proteins were purified with protein A–Sepharose beads. Proteins were quantitated by dot immunoblot analysis by using conjugated antibody to human IgG–horseradish peroxidase (Jackson ImmunoResearch Lab). Protein purity was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and silver staining. Ligand binding specificity of the proteins was assessed with NIH 3T3 cells. Briefly, serum-starved cells were stimulated with FGFs in the presence of either FR1-IgG or FR4-IgG for 5 hours. Total cell lysates were prepared and the changes in the protein phosphorylation profile were determined by immunoblot analysis with an antibody to phosphotyrosine (Santa Cruz).

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Identification of Both Shared and Distinct Proteins in the Major and Minor Spliceosomes

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In metazoans, two distinct spliceosomes catalyzing pre–messenger RNA splicing have been identified. Here, the human U11/U12 small nuclear ribonucleoprotein (snRNP), a subunit of the minor (U12-dependent) spliceosome, was isolated. Twenty U11/U12 proteins were identified, including subsets unique to the minor spliceosome or common to both spliceosomes. Common proteins include four U2 snRNP polypeptides that constitute the essential splicing factor SF3b. A 35-kilodalton U11-associated protein homologous to the U1 snRNP 70K protein was also identified. These data provide fundamental information about proteins of the minor spliceosome and shed light on its evolutionary relationship to the major spliceosome.

The minor (U12-dependent) spliceosome is required for splicing a rare class of nuclear pre-mRNA introns (1). These so-called U12-type introns contain highly conserved sequence elements at the 5' splice site and branch site that are distinct from the weakly conserved sequence elements found in the major class of pre-mRNA introns (1, 2). During assembly of the minor spliceosome, the U11 snRNP forms base pairs with the 5' splice site, and the U12 snRNP forms base pairs with the branch site, analogous to the U1 and U2 snRNPs in the major spliceosome (3). Subsequently, mature spliceosomes are formed by the association of the U5 and U4atac/U6atac snRNPs (4). In the major spliceosome, 5' splice site and branch site recognition are mediated by multiple interactions involving both RNA and protein (5). In metazoans, formation of the U1 snRNA/5' splice site duplex is facilitated by several polypeptides, including the U1 snRNP 70K and C proteins, as well as members of the SR (serine- and arginine-rich) protein family (6). Similarly, base pairing between U2 snRNA and the branch site requires numerous U2 snRNP proteins, in particular the subunits of the heteromeric splicing factors SF3a and SF3b (5–7).

In contrast to their counterparts in the major spliceosome, the U11 and U12 snRNPs are present in nuclear extract not only as individual monoparticles, but also as a highly stable 18S U11/U12 complex (8). Recent in vitro binding studies suggest that U11 and U12 interact with the pre-mRNA as a preformed complex (9). This observation, coupled with the fact that U12-type introns lack the essential pyrimidine tract found at the 3' splice site of the major class of introns, suggests that differences may exist in the mechanism of initial splice site recognition and pairing in the two types of spliceosomes. To identify proteins involved in this process in the minor spliceosome, we have characterized polypeptides associated with the human 18S U11/U12 snRNP complex.
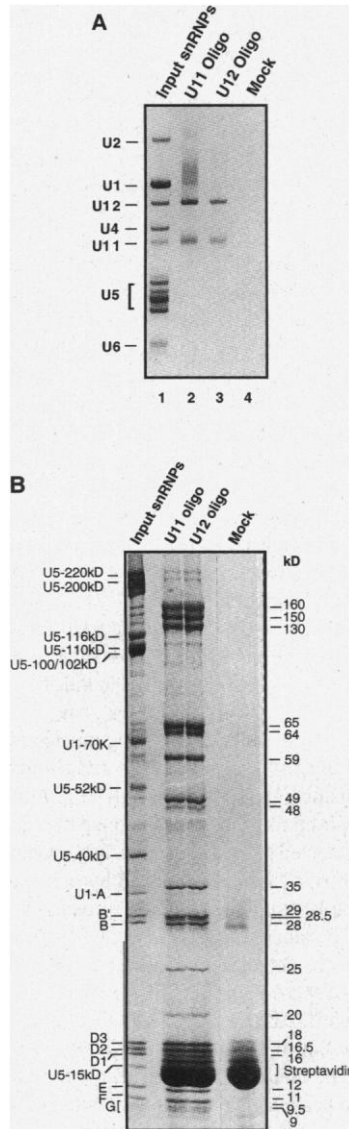
Spliceosomal snRNPs were immunoaffinity-purified from HeLa nuclear extract and fractionated by glycerol gradient sedimentation. From the 18S region of the gradient, U11/U12 snRNP complexes were then affinity-selected using biotinylated 2'-O-methyl oligonucleotides and streptavidin agarose (10). snRNPs containing predominantly U11 and U12 snRNA were selected by oligonucleotides complementary to nucleotides 2 to 18 of the U11 snRNA (Fig. 1A, lane 2) or nucleotides 11 to 28 of the U12 snRNA (Fig. 1A, lane 3). The coselection of U12 with an oligonucleotide directed against U11, and vice versa, indicated that mainly 18S U11/U12 snRNP complexes (as opposed to U11 or U12 monoparticles) had been selected. Consistent with this conclusion, the U11/U12 snRNPs selected by either oligonucleotide exhibited identical protein patterns (Fig. 1B, lanes 2 and 3). Twenty distinct proteins were detected in the U11/U12 complex. Eight of these comigrated with the snRNP Sm proteins B', B, D3, D2, D1, E, F, and G, which are present in the major spliceosomal snRNPs (Fig. 1B, lanes 1 to 3; see also Fig. 2) (11). Antibodies reacting specifically with B'/B, D3, D2, F, or G also recognized proteins of identical molecular mass on immunoblots of the U11/U12 complex (12). These data indicate that U11/U12 contains the same eight Sm proteins

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Table 1. U2 snRNP proteins are also present in U11/U12 snRNPs. X indicates an unidentified amino acid (28). Mismatches (indicated by boldface letters) were observed between the U11/U12-160kD and U2-160kD proteins at position 149 (V versus A) and between the U11/U12-130kD and U2-120kD proteins at positions 1209, 1211, and 1212 (D versus N, R versus A, and T versus Q, respectively).

U11/U12 protein	Peptides	Identity	Reference	Amino acids
160 kD	KMN**A**RTYMDVMREQHLTK KLTATPTPLGGMTGF KAIVNVIGMH	U2-160kD	(16)	146–162 428–442 997–1007
150 kD	KRIFEAFK KLRRMNRFTVAE KRTGIQEMREALQEK KLTIHGDLYYEG	U2-150kD	(17)	364–371 430–441 506–520 560–571
130 kD	KLGAVFNQVAFPLQYT KLLRVYDLGK KNVSEELDRTPPEVSK KLE**N**IA**Q**R**Y**AF	U2-120kD	(29)	768–783 964–973 1190–1205 1206–1217
49 kD	KVSEPLLXELFLQ KDRVTGQHQGYGFVEFLSEE	U2-53kD	(18)	23–35 46–65

Fig. 1. Proteins associated with 18S U11/U12 snRNP complexes. (**A**) snRNA composition of purified snRNPs. snRNAs were isolated from snRNPs precipitated in the absence (Mock) or presence of the indicated oligonucleotide. (**B**) Protein composition of oligonucleotide-selected U11/U12 snRNPs. Molecular masses of the U11/U12 proteins are shown on the right, and the identity of the major proteins in the 18S gradient fractions (Input) is indicated on the left.

found in the major spliceosome.

We next asked whether any of the 12 remaining U11/U12 proteins correspond to U1- or U2-specific proteins. Interestingly, the 160-, 150-, 130-, and 49-kD U11/U12 proteins comigrated with four of the 17S U2-specific proteins that constitute the essential splicing factor SF3b (that is, U2-160kD, U2-150kD, U2-120kD, and U2-53kD) (Fig. 2) (7, 13). Moreover, antibodies directed against the U2-160kD, U2-150kD, or U2-120kD proteins reacted strongly with the 160-, 150-, and
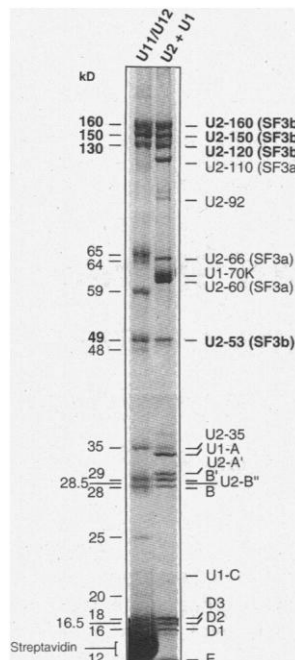
Fig. 2. SF3b proteins are present in U11/U12 snRNPs. Proteins associated with 18S U11/U12 or 17S U2 and 12S U1 snRNPs were fractionated by SDS-PAGE and visualized by Coomassie staining.

130-kD U11/U12 proteins, respectively (12). Finally, peptide sequences of the 160-, 150-, 130-, and 49-kD U11/U12 proteins were obtained by microsequencing (Table 1) (14). These sequences were identical to the known sequence of the corresponding SF3b protein, except for two peptides that had one or more mismatches (15). Thus, proteins that are most likely identical to the SF3b components in 17S U2 snRNP are present in the U11/U12 snRNP complex. In contrast, none of the U11/U12 proteins comigrated with the U2 snRNP proteins that constitute the essential splicing factor SF3a (that is, U2-110kD, U2-66kD, and U2-60kD) (7). Thus, either SF3a is not a component of the U11/U12 complex, or this factor has dissociated during the U11/U12 selection procedure. The U2-160kD, U2-150kD, and U2-53kD SF3b subunits interact with U2-type introns in the vicinity of the branch site, suggesting that they are core components of the major spliceosome (16–18). Similar interactions involving these same proteins may help to stabilize the association of U12 with the branch site of U12-

type introns.

Protein immunoblot analysis and sequence information from six of the eight other U11/U12-specific proteins failed to identify any additional U1 or U2 snRNP proteins or intron-bridging proteins such as SF1/mBBP or FBP21 (19, 20). Instead, several previously uncharacterized polypeptides were detected. However, a database search with peptide sequences obtained from one of these proteins (U11/U12-35kD) suggested that it may be related to the U1 snRNP-specific 70K protein. To further characterize the U11/U12-35kD protein, we isolated a full-length cDNA (21). This cDNA encodes a 246–amino acid protein with a predicted molecular mass of 29 kD and an isoelectric point of 9.88. A potential ortholog in *Arabidopsis thaliana* (GenBank accession number AC002335, PID g228900) is 47% identical (60% similar); this high degree of conservation between organisms as diverse as humans and plants suggests that the 35-kD protein performs an important function. Consistent with the fact that U12-type introns are not present in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (22), 35-kD orthologs were not detected in these organisms.

The U11/U12-35kD protein contains an RNA recognition motif (amino acids 51 to 129), and this region and the adjacent glycine-rich region are the most similar between U1-70K and U11/U12-35kD (U1-70K positions 99 to 203; 49% identity, 65% similarity) (Fig. 3) (23). In addition, the COOH-terminal half of both proteins is rich in Arg-Asp and Arg-Glu dipeptide repeats (a so-called alternating arginine domain) (24). The similarity between these proteins suggested that they may be functional analogs. Thus, we next asked whether U11/U12-35kD is associated with U11 snRNPs. Indeed, antiserum against the 35-kD protein specifically and efficiently immunoprecipitates U11 from a mixture of gradient-fractionated snRNPs containing U11 monoparticles (Fig. 4, lanes 3 and 4) (25). Thus, analogous to U1-70K, the U11-35kD protein may facilitate 5' splice site recognition in the minor spliceosome. Likewise, U11-35kD may be involved in exon bridging, interacting with components of the major spliceosome bound to the pyrimidine tract of an upstream U2-type intron. Consistent with this idea, binding of the U1 snRNP to a U2-type 5' splice site enhances splicing of an upstream U12-type intron, indicating that components of both types of spliceosome can interact with each other (26).

The identification of proteins unique to

Fig. 3. Schematic of U11/U12-35kD and U1-70K proteins. The U11/U12-35kD protein is homologous to the U1-70K protein. RRM, RNA recognition motif; Gly, glycine-rich; Alternating Arg (Alt Arg), rich in Arg-Asp and Arg-Glu dipeptides; Gly/Pro, glycine/proline-rich.
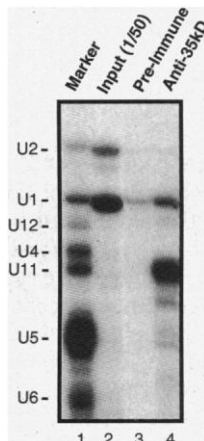
Fig. 4. The 35-kD protein is associated with U11 snRNPs. Immunoprecipitated snRNAs were labeled with [^{32}P]cytidine 3',5'-bisphosphate (pCp) fractionated by denaturing PAGE, and visualized by autoradiography.

the minor spliceosome is consistent with apparent differences in its mechanism of early spliceosome formation. In the major spliceosome, U1 and U2 bind to the pre-mRNA sequentially, whereas in the minor spliceosome U11 and U12 appear to bind as a preformed complex, implicating U11/U12 proteins in bridging the 5' and 3' ends of U12-type introns (9). The identification of proteins shared by both spliceosomes (the Sm proteins and SF3b) supports proposed models for evolutionary relationships between the major and minor spliceosome (1, 22). Analyses of the distribution of U12-type introns in various species favor a so-called fission-fusion model in which each spliceosome (derived from a common ancestor) evolved in separate lineages before being fused in a eukaryotic progenitor (22). In this model, only one version of those proteins that did not diverge considerably before the fusion event would ultimately be retained by both spliceosomes, resulting in shared spliceosomal components. However, preliminary immunoprecipitation experiments suggest that most of the [U4/U6.U5] tri-snRNP proteins may also be shared by both spliceosomes (27). Conservation of such a large number of proteins is more readily explained by an alternative model in which the U12-dependent spliceosome evolved in the presence of the U2-dependent one. In this case, a new class of introns and snRNAs, derived from the fragmentation of a parasitic group II intron, were introduced into an organism with a preexisting spliceosome (1, 22). As a result of convergent evolution, facilitated by the sharing of most of the spliceosomal proteins, a distinct but similar spliceosome would be generated (1, 22).

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14. Sequencing of tryptic peptides generated from each protein was performed by Toplab (Martinsried, Germany).
15. A database search with the U11/U12-130kD peptide containing three mismatches did not detect, among a total of 36 positives, any expressed sequence tags (ESTs) whose open reading frame (ORF) contained one or more of the observed amino acid changes. This indicates that U11/U12-130kD is probably not a splice variant of the U2-120kD protein; rather, these discrepancies most likely represent peptide sequencing errors.
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21. Peptide sequences were obtained from gel-fractionated, trypsin-digested U11/U12 proteins by microsequencing. The 35-kD protein peptides KEYDPLK and KRWQEREPTRVWPDND (28) were used to search the National Center for Biotechnology Information EST database for full-length cDNAs by means of the program TBLASTN. Both peptides were found in the ORF of a cDNA from human macrophage cells (GenBank accession number U44798) encoding a protein of unknown function that was noted to share homology with the U1-70K protein. An EST cDNA from human muscle cells that contains an identical ORF (GenBank accession number AA211268) was cloned and sequenced in its entirety. Protein generated by in vitro translation of this cDNA comigrated with the purified U11/U12-35kD protein on SDS-polyacrylamide gels, confirming that it encodes full-length protein (12).
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