The HeLa 200 kDa U5 snRNP-specific protein and its homologue in *Saccharomyces cerevisiae* are members of the DEXH-box protein family of putative RNA helicases

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The primary structure of the 200 kDa protein of purified HeLa U5 snRNPs (U5-200kD) was characterized by cloning and sequencing of its cDNA. In order to confirm that U5-200kD is distinct from U5-220kD we demonstrate by protein sequencing that the human U5-specific 220 kDa protein is homologous to the yeast U5-specific protein Prp8p. A 246 kDa protein (Snu246p) homologous to U5-200kD was identified in *Saccharomyces cerevisiae*. Both proteins contain two conserved domains characteristic of the DEXH-box protein family of putative RNA helicases and RNA-stimulated ATPases. Antibodies raised against fusion proteins produced from fragments of the cloned mammalian cDNA interact specifically with the HeLa U5-200kD protein on Western blots and co-immunoprecipitate U5 snRNA and to a lesser extent U4 and U6 snRNAs from HeLa snRNPs. Similarly, U4 and U6 snRNAs can be co-immunoprecipitated from yeast splicing extracts containing an HA-tagged derivative of Snu246p with HA-tag specific antibodies. U5-200kD and Snu246p are thus the first putative RNA helicases shown to be intrinsic components of snRNPs. Disruption of the *SNU246* gene in yeast is lethal and leads to a splicing defect *in vivo*, indicating that the protein is essential for splicing. Anti-U5-200kD antibodies specifically block the second step of mammalian splicing *in vitro*, demonstrating for the first time that a DEXH-box protein is involved in mammalian splicing. We propose that U5-200kD and Snu246p promote one or more conformational changes in the dynamic network of RNA–RNA interactions in the spliceosome.

Keywords: ATPase/DEXH-box/RNA helicase/RNA splicing/U5 snRNP

Introduction

Nuclear pre-mRNA splicing proceeds via two successive transesterification steps. In the first step, the 2' hydroxyl of the branch-point adenosine attacks the 5' splice site, thereby forming the splicing intermediates, exon 1 and lariat-exon 2. The second step involves a nucleophilic attack by the 3' hydroxyl of exon 1 at the 3' splice site

resulting in ligated exons 1 and 2 and excised intron in the form of a lariat. The splicing reaction is catalysed by a macromolecular complex denoted the spliceosome. The spliceosome assembles in an orderly fashion on the pre-mRNA and consists of four small nuclear ribonucleoprotein particles (snRNPs), U1, U2, U4/U6 and U5, and a large but as yet undefined number of non-snRNP splicing factors (for review, see Green, 1991; Guthrie, 1991; Rymond and Rosbash, 1992; Moore *et al.*, 1993). The biochemical composition of mammalian spliceosomal snRNPs has been investigated in much detail and more than 40 distinct snRNP proteins have been detected. Two classes of snRNP proteins can be distinguished: the common proteins (also denoted Sm-proteins) which are associated with all snRNPs, and the snRNP-specific proteins. The number of specific proteins associated with individual snRNPs varies considerably. While U1 snRNP contains only three specific proteins, the 17S U2 snRNP contains 11, nine of which are bound in a highly salt-sensitive manner (Behrens *et al.*, 1993). The most complex protein composition is observed for the 25S [U4/U6.U5] tri-snRNP complex. The 20S U5 snRNP contains nine specific proteins with apparent molecular weights of 15, 40, 52, 100, 102, 110, 116, 200 and 220 kDa (Behrens and Lührmann, 1991), while two proteins with apparent molecular weights of 60 and 90 kDa are associated with the 12S U4/U6 snRNP (Gozani *et al.*, 1994; J.Lauber and R.Lührmann, manuscript in preparation). The 25S [U4/U6.U5] tri-snRNP complex contains an additional five proteins with apparent molecular weights of 15.5, 20, 27, 61 and 63 kDa (Behrens and Lührmann, 1991; J.Lauber and R.Lührmann, in preparation).

Like their snRNA counterparts, a number of snRNP proteins have been shown to be evolutionarily conserved between yeast and man. The first indication for evolutionary conservation among snRNP proteins came from studies investigating the immunological cross-reactivity of antibodies directed against the yeast U5-specific Prp8 protein; surprisingly, anti-Prp8p antibodies recognized the 220 kDa protein of mammalian U5 snRNPs, indicating that the two proteins are structurally related (Anderson *et al.*, 1989; Pinto and Steitz, 1989). Over the past few years, based on antibody cross-reactivity and/or sequence comparisons, numerous additional human snRNP protein homologues have been identified in yeast (for review, see Beggs, 1995; Krämer, 1995; Will *et al.*, 1995). In addition, biochemical purification of yeast 25S [U4/U6.U5] tri-snRNPs not only revealed a similarly complex protein composition but also, as demonstrated by electron microscopy, a similar ultrastructure to the human [U4/U6.U5] tri-snRNP (Fabrizio *et al.*, 1994), supporting the idea that the tri-snRNP complex is functionally equivalent in both species.

The snRNPs serve at least three functions in pre-mRNA splicing: (i) they help distinguish exons from introns by

recognizing and binding the 5' and 3' splice sites as well as the branch site; (ii) they impart a three-dimensional structure to the intron such that the reactive sites of the pre-mRNA are juxtaposed; and (iii) they contribute to the catalysis of pre-mRNA splicing. The snRNPs accomplish these functions, at least in part, by forming a network of RNA–RNA interactions, both with the pre-mRNA substrate and with each other. These RNA–RNA interactions are also evolutionarily highly conserved between yeast and man (Guthrie, 1991; Moore *et al.*, 1993; Nilsen, 1994). U1 and U2 snRNP initially base-pair with the 5' splice site and the branch site, respectively. U4/U6 and U5 interact subsequently with the pre-mRNA as part of a preformed 25S [U4/U6.U5] tri-snRNP complex. U5 snRNA, through its conserved loop 1, interacts with exonic sequences at both splice sites (Newman and Norman, 1991, 1992; Wyatt *et al.*, 1992; Cortes *et al.*, 1993; Sontheimer and Steitz, 1993), while the conserved ACA-GAG sequence of U6 snRNA interacts with intron sequences at the 5' splice site (Sawa and Abelson, 1992; Kandels-Lewis and Séraphin, 1993; Lesser and Guthrie, 1993; Sontheimer and Steitz, 1993). Before catalysis the snRNAs undergo a number of conformational transitions. For example, the two intermolecular helices between the U4 and U6 snRNAs within the U4/U6 snRNP particle dissociate (Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Konarska and Sharp, 1987; Siliciano *et al.*, 1987; Brow and Guthrie, 1988; Blencowe *et al.*, 1989.) The dissociation of U4/U6 is accompanied by the formation of U6/U2 snRNA intermolecular helices (Hausner *et al.*, 1990; Madhani *et al.*, 1990; Datta and Weiner, 1991; Wu and Manley, 1991; Madhani and Guthrie, 1992; Sun and Manley, 1995).

As described above, the RNA network in the spliceosome is highly dynamic and the snRNAs undergo many conformational changes not only during spliceosome assembly, but also as the splicing reaction proceeds (for review, see Wise, 1993; Madhani and Guthrie, 1994; Nilsen, 1994). The driving forces behind these RNA conformational changes and how the disruption and formation of RNA–RNA helices is regulated, are poorly understood. Interestingly, a group of non-snRNP splicing factors, identified by genetic means in yeast, has been postulated to play an important role in the dynamics of the RNA–RNA network. These proteins, which all contain putative ATP-binding domains, fall into two related classes. Specifically, two proteins, Prp5p (Dalbadie-McFarland and Abelson, 1990) and Prp28p (Strauss and Guthrie, 1991), belong to the DEAD-box family of putative ATP-dependent RNA helicases, while Prp2p (Chen and Lin, 1990), Prp16p (Burgess *et al.*, 1990) and Prp22p (Company *et al.*, 1991) fall into a subfamily of putative RNA helicases that is characterized by a DEAH-box (Wassarman and Steitz, 1991; Schmid and Linder, 1992). The DEAH-box proteins contain motifs which are related to but distinct from the DEAD-box motif (e.g. DEAH versus DEAD, GRAGR versus HRIGR) (Burgess *et al.*, 1990; Chen and Lin, 1990; Company *et al.*, 1991). The putative ATP-binding domain of both DEAH and DEAD proteins contains the motif G/AX₄GKT). While Prp5p, Prp28p and Prp2p most likely act before catalysis, Prp16p acts after step 1 but before step 2 of splicing. Prp22p is required for the release of

the spliced exons from the spliceosome after step 2 of splicing (Company *et al.*, 1991). While an *in vitro* helicase activity has not as yet been demonstrated for any of the above proteins, Prp2p and Prp16p have been shown to exhibit RNA-dependent ATPase activity *in vitro* (Schwer and Guthrie, 1991; Kim *et al.*, 1992). Moreover, there is strong evidence that Prp2p- and Prp16p-catalysed ATP hydrolysis is accompanied by a conformational rearrangement in the spliceosome (Schwer and Guthrie, 1992; Kim and Lin, 1993; Teigelkamp *et al.*, 1994). Interestingly, the DEAD/DEAH-box Prp proteins are not stably bound to snRNPs and, as has been demonstrated for Prp2p and Prp16p, apparently interact only transiently with the spliceosome (King and Beggs, 1990; Schwer and Guthrie, 1991; Teigelkamp *et al.*, 1994).

While the splicing machinery appears to be highly conserved between yeast and man, homologues of the above-described DEAD/DEAH PRP splicing factors have not yet been identified in mammalian splicing systems. A mammalian homologue of Prp22p has been reported (Ono *et al.*, 1994); however, involvement of this protein in mammalian pre-mRNA splicing has yet to be demonstrated. A potential mammalian homologue of a putative yeast RNA helicase has recently been identified by studies investigating enzymatic activities associated with purified HeLa spliceosomal snRNPs (Laggerbauer *et al.*, 1996). In these studies, we observed that purified 25S [U4/U6.U5] tri-snRNPs as well as purified 20S U5 snRNPs, contain an RNA-dependent ATPase activity. Moreover, it was possible to cross-link the U5-specific 200 kDa protein to radiolabelled ATP, suggesting that this protein might harbour an ATP-binding domain.

Here, we describe the molecular cloning of the HeLa U5-specific 200 kDa protein and demonstrate that it belongs to the DEXH-box family of putative RNA helicases. Interestingly, the 200 kDa protein harbours one DEIH and one DEVH domain each of which contains, in addition to the DEXH-sequence, all other sequence motifs necessary for a functional helicase domain. To our knowledge, this is the first example of a protein containing two putative RNA helicase domains. A database search revealed a close homologue of the human U5-specific 200 kDa protein in yeast, which has a predicted molecular weight of 246 kDa and also possesses two DEXH-box RNA helicase domains. We further show that the yeast protein, which we denote Snu246p, is an intrinsic component of the yeast 25S [U4/U6.U5] tri-snRNP complex and that it is essential for yeast cell viability. Genetic depletion of *SNU246* indicated that it is involved in pre-mRNA splicing *in vivo*. The participation of the U5-specific, putative RNA helicase in pre-mRNA splicing was investigated in more detail in the mammalian splicing system by antibody inhibition studies. Antibodies raised against the human U5 200 kDa protein specifically inhibit the second step of pre-mRNA splicing *in vitro*, as evidenced by the accumulation of splicing intermediates (i.e. exon 1 and exon 2 intron lariat).

Results

Cloning of a cDNA for the human U5-specific 200 kDa protein

Peptide sequences obtained by microsequencing of the purified human U5-specific 200 kDa protein (hereafter

termed U5-200kD protein) allowed a database search for corresponding open reading frames. Two of five peptide sequences were 100% identical with parts of an open reading frame of an expressed sequence tag (EST). The EST resulted from sequencing of clone HFBCN20 (accession No.: M85756) which had been derived from a human foetal brain cDNA library. Oligonucleotides complementary to the EST sequence were designed to amplify a 320 bp DNA fragment by PCR. Screening of a HeLa cell cDNA library with this probe led to the isolation of a 4.94 kb cDNA clone. Sequence missing at the 5' end of the clone was obtained by PCR amplification of reverse-transcribed HeLa mRNA with specific primers, which resulted in a cDNA clone of 5.4 kb. The open reading frame of the cDNA encodes a protein with a predicted molecular weight of 194.4 kDa and a calculated isoelectric point of 6.53. A polyadenylation signal (nucleotides 5324–5329) is present 218 nucleotides downstream of the stop codon (nucleotides 5104–5106), and a poly(A) tail is present 22 nucleotides downstream of the AAUAAA sequence (nucleotides 5351–5395), indicating that the cDNA comprises the complete carboxy-terminus of the protein. Since the primary amino acid sequence does not contain a methionine at the amino-terminus, the cDNA may still be incomplete. Unfortunately, several attempts to identify the 5' end of the cDNA (e.g. by RACE) in two different libraries failed. It appears, however, that only a minor portion of the sequence is missing since the calculated molecular weight of the protein is close to the apparent molecular weight (~200 kDa) observed by SDS-PAGE (Figure 1).

It was verified by two criteria that the putative protein product of this clone is the U5-200kD protein. First, all of the peptide sequences obtained by microsequencing were found in the predicted amino acid sequence (Figure 1). Two out of five peptide sequences were found in the newly cloned cDNA upstream of the start codon of the EST (Figure 1). Second, antibodies raised against two regions of the protein encoded by the cloned cDNA interact specifically with the HeLa U5-200kD protein from HeLa cells on Western blots (see below, Figure 2).

The U5-200kD protein contains two putative RNA helicase domains of the DEXH-box protein family

The predicted primary structure encoded by the U5-200kD cDNA places the protein in the DEAD/DEXH-box protein family of RNA-stimulated ATPases and putative RNA helicases. Strikingly, the domain harbouring the conserved motifs which characterize this protein family (Fuller-Pace and Lane, 1992; Schmid and Linder, 1992) is present twice in the predicted amino acid sequence (hereafter referred to as DEXH-domains I and II; see Figures 1 and 4B). The conserved DEXH-domain I is located from amino acid positions 68 to 423, whereas DEXH-domain II is located between amino acids 915 and 1258 (Figures 1 and 4A). Both domains contain the conserved motifs characteristic of putative RNA helicases of the DEXH-box protein family (Gorbalenya *et al.*, 1989), suggesting that both domains bind ATP (for a more detailed comparison of the two domains see below and Discussion). The most prominent conservation is found in a p-loop sequence (G/AX₄GKT) at positions 68–75 and 915–922, a DEIH motif at position 180–183 in DEXH-domain I

	10	20	30	40	50
1	RRQRKGYEEV	HVPALKPKPF	GSEEQLLPVE	KLPKYAQAGF	EGFQTLNRIG
51	SKLYRAALET	DENLLLCAPT	GAGKTIN	VALM	CMLREIGKHI
101	FKIIYIA PMR	SLVQEMVGSF	GKRLATYGIT	VAELTGDHQL	CKEEISATQI
151	IVCT TEPKWDI	ITRKGGERTY	TQLVRLIVLD	ETIHLHDDRG	PVLEALVARA
201	IRNIEMTQED	VRLLIGLSATL	PNYEDVATFL	RVDPAKGLFY	FDNSFRPVP
251	EQTYVGITEK	KAIKRFQIMN	EIVYEKIMEH	AGKNQVLVVF	HSRKETGKTA
301	RAIROMCLEK	DTLGLFLREG	SASTEVLRT	AEQCKNLELK	DLPLYGFAIH
351	HAEVTRVDRT	LVEDLFGDKH	IQVLVSTATL	AWGVNLPANT	VIKIGTQVYS
401	PEKGRWTELG	ALDILLQMLGR	AGRPQYDTKG	EGILITSHGE	LQYYLSLLNQ
451	QLPIESQMVS	KLPDMLNAEI	VLGNVQNAKD	AVNWLGYAYL	YIRMLRSPTL
501	YGISHDDLKG	DPLLDQRRLD	LVHTAALMLD	KNNLVKYDKK	TGNFQVTELG
551	RTASHYYITN	DTVQTYNQLL	KPTLSEIELF	RVFSLSSEFK	NITVREEEKL
601	ELQKLLERVP	IPVKESIEEP	SAKINVLLQA	FISQLKLEGF	ALMADMVYVT
651	QSAGRLMRAI	FEIVLNRGWA	QLTDKTLNLC	KMIDKRMWQS	MCPLRQFRKL
701	PEEVVKKIEK	KNFPEFLRYD	LNHNEIGELI	RMPKMGKTIH	KYVHLFPKLE
751	LSVHLQPIR	STLKVLTIT	PDFQNDKEVH	GSSEAFWILV	EDVDSEVILH
801	HEYFLLKAKY	AQDEHLITFF	VPVFEPLPPQ	YFIRVVSDFW	LSCETQLPVS
851	FRHLT LEKY	PPFTELLDLQ	PLPVSAALNS	AFESLYQDKF	PFNFPIQTQV
901	FNTVNSDDN	VFVQAPTGSQ	KTLCAEFAIL	RMLQNSEGR	CVYTTPNRLW
951	QEQVYMDWYE	KFQDRLNKKV	VLLTGETSTD	LKLLGKGNII	ISTTEPKWDIL
1001	SRRWQRKNV	QNINLFVVDE	VHLIGGENP	VLEVICSRMR	YISSQIERPT
1051	RIVALSSLS	NAKDVAHWLG	CSATSTENF	PNVRVPLEL	HIQGFNISHT
1101	QTRLLSMAKP	VEHAITKHSP	KKPVIVEVPS	RKQRLTAID	LITTCADIQ
1151	RQRFLHCTEK	DLIPYLEKLS	DSTLKETLLN	GVGYLHEGLS	PMERRLVEQI
1201	FSSGAIQVVV	ASRSICWGMN	VAAHLVIIMD	TLYYNGKIHA	YVDYPIYDVL
1251	QMVGHANRPL	QDDEGRVIM	CQGSKKDFFK	KFLYELPVE	SHLDHCHMDH
1301	FNAEIVTKTI	ENKQDAVDYL	TWTFLYRMT	QNPNNYNLQG	ISHRHLSDEL
1351	SELVEQTLSD	LEQSKCISIE	DEMDEVAPNL	GMAAYYYIN	YTTIELFSMS
1401	LNAKTKVRGL	IEIISNAEY	ENIPIRHHEE	NLLRLAQKV	PHKLNNPKFN
1451	DPHVNTLLL	QAHLSRMQLS	AELQSDTEEI	LSKAIRLIQA	CVVDLSNSGW
1501	LSPALAAMEL	AQMVTQAMWS	EDSYLRRLPP	FPSGLFKRCT	DKGVESVFDI
1551	MEMEDEERNA	LLQLTDSQIA	DVARFCNRYP	NIELSYEVVD	KDSIRSGGPV
1601	VVLVQLEREE	EVTGPVIAPL	FPQKREEGWW	VVIGDAKSNS	LISIKRLTLQ
1651	QKAKVKLDFV	APATGGRHNT	LYFMSDAYMG	CDQEYKESVD	VKEAETSDSDS
1701	D				

Fig. 1. Amino acid sequence (single letter code) of the U5-200kD protein. Conserved motifs common to the DEXH-box protein family are indicated by shaded boxes. Amino acid sequences obtained by microsequencing of U5-200kD tryptic peptides are in italics and underlined. The arrow indicates the start of the expressed sequence tag of clone HFBCN20. The EMBL/GenBank accession number for the U5-200kD cDNA is Z70200.

and a corresponding DEVH motif at position 1019–1022 in DEXH-domain II, a SAT box at position 217–219 in DEXH-domain I and a corresponding SSS motif at position 1056–1058, and degenerate QRIGRXXX sequences at position 416–423 (QMLGRAGR) in DEXH-domain I and at position 1251–1258 (QMVGHANR) in DEXH-domain II (Figure 1).

Antibodies raised against the recombinant DEXH-box protein interact with the U5-200kD protein

For further structural and functional characterization of the U5-200kD protein, polyclonal antibodies were raised against fusion proteins containing glutathione S-transferase (GST) and fragments of the recombinant U5-200kD protein. Specifically, antisera were raised against a GST fusion protein containing either the amino-terminal amino acids 157–934 or the carboxy-terminal amino acids 935–1701. In contrast to the preimmune serum both immune sera bind specifically to the U5-200kD protein on immunoblots of affinity-purified HeLa snRNP proteins (Figure 2A, compare lanes 4, 6 and 7). The faint staining of

several bands migrating more quickly than the U5-200kD protein is most likely due to non-specific degradation of U5-200kD. Identical results were obtained with immunoblots of crude nuclear extract (Figure 2A, lanes 8–10). Cross-reactivity of the antisera with any of the known [U4/U6.U5] tri-snRNP-specific proteins or other nuclear proteins was not detected, suggesting that U5-200kD is structurally different from the other [U4/U6.U5] tri-snRNP specific proteins. This is of particular interest with regard to the U5 snRNP-specific 220 kDa protein, the putative homologue of the yeast splicing factor Prp8p (Anderson *et al.*, 1989). In previous immunoblotting experiments carried out with antibodies directed against Prp8p, it was unclear whether the U5-200kD protein is distinct from U5-220kD. For comparison, immunoblotting of affinity-purified snRNPs was carried out with antibodies directed against a synthetic peptide of the U5 snRNP protein migrating at 220 kDa (see below). These antibodies recognized a protein with an apparent molecular weight of 220 kDa migrating above U5-200kD (Figure 2A, lane 5), and a faint band migrating more quickly than both U5-220kD and U5-200kD. The lower band most likely represents a degradation product of U5-220kD and has been previously observed with anti-Prp8p antibodies

(Anderson *et al.*, 1989). Neither band was recognized by preimmune serum (Figure 2A, lane 3). A cross-reaction of antibodies against the 220 kDa protein with U5-200kD was not observed (Figure 2A, lanes 5–7), confirming the distinct character of both proteins.

In order to investigate whether the U5-200kD protein is exposed on the surface of the [U4/U6.U5] tri-snRNP, immunoprecipitation studies were carried out with anti-U5-200kD antibodies and purified snRNP particles that had been enriched for [U4/U6.U5] tri-snRNPs and 20S U5 snRNPs by glycerol gradient centrifugation. The monoclonal antibody Y12 was also included to monitor the total amount of snRNPs in the immunoprecipitation reactions (Figure 2B, lane 1). As shown in Figure 2B (lanes 3 and 4), anti-U5-200kD antisera precipitated predominantly U5 snRNPs, indicating that the 200 kDa protein is accessible for the antibodies in this particle. In contrast, only background precipitation of U5 snRNPs was observed with the preimmune serum (Figure 2B, lane 2). As compared with U5 snRNP, significantly less U4/U6 snRNPs were co-precipitated by the anti-U5-200kD antibodies. This is particularly true for the anti-200kD C antibodies (Figure 2B, lane 3). At present, we do not know whether binding of the antibodies to U5-200kD destabilizes the [U4/U6.U5] tri-snRNP complex or whether U5-200kD is less accessible in the 25S [U4/U6.U5] tri-snRNP as compared with the 20S U5 snRNP particle. Similar results were obtained when immunoprecipitations were carried out with total nuclear extract (data not shown).

Additional evidence that the 200 and 220 kDa proteins are distinct was obtained by microsequencing peptides derived from the purified human 220 kDa protein. Interestingly, three peptide sequences (220K b; 220K c; 220K d) are highly homologous to the yeast splicing factor Prp8p (55–83% identity; 82–100% similarity) and its homologue in *Caenorhabditis elegans* (88–100% identity; 88–100% similarity) (Figure 3). One peptide sequence (220K a) shows less, but still significant, homology to Prp8p. The

Fig. 2. Immunological characterization of the U5-200kD protein. (A) Anti-U5-200kD antibodies react specifically with U5-200kD on immunoblots. Total snRNP proteins affinity-purified with monoclonal antibody H20 (10 µg each lane; lanes 2–7) or HeLa nuclear extract (2 µl each lane; lanes 8–10) were separated on a 10% SDS–polyacrylamide gel and electroblotted (lanes 3–10) onto nitrocellulose. The position of size markers stained with Coomassie blue is shown in lane 1 with the individual molecular weights given on the left. Total snRNP proteins stained with Coomassie blue are shown in lane 2. Electroblotted snRNP proteins were probed with anti-U5-220kD preimmune serum (lane 3) or anti-U5-200kD preimmune serum (lane 4), with U5-220kD-specific antibodies (lane 5) and antibodies directed against the carboxy-terminal half (lane 6) or amino-terminal half (lane 7) of U5-200kD. Similarly, HeLa nuclear extract was analysed with anti-U5-200kD preimmune serum (lane 8) and antibodies directed against the carboxy-terminal half (lane 9) or amino-terminal half (lane 10) of U5-200kD. The position of the U5-220kD protein and the U5-200kD protein are indicated. (B) U4, U5 and U6 snRNPs are specifically precipitated by anti-U5-200kD antibodies. SnRNP fractions enriched for the [U4/U6.U5] tri-snRNP by glycerol gradient centrifugation were used for immunoprecipitation experiments. Individual snRNP particles were detected by extraction of the corresponding snRNAs, 3' end labelling with [³²P]pCp and separation of the snRNAs on a denaturing 10% polyacrylamide gel. snRNAs co-immunoprecipitated by monoclonal antibody Y12 (lane 1), anti-U5-200kD preimmune serum (lane 2) and antibodies directed against the carboxy-terminal half (lane 3) or amino-terminal half (lane 4) of U5-200kD are shown. The identity of the respective snRNAs is indicated on the right.

Fig. 3. Comparison of partial U5-220kD amino acid sequences with yeast Prp8p. The amino acid sequence of four U5-220kD fragments is shown, with the corresponding Prp8p sequence presented above. Asterisks indicate amino acids identical in U5-220kD and Prp8p, and dots indicate conservative changes. The positions of the individual homology regions in the full-length Prp8p sequence is shown as dark blocks on the map on top of the figure.

homologous peptide sequences are spread throughout the Prp8 protein, suggesting that homology between U5-220kD and Prp8p is not restricted to a particular domain. Homology of the U5-200kD protein with Prp8p and its homologue in *C.elegans* was not detected.

U5-200kD is highly homologous to a 246 kDa DEXH-box protein in *S.cerevisiae* (Snu246p)

Since U5-200kD is the first putative RNA helicase identified as an intrinsic component of snRNP particles, we were interested in determining whether a homologous, snRNP-associated protein is also present in other eukaryotes. A homology search of the gene bank with the amino acid sequence of U5-200kD revealed an uncharacterized open reading frame (*YER172c*), present on chromosome V of *S.cerevisiae* between the genes *YER171w* (identical to *RAD3*) and *YER173w* (Figure 4A) in the antiparallel orientation relative to *YER171w* and *YER173w*. *YER172c* potentially codes for a 2163 amino acid protein with a predicted molecular weight of 246.2 kDa and a calculated isoelectric point of 5.23. The amino acid sequence corresponding to the open reading frame of *YER172c* is highly homologous to the human U5-200kD protein (43.6% identity; 64.2% similarity), suggesting that it carries out an important function. Homology was not restricted to a particular domain, but rather was found throughout the entire U5-200kD sequence (Figure 4A). As compared with the human U5-200kD, the yeast protein contains additional amino acids at the amino-terminus, and, thus, its molecular weight is ~50 kDa greater. Closer inspection of the yeast protein sequence revealed that it possesses the same two-domain structure and a similar spacing of the conserved motifs, as found in the mammalian U5-200kD (Figure 4B). These include: a p-loop (APTGSGKT) at position 521–528, a DEIH motif at position 634–637, a SAT box at position 671–673 and a degenerate QRIGRXR motif (QMLGRAGR) at position 870–877. In contrast to U5-200kD, conserved motifs in DEXH domain II are more degenerate in the yeast amino acid sequence. For example, the first alanine of the p-loop is replaced by serine, DEVH is replaced by DDAH in the yeast protein and a SAT box is completely missing. An additional p-loop (AVEKTGKS) is present close to the amino-terminus of the yeast protein (position 79–86), a region which has no corresponding sequence in U5-200kD. Finally, the yeast protein contains a stretch of leucines and isoleucines between DEXH domains I and II (position 1073–1115) with a spacing reminiscent of a leucine zipper. However, formation of an amphipathic helix is not predicted by secondary structure

analysis. As this motif is not present in U5-200kD, and thus not conserved, it may not be significant.

Because the protein encoded by *YER172c* is a snRNP component (see below) it has been named Snu246p (Snurp 246 kDa protein).

The 246 kDa protein is encoded by a single copy gene essential for cell growth

As a prerequisite for a more detailed characterization of the role of Snu246p in splicing, the *SNU246* gene was cloned by PCR amplification using gene-specific primers and yeast genomic DNA as template (see Materials and methods). Subsequently, the *SNU246* gene was disrupted in a diploid strain by homologous recombination (Scherer and Davis, 1979). A 2.7 kb fragment of the *SNU246* gene was replaced by the selective marker *HIS3* (Figure 5A; see Materials and methods). The partially deleted *snu246Δ::HIS3* allele was inserted at the *SNU246* locus in a diploid strain by homologous recombination. Correct integration at the *SNU246* locus was verified by Southern analysis. Whereas digestion of the non-transformed strain with *Bam*HI and *Bsa*HI yielded a 6.5 kb fragment, corresponding to the wild-type *SNU246* gene, digestion of three independent transformants yielded both a 6.5 kb and 4.9 kb fragment (Figure 5B). The latter is the predicted size for a recombinant chromosome containing the *snu246Δ::HIS3* gene, instead of the normal *SNU246* sequence (Figure 5B). Diploid cells were viable, indicating that the mutation was recessive, while haploid progeny containing only the disrupted *SNU246* gene were non-viable (Figure 5C). The non-viable spores germinated and divided two or three times before final cell death, indicating that the *SNU246* gene is essential for yeast cell growth and not for spore germination. All viable cells were *his⁺*, and the viability of cells containing the disrupted *SNU246* gene was restored by transformation with a single-copy plasmid (ARS/CEN6-URA3 plasmid) containing a tagged derivative (see below) of the wild-type *SNU246* gene under control of the *GAL1* promoter (Johnston and Davis, 1984; see Table I and Figure 5D).

Snu246p is associated with the yeast [U4/U6.U5] tri-snRNP complex

A tagged derivative of Snu246p was constructed to test its ability to associate with snRNPs. A sequence encoding the nine amino acid long influenza virus haemagglutinin (HA) epitope (Kolodziej and Young, 1991) was fused in-frame downstream of the sequence coding for the Snu246p protein (see Materials and methods). The construct was

Fig. 4. Primary structure comparison of human U5-200kD and the yeast homologue Snu246. (A) Amino acid sequence alignment of U5-200kD (top) and Snu246p (bottom). Asterisks indicate amino acids identical in U5-200kD and Snu246p, and dots indicate conservative changes. Conserved motifs characteristic for DEXH-box proteins are shown as boxes. (B) Map of the primary structure of the U5-200kD protein (top) and Snu246p (bottom). The positions of the most prominent conserved motifs within the protein are boxed; all spacing is according to scale. The sequence of the conserved motifs in U5-200kD is indicated above and that of those in Snu246p below.

Fig. 5. The *SNU246* gene is a single copy gene essential for cell growth. (A) Strategy for interrupting *SNU246* with the *HIS3* selectable marker. A 2.7 kb *SnaBI*–*Sall* fragment was deleted and replaced by a 1.1 kb *HIS3* gene. (B) Southern blot analysis of the *SNU246* gene. wt, genomic DNA from the parental diploid strain YPF1. Lanes 1, 2 and 3 contain DNA from three different diploid transformants heterozygous for the *SNU246* allele. The positions of the 6.5 kb *Bam*HI–*Bsa*HI fragment containing the *SNU246* gene and of the 4.9 kb *Bam*HI–*Bsa*HI fragment containing the deleted *snu246* gene interrupted by the 1.1 kb *HIS3* gene are indicated by arrows. The position of DNA size markers is indicated on the right. (C) Tetrad analysis of YPF7 (*SNU246/snu246Δ::HIS3*). The four spores (1–4) from 12 individual tetrads (a–n) were dissected in YP plates containing 2% glucose and incubated for 2 days at 30°C. A 2:2 segregation was found and the two viable spores exhibited a *his*[−] genotype. (D) Complementation of the haploid *snu246Δ::HIS3* mutant by transformation with a single-copy plasmid (*CEN6*) containing a tagged version of the wild-type *SNU246* under control of the *GAL1* promoter. The four spores (1–4) from 12 individual tetrads (a–n) were dissected in YP plates containing 2% galactose and incubated for 4 days at 30°C. A 3:1 segregation was found. Here one out of three spores was always *HIS*⁺. A haploid cell colony containing *snu246Δ::HIS3* allele and the plasmid carrying the wild-type *SNU246* gene was selected and named YPF15 (Table I).

inserted into the high copy number plasmid pRS424-*GAL1* (Mumberg *et al.*, 1994) which contains a 2μ origin of replication and a *TRP1* marker for selection; expression of the fusion protein was under the control of the *GAL1* promoter. The plasmid was introduced into the diploid strain YPF7, which is heterozygous at the *SNU246* locus (Table I). Three independent diploid transformants were sporulated and the meiotic product dissected to obtain haploid strains expressing only the tagged *GAL1::SNU246* derivative of the fused gene. One of the strains (YPF12; see Table I) was selected and total cell extracts were prepared (Séraphin and Rosbash, 1989). Control extracts were prepared from isogenic haploid cells containing a chromosomal wild-type *SNU246* gene and a HA-tagged U1-70K protein (Snp1p). HA tag-containing complexes present in these extracts were immunoprecipitated using HA tag-specific polyclonal antibodies coupled to protein A–Sepharose beads. Subsequently, the presence of co-immunoprecipitated RNAs in the pellet was assayed by Northern analysis using probes specific for U1, U2, U4, U5 and U6 snRNAs. Because Prp4p is stably associated with the [U4/U6.U5] tri-snRNP at 200 mM NaCl concentration (Banroques and Abelson, 1989; Horowitz and Abelson, 1993b), immunoprecipitation was carried out with anti-Prp4p antibodies in order to demonstrate that a sufficient amount of the [U4/U6.U5] tri-snRNP was present in all extracts used in the following experiments (data not shown). The U1 snRNP could be immunoprecipitated with anti-HA antibodies at NaCl concentrations ranging from 150 to 500 mM NaCl (Figure 6, lanes 4–6 and data not shown), indicating that the antibodies bind efficiently to the HA-tagged U1-70K at NaCl concentrations up to 500 mM. In contrast, [U4/U6.U5] tri-snRNPs containing HA-tagged Snu246p could be immunoprecipitated at NaCl concentrations ranging from 150 to 300 mM NaCl (Figure 6, lane 1 and data not shown). At NaCl concentrations >300 mM, immunoprecipitation of [U4/U6.U5] tri-snRNPs by anti-HA antibodies was less efficient (Figure 6, lanes 2 and 3). Since anti-HA antibodies stably interact with the HA tag in the presence of up to 500 mM NaCl (Figure 6, lane 6), the decrease in immunoprecipitation efficiency is most likely due to dissociation of Snu246p from the [U4/U6.U5] tri-snRNP. No immunoprecipitation could be detected with anti-HA antibodies and an extract containing untagged Snu246p (Figure 6, lane 7).

Snu246p is required for pre-mRNA splicing in vivo

To analyse the role of Snu246p in pre-mRNA processing, we constructed the strain YPF15, which allowed us specifically to deplete Snu246p from the cell (Table I). YPF15 contains a conditional allele of *SNU246* cloned into the low copy number plasmid, pRS416-*GAL1*, which

Table I. Yeast strains used in this work

Strain	Genotype
YPF1	MATa/α, <i>trp1-Δ1/trp1-Δ1</i> ; <i>his3-Δ/his3-Δ</i> ; <i>ura3-52/ura3-52</i> ; <i>lys2-801/lys2-801</i> ; <i>ade2-101/ade2-101</i> (Sikorski and Hieter, 1989)
YPF2	MATa, <i>trp1-Δ1</i> ; <i>his3-Δ</i> ; <i>ura3-52</i> ; <i>lys2-801</i> ; <i>ade2-101</i> ; <i>SNU246</i>
YPF7	MATa/α, <i>trp1-Δ1/trp1-Δ1</i> ; <i>his3-Δ/his3-Δ</i> ; <i>ura3-52/ura3-52</i> ; <i>lys2-801/lys2-801</i> ; <i>ade2-101/ade2-101</i> ; <i>SNU246/snu246Δ::HIS3</i>
YPF12	MATa, <i>trp1-Δ1</i> ; <i>his3-Δ</i> ; <i>ura3-52</i> ; <i>lys2-801</i> ; <i>ade2-101</i> ; <i>snu246Δ::HIS3</i> (<i>TRP1</i> pRS424- <i>GAL1/SNU246</i> HAtag, 2μ)
YPF15	MATa, <i>trp1-Δ1</i> ; <i>his3-Δ</i> ; <i>ura3-52</i> ; <i>lys2-801</i> ; <i>ade2-101</i> ; <i>snu246Δ::HIS3</i> (<i>URA3</i> pRS416- <i>GAL1/SNU246</i> HAtag, <i>ARS.CEN6</i>)

Fig. 6. Snu246p is associated with the [U4/U6.U5] tri-snRNP. Splicing extracts from strain YPF12 expressing epitope-tagged Snu246pHA (lanes 1–3), or epitope-tagged Snp1pHA (yeast homologue of the human U1-70k protein; lanes 4–6) and from the YPF2 haploid strain isogenic to YPF12 containing wild-type, untagged Snu246p (lane 7), were immunoprecipitated with anti-HA antibodies and washed with 150 (for the control strain), 300, 400 and 500 mM NaCl, as indicated above each lane. Lane 8 shows marker snRNAs before immunoprecipitation (a shorter exposure of the marker is shown). The RNAs were recovered from the immunocomplexes, fractionated on an 8% denaturing polyacrylamide gel, blotted and probed with radiolabelled U1, U2, U4, U5 and U6 DNAs. The positions of the respective snRNAs are indicated on the right.

contains an ARS-CEN6 origin of replication and a URA3-selectable marker. A construct was made in which the authentic *SNU246* promoter was replaced by the *GAL1* promoter. This promoter is active when the cells are grown in galactose, but repressed by growth in glucose. The *GAL1::SNU246* allele is fully functional since it can complement the lethality of the *snu246Δ::HIS3* null allele. Growth of strain YPF15 is galactose-dependent, i.e. the cells grow on galactose as the sole carbon source but not in glucose (data not shown). When the growth rate of YPF15 in galactose versus glucose media was compared, the doubling times were almost indistinguishable for the first 12 h. However, cell growth was reduced at ~16–24 h following the shift to glucose medium and stopped after 28 h, indicating that depletion of Snu246p arrests cell growth (Figure 7A).

To investigate the effect of Snu246p depletion on pre-mRNA splicing, total RNA was extracted from YPF15 cells grown continuously in galactose or shifted to glucose medium for various lengths of time. Splicing efficiency was analysed by probing Northern blots with the yeast *RP51A* ribosomal protein gene. Spliced *RP51A* mRNA and only residual amounts of unspliced *RP51A* pre-mRNA were detected in cells grown in galactose medium at all time points (Figure 7B, lanes 5–8) or up to 4 h after shifting to glucose medium (Figure 7B, lanes 1 and 2). After 8 and 12 h, only *RP51A* pre-mRNA was detected in cells grown in glucose (Figure 7B, lanes 3 and 4),

Fig. 7. (A) Depletion of Snu246p arrests cell growth. Strain YPF15 was grown to mid-logarithmic phase in 2% galactose medium, harvested, washed and resuspended in sterile water. Half of this suspension was used to inoculate either pre-warmed galactose or glucose media to an initial OD₆₀₀ of 0.05. Cultures were diluted to keep all OD readings below 0.6, maintaining the cells in logarithmic growth. The OD₆₀₀ of the cultures has been monitored and plotted as a function of time. (B) Snu246p is required for pre-mRNA splicing *in vivo*. Total RNA was extracted from YPF15 cells grown at various times in galactose or at various times following a shift to glucose medium, resolved on a 1.2% denaturing agarose gel, blotted onto nylon membranes and probed with radiolabelled *RP15A* DNA. The positions of pre-mRNA and mature mRNA are indicated on the right.

suggesting that Snu246p is required for pre-mRNA splicing *in vivo*.

Anti-U5-200kD antibodies inhibit the second catalytic step of HeLa splicing *in vitro*

Since the anti-U5-200kD antibodies specifically recognized the U5-200kD protein which is available on the surface of [U4/U6.U5] tri-snRNPs, they appeared ideally suited to test whether, like its yeast homologue, the U5-200kD protein is also an essential splicing factor in higher eukaryotes. To test this, affinity-purified antibodies directed against the amino-terminal region of U5-200kD were incubated with nuclear extracts in the presence of ATP. Increasing concentrations of the antibody led to the accumulation of the first step intermediates of the splicing reaction (i.e. lariat-intron–exon 2 and excised exon 1), while the concentration of the second step products (i.e. lariat-intron and ligated exons) decreased (Figure 8, lanes 4–8), indicating that U5-200kD is required either directly or indirectly for the second transesterification reaction in mammalian splicing *in vitro*. Identical concentrations of non-specific immunoglobulin (Figure 8, lanes 1–3) and the changed buffer conditions due to antibody addition

Fig. 8. Antibodies raised against the U5-200kD protein inhibit step 2 of splicing in HeLa nuclear extract. Standard 12.5 μ l *in vitro* splicing reactions were incubated for 30 min on ice with 0.5 μ l (lane 1), 1.0 μ l (lane 2) or 2.0 μ l (lane 3) of protein A-Sepharose-purified immunoglobulin (5.3 μ g/ μ l) and 0.5 μ l (lane 4), 1.0 μ l (lane 5), 2.0 μ l (lane 6), 4.0 μ l (lane 7) or 6.3 μ l (lane 8) of affinity-purified antibody (0.09 μ g/ μ l) directed against the amino-terminus of U5-200kD. The reactions were subsequently incubated for 60 min at 30°C. A splicing assay in the absence of ATP is shown in lane 9. The RNA was recovered by phenol extraction and ethanol precipitation, followed by separation on a 12.5% denaturing polyacrylamide gel. The position of the pre-mRNA, splicing intermediates and products is indicated on the right.

(data not shown) had no effect. Similar results were obtained with affinity-purified antibodies directed against the carboxy-terminal region of U5-200kD (data not shown).

Discussion

In the present work we describe the characterization of the human U5 snRNP-specific 200 kDa protein (U5-200kD) as a putative RNA helicase of the DEAD/DEXH-box protein family. It is the first time that a putative RNA helicase is shown to be an intrinsic component of snRNPs and also to be involved in human splicing. Inhibition studies using affinity-purified antibodies indicate that U5-200kD is present in functional spliceosomes at the second step of splicing *in vitro*. These results are in good agreement with recent data demonstrating that purified 25S [U4/U6.U5] tri-snRNPs and 20S U5 snRNPs contain RNA-stimulated ATPase activity and that the U5 snRNP-specific 200 kDa protein can be cross-linked to ATP in an RNA-dependent manner (Laggerbauer *et al.*, 1996). Moreover, a homologous protein with a predicted molecular weight of 246 kDa was also identified in *S.cerevisiae*. This protein, denoted Snu246p, is an intrinsic component of the yeast [U4/U6.U5] tri-snRNP and is essential for cell viability and splicing *in vivo*. The evolutionary conservation of this snRNP-associated, putative RNA helicase and its presence in the splicing complexes in such diverse organisms as yeast and man, emphasizes its functional importance in the splicing process.

Two DEXH-domains in the U5-specific putative RNA helicase

The predominant feature of U5-200kD and its yeast homologue Snu246p, which is unique among DEAD/

DEXH-box proteins, is the presence of two DEXH-box domains (DEXH-domain I and II). The crucial motifs, AX₄GKT, DEXH and SAT, are fully conserved in domains I and II of U5-200kD (Figure 9). These motifs are highly conserved in domain I of Snu246p. In contrast to the strong conservation of DEXH-domain I in Snu246p, conserved motifs in DEXH domain II are more degenerate (AX₄GKT→SX₄GKT; DEXH→DDAH; SAT→SNC), although homology to the DEXH-box domain II of U5-200kD is clearly visible (Figure 9). The E to D exchange in position 2 of the DEXH motif (DDAH) is a conservative replacement and an A to S exchange in position 1 of the p-loop has also been reported for Prp5p (Dalbadie-McFarland and Abelson, 1990); thus, these replacements might not affect the functional integrity of the motifs. The degree of homology of the human U5-200kD DEXH-domain I is higher with DEXH-domain I of Snu246p than with its own DEXH domain II, suggesting that the two domains were duplicated before the divergence of the yeast and human lineage (Figure 9). Although DEXH-domain II is less conserved in Snu246p than in U5-200kD, the overall phylogenetic conservation of the primary structure is high, suggesting a similar or subtly different function of domain II of the proteins in yeast and mammalian splicing. Eventually, mutational and deletion analysis of the conserved motifs may provide answers to some of these questions. Other motifs assigned to the conserved domains of putative RNA helicases in both proteins are degenerate, as expected for proteins of the DEXH subfamily of DEAD-box proteins (Schmid and Linder, 1992; Pause and Sonenberg, 1993). The GG and TPGR motifs, which are present in DEAD-box proteins but have thus far not been detected in DEXH-box proteins (Pause and Sonenberg, 1993), were found in a degenerate form (GX and TPXX) in domains I and II of both U5-200kD and Snu246p, as was the QRXGRXGR box.

A subgroup of seven DEXH-box proteins [Hfmlp (U22156); HumORFA02_1 (D29641); ScyJL050W (Z49325); Ski2p (P35207); YY02 (P42285)] shares significant homology with U5-200kD and Snu246p predominantly in their DEXH-domains (Figure 9). In contrast to U5-200kD and Snu246p, all of these proteins contain only one DEXH-domain; however, like U5-200kD and Snu246p, exhibit partial conservation of the TPGR, QRXGRXGR and GG motifs. We thus propose that the proteins shown in Figure 9 form a new subgroup within the DEAD/DEXH-box protein family of putative RNA helicases. The motifs, although degenerate as compared with the DEAD-box proteins, are highly homologous within this subgroup. For example, most of them contain a GD instead of GG motif and the QRIGRXGR box is QMLGRAGR. The TPGR consensus sequence is either TPEKWD (U5-200kD, Snu246p, Hfmlp) or TEILRS (others; see Figure 9). The sequence divergence of these motifs, as compared with the DEAD and DEAH-box proteins, might account for a subtle change in function and/or activity. Nothing is known about the function of these proteins. The DEAD/H box proteins known to be involved in yeast splicing (i.e. Prp5p, Prp28p, Prp2p, Prp16p and Prp22p) and eIF4a are less related to this subgroup, also with respect to homology outside this conserved domain.

The function of an additional p-loop sequence towards

Fig. 9. Amino acid sequence alignment of DEXH-domains I and II of U5-200kD and SnU246p with the DEXH-domains of Hfm1p, HumORFA02_1, ScyJL050W, YY02 and Ski2p. Sequences were aligned using the ClustalV program. Identification of the proteins is shown on the left with DEXH-domains I and II indicated with roman numeral following 200K and SnU246p. Conserved motifs are shown as shaded boxes, and individual conserved amino acids are marked by asterisks and dots.

the amino-terminus of Snu246p is also unclear. Evidence that this motif (among others) is functionally significant will require a detailed mutational analysis of Snu246p.

Phylogenetic conservation of protein components of the [U4/U6.U5] tri-snRNP

Electron microscopic analysis of snRNPs has demonstrated structural similarity of [U4/U6.U5] tri-snRNPs isolated from yeast and HeLa cells (Fabrizio *et al.*, 1994), suggesting possible conservation of [U4/U6.U5] tri-snRNP-specific splicing factors. In addition, particles in both organisms have a complex protein composition. The HeLa U5 snRNP-specific protein migrating at 220 kDa has been shown to be homologous to the 280 kDa yeast splicing factor Prp8p (Anderson *et al.*, 1989; Pinto and Steitz, 1989), based on the cross-reactivity of antibodies raised against Prp8p with mammalian U5-220kD. High homology of peptide sequences derived from the human 220 kDa protein with Prp8p confirmed the homology (see Figure 3). Human U5-200kD and yeast Snu246p are the second

(and probably not the last) example of structurally highly conserved U5 snRNP components.

The putative U5 snRNP RNA helicase is essential for splicing

So far, the involvement of putative RNA helicases of the DEAD-box family in mammalian splicing has not been demonstrated. The fact that pre-mRNA-snRNA interactions are less stable in mammals as compared with yeast due to lower conservation of consensus sequences in mammalian introns, has led to the speculation that the strand displacement activity of RNA helicases may not be necessary for mammalian splicing. Alternatively, the biochemical methods employed in mammalian splicing may be inappropriate to detect protein functions as described for the DEAD/DEAH-box proteins required for splicing in *S.cerevisiae* (see Introduction). Although a cDNA encoding the human homologue of Prp22p (an essential DEAH-box protein in yeast) has recently been isolated from HeLa cells, the association of this Prp22p

homologue with spliceosomes or its requirement in mammalian splicing has not been demonstrated (Ono *et al.*, 1994). Thus, U5-200kD is the first putative RNA helicase shown to be involved in human splicing. In contrast to the DEAD/DEAH-box proteins involved in yeast splicing, U5-200kD is an intrinsic component of 20S U5 snRNPs. In this respect, the identification of the yeast homologue Snu246p as an integral component of yeast [U4/U6.U5] tri-snRNPs is particularly interesting, suggesting that the association of at least one putative RNA helicase with snRNPs is conserved among eukaryotes. Snu246p is an important yeast DEAD/DEXH-box protein not only with respect to its association behaviour, but also in terms of its activity; a *SNU246* null allele affects the formation of mature mRNA in yeast cells. Conversely, transiently interacting DEAD/DEAH-box proteins as described in yeast splicing may be conserved as well, and the mammalian homologues of these proteins remain to be discovered.

The step which requires the presence of U5-200kD was more precisely investigated in HeLa splicing by inhibition experiments using affinity-purified anti-U5-200kD antibodies. Interestingly, the second transesterification reaction was severely impaired by the presence of anti-U5-200kD antibodies in an *in vitro* splicing reaction; step 1 of splicing was not affected (see Figure 8). Binding of the antibody could inhibit the function of U5-200kD or prevent its interaction with other spliceosomal components. Alternatively, the function of the entire U5-snRNP could be inhibited by steric hindrance due to antibody binding, and the U5-200kD protein activity may not be affected by the antibody. At present, we are not able to distinguish between these possibilities. However, steric hindrance of interactions due to the bulky size of antibodies bound to U5-200kD is a rather general effect and would probably lead to a block of all splicing activity. Considering the specific inhibition of the second transesterification reaction by anti-U5-200kD antibodies, our data suggest a more direct role for the U5-200kD protein in this step. Thus, U5-200kD/Snu246p may be second-step splicing factors like Prp16p, Prp17p, Prp18p, Slu4, and Slu7, which have been identified previously by yeast genetics (Vijayraghavan and Abelson, 1990; Schwer and Guthrie, 1991; Frank and Guthrie, 1992; Horowitz and Abelson, 1993a,b; Ansari and Schwer, 1995; Haltiner Jones *et al.*, 1995).

As the anti-U5-200kD antibodies may only partially inhibit the function of the 200 kDa protein (note that U5-200kD contains two RNA helicase domains), it is possible that U5-200kD is also required for the first step of the splicing reaction. It could, for example, be involved in the disruption of the U1 snRNA interaction with the 5' splice site or destabilization of the U4–U6 interaction which occur during spliceosome assembly before step 1. As the spliceosome is known to undergo significant conformational changes also between the first and second transesterification reaction (e.g. rearrangements of U2, U5 and U6 snRNA interactions with each other and/or with the pre-mRNA; see Introduction), U5-200kD and Snu246p may be actively involved in promoting at least some of these conformational changes. Mutagenesis of the two DEXH domains of Snu246p should help to clarify these questions.

Materials and methods

Isolation and microsequencing of snRNP proteins

HeLa cells were grown in suspension culture as described previously (Bringmann *et al.*, 1983) or obtained from Computer Cell Culture Company (Mons, Belgium). Nuclear extracts were prepared by the method of Dignam *et al.* (1983). U1, U2, U5 and U4/U6 snRNPs were purified from nuclear extract in buffer C-5 (20 mM HEPES–KOH pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA pH 8, 0.5 mM DTE, 0.5 mM PMSF, 4 µg/ml leupeptin, 5% glycerol) by affinity chromatography using the monoclonal antibody H20, covalently attached to CNBr-activated Sepharose 4B (Bochnig *et al.*, 1987; Pharmacia). SnRNPs were eluted with the nucleoside m⁷G, with buffer C-5 being used in all steps (Bach *et al.*, 1990).

SnRNP proteins were extracted from 20 mg of purified snRNPs with 1 vol phenol and proteins were precipitated from the phenolic phase with 5 vols acetone. The precipitated proteins were separated by preparative SDS–PAGE, followed by electroelution of excised, Coomassie-stained, gel slices containing a single protein band. The purified proteins were precipitated with acetone, subjected to SDS–PAGE and electroblotted onto sequencing grade PVDF-membrane (Bio-Rad). Partial amino acid sequences of tryptic peptides of the snRNP proteins were determined by microsequencing on an ABI 477A protein sequencer.

Peptide sequences obtained from U5-220kD were:

220K a	:RQEIADLL
220K b	:AEQERQHNYLK
220K c	:IGLNSK
220K d	:FAEEVRALIRSLPVEEQ

Peptide sequences obtained from U5-200kD were:

200-24I	:VELTITPDFQXDEK
200-24II	:FLYXPLPVESHLD
200-43	:GLFYFDNSFRPVXLXQTY
200-54I	:YPPPTLELLDQLPVSALRNSAF
200-54II	:PFFNPIQTQVFNT

Database search

A database search was carried out on the NIH mailserver using the TBLASTN and BLASTP programs (Altschul *et al.*, 1990). An expressed sequence tag with an open reading frame matching partial peptide sequences of U5-200kD is part of the clone HFBCN20, which has the accession number M85756. An ORF derived from chromosome V of *S.cerevisiae* (YER172c) codes for the Snu246p protein (accession number U18922), which is homologous to U5-200kD.

cDNA cloning and sequencing

A HeLa cell cDNA library was constructed using a cDNA synthesis kit as described by the manufacturer (Stratagene, Heidelberg). A 320 bp DNA fragment, matching the 5'-region of clone HFBCN20, was isolated from the HeLa cDNA library by PCR with gene-specific primers (200for and 200rev, see below). Screening of the cDNA library with this fragment resulted in the isolation of two clones, 3.84 kb (200.1) and 4.94 kb (200.20) in length, which exhibit a similar restriction pattern. Sequence analysis was carried out on exonuclease III/nuclease S1 deletion sub-clones (Henikoff, 1984) using a nested deletion kit (Pharmacia, Freiburg) or by using primers designed from the new sequence. Sequences were determined manually by the dideoxy termination method (Sanger *et al.*, 1977) and with an automated DNA sequencer (Applied Biosystems) using *Taq* polymerase and double-stranded templates (PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit, Pharmacia). For identification of the cDNA sequence missing at the 5' end of clone 200.20, nested PCR amplification from reverse-transcribed HeLa mRNA was performed with two gene-specific primers (200GSP1 and 200GSP2, see below) and one vector-specific primer (λgt10for). The resulting 570 bp DNA fragment was sequenced as described above.

Antibody production

Antibodies against U5-220kD were raised by immunization of New Zealand White rabbits with a synthetic peptide, comprising the sequence of the tryptic peptide 220K d, coupled to chicken ovalbumin or keyhole limpet haemocyanin (KLH). The peptide contained an additional cysteine and was coupled to the carrier protein at a molar ratio of ~1:40 using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS), following the method of Liu (1979).

Antibodies to U5-200kD were raised against two fragments of clone 200.20 expressed as glutathione *S*-transferase fusion proteins. Expression vectors were generated by cloning the 5'-half of clone 200.20 (a 2351 bp *EcoRI*–*EcoRI* fragment) into pGEX-4T2 (Pharmacia) and the 3'-half of clone 200.20 (a 2590 bp *EcoRI*–*XhoI* fragment with 2299 bp of the coding region) into pGEX-4T3 (Pharmacia). Overexpression of the GST fusion proteins in *Escherichia coli* DH5 α was induced by incubation with isopropyl- β -D-thiogalactoside (1 mM) for 2 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 pH 7.5/10% SDS. Fusion proteins were purified by preparative SDS–PAGE, followed by electroelution, and subsequently used for immunization (Harlow and Lane, 1988).

Anti-U5-200kD antibodies were affinity-purified on columns containing gel-purified fusion proteins coupled to CNBr-activated Sepharose as described by the manufacturer (Pharmacia). All steps were carried out at 4°C. Crude antisera were loaded onto the affinity column in PBS (pH 8.0) and the column was washed with 10 bed volumes of PBS pH 8.0. Antibody was eluted first with glycine–HCl (100 mM, pH 2.9) and, following a wash with PBS pH 8.0, with triethanolamine (100 mM, pH 11.5). The pH of the collected fractions was readjusted to pH 8.0 with 1 M Tris–HCl. The protein containing fractions were pooled, dialysed against 1 mM Tris–HCl pH 8.0, lyophilized and redissolved in 1/40 vol H₂O.

Polyclonal antibody HA.11 was obtained from the Berkeley Anti-body Company.

In vitro splicing

Splicing was performed with ³²P-labelled pre-mRNA derived from the adenovirus 2 major late transcription unit. SP6 run-off transcripts, which were synthesized *in vitro* from pSP62Δ1 (Freundewey and Keller, 1985), were capped with G(5')ppp(5')G (Pharmacia) and had a specific activity of 4 × 10⁶ c.p.m./pmol. *In vitro* splicing reactions were carried out according to Krainer *et al.* (1984). Standard assays contained 2.5 μl nuclear extract (20% final concentration), 0.6 μl MgCl₂ (50 mM), 1.25 μl KCl (400 mM), 0.125 μl creatine phosphate (1 M), 0.25 μl ATP (100 mM), 1.5 μl ³²P-labelled pre-mRNA (3 × 10⁴ c.p.m.) and Tris–HCl (40 mM, pH 7.4) or antibody solution to a final volume of 12.5 μl. For inhibition of *in vitro* splicing activity, standard splicing reactions were pre-incubated with increasing concentrations of affinity-purified anti-U5-200kD antibodies (1–13 μg) for 30 min on ice, followed by incubation at 30°C for 60 min. The reactions were stopped by addition of 400 μl PK buffer (100 mM Tris–HCl pH 7.4; 12.5 mM EDTA; 150 mM NaCl; 1% SDS). Samples were extracted with 1 vol phenol/chloroform and the aqueous phase was precipitated with 2.5 vols ethanol. RNA was separated on a denaturing 12.5% polyacrylamide–7 M urea gel and visualized by autoradiography.

Immunoprecipitation of snRNP particles

For immunological detection of proteins, affinity-purified snRNPs or immunoprecipitates were separated on SDS–polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (Schleicher and Schüll). The blots were probed with anti-U5-200kD and anti-U5-220kD antibodies as described by Zeller *et al.* (1983).

To co-immunoprecipitate snRNP particles with anti-U5-200kD antibodies, 10 μl antiserum per sample was coupled to 20 μl pre-swollen protein A–Sepharose beads (PAS; Pharmacia) in 300 μl PBS pH 8 at 4°C overnight. The beads were then washed three times with PBS and resuspended in 300 μl of the same buffer. 5–10 μg of affinity-purified snRNPs were incubated with the PAS–antibody conjugate for 2 h at 4°C and washed four times with ice-cold TBSN [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% (v/v) NP-40]. The antibody complexes, in 300 μl TBSN, were extracted twice with phenol/chloroform and the snRNAs were precipitated from the aqueous phase with ethanol. The precipitated snRNAs were labelled at the 3' end with [³²P]pCp as described by England and Uhlenbeck (1978), separated on a 10% polyacrylamide–8 M urea gel and visualized by autoradiography.

Immunoprecipitation from yeast splicing extracts was carried out by coupling 2 μl antiserum in 500 μl TBSN to PAS beads for 1 h at 4°C. Subsequently, the beads were washed three times with TBSN. The conjugate was incubated with 15 μl of yeast extract for 2 h at 4°C, and then washed four times with TBSN containing 150 to 500 mM NaCl. The immunoprecipitate was digested with proteinase K (2 mg/ml in 50 mM EDTA pH 8.0, 1% SDS) for 15 min at 37°C, extracted with phenol, and the RNA precipitated with ethanol. Co-immunoprecipitated snRNAs were separated on an 8% polyacrylamide–8 M urea gel,

electroblotted to a nylon membrane and detected by Northern hybridization (see below).

Yeast strains, growth curves and splicing extracts

Details about the strains used in this work are presented in Table I; standard methods were used for growth and maintenance of yeast (Guthrie and Fink, 1991). Transformation of yeast cells with DNA was carried out by the lithium acetate method (Ito *et al.*, 1983). To monitor growth, YPF15 cells were grown in medium containing 2% galactose, pelleted, washed and resuspended in YP medium. The suspension was used to inoculate fresh pre-warmed YP-medium containing 2% galactose or 2% glucose to an initial OD₆₀₀ of 0.05. In order to maintain logarithmic growth, cultures were diluted as necessary to keep all OD₆₀₀ readings below 0.6.

For preparation of mini-extracts, strains YPF12 and YPF2 were grown in 40 ml of galactose-based medium to an OD₆₀₀ of 3, and extracts were prepared as described previously (Séraphin and Rosbash, 1989). Extracts from the strains U1-70kHA and EJ101 were prepared from 16 l cultures as described previously (Fabrizio *et al.*, 1994).

Isolation, blotting and hybridization of DNA and RNA

Yeast genomic DNA was isolated according to Kaise *et al.* (1994) and used for PCR amplification or Southern blotting (Sambrook *et al.*, 1989). After digesting with *Bam*HI and *Bsa*HI, DNA fragments were subjected to electrophoresis, stained, transferred to nylon membranes (Qiagen, Hilden), and irradiated with UV-light for 2.5 min. Pre-hybridization, hybridization and washing of the membrane were carried out as described previously (Fabrizio *et al.*, 1989). A 6.5 kb *Bam*HI–*Bsa*HI DNA fragment of *SNU246* was used as a probe for Southern hybridization. Northern blotting and hybridization with probes for U1, U2, U4, U5 and U6 snRNAs were carried out as described previously (Fabrizio *et al.*, 1989).

For Northern blot analysis of mRNA, total RNA was extracted from strain YPF15 by the method of Vijayraghavan *et al.* (1989). Following denaturing agarose gel electrophoresis, the RNA was transferred to a nylon membrane (Sambrook *et al.*, 1989). A 0.2 kb PCR fragment was amplified from the plasmid pBS195 (Séraphin and Rosbash, 1991) using oligonucleotides 7 and 8 (see below) as primers. The fragment was radiolabelled by random priming (prime-it II random primer labelling kit; Stratagene) and used as a probe for Northern hybridization.

Cloning, plasmids and disruption of *SNU246*

The *SNU246* gene was cloned by PCR amplification of yeast genomic DNA using two primers complementary to the 5' and 3' untranslated regions of the gene (oligonucleotides 1 and 2, see below). The PCR was carried out as follows: 1 cycle (5 min at 95°C), 35 cycles (30 s at 95°C, 30 s at 55°C, 5 min at 72°C), 1 cycle (5 min at 72°C). *Taq*-Plus polymerase (Stratagene) was used to ensure high-fidelity polymerization. PCR amplification resulted in a unique product with the expected size of 8234 nucleotides, which was cloned into pRS316 (Sikorski and Hieter, 1989) at the *Xho*I and *Sac*II sites to generate pRS316/*SNU246*. The identity of the insert was verified by partial sequencing in both directions and digestion with several restriction enzymes.

A null *SNU246* allele was created from pRS316/*SNU246* by deletion of a central 2.7 kb *Sal*I–*Sna*BI fragment from the *SNU246* ORF (amino acids 1044–1942), and blunt ends were obtained by incubation with Klenow polymerase at room temperature for 15 min. A 1.1 kb *Sna*BI–*Xba*I fragment containing the full-length *HIS3* gene was obtained from the plasmid pRS313 (Sikorski and Hieter, 1989) by PCR using oligonucleotides 5 and 6 as primers and cloned into *SNU246*, after digestion with *Sna*BI and *Eco*47III. The resulting plasmid is pRS316/*snu246Δ::HIS3*. A 2.7 kb *Xmn*I–*Hpa*I fragment from pRS316/*snu246Δ::HIS3* containing *snu246Δ::HIS3* was used to replace one copy of *SNU246* in the diploid yeast strain YPF1 by homologous recombination (Scherer and Davis, 1979). Viable *HIS*⁺ transformants were obtained and correct integration of the *snu246Δ::HIS3* gene at the homologous locus was confirmed by Southern analysis. One correct integrant, YPF7, was sporulated on YPA plates and tetrads were dissected on YPD plates.

Fusion to the *GAL1* promoter and epitope tagging of *SNU246*

The *SNU246* coding sequence was amplified from yeast genomic DNA by PCR using specific primers (oligonucleotides 3 and 4, see below). The forward primer contained an *Spe*I restriction site, while the reverse primer contained an *Xho*I restriction site and a sequence encoding the nine amino acid-long influenza virus haemagglutinin (HA) epitope. The 6.5 kb *Spe*I–*Xho*I PCR-product harbouring the ORF of *SNU246* and the HA epitope at the 3'-end was cloned into *Spe*I–*Xho*I-digested pRS424-

GAL1 (Mumberg *et al.*, 1994) and, for sequencing, into *SpeI*-*XhoI*-digested pBSIIKS(-). The presence of the HA epitope coding sequence was confirmed by sequence analysis. A *SacI*-*KpnI* fragment from pRS424-*GAL1/SNU246HA*, containing the *GAL1* promoter, the *SNU246* HA-tagged ORF and the CRYI-terminator, was inserted into the *SacI*-*KpnI* sites of pRS416 (Christianson *et al.*, 1992), to generate pRS416-*GAL1/SNU246HA*. In these constructs, the ATG of *SNU246* is directly 3' of the *GAL1* transcription start site, such that the transcription of *SNU246* is under the control of the *GAL1* promoter. The plasmids containing the *GAL1::SNU246* allele were separately transformed into the heterozygous diploid strain YPF7 (*SNU246/snu246Δ::HIS3*). Viable *HIS⁺/TRP1⁺* or *HIS⁺/URA⁺* transformants were sporulated on YPA plates and dissected on galactose-based YP medium. The haploid progeny were analysed for the presence of the chromosomal *snu246Δ::HIS3* disruption and for the presence of pRS424-*GAL1/SNU246HA* on synthetic media lacking histidine and tryptophan; similarly, the presence of pRS416-*GAL1/SNU246HA* was analysed on synthetic media lacking histidine and uracil. Growth of all strains was monitored on galactose-based and glucose-based media. Strain YPF12 and YPF15 were identified among the meiotic progeny which possessed the desired phenotype.

Oligonucleotides and primers used in this work

200for: 5'-CCCGTGTCTGCTCTGAGAA-3'
 200rev: 5'-AGGTCTGTGCTGGTCTCG-3'
 200GSP1: 5'-AGCACTGAGACCAATGAG-3'
 200GSP2: 5'-ACCTCTGTCATCATGTAGAA-3'
 λgt10for: 5'-AGCAAGTTCAGCGTGGTTAAG-3'
 1: 5'-CTGGGGCTCGAGCGTCTGGTATCTATATGG-3'
 2: 5'-GCACAACCGCGGGGATCACACCTGATGGAAT-3'
 3: 5'-GGGGGACTAGTATGACTGAGCATGAAACGA-3'
 4: 5'-GGGGGCTCGAGTTAAGCGTAGTCTGGGACGTCGTAT-
 GGGTATTTACATTTATTTCAAA-3'
 5: 5'-CTGGGGTACGTATCGAGTTCAAGAGAAAAA
 6: 5'-GCTCGGTCTAGAAAGAGCTTGGTGAGCGCT
 7: 5'-GGGAAAGCTAGCTTGGATCTCGAGACTAG
 8: 5'-GCCTTAGAAGCACGCTTGACGGTCTTGGTT

Underlined sequences indicate the following restriction sites: (i) *XhoI*; (ii) *SacII*; (iii) *SpeI*; (iv) *XhoI*; (v) *SnaBI*; (vi) *XbaI*.

Acknowledgements

We are most grateful to Cindy Will for carefully reading the manuscript and making helpful suggestions, and to S.Becker, D.Meyer, I.Öchsner and M.Wicke for excellent technical assistance. We thank M.Funk for helpful discussions and the kind gift of various plasmids. The yeast strain U1-70kHA was provided by M.Rosbash, and antibody Y12 by J.Steitz. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 272), the Fonds der Chemischen Industrie and the Alexander von Humboldt-Stiftung to R.L.

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Received on February 5, 1996; revised on March 15, 1996

Note added in proof

While this work was under revision we were pleased to learn that the yeast *SNU246* gene was also identified by S.M.Noble and C.Guthrie (*Genetics*, **143**, 67–80, 1996) D.Xu *et al.* (*Nature*, in press) and J.Rossi and coworkers (personal communication). At the same time, the DEAH box protein HRH1 was shown to be a functional mammalian homologue of yeast Prp22p by M.Ohno and Y.Shimura (*Genes Dev.*, **10**, 997–1007, 1996).