

A subset of human 35S U5 proteins, including Prp19, function prior to catalytic step 1 of splicing

Olga V Makarova^{1,3,4}, Evgeny M Makarov^{1,3}, Henning Urlaub¹, Cindy L Will¹, Marc Gentzel², Matthias Wilm² and Reinhard Lührmann^{*,1}

¹Department of Cellular Biochemistry, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany and ²EMBL, Bioanalytical Research Group, Heidelberg, Germany

During catalytic activation of the spliceosome, snRNP remodeling events occur, leading to the formation of a 35S U5 snRNP that contains a large group of proteins, including Prp19 and CDC5, not found in 20S U5 snRNPs. To investigate the function of 35S U5 proteins, we immunoaffinity purified human spliceosomes that had not yet undergone catalytic activation (designated BAU1), which contained U2, U4, U5, and U6, but lacked U1 snRNA. Comparison of the protein compositions of BAU1 and activated B* spliceosomes revealed that, whereas U4/U6 snRNP proteins are stably associated with BAU1 spliceosomes, 35S U5-associated proteins (which are present in B*) are largely absent, suggesting that they are dispensable for complex B formation. Indeed, immunodepletion/complementation experiments demonstrated that a subset of 35S U5 proteins including Prp19, which form a stable heteromeric complex, are required prior to catalytic step 1 of splicing, but not for stable integration of U4/U6.U5 tri-snRNPs. Thus, comparison of the proteomes of spliceosomal complexes at defined stages can provide information as to which proteins function as a group at a particular step of splicing.

The EMBO Journal (2004) 23, 2381–2391. doi:10.1038/sj.emboj.7600241; Published online 3 June 2004

Subject Categories: RNA

Keywords: mass spectrometry; proteomics; Prp19/CDC5 35S U5 snRNP; spliceosome

Introduction

Pre-mRNA splicing is catalyzed by the spliceosome, a highly dynamic RNP machine. The main components of the major spliceosome are the U1, U2, U4, U5, and U6 snRNPs (Burge *et al.*, 1999). These RNA–protein complexes together with a large number of non-snRNP splicing factors totaling well over 120 distinct proteins (Hartmuth *et al.*, 2002; Jurica *et al.*, 2002;

Makarov *et al.*, 2002; Rappsilber *et al.*, 2002; Zhou *et al.*, 2002) associate with the pre-mRNA in an ordered manner (Burge *et al.*, 1999). First, the U1 snRNP interacts with the 5' splice site, followed by the stable association of U2 snRNP with the branch site to form spliceosomal complex A. The pre-assembled 25S U4/U6.U5 tri-snRNP is then recruited to form complex B. The latter is structurally rearranged to form the catalytically activated spliceosome, which subsequently catalyzes the first transesterification reaction, generating complex C. After the second step of splicing, the mRNA is released, the post-spliceosomal complex disassembles, and the snRNPs are recycled for new rounds of splicing. Thus, spliceosome formation goes through many intermediate stages, the most stable of which (e.g., A, B, and C complexes) can be detected biochemically, for example, by native gel electrophoresis. Many additional stable intermediates, such as the recently purified activated spliceosome B*, also appear to exist (Makarov *et al.*, 2002; reviewed by Brow, 2002).

The most decisive step during the spliceosome maturation process is the conversion of complex B, which does not contain an active site, into the catalytically activated spliceosome B*, which is poised to catalyze both steps of splicing. Activation of the spliceosome entails major structural changes, including the displacement of U1 from the 5' splice site, unwinding of the U4 and U6 base-pairing interaction, and the subsequent base pairing of U6 with the 5' splice site and the U2 snRNA (Nilsen, 1994; Staley and Guthrie, 1998). These rearrangements result in the dissociation of the U1 and U4 snRNPs during the activation step. The resulting RNA network forms the core of the newly formed catalytic center of the spliceosome.

While spliceosome activation is well characterized at the RNA level, the role of proteins at this stage, as well as RNP remodeling events accompanying activation, is only poorly understood. Proteins of the DEAD/H-box family of RNA/RNP unwindases appear to be one of the driving forces that mediate RNA rearrangements at this step. For example, Prp28/U5-100K has been implicated in displacing U1 from the 5' splice site (Staley and Guthrie, 1999; Chen *et al.*, 2001), whereas Brr2/U5-200K appears to facilitate U4/U6 unwinding (Laggerbauer *et al.*, 1998; Raghunathan and Guthrie, 1998). In yeast, evidence has been provided that Prp19p plays an essential role in spliceosome activation (Tarn *et al.*, 1993a, b). Prp19p is present in a heteromeric protein complex that consists of at least eight proteins (the NTC, nineteen complex) (Tarn *et al.*, 1994; Tsai *et al.*, 1999; Chen *et al.*, 2002), and more recent data suggest that it is part of a very large RNP complex containing U2, U5, and U6 snRNA, as well as many additional proteins (Ohi *et al.*, 2002). The NTC complex acts subsequent to U4 dissociation, stabilizing the association of U5 and U6 with the activated spliceosome (Chan *et al.*, 2003). In humans, hPrp19p co-purifies with CDC5 in a larger complex containing approximately 30 proteins, and this CDC5/Prp19 complex was reported to play a role in the second step of splicing (Ajuh *et al.*, 2000).

*Corresponding author. Department of Cellular Biochemistry, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany. Tel.: +49 551 201 1407; Fax: +49 551 201 1197; E-mail: Reinhard.Luehrmann@mpi-bpc.mpg.de

³These authors contributed equally to this work

⁴Present address: Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK

Received: 9 March 2004; accepted: 27 April 2004; published online: 3 June 2004

We recently immunoaffinity purified the activated B* spliceosome using antipeptide antibodies against the splicing factor SKIP (Makarov *et al*, 2002). We intentionally purified spliceosomal complexes under stringent conditions (i.e., in the presence of heparin) so that only stably bound proteins would be present. Consistent with the absence of U4, the purified activated spliceosome lacked all U4/U6-associated proteins. In addition, a group of 16 proteins (see Table I), including CDC5, Prp19 and additional proteins that were previously shown to be physically associated with Prp19 (Tarn *et al*, 1994; Tsai *et al*, 1999; Ben-Yehuda *et al*, 2000; Chen *et al*, 2002; Ohi and Gould, 2002), were also stably associated with the activated spliceosome, as well as a novel 35S form of the U5 snRNP (Makarov *et al*, 2002). These results indicated that the U5 snRNP is remodeled during activation, and that the 16 proteins that are stably associated with it within the activated spliceosome may play a role in activation or potentially at an earlier stage, for example during tri-snRNP integration into the spliceosome.

To learn more about the driving forces behind spliceosome activation, and in particular the contribution of 35S U5-associated proteins to this process, we set out to isolate spliceosomal complexes prior to activation but after stable recruitment of the U4/U6.U5 tri-snRNP. Indeed, using antibodies against the U4/U6-specific 61K protein, we affinity purified spliceosomal B complexes that contain stably integrated, intact U4/U6.U5 tri-snRNPs but lack U1 snRNPs (henceforth denoted BΔU1). The protein composition of these complexes differed dramatically from that of the activated spliceosome; all U4/U6-specific proteins were present in the BΔU1 complexes, whereas essentially all 35S U5-specific proteins were largely absent. We addressed a possible role of 35S U5-specific proteins in spliceosome activation by performing immunodepletion/complementation assays in splicing active HeLa nuclear extract, and demonstrate that a subset of them, including CDC5 and Prp19, function after tri-snRNP addition but prior to/during the first catalytic step of splicing.

Results

Purification of BΔU1 spliceosomes

To isolate spliceosomes just prior to their activation, but after stable integration of the U4/U6.U5 tri-snRNP, we used antipeptide antibodies against the U4/U6 protein 61K (hPrp31), which had previously been shown to be required for formation of stable tri-snRNPs (Makarova *et al*, 2002). Splicing complexes were allowed to form after incubation of pre-mRNA (MINX) with splicing active HeLa nuclear extract. After addition of heparin, immunoprecipitation was performed with anti-61K antibodies at different time points. Unspliced pre-mRNA was efficiently precipitated after 10 min (when B complex is efficiently formed) and also at later time points, whereas no precipitation of splicing intermediates or products was observed even at time points when efficient splicing had occurred (not shown). Thus, there is a window during spliceosome assembly (i.e., after tri-snRNP addition but prior to activation) when the 61K protein, and thus the U4/U6.U5 tri-snRNP as well (see below), is stably associated with the spliceosome, such that it remains bound even in the presence of heparin. Notably, under the same stringent conditions, 25S U4/U6.U5 tri-snRNPs present in the extract completely dissociate (not shown).

For biochemical analyses, we immunoaffinity purified preparative amounts of spliceosomes formed after 10 min of splicing. Bound spliceosomal complexes were then eluted with antigenic 61K peptide and further purified by glycerol gradient centrifugation. Purified spliceosomes contained stoichiometric amounts of uncleaved pre-mRNA, U2, U4, U5, and U6 snRNA but lacked U1 snRNA (Figure 1A, lane 1). Thus, in contrast to the activated spliceosome, which was isolated under identical conditions but with anti-SKIP antibodies (Figure 1A, lane 2), spliceosomes purified with anti-61K antibodies contain U4 snRNA. They, therefore, resemble complex B, but differ from it in that they lack the U1 snRNP. We therefore refer to these complexes as BΔU1 spliceosomes.

To determine whether U4 is base paired with U6 in BΔU1 spliceosomes, psoralen crosslinking studies were performed with affinity-purified complexes that had been fractionated on a glycerol gradient. Gradient fractions containing BΔU1 or B* complexes, or purified tri-snRNPs (which served as a positive control for a U4/U6 crosslink), were UV irradiated in the presence or absence of psoralen (AMT), and RNA–RNA crosslinks were analyzed by Northern blotting, probing sequentially for U6 and U2 snRNA (Figure 1B). Multiple U6 snRNA-containing crosslinks were observed, one of which (designated U4 × U6) was clearly present in BΔU1 complexes but only weakly detected in the activated spliceosome (Figure 1B, compare lanes 5 and 7). This band was the major crosslink obtained with purified U4/U6.U5 tri-snRNPs (lane 9), demonstrating that it is a U4/U6 crosslink. Thus, U4 and U6 are still base paired in BΔU1 spliceosomes, confirming that they have not undergone catalytic activation.

Two of the U6-containing bands were also detected with a probe specific for the U2 snRNA (lanes 11 and 13), indicating that they represent U2/U6 crosslinks. Based on their migration behavior, the major U2/U6 crosslinks observed in both BΔU1 and B* appear to correspond to the well-characterized U2/U6 interaction between complementary regions of the 5' end of U2 and the 3' end of U6 snRNA (Hausner *et al*, 1990). This so-called U2/U6 helix II base-pairing interaction does not require U4/U6 unwinding. As previous crosslinking studies analyzing spliceosomes were performed in the presence of nuclear extract and large amounts of a U2/U6 helix II crosslink are observed in extracts even in the absence of splicing (Hausner *et al*, 1990), it was previously not possible to determine precisely at what stage of spliceosome assembly this base-pairing interaction first occurs. Thus, our psoralen crosslinking studies performed with purified spliceosomal complexes suggest that U2/U6 helix II is formed at the time of B complex formation.

A crosslink containing U6 that precisely co-migrated with a pre-mRNA-containing crosslink (designated Pre-mRNAXU6) was also observed (Figure 1B, compare lanes 1 and 5), indicating that U6 contacts the pre-mRNA in BΔU1 spliceosomes. The fact that this band was not observed after probing for U2 (lane 11), and that the signals of the non-crosslinked ³²P-labeled pre-mRNA are of equal intensity in both the U2 and U6 panels, confirms that the band designated Pre-mRNAXU6 in lane 5 does not contain solely ³²P-labeled pre-mRNA. Although the crosslinking site has not been precisely mapped, the presence of this crosslink in activated B* spliceosomes (lanes 3 and 7) raises the possibility that it may represent a base-pairing interaction between the region of U6 snRNA containing the ACAGA box and the 5' end of the

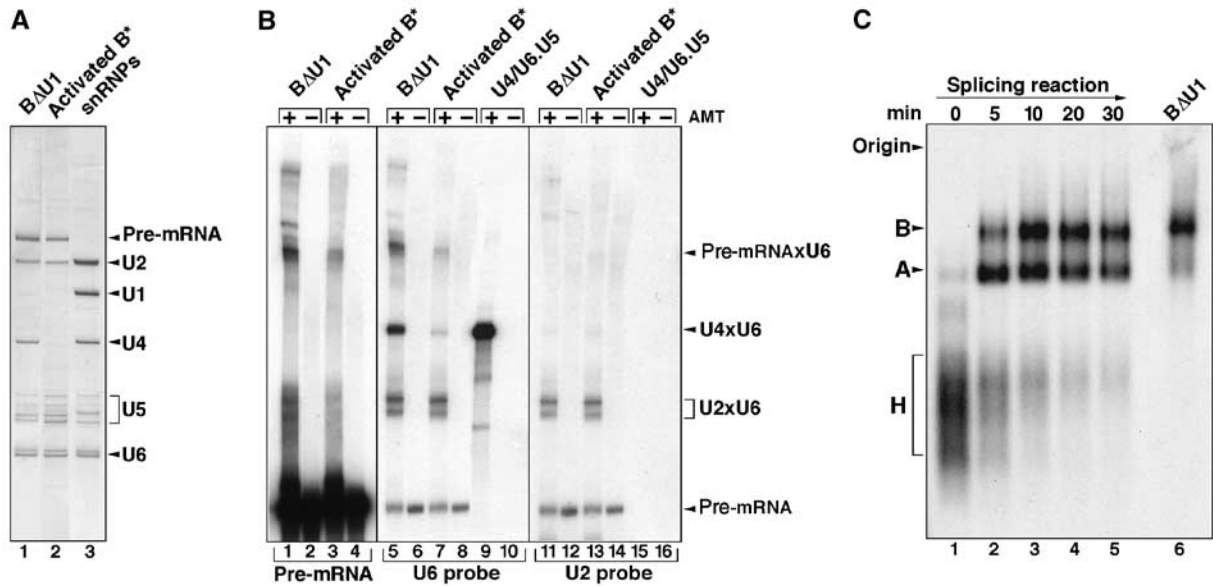


Figure 1 Characterization of immunoaffinity-purified BAU1 spliceosomes. (A) RNA composition of BAU1 versus activated B* spliceosomes. RNAs extracted from gradient-purified BAU1 (lane 1), B* (lane 2) or total snRNPs (lane 3) were analyzed by denaturing PAGE and visualized by silver staining. (B) Identification of RNA base-pairing interactions in BAU1. Gradient-fractionated BAU1 (lanes 1–2, 5–6, and 11–12), B* (lanes 3–4, 7–8, and 13–14), or U4/U6.U5 tri-snRNPs (lanes 9–10 and 15–16) were incubated in the absence (even lanes) or presence (odd lanes) of psoralen (AMT) prior to UV irradiation. RNA was analyzed by denaturing PAGE, followed by either autoradiography to directly detect the ³²P-labeled pre-mRNA present in the BAU1 and B* spliceosomes (lanes 1–4), or by Northern blotting with ³²P-labeled probes specific for U6 (lanes 5–10) or U2 (lanes 11–16). (C) Migration behavior of purified BAU1 spliceosomes on native agarose gels. ³²P-labeled MINX pre-mRNA was incubated under splicing conditions with HeLa nuclear extract for 0–30 min (lanes 1–5), and spliceosome assembly was analyzed on a 2% agarose gel, together with immunoaffinity-purified BAU1 (lane 6). The positions of the H, A, and B complexes are indicated on the left.

intron (for a summary of known RNA interactions in spliceosome, see Nilsen, 1994; Staley and Guthrie, 1998). We note that a U6/5' splice site interaction can potentially occur concomitantly with U4/U6 base pairing.

Consistent with their designation as B complexes, which have been reported to sediment approximately as a 50S particle (Freundewey and Keller, 1985), purified BAU1 spliceosomes sedimented as a 45S complex on glycerol gradients (not shown). They also exhibited a migration behavior similar to spliceosomal B complexes formed *in vitro* in HeLa nuclear extract, when analyzed by native gel electrophoresis (Figure 1C, cf lane 6 with lanes 1–5). Note that, under the conditions used for native gel electrophoresis (i.e., in the presence of heparin), the U1 snRNP has been reported to dissociate from the spliceosomal B complex (Konarska and Sharp, 1986). By eluting this complex from the gel and analyzing its snRNA composition, we have confirmed that the spliceosomal complex designated B that we observe after native gel electrophoresis of splicing reactions in fact represents a BAU1 spliceosome; it contains U2, U4, U5, and U6 snRNA, but U1 is clearly absent (not shown).

In the presence of nuclear extract in which endogenous snRNPs had been destroyed by micrococcal nuclease treatment, the purified BAU1 complex catalyzed both steps of splicing, whereas naked pre-mRNA did not (Figure 2). These experiments demonstrate that the purified BAU1 spliceosome does not require complementation with U4/U6.U5 tri-snRNPs or U1 snRNPs for its activity, and that it is thus functionally committed for subsequent activation and the catalytic steps of splicing.

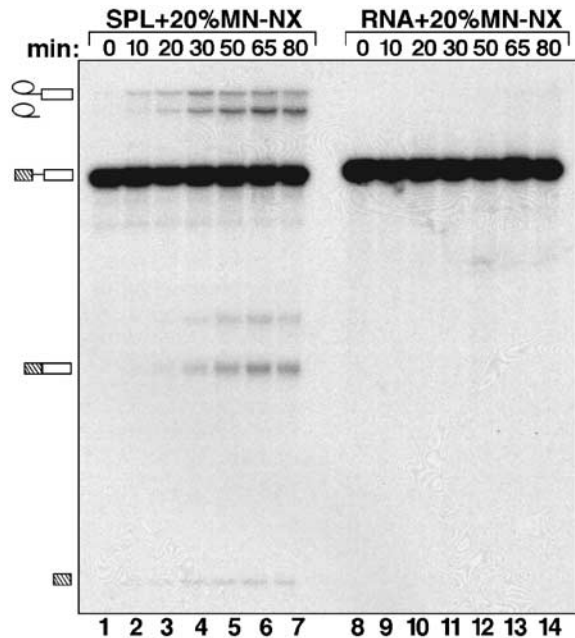


Figure 2 BAU1 spliceosomes catalyze splicing in the absence of exogenously added snRNPs. Immunoaffinity-purified BAU1 spliceosomes (SPL) (lanes 1–7) or ³²P-labeled MINX pre-mRNA (RNA) (lanes 8–14) were incubated for 0–80 min (as indicated) under splicing conditions in the presence of 20% micrococcal nuclease-treated nuclear extract. RNAs were analyzed by denaturing PAGE and visualized by autoradiography. The positions of the pre-mRNA, and the splicing intermediates and products, are indicated on the left.

Table I Comparison of the proteome of the purified BAU1 spliceosome with that of the activated spliceosome (complex B*), the 35S U5 snRNP, and the 25S U4/U6.U5 tri-snRNP particle

BAU1		B*		35S U5 snRNP	[U4/U6.U5] tri-snRNP
Sm proteins B, D1, D2, D3, E, F, G		Sm proteins B, D1, D2, D3, E, F, G		Sm proteins B,D1,D2,D3,E,F,G	Sm proteins B,D1,D2,D3,E,F,G
LSm proteins LSm 3, 4, 7, 8		LSm proteins LSm 2, 3			LSm proteins LSm 2,3,4,5,6,7,8
17S U2	U5 snRNP	17S U2	U5 snRNP	U5 snRNP	U5 snRNP
SF3b155	220K	SF3b155	220K	220K	220K
SF3b145	200K	SF3b145	200K	200K	200K
SF3b130	116K	SF3b130	116K	116K	116K
SF3b125	40K	SF3b49	40K	40K	40K
SF3b49	102K	p14	102K		102K
p14	15K	SF3b14b			15K
SF3b14b		SF3b10			100K
SF3b10	tri-snRNP	SF3a120	tri-snRNP	tri-snRNP	tri-snRNP
SF3a120	110K	SF3a66	110K		110K
SF3a66		SF3a60			65K
SF3a60		U2A'			27K
U2A'	U4/U6 snRNP	U2B''	U4/U6 snRNP	U4/U6 snRNP	U4/U6 snRNP
U2B''	90K	17S U2 AP			90K
17S U2 AP	60K	hPrp43			60K
hPrp43	20K	SR140			20K
SPF45	61K	CBP80			61K
SPF30	15.5K	CBP20			15.5K
CBP80	<i>hPrp19</i>	ALY	<i>hPrp19</i>	<i>hPrp19</i>	<i>hPrp19</i>
CBP20	<i>CDC5</i>	hnRNP	<i>CDC5</i>	<i>CDC5</i>	<i>CDC5</i>
ALY		L	L	SPF27	SPF27
hnRNP		G	G	PRL1	PRL1
M		C1/C2	C1/C2	AD-002	AD-002
C1/C2	<i>SKIP</i>	A2/B1	A2/B1	SKIP	SKIP
SR	<i>hSyf3</i>	SR	SR	hSyf3	hSyf3
9G8		hTra-2	hTra-2	hSyf1	hSyf1
Srp20		9G8	9G8	hlsy1	hlsy1
		SRp30c	SRp30c	hECM2	hECM2
MGC13125		Srp20	Srp20	KIAA0560	KIAA0560
p68		PPlase-like 3	PPlase-like 3	GCIP p29	GCIPp29
hSnu23		MGC13125	MGC13125	MGC23918	MGC23918
		hPrp22	hPrp22	G10	G10
		p68	p68	Cyp-E	Cyp-E
		hDBP5	hDBP5	PPlase-like 1	PPlase-like 1
		PSF	PSF		
	KIAA1590	hSnu-23	hSnu-23	hPrp17	hPrp17
	Tip-48	CA150	CA150	DDX35	DDX35
PABP1		CDC2L2p110	CDC2L2p110	HSP73	HSP73
		CPSF160	CPSF160	FLJ10206	FLJ10206
RED		PABP1	PABP1	KIAA0773	KIAA0773
MFAP1		PABP2	PABP2	C25A1.10p	C25A1.10p
hSmu-1		TFIP11	TFIP11	S100 A8	S100 A8
pinin		RED	RED	S100 A9	S100 A9
	UBL5p	MFAP1	MFAP1		
		hSmu-1	hSmu-1		
HsKin17		pinin	pinin		
	Npw38BP	HDB/DICE1	HDB/DICE1		
	Npw38	Cyp-60	Cyp-60		
	N-CoR1	HsKin17	HsKin17		
	hPrp4 kinase				
	FBP-21				
	TBLR1				
	SMC1				
	p220(NPAT)				

Proteins in the dark blue box, namely NPW38BP, NPW38, NCoR-1, hPrp4 kinase, FBP-21, TBLR-1, SMC1, and p220/NPAT, are not found in the activated spliceosome. Most of the 35S U5 snRNP proteins highlighted red are either absent or significantly under-represented (indicated by italics) in the BAU1 complex. The protein compositions of complex B*, the 35S U5 snRNP, and the tri-snRNP, as well as the color code for the various groups of proteins, was taken from Makarov *et al* (2002). In this manuscript, the group of proteins highlighted red are referred to as 35S U5 snRNP proteins. For accession numbers of proteins from complexes BAU1 and B*, the 35S U5 snRNP, and tri-snRNP, see Table S2 in Supplementary data.

Protein composition of BAU1 versus activated spliceosomes

To determine its protein composition, the purified BAU1 complex was fractionated by glycerol gradient centrifugation and proteins isolated from the 45S peak fractions were

separated by SDS-PAGE and characterized by MALDI mass spectrometry (MS). More than 70 proteins were identified and are summarized in Table I. Thus, compared to the activated B* spliceosome (Table I; Makarov *et al*, 2002), BAU1 contains significantly fewer proteins. Consistent with the absence of

U1 snRNA, no U1 snRNP-specific proteins (i.e., 70K, A and C) were identified. Most U2 snRNP proteins, such as A', B'', SF3a and SF3b subunits, SPF30 and hPrp43 that were previously found in isolated 17S U2 snRNPs and the purified complex A (Hartmuth *et al*, 2002; Will *et al*, 2002), were present in the BAU1 complex.

The protein compositions of the BAU1 and activated B* spliceosome differ significantly with respect to the presence/absence of two groups of proteins (Table I). First, BAU1 contains the U4/U6-specific proteins 90K, 61K, 60K, 15.5K, and 20K (CypH), while these are essentially absent in the activated B* complex (Table I). This finding, together with the fact that U4 and U6 are still base-paired, as evidenced by psoralen crosslinking (see above), strongly supports the idea that an intact U4/U6 snRNP is present in the BAU1 spliceosome. Moreover, the U4/U6.U5 tri-snRNP also appears to be essentially intact in BAU1. That is, the only U5 protein missing is 100K (hPrp28). Furthermore, both the U4/U6-61K and U5-102K proteins, which interact with each other and mediate stable tri-snRNP formation (Makarova *et al*, 2002), are present, suggesting that the two particles are still bridged in BAU1.

The second major difference in protein composition between BAU1 and B* is that all of the 35S U5-specific proteins, including Prp19 and CDC5 (highlighted red in Table I) that are found in the activated spliceosome, are absent or under-represented in BAU1 (i.e., the sequence coverage is two- to three-fold lower compared to that of U2 or U5 proteins). To perform a more accurate comparison, activated and BAU1 spliceosomes were immunoaffinity purified in parallel under the same stringent conditions, and the amount of material loaded onto the gel was normalized. LC-MSMS, focusing on the U4/U6 snRNP proteins and 35S U5 proteins, was subsequently performed and confirmed that the 35S U5 proteins are largely absent in the BAU1 complex; only hPrp19 and SKIP were identified by two peptides each and CDC5 and hSyf3 by one peptide (Supplementary Table S1). In contrast, nearly all of the 35S U5 proteins, with the exception of hSyf1, were clearly more abundant in the purified B* complex. For example, eight peptides were obtained for hPrp19 and CDC5, and 11 for SKIP. The difference in the abundance of the U4/U6 snRNP proteins between BAU1 and B* was similarly pronounced but in the opposite way; that is, two to 11 peptides were obtained for the U4/U6 proteins in the BAU1 spliceosome, while none were obtained from B* (Supplementary Table S1). For comparison, the sequence coverage of U5 snRNP proteins, such as 220K, 200K, 116K, and 102K, was very similar in both complexes, confirming that equal molar amounts of both spliceosomes had been analyzed (Supplementary Table S1).

To provide more quantitative information about the absence or presence of the 35S U5-associated proteins, as well as U4/U6-specific proteins, we performed immunoblotting experiments with selected antibodies (Figure 3). We normalized the amount of each complex applied to the blot according to the amount of ³²P-labeled pre-mRNA. Indeed, antibodies against the stably associated U5-116K and U5-40K proteins confirmed that equal molar amounts of each spliceosome were present (Figure 3). Subsequent immunoblotting experiments demonstrated that the U4/U6-specific 90K and 60K proteins, as well as the U2-associated protein SPF30, are present in BAU1 but not in the activated spliceosome.

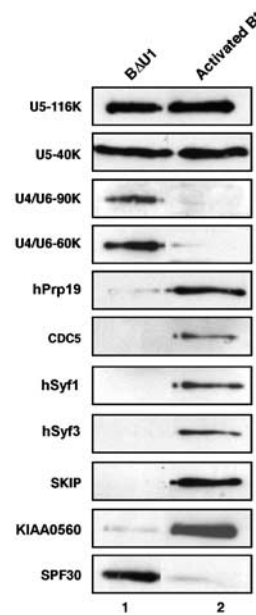


Figure 3 Protein composition of BAU1 versus activated B* spliceosomes. BAU1 and B* spliceosomes were immunoaffinity purified under identical conditions and subjected to glycerol gradient centrifugation. Proteins from BAU1 or B* were separated by SDS-PAGE, blotted onto a membrane and immunostained with affinity-purified antibodies against selected proteins, as indicated on the right.

some. Conversely, the 35S U5 proteins Prp19, CDC5L, Syf1, Syf3, and SKIP were essentially absent from BAU1 but present in the activated spliceosome. Thus, whereas the U4/U6 proteins are stably associated with the spliceosome prior to activation, they dissociate or are destabilized at the time of activation. In contrast, the 35S U5-associated group of proteins become stably integrated into the spliceosome during transition from the B complex to the activated spliceosome. This observation suggests that the latter group of proteins may play a major role in the activation of the spliceosome rather than in the stable integration of the tri-snRNP. However, it is also conceivable that, despite the fact that they may only be loosely associated with the BAU1 spliceosome (and thus lost due to heparin treatment), one or more of the 35S U5 snRNP proteins may play a role in tri-snRNP integration and thus B complex formation.

Spliceosomal complex B forms in the absence of a subset of 35S U5 proteins

To determine whether one or more of the 35S U5-specific proteins play a role in tri-snRNP integration, we carried out immunodepletion/complementation studies with HeLa nuclear extract. For this purpose, we raised antibodies against the 35S U5 protein CDC5. To define more precisely which proteins are stably associated with CDC5 and thus potentially co-immunodepleted, we first performed immunoaffinity chromatography with nuclear extract and antipeptide antibodies raised against CDC5. The bound complexes were eluted with an excess of CDC5 peptide and the eluate fractionated on a 5–20% glycerol gradient. Proteins were isolated from fractions across the gradient and analyzed by SDS-PAGE (Figure 4). A group of seven proteins co-migrated in fractions 13–15, indicating that they form a complex that

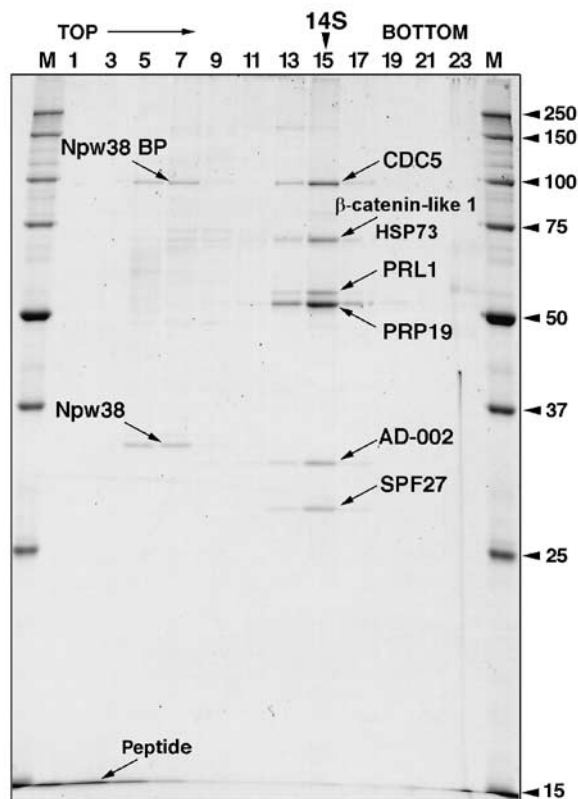


Figure 4 Sedimentation behavior and protein composition of CDC5/Prp19-containing complexes. Complexes were immunoaffinity purified from HeLa nuclear extract with anti-CDC5 antibodies and subjected to 5–20% glycerol gradient centrifugation. Proteins from uneven fractions were separated by SDS–PAGE and stained with Coomassie. The following proteins (indicated with arrows) were identified by MS: CDC5 (NP_001244), HSP73 (NP_006588), β -catenin-like 1 (NP_110517), PRL1 (NP_002660), Prp19 (NP_055317), AD002 (AAF14858), SPF27 (NP_005863), Npw38 (NP_005701), and Npw38BP (NP_057396).

sediments with a Svedberg value of 14S. Proteins present in the 14S peak were analyzed by MALDI-MS and identified as CDC5, Hsp73, β -catenin-like 1, PRL1, Prp19, AD002, and SPF27. MS analyses further revealed that the proteins Npw38 and Npw38BP co-migrate at the top of the gradient, suggesting that they were co-isolated with the CDC5/Prp19 complex but then dissociated from it during centrifugation.

With the exception of the β -catenin-like 1 protein, 14S CDC5/Prp19 complex consists primarily of a subset of 35S U5 proteins (i.e., CDC5, Hsp73, PRL1, Prp19, AD002, and SPF27) (Makarova *et al*, 2002). To determine whether this subset of 35S U5-associated proteins play a role in splicing, we immunodepleted nuclear extract of the CDC5/Prp19 complex using anti-CDC5 and anti-AD002 antibodies. The level of each protein was significantly reduced in their respective immunodepleted extract when compared with the mock-depleted extract, as evidenced by immunoblotting (Figure 5A). Consistent with the fact that they are present together in a stable complex, depletion of CDC5 also led to a reduction in AD002 and vice versa (Figure 5A, lanes 2 and 3). *In vitro* splicing was subsequently performed with mock-depleted extract and extract depleted with both anti-CDC5 and anti-AD002 antibodies. As shown in Figure 5B, splicing activity

was significantly reduced in the CDC5/AD002-depleted as compared to mock-depleted extract (cf lanes 1–4 and 5–8). Significantly, addition of immunoaffinity-purified 14S CDC5/Prp19 complex (i.e., gradient fraction 14 from Figure 4) partially restored splicing activity to the depleted extract (lanes 8–12).

To determine at what step of the splicing reaction the 14S CDC5/Prp19 complex first acts, we analyzed splicing complex formation in mock- and CDC5/AD002-depleted extract by agarose gel electrophoresis. Relative to the mock-depleted extract (Figure 5C, lanes 1–4), no significant decrease in the formation of A or B complex was observed with the CDC5/AD002-depleted extract (Figure 5C, lanes 5–8). Due to the fact that the catalytic steps of splicing occur very rapidly with the MINX pre-mRNA, C complex is difficult to detect (Das and Reed, 1999).

To determine whether or not the complex designated B that we observe in the absence of the 14S CDC5/Prp19 complex has undergone activation (i.e., release of U4), we performed immunoprecipitation experiments under the same conditions used to isolate Δ U1 spliceosomes with anti-61K antibodies. After allowing for splicing complex formation, spliceosomes were immunoprecipitated from mock- or CDC5/AD002-depleted extract and their RNA composition was analyzed by 3'-end labeling with 32 P-pCp, followed by denaturing PAGE. The RNA compositions of spliceosomes immunoprecipitated from mock- and CDC5/AD002-depleted extract were essentially identical (Figure 5D); when antibodies were preblocked with cognate peptide, essentially no RNA was precipitated (not shown). Importantly, in addition to U2 and U5 (due to inefficient end labeling, the U6 snRNA is difficult to detect), a similar level of U4 snRNA was detected in both cases. These results demonstrate that the majority of spliceosomes present have not undergone activation and, thus, spliceosomal complex B can form even in the absence of the CDC5/Prp19 complex, but splicing is blocked prior to step 1 of splicing.

Discussion

To gain insight into the multiple RNP rearrangements and changes in protein composition of the spliceosome during its maturation, catalytic activity, and subsequent disassembly, we have set out to isolate various spliceosomal complexes under identical stringent conditions at defined assembly/functional stages. Using anti-peptide antibodies directed against spliceosomal proteins that are transiently stably associated with the spliceosome, we have now been able to isolate two spliceosomal complexes (Δ U1 and the activated spliceosome) under the same conditions, allowing a meaningful comparison of their protein compositions via MS. These results demonstrate that, in addition to the highly stable spliceosomal complexes detected by standard biochemical methods, there exist additional spliceosomal intermediates that transiently form configurations of high stability, and that such intermediates can be isolated by targeting the suitable epitope for immunoaffinity selection. This method can theoretically be extended to isolate additional spliceosomal complexes and thus generate additional 'snapshots' of the spliceosome's changing protein composition and RNP structure.

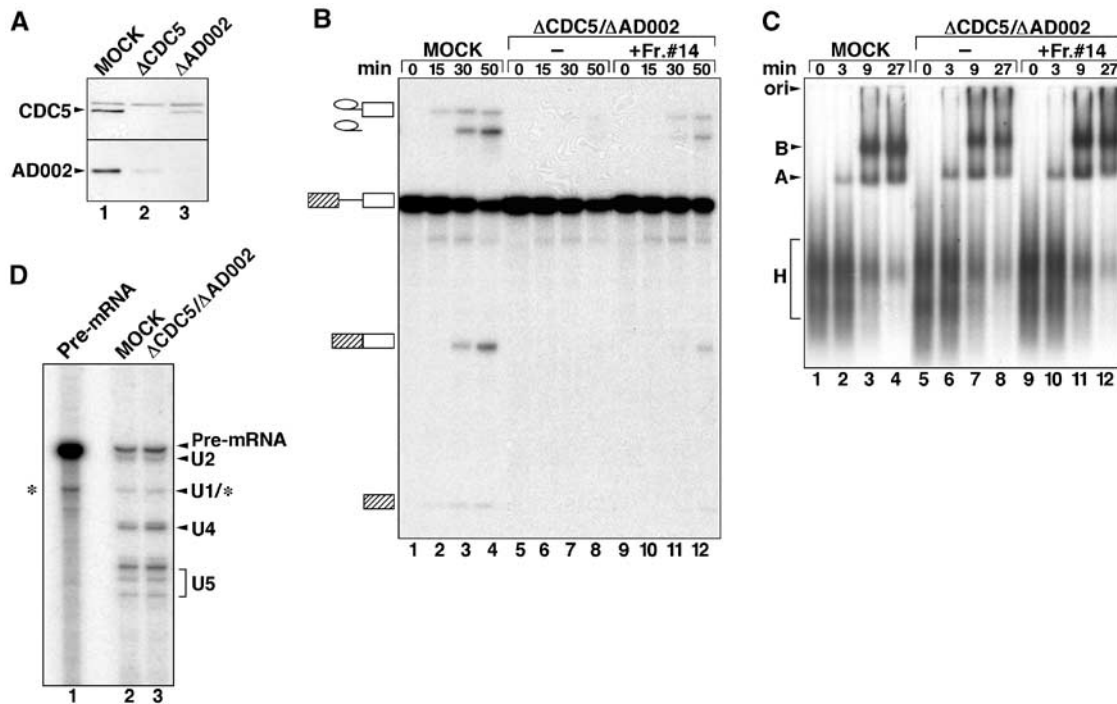


Figure 5 Spliceosomal B complex forms in the absence of the CDC5/PRP19 complex. (A) Nuclear extracts are efficiently immunodepleted of CDC5 and AD002. Western blot of mock-depleted (lane 1), CDC5-depleted (lane 2) or AD002-depleted (lane 3) extract probed with anti-CDC5 (upper panel) or anti-AD002 (lower panel) antibodies. (B) Time course of splicing of [³²P] MINX pre-mRNA in mock-depleted extract (MOCK, lanes 1–4) or extract depleted with CDC5 and AD002 (Δ CDC5/ Δ AD002, lanes 5–8), or CDC5/AD002-depleted extract complemented with purified CDC5/PRP19 complex (fraction no. 14, from the gradient in Figure 4) (lanes 9–12). RNA was analyzed by denaturing PAGE and visualized by autoradiography. The pre-mRNA, and splicing intermediates and products are indicated on the left. (C) The CDC5/PRP19 complex is not required for B-complex formation. Spliceosome assembly in mock-depleted extract, CDC5/AD002-depleted extract, or CDC5/AD002-depleted extract complemented with the CDC5/PRP19 complex (lanes marked as in (B)) was analyzed at the indicated times by native gel electrophoresis and visualized by autoradiography. The position of the H, A, and B complexes is indicated on the left. (D) Spliceosomes formed in CDC5/AD002-depleted extract contain U4 snRNA. Spliceosomes were immunoprecipitated from mock- (lane 2) or CDC5/AD002-depleted extract (lane 3) with anti-61K antibodies. RNA was isolated, end labeled with ³²P-pCp, fractionated by denaturing PAGE, and visualized by autoradiography. The identities of the RNAs are indicated on the right. Internally radiolabeled MINX pre-mRNA (lane 1) was analyzed in parallel. Note that the band migrating at the position of U1 may also contain a pre-mRNA degradation product (*).

Composition of the B Δ U1 spliceosome

The protein composition of the B Δ U1 spliceosome differed markedly from that of the activated B* spliceosome in that U4/U6-associated proteins (which were not found in B*) were present, but a group of 16 35S U5-associated proteins that are stably associated with B* were largely absent. Only minimal amounts of a subset of these proteins were detected by MS or immunoblotting. That some proteins appear to be present at a very low level in B Δ U1 could reflect the existence of a subpopulation of spliceosomes in which the 61K protein is still stably associated and several 35S U5 snRNP proteins have also been stably integrated. B Δ U1 contained a group of eight proteins, including Npw38, Npw38BP, N-CoR1, hPrp4 kinase, FBP-21, TBLR-1, SMC1, and p220(NPAT), not detected in the pre-spliceosome or activated spliceosome (Table 1) (Hartmuth *et al*, 2002; Makarov *et al*, 2002). Thus, these proteins could potentially function specifically at this stage of spliceosome formation. Consistent with these proteins being present in the B Δ U1 spliceosome, hPrp4 kinase was previously shown to interact with N-CoR (Dellaire *et al*, 2002). Interestingly, the *Saccharomyces pombe* ortholog of hPrp4 kinase has been shown to be essential for splicing and to phosphorylate the *S. pombe* prp1 protein (human U5-102K protein) (Schwelnus *et al*, 2001). Thus, hPrp4 kinase, via

phosphorylation of the U5-102K protein, could play a role in tri-snRNP rearrangement during spliceosome activation.

Interestingly, anti-61K affinity-purified spliceosomes lacked the U1 snRNP. The absence of U1 may simply be due to the presence of heparin during the isolation procedure, and thus anti-61K affinity-purified spliceosomes likely represent spliceosomal complex B that has been depleted of U1. Alternatively, the B Δ U1 spliceosome could represent a bona fide spliceosome intermediate in which the U1 snRNP/5' splice site interaction has been actively disrupted, but the U4/U6 base-pairing interaction remains intact. The detection of a pre-mRNA/U6 snRNA crosslink (Figure 1B) is a first indication that this may indeed be the case. However, more detailed analyses are required to map the precise U6 cross-linking site on the pre-mRNA. In this respect, it is interesting to note that, in the minor U12-type spliceosome, interaction of U6atac with the 5' splice site was observed prior to complete unwinding of the U4atac/U6atac base-pairing interaction (Ferland and Steitz, 2001). Complementation experiments with micrococcal nuclease-treated nuclear extract demonstrated that B Δ U1 can be chased into a catalytically active complex in the absence of snRNPs. Thus, the presence of U1 is not required for the subsequent activation of the spliceosome. Displacement of U1 from the 5' splice site is a

Table II Comparison of Prp19/CDC5 complexes

14S CDC5/hPrp19 complex (this study) ^a	Human CDC5 complex (Ajuh <i>et al</i> , 2000) ^b	Prp19 complex (Chen <i>et al</i> , 2002) ^c	Prp19 complex (Ohi <i>et al</i> , 2002) ^d
hPrp19	hPrp19/CCAP3 ^e	Prp19	Prp19
CDC5	CDC5L ^e	Ntc85/Cef1/Cdc5	Cef1/Cdc5
PRL1	PRL1/hPrp46 ^e		Cwc1/Prp46
AD-002	AD-002/CCAP2		
HSP73	HSP73/CCAP1 ^e		
SPF27	SPF27 ^e	Ntc25/Snt309 ^f	Snt309 ^f
β-catenin like	CCAP4		
	CCAP5		
	CCAP6 ^e		
	CCAP7		
	CCAP8		
	ASF/SF2		
	PSF		
	SRm160		
	SC35		
	NF45		
	TOPIIa		
	U1A		
	hnRNPG		
	PP2c (WIP1)		
	DDP kinase		
	PP1-α		
	Sm proteins		Sm proteins
	U2-specific proteins		U2-specific proteins
		Ntc77/Clf1/Syf3	U5-specific proteins
		Ntc90/Syf1	Clf1/Syf3
		Ntc30/Isy1	Syf1
		Ntc31/Syf2	Isy1
		Ntc20	Syf2
			Ntc20
			Slu7
			Ecm2
			Spp2
			Cwc2, Cwc14, Cwc15
			Cwc16 Cwc21, Cwc22
			Cwc23, Cwc24, Cwc25
			Cwc26, Cwc27
			Prp17, Prp22, Prp45

^aComplexes were immunoaffinity purified from HeLa nuclear extract with anti-CDC5 antibodies at 200 mM salt and further purified by glycerol gradient centrifugation.

^bComplexes were purified from HeLa nuclear extracts either by immunoaffinity chromatography with anti-CDC5 antibodies at 150 mM salt or by tandem chromatographic steps involving gel filtration and ion exchange chromatography (Ajuh *et al*, 2000).

^cComplexes were isolated from *S. cerevisiae* total cell extracts at 60 mM salt by tagging the Prp19 protein with an HA-tag and precipitating with anti-HA antibodies (Tarn *et al*, 1994).

^dComplexes were isolated from *S. cerevisiae* total cell extracts using the TAP method (150 mM salt) after tagging the Prp19 protein (Ohi *et al*, 2002).

^eStably associated core protein in the CDC5 protein complex (Ajuh *et al*, 2000).

^fSnt309 is a functional counterpart of *S. pombe* Cwf7 that is homologous to SPF27 (Ohi and Gould, 2002).

prerequisite for the subsequent base-pairing interaction of the U6 snRNA, and appears to be facilitated by the DEAD-box protein hPrp28/U5-100K (Staley and Guthrie, 1999; Chen *et al*, 2001). Intriguingly, hPrp28p/U5-100K is also no longer stably associated with the BAU1 spliceosome, and thus may dissociate together with U1 after catalyzing its displacement from the 5' splice site.

Isolation of a well-defined, human 14S CDC5/Prp19 heteromeric complex

Using antibodies against CDC5, we isolated a heteromeric, 14S protein complex consisting of CDC5, Prp19, Hsp73, β-catenin-like 1, PRL1, AD002, and SPF27. This CDC5/Prp19 complex contains only a subset of the 35S U5 proteins that are stably integrated first in the activated spliceosome.

Whether additional 35S U5 proteins associate and/or are stably integrated into the spliceosome as a larger group, together with the CDC5/Prp19 heptameric complex, is presently not clear. Previous studies characterizing the human CDC5/Prp19 complex did not allow precise conclusions regarding its protein composition (Ajuh *et al*, 2000). In the latter studies, several CDC5-containing complexes were isolated from HeLa nuclear extract either by immunoaffinity chromatography or by size exclusion, followed by anion exchange chromatography; in each case approximately 30 proteins appeared to co-purify with CDC5 (Table II). Only six of these proteins, that is, CDC5, Prp19, PRL1, Hsp73 (CCAP1), SPF27, and CCAP6, were found in all preparations, and thus were denoted core components of the CDC5-containing complex (Ajuh *et al*, 2000; Table II). While five of

these are also found in our 14S CDC5/Prp19 complex, CCAP6 is not. Furthermore, AD002, which is present in our complex, was not previously reported to be a core component of CDC5 complex, although it co-purifies with CDC5 (AD002 is denoted CCAP2 by Ajuh *et al*, 2000).

The organization of the human 14S CDC5/Prp19 complex differs considerably from the NTC complex in *S. cerevisiae* (Table II). The latter complex has not been characterized in its entirety; it consists of at least eight proteins with several additional proteins also thought to be present (Chan *et al*, 2003). Of the known eight NTC components, only two, Prp19 and CDC5/Cef1, are found in our 14S CDC5/Prp19 complex. A third candidate is Snt309, which may be a functional counterpart of SPF27 (Ohi and Gould, 2002). That the NTC complex likely consists of a much larger group of proteins is further suggested by more recent studies (Ohi *et al*, 2002). Using the TAP method, Prp19 in *S. cerevisiae* was shown to be part of a large RNP complex containing numerous proteins (Table II) as well as U2, U5, and U6 snRNAs. A comparison of this complex with our 14S CDC5/Prp19 complex reveals that their protein compositions are significantly different.

Interestingly, Npw38 and Npw38BP, which interact with one another (Komuro *et al*, 1999), were reproducibly co-isolated with the CDC5/Prp19 complex, but appeared to dissociate during glycerol gradient centrifugation. Thus, these proteins, which have no apparent orthologs in *S. cerevisiae* (Supplementary Table S2), likely exhibit affinity for one or more components of the CDC5/Prp19 complex, and may play a role in recruiting this complex to the spliceosome, at least in higher eucaryotes. Indeed, Npw38 and Npw38BP are stably associated with the BAU1 spliceosome and could thus act as a docking site for the CDC5/Prp19 complex. Npw38 has been shown to interact with the U5-15K (Dib1) protein (Zhang *et al*, 2000), and both of these proteins together with Npw38BP are no longer stably associated with the activated spliceosome (Table I), suggesting that they are destabilized together during activation.

Function of the human CDC5/Prp19 complex

Previous functional analyses of the CDC5/Prp19 complex in both yeast and humans were performed with a poorly defined, large group of proteins (see above) (Ajuh *et al*, 2000; Chan *et al*, 2003). Thus, these studies do not allow clear-cut conclusions as to precisely which components of the CDC5/Prp19 complex are required for its function. We have performed depletion/complementation studies with a well-defined complex containing CDC5, Prp19, Hsp73, β -catenin-like 1, PRL1, AD002, and SPF27, and thus can narrow down the number of essential Prp19/CDC5 proteins to a very small subset. The CDC5/Prp19 complex as defined here does not play a role in tethering the tri-snRNP within the spliceosome, as evidenced by the fact that this group of proteins is not found in the BAU1 complex, where the tri-snRNP is stably associated. Moreover, by physically depleting this complex, we have further demonstrated that these proteins are not required for the initial integration of the tri-snRNP. The 17S U2-associated protein SPF30, which is present in the BAU1 spliceosome, likely plays an important role in recruiting/tethering the U4/U6.U5 tri-snRNP complex; indeed SPF30 has been shown to interact with the U4/U6-90K protein (Meister *et al*, 2001; Rappsilber *et al*, 2001). Interestingly, SPF30 is no longer stably associated with the activated

spliceosome, consistent with the fact that the U4 snRNA and the U4/U6-associated proteins (15.5K, 20K, 60K, 61K, and 90K) dissociate or are destabilized at this time. This finding underscores how comparison of the stable proteomes of spliceosomal complexes at defined stages may help identify those proteins that are interaction partners and/or function as a group at a particular step of the splicing process.

Depletion/complementation studies provided evidence that the CDC5/Prp19 heptameric complex is required for the first step of splicing. Thus, one or more members of this complex initially function in a time window encompassing the spliceosome activation step (i.e., after B-complex formation but prior to step one of splicing). These results differ from previous reports where immunodepletion of human CDC5 and its associated proteins led predominantly to a block in the second step of splicing (Ajuh *et al*, 2000). One possible explanation for this difference is that a more efficient depletion of the CDC5/Prp19 complex was achieved in our hands due to the double depletion with both anti-CDC5 and anti-Ad002 antibodies. Although spliceosomal B complex was observed in the absence of the CDC5/Prp19 complex, it is presently not clear whether it functions prior to, during, or after U4/U6 unwinding. A role in activation and/or the first step of splicing, but not in tri-snRNP integration, likely also holds for the nine other 35S U5 proteins that are present in the activated spliceosome but were not present in the immunoaffinity-purified CDC5/Prp19 complex. Although direct evidence that they function at this stage is lacking, the fact that they are stably integrated into the spliceosome at the same time as proteins of the CDC5/Prp19 complex is a first indication that they may also function at a similar step. Additional studies are clearly required to address this question in more detail.

In yeast, the NTC complex acts subsequent to U4 dissociation, stabilizing the association of U5 and U6 with the activated spliceosome (Chan *et al*, 2003). Recent studies in yeast have also indicated that the U6-associated Lsm proteins (Lsm2–8) are destabilized during activation of the spliceosome and that the NTC complex is required for their destabilization (Chan *et al*, 2003). Our MS, as well as initial immunoblotting data, suggests that Lsm proteins are not destabilized during spliceosome activation in humans. That is, several Lsm proteins were detected by MS both in BAU1 and activated B* spliceosomes, despite the fact that they are generally difficult to detect by MS (Table I). In addition, in an initial immunoblotting experiment with antibodies against LSm4, we did not observe a decrease in signal between the BAU1 and B*-activated spliceosome; in both complexes, significant amounts of Lsm4 were detected (not shown). However, additional experiments are required to clarify whether the situation in humans indeed differs from that in yeast.

The ability to isolate both BAU1 and activated spliceosomes under identical conditions allows us to address other interesting questions. For example, what triggers U4 release and other RNP rearrangements during spliceosome activation? Aside from RNA helicase activity, post-translational modifications might also be a driving force that alters the stability of protein–protein interactions within the spliceosome during its activation. To address this question, we have begun to compare the modification states of proteins in the BAU1 versus activated spliceosome.

Materials and methods

In vitro splicing

HeLa nuclear extract was prepared according to Dignam *et al* (1983). *In vitro* splicing, analytical immunoprecipitation of spliceosomes, and micrococcal nuclease (MN) digestions were performed as described (Makarova *et al*, 2001, 2002). For analysis of splicing complexes, heparin (5 mg/ml) was added to 5 μ l of a splicing reaction or 10 μ l of immunoaffinity-purified BAU1 spliceosomes to a final concentration of 1 mg/ml, and samples were placed on ice. Complexes were analyzed on 2% agarose gels (Das and Reed, 1999).

Preparative immunoaffinity purification of spliceosomes and MS

Antibodies raised against a peptide (aa 484–497) of U4/U6-61K or the splicing factor SKIP (Makarov *et al*, 2002) were affinity purified using a SulfoLink column (Pierce) containing the cognate peptide. A 4.0-ml splicing reaction containing 40% HeLa nuclear extract and 10–20 nM ³²P-labeled MINX pre-mRNA (2000–4000 cpm/pmol) was incubated at 30°C for 10 min. Heparin was added to a final concentration of 0.5 mg/ml and incubation at 30°C continued for 5 min. Subsequent procedures were performed at 4°C. The splicing reaction was diluted 10-fold with IP buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTT) containing 0.05% NP-40 and incubated for 1 h with 1 ml of protein A-sepharose (PAS) preblocked with 0.5 mg/ml of BSA and 50 μ g/ml of yeast tRNA. The reaction was then incubated for 2 h with 1 ml of PAS charged with affinity-purified anti-61K or anti-SKIP antibodies. Beads were washed 6 \times with IP buffer containing 0.05% NP-40 and the bound material was eluted by incubating for 1 h with 2.5 ml IP buffer containing 5% glycerol, 0.05% NP-40, and 0.6 mg/ml of cognate peptide. The eluate was loaded onto a 10–30% glycerol gradient containing IP buffer and centrifuged in a TST41.14 rotor (Sorvall) for 14 h at 31 000 rpm. Gradients were fractionated into 24 0.5-ml aliquots. Five fractions containing the peak of the BAU1 or B* spliceosome were combined and complexes were pelleted by centrifugation in a TH660 rotor (Sorvall) for 4 h at 60 000 rpm. Pellets were resuspended in SDS-PAGE loading buffer, subjected to 10/13% SDS-PAGE, and stained with Coomassie. Individual bands were excised from the gel and analyzed by both MALDI-MS and LC-MSMS (Hartmuth *et al*, 2002). MS analyses of BAU1 were performed twice: once alone and then subsequently in direct comparison with an equal molar amount of B*.

Isolation of the CDC5/Prp19 complex

PAS beads (0.4 ml) were charged with affinity-purified antibodies against a peptide of CDC5 (aa 106–124), preblocked with 0.5 mg/ml of BSA and 50 μ l/ml of yeast tRNA, and incubated with 1 ml of HeLa nuclear extract in C buffer (Dignam *et al*, 1983) for 2 h at 4°C. Beads were washed 6 \times with G200 buffer (20 mM HEPES, pH 7.9, 200 mM NaCl, 1.5 mM MgCl₂, 0.05% NP-40). The bound material was eluted with 400 μ l of IP buffer containing 200 mM NaCl, 0.5 mg/ml antigenic peptide, and 0.5 mM DTT, and loaded on a 5–20% glycerol gradient containing IP buffer. Gradients were centrifuged for 14.5 h at 35 000 rpm in a TH-660 rotor. 12S U1 and 20S U5 snRNPs served as S-value markers.

Psoralen crosslinking

A 1.25 ml splicing reaction containing ³²P-labeled MINX pre-mRNA was incubated for 10 min at 30°C, heparin was added to a final concentration of 0.5 mg/ml, and the reaction incubated for an additional 5 min. The splicing reaction was diluted eight-fold with IP buffer, precleared by incubating with 300 μ l PAS preblocked with BSA and tRNA, and then incubated with 300 μ l PAS charged with anti-61K antibodies (to isolate BAU1) or anti-SKIP antibodies (to isolate activated B* spliceosomes). Complexes were eluted with cognate peptide as described above and further purified by centrifugation on a 10–30% glycerol gradient prepared with IP

buffer. Peak fractions from each gradient were combined and split into two aliquots. Psoralen (AMT) was added to one aliquot to a final concentration of 25 μ g/ml and incubated on ice for 10 min. Both the psoralen-treated and nontreated aliquots were subsequently irradiated for 15 min at 365 nm on ice. After proteinase K digestion, RNA was recovered by phenol–chloroform extraction and ethanol precipitation, and separated on a 5% polyacrylamide, 7 M urea gel (Hausner *et al*, 1990). The ³²P-labeled pre-mRNA was detected directly by autoradiography, whereas the U2 and U6 snRNAs were visualized by Northern blotting (Hartmuth *et al*, 2002). Psoralen crosslinking was performed in parallel with anti-m3G affinity-purified, gradient-fractionated U4/U6.U5 tri-snRNPs (Laggerbauer *et al*, 1998).

Immunoblotting

Proteins from BAU1 or B* spliceosomes were separated by SDS-PAGE, transferred to Hybond P membrane and immunostained with affinity-purified antibodies, using an ECL detection kit (Amersham). Antibodies were raised in rabbits against the following antigens: U5-116K (aa 1–117), U5-40K (full length); U4/U6-90K (aa 287–302); U4/U6-60K (aa 58–94), Prp19 (aa 176–191), CDC5 (aa 106–124), hSyf1 (aa 6–21), hSyf3 (aa 47–60), SKIP (aa 516–531), and KIAA0560 (aa 1470–1485).

Immunodepletion/complementation assays

HeLa nuclear extract (0.5 ml) in C buffer was incubated 2 \times with 75 μ l of PAS beads charged with affinity-purified antibodies against CDC5 or a peptide of AD002 (aa 71–86) for 2 h at 4°C with HOT rotation. For double depletion, incubation with anti-CDC5 beads was followed by anti-AD002 beads. The extract was then dialyzed for 4 h against D buffer (Dignam *et al*, 1983) in a Slide-A-Lyzer 3.5K (Pierce). Mock-depleted extracts were treated in an identical manner, except that antibody was omitted. For complementation, prior to dialysis, 100 μ l of depleted extract was mixed with 56 μ l of gradient fraction no. 14 (Figure 4) containing the CDC5/Prp19 complex at a concentration of \sim 0.1 mg/ml, and then dialyzed as above. Splicing reactions (10–20 μ l) contained 35% mock-, depleted- or CDC/Prp19-complemented extract and 10 nM MINX ³²P-labeled pre-mRNA (10 000–50 000 cpm/pmol).

Analysis of spliceosomal complexes formed in CDC5/AD002-depleted extract

A 150 μ l splicing reaction containing mock- or CDC5/AD002-depleted extract and 10 nM MINX pre-mRNA was incubated at 30°C for 10 min and subsequently treated with heparin as described above. The reaction was diluted eight-fold with IP buffer containing 0.05% NP-40, incubated for 2 h at 4°C with 30 μ l PAS charged with anti-61K antibodies, and washed 6 \times with IP buffer containing 0.05% NP-40. RNA was isolated from the immunoprecipitated spliceosomal complexes, 3'-end-labeled with [³²P]pCp, analyzed on a 14% polyacrylamide/7 M urea gel, and visualized by autoradiography.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Monika Raabe, Gabi Heyne, Irene Öchsner, and Peter Kempkes for excellent technical assistance, and Klaus Hartmuth for helpful advice and discussions regarding psoralen crosslinking. We are grateful to Utz Fischer for kindly providing antibodies against SPF30. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Lu294/12-1), BMBF (031U215B), Ernst Jung Stiftung, and the Fonds der Chemischen Industrie to RL, and a grant from the BMBF (031U215A) to MW.

References

- Ajuh P, Kuster B, Panov K, Zomerdijk JC, Mann M, Lamond AI (2000) Functional analysis of the human CDC5L complex and identification of its components by mass spectrometry. *EMBO J* **19**: 6569–6581
- Ben-Yehuda S, Dix I, Russell CS, McGarvey M, Beggs JD, Kupiec M (2000) Genetic and physical interactions between factors involved in both cell cycle progression and pre-mRNA splicing in *Saccharomyces cerevisiae*. *Genetics* **156**: 1503–1517
- Brow DA (2002) Allosteric cascade of spliceosome activation. *Annu Rev Genet* **36**: 333–360
- Burge CB, Tuschl T, Sharp PA (1999) Splicing of precursors to mRNAs by the spliceosomes. In *The RNA World*, Gesteland C, Atkins (eds), 2nd edn, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press pp 525–560
- Chan SP, Kao DI, Tsai WY, Cheng SC (2003) The Prp19p-associated complex in spliceosome activation. *Science* **302**: 279–282
- Chen CH, Yu WC, Tsao TY, Wang LY, Chen HR, Lin JY, Tsai WY, Cheng SC (2002) Functional and physical interactions between components of the Prp19p-associated complex. *Nucleic Acids Res* **30**: 1029–1037
- Chen JY, Stands L, Staley JP, Jackups RR, Latus LJ, Chang TH (2001) Specific alterations of U1-C protein or U1 small nuclear RNA can eliminate the requirement of Prp28p, an essential DEAD box splicing factor. *Mol Cell* **7**: 227–232
- Das R, Reed R (1999) Resolution of the mammalian E complex and the ATP-dependent spliceosomal complexes on native agarose mini-gels. *RNA* **5**: 1504–1508
- Dellaire G, Makarov EM, Cowger JJ, Longman D, Sutherland HG, Lührmann R, Torchia J, Bickmore WA (2002) Mammalian PRP4 kinase copurifies and interacts with components of both the U5 snRNP and the N-CoR deacetylase complexes. *Mol Cell Biol* **22**: 5141–5156
- Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11**: 1475–1489
- Friendewey D, Keller W (1985) Stepwise assembly of a pre-mRNA splicing complex requires U-snRNPs and specific intron sequences. *Cell* **42**: 355–367
- Frilander MJ, Steitz JA (2001) Dynamic exchanges of RNA interactions leading to catalytic core formation in the U12-dependent spliceosome. *Mol Cell* **7**: 217–226
- Hartmuth K, Urlaub H, Vornlocher HP, Will CL, Gentzel M, Wilm M, Lührmann R (2002) Protein composition of human prespliceosomes isolated by a tobamycin affinity-selection method. *Proc Natl Acad Sci USA* **99**: 16719–16724
- Hausner TP, Giglio LM, Weiner AM (1990) Evidence for base-pairing between mammalian U2 and U6 small nuclear ribonucleoprotein particles. *Genes Dev* **4**: 2146–2156
- Jurica MS, Licklider LJ, Gygi SR, Grigorieff N, Moore MJ (2002) Purification and characterization of native spliceosomes suitable for three-dimensional structural analysis. *RNA* **8**: 426–439
- Komuro A, Saeki M, Kato S (1999) Association of two nuclear proteins, Npw38 and NpwBP, via the interaction between the WW domain and a novel proline-rich motif containing glycine and arginine. *J Biol Chem* **274**: 36513–36519
- Konarska MM, Sharp PA (1986) Electrophoretic separation of complexes involved in the splicing of precursors to mRNAs. *Cell* **46**: 845–855
- Laggerbauer B, Achsel T, Lührmann R (1998) The human U5-200kD DEXH-box protein unwinds U4/U6 RNA duplexes *in vitro*. *Proc Natl Acad Sci USA* **95**: 4188–4192
- Makarov EM, Makarova OV, Urlaub H, Gentzel M, Will CL, Wilm M, Lührmann R (2002) Small nuclear ribonucleoprotein remodeling during catalytic activation of the spliceosome. *Science* **298**: 2205–2208
- Makarova OV, Makarov EM, Liu S, Vornlocher HP, Lührmann R (2002) Protein 61K, encoded by a gene (PRPF31) linked to autosomal dominant retinitis pigmentosa, is required for U4/U6*U5 tri-snRNP formation and pre-mRNA splicing. *EMBO J* **21**: 1148–1157
- Makarova OV, Makarov EM, Lührmann R (2001) The 65 and 110 kDa SR-related proteins of the U4/U6.U5 tri-snRNP are essential for the assembly of mature spliceosomes. *EMBO J* **20**: 2553–2563
- Meister G, Hannus S, Plottner O, Baars T, Hartmann E, Fakan S, Laggerbauer B, Fischer U (2001) SMNrp is an essential pre-mRNA splicing factor required for the formation of the mature spliceosome. *EMBO J* **20**: 2304–2314
- Nilsen TW (1994) RNA–RNA interactions in the spliceosome: unraveling the ties that bind. *Cell* **78**: 1–4
- Ohi MD, Gould KL (2002) Characterization of interactions among the Cef1p-Prp19p-associated splicing complex. *RNA* **8**: 798–815
- Ohi MD, Link AJ, Ren L, Jennings JL, McDonald WH, Gould KL (2002) Proteomics analysis reveals stable multiprotein complexes in both fission and budding yeasts containing Myb-related Cdc5p/Cef1p, novel pre-mRNA splicing factors, and snRNAs. *Mol Cell Biol* **22**: 2011–2024
- Raghuathan PL, Guthrie C (1998) RNA unwinding in U4/U6 snRNPs requires ATP hydrolysis and the DEIH-box splicing factor Brr2. *Curr Biol* **8**: 847–855
- Rappsilber J, Ajuh P, Lamond AI, Mann M (2001) SPF30 is an essential human splicing factor required for assembly of the U4/U5/U6 tri-small nuclear ribonucleoprotein into the spliceosome. *J Biol Chem* **276**: 31142–31150
- Rappsilber J, Ryder U, Lamond AI, Mann M (2002) Large-scale proteomic analysis of the human spliceosome. *Genome Res* **12**: 1231–1245
- Schwelnus W, Richert K, Opitz F, Gross T, Habara Y, Tani T, Kaufner NF (2001) Fission yeast Prp4p kinase regulates pre-mRNA splicing by phosphorylating a non-SR-splicing factor. *EMBO Rep* **2**: 35–41
- Staley JP, Guthrie C (1998) Mechanical devices of the spliceosome: motors, clocks, springs, and things. *Cell* **92**: 315–326
- Staley JP, Guthrie C (1999) An RNA switch at the 5' splice site requires ATP and the DEAD box protein Prp28p. *Mol Cell* **3**: 55–64
- Tarn WY, Hsu CH, Huang KT, Chen HR, Kao HY, Lee KR, Cheng SC (1994) Functional association of essential splicing factor(s) with PRP19 in a protein complex. *EMBO J* **13**: 2421–2431
- Tarn WY, Lee KR, Cheng SC (1993a) Yeast precursor mRNA processing protein PRP19 associates with the spliceosome concomitant with or just after dissociation of U4 small nuclear RNA. *Proc Natl Acad Sci USA* **90**: 10821–10825
- Tarn WY, Lee KR, Cheng SC (1993b) The yeast PRP19 protein is not tightly associated with small nuclear RNAs, but appears to associate with the spliceosome after binding of U2 to the pre-mRNA and prior to formation of the functional spliceosome. *Mol Cell Biol* **13**: 1883–1891
- Tsai WY, Chow YT, Chen HR, Huang KT, Hong RI, Jan SP, Kuo NY, Tsao TY, Chen CH, Cheng SC (1999) Cef1p is a component of the Prp19p-associated complex and essential for pre-mRNA splicing. *J Biol Chem* **274**: 9455–9462
- Will CL, Urlaub H, Achsel T, Gentzel M, Wilm M, Lührmann R (2002) Characterization of novel SF3b and 17S U2 snRNP proteins, including a human Prp5p homologue and an SF3b DEAD-box protein. *EMBO J* **21**: 4978–4988
- Zhang Y, Lindblom T, Chang A, Sudol M, Sluder AE, Golemis EA (2000) Evidence that dim1 associates with proteins involved in pre-mRNA splicing, and delineation of residues essential for dim1 interactions with hnRNP F and Npw38/PQBP-1. *Gene* **257**: 33–43
- Zhou Z, Licklider LJ, Gygi SP, Reed R (2002) Comprehensive proteomic analysis of the human spliceosome. *Nature* **419**: 182–185