Differential Isoform Expression and Interaction with the P32 Regulatory Protein Controls the Subcellular Localization of the Splicing Factor U2AF26*

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The U2 auxiliary factor (U2AF) is an integral part of the spliceosome that is important for the recognition of the 3′ splice site. U2AF consists of a large and a small subunit, the prototypes of which are U2AF65 and U2AF35. Recent evidence suggests that several homologs of both U2AF subunits exist that are able to regulate alternative splicing. Here we have investigated the expression, intracellular localization, and nucleo-cytoplasmic shuttling of one homolog of the small U2AF subunit, U2AF26, and a splice variant lacking exon 7, U2AF26ΔE7. In contrast to the nuclear U2AF26, which displays active nucleo-cytoplasmic shuttling, U2AF26ΔE7 is localized in the cytoplasm. Our studies reveal a nuclear localization sequence in the C-terminal exons 7 and 8 of U2AF26 that differs from the known nuclear localization sequence in U2AF35. In addition, we could identify P32 as a protein that is able to interact with U2AF26 through this domain, and we demonstrate that this interaction is required for the nuclear translocation of U2AF26. Our results suggest the existence of two distinct nuclear import pathways for U2AF26 and U2AF35 that could independently control their intracellular distribution and availability to the splicing machinery. Such a mechanism could work in addition to the differential expression of U2AF homologs to contribute to the regulation of alternative splicing.

With the genomes of several species being sequenced, the interest to understand the mechanisms that increase protein diversity independent of the number of protein-coding genes has risen considerably in recent years. Because of its potential to generate multiple, functionally distinct proteins from one pre-mRNA, alternative splicing is one of the mechanisms that has gained much attention in the post-genomic era (for recent reviews see Refs. 1 and 2). The decision of whether or not an exon is included in the mature mRNA is taken by the assembly of the spliceosome at a given splice site (ss), which leads to the excision of the neighboring intron and inclusion of the respective exon. In the case of alternatively spliced exons, the assembly of the spliceosome is a regulated process that can either take place or not, leading to inclusion or exclusion of the exon from the final message (1).

One of the early steps in spliceosome assembly is the recognition of the 3′ ss by the heterodimeric splicing factor U2AF, which subsequently recruits the U2snRNP to the pre-mRNA (3). U2AF consists of a 35-kDa subunit that binds to the AG dinucleotide at the 3′ splice site (4–6) and a 65-kDa subunit that binds the adjacent polypyrimidine tract upstream of the 3′ splice junction (7). Although the vast majority of research has been devoted to the U2AF35 and U2AF65 subunits, bioinformatic approaches have demonstrated the presence of several homologs of both U2AF subunits in mammalian genomes, some of which have been cloned and functionally tested (8–10). Furthermore, expressed sequence tag data base searches and experimental evidence suggest that most of these subunits are themselves subject to alternative splicing, giving rise to a potentially very large variety of differentially composed U2AF splicing factors (11). Initial studies have suggested a tissue-specific expression of different U2AF subunits (9), but the functional properties of the different U2AF homologs remain to be fully elucidated to understand their individual contribution to tissue-specific alternative splicing.

U2AF26, also called U2AF1-like 4, is a homolog of U2AF35, and both proteins share over 90% of their first 187 amino acids. Consistent with this, U2AF26 binds RNA and forms a heterodimer with U2AF65 through domains that fall into the N-terminal part of the protein (8). In contrast, the C-terminal part of U2AF35, which contains the RS domain, is only weakly conserved in U2AF26. Despite the lack of an RS domain, U2AF26 was able to substitute for U2AF35 in constitutive and enhancer-dependent splicing. A comparison of the RNA binding specificities of U2AF26 and U2AF35 demonstrated that U2AF35 binds with higher affinity to the 3′ consensus ss AGG, whereas U2AF26 shows a preference for AGC or AGA (12). Interestingly, AGC and AGA 3′ ss have been reported to be enriched in alternatively spliced exons in tissues such as brain or muscle (13), pointing to a role of U2AF26 in regulating tissue-specific alternative splicing.

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§ The abbreviations used are: ss, splice site; U2AF, U2 auxiliary factor; NLS, nuclear localization sequence; BSA, bovine serum albumin; hnRNP, heterogeneous nuclear ribonucleoprotein; siRNA, short interfering RNA; GFP, green fluorescent protein; HIV, human immunodeficiency virus; IP, immunoprecipitation.
Many splicing factors have been shown to constitutively shuttle between the nucleus and cytoplasm and to be involved in RNA transport, turnover, and translation (14–16). Both U2AF35 and U2AF65 have been shown to be shuttling proteins (17) and have been implicated in regulating the export of spliced mRNA from the nucleus by recruiting the export factor TAP (18). A role for the large U2AF subunit in RNA transport has also been shown in Drosophila melanogaster, where it associates with intronless RNAs and facilitates their export from the nucleus (19). For both U2AF35 and U2AF65, the nuclear import that might be a mechanism to selectively control the intracellular distribution of different U2AF homologs, which may have important implications in the regulation of alternative splicing.

The protein P32, also known as C1qbp and HABP1, has been implicated in a variety of functions, including binding of the complement component C1q (20) and also binding of the splicing factor ASF/SF2 and the regulation of alternative splicing (21). In addition, P32 plays a role in the regulation of the life cycle of several viruses such as HIV or cytomegalovirus (22, 23). Consistent with the large diversity of its functions, P32 is localized to different subcellular compartments, such as mitochondria, to the outer cell membrane, or to the nucleus (24).

Here we show that P32 interacts with U2AF26, and we provide evidence that this interaction leads to a nuclear localization of U2AF26. Our results define a new mode of P32-dependent nuclear import that might be a mechanism to selectively control the intracellular distribution of different U2AF homologs, which may have important implications in the regulation of alternative splicing.

EXPERIMENTAL PROCEDURES

Cell Culture and Reverse Transcription-PCR—HeLa, COS7, and NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. RNA was extracted from sub-confluent NIH 3T3 fibroblasts using TRIzol, and cDNA was prepared with SuperscriptII reverse transcriptase (Invitrogen). PCRs were performed using the U2AF26-specific primers U2AF26for, atggctgaatatttagcttcg, and U2AF26rev, gaagcgaccatgccggtggtct. PCRs were performed using the U2AF26-specific primers U2AF26for, atggctgaatatttagcttcg, and U2AF26rev, gaagcgaccatgccggtggtct.

Plasmids, Immunoprecipitation and Immunoblotting—Expression plasmids were generated by cloning the respective cDNA in-frame with the sequence encoding enhanced GFP or FLAG to yield C-terminally tagged proteins. The vector backbone used was pEGFP-N3 (Clontech). U2AF26 deletion mutants were generated by PCR with a WT U2AF26 expression plasmid as template (25). P32 open reading frame was amplified from thymus cDNA and cloned in the vectors mentioned above. All constructs were verified by sequencing; primer sequences are available upon request.

COS7, HeLa, or 3T3 fibroblasts were transfected with the indicated plasmids and lysed 24 h later in lysis buffer (25 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1% (v/v) Triton X-100) containing 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 1 mM Na2VO4. For immunoprecipitation, the lysate volume equivalent to 100 µg of protein was dissolved in 0.5 ml of lysis buffer containing 3% BSA and protease inhibitors as above. Precipitation was performed overnight at 4 °C with 1 µg of the indicated antibody and pre-equilibrated protein G-Sepharose or M2 affinity gel suspension, respectively. Beads were washed extensively with lysis buffer, and samples were then separated by SDS-PAGE. Proteins were transferred to Hybond-C membrane (Amersham Biosciences) and blocked with phosphate-buffered saline, 0.05% Tween 20, 1% BSA. Incubation with primary antibody was performed overnight at 4 °C in blocking solution. After washing, the membrane was incubated with secondary horseradish peroxidase-coupled antibody (Dianova) for 1 h at 22 °C in phosphate-buffered saline, 0.05% Tween 20, 2% milk powder, and developed using the SuperSignal ECL system (Pierce). The following primary antibodies were used: anti-FLAG (Sigma), anti-hnRNP C (Abcam), anti-P32 (CeMines), and anti-GFP (Santa Cruz Biotechnology).

Confocal Microscopy, Shuttling and Temperature Shift Assay—1 × 104 HeLa cells or 3T3 fibroblasts per well were seeded in 12-well plates on glass coverslips and transfected 24 h later using Lipofectamine 2000 with 1 µg of the indicated plasmid overnight. 24 h later cells were fixed in 4% paraformaldehyde or methanol and blocked with 1% BSA in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween 20. Cells were then stained with the indicated antibodies and Topro3 to visualize the nucleus and mounted using the ProLong antifade kit (Molecular Probes). Analysis was performed using a confocal microscope (Zeiss, LSM 510) and Zeiss LSM 5 Image Browser software.

Shuttling and temperature shift assays were performed as described (17). Briefly, HeLa cells in 12-well plates on glass coverslips were transfected as above. 24 h later they were overlaid with a suspension containing 2.5 × 105 3T3 fibroblasts and 50 µg/ml cycloheximide for 3 h. Cell fusion was then induced by PEG2000, and shuttling was allowed to take place for 3 h in the presence of cycloheximide before fixation, staining, and analysis as above. For temperature shift assays, transfected HeLa cells were placed at 4 °C in culture medium containing 20 mM HEPES and 50 µg/ml cycloheximide for 3 h and then analyzed as above.

siRNA Transfection—5 × 104 HeLa cells or 3T3 fibroblasts per well were seeded in 12-well plates and transfected 24 h later using Lipofectamine 2000. Cells were incubated with the transfection mixture containing 40 pmol of siRNA overnight. Following transfection, cells were maintained in growth medium for 8 h and then transfected with the indicated expression plasmid using Lipofectamine 2000 overnight. Cells were harvested 8 h later and analyzed by confocal microscopy. Nonsilencing control or P32-specific (Hs_C1QBP_6; Mm_C1qbp_3) siRNAs were purchased from Qiagen.

Yeast Two-hybrid Assay—U2AF26 exons 7 and 8 were cloned in pGBK7T7 (Clontech) in-frame with the Gal4-DNA-binding domain and used as bait to screen a mouse lymphoma cDNA library (Matchmaker Gal4 cDNA library, Clontech). Screening was performed as described by the manufacturer. Briefly, the yeast strain AH109 was sequentially transfected with the bait vector and the library using the lithium-acetate method and
plated on selective plates containing 5 mM 3-aminotriazole. Colonies that grew on −His-Ade-Trp-Leu plates (−4) were transferred to −4 plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to assay β-galactosidase activity. Interaction of clones developing blue color was confirmed by retransfection of isolated library plasmids into yeast cells containing the bait plasmid. To assay the interaction between U2AF26 exon 7 and exon 8 with P32, the respective DNA fragments were cloned in pGBK7, cotransfected with a P32 containing library plasmid into AH109, and assayed for growth on selective plates as above.

RESULTS

Two U2AF26 Isoforms Show Distinct Intracellular Localization—In a recent report we have described a U2AF26 isoform that lacks exon 7 (hereafter U2AF26\(\Delta E7\)) and appears to be nonfunctional with respect to the regulation of CD45 alternative splicing (25). This initial observation prompted us to investigate the isoform expression of U2AF26 in more detail. Using PCR primers that amplify the whole U2AF26 coding region from cDNA of murine 3T3 fibroblasts, we observed two faster migrating bands in addition to the full-length U2AF26. DNA sequencing showed that these products correspond to U2AF26 isoforms generated by alternative splicing, lacking either exon 7 or exons 6 and 7, respectively (Fig. 1A). As the isoform lacking both exons 6 and 7 induces a frameshift thereby altering the amino acid sequence of exon 8 and is present to only a minor proportion, we focused our studies on the \(\Delta E7\) isoform. To quantitate U2AF26 isoform expression, we performed real time PCR assays to detect total U2AF26 with primers and a probe in exons 1 and 2 of the gene or the \(\Delta E7\) variant. A \(\Delta C T\) was calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference (\(\Delta C T[U2AF26] - \Delta C T[GAPDH]\)) and the value was set to 100% for total U2AF26. A fold difference was calculated assuming that a \(\Delta C T\) of 1 equals a 2-fold difference in the presence of the respective mRNAs. Intracellular distribution of U2AF26 and U2AF26\(\Delta E7\) was analyzed using immunohistochemistry. Expression plasmids containing the respective cDNAs were transfected in 3T3 fibroblasts. 24 h post-transfection, cells were fixed, stained with the respective antibodies and 4',6-diamidino-2-phenylindole (DAPI), and analyzed using confocal microscopy.

TABLE 1
Primer and probes for U2AF26 isoform-specific real time PCR

| Primer 1 | Primer 2 | Probe |
|----------|----------|-------|
| U2AF26total | TGGCTGAATATTTGCTCTCCAATCTTTAAAGTAAA | CTGGAAAGCAAGCTTTAACT |
| U2AF26ΔE7 | GCTGCCCTTCCTGACCTGACTTC | GGGAAAGCTGGACCTGTTGAT | CTTGGAAAGCTGCCCAT |
bility would be an altered intracellular localization of U2AF26ΔE7, and we therefore analyzed the distribution of both isoforms in 3T3 fibroblasts using immunohistochemistry. As expected, U2AF26FLAG was exclusively localized to the nucleus, where it showed a punctated staining pattern. In contrast, U2AF26ΔE7FLAG was predominantly localized in the cytoplasm of transfected cells (Fig. 1C), suggesting that loss of exon 7 interferes with the presence of an NLS. Interestingly, U2AF26ΔE7 was able to interact with U2AF65 (see below) arguing that the interaction with endogenous U2AF65 is not sufficient U2AF26 exon 7 and 8 was exclusively found in the nucleus (Fig. 2A), whereas fusions between regions encoded by either exon 7 or exon 8 alone and GFP showed a distribution comparable with GFP. These experiments suggested a new NLS that includes sequences of both U2AF26 exons 7 and 8, because neither of the exons alone was sufficient to induce nuclear localization of U2AF26ΔE7, which has been shown to be the case for U2AF35 (17).

Exon 7 and the C-terminal Exon 8 of U2AF26 Are Both Required for Nuclear Localization—A classical nuclear localization signal (NLS) was absent from U2AF26 exon 7 or any other part of the protein (see Fig. 1A). Moreover, U2AF26ΔE7 remained in the cytoplasm also in the presence of leptomycin B, which inhibits nuclear export (supplemental Fig. 1), suggesting that the lack of exon 7 interferes with nuclear import rather than creating a nuclear export signal. We constructed vectors that allow the expression of GFP fusion proteins to determine the domain responsible for the nuclear localization of U2AF26. As expected, the full-length U2AF26GFP showed a complete nuclear localization, whereas U2AF26ΔE7GFP was localized to the cytoplasm and the nucleus (Fig. 2A, see supplemental Fig. 2 for an additional set of cells) independently from the amount of overexpressed protein. This distribution matched the distribution of GFP alone (Fig. 2A, bottom), which differs from the predominant cytoplasmic localization of U2AF26ΔE7FLAG (Fig. 1C). This suggests that the stronger nuclear localization of U2AF26ΔE7GFP is because of the GFP tag and is not mediated by the U2AF part of the fusion protein. A deletion mutant lacking the first RNP domain in U2AF26 was still localized to the nucleus (Δ60), as was a deletion mutant lacking the first 120 amino acids comprising both RNP-domains (not shown), indicating that nuclear localization was independent of RNA binding. A fusion protein between GFP and the region encoded by U2AF26 exon 7 and 8 was exclusively found in the nucleus (Fig. 2A), whereas fusions between regions encoded by either exon 7 or exon 8 alone and GFP showed a distribution comparable with GFP. These experiments suggested a new NLS that includes sequences of both U2AF26 exons 7 and 8, because neither of the exons alone was sufficient to induce nuclear localization. Consistently, a U2AF26 mutant containing the first 153 amino acids but lacking sequences encoded by exons 7 and 8 of the protein was evenly distributed between the cytoplasm and the nucleus (Fig. 2A). The expression of all fusion proteins used for this study was
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confirmed by immunoblot, which revealed the expression of shorter fusion proteins because of the presence of internal ATGs (Fig. 2B) to only a minor proportion, validating the results obtained in our assay. A summary of the constructs used and their respective localization is given in Fig. 2C.

We noted the presence of several proline residues at the junction between U2AF26 exons 7 and 8, and we hypothesized that they might be critical components of the NLS, as proline-rich sequences have been shown to serve that function (27). However, proline to alanine mutations of proline 191 or 195 and 196 did not impair nuclear localization, suggesting that these residues are not involved in nuclear import of U2AF26 (data not shown).

To confirm the NLS activity of the U2AF26 exons 7 and 8, we fused this domain to the pseudokinase Trb3, which displays cytoplasmic localization when expressed as WT FLAG-tagged protein (Fig. 3A). However, when U2AF26 E7E8 was inserted between Trb3 and the FLAG tag to yield Trb3-E7E8FLAG, the fusion protein showed a nuclear localization. The ability of the E7E8 domain to induce nuclear localization was further confirmed by the use of Trb3-E7E8GFP, which also showed exclusive nuclear staining (Fig. 3C). Expression of fusion proteins was confirmed by immunoblotting (Fig. 3D), which showed minor production of proteins smaller than the full-length products for the GFP fusions, likely due to the presence of internal ATGs. Interestingly, for both Trb3-E7E8FLAG and Trb3-E7E8GFP, we observed a punctated staining pattern that was also observed for WT U2AF26 (Fig. 2A). Taken together, we conclude that U2AF26 exons 7 and 8 together act as a NLS and additionally may lead to localization in nuclear dot-like structures.

U2AF26 Displays Active Nucleo-cytoplasmic Shuttling—Numerous recent reports suggest that proteins primarily known to be involved in splicing also have cytoplasmic functions, and a subset of RNA-binding proteins has been shown to continuously shuttle between the nucleus and the cytoplasm. Among others, U2AF35 has been shown to be a shuttling protein, which is dependent on the presence of its C-terminal RS domain (17). We performed a heterokaryon assay to investigate whether the lack of an RS domain in U2AF26 would impair its ability of nucleo-cytoplasmic shuttling. Interestingly, we found that U2AF26GFP was present in nuclei of 3T3 fibroblasts that were fused with HeLa cells transiently expressing the fusion protein, suggesting that U2AF26 retains the ability to shuttle between the nucleus and the cytoplasm (Fig. 4A). Nuclei of HeLa cells were identified by staining the nonshuttling hnRNP C with an antibody that specifically recognizes the human protein and by the absence of spots of densely packed heterochromatin, which is typical for murine cells. The absence of hnRNP C in 3T3 nuclei also demonstrated that cycloheximide treatment resulted in an efficient block of transcription in the heterokaryons, as de novo transcription would have resulted in the presence of hnRNP C in 3T3 nuclei (Fig. 4A).

As we have shown that exons 7 and 8 from U2AF26 are sufficient to induce nuclear localization of a normally cytoplasmic protein (Fig. 3), we were interested to test whether the same domain would also confer nucleo-cytoplasmic shuttling to that protein. To this end, we transfected the Trb3-E7E8GFP construct in HeLa cells and performed a heterokaryon assay as above. As shown by the presence of Trb3-E7E8GFP in the nuclei of 3T3 cells (Fig. 4B), the fusion protein was able to shuttle between the nucleus and the cytoplasm. Therefore, exons 7 and 8 of U2AF26 do not only represent an NLS but also confer the ability of nucleo-cytoplasmic shuttling.
As some smaller proteins may passively diffuse through the nuclear pore complex (28), we performed a temperature shift assay to test whether the shuttling of U2AF26 is an active process. By decreasing the temperature to 4 °C, processes such as active nuclear transport are blocked, and nuclear proteins that are small enough to passively diffuse into the cytoplasm accumulate there because of the lack of active reimport. Under these conditions both U2AF26GFP (Fig. 4C, left) and Trb3-E7E8GFP (Fig. 4C, middle) were still found in the nucleus of HeLa cells, confirming that the E7E8-mediated shuttling is an active process. In contrast, the shorter E7E8GFP fusion protein (38 kDa) was small enough to leak through the nuclear pore, which served as a positive control for our experimental set up (Fig. 4C).

The C Terminus of U2AF26 Interacts with P32-C1QBP—From the experiments described thus far, we concluded that the C-terminal domain of U2AF26 is sufficient to mediate nuclear localization and nucleo-cytoplasmic shuttling, despite the absence of any known NLS. In an attempt to identify components of the nuclear import machinery that are associated with the nuclear import of U2AF26, we performed a yeast two-hybrid screen with a C-terminal part of U2AF26 consisting of exons 7 and 8 as bait. From a total number of 3 × 10^6 independent clones that were screened, 44 showed an interaction under the most stringent conditions. 39 of these 44 clones contained overlapping cDNA fragments of the ubiquitously expressed P32/C1QBP (hereafter P32). P32 has been implicated in a variety of functions ranging from regulation of the complement system at the cell surface to the assembly of viral particles in the nucleus. Intriguingly, using the drug leptomycin B, which blocks nuclear export, it was shown that P32 also transiently enters the nucleus before being redistributed to other cellular compartments such as mitochondria (24) (supplemental Fig. 3).

To investigate the U2AF26-P32 interaction more closely, we performed coimmunoprecipitation experiments with cell extracts from transfected cells containing P32 GFP or FLAG fusion proteins and U2AF26 deletion mutants. Using an antibody against P32, a coprecipitation of U2AF26 was observed, confirming an interaction of the WT proteins in mammalian cells (Fig. 5A). When anti-P32 was used for IP, we repeatedly found small amounts of U2AF26FLAG in precipitates from COS7 cells that did not overexpress P32, indicating that endogenous P32 precipitated overexpressed U2AF26FLAG (Fig. 5A, top panel, right lane). In the same assay, we did not observe an interaction between U2AF26ΔE7 with P32 despite the presence of P32 in the precipitate, suggesting an essential role of exon 7 in this protein-protein interaction (Fig. 5A). This was confirmed in a further experiment, where we used U2AF26 E7-, E8-, or E7E8GFP fusion proteins and P32FLAG. An interaction was only observed between E7E8GFP and P32FLAG, whereas E7 or E8 alone were not sufficient to mediate an interaction (Fig. 5B). The same result was obtained in an interaction assay in yeast, where we transformed either the bait plasmid used for the two-hybrid screen (E7E8) or bait vectors containing only E7 or E8 together with a P32 containing library plasmid. Under conditions that allow growth only in the presence of a protein-protein interaction, we found that only the combined E7E8 exons were able to interact with P32 (Fig. 5C). As only the P32-interacting E7E8GFP fusion protein showed nuclear localization, these results point to a role of P32 in nuclear translocation of U2AF26.
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To quantify the effect, we counted HeLa cells that were transfected with control siRNA or siRNA specific for P32 with regard to the localization of U2AF26GFP. We found that 26% of cells transfected with P32 siRNA showed partly cytoplasmic staining, whereas this was only the case for 3% of cells treated with nonsilencing control siRNA (Fig. 6E). A similar percentage, 4.5%, of cells expressing U2AF35GFP showed cytoplasmic staining when treated with P32 siRNA, suggesting that P32 is not required for nuclear import of U2AF35.

**DISCUSSION**

In this work, we have characterized a new cytoplasmic isoform of the splicing factor U2AF26, which we have named U2AF26ΔE7. We have determined a region in U2AF26 encoded by exon 7 and 8 that is essential for its nuclear import, and we have identified P32 as a factor that interacts with U2AF26 in this region. Two findings suggest that the interaction with P32 is required for U2AF26 nuclear translocation. First, a nuclear localization sequence in U2AF26 is present in the C-terminal exon 7 and 8, the same region required for interaction with P32. Second, upon reduced expression of P32, achieved by siRNA-mediated knockdown, U2AF26 becomes partially cytoplasmic.

The differential expression of U2AF26 and U2AF35 in murine tissues has been suggested to be involved in regulating alternative splicing (8). Our results presented here reveal another possibility how both proteins might regulate the splicing process, namely through their subcellular distribution. We demonstrate that the major nuclear import pathway for U2AF26 requires an interaction with P32, whereas this is not the case for U2AF35, which contains a classical NLS and is imported independently of P32. As the nuclear import of both proteins seems to be regulated by different mechanisms, the availability of either of the two proteins to the splicing machinery could represent a new way to control alternative splicing. Because both U2AF26 and U2AF35 are continuously exported from the nucleus (17; and this report), regulating their reimport would represent a very rapid way to change their nuclear presence, for instance in response to external stimuli, without the need for resynthesis or degradation. A similar model has been proposed for hnRNP and SR proteins, which are critically involved in regulating alternative

**FIGURE 5.** U2AF26 interacts with P32 via its C-terminal exons 7 and 8. **A,** P32GFP and U2AF26FLAG or U2AF26ΔE7FLAG were overexpressed in COS7 cells (input, bottom). Lysates were mixed, and IP was performed using P32 antibody. Precipitates were analyzed using Western blot (WB) with the indicated antibodies (top and middle). The presence of minor amounts of U2AF26FLAG in precipitates in the absence of P32GFP (right lane) is most likely because of the interaction between endogenous P32 and U2AF26FLAG. Note that a control IP with U2AF35FLAG was not included as we have observed reduced stability of U2AF35 when compared with U2AF26, thus preventing a meaningful comparison of the two proteins in our IPs. **B,** the indicated expression constructs were transfected into COS7 cells, and whole cell lysates were prepared 24 h later (input, bottom). Lysates were then mixed; IP was performed using P32 antibody, and the precipitates were analyzed using immunoblot with anti-GFP (top) or anti-P32 (middle). Only full-length E7E8GFP was coprecipitated with P32 (top panel); the absence of shorter products resulting from the usage of internal ATGs (bottom panel) in the precipitate is consistent with E7 and E8 being both essential for this interaction. As P32 migrates close to the IgG light chain, P32 blots are underexposed. C, a two-hybrid bait vector containing U2AF26-E7, E7E8, or E8 was cotransformed into yeast with a library vector containing P32 cDNA. Yeast growth was assayed under conditions that allowed growth only in the presence of protein-protein interaction. Dotted circles show the position of plated yeast.

U2AF26, we used siRNA-mediated knockdown of P32 and investigated the effect on overexpressed U2AF26GFP. Cells treated with a nonsilencing control siRNA showed robust staining of endogenous P32, and U2AF26GFP was exclusively localized to the nucleus (Fig. 6A). However, when cells were treated with a P32-specific siRNA, P32 protein levels were reduced as evidenced by immunoblot and immunohistochemistry (Fig. 6, B and C). In samples treated with P32 siRNA, U2AF26 partly accumulated in the cytoplasm (Fig. 6B), which was not observed for U2AF35 (Fig. 6D), indicating a role of P32 in mediating nuclear localization of U2AF26 but not U2AF35. Similar results were obtained in murine 3T3 fibroblasts (not shown). To quantify the effect, we counted HeLa cells that were transfected with control siRNA or siRNA specific for P32 with regard
intranuclear shuttling, thus recapitulating all known aspects of nuclear localization, this domain was also sufficient to induce a punctated nuclear staining pattern and nucleo-cytoplasmic shuttling, thus recapitulating all known aspects of nuclear localization by siRNA-mediated knockdown of P32 leading to a cytoplasmic distribution of splicing regulators might be a common regulatory mechanism.

The cytoplasmic localization of U2AF26ΔE7 has led us to define a new nuclear localization signal that includes sequences of exons 7 and 8 of U2AF26, which are neither involved in RNA binding nor in the interaction of U2AF26 with U2AF65 (26). Furthermore, blast searches using the amino acid sequence of exons 7 and 8 did not reveal the presence of other proteins with significant homology. It therefore appears that this NLS is either unique to U2AF26 or depends on the three-dimensional structure that would prevent the identification of its presence in other proteins by sequence-based approaches. In addition to the nuclear localization, this domain was also sufficient to induce a punctated nuclear staining pattern and nucleo-cytoplasmic shuttling, thus recapitulating all known aspects of the nuclear localization of U2AF26 in 26% of the treated cells. The percent-age could be explained by the knockdown efficiency, which was not 100%, as remaining P32 protein was clearly visible in an immunoblot. Alternatively, other nuclear import pathways might lead to nuclear localization of U2AF26 in the absence of intranuclear protein interactions.

![FIGURE 6. P32 is required for nuclear localization of U2AF26.](image)

We were able to identify P32 as a protein that interacts with the domain of U2AF26 encoded by exons 7 and 8 by means of a yeast two-hybrid screen. Coimmunoprecipitation and further interaction assays in yeast provided additional evidence that both proteins bind to each other and confirmed that the regions of U2AF26 encoded by exon 7 and exon 8 are required for this interaction. This finding already suggested a role of the P32-U2AF26 interaction in mediating U2AF26 nuclear localization, as it coincides with the nuclear localization of E7E8GFP but not the single domains. This hypothesis was further corroborated by siRNA-mediated knockdown of P32 leading to a cytoplasmic localization of U2AF26 in 26% of the treated cells. The percent-age could be explained by the knockdown efficiency, which was not 100%, as remaining P32 protein was clearly visible in an immunoblot. Alternatively, other nuclear import pathways might lead to nuclear localization of U2AF26 in the absence of intranuclear protein interactions.
P32. It has been demonstrated that U2AF35 enters the nucleus as a heterodimer with U2AF65 (17), and a version of U2AF35 lacking the NLS is still found in the nucleus, which depends on its ability to interact with U2AF65. Considering that U2AF26ΔE7 is able to interact with U2AF65 (supplemental Fig. 4), we examined the effect of overexpressed U2AF65 on the intracellular distribution of U2AF26ΔE7. Indeed, overexpression of U2AF65 led to accumulation of U2AF26ΔE7 in the nucleus, pointing to the presence of an additional nuclear import pathway for U2AF26 that involves interaction with U2AF65. However, as overexpressed U2AF26 is imported into the nucleus also in the absence of overexpressed U2AF65 and U2AF26ΔE7/E8 localizes to the nucleus despite it lacks the domain responsible for interaction with U2AF65, we consider this pathway to be of only minor importance.

Following the classical model of import receptors, the interaction between P32 and U2AF26 would have to be stable in the cytoplasm, whereas both proteins would separate in the nucleus followed by a re-export of P32 into the cytoplasm. Although P32 itself is predominantly localized in the cytoplasm, our experiment with leptomycin B-treated cells clearly shows that, consistent with this model and earlier studies, P32 can enter the nucleus in a transitory fashion (24). How the interaction between U2AF26 and P32 is regulated is currently unknown, but the E7/E8 domain in U2AF26 contains several serine and threonine residues leaving room for speculation that phosphorylation may be important. It has also been shown that P32 can be phosphorylated (22), which might also play a role in regulating its interaction with U2AF26. As the E7/E8 domain within U2AF26 is also sufficient to induce nucleo-cytoplasmic shuttling, it is conceivable that P32 is not only required for nuclear localization of U2AF26 but that it also mediates shuttling between the nucleus and the cytoplasm. In this model, P32 could, under certain conditions, also bind nuclear U2AF26 and transport it across the nuclear membrane into the cytoplasm. This would be consistent with our observation that the nucleo-cytoplasmic shuttling is an active process and is not based on passive diffusion, but the involvement of another protein than p32 cannot be ruled out at this point.

It is of interest to note that P32 has already been described as a regulator of alternative splicing, as it interacts with the splicing factor ASF/SF2 (30), and it is of course conceivable that the intracellular distribution of these proteins is regulated by P32. This appears highly possible, considering the localization of P32 to diverse cellular compartments, which might facilitate the traffic of P32-bound proteins between these compartments, as we have shown here for the nuclear import of U2AF26.

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