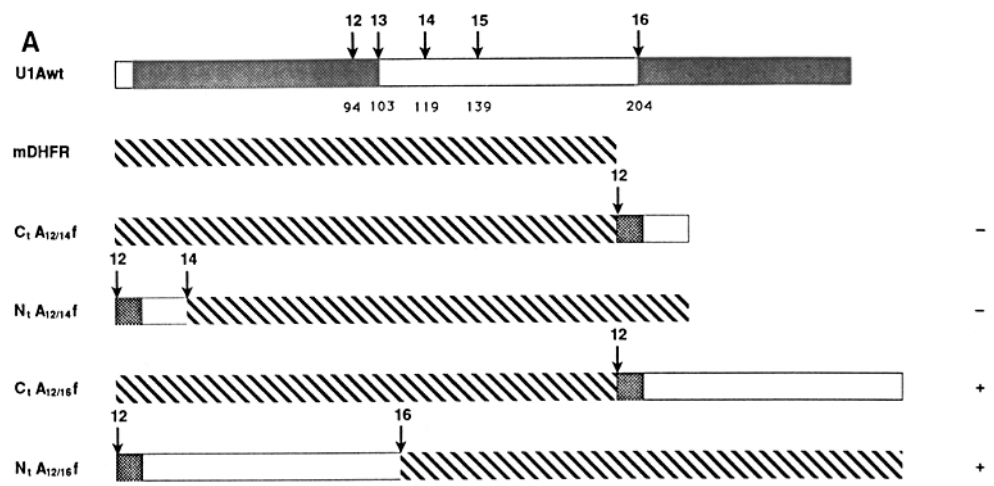
As a next step in the analysis, NH_2- and COOH-terminal fragments of the U1A protein were tested as truncations as well as in fusion constructs with the cytoplasmic protein mouse dihydrofolate reductase (mDHFR, Fig. 4, *B* and *C*). NH_2-terminal U1A fragments increasing in length (whether as truncations, data not shown, or as fusions, Fig. 4 *B*) showed a continuous increase in transport activity, ranging from negative for a mutant ending at amino acid 94 (N_tA_{12}f) through intermediate to positive for mutants ending at positions 139 and 204 (N_tA_{15}f and N_tA_{16}f; Fig. 4 *B*). A corresponding series of COOH-terminal fusion proteins (Fig. 4 *C*) showed the complementary behavior, namely a transport-positive phenotype for the fragment starting at position 94 (C_tA_{12}f), reduction in transport activity as the fragments got shorter, and no transport for the mutants starting at positions 119, 139, and 204 (C_tA_{14}f, C_tA_{15}f, and C_tA_{16}f; Fig. 4 *C*). The results presented so far with the deletion and fusion derivatives of U1A are all consistent with the picture that the region between amino acids 94 and 204 directs nuclear transport. The effect of removal of several non-overlapping subfragments of this region suggests that the more of this region that is present, the stronger is the NLS activity. Two points, however, have to be made in this context. First, the results in Fig. 4 *C* were obtained with COOH-terminal fragments tested as fusion mutants with mDHFR. Mutant proteins that were truncated, beginning either at positions 94 or 102, were transport defective. Second, comparison of $A_{\Delta 94/103}$ and $A_{\Delta 94/119}$ with C_tA_{13}f and C_tA_{14}f, respectively (Fig. 4 *A* and *C*), reveals that the fusion proteins accumulate in the nucleus to a lesser extent than the deletion derivatives. We have no good explanation for these differences and can only suggest that structural effects due to having the U1A NLS, or parts of it, in different contexts, can influence its activity.

Thus far, the results obtained with the internal deletion mutants (Fig. 4 *A*), the NH_2-terminal and the COOH-terminal U1A fragments (as mDHFR-fusions, Fig. 4, *B* and *C*) are consistent with the hypothesis that the central 110 amino acids (from amino acid 94 to 204) are necessary and sufficient to confer transport competence to the U1A protein. Several mutants tested did not fit into this picture, however (Fig. 4 *D*). They include internal deletion mutants ($A_{\Delta 102/119}$, $A_{\Delta 102/139}$, and $A_{\Delta 102/204}$), an NH_2-terminal mDHFR fusion mutant (N_tA_{13}f) and a truncation mutant (A13), and all accumulate in the nucleus to a higher level than the wt protein (data not shown). In all these mutants, the NH_2-terminal section of U1A ends at amino acid 102. Two possible explanations for this phenomenon can be considered: first, sequences COOH-terminal of position 102 might downregulate the activity of a transport signal present in the vicinity of this site, such that mutants missing the downstream sequences would be transported to a higher level than mutants containing them. Second, an artificial nuclear transport signal might be created by mutagenic manipulations of the U1A sequence at this position.

To discriminate between these possibilities and to assay directly for the NLS activity of the U1A fragment between amino acids 94 and 204, we fused both this and a shorter fragment (from amino acid 94 to 119) to both ends of mDHFR and tested them in the transport assay (Fig. 5). Conferring nuclear transport to a non-nuclear protein is one of the criteria which have been used to define NLSs (Silver, 1991; and references therein). The shorter fragments ($C_tA_{12/14}$f and $N_tA_{12/14}$f) do not confer active transport on mDHFR, whereas the longer ones ($C_tA_{12/16}$f and $N_tA_{12/16}$f) do. The corresponding gels are depicted in Fig. 5, *B–E*. There is essentially no difference in the nuclear accumulation of $C_tA_{12/14}$f and $N_tA_{12/14}$f at 19°C (Fig. 5 *B*, lanes *6* and *9*) and at 0°C (Fig. 5 *C*, lanes 6 and 9), showing that they are not actively transported. $C_tA_{12/16}$f and $N_tA_{12/16}$f, on the other hand, show a marked difference between the transport level at 19°C (Fig. 5 *D*, lanes *3* and *6*) and at 0°C (Fig. 5 *E*, lanes *3* and *6*). Together with the earlier results this defines the fragment from amino acid 94 to 204 as the NLS of the U1A protein. Note that this region is almost completely non-overlapping with the sequences of U1A required for interaction with U1 snRNA (Scherly et al., 1989; Lutz-Freyermuth et al., 1990; Nagai et al., 1990).

The Nucleo-cytoplasmic Distribution of U1A Depends on U1A Binding Sites

The fraction of U1A protein found in the nucleus varies between 20 and 50% of the total injected material in different experiments, dependent on the batch of oocytes used. Most



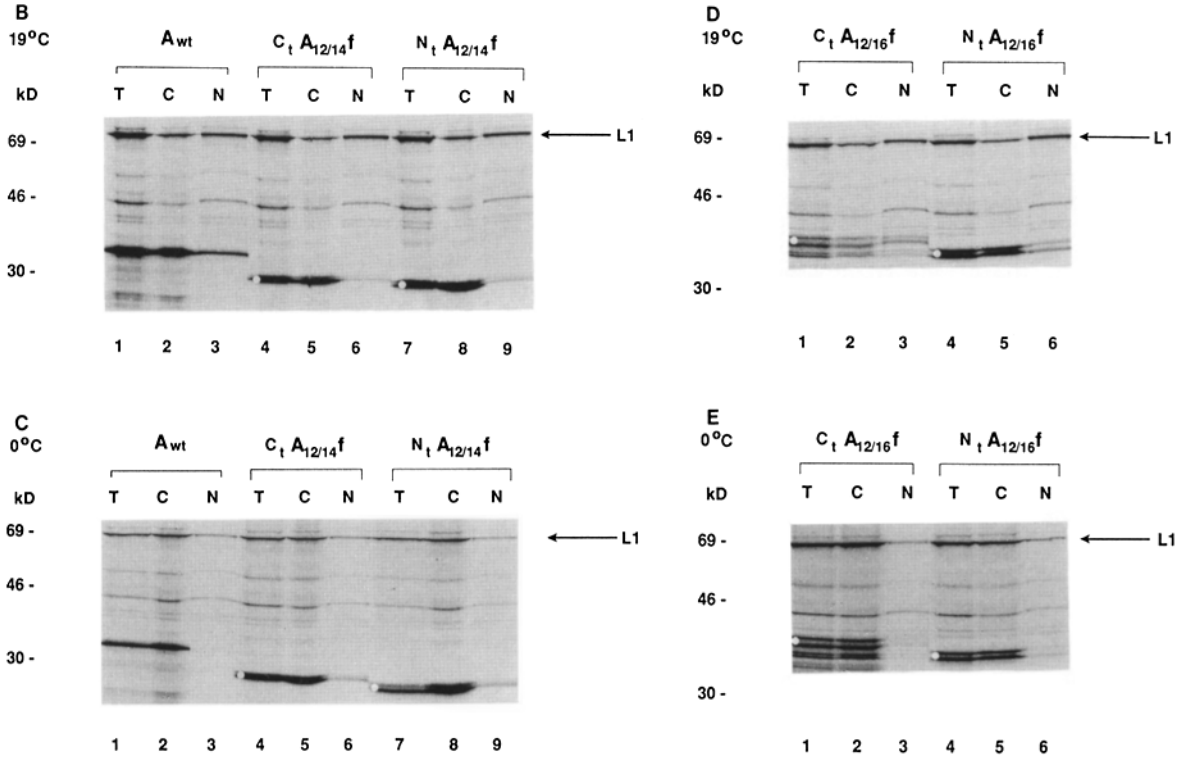
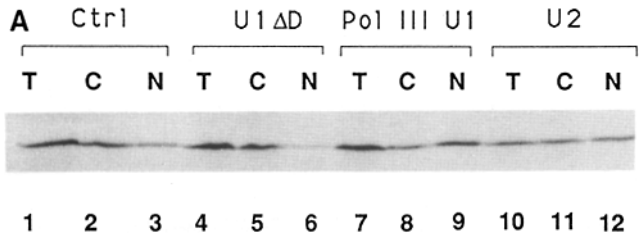
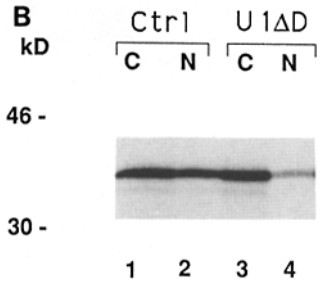


Figure 5. (*A*) Scheme for internal fragments of U1A protein transferred to the COOH-terminal ($C_tA_{12/14}f$, $C_tA_{12/16}f$) or NH_2-terminal ($N_tA_{12/14}f$, $N_tA_{12/16}f$) end of mouse dihydrofolate reductase (*mDHFR*). See legend to Fig. 4 for explanation of symbols. (*B*) Transport activity of U1A wt, $C_tA_{12/14}f$ and $N_tA_{12/14}f$ at 19°C. *Xenopus laevis* oocytes were injected with a mixture of lamin L1 and U1A wt or one of the above U1A mutants. After 14 h incubation at 19°C, oocytes were dissected and the subfractions analyzed. (*C*) Corresponding experiment for $C_tA_{12/14}f$ and $N_tA_{12/14}f$ at 0°C. (*D*) Corresponding experiment for $C_tA_{12/16}f$ and $N_tA_{12/16}f$ at 19°C. (*E*) Corresponding experiment for $C_tA_{12/16}f$ and $N_tA_{12/16}f$ at 0°C. All mutants (see also Fig. 5, *B*, *C*, and *D*) run as doublets. The relevant bands are marked with white dots.

nuclear proteins migrate to the nucleus to a higher level, including the lamin L1 used as internal control in this study, accumulating in the nucleus to between 50 and 70% of total protein. We investigated possible reasons for this difference. To determine whether slow kinetics was responsible for the comparatively low nuclear accumulation, a time course over 72 h was performed. Oocytes were injected with U1A and *Xenopus* N1 protein (Kleinschmidt et al., 1986) as internal control and incubated at 19°C. After 0, 8, 16, 32, 48, and 72 h, five oocytes were dissected into cytoplasm and nu-





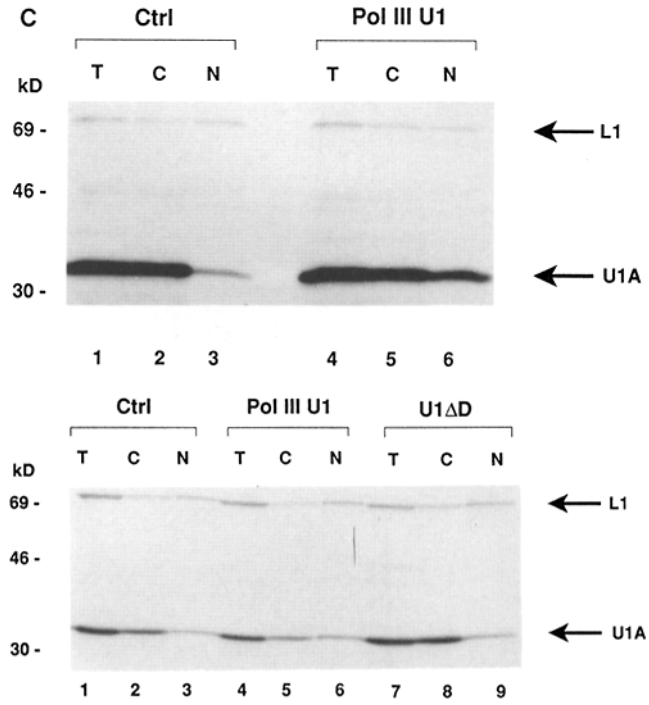


Figure 6. Effect of cytoplasmic and nuclear binding sites for U1A protein on its nucleocytoplasmic distribution. (*A*) The genes for the U1 snRNA mutants U1ΔD (a substitution mutant in the Sm site which does not bind the core proteins and is incapable of nuclear migration, lanes *4–6*) and Pol III U1 (a U1 wt snRNA transcribed by RNA polymerase III, incapable of leaving the nucleus, lanes *7–9*) and U2 snRNA (lanes *10–12*) were injected together with a DNA oligonucleotide complementary to the 5′ end of U1 snRNA into the nuclei of *Xenopus laevis* oocytes. The oocytes were incubated for 2 h at room temperature and then injected with in vitro–translated U1A protein. Control oocytes in lanes *1–3* were not injected with a U snRNA gene. *T*, *C*, and *N*, protein extracted from total oocytes or cytoplasmic or nuclear fractions, respectively, 14 h after injection. (*B*) As in Fig. 5 *A*, showing an example where the nuclear/cytoplasmic ratio of U1A wt reached an exceptionally high level in control oocytes (lanes *1* and *2*). (*C*) As in Fig. 5 *A*, showing an example where the nuclear/cytoplasmic ratio of U1A wt reached an exceptionally low level in control oocytes (lanes *1–3*). (*D*) U1A wt was injected into the cytoplasm of *Xenopus laevis* oocytes. After 14 h incubation, the Pol III U1 gene (lanes *4–6*), or the gene for U1ΔD (lanes *7–9*, both genes at 1 mg/ml) were injected into the nuclei of the oocytes. All oocytes were then incubated for additional 12 h, dissected, and the fractions analyzed. Oocytes in lanes *1–3* were not injected with a U1 snRNA gene. The figures from the quantitation by the PhosphorImager are as follows: *PolIII U1*, 139% transport of A wt control; *U1ΔD*, 77% transport of A wt control. The values for the lamin L1 internal control are 106% (*Pol III U1*) and 107% (*U1ΔD*) transport of L1 external control.

cleus, the fractions pooled, and analyzed together with extracts from total oocytes on denaturing protein gels. The level of nuclear accumulation of both U1A and N1 (as judged by comparison of signal intensities from nuclear and cytoplasmic fractions) reached levels of about 30 and 80%, respectively, of the total amount injected after 12 h, and this level did not change markedly after longer incubation periods (data not shown). Thus, slow kinetics does not seem to be responsible for the difference in nuclear accumulation levels of U1A and other karyophilic proteins.

Several explanations for the transport behavior of U1A are possible. The in vitro–translated protein might only be functional in terms of transport activity to a limited extent, because of the lack of posttranslational modifications. Alternatively, the protein may be anchored in the cytoplasm by binding to some component, or it may be shuttling between cytoplasm and nucleus. In the latter case, it should be possible to influence the nucleo-cytoplasmic distribution of U1A by introduction of binding sites for the protein in the cytoplasm or the nucleus. To test this, two different U1 snRNA mutants were introduced into oocytes to create additional U1A binding sites in cytoplasm or nucleus, respectively.

The first U1 snRNA tested for its effect on U1A protein distribution was U1ΔD. This RNA cannot bind the common U snRNP proteins, and is therefore unable to enter the nucleus (Hamm et al., 1990*b*). The second mutant tested was a U1 snRNA transcribed by RNA polymerase III (Pol III U1) due to the altered promoter structure of its gene. This transcript cannot leave the nucleus (Hamm and Mattaj, 1990). Both RNAs, however, retain the binding site for the U1A protein (Scherly et al., 1989). After in vivo transcription of these mutants, U1A protein was injected and the cytoplasmic/nuclear ratio of U1A protein determined after overnight incubation. SnRNA distribution was determined by Northern analysis to check for efficient transcription of the injected snRNA genes (data not shown). The effect of these mutant RNAs on nuclear accumulation levels of U1A is depicted in Fig. 6 *A*. The presence of additional binding sites for U1A in the cytoplasm due to the introduction of U1ΔD shifts the cytoplasmic/nuclear ratio to higher values compared with untreated control oocytes (compare the signal ratio of lanes *5* and *6* with the ratio of lanes *2* and *3*). Conversely, the introduction of additional nuclear binding sites by injection of Pol III U1wt increases nuclear accumulation of U1A when compared with the control (compare the signal ratio of lanes *8* and *9* with the signal ratio of lanes *2* and *3*). Introduction of U2 snRNA has no effect (compare the signal ratio of lanes *11* and *12* with the signal ratio of lanes *2* and *3*). These effects were reproducible, although not large. We noted, however, that the degree to which the introduction of the mutant U1 snRNAs influenced the distribution of U1A depended on the basic transport level of the experiment. Fig. 6 *B* depicts a case where a high basic level of U1A nuclear accumulation is significantly reduced upon introduction of U1ΔD (com-

pare lanes *2* and *4*), whereas Fig. 6 *C* shows a marked increase in nuclear accumulation of U1A by the introduction of Pol III U1 in an experiment with a low basic transport rate (compare lanes *3* and *6*).

The above results could still be explained either by the assumption that the U1 snRNA molecules present in cytoplasm or nucleus could act as competitors for the transport reaction and thereby influence the nucleo-cytoplasmic distribution of U1A through simple mass action, or by the U1A shuttling between cytoplasm and nucleus and reaching an equilibrium dependent upon the number of binding sites in each compartment. To differentiate between these two possibilities, the time order of the above experiments was reversed. First, U1A protein was injected into the cytoplasm of oocytes. After overnight incubation, the gene for Pol III U1 or U1ΔD, respectively, was injected, and, after 12 h of additional incubation, their effect on U1A distribution was determined by comparison with control oocytes. The nuclear/cytoplasmic ratio of U1A was increased to 139% in the case of Pol III U1 (Fig. 6 *D*, compare signal ratios of lanes *2* and *3* with *5* and *6*, and see figure legend for details of quantitation). The injection of U1ΔD gene decreased the nuclear/cytoplasmic ratio of U1A to 77% (Fig. 6 *D*, compare signal ratios of lanes *2* and *3* with *8* and *9*). The effect was reproducible both qualitatively and quantitatively in five independent experiments. The conclusion is that U1A must be shuttling between cytoplasm and nucleus, since its nucleo-cytoplasmic distribution can be disturbed by the introduction of nuclear binding sites even after it has reached equilibrium.

Discussion

U1A Nuclear Transport and Binding to U1 snRNA

A series of experiments that address several aspects of the nuclear transport of the U1 snRNP-specific A protein have been presented. First, it was shown that nuclear migration of U1A does not depend on its binding to U1 snRNA. This is consistent with previous results obtained in mouse fibroblasts where nuclear accumulation of U1A and U1C was shown to be unaffected by inhibition of U1 snRNA synthesis (Feeney et al., 1989). The concept of two independent transport pathways for U1 snRNA and U1A is supported by the differential inhibition of nuclear transport of the two by WGA. U1 snRNA transport is not affected by WGA under the conditions used here (Fischer et al., 1991, Fig. 3 *B*). (There is evidence that WGA can affect U1 transport under some conditions; Michaud and Goldfarb, 1992 and E. Lund, personal communication.) On the other hand, U1A protein transport is markedly inhibited by the lectin and thus behaves like a variety of other non-RNA-associated nuclear proteins (Finlay et al., 1987; Dabauvalle et al., 1988).

The snRNA-independent transport pathway for U1A could be shown here to be an active process: nuclear accumulation of U1A is drastically reduced by cooling to 0°C. Particularly notable in this context is the finding that transport-defective U1A mutants (such as $C_tA_{12/14}f$ and $N_tA_{12/14}f$) enter the nucleus to a significant extent at 0°C, whereas transport-competent mutants (such as $C_tA_{12/16}f$ and $N_tA_{12/16}f$) and the wt protein do not. Transport arrest at 0°C for nuclear proteins that are smaller than the diffusion limit across the nuclear pore complex has been observed previously (Breeuwer and Goldfarb, 1990). The proposed explanation was that NLS-containing proteins bind to some cytoplasmic "receptor", and the resulting complex is retained in the cytoplasm at 0°C (Breeuwer and Goldfarb, 1990).

The U1A NLS

The assay for transport competence of a series of U1A mutants, including internal deletions and fusions to the non-nuclear mouse DHFR protein, determined the sequence elements within U1A necessary and sufficient for nuclear transport. Neither the NH_2-terminal nor the COOH-terminal copy of the RNP80 motif (Sillekens et al., 1987) are required for transport activity. Rather, the results from the internal deletion mutants, and in particular from the fusion of internal U1A fragments to mDHFR suggest that the sequence spanning the region from amino acids 94 to 204 is responsible for nuclear migration of U1A. The overall transport activity of U1A appears to be the result of cumulative effects encoded by sequence elements dispersed throughout this segment. Evidence for this interpretation stems from the fact that nonoverlapping deletions of this region all led to reduced, but not abolished, transport activity, whereas removal of the whole element results in a completely transport-defective mutant. The sequence of amino acids 94–204 is shown in Fig. 7. Positively charged amino acids, found in other defined NLSs (see below), are underlined. Comparison of Figs. *4* and *5* shows that none of these positively charged amino acids are either essential, or sufficient, for nuclear accumulation of U1A. The similarity between U1A and the highly related U2B″ protein is particularly low in this region (Sillekens et al., 1987). Mechanistic differences between nuclear transport of these two proteins are therefore possible. The fact that there is almost no overlap between the sequences required for the binding of U1A to U1 snRNA (Scherly et al., 1989; Lutz-Freyermuth et al., 1990; Nagai et al., 1990) and nuclear transport activity (this study) fits well with the apparent existence of two independent migration pathways for U1 snRNA and U1A.

Data collected from U1A mutants ending at amino acid 102 were not consistent with the conclusions drawn above. These mutants were highly active in transport, irrespective of the presence or absence of other sequence elements shown

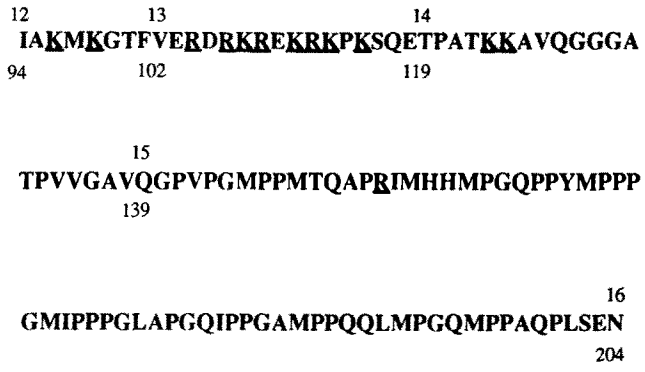


Figure 7. Sequence of the region of U1A required for efficient nuclear targeting. Basic residues are underlined. The positions of various residues in the amino acid sequence are below the line. The numbering system above the sequence is that of the mutant proteins.

to be required for transport competence in other mutants. The fact that this behavior does not depend on the context (it is displayed by the corresponding fusion, truncation, and internal deletion mutants) argues strongly for the creation of an artificial NLS by the introduction of a foreign sequence (gly ser) at this position. In particular, the fact that amino acids 94–119, when fused to mDHFR, have no NLS activity argues strongly that the sequence surrounding amino acid 102 is not, on its own, capable of directing nuclear accumulation. An alternative possible explanation, based on the fact that the U1A protein shuttles between the nucleus and the cytoplasm (see below), is that this collection of mutants allows nuclear import but prevents nuclear export. Many results are difficult to reconcile with this hypothesis, e.g., the difference between Δ102/119 and Δ94/119, but it cannot be completely ruled out at this point.

When compared with "classical" NLSs, like the SV-40 T antigen NLS (Kalderon et al., 1984*a*,*b*; Lanford and Butel, 1984) or the more complex Nucleoplasmin NLS (Dingwall et al., 1982; Robbins et al., 1991), the U1A NLS displays some specific features. First, it is not a "peptide NLS", i.e., a segment of 5 to 8 basic amino acids interrupted by a proline, as is the case for the signals of a whole class of karyophilic proteins (for reviews see Goldfarb, 1989; Garcia-Bustos et al., 1991; Silver, 1991). Though the central 110 amino acids of U1A identified as its NLS bear a net positive charge (Sillekens et al., 1987, Fig. 7), the fact that transport activity depends in a cumulative way on the presence of the segment as a whole indicates a marked structural difference between the U1A NLS and the aforementioned NLSs. Proteins that contain more than one independent NLS (e.g., Polyoma T antigen; Richardson et al., 1988) or a single bipartite NLS (Nucleoplasmin; Robbins et al., 1991) have been documented. The large size and apparent internal redundancy of the U1A NLS does not seem similar to either of these classes. The cumulative negative effect of combining non-overlapping deletions, or the similar positive effects of including more of the NLS segment in fusion proteins, suggests that much or all of the 110-amino acid segment of the protein is required for normal NLS function. It may be that correct folding of this segment of the protein is required to generate the NLS. The only other reported NLS that may be similar is that of the yeast Gal 4 protein. The NH_2-terminal 74 amino acids of Gal 4 contain an NLS, and several point mutations scattered throughout this region of the protein affect the efficiency of nuclear localization of the Gal 4 protein (Silver et al., 1988). It should be borne in mind that the apparent complexity of the U1A NLS may be due to the fact that the protein shuttles between the cytoplasm and the nucleus. If there is also a signal for nuclear export in U1A it could complicate the analysis of the NLS. We have not, however, found a class of mutants whose phenotype is easily explicable by the destruction of such an export signal. It will nevertheless be of interest to compare the NLS of U1A with that of other shuttling proteins when they are characterized. It may well be that the import and export signals are interdigitated and difficult to separate.

Establishment of the Nuclear/Cytoplasmic Equilibrium of U1A Protein

U1A consistently accumulated in the nucleus to a lesser extent than other karyophilic proteins used as internal controls. A time course experiment revealed that this was not due to slow transport kinetics. What factor(s) then determined the nucleo-cytoplasmic distribution of U1A? As we could demonstrate, the introduction of additional binding sites for U1A in the cytoplasm or the nucleus in the form of U1 snRNA mutants restricted to one of the two compartments influenced the intracellular distribution of U1A. These effects were independent of the order in which protein and RNA were introduced, i.e., a pre-established protein distribution was altered by artificially increasing the number of nuclear or cytoplasmic binding sites for the protein. Together, these results suggest that the nucleo-cytoplasmic distribution of U1A at equilibrium is not determined by its transport rate, but rather by the number of free binding sites in the two compartments. The binding site of the U1A protein is the second hairpin loop of U1 snRNA (Scherly et al., 1989). U1 snRNA leaves the nucleus immediately after its transcription, but then rapidly reaccumulates in the nucleus after assembly in the cytoplasm with common U snRNP proteins (Mattaj, 1988; Zieve et al., 1988). Our results suggest that the number of free nuclear and cytoplasmic U1A binding sites will determine the intracellular distribution of the U1A protein. The physiological relevance of this is not immediately clear, but the effect will be to ensure that there will always be a moderate excess of U1A protein over U1 snRNP in the nucleus. Thus, if U1A should dissociate from an snRNP, there will be an excess of free protein helping to ensure that the full complement of U1 proteins are restored. On the other hand, the nuclear excess of free U1A protein will be maintained at a relatively low level. This might be important to prevent its interaction with RNAs other than U1 in the nucleus.

For many years it was a matter of debate whether nuclear proteins reached the nucleus by active transport or by diffusion and subsequent binding to (nondiffusible) nuclear components (for discussion see Dingwall et al., 1982). While the distribution of U1A is probably determined by binding interactions, it is of interest that it reaches the nucleus by an active transport mechanism. Whether export from the nucleus is also an active process is currently unknown. Attempts have been made to determine this experimentally by direct injection of the protein into the nuclei of oocytes followed by incubation at 0 or 19°C and subsequent dissection, analogous to the recent experiments with the B3 and B4 proteins (Mandell and Feldherr, 1990). However, in the case of U1A, the results obtained were very inconsistent (our own unpublished data). Insight into this question is therefore only to be expected from the use of other experimental systems.

Whether the binding of U1A to U1 snRNA prevents reexport of the protein directly, by covering up an "export signal," or is because of the attachment of U1A to an RNP which is either too large or too immobile to be exported from the nucleus, thus remains an open question.

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