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The human U5 snRNP-specific 100-kD protein is an RS domain-containing, putative RNA helicase with significant homology to the yeast splicing factor Prp28p

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ABSTRACT

Through UV-crosslinking experiments, we previously provided evidence suggesting that a U5 snRNP protein with a molecular weight in the 100-kDa range is an ATP-binding protein (Laggerbauer B, Lauber J, Lührmann R, 1996, *Nucleic Acid Res 24*:868–875). Separation of HeLa U5 snRNP proteins on 2D gels revealed multiple variants with apparent molecular masses of 100 kDa. Subsequent microsequencing of these variants led to the isolation of a cDNA encoding a protein with an N-terminal RS domain and a C-terminal domain that contains all of the conserved motifs characteristic of members of the DEAD-box family of RNA-stimulated ATPases and RNA helicases. Antibodies raised against cDNA-encoded 100-kDa protein specifically recognized native U5-100kD both on immunoblots and in purified HeLa U5 snRNPs or [U4/U6.U5] tri-snRNP complexes, confirming that the bona fide 100-kDa cDNA had been isolated. In vitro phosphorylation studies demonstrated that U5-100kD can serve as a substrate for both Clk/Sty and the U1 snRNP-associated kinase, and further suggested that the multiple U5-100kD variants observed on 2D gels represent differentially phosphorylated forms of the protein. A database homology search revealed a significant degree of homology (60% similarity, 37% identity) between the *Saccharomyces cerevisiae* splicing factor, Prp28p, which lacks an N-terminal RS domain, and the C-terminal domain of U5-100kD. Consistent with their designation as structural homologues, anti-Prp28 antibodies recognized specifically the human U5-100kD protein on immunoblots. Together with the DEXH-box U5-200kD protein (Lauber J et al., 1996, *EMBO J 15*:4001–4015), U5-100kD is the second example of a putative RNA helicase that is tightly associated with the U5 snRNP. Given the recent identification of the U5-116kD protein as a homologue of the ribosomal translocase EF-2 (Fabrizio P, Laggerbauer B, Lauber J, Lane WS, Lührmann R, 1997, *EMBO J 16*:4092–4106), at least three integral U5 snRNP proteins thus potentially facilitate conformational changes in the spliceosome during nuclear pre-mRNA splicing.

Keywords: DEAD-box protein; pre-mRNA splicing; snRNP protein; SR protein

INTRODUCTION

Catalysis of the two transesterification steps of the pre-mRNA splicing reaction is conducted by the spliceosome, a large ribonucleoprotein complex that is formed by the ordered interaction of the U1, U2, U5, and U4/U6 snRNPs and numerous splicing factors with the pre-mRNA (for review see Moore et al., 1993; Krämer, 1995). Splicing complex formation involves the initial interaction of the U1 snRNP with the 5′ splice site, followed by U2 snRNP recognition of the branch site to form the so-called pre-spliceosome. Spliceosome assembly is completed by the subsequent interaction of a preformed tri-snRNP complex, which contains the U5 and U4/U6 snRNPs.

During spliceosome assembly, a complex network of RNA–RNA interactions is formed between the U1, U2, U4/U6, and U5 snRNAs and the pre-mRNA, as well as between a subset of the snRNAs themselves (reviewed by Madhani & Guthrie, 1994; Nilsen, 1997). During pre-spliceosome assembly, the U1 and U2

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snRNAs base pair with the 5′ splice site and the branch site, respectively. Upon integration of the [U4/U6.U5] tri-snRNP complex, several RNA conformational changes occur. For example, the U4 and U6 snRNAs, which are base paired within the tri-snRNP complex (Bringmann et al., 1984; Hashimoto & Steitz, 1984; Rinke et al., 1985; Brow & Guthrie, 1988), dissociate, allowing U6 to base pair with the 5′ end of the U2 snRNA (Datta & Weiner, 1991; Wu & Manley, 1991; Madhani & Guthrie, 1992; Sun & Manley, 1995) and also with intron sequences at the 5′ splice site (Sawa & Abelson, 1992; Sawa & Shimura, 1992; Wassarman & Steitz, 1992; Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993). The latter interactions result in structural rearrangements of both the U6 and U2 snRNAs. Prior to the first catalytic step of splicing, U1 snRNA appears to dissociate from the pre-mRNA, whereas the U5 snRNA interacts with exon nucleotides at the 5′ splice site (Newman & Norman, 1991, 1992; Wyatt et al., 1992; Cortes et al., 1993; Sontheimer & Steitz, 1993). U5 interacts additionally with exon nucleotides at the 3′ splice site prior to the second step of splicing (Newman & Norman, 1992; Sontheimer & Steitz, 1993). After dissociation of the spliceosome, the U4/U6 snRNA duplex is re-formed and the [U4/U6.U5] tri-snRNP complex is regenerated and thought to take part in subsequent rounds of splicing.

The driving forces mediating the many RNA conformational changes occurring throughout the splicing process are poorly understood. In yeast, a number of essential splicing factors have been identified as members of the DEAD-box superfamily of putative RNA helicases, or of a subfamily that contains a DEAH-box, and they appear to play an important role in the dynamics of the splicing process (reviewed by Wassarman & Steitz, 1991; Fuller-Pace, 1994; Beggs, 1995). These factors include the DEAD-box proteins Prp5p and Prp28p, which are involved in spliceosome assembly (Lin et al., 1987; Dalbadie-McFarland & Abelson, 1990; Strauss & Guthrie, 1991), the DEAH-box proteins Prp2p and Prp16p, which are required for the first and second catalytic step, respectively (Chen & Lin, 1990; King & Beggs, 1990; Schwer & Guthrie 1991), and the DEAH-box protein Prp22p, which mediates the release of mRNA from the spliceosome (Company et al., 1991). Prp2p, Prp5p, and Prp16p exhibit RNA-stimulated ATPase activity (Schwer & Guthrie, 1991; Kim et al., 1992; O'Day et al., 1996). In addition, consistent with their proposed role as RNA helicases, conformational changes in the spliceosome have been detected after ATP hydrolysis by Prp16p and Prp2p (Schwer & Guthrie, 1992; Teigelkamp et al., 1994; Kim & Lin, 1996). Interestingly, the latter two proteins interact only transiently with the spliceosome, whereas Prp5p, Prp22p, and Prp28p are more stably associated but do not appear to be integral components of the spliceosomal snRNPs.

As compared to yeast, in higher eukaryotes, relatively little is known about spliceosomal proteins potentially catalyzing conformational changes during splicing. A human homologue of Prp22p, HRH1, has been identified and shown to function in a manner similar to its yeast counterpart, namely to release mRNA from the spliceosome (Ono et al., 1994; Ohno & Shimura, 1996). Interestingly, in addition to containing those motifs characteristic of ATP-dependent RNA helicases of the DEAH-box protein family, HRH1 also possesses an arginine–serine-rich (RS) domain not present in Prp22p, that is characteristic of members of the SR protein family (Ono et al., 1994). The RS domains of various splicing factors are involved in a number of functionally important protein–protein interactions, particularly during the early stages of spliceosome assembly (reviewed by Fu, 1995; Manley & Tacke, 1996). RS domain interactions can be regulated by phosphorylation–dephosphorylation cycles (Xiao & Manley, 1997), and the phosphorylation state of SR proteins is known to affect both spliceosome assembly and the subsequent catalytic steps of splicing (Tazi et al., 1993; Gui et al., 1994; Mermoud et al., 1994; Roscigno & Garcia-Blanco, 1995).

Recently, a component of the human and yeast U5 snRNP (the human U5-200kD protein) was identified as a novel, putative RNA helicase that belongs to the DEXH subgroup of the DEAD-box protein family (Lauber et al., 1996; Lin & Rossi, 1996; Noble & Guthrie, 1996; Xu et al., 1996). The U5-200kD protein is required for pre-mRNA splicing and is unique among DEAD/DEXH-box proteins in that it contains two putative RNA helicase domains. Because it joins the spliceosome together with the 25S [U4/U6.U5] tri-snRNP, U5-200kD is most likely involved in one or more of the RNA structural rearrangements occurring upon tri-snRNP integration into the spliceosome. The 25S tri-snRNP complex contains a large number of additional proteins, some of which probably also contribute to the dynamics of the splicing process. At least nine polypeptides specifically associated with U5 snRNPs and two with U4/U6 snRNPs have been identified (Bach et al., 1989; Gozani et al., 1994; Lauber et al., 1997). Moreover, the 25S tri-snRNP particle contains, in addition to these U5 and U4/U6-associated proteins, five tri-snRNP-specific proteins (Behrens & Lührmann, 1991; Lauber et al., 1997).

Molecular cloning of the genes encoding proteins of the [U4/U6.U5] tri-snRNP complex has recently uncovered a number of proteins with potential enzymatic activities, including RNA helicase (as discussed above) and GTPase activity (Fabrizio et al., 1997; reviewed by Will & Lührmann, 1997). We were particularly interested in characterizing tri-snRNP proteins with molecular masses in the 100-kDa range, because previous UV crosslinking experiments suggested that a U5 snRNP protein of this size might be an ATP-

binding protein (Laggerbauer et al., 1996). In addition, earlier in vitro phosphorylation studies indicated that the U5-specific 100-, 102-, or 110-kD protein probably possesses an RS domain (Fetzer et al., 1997). Here, we describe the cloning and characterization of the U5-100kD protein and provide evidence that it, too, potentially catalyzes structural changes during splicing. Specifically, using partial peptide sequence data derived by microsequencing 2D gel-purified tri-snRNP proteins, a cDNA encoding the human 100-kD protein was isolated. Subsequent sequencing revealed that it is a member of the DEAD-box family of ATP-dependent RNA helicases and that it additionally contains an N-terminal RS domain. A database homology search revealed a significant degree of overall homology (60% similarity, 37% identity) between U5-100kD and the yeast DEAD-box protein, Prp28p, suggesting that U5-100kD, like Prp28p, may perform an essential function during mammalian pre-mRNA splicing. Interestingly, U5-100kD can be phosphorylated in vitro by Clk/Sty and the U1 snRNP-associated kinase, both of which have been shown previously to phosphorylate the RS domains of a number of splicing factors (Woppmann et al., 1993; Colwill et al., 1996; Fetzer et al., 1997). These results raise the interesting possibility that this putative helicase interacts with and is activated by other SR proteins, and further that its activity might be regulated by a phosphorylation–dephosphorylation cycle.

RESULTS

Analysis of high molecular weight tri-snRNP proteins by 2D gel electrophoresis

In order to characterize proteins associated with the 25S [U4/U6.U5] tri-snRNP complex in more detail, spliceosomal snRNPs (U1, U2, U4/U6, and U5) were purified from HeLa cell nuclear extract by immunoaffinity chromatography and subsequently separated by glycerol gradient centrifugation. To clearly separate tri-snRNP proteins with molecular masses in the 100-kDa range, protein components of purified 25S [U4/U6.U5] tri-snRNP particles were subsequently fractionated in the first dimension by nonequilibrium pH-gradient gel electrophoresis (NEPHGE) and in the second dimension by SDS-PAGE. As shown in Figure 1, the U5-110kD and U5-102kD proteins migrated as distinct spots after 2D gel electrophoresis, whereas proteins with a molecular mass of 100 kDa resolved into a cluster of spots and two individual spots (designated d and e). Spots d and e, as well as the a, b, and c regions of the cluster, were excised from the gel. Proteins derived from each of the gel pieces were analyzed subsequently by microsequencing. One peptide sequence (20 amino acids in length) was obtained from spot d and two sequences (7 and 8 amino acids) from spot e. These sequences were 100% identical to regions of the human splicing factor PSF (Patton et al., 1993), indicating that the latter protein is associated

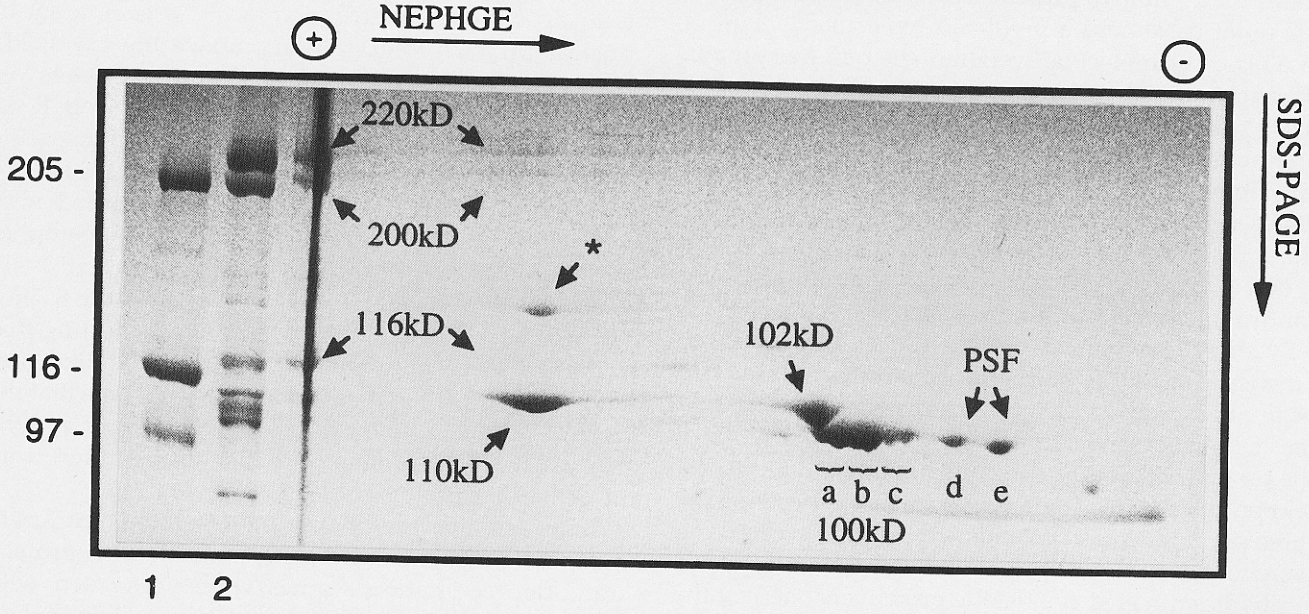

FIGURE 1. Analysis of [U4/U6.U5] tri-snRNP high molecular weight proteins by 2D gel electrophoresis. Proteins were separated in the first dimension by NEPHGE with a pH gradient ranging from 3 to 11, as indicated at the top of the gel, and in the second dimension on a 9% SDS-polyacrylamide gel. Spots of interest are labeled. In this gel, the U5-specific 220-kD, 200-kD, and 116-kD proteins have partially precipitated at the acidic end of the first dimension. The asterisk denotes a minor protein of unknown identity that is consistently associated with snRNP preparations. Molecular weight markers, whose sizes in kDa are indicated on the left, and [U4/U6.U5] tri-snRNP proteins are shown in lanes 1 and 2, respectively. Proteins were visualized by staining with Coomassie brilliant blue.

with the [U4/U6.U5] tri-snRNP particle. The separation of PSF into two isoelectric variants is consistent with the fact that two alternatively spliced forms of this protein, with slightly different sizes and isoelectric points, have been identified (Patton et al., 1993). Three peptide sequences, none of which matched any known protein sequence, were obtained after microsequencing the a, b, and c regions of the 100-kDa cluster, indicating that this cluster contains a novel protein that is different from PSF.

The U5-100kD cDNA encodes a DEAD-box protein containing an RS domain

Peptide sequences obtained by microsequencing of the 100-kDa cluster were used for a database search for corresponding open reading frames. The peptide sequence 100kD-3 was 100% identical to part of the open reading frame of an expressed sequence tag (EST) derived from the human infant brain clone 51919, and the peptide sequence 100kD-2 was identical to parts of three additional ESTs derived from cDNA clones of different human tissues. All of the ESTs contained overlapping open reading frames and coded for an identical protein product, but were incomplete at their 5′ end. Oligonucleotides were designed to amplify a 304-bp DNA fragment of the EST clone 51919 by PCR. Screening of a HeLa cDNA library with this probe led to the isolation of a 3.3-kb full-length clone. The open reading frame of this clone is 2,463 nt in length and codes for a putative protein with a calculated isoelectric point of 9.6 and a predicted molecular weight of 95.6 kDa. The molecular weight of the putative protein product is thus in good agreement with the apparent size of the 100-kD protein. The observed isoelectric point of 8.0–8.5 (Fig. 1) is below the calculated value, but could be due to phosphorylation of the native protein (see below). An AUG codon is located 40-nt downstream of the 5′ end of the cDNA and an inframe stop codon is found 33-nt upstream of this initiation codon, confirming that it codes for the first methionine. A polyadenylation signal (AAUAAA) is located 698-nt downstream of the stop codon, with a poly(A) tail beginning 22-nt further downstream, indicating that the cDNA comprises the complete carboxy terminus of the protein. Two criteria indicate that the putative protein product of this clone is the U5-100kD protein. The three peptide sequences obtained by microsequencing were found in the predicted amino acid sequence, and antibodies directed against the protein encoded by the cloned cDNA interact specifically with the U5-100kD protein from HeLa cells on western blots (see below, Fig. 3A).

The primary structure of the protein encoded by the U5-100kD cDNA indicates that it is a novel member of the DEAD-box protein family of RNA-stimulated ATPases and putative RNA helicases, which contains an RS domain at its amino terminus (Fig. 2A,B). The RS domain comprises approximately the first 220 amino acids of the protein. The helicase domain encompasses a 319-amino acid long region, beginning at position 435 with a conserved P-loop (AxxxxGKT) and ending at position 754 with an HRIGR motif (Fig. 2B). All of the RNA helicase motifs characteristic of DEAD-box proteins are present when compared with eIF-4A, the prototype of this protein family (Schmid & Linder, 1992; Fuller-Pace, 1994). The majority of these motifs are 100% conserved; exceptions include single conservative exchanges in the SAT-motif, which is replaced by TAT, and in the ARGID-motif, which is replaced by GRGID (Fig. 2).

Antibodies raised against the recombinant 100-kDa DEAD-box protein interact with U5-100kD

For further characterization of the U5-100kD protein, polyclonal antibodies were raised against a fusion protein containing glutathione S-transferase (GST) and a 326-amino acid fragment comprising the central region (amino acids 366–692) of the recombinant 100-kD protein. In contrast to the nonimmune serum, immune serum reacted specifically with the U5-100kD protein on immunoblots of HeLa nuclear extract, affinity-purified HeLa snRNPs, glycerol gradient-purified [U4/U6.U5] tri-snRNPs, and U5-snRNPs purified by anion exchange chromatography (Fig. 3A, lanes 3–8). The faster migration of the U5-100kD protein in crude nuclear extract can be attributed to the large amount of nuclear proteins migrating just above the U5-100kD band (Fig. 3A, lane 3). The faint staining of two lower molecular weight bands in total snRNPs and [U4/U6.U5] tri-snRNPs is most likely due to nonspecific degradation of the U5-100kD protein (Fig. 3A, lanes 4 and 5). When immunoblotting was performed with tri-snRNP proteins separated by 2D gel electrophoresis, anti-100kD antibodies recognized exclusively those spots (a–c) migrating as a cluster in the 8.0–8.5 pH range (see Fig. 1; data not shown), indicating that these proteins represent variants of the U5-100kD protein. Taken together, these results thus indicate that the protein product of the cloned cDNA is identical to the HeLa U5-100kD protein and that it is distinct from other snRNP proteins.

To investigate whether the anti-U5-100kD antiserum recognizes native, particle-associated 100-kD protein, immunoprecipitation studies were performed with crude nuclear extract or immunoaffinity-purified spliceosomal snRNPs (Fig. 3B). The monoclonal antibody Y12, which reacts with the snRNP Sm proteins, was also included to monitor the total amount of snRNPs in the immunoprecipitation reactions (Fig. 3B, lane 3). U5-100kD-specific immune serum, but not nonim-

A

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MAGELADKKD RDASPSKEER KRSRTPDRER DRDRDRKSSP SKDRKRHRSR    50
DRRRGGSRSR SRSRSKSAER ERRHKERERD KERDRNKKDR DRDKDGHRRD   100
KDRKRSSLSP GRGKDFKSRK DRDSKKDEED EHGDKKLKAQ PLSLEELLAK   150
KKAEEEAEAK PKFLSKAERE AEALKRRQQE VEERQRMLEE ERKKRKQFQD   200
LGRKMLEDPQ ERERRERRER MERETNGNED EEGRQKIREE KDKSKELHAI   250
KERYLGGIKK RRRTRHLNDR KFVFEWDASE ETSIDYNPLY KERHQVQLLG   300
RGFIAGIDFK QQKREQSRFY GDLMEKRRTL EEKEQEEARL RKLRKKEAKQ   350
RWDDRHWSQK KLDEMTDRDW RIFREDYSIT TKGGKIPNPI RSWKDSSLPP   400
HILEVIDKCG YKEPTPIQRQ AIPIGLQNRD IIGVAETGSG KTAAFLIPLL   450
VWITTLPKID RIEESDQGPY AIILAPTREL AQQIEEETIK FGKPLGIRTV   500
AVIGGISRED QGFRLRMGCE IVIATPGRLI DVLENRYLVL SRCTYVVLDE   550
ADRMIDMGFE PDVQKILEHM PVSNQKPDTD EAEDPEKMLA NFESGKHKYR   600
QTVMFTATMP PAVERLARSY LRRPAVVYIG SAGKPHERVE QKVFLMSESE   650
KRKKLLAILE QGFDPPIIIF VNQKKGCDVL AKSLEKMGYN ACTLHGGKGQ   700
EQREFALSNL KAGAKDILVA TDVAGRGIDI QDVSMVVNYD MAKNIEDYIH   750
RIGRTGRAGK SGVAITFLTK EDSAVFYELK QAILESPVSS CPPELANHPD   800
AQHKPGTILT KKRREETIFA                                    820
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B

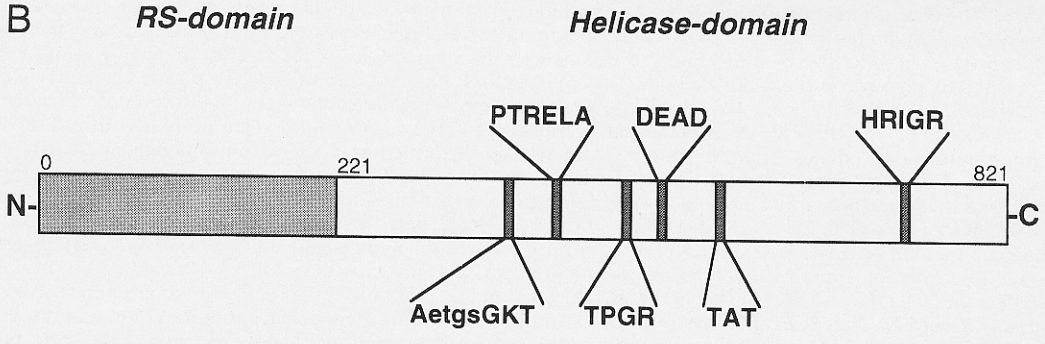


FIGURE 2. Primary structure of the U5-100kD protein. **A:** Amino acid sequence of the U5-100kD protein. Arginine-serine (bold faced letters) and arginine-aspartic/glutamic acid dipeptides are shaded. Conserved motifs characteristic of DEAD-box proteins are indicated by black boxes, and partial peptide sequences obtained by microsequencing of the purified protein are underlined. **B:** Map of the primary structure of U5-100kD. The RS domain (amino acids 1–221) is shaded. The position of the conserved motifs of the RNA-helicase domain (amino acids 221–821) are boxed and the sequences of these motifs are indicated above or below each box. All spacing is according to scale.

mune serum, precipitated U4, U5, and U6 snRNPs from crude nuclear extract (Fig. 3B, compare lanes 1 and 2) and from immunoaffinity-purified snRNPs (Fig. 3B, compare lanes 4 and 5) in the presence of 150 mM NaCl. At concentrations of 450 mM and 600 mM NaCl (Fig. 3B, lanes 6 and 7), anti-U5-100kD antibodies precipitated predominantly U5 snRNPs. Because the [U4/U6.U5] tri-snRNP dissociates at salt concentrations above 300 mM NaCl (Behrens & Lührmann, 1991), these results confirm that the 100-kD protein is a U5 snRNP-specific component and indicate that the epitope recognized by the anti-U5-100kD antibodies is accessible in the U5-snRNP, as well as in the 25S [U4/U6.U5] tri-snRNP complex.

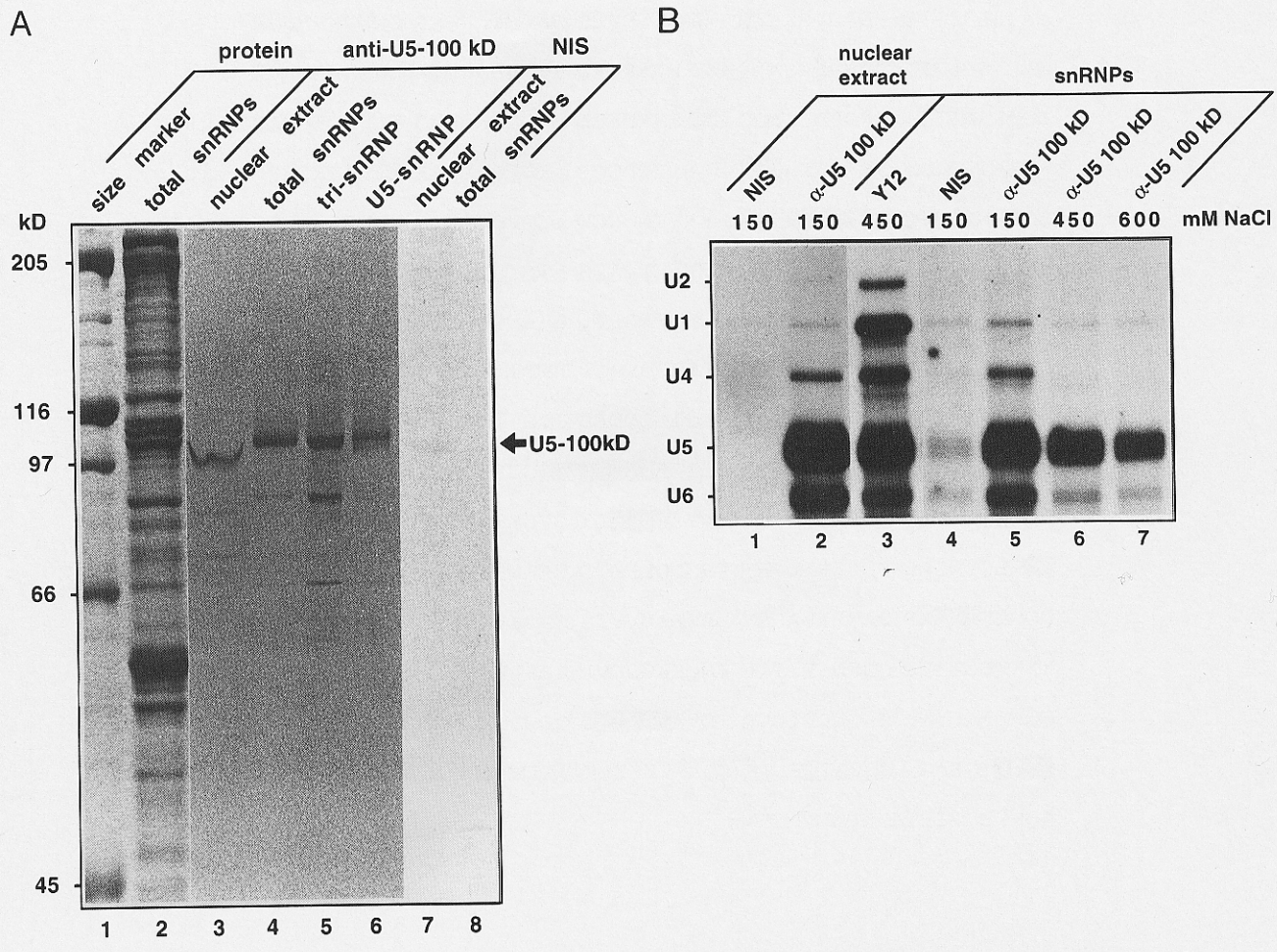
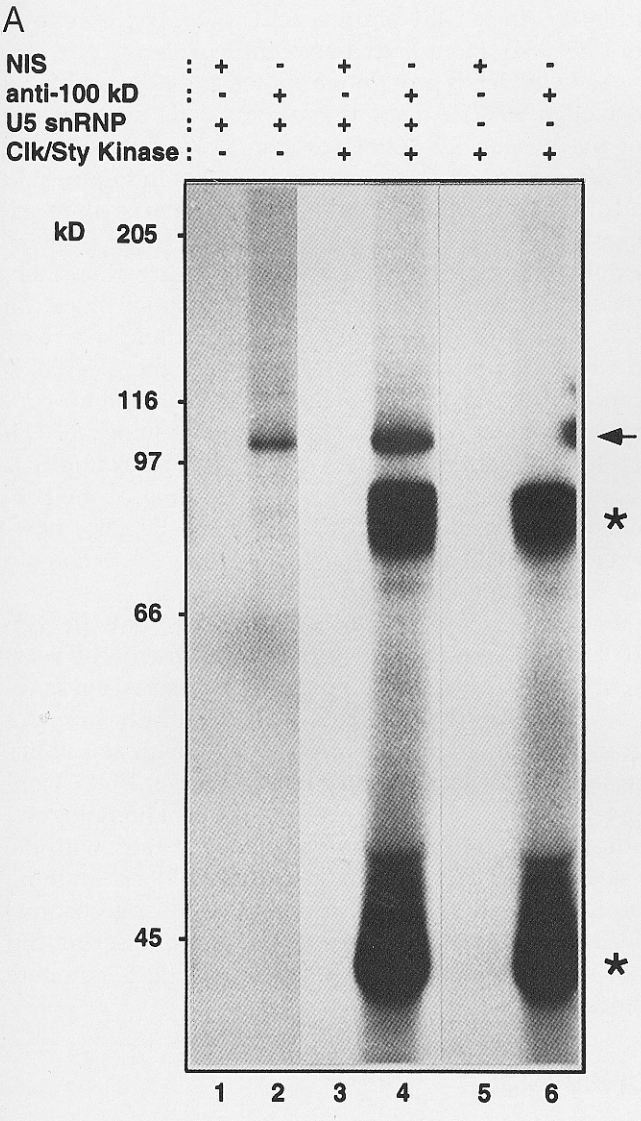


FIGURE 3. Characterization of antibodies raised against the recombinant U5-100kD protein. **A:** Anti-U5-100kD antibodies interact specifically with the HeLa U5-100kD protein on immunoblots. Proteins isolated from HeLa nuclear extract (lanes 3 and 7), spliceosomal snRNPs (U1-U6) affinity purified with the monoclonal antibody H20 under low-salt conditions (lanes 2, 4, and 8), glycerol gradient-purified [U4/U6.U5] tri-snRNPs (lane 5), and U5 snRNPs purified by anion exchange chromatography (lane 6), were separated on a 9% polyacrylamide gel and electroblotted onto nitrocellulose. Electroblotted proteins were incubated with anti-U5-100kD-specific antibodies (lanes 3–6) or on a separate blot with anti-U5-100kD nonimmune serum (NIS) (lanes 7 and 8). Immunostaining was performed as described in Materials and Methods. The position of the U5-100kD protein is indicated by an arrow on the right. Size markers stained with Coomassie blue are shown in lane 1 with the individual molecular weights indicated on the left. Total snRNP proteins stained with Coomassie blue are shown in lane 2. **B:** U5 and [U4/U6.U5] snRNPs are precipitated by anti-U5-100kD antibodies. HeLa nuclear extract (lanes 1 and 2) or spliceosomal snRNPs affinity purified with the monoclonal antibody H20 (lanes 3–7) were used for immunoprecipitation experiments. Immunoprecipitation was performed with anti-U5-100kD antibodies in the presence of 150 mM NaCl (lanes 2 and 5), 450 mM NaCl (lane 6), and 600 mM NaCl (lane 7), with nonimmune serum in the presence of 150 mM NaCl as a negative control (lanes 1 and 4) and with the monoclonal antibody Y12 to monitor the total amount of snRNPs present (lane 3). Individual snRNP particles were detected by phenol extraction of the corresponding snRNAs, 3′ end-labeling with [^{32}P]pCp, and fractionation on an 8 M urea/10% polyacrylamide gel followed by autoradiography. The identity of the snRNAs is indicated on the left.

U5-100kD protein can be phosphorylated in vitro by Clk/Sty and the snRNP-associated kinase

The fact that U5-100kD contains an RS domain, suggested that it, like most SR proteins, might be a phosphoprotein. To test whether the U5-100kD protein can be phosphorylated in vitro, glycerol gradient-purified U5 snRNPs were incubated with [^{32}P]-γ-ATP and, after dissociation of the particles under denaturing conditions, immunoprecipitation was performed with anti-U5-100kD antibodies. Immunoprecipitates were analyzed subsequently by SDS-PAGE and autoradiography (Fig. 4A). A single radiolabeled band migrating at 100 kDa was detected after immunoprecipitation with immune serum (Fig. 4A, lane 2), whereas no signal was observed with the nonimmune serum (Fig. 4A, lane 1). This phosphorylation is most likely due to the activity of a kinase, which was shown to be associated predominantly with the U1 snRNP, but is also present to a lesser extent in other snRNP preparations (Woppmann et al., 1993). The ability of U5-100kD to serve as



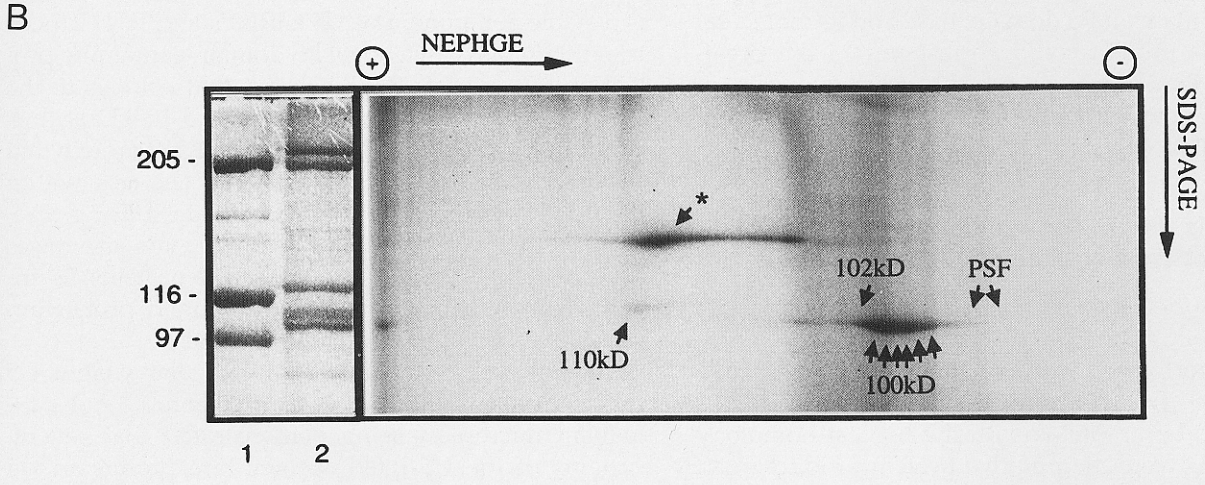


FIGURE 4. In vitro phosphorylation of the U5-100kD protein. **A:** U5 snRNP particles purified by glycerol gradient centrifugation were incubatd with [^{32}P]-γ-ATP in the absence (lanes 1 and 2) and presence (lanes 3–6) of recombinant Clk/Sty kinase. U5 particles were disrupted by denaturation with SDS and Triton X-100 (see Materials and Methods) and immunoprecipitation was performed with anti-U5-100kD antibodies (lanes 2, 4, and 6) and nonimmune serum (lanes 1, 3, and 5). To monitor autophosphorylation of the Clk/Sty kinase (indicated by asterisks), reactions shown in lanes 3 and 4 were performed in the absence of U5 snRNP particles (lanes 5 and 6). Immunoprecipitates were analyzed on a 9% SDS-polyacrylamide gel and visualized by autoradiography. The position of molecular weight markers is indicated on the left, and that of the phosphorylated U5-100kD protein by an arrow on the right. **B:** U5 snRNP particles were incubated with [^{32}P]-γ-ATP and kinase-active U1 snRNPs, and analyzed by 2D gel electrophoresis. Proteins were separated in the first dimension by NEPHGE with a pH-gradient ranging from 3 to 11 and in the second dimension on a 9% SDS-polyacrylamide gel. Proteins were visualized by staining with Coomassie brilliant blue (compare with Fig. 1), followed by autoradiography. An autoradiograph, where the positions of proteins of interest are indicated by arrows, is presented in the right panel. The asterisk denotes a minor protein of unknown identity that is consistently associated with snRNP preparations. Coomassie-stained marker proteins (lane 1), whose sizes are indicated on the left, and U5 snRNP proteins (lane 2) are shown in the left panel.

a substrate for the Clk/Sty kinase, which has been shown to phosphorylate both serine/threonine and tyrosine residues, and whose substrates include splicing factors containing an RS domain, was also tested (Colwill et al., 1996). Addition of a recombinant GST fusion protein containing the Clk/Sty kinase resulted in a greater than 10-fold increase in U5-100kD phosphorylation (Fig. 4A, lanes 3 and 4; note that lanes 1 and 2

are a 3-day exposure, whereas lanes 3–6 are a 12-h exposure). Because anti-U5-100kD antibodies were raised against a GST-100kD fusion protein and thus contain anti-GST antibodies, an autophosphorylated form of the GST-Clk/Sty fusion protein with a molecular weight of 90 kDa, as well as the 45-kDa breakdown product, also were co-immunoprecipitated in the presence and absence of U5 snRNPs (Fig. 4A, lanes 3–6).

A similar increase in phosphorylation was observed after incubation of U5 snRNPs with kinase-active U1 snRNPs (Fig. 4B). Gradient-purified U5 snRNPs were incubated with [^{32}P]-γ-ATP in the presence and absence of U1 snRNPs and phosphoproteins were analyzed by 2D gel electrophoresis. Consistent with the presence of kinase activity in purified U5 snRNPs, a low level of phosphorylation of the 110- and 100-kD U5-specific proteins, as well as of a minor unidentified, 150-kDa protein (labeled with an asterisk), was detected after incubation with [^{32}P]-γ-ATP alone (data not shown). Upon addition of kinase-active U1 snRNPs to the reaction, U5-100kD and the unidentified protein became heavily phosphorylated (Fig. 4B). Significantly, in vitro-phosphorylated U5-100kD resolved into a cluster of radioactive spots that migrated in the same positions as the Coomassie-stained U5-100kD variants (see Fig. 1; data not shown), suggesting that these variants could be differentially phosphorylated forms of the native U5-100kD protein. Because the kinase activity associated with U1 snRNPs phosphorylates specifically serine residues in an arginine-rich context (S. Fetzer & R. Lührmann, in prep), these results indicate that residues within the U5-100kD's RS domain can be phosphorylated in vitro and thus further suggest that the U5-100kD protein might be phosphorylated in vivo. They also suggest that the unidentified 150-kDa protein may contain an RS domain. Based on its molecular weight, this protein could correspond to the previously described autoantigen p150, which is present in substoichiometric amounts in U2, U5, and tri-snRNP particles (Blencowe et al., 1993).

U5-100kD is the human homologue of the yeast splicing factor Prp28p

In order to identify putative homologues of the U5-100kD protein, a database search using the FASTA program was performed with the amino acid sequence of the U5-100kD DEAD-box region (i.e., amino acids 221–820). DEAD-box proteins with the highest homology to U5-100kD were the putative protein product of an open reading frame of a *Caenorhabditis elegans* cosmid clone (67% identity in a 559-amino acid overlap; accession no. U13070), the DDHEL-2B protein product from *Dictyostelium dyscoideum* (47% identity in a 571-amino acid overlap; accession no. X81824), and the splicing factor Prp28p of *Saccharomyces cerevisiae* (37% identity in a 451-amino acid overlap; accession no. X56934). Homology between Prp28p and the *Dictyostelium* Hel2B protein was noted previously (Mahal & Nellen, 1994). An alignment comparing the sequence of the human U5-100kD protein with that of the *C. elegans* and *S. cerevisiae* protein is shown in Figure 5. In addition to the conserved motifs common to all members of the DEAD-box protein family, these proteins exhibit homology throughout their entire RNA helicase domains and contain several distinct blocks of extraordinarily high identity (Fig. 5). Strikingly, in contrast to all other DEAD-box proteins, the U5-100kD protein, like Prp28p and the *C. elegans* protein, contains a TAT rather than SAT motif. Homology of U5-100kD with other members of the DEAD-box family is significant, but less pronounced than that of the proteins mentioned above. In contrast to Prp28p, however, U5-100kD and the *C. elegans* protein both contain an RS domain at their amino termini.

Based on these results, we propose that the U5-100kD protein is the structural homologue of the yeast splicing factor Prp28p. To provide additional evidence that the latter two proteins are structurally homologous, immunoblotting was performed with antibodies raised against Prp28p (Strauss & Guthrie, 1994). Consistent with their designation as structural homologues, anti-Prp28p antibodies interacted specifically with the human U5-100kD protein on western blots containing proteins isolated from affinity-purified spliceosomal snRNPs, whereas anti-Prp28p nonimmune serum did not react with any of these proteins (Fig. 6, compare lanes 2 and 4).

DISCUSSION

We have identified the human U5-100kD protein, which is a stable component of U5 snRNPs and [U4/U6.U5] tri-snRNPs, as both a novel RS domain-containing protein and a putative RNA helicase that belongs to the DEAD-box protein family (Fig. 2). U5-100kD is thus the third example of an integral U5 snRNP protein that potentially catalyzes conformational changes within the spliceosome during the mammalian splicing reaction. The fact that it contains an RS domain also raises the possibility that it, like other SR proteins, is involved in functionally important protein–protein interactions during spliceosome assembly.

Characterization of the high molecular weight U5 snRNP proteins by 2D gel electrophoresis and subsequent microsequencing demonstrated that several variants of the U5-100kD protein are present in U5 snRNPs (Fig. 1). These variants could represent U5-100kD that is phosphorylated to varying degrees; in vitro-phosphorylation studies demonstrated that each of the spots present in the cluster of U5-100kD variants are phosphorylated (Fig. 4B). Interestingly, in the course of these initial experiments, we also identified the es-

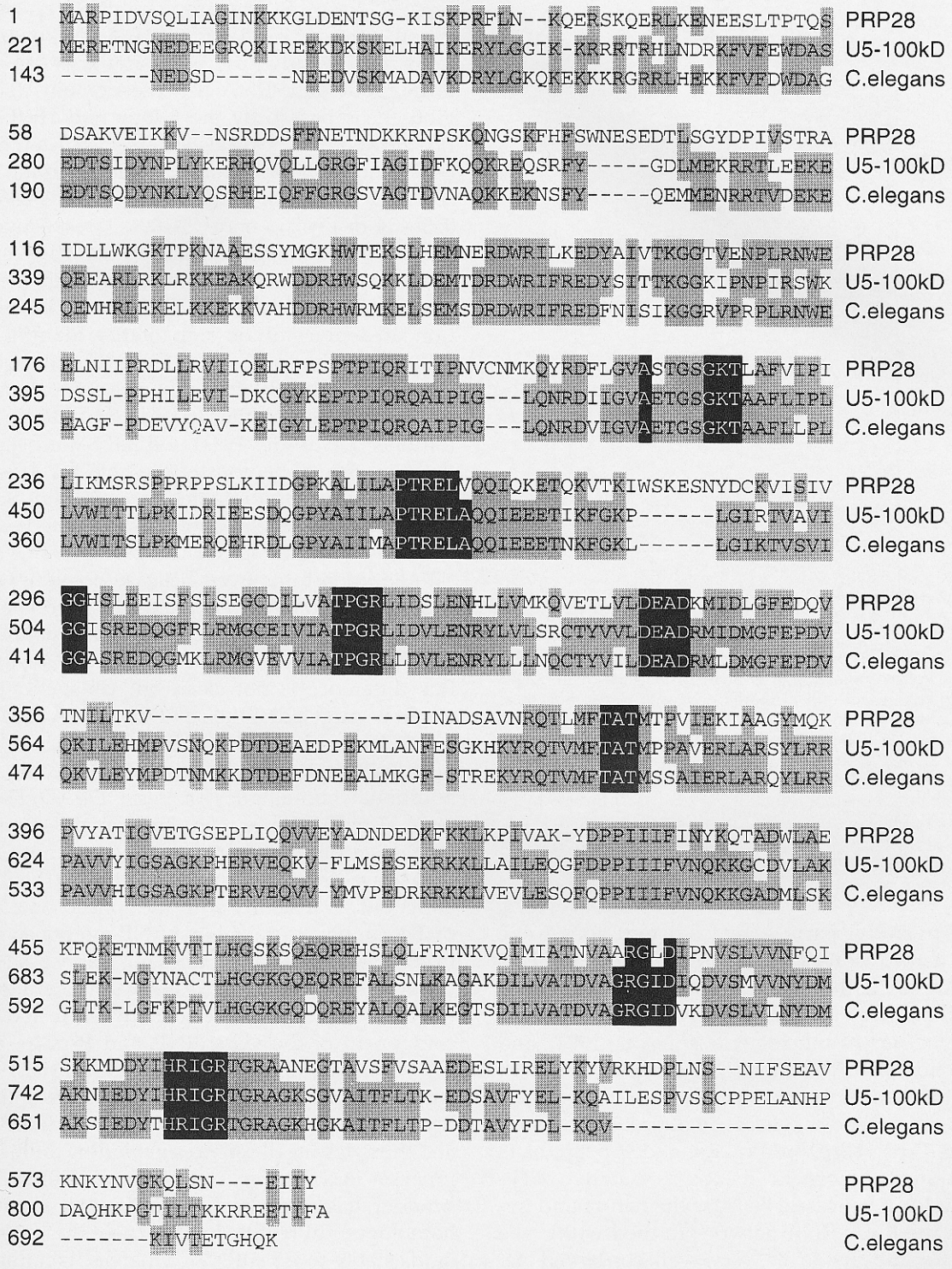


FIGURE 5. Amino acid sequence alignment of the DEAD-box domain of U5-100kD (amino acids 221–820) (Genbank accession no. AF026402) with homologues from *C. elegans* (accession no. U13070) and *S. cerevisiae* (Prp28p) (accession no. X56934). Sequences were aligned using the ClustalV program. The identity of the proteins is indicated on the right and positions of amino acids are given on the left. Identical residues shared by two or more proteins are indicated as shaded boxes, and conserved motifs characteristic of the DEAD-box protein family are marked by black boxes.

sential splicing factor PSF as a component of highly purified [U4/U6.U5] tri-snRNPs. The amount of PSF co-isolated with these complexes varies from batch to batch, but is reproducibly observed in all tri-snRNP preparations. Because PSF has been shown to be specifically associated with spliceosomal C complexes (i.e., it interacts with the spliceosome subsequent to tri-snRNP incorporation) (Gozani et al., 1994), its associ-

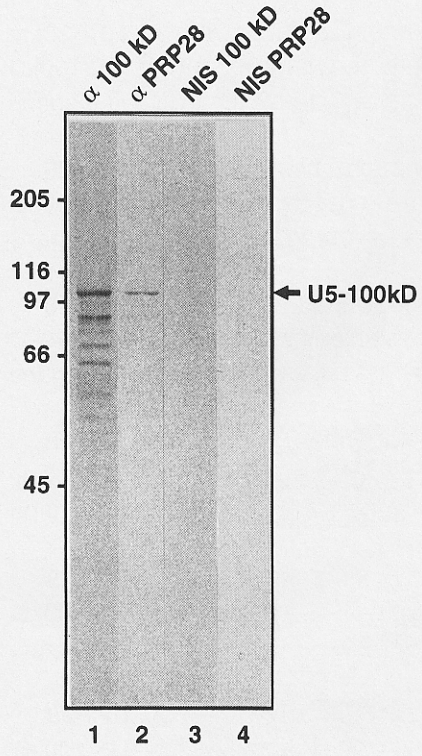


FIGURE 6. Prp28p-specific antibodies recognize the HeLa U5-100kD protein. Total spliceosomal snRNP proteins were separated on a 9% SDS-polyacrylamide gel and electroblotted onto nitrocellulose as described in Materials and Methods. Electroblotted proteins were probed with anti-U5-100kD-specific immune (lane 1) or nonimmune serum (lane 3), and with anti-Prp28p-specific immune (lane 2) or nonimmune serum (lane 4). The position of molecular weight markers is indicated on the left and that of the U5-100kD protein by an arrow on the right.

ation with tri-snRNP particles may be a fortuitous event, owing to its proximity with one or more components of this complex within the spliceosome. On the other hand, PSF has been shown to be required for the second catalytic step of splicing (Gozani et al., 1994), and its interaction with the tri-snRNP complex could be essential for its activity during splicing.

In yeast, a number of spliceosomal proteins, including Prp2p, Prp5p, Prp16p, Prp22p, and Prp28p, have been identified by genetic means as putative ATP-dependent RNA helicases. Because the nature and dynamics of the RNA–RNA network formed within the spliceosome appears to be very well conserved between yeast and higher eukaryotes, it has long been assumed that the metazoan splicing machinery contains homologues of these essential yeast splicing factors. However, the first metazoan counterparts of these proteins have only recently been identified. The human homologue of Prp22p, HRH1, was the first of these to be elucidated (Ono et al., 1994; Ohno & Shimura, 1996). Here, we have demonstrated that U5-100kD is structurally homologous to the yeast DEAD-box protein Prp28p (Fig. 5), which is an essential factor required prior to the first catalytic step of splicing (Strauss & Guthrie, 1991, 1994). A high degree of homology (50% identity, 66% similarity) is observed throughout their RNA helicase domains (i.e, the region encompassed by the P-loop and HRIGR motif). Prp28p and U5-100kD also share significant homology (45% identity, 64% similarity) in an 85-amino acid long region upstream of the P-loop (positions 136–221 and 355–434 of Prp28p and U5-100kD, respectively), suggesting that this region is also functionally important. Based on their shared amino acid sequence homology, U5-100kD most likely also performs an essential function at a precatalytic step of the mammalian pre-mRNA splicing process. Although immunodepletion studies with anti-PRP28 antibodies demonstrated a role for PRP28 prior to the first catalytic step of splicing (Strauss & Guthrie, 1991), we have not been able, to date, to clearly demonstrate a role for U5-100kD in splicing by immuno-depletion or immuno-inhibition experiments with the anti-100kD antibodies that are currently available in our laboratory.

In addition to its RNA helicase domain, U5-100kD, as well as HRH1, also contains an RS domain that is missing from its yeast homologue (i.e., neither Prp28p nor Prp22p contain an RS domain). The significance of this difference is not clear, but U5-100kD or HRH1 might be targeted to their putative RNA substrates via an RS domain interaction with other spliceosomal components (see below). It will be interesting to see if additional homologues of yeast DEAD/H-box proteins also contain a modular domain structure that includes a protein–protein interaction motif.

In yeast, several of these putative PRP helicases interact only transiently with the spliceosome and none of them appear to be stable snRNP components. Prp28p, in contrast to U5-100kD, does not stably associate with U5 or tri-snRNP complexes in yeast, but a genetic interaction has been detected between it and Prp8p, a component of the U5 snRNP, and Prp24p, a component of the U6 snRNP (Strauss & Guthrie, 1991). This suggests that, within the context of the spliceosome, Prp28p also interacts with U5 or tri-snRNP proteins and thus, despite differences in binding affinities, could function in a manner similar to U5-100kD. A similar situation has been observed for Prp18p and its human homologue, hPrp18p. In this case, the yeast protein binds stably to U5 snRNPs, whereas its human homologue is only transiently associated with the spliceosome; nonetheless, both proteins are required for the second catalytic step of splicing (Horowitz & Abelson, 1993; Horowitz & Krainer, 1997). A third putative spliceosomal RNA helicase that has been identified in higher eukaryotes, the U5-200kD DEXH-box protein, is an integral component of [U4/U6.U5] tri-snRNPs both in yeast and man (Lauber et al., 1996).

Evidence that Prp28p or its human homologue, U5-100kD, exhibits ATP-dependent RNA helicase activity

is presently lacking. However, it has generally proven difficult to demonstrate RNA unwinding activity for any of the known spliceosomal DEAD/H-box proteins in a purified system. One potential explanation for this is that these proteins require the complex environment of the spliceosome and/or helicase-activating proteins for activity. Previous biochemical studies demonstrating that highly purified U5 snRNPs and [U4/U6.U5] tri-snRNPs exhibit ATPase activity are consistent with the hypothesis that U5-100kD acts as an ATP-dependent RNA helicase (Laggerbauer et al., 1996). Although the U5-200kD protein was identified by UV crosslinking as the major ATP-binding protein in the U5 snRNP particle, minor crosslinks were also observed between ATP and several proteins in the 100-kD molecular weight range (Laggerbauer et al., 1996).

In light of the fact that U5-100kD is an integral U5 snRNP component, a number of RNA helices formed within the spliceosome come to mind as candidate substrates for its putative RNA unwinding activity. Because U5-100kD is incorporated into the spliceosome at the time of tri-snRNP addition, it most likely facilitates structural rearrangements occurring during or after the formation of catalytically active spliceosomes. Prp28p, for example, is required prior to the first catalytic step of splicing and, due to its genetic interactions with Prp24 and Prp8p, it has been proposed to play a role in destabilizing the U4/U6 intermolecular helices during spliceosome formation (Strauss & Guthrie, 1991). Based on the fact that U5-100kD is a component of the [U4/U6.U5] tri-snRNP, it is attractive to speculate that it, too, might facilitate unwinding of the U4/U6 snRNAs prior to the first step of splicing. However, in vitro RNA helicase assays suggest that the U5-200kD protein, and not U5-100kD, is responsible for unwinding the U4/U6 intermolecular helix (B. Laggerbauer, T. Achsel, & R. Lührmann, unpubl.). A number of alternative functions can also be envisaged for U5-100kD. For example, it could be involved in destabilizing the U1/5′ splice site interaction, thereby facilitating the subsequent interactions of U6 and U5 snRNA with the 5′ splice site, or it could conceivably catalyze conformational changes in the U2 and U6 snRNAs.

The demonstration that U5-100kD contains an RS domain suggests that it may perform additional functions during splicing. RS domains are known to interact with one another, and a number of functionally important protein–protein interactions involve the association of two SR proteins via their RS domains. Foremost in the function of this domain is the facilitation of intermolecular interactions during spliceosome formation, particularly at the early stages of this process (reviewed by Fu 1995; Manley & Tacke, 1996). For example, the U1/5′ splice site interaction is stabilized by an interaction between the RS domain of the U1-70K protein and that of the SR protein ASF/SF2 (Kohtz et al., 1994). The recent demonstration that SR proteins promote the integration of tri-snRNPs into the spliceosome (Roscigno & Garcia-Blanco, 1995) and the identification of an RS domain in the tri-snRNP-specific 27K protein (Fetzer et al., 1997) suggest that protein–protein interactions involving SR proteins probably also facilitate the stable association of the [U4/U6.U5] tri-snRNP complex with the pre-spliceosome. The U5-100kD protein could thus contribute to this process by interacting with SR proteins present in the pre-spliceosome. Because the U5 snRNP interacts with the 5′ splice site subsequent to U1, U5-100kD could conceivably replace U1-70K in its interaction with other SR proteins. Consistent with this hypothesis, the biochemical character of U5-100kD's RS domain is similar to that of the U1-70K protein. Both RS domains contain a high percentage of alternating mixed-charged residues (e.g., RD and RE dipeptides) and only short streches of RS dipeptides (Fig. 2A). Interestingly, a U1-70K-like RS domain is also found in the putative RNA helicase, HRH1 (Ono et al., 1994). The interaction of U5-100kD's RS domain with other spliceosomal proteins could also target this putative RNA helicase to its substrate. An interaction with SR proteins at the 5′ splice site, for example, could favorably position it to unwind the U1 snRNA base pairing interaction with the pre-mRNA. It is also possible that an RS-RS domain interaction could trigger its putative RNA helicase activity. Finally, consistent with the known genetic interaction of Prp28p with both U5 and U6 components (Strauss & Guthrie, 1991), protein–protein interactions involving the RS domain of U5-100kD could potentially mediate the association of U5 with U4/U6 to form the tri-snRNP complex.

We have also demonstrated that U5-100kD can be phosphorylated in vitro by Clk/Sty and the U1 snRNP-associated kinase (Fig. 4), both of which are known to phosphorylate the RS domains of SR proteins (Woppmann et al., 1993; Colwill et al., 1996; Fetzer et al., 1997). RS domain interactions can be modulated by phosphorylation (Xiao & Manley, 1997) and thus the interaction of the U5-100kD protein with other spliceosomal proteins could be a regulated process. The activity of SR protein kinases/phosphatases therefore also could indirectly modulate the putative helicase activity of this protein by preventing or promoting interactions with its substrate. Future biochemical studies are clearly needed to identify potential interaction partners of the U5-100kD protein, as well as to identify its substrate RNA.

MATERIALS AND METHODS

Isolation and microsequencing of snRNP proteins

Nuclear extracts were prepared from HeLa cells (Computer Cell Culture Company, Mons, Belgium) by the method of

Dignam et al. (1983). U1, U2, U5, and U4/U6 snRNPs were purified from HeLa nuclear extract by immunoaffinity chromatography using the monoclonal antibody H20 as described previously (Bach et al., 1990). Affinity-purified snRNPs were separated by centrifugation on a 10–30% glycerol gradient containing 150 mM KCl, 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 1 mM DTE, and 0.5 mM PMSF (Behrens & Lührmann, 1991). SnRNP proteins were extracted from 20 μg of affinity-purified snRNPs with 1 volume phenol and precipitated from the phenolic phase with 5 volumes of acetone. The precipitated proteins were fractionated in the first dimension by NEPHGE according to the manufacturer's instructions (Bio-Rad) and in the second dimension by SDS-PAGE. Coomassie-stained spots of individual snRNP proteins were excised from the gel, concentrated by funnel well SDS-PAGE, and electroblotted onto sequencing-grade PVDF-membrane (Bio-Rad). Partial amino acid sequences of tryptic peptides of the snRNP proteins were obtained by microsequencing on an ABI 477A protein sequencer.

Partial peptide sequences obtained from the 100-kD protein were:

100kD-1: FVFEWDASEDTSIDYNPLY
100kD-2: GVAITFLTK
100kD-3: CGYKEPTPIQR.

Partial peptide sequences obtained from PSF were:

PSF-1: TYTQRCRLFVGNLPADITED
PSF-2: QQREQVEK
PSF-3: IVEFASK.

Database search, cDNA cloning, and sequencing

A database search was performed on the NIH mailserver using the TBLASTN and FASTA programs (Altschul et al., 1990). An EST with an open reading frame matching the partial peptide sequence 100kD-3 was part of the human infant brain cDNA clone 51919 (GenBank accession no. H24106), which is incomplete at the 5′ end. Further database searches in the course of this work revealed three additional ESTs matching the partial peptide sequence 100kD-2 (GenBank accession nos. R46313, T89754, H65151). A 304-bp fragment matching the 5′-region of clone H24106 was isolated from the infant brain cDNA clone by PCR using gene-specific primers (100Kfor1 and 100Krev2). Screening of a HeLa cell λ-Zap cDNA library with this fragment resulted in the isolation of three identical 3.3-kb clones. Sequence analysis was performed with an automated DNA sequencer (Applied Biosystems) using *Taq* polymerase and double-stranded templates (PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit, Pharmacia). Sequence alignment of homologous proteins was performed with the ClustalV program. Similar residues are grouped as follows: (DE), (HKR), (GNQST), and (AVILPFYWCM).

Antibody production and immunoprecipitation of snRNP particles

Antibodies to U5-100kD were raised against two fragments of clone 51919 expressed as glutathione-S-transferase fusion proteins. Expression vectors were generated by cloning a 309-bp *Bam*H I-blunt end fragment (amplified by PCR using the primers 100KBamHI and 100Krev2) and a 994-bp *Bam*H I-blunt end fragment (amplified by PCR using the primers 100KBamHI and 100Krev3) into pGEX-4T2 (Pharmacia). Overexpression of the GST-fusion proteins in *Escherichia coli* DH5α was induced by incubation with isopropyl β-D-thiogalactoside (1 mM) for 4 h at 30 °C. Cells were disrupted by sonication, and inclusion bodies, which contained at least 90% of the fusion proteins, were redissolved in PBS (20 mM potassium phosphate and 130 mM NaCl, pH 7.5) containing 10% SDS. Fusion proteins were purified by preparative SDS-PAGE, followed by electroelution, and used subsequently for immunization (Harlow & Lane, 1988). For immunological detection of proteins, nuclear extract and affinity-purified snRNPs were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes (Schleicher and Schüll). The blots were probed with anti-100kD and anti-PRP28 antibodies and immunostained as described by Lehmeier et al. (1990).

Immunoprecipitation of snRNP particles

To immunoprecipitate snRNP particles with anti-U5-100kD antibodies, 30 μL of antiserum in 300 μL PBS (per sample) were coupled overnight at 4 °C to 20 μL pre-swollen protein A Sepharose beads (PAS; Pharmacia). The beads were then washed three times with PBS and resuspended in 300 mL of TBSN [50 mM Tris/HCl, pH 7.4, 150 mM NaCl; 0.05% (v/v) NP-40]. The stringency of the immunoprecipitation was varied by altering the concentration of NaCl from 150 to 600 mM. Affinity-purified snRNPs (5–10 μg) were incubated with the PAS-antibody conjugate for 2 h at 4 °C and washed four times with ice-cold TBSN. The antibody complexes, in 300 μL TBSN, were extracted twice with phenol/chloroform and the snRNAs were precipitated from the aqueous phase with ethanol. Precipitated snRNAs were 3′ end-labeled with [^{32}P]-pCp as described by England and Uhlenbeck (1978), separated on a 10% polyacrylamide–8 M urea gel, and visualized by autoradiography.

Phosphorylation of the U5-100kD protein

For in vitro phosphorylation studies with Clk/Sty kinase, 10 pmol of U5 snRNP particles, purified by immunoaffinity chromatography and glycerol gradient centrifugation, were incubated in a total volume of 60 μL containing 50 mM triethanolamine, pH 7.8, 15 mM $MgCl_2$, 5 mM DTT, 100 mM NaCl, and 50 μCi [^{32}P]-γ-ATP (5,000 Ci/mmol) for 30 min at 37 °C. In some cases, 2 μL of purified recombinant Clk/Sty kinase were added to the reaction mixture. Subsequently, U5 snRNPs were disrupted by adding 30 μL denaturing cocktail (5% SDS, 2.7% Triton-X-100, and 233 μM DTT) and incubating at 90 °C for 2 min. The reaction was diluted 10-fold by addition of 820 μL PBS, pH 8.0, and the U5-100kD protein was immunoprecipitated with anti-100kD antibodies as described above. Precipitated antibody complexes were boiled for 10 min in SDS-PAGE sample buffer and separated on a 9% SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue R250 and radiolabeled proteins were visualized by autoradiography. For phosphorylation with snRNP-associated kinase, 20 pmol of U5 snRNP particles

were incubated with 1 μM [^{32}P]-γ-ATP (5,000 Ci/mmol) in the absence or presence of 10 μg kinase active U1 snRNPs for 30 min at 37 °C. Proteins were isolated by phenol/chloroform extraction, fractionated by NEPHGE/SDS-PAGE as described above, and visualized by Coomassie staining and/or autoradiography.

Oligonucleotides and primers used in this work

100Kfor1 5′-GACAGGGACTGGCGGATCTT-3′
100Krev2 5′-CCTTGGTCTGACTCTTCGAT-3′
100KBamHI 5′-GGGGATCCGACAGGGACTGGCGGATCTT-3′
100Krev3 5′-CCGTGCAGTGTGCAAGCATT-3′
pGEX 5′-sequencing primer 5′-GGGCTGGCAAGCCACGTTTGGTG-3′.

Gene-specific sequencing primers are not listed.

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