Nuclear Import of the U1A Spliceosome Protein Is Mediated by Importin α/β and Ran in Living Mammalian Cells*

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U1A is a component of the uracil-rich small nuclear ribonucleoprotein. The molecular mechanism of nuclear import of U1A was investigated in vivo and in vitro. When recombinant deletion mutants of U1A are injected into the BHK21 cell cytoplasm, the nuclear localization signal (NLS) of U1A is found in the N-terminal half of the central domain (residues 100–144 in mouse U1A). In an in vitro assay, it was found that the U1A-NLS accumulated in only a portion of the nuclei in the absence of cytosolic extract. In contrast, the addition of importin α/β and Ran induced the uniform nuclear accumulation of U1A-NLS in all cells. Furthermore, U1A was found to bind the C-terminal portion of importin α. In addition, the in vitro nuclear migration of full-length U1A was found to be exclusively dependent on importin α/β and Ran. Moreover, in living cells, the full-length U1A accumulated in the nucleus in a Ran-dependent manner, and nuclear accumulation was inhibited by the importin β binding domain of importin α. These results suggest that the nuclear import of U1A is mediated by at least two distinct pathways, an importin α/β and Ran-dependent and an -independent pathway in permeabilized cells, and that the latter pathway may be suppressed in intact cells.

Cellular activities in eukaryotic cells are coordinated via the continuous and bi-directional transport of macromolecules between the nucleus and the cytoplasm, which occurs through the nuclear pore complexes (NPCs). The translocation of proteins through the NPCs is mediated by an active and selective mechanism that is controlled by saturable receptors and signals that are termed nuclear localization signals (NLSs) and nuclear export signals (reviewed in Refs. 1 and 2).

The best characterized active nuclear protein import is mediated by a basic type NLS, referred to as “classical NLS,” and which contains one or more clusters of basic amino acids. The import of substrates containing classical NLS such as SV40 large T antigen is initiated by the formation of an NLS-dependent complex with importin α/β in the cytoplasm, which is referred to as the nuclear pore-targeting complex. Importin α recognizes the NLS and binds to importin β via its N-terminal sequences, which are rich in basic amino acids, and is referred to as the importin β binding (IBB) domain (3). Importin β accounts for the targeting of the complex to the NPC. In addition to this classical import pathway, several different types of pathways have been identified, which involve importin β or importin β family members that bind directly to their cognate cargoes without importin α (4–6).

It has been shown that there are at least five different forms of importin α in the human and mouse that display significantly different tissue expression patterns (7, 8). It has also been shown that these importin α isoforms interact differentially with specific NLS (9–11). As evidenced by the primary sequences, these molecules can be grouped into three subfamilies, which are referred to Rch1, NPI-1, and Qip1, and which show >50% amino acid identity with one another. Individuals within the same subfamily show more than an 80% amino acid identity. The alignment of these importin α isoforms indicates a rough structural domain organization of importin α (12). There are three conserved domains as follows: the N-terminal IBB domain, the hydrophilic C-terminal regions, and a large central domain that consists of tandemly repeated relatively hydrophobic modules known as armadillo (Arm) motifs. It has been proposed that the Arm repeat domain of importin α harbors the binding site for NLSs (10, 12–14).

A small GTPase Ran and its interacting protein NTF2 (nuclear transport factor 2) are involved in the subsequent translocation step of the complex through the NPC. It is also known that Ran is essential for various active transport pathways including the import of snRNPs, as well as the export of nuclear export signal-containing proteins and several mRNAs (reviewed in Refs. 1 and 15). In the case of the nuclear import of proteins, nuclear Ran GTP functions to terminate the import reaction. For example, after the translocation of the trimeric complex, which is composed of importin α/β and a karyophile with the classical NLS, through the nuclear pores, the direct binding of nuclear Ran GTP to importin β causes the dissociation of the trimeric complex, thus releasing the cargo (16–18).

Nuclear pre-mRNA splicing is dependent upon the activity of a number of trans-acting splicing factors. Small nuclear ribonucleoprotein particles (snRNPs) play a central role in the recognition and alignment of splice sites. Proteins contained in the snRNP particles can generally be divided into the following two classes: those that are common to all snRNP particles and others that are associated with a specific snRNP particle (re-
Nuclear Import of U1A

viewed in Ref. 19). With the exception of U6, which does not leave the nucleus, the biogenesis of these U snRNPs requires the bi-directional transport of the snRNA across the nuclear envelope. Newly synthesized snRNAs exit to the cytoplasm immediately after transcription in the nucleus where they undergo RNA processing and assemble with the snRNP common core proteins prior to returning to the nucleus.

Previous studies of the behavior of specific snRNP proteins, using pulse-chase labeling techniques and cultured somatic cell fractionations, showed that U1 snRNP proteins, U1A and U1C, move to the nucleus independently of de novo snRNA synthesis, i.e. they are transported separately from the rest of the snRNP (20). The study in Xenopus laevis oocytes confirmed that U1A is targeted to the nucleus independently of its association with snRNA (21). U1A contains two RNA binding domains (RNP motifs or RNA recognition motifs). The N-terminal RNA binding domain, along with a small number of flanking amino acids, is required for binding to U1 snRNA. The remaining central domain, which contains 110 amino acids residues, is responsible for the nuclear import of U1A (21). It has been recently reported that the central 110 amino acids can enter the nucleus in an ATP-dependent and cytosol-independent manner in an in vitro transport assay system (22). However, the precise molecular mechanism of the U1A nuclear import and the in vivo behavior remain unclear.

In this study, we show that the central 45-amino acid region of U1A, which exhibits considerable sequence similarity with the corresponding region of U2B, is sufficient for the active nuclear accumulation of U1A in vivo, which results in a narrowing of the NLS domain of U1A. The nuclear import mechanism was also investigated using a recombinant U1A-NLS containing protein and full-length U1A in an in vitro transport assay system and in living cells. The results suggest that at least two distinct import pathways, an importin α/β and Ran-dependent pathway and an independent pathway, are involved in the mediation of the nuclear import of U1A-NLS in vitro. However, it was demonstrated that the nuclear import of full-length U1A is exclusively dependent on importin α/β and Ran in living cells, which poses a new question as to how these two pathways are regulated in the mediation of the import of U1A appropriately in cells.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins—The full length of the mouse U1A gene was amplified from the mouse Ehrlich ascites tumor cells cDNA library by PCR using the synthetic oligonucleotide primers 5′-CTCTTGGATCCATGCCGACCATGCTCATGTCGAG-3′ and 5′-ACGCGGGATCCATTTCTTCGCTTGGACCAAGATATCCT-3′. The PCR product was inserted into the BamHI and EcoRI site of a pGEX-2P-2-hGFP vector that has been described previously (23). Recombinant GST-GFP-U1A fusion protein was expressed by 1 mM isopropyl-β-D-thiogalactopyranoside for 20 h at 20 °C in Escherichia coli strain BL21 (DE3) and purified with glutathione-Sepharose (Amersham Pharmacia Biotech) following the manufacturer’s recommendations. The GST portion was cleaved with PreScission protease (Amersham Pharmacia Biotech) overnight in transport buffer (TB, 20 mM Tris, pH 7.4, 5 mM MgCl2, 0.25 mM sucrose, 1 mM dithiothreitol, and protease inhibitors) containing 200 μM GTP and RNAse to a final concentration of 100 units/ml (1 unit = 3 × 10⁷ nuclei). The suspension was allowed to stand for 1 h at room temperature and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was used as nuclear extract in this study.

Microinjection and in Vivo Import Assay—BHKK21 and tsBN2 cells were grown on coverslips at the temperatures indicated in the figure legends. The microinjection of proteins was performed as described previously (31). Purified GFP-fused U1A and deletion mutants of U1A were used at a concentration of 2 μg/ml in capillary for microinjection experiments. Wild type Ran-GFP and GFP-Ran-GTP were used at a concentration of 5 μg/ml. After incubation for the times indicated in each figure legend, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline. GST-tagged U1A-(1–108) was probed with anti-GST antibodies and fluorescein isothiocyanate-labeled anti-rabbit IgG. The injected GFP-tagged protein and fluorescent labeled 2nd antibodies were detected by fluorescent microscopy (Axiophot II; Carl Zeiss, Inc.). The other details are given in the figure legends.

Solution Binding Assay—The binding ability of purified recombinant U1A to recombinant importin α and importin β was examined using solution binding assay. All assays were performed in TB (total volume of 300 μl). 100 pmol of GST-U1A-NLS was immobilized on 10 μl of a packed volume of glutathione-Sepharose beads by batchwise incubation for 1 h at 4 °C. 100 pmol of recombinant importin β and/or importin α were added to the immobilized GST-U1A-NLS, and the resulting suspension was incubated for 1 h at 4 °C in 300 μl of TB containing 1% of bovine serum albumin and 0.05% of Nonidet P-40 in the presence or absence of 200 pmol of Ran-GTP or Ran-GDP. The beads were then washed with TB extensively and eluted with 10 mM glutathione. The eluted material was analyzed by SDS-PAGE. For the pull-down experiments using a nuclear extract, 100 pmol of GST-tagged NPI-1 deletion mutants were immobilized on glutathione-Sepharose, and 300 μl of the nuclear extract of mouse Ehrlich ascites tumor cells was added in the presence or absence of 100 pmol of importin β. After a 1-h incubation at 4 °C, the bound fractions were analyzed by immunoblotting with anti-U1A monoclonal antibody.

Cell-free Transport Assay—In vitro transport assays were performed essentially as described previously (32) with some modifications. In order to decrease the remaining factors in the cytoplasm after digoxigenin permeabilization, cells were incubated at 25 °C for 10 min after digito- nin permeabilization and then washed three times with TB. The assay solution (10 μl) in TB containing 1% bovine serum albumin was incubated with permeabilized cells for 15 min with or without 1 μM ATP, 5 mM creatine phosphate, and 20 units/ml creatine phosphokinase at 30 °C, or on ice, as described in the respective figure legends. The assay solution contained import factors, substrate proteins, and a Ran mixture (4 μM Ran-GDP, 0.4 μM RanBP1, 0.4 μM RanGAP1, and 0.4 μM NTF2) or a cytosolic extract as described in each figure legend. After the import reaction, the cells were fixed with 3.7% formaldehyde. T-NLS was prepared as a control transport substrate by chemical cross-linking of allophycocyanin (Calbiochem) with SV40 T antigen NLS-containing peptides as described previously (30).

RESULTS

The Central 45-Amino Acid Domain of U1A Is Sufficient for the Nuclear Localization in Living Cells—In order to investigate the nuclear import mechanism of U1A in living somatic cells, recombinant GFP-tagged mouse U1A (Fig. 2A, construct α) was injected into the cytoplasm of cultured BHKK21 cells. As shown in Fig. 1, U1A efficiently accumulated in the nucleus after only 5 min of incubation at 37 °C, whereas no nuclear import of U1A was observed when the cells were incubated on ice. In order to clarify the region that is responsible for nuclear...
accumulation in living somatic cells, we next constructed four deletion mutants (Fig. 2A, constructs b–e). U1A protein can be divided into three domains, the N-terminal and C-terminal RNA binding domains and the central 110-amino acid domain, that was reported to be necessary and sufficient for nuclear transport in Xenopus oocytes (21). Twenty min after the injection, the subcellular localization of these mutant proteins was examined. As shown in Fig. 2B, both the N-terminal and C-terminal RNA binding domains were located in the cytoplasm, whereas the two mutants that contain the central domains accumulated in the nucleus. Since these two mutants contain both GFP and GST tags, they are too large to diffuse passively through the nuclear pores. Moreover, the accumulation of these deletion mutants was not observed when the cells were incubated on ice (Fig. 2B, d, and data not shown for construct e). Therefore, we conclude that the N-terminal half of the central domain (residues 100–144 in mouse U1A) is sufficient for the active nuclear import of U1A in living somatic cells.

Nuclear Accumulation of U1A-NLS in an in Vitro Transport Assay System—In order to investigate the molecular mechanism of U1A import, the central 110-amino acid region, fused to GST and GFP (U1A-NLS), was used as a transport substrate (Fig. 2A, construct d) in the following experiments. When U1A-NLS was incubated with digitonin-permeabilized cells (32) in the presence of an ATP regeneration system, a portion (∼10–20%) of the cells showed nuclear accumulation of U1A-NLS in the absence of exogenous cytosolic extracts (Fig. 3A, a). In contrast, when the U1A-NLS was incubated with crude cytosolic extracts in digitonin-permeabilized cells, an obvious and uniform nuclear accumulation in all cells was observed (Fig. 3A, b). When the cells were incubated on ice or in the absence of ATP, no nuclear import was observed (Fig. 3A, c–f). Furthermore, wheat germ agglutinin treatment completely inhibited the nuclear import of U1A-NLS (data not shown). These results were highly reproducible, strongly suggesting that the nuclear import of U1A-NLS can occur via two distinct pathways, cytosolic extract-dependent and -independent ones. Moreover, it should be noted that when GST-U1A-(100–144)-GFP was used as an import substrate, the same results were obtained in the in vitro transport assay (data not shown).

Importin α/β and Ran Mediate the Cytosolic-dependent Import Pathway of U1A-NLS—We next attempted to analyze the cytosolic extract-dependent pathway and to understand better the transport factors for U1A-NLS. The central domain of U1A contains some basic amino acid residues (see Fig. 2), although 100–125 in mouse U1A, which corresponds to 94–119 in human, has been shown not to be sufficient for nuclear accumulation of U1A-NLS in Xenopus oocytes and in vitro (21, 22). To determine whether importin α/β is able to mediate the nuclear import of U1A-NLS, we examined the effect of the IBB domain, which is the importin β binding domain of importin α and which is well known to inhibit competitively the importin α/β-dependent import pathway. The IBB domain was added to the in vitro import assay in the presence of a cytosolic extract. As shown in Fig. 4a, the IBB domain strongly inhibited the nuclear accumulation of U1A-NLS except for 10–20% of the cells. In addition, the issue of whether the nuclear import was reconstituted by recombinant importin α/β and small GTPase Ran was tested. After a 15-min incubation at 30°C with Rch1 (one of the importin α family molecules), importin β, and a Ran mixture (see “Experimental Procedures”), the U1A-NLS accumulated in the nucleus efficiently (Fig. 4c). When Rch1 or importin β alone was added in the presence of a Ran mixture, or when Rch1/importin β were added in the absence of the Ran mixture, the U1A-NLS only accumulated in 10–20% of the nuclei, and no stimulation of nuclear uptake of U1A-NLS was observed (data not shown). These results indicate that Rch1/importin β, in conjunction with Ran, is able to mediate the import of U1A-NLS into the nucleus. It should be noted that the nuclear import of the U1A-(100–144) region was reconstituted with importin α/β and Ran to a similar extent to that of U1A-(100–209) in the in vitro transport assay system (data not shown).

U1A-NLS Forms a Complex with Rch1/Importin β in the Absence of Ran-GTP—It is well known that the classical basic type NLS directly binds to importin α, forming a ternary complex with importin α and β in the absence of Ran-GTP and that this complex targets the nuclear envelope to translocate through the nuclear pores. In order to confirm that the U1A-NLS binds to importins, a solution binding assay with recombinant U1A-NLS and importins was performed. As shown in Fig. 5A, although the interaction of the U1A-NLS with Rch1 or with importin β was not detected under our assay conditions, it obviously formed a ternary complex with Rch1 and importin β. On the other hand, GST-C-terminal RNA binding domain-GFP (Fig. 2A, construct c) did not bind to the importin β and Rch1/ importin β complex (data not shown). Consistent with the previous reports, the GST-SV40 T antigen-NLS-GFP, which was used as a positive control, bound directly to Rch1 but not to importin β and formed a ternary complex with Rch1 and importin β. In the case of U1A-NLS, although high concentrations (up to 1 μM) of recombinant proteins were used, no obvious direct binding between U1A-NLS and Rch1 was detected (data not shown). It has been demonstrated that the direct binding of Ran-GTP to importin β causes the dissociation of the ternary complex including importin α/β and the NLS substrates (16, 18). Therefore, to determine whether Ran-GTP dissociates the Rch1/importin β/U1A-NLS, a solution binding assay was performed in the presence of Ran-GTP or Ran-GDP. The results clearly demonstrated that Ran-GTP but not Ran-GDP induced the dissociation of the Rch1-importin β-U1A-NLS complex (Fig. 5B). These results support the view that the nuclear import of U1A is mediated by Rch1/importin β and Ran.

U1A-NLS Forms a Complex with All of Three Importin α Subfamilies—It has been reported that three types of importin α subfamilies show distinct substrate specificity (8, 9, 26). As a result, we examined the issue of whether other importin α subfamily molecules, NPI-1 and Qip1, are able to form a complex with U1A-NLS in the presence or absence of importin β. The results clearly showed that, whereas the U1A-NLS did not bind to NPI-1 and Qip1 directly, in the presence of importin β, U1A-NLS formed a complex with NPI-1/importin β or Qip1/importin β as efficiently as with Rch1/importin β (Fig. 5C). GST-U1A-RBD2-GFP, used as a negative control, did not form complex with importin α/β (data not shown). Identical results were obtained by using the U1A-NLS-(100–144) construct (data not shown) suggesting that the nuclear import of U1A can be mediated by all of three distinct importin α subfamilies.

U1A Binds to a Domain That Is Distinct from SV40 T-NLS-binding Site of Importin α—As described above, importin α is divided into three functional domains as follows: the N-termi-
nal IBB domain, the central Arm repeat domain, and the C-terminal region. The central portion lacking the first 77 amino acids and the last 63 amino acids retains the ability to bind to the monopartite SV40 T antigen NLS and the bipartite nucleoplasmic NLS, indicating that the conventional basic type NLS binding domains map to the Arm repeat region of importin-a (26). U1A-NLS-(100–144) contains not only positively but negatively charged amino acids and differs from the conventional NLSs. To understand more clearly the binding domain of importin-a for U1A, we assessed the binding ability of several NPI-1 deletion mutants (Fig. 6A) to endogenous U1A in a mouse Ehrlich ascites tumor cell nuclear extract, since U1A is mainly contained in the nuclear extract but not the cytosol. It has previously been reported that residues 1–538 (full-length NPI-1), 78–538 (IBB-deleted NPI-1), and 1–475 (C-terminal region-deleted NPI-1) bind to the conventional NLSs, whereas residues 404–538 (C-terminal fragment of NPI-1) do not (26). On the other hand, as shown in Fig. 6B, the full-length NPI-1 and C-terminal region-deleted NPI-1 interacted with endogenous U1A very weakly. In contrast, the IBB-deleted NPI-1 and the C-terminal fragment of NPI-1 significantly bound to U1A (Fig. 6B), whereas it did not bind to the classical basic type NLS as shown previously (26). From these results, we conclude that the region 404–475 of NPI-1 is required for the interaction with U1A. This region is obviously distinct from the classical basic type NLS-binding site (see “Discussion”).

We next examined the effect of classical NLS on the trimeric complex formation of U1A-NLS/importin-αβ. When an excess amount of SV40 T-NLS peptide was added to the solution binding assay with U1A-NLS, importin-α and importin-β, complex formation of U1A-NLS/importin-αβ was not detected (data not shown), indicating that importin-α is unable to bind simultaneously to U1A-NLS and T-NLS.

Cytosol-independent Nuclear Import of U1A-NLS—In order to determine the exact ratio of cells that import the U1-NLS in the absence of cytosol, we counted the nuclei in which the U1A-NLS accumulated in a cytosol-independent manner. As a result, it was found that the U1A-NLS accumulated in the nuclear IBB domain, the central Arm repeat domain, and the C-terminal region. The central portion lacking the first 77 amino acids and the last 63 amino acids retains the ability to bind to the monopartite SV40 T antigen NLS and the bipartite nucleoplasmic NLS, indicating that the conventional basic type NLS binding domains map to the Arm repeat region of importin-a (26). U1A-NLS-(100–144) contains not only positively but negatively charged amino acids and differs from the conventional NLSs. To understand more clearly the binding domain of importin-a for U1A, we assessed the binding ability of several NPI-1 deletion mutants (Fig. 6A) to endogenous U1A in a mouse Ehrlich ascites tumor cell nuclear extract, since U1A is mainly contained in the nuclear extract but not the cytosol. It has previously been reported that residues 1–538 (full-length NPI-1), 78–538 (IBB-deleted NPI-1), and 1–475 (C-terminal region-deleted NPI-1) bind to the conventional NLSs, whereas residues 404–538 (C-terminal fragment of NPI-1) do not (26). On the other hand, as shown in Fig. 6B, the full-length NPI-1 and C-terminal region-deleted NPI-1 interacted with endogenous U1A very weakly. In contrast, the IBB-deleted NPI-1 and the C-terminal fragment of NPI-1 significantly bound to U1A (Fig. 6B), whereas it did not bind to the classical basic type NLS as shown previously (26). From these results, we conclude that the region 404–475 of NPI-1 is required for the interaction with U1A. This region is obviously distinct from the classical basic type NLS-binding site (see “Discussion”).

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Cytosol-independent Nuclear Import of U1A-NLS—In order to determine the exact ratio of cells that import the U1-NLS in the absence of cytosol, we counted the nuclei in which the U1A-NLS accumulated in a cytosol-independent manner. As a result, it was found that the U1A-NLS accumulated in the

Fig. 2. The central 45-amino acid domain is responsible for the nuclear localization of U1A. A, schematic presentation of mouse U1A and its deletion mutants for the analysis of nuclear accumulation activity, expressed in E. coli as recombinant GFP and/or GST fusion proteins. The numbers below the proteins refer to amino acid positions. The lower part shows an amino acid comparison between U1A-NLS and U2B-NLS. Bold letters show basic amino acids. Asterisks indicate acidic amino acids. B, each fragment (construct b–e in A) was injected into the cytoplasm of cultured BHK21 cells. After a 20-min incubation at the indicated temperature, cells were fixed, and the subcellular localization of the injected proteins was detected by GFP or polyclonal anti-GST antibodies and fluorescein isothiocyanate-conjugated anti-rabbit IgG.
FIG. 3. Nuclear import of U1A-NLS in an in vitro transport assay. A, GST-U1A-(100–209)-GFP (Fig. 2A, construct d) was used as a substrate for the permeabilized Mandin Darbey bovine kidney cells. The import reaction was performed for 15 min in the absence (a, c, and e) or presence (b, d, and f) of an Ehrlich ascites tumor cell cytosolic extract at 30 °C (a, b, e, and f) or on ice (c and d). An energy-regenerating system was added in the upper panel. The reactions were terminated by fixation, and import was analyzed by fluorescence microscopy. B, GST-U1A-(100–209)-GFP or T-NLS (SV40 T antigen NLS-conjugated allophycocyanin, see “Experimental Procedures”) was added to digitonin-permeabilized cells and incubated with an energy-regenerating system in the absence (upper panels) or presence (lower panels) of the cytosolic extract. GST-U1A(100–209)-GFP but not T-NLS accumulated in a portion of the nuclei even in the absence of the cytosol.

nuclei of 19.1 ± 8.5% of the digitonin-permeabilized cells (total ~740 cells/12 independent experiments) in the absence of cytosolic extracts. We next attempted to exclude the possibility that this is due to the impairment of nuclear membrane integrity. First, SV40 T antigen NLS substrates did not enter the nuclei, in which the U1A-NLS accumulated in the absence of cytosol (Fig. 3B). Second, cytosol-independent nuclear accumulation was completely inhibited when the cells were incubated on ice or in the absence of ATP (Fig. 3A), which is consistent with the previous report (22). Third, cells in which the U1A-NLS accumulated in the nucleus in a cytosol-independent manner were not stained with anti-lamin monoclonal antibodies without permeabilization with Triton X-100 (data not shown).

In order to exclude the possibility that the permeabilized cells still contained a significant amount of transport factors, i.e. importin α and β, the IBB domain was added to the in vitro import assay system, which was performed in the absence of the exogenous cytosol. The nuclear entry of U1A-NLS was still evident in ~20% of the cells, indicating that the cytosol-independent import of U1A-NLS was not dependent on importin α/β (Fig. 4B). Moreover, when the nuclear import was reconstituted with recombinant importin α/β and Ran, the IBB domain failed to inhibit the nuclear localization of U1A-NLS in some portion of the cells (Fig. 4d). Such cytosol-independent and ATP-dependent import is compatible with previously reported results (22). However, they obviously showed that the U1A-NLS accumulated in the nucleus of nearly all cells in a cytosol-independent manner. The reason for this discrepancy remains to be elucidated.

Full-length U1A Does Not Accumulate in the Nucleus in a Cytosol-independent Manner—The above data, which were obtained using the U1A-NLS, suggest that U1A is able to accumulate in the nucleus by two distinct import pathways, importin α/β-dependent and -independent. To determine whether these pathways actually function in cells, we examined the import of full-length U1A in the in vitro transport assay system. In the absence of a cytoplasmic extract, the nuclear accumulation of GFP-U1A (Fig. 2A, construct a) was not observed at all, whereas the addition of cytosolic extract induced a remarkable nuclear localization of U1A (Fig. 7A). We next attempted to reconstitute the nuclear import of U1A with recombinant importin α/β and Ran. Rch1, NPI-1, and Qip1 were used in this assay because, as shown in Fig. 5C, U1A-NLS efficiently formed a complex with all of the importin α subfamily members in the presence of importin β. The results indicate that all the subfamilies of importin α mediated the nuclear accumulation of the full-length U1A in the presence of importin β and Ran (Fig. 7, d–f). The elimination of the Ran mixture suppressed the import of U1A (Fig. 7c and data not shown). These results
blocked the nuclear import of U1A, whereas wild type Ran did not.

To confirm further the involvement of Ran in the nuclear import of U1A in living cells, tsBN2, which is a temperature-sensitive mutant derived from the BHK21 cell line (34) and has a point mutation in rec1 gene, was used (35). We showed previously that the loss of RCC1 gene leads to the suppression of nuclear import of classical NLS-containing proteins in living cells (36). The tsBN2 cells were incubated at the permissive or non-permissive temperature, and GFP-U1A was injected into the cytoplasm. The nuclear import of U1A was clearly inhibited in the cells that had been incubated at the non-permissive temperature (Fig. 8), whereas U1A migrated into the nucleus of the cells cultured at the permissive temperature (data not shown). Based on these data, we conclude that Ran is involved in the nuclear import of U1A, and the Ran-independent pathway for U1A is negatively regulated in living mammalian cultured cells.

**IBB Domain Inhibits the Nuclear Accumulation of U1A in Living Cells**—Finally, to determine whether the nuclear import of full-length U1A is dependent upon importin αβ in living cells, we co-injected the IBB domain with full-length U1A into the cell cytoplasm. As shown in Fig. 8B, although MBP alone did not inhibit the nuclear import of U1A, the same concentration of MBP-IBB strongly suppressed the nuclear accumulation of U1A as well as T-NLS substrate. From these findings, we conclude that importin αβ mediates the nuclear import of full-length U1A in living cells.

**DISCUSSION**

Is U1A-NLS One of the Conventional NLSs?—In this study, we showed that the 100–144-amino acid region in mouse U1A is sufficient for targeting to the nucleus in living mammalian cells. Furthermore, it was found that this domain forms a complex with importin αβ in the absence of Ran-GTP. Although a number of basic type NLSs have been reported to be recognized by importin α with a high degree of specificity, these classical NLSs lack a stringent consensus, except for general features of short stretches of amino acids that contain a high proportion of positively charged residues (37). Although U1A-NLS is recognized by all of the importin α subfamilies, it was found that the U1A-NLS has quite unique features that are different from the known conventional NLSs such as the SV40 T-NLS. The U1A-NLS was transported into the nucleus in an importin αβ-dependent and -independent manner. Moreover, the basic amino acids cluster (PKSQETP119) in human U1A-NLS, which corresponds to residues 100–125 in mouse U1A) was not sufficient for nuclear accumulation in vivo and in vitro (21, 22). Therefore, the NLS activity of U1A required a much longer domain.

In addition, it was demonstrated that U1A-NLS binds to a unique site of importin α that is distinct from the binding site for the conventional monopartite and bipartite NLSs, such as those of SV40 large T antigen and Xenopus nucleoplasmin. The crystal structure of yeast importin α revealed that nucleoplasmin NLS and SV40-T antigen NLS interact with importin α at two sites in the Arm motifs, namely the second through fourth and seventh and eighth (14, 38). On the other hand, in this study, it was found that the binding site of importin α for U1A is located in the C-terminal portion of the Arm repeats (residues 404–475) probably corresponding to Arm 9 and its flanking regions (Fig. 6). Moreover, we were able to detect the binding of U1A-NLS to importin α only in conjunction of importin β. It is known that although the interaction of the classical NLS to importin α is considerably enhanced in the presence of importin β, the classical NLS is able to bind importin α alone. From these findings, we conclude that the U1A-NLS
is a novel basic type NLS, which interacts with importin α at a site that is distinct from the conventional NLS. U1A and U2B⁺ are closely related and components of U1 snRNP and U2 snRNP, respectively. Both proteins consist of two RNA binding domains at the N- and C-terminal ends that are highly conserved, whereas the central domains show relatively poor conservation. The NLSs of the two snRNP proteins, which are located in the poorly conserved central domain, showed efficient cross-competition and behaved similarly in the in vitro nuclear transport assay (22). The comparison of U1A-NLS (45 amino acids as demonstrated in this study) with U2B⁺-NLS (56 amino acids as reported previously (22)) revealed characteristic amino acid sequence similarity, which has a basic charged cluster divided by acidic amino acids and is surrounded by polar amino acids (Fig. 2A). Although the issue of whether U2B⁺-NLS is transported into the nucleus in an importin α/β-dependent manner was not confirmed, both of the two snRNP proteins might be imported into the nucleus in a similar fashion.

A Possible Involvement of Two Distinct Nuclear Import Pathways for U1A—It was found that in digitonin-permeabilized cells, GST/GFP-tagged U1A-NLS accumulated in the nuclei in a portion of the cells in the absence of a cytosolic extract. This accumulation was not inhibited by the IBB domain of importin α but was completely inhibited by low temperature and ATP depletion, indicating that the cytosolic extract-independent nuclear accumulation does not involve passive diffusion. A similar observation was reported by using the 110-amino acid central portion of U1A fused to nucleoplasmin core region (22). These investigators reported that the central domain of U1A entered the nuclei in a Ran, cytosolic extract-independent but an ATP-dependent manner. On the other hand, in our in vitro transport assay, it was found that only when the cytosolic extract was added did all cells show a nuclear accumulation of U1A-NLS, which was inhibited by the IBB domain of importin α. Moreover, importin α/β formed a complex with U1A-NLS and reconstituted nuclear entry in digitonin-permeabilized cells. These findings suggest that U1A-NLS is able to accumulate in the nucleus via two distinct active import pathways, importin α/β and Ran-dependent and -independent. Nonetheless, when the full-length U1A was used as an import substrate, surprisingly, cytosolic extract-independent nuclear migration was not observed and the import of the full-length U1A was exclusively

![Figure 6](http://www.jbc.org/Downloadedfrom)

**FIG. 6.** U1A-NLS binds to the C-terminal Armadillo repeat motif in importin α. A, schematic presentation of NPI-1 deletion mutants. B, recombinant GST-tagged deletion mutants of NPI-1 were immobilized to glutathione-Sepharose and incubated with nuclear extract of mouse Ehrlich ascites tumor cells. The bound fractions were analyzed by immunoblotting with an anti-U1A monoclonal antibody. C, the C-terminal deleted fragment of NPI-1 is able to interact with U1A in the presence of importin β. The same pull-down assay as described in B was performed with GST-tagged C-terminal deleted fragment of NPI-1 in the absence (lanes 1–3) or presence (lanes 4–6) of importin β. The bound U1A was detected with anti-U1A monoclonal antibody.

![Figure 7](http://www.jbc.org/Downloadedfrom)

**FIG. 7.** Nuclear accumulation of full-length U1A is completely dependent on the cytosolic extract. GFP-U1A was added to the permeabilized cells, followed by incubation for 15 min at 30 °C in the absence (a) or presence (b) of cytosolic extract. Nuclear import of full-length U1A was reconstituted by Rch1 (d), NPI-1 (c), or Qip1 (e and f) with importin β in the presence (d–f) or absence (c) of a Ran mixture.
mediated by importin αβ and Ran in the *in vitro* transport assay. Furthermore, in living cells, the dominant-negative form of Ran mutant and the IBB domain of importin α suppressed the nuclear accumulation of full-length U1A. Collectively, these data point to the fact that importin αβ and Ran mediate the nuclear import of U1A *in vivo* and that cytosolic extract-independent import pathway might be negatively regulated under ordinary cell conditions.

**Why Should Cells Provide Two Distinct Import Pathways for U1A?**—Human hnRNK K protein contains two different NLSs, a conventional basic type NLS (39) and KNS (hnRNK K nuclear shuttling domain), that mediate nuclear entry in a saturable but cytosol-independent and an ongoing RNA polymerase II transcription-dependent manner (40). However, in the wild type K protein, the classical NLS overrides KNS, and the nuclear import of the wild type K protein requires importin αβ and Ran *in vitro* and is independent of polymerase II transcription *in vivo* (8). Therefore, the removal of the classical NLS in the K protein induces KNS activity. Recently, Nemergut and Macara (41) reported that the import of RCC1 can proceed by at least two distinct mechanisms. The first is mediated by the N-terminal domain of RCC1 and is dependent on importin α and β. This pathway preferentially uses the importin α 3 isoform, Qip1. The nuclear import of wild type RCC1 is, however, also mediated by the second pathway that is saturable and temperature-sensitive but does not require soluble transport factors *in vitro* and the Ran-GTP gradient *in vivo*.

Thus, although RCC1 and the K protein show two NLS activities and appear to be mediated by two distinct pathways *in vitro*, both of them as well as U1A preferentially utilize one of the two pathways. Why do cells utilize and regulate two distinct import pathways for one karyophile? Michael et al. (40) suggested that the K protein has more than one function in the nucleus and that each function may be achieved by being targeted to different subnuclear domains by distinct pathways. In fact, RCC1 has two distinct biological functions. One is to generate a Ran-GTP gradient across the nuclear pores (42), and the other is to create a Ran-GTP gradient near the chromatin surface, which is required for mitotic spindle formation (43–45). O’Connor et al. (46) found a unique form of U1A that is not associated with the U1 snRNP (termed snRNP-free U1A or SF-A) and that is complexed with a novel set of non-snRNP proteins. This non-snRNP complex, which contains the U1A, has been suggested to perform an important function in both splicing and polyadenylation of pre-mRNA (47). These findings show that the U1A has two distinct functions in the nucleus, suggesting a localization in the distinct subdomains for each function properly, which supports the suggestion by Michael et al. (40). Alternatively, the fact that U1A, K protein, and RCC1 are important for cell function and extremely abundant in cells may explain the existence of the two import pathways, that is the buffer system may be required for the nuclear import of certain karyophiles. Consistent with this notion, it has been demonstrated that various importins overlap in their ability to import ribosomal proteins. In mammals, ribosomal proteins L23a, S7, and L5 can be imported alternatively by any of the four receptors, importin β, transportin, RanBP5, and RanBP7 (33). In yeast, both Kap123p and another related importin β, Pse1p, are able to mediate the import of ribosomal NLS-bearing substrates into the nucleus *in vivo* (40). In summary, our study demonstrates that 1) U1A has the ability to be transported into the nucleus by at least two distinct pathways and 2) that the import of U1A is regulated to be mediated by an importin αβ-dependent pathway in living mammalian cultured cells under ordinary conditions. Further studies will be required to completely understand how these two pathways are regulated *in vivo*.

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Nuclear Import of the U1A Splicesome Protein Is Mediated by Importin α/β and Ran in Living Mammalian Cells
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The image of the Madin Darby bovine kidney cells incubated without Ehrlich ascites tumor cell cytosolic extract in Fig. 3Ac, incorrectly duplicated the image of cells incubated with extract in Fig. 3Ad. The correct images are shown. This correction does not change the interpretation of the results or the conclusions of this work.