Human homologs of yeast Prp16 and Prp17 reveal conservation of the mechanism for catalytic step II of pre-mRNA splicing

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Pre-mRNA splicing takes place in two catalytic steps. The second step is poorly understood, especially in mammals. In yeast, the splicing factors, Prps 16, 17, 18 and Slu7 function exclusively in step II. Here we report the isolation of cDNAs encoding human Prps 16 and 17 which are 41 and 36% identical to their yeast counterparts. The Prp16 gene is essential in yeast, and we show that a chimeric yeast–human Prp16 protein rescues a yeast Prp16 knockout strain. Immunodepletion of hPrp16 from splicing extracts specifically blocks step II, and the activity can be fully restored with recombinant hPrp16. Moreover, both hPrps 16 and 17 associate with the spliceosome late in the splicing pathway. Mutations at the 3′ splice site that specifically block step II do not affect the association of hPrps 16 and 17 with the spliceosome, indicating that these factors may function at a stage of step II prior to recognition of the 3′ splice site. Recently, the human homologs of Prp18 and Slu7 were identified. The observation that humans contain homologs of all four known step II proteins in yeast indicates that the mechanism for catalytic step II is highly conserved.
Keywords: catalytic step II/hPrp16/hPrp17/spliceosome/splicing factor

Introduction

Pre-mRNA splicing *in vitro* requires the formation of series of spliceosomal complexes which assemble in the order E, A, B and C. The two catalytic steps of splicing take place in the C complex. The spliceosomal complexes and the functions of the five spliceosomal snRNAs (U1, U2, U4, U5 and U6) are conserved from *Saccharomyces cerevisiae* to humans (for reviews, see Rymond and Rosbash, 1992; Moore *et al.*, 1993; Madhani and Guthrie, 1994). More recently, a number of spliceosomal proteins have also been shown to be conserved from yeast to humans. In yeast, genetic screens have identified ~50 protein-coding genes involved in splicing (for reviews, see Krämer, 1996; Hodges *et al.*, 1997; Will and Lührmann, 1997). Likewise, ~50 proteins have been identified on two-dimensional gels of purified human spliceosomes. So far, about half of these have been identified as components of U1, U2, U4/U6 and U5 snRNPs (for reviews, see Krämer, 1996; Reed and Palandjian, 1997; Will and Lührmann, 1997). Most of the human spliceosomal proteins that have counterparts in yeast first associate with

pre-mRNA in the E, A or B spliceosomal complexes which assemble prior to catalytic step I (Reed and Palandjian, 1997). The conserved proteins in these complexes include three U1 snRNP components, six U2 snRNP proteins, four U5 snRNP proteins and two U4/U6 snRNP proteins (Lauber *et al.*, 1997; Tang *et al.*, 1997; for reviews, see Krämer, 1996; Will and Lührmann, 1997). Among the non-snRNP spliceosomal proteins that have yeast homologs are the E complex components SF1/mBBP and U2AF^{65} (Zamore *et al.*, 1992; Abovich *et al.*, 1994; Arning *et al.*, 1996; Abovich and Rosbash, 1997).

In contrast to the progress in identifying proteins required for early stages in spliceosome assembly, comparatively little is known about the proteins involved in catalytic step II. A general picture that has emerged from studies in both humans and yeast is that the spliceosome undergoes substantial remodeling prior to step II (for review, see Umen and Guthrie, 1995c). For example, in humans, 14 new spliceosomal proteins associate with the C complex prior to step II (Gozani *et al.*, 1994; for review, see Umen and Guthrie, 1995c). Moreover, U5 snRNP, which interacts at the 5′ splice site prior to step I (Newman and Norman, 1992; Wyatt *et al.*, 1992; Cortes *et al.*, 1993; Sontheimer and Steitz, 1993; Newman *et al.*, 1995; Teigelkamp *et al.*, 1995b; Chiara *et al.*, 1996; Reyes *et al.*, 1996), binds to the 3′ splice site prior to step II, where it is thought to play a key role in 3′ splice site recognition for step II (Umen and Guthrie, 1995a; Chiara *et al.*, 1997). Finally, both the U5 snRNP protein, U5^{220} (Prp8 in yeast) and U5 snRNA contact exon sequences adjacent to the 5′ splice site prior to step I and then, prior to step II, also contact intron and exon sequences next to the 3′ splice site (Sontheimer and Steitz, 1993; Newman *et al.*, 1995; Teigelkamp *et al.*, 1995a; Umen and Guthrie, 1995a,b, 1996; O'Keefe *et al.*, 1996; Chiara *et al.*, 1997; for reviews, see Umen and Guthrie, 1995c; Newman, 1997). These interactions are thought to align the exons for ligation during step II.

In order to understand how the spliceosome is remodeled for step II and how the active site is formed for this step in splicing, it is necessary to identify and characterize individual proteins involved in these processes. Most of the progress towards this goal has been made in yeast, where four proteins that function exclusively in catalytic step II have been identified (for review, see Umen and Guthrie, 1995c). These are Prp16 (Couto *et al.*, 1987; Burgess *et al.*, 1990; Schwer and Guthrie, 1991, 1992a,b), Prp17 (Vijayraghavan *et al.*, 1989; Jones *et al.*, 1995), Prp18 (Vijayraghavan and Abelson, 1990; Horowitz and Abelson, 1993a,b) and Slu7 (Frank and Guthrie, 1992; Ansari and Schwer, 1995; Jones *et al.*, 1995). Prp16 and Slu7 are essential for viability in yeast, whereas Prps 17 and 18 are not (for review, see Umen and Guthrie, 1995c). As an approach for identifying mammalian proteins func-
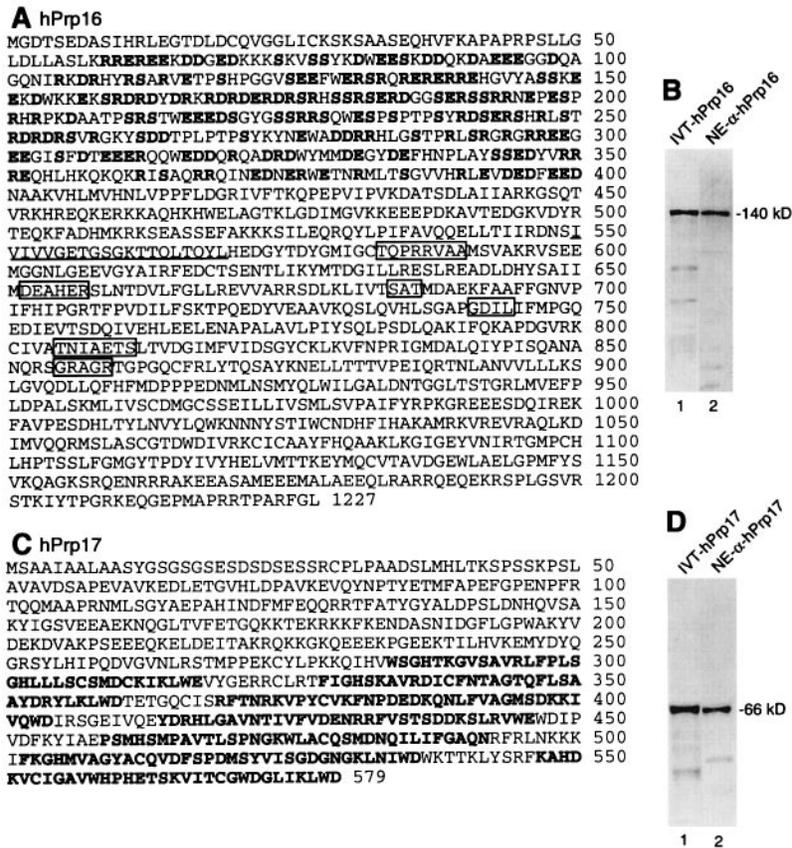
A hPrp16

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MGDTSEDASIHRLEGTDLDCQVGGLICKSKSAASEQHVFKAPAPRPSLLG  50
LDLLASLKRRREREEKDDGEDKKKSKVSSYKDWEESKDDQKDAEEEGGDQA 100
GQNIRKDRHYRSARVETPSHPGGVSEEFWERSRQRERERREHGVYASSKE  150
EKDWKKEKSRDRDYDRKRDRDERDRSRHSSRSERDGGSERSSRRNEPESP  200
RHRPKDAATPSRSTWEEEDSGYGSSRRSQWESPSPTPSYRDSERSHRLST  250
RDRDRSVRGKYSDDTPLPTPSYKYNEWADDRRHLGSTPRLSRGRGRREEG  300
EEGISFDTEEERQQWEDDQRQADRDWYMMDEGYDEFHNPLAYSSEDYVRR  350
REQHLHKQKQKRISAQRRQINEDNERWETNRMLTSGVVHRLEVDEDFEED  400
NAAKVHLMVHNLVPPFLDGRIVFTKQPPEVIPVKDATSDLAIIARKGSQT  450
VRKHREQKERKKAQHKHWELAGTKLGDIMGVKKEEEPDKAVTEDGKVDYR  500
TEQKFADHMKRKSEASSEFAKKKSILEQRQYLPIFAVQQELLTIIRDNSI  550
VIVVGETGSGKTTQLTQYLHEDGYTDYGMIGCTQPRRVAAMSVAKRVSEE  600
MGGNLGEEVGYAIRFEDCTSENTLIKYMTDGILLRESLREADLDHYSAII  650
MDEAHERSLNTDVLFGLLREVVARRSDLKLIVTSATMDAEKFAAFFGNVP  700
IFHIPGRTFPVDILFSKTPQEDYVEAAVKQSLQVHLSGAPGDILIFMPGQ  750
EDIEVTSDQIVEHLEELENAPALAVLPIYSQLPSDLQAKIFQKAPDGVRK  800
CIVATNIAETSLTVDGIMFVIDSGYCKLKVFNPRIGMDALQIYPISQANA  850
NQRSGRAGRTGPGQCFRLYTQSAYKNELLTTTVPEIQRTNLANVVLLLKS  900
LGVQDLLQFHFMDPPPEDNMLNSMYQLWILGALDNTGGLTSTGRLMVEFP  950
LDPALSKMLIVSCDMGCSSEILLIVSMLSVPAIFYRPKGREEESDQIREK 1000
FAVPESDHLTYLNVYLQWKNNNYSTIWCNDHFIHAKAMRKVREVRAQLKD 1050
IMVQQRMSLASCGTDWDIVRKCICAAYFHQAAKLKGIGEYVNIRTGMPCH 1100
LHPTSSLFGMGYTPDYIVYHELVMTTKEYMQCVTAVDGEWLAELGPMFYS 1150
VKQAGKSRQENRRRAKEEASAMEEEMALAEEQLRARRQEQEKRSPLGSVR 1200
STKIYTPGRKEQGEPMAPRRTPARFGL 1227
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C hPrp17

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MSAAIAALAASYGSGSGSESDSDSESSRCPLPAADSLMHLTKSPSSKPSL  50
AVAVDSAPEVAVKEDLETGVHLDPAVKEVQYNPTYETMFAPEFGPENPFR 100
TQQMAAPRNMLSGYAEPAHINDFMFEQQRRTFATYGYALDPSLDNHQVSA 150
KYIGSVEEAEKNQGLTVFETGQKKTEKRKKFKENDASNIDGFLGPWAKYV 200
DEKDVAKPSEEEQKELDEITAKRQKKGKQEEEKPGEEKTILHVKEMYDYQ 250
GRSYLHIPQDVGVNLRSTMPPEKCYLPKKQIHVWSGHTKGVSAVRLFPLS 300
GHLLLSCSMDCKIKLWEVYGERRCLRTFIGHSKAVRDICFNTAGTQFLSA 350
AYDRYLKLWDTETGQCISRFTNRKVPYCVKFNPDEDKQNLFVAGMSDKKI 400
VQWDIRSGEIVQEYDRHLGAVNTIVFVDENRRFVSTSDDKSLRVWEWDIP 450
VDFKYIAEPSMHSMPAVTLSPNGKWLACQSMDNQILIFGAQNRFRLNKKK 500
IFKGHMVAGYACQVDFSPDMSYVISGDGNGKLNIWDWKTTKLYSRFKAHD 550
KVCIGAVWHPHETSKVITCGWDGLIKLWD 579
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B

IVT-hPrp16   NE-α-hPrp16

-140 kD

1   2

D

IVT-hPrp17   NE-α-hPrp17

-66 kD

1   2

Fig. 1. Characterization of hPrp16 and hPrp17 cDNAs. (**A**) Predicted amino acid sequence of hPrp16. The 3703 bp cDNA sequence of the hPrp16 gene has been deposited in DDBJ/EMBL/GenBank under accession No. AF038391. The N-terminal REDS residues are in bold, conserved motifs identical to the DEAH box protein family are boxed, and the NTP-binding fold is underlined. (**B**) Co-migration of *in vitro* translated (IVT) hPrp16 and hPrp16 detected in nuclear extract by hPrp16 antibody. Two µl of nuclear extract was mixed with 1 µl of [^{35}S]methionine-labeled IVT-hPrp16, separated by SDS–PAGE and then proteins were immobilized on a membrane. The membrane was probed with hPrp16 antibodies and bands were detected using a horseradish perioxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL) (lane 2) followed by detection of the ^{35}S-labeled protein by autoradiography (lane 1). Note that the IVT-hPrp16 is below the level of detection of the antibody (data not shown). (**C**) Predicted amino acid sequence of hPrp17. The 1979 bp cDNA sequence of the hPrp17 gene has been deposited in DDBJ/EMBL/GenBank under accession No.AF038392. Seven conserved WD repeats (GH-X$_{27-35}$-WD) identified by the PROSITE Program are in bold. (**D**) Co-migration of IVT-hPrp17 and hPrp17 detected in nuclear extract by hPrp17 antibodies. Samples were analyzed as in (B). The IVT-hPrp17 is below the level of detection of the antibody (data not shown).

tionally important for step II, we sought to identify human homologs of these yeast step II proteins. Recently, the human homologs of Prp18 and Slu7 were identified and shown to function in step II in humans (Horowitz and Krainer, 1997; K.Chua and R.Reed, in preparation). We report here the identification of the human homologs of Prps 16 and 17. Our data indicate that these proteins are both structurally and functionally conserved with their yeast homologs.

Results

Characterization of hPrp16 and hPrp17 cDNAs

The probable human homologs of yeast Prp16 (Burgess *et al.*, 1990) and Prp17 (M.Company and J.Abelson, personal communication) were identified by a BLAST search of the GenBank database, and full-length cDNA clones were isolated using PCR strategies (see Materials and methods). The human clones are designated hPrp16 and hPrp17. The open reading frame (ORF) of the hPrp16 cDNA predicts a protein of 1227 amino acids with an expected mol. wt of 140.5 kDa and an isoelectric point of 6.35 (Figure 1A). The

hPrp17 cDNA encodes a 579 amino acid protein with a calculated mol. wt of 66 kDa and an isoelectric point of 6.89 (Figure 1C). To characterize the proteins encoded by these cDNAs, we raised rabbit polyclonal antibodies to GST fusions of each protein (see Materials and methods). One main band of 140 kDa is detected by hPrp16 antibodies on a Western blot of nuclear extract (Figure 1B, lane 2). This band co-migrates with the *in vitro* translated (IVT) protein produced from the hPrp16 cDNA (Figure 1B, lane 1). Similarly, one main band of ~66 kDa, which co-migrates with IVT-hPrp17, is detected by hPrp17 antibodies in nuclear extract (Figure 1D). These observations, together with the fact that there is a consensus Kozak sequence (Kozak, 1986) upstream of the designated initiator methionine in the hPrp16 and 17 cDNAs, indicate that these cDNAs encode full-length proteins.

As shown in Figure 1A, hPrp16 contains an NTP-binding motif (underlined) and six RNA helicase motifs (boxed) that are present in yeast Prp16 and all other members of the DEAH box family of putative RNA helicases (for review, see Wassarman and Steitz, 1991; Schmid and Linder, 1992). These motifs are strikingly

A hPrp16/Prp16

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1     MGDTSEDASIHRLEGTDLDCQVGGLICKSKSAASEQHVFKAPAPRPSLIGLDLLASIKRREREEKDDGEDKKKSKVSSYKDWEESKDDQK  hPrp16
1     MGHSGREERI------------------------KDIPKELTSKE--LTPGLLLTLQKLAQKPNTNLEQF----IASCK----------  Prp16

91    DAEEEGGDQAGQNIRKDRHYRSARVETPSHPGGVSBEFWERSRQRERERREHGVYASSKEEKDWKKEKSRDRDYDRKRIRIERDRSRHSS  hPrp16
50    --------------------ALTKLSSNNPIIFNELLELLKNKSEE--------DSTGPKKIAPSINKRKKFKIQLDLLDNE------  Prp16

181   RSERDGGSERSSRRNEPESPRHRPKDAATPSRSTWEEEDSGYGSSRRSQWESESPIPS-YRDSEFSHRISTRDRDRSVFGKVSDDTPLIT  hPrp16
104   --------------DELDSPVQK-------------------------KFAPIRTLFK---RIDKIKAKQ----LR-QVSPTVKDIS  Prp16

270   ESYKYNEWADDRRHLGSTPRLSRGRGFREEGEEGISFDTEEERQQWEDDQRQADRDWYMMDEGYDEF-HNPIAYSSEDYVRRREQHLHKQ  hPrp16
144   EN------SEQQTQNCHAETKDYEPTRSEVVEE-------------------DREWYDNDDDYGNLVPBPIISELPEE------------  Prp16

359   KQKRISAQRFQINEDNERWETNRMLTSGVVHFLEVDEDEEEDNAAKVHLMVHNLVPPFLDGRIVFTKQPEPVIEVKDATSDLAIIARKGS  hPrp16
196   --AKLLPVIRNIDNDDALRNTVQLYPIPLKQRMEWIPPELSKFALENKVPTSIIIGSISETSSQVSALSM-VNEFRNPDSEFSANAKRGS  Prp16

449   QTVRKHREQKERKKAQHKHWELAGTKLGDIMGVKKEEEPDKAVTEDGKVDYRTEQKFADHMKRKSEASSEFAKKKSILEQ-RQYLEIEAV  hPrp16
283   KLVALRRINMPHIQQSRDNTTVLNTAMGEVIGLENNN---------KAKDKSNQKICDD-------TALFTPSKDDIKHTKEQLEVRC  Prp16

538   QQELLTIIRDNSIVIVVGETGSGKTTQLTQYIHEDGYT-LYGM-IGCTQPRRVAAMSVAKRVSBEMGGNLGEEVGYAIRFEDCT-SENIL  hPrp16
356   RSQLLSLIRENQVVVIIGETGSGKTTQLAQYIYEEGYANDRGKSIVVTQPRRVAAISVAKRVAMEMQVPLGKEVGYSIRFEDVIDSECTK  Prp16

625   IKYMTDGILLRESIREADLDHYSAIIMDEAHERSLNTDVIFGLLREVVARRSDLKLIVTSATMDAEKFAAFFGNVFIGH-IPGRTFPVDIL  hPrp16
446   LRFVTDGILLRETILDDTLDKYSCVIIDEAHERSLNTDIILGFFKILLARRFRDLKLIITSATMNAKKFSAFFGAEQFIIPGRTFPVQTI  Prp16

715   FSKTEQEDYVEAAVKCSLQVHISG--APGDILIFMFGQEDIEVISEQIVEHL-----------EELENAPALAVLPIYSQLFSDLQAKIFQ  hPrp16
536   YTSNEVCDYVEAAVSQAVKIHLANDCSSGDILIFMTGQEDIETIFLTLQEKFLQVYSKKFGTANFEEINDIEILPIYSALEADLQFKIFQ  Prp16

793   KAPDGVRKCIVATNIAETSLTVDGIMFVIDSGYCKLKVFNERIGMDALQIYPISQANANQRSGRAGRTGPGQCFRLYTQSAYKNELLTTI  hPrp16
626   DLHGTKRKIIIATNIAETSLTIKGIRYVIDCGYSKLKVYNEKIGLDSIVITPISKANADQRSGRAGRTAPGTRYRLYTEDTFKEDMYLQT  Prp16

883   VPEIQRTNLANVVLLLKSLGV-QDLLQEHFMEPPPEDNMLNSMYQLWIILGALDNTGGLTSTCRLMVEFPLDEALSKMLIVSCDMGCSSEI  hPrp16
716   IPEIQRTNLSNTLLLLKSIDVTDBLSKFPEILKPPLQTFLSSLYBLMFIGAIDTSCQLTPLCLQMAKFPLQESLSKILLIAVRNGCSDEM  Prp16

972   LLIVSMLSVEAIFYRPKGREEESEQIFEBKFAVPESDHLTYLNVYLQWKNNNYSTIWCNDHEIHAKAMRKVREVRAQIKDIMVQCRMSLAS  hPrp16
806   LTIVSMLSVEQVFYRPKERQKEAEIARNKEFIAKSDHLTLLNVFBQWRANNFSSHWCNKHEVQYKSLVRARDIRDQLLTILKSCKIPVIS  Prp16

1062  CGTDWDIVRKCICAAYFHQAAKLKGIGEYVNIRTGMPCHLHPTSSIFGMGYTEDYIVYHEIVMTTKEYMQCVTAVLGBWLAELCPMFYSV  hPrp16
896   SGKDWDIIKKCICSGFAHQAAKITGLRNYVHLKTGVSVQLHPTSALHGLGDLEFYVVYHEILMTSKEYICCVTSVLPFWLMEYCGLLYDI  Prp16

1152  KQAGKSRQENRRRAKEEASAMEEEMALAEEQLRARRQEQEKRSPLGSVRSIKIYTPGRKEC-----------GEPVAERRTPARFGL   hPrp16
986   KRIKNDQEATTTGLFGEHYEHTLDKVEDDIDINIRRCKDMRDSVIQELKMTDNSNKEDKKQKTKKQNILNGKENSVKEFKRRKFE-F   Prp16
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B hPrp17/Prp17

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1     MSAAIAALAASYGSGSGSESDSDSESSRCPLPAADSLMELTKSPSSKPSLAVAVDSAPEVAVKEDLETGVHLDEAVKEVQYNPTYETMFA  hPrp17
1     MG-----LVDGYDTSSDSDLNFDEGKS----------VHEKKN-------------------GNLHEDTSYEESSNNIH----------  Prp17

91    PEFGPENPFRTQQMAAPRNMLSGYAEPAHINDFMFEQQRRTEATYGYALDPSLDNHQVSAKYIGSVEEAEKNQGLTVFETGQKKTEKRKK  hPrp17
46    -----------------------------------KRKSHET-----------------------------------KSELKRRRK  Prp17

181   FKENEASNIDGFLGPWAKYVDEKDVAKPSBEEQKELDETTAKRQKKGKQEBEKPG-EBKTILHVKEMYDYQGRSYLHIEQDVGVNLFSTM  hPrp17
62    TRKGD--------GPMGSWSSSDEETSQASFTQKEDQDIFVHALAEDNLDSFQIEVEBVSHFYGKSEKDYQGRGYLYPFNDVDVDLREER  Prp17

270   PPEKCYLPKKQIHVWSGHTKGVSAVRLFFLSGHILLSCSMDCKIKLMEVYGERRCLRTFIGHSKAVRDICFNTAGTQFLSAAYDRYLKLW  hPrp17
144   ISFRCYLPKKVIRNYPGHPEGTTALKFLEKTGHIILSGGNDHTIKIADFYHDYECLRDEQGINKPIKALRFTEDCQSFLSSSFDRSVKIW  Prp17

360   DTETGQCISRFTNRKVEYCVKFNEDEDKQNLFVAGMSDKKIVQWDIFSGE---IVGEYDRHIGAVNTIVFVDENRRFVSTSEDKSLRVWE  hPrp17
234   DTETGKVKTRLHLNSTEADVESRFTNPHE--EIVGLSNSKILHYDDRVSENQGLVGTYDHHISSILALKYFPDGSKFISSSEDKTVRIWE  Prp17

447   WDIFVDFKYIAEPSMHSMEAVTLSENGKWLACQSMDNQILIEGAQNRFRLNKKKIFKGHMVAGYACQVDFSHDMSYVISGIGNGKLNIWD  hPrp17
322   NQINVPIKQISDTAQHSMEFLNVHPSQNYFCAQSMDNRTYSESLKPKYKRHEKKKIFKGHSSAGYGISLTFSGDGRYICSGDSKSRLFTWD  Prp17

537   WKITKLYSRFKAHDKVCIGAV-WHPHETSKVITCGWDGLIKLWD   hPrp17
412   WNTSRLLNNIKIPGNKPITQVDWHPQETSKVICSCAAGKIYVCD   Prp17
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C WD repeats

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REPEAT 1
            290         300           310
hPrp17   WSGHTKGVSAVRLFPLSGHLLLSCSMDCKIKLWE
         .:GH..G.:A:::.P.:GHL:LS.:.D..IK:W:
Prp17    YPGHPEGTTALKFLPKTGHLILSGGNDHTIKIWD
            160         170           180
REPEAT 2
            330         340           350
hPrp17   PIGHSKAVRDICFNTAGTQFLSAAYDRYLKLWD
         F.GH:K:::.:F.......FLS:::DR.:K:WD
Prp17    PQGHNKPIKALRFTEDCQSFLSSSFDRSVKIWD
            210         220           230
REPEAT 3
            370         380         390          400
hPrp17   RFTNRKVPYCVKFNPDEDKQNLFVAGMSDKKIVQWD
         R:.:.:.P..V..P.....:.F::G:S:.KI:::D
Prp17    RLHLNSTPADVESRP--TNPHEFIVGLSNSKILHYD
            250         260         270
REPEAT 4
            420         430           440
hPrp17   YDRHLGAVNTIVFVDENRRFVSTSDDKSLRVWE
         YD:HL:::.::.:..:..:F:S:S:DK::R:WE
Prp17    YDHHLSSILALKYFPDGSKFISSSEDKTVRIWE
            290         300           310          320
REPEAT 5
            470         480           490
hPrp17   PSMHSMPAVTLSPNGKWLACQSMDNQILIFGAQN
         .:HSMP.:.:P.:..:QSMDN:I..F:.:
Prp17    TAQHSMPFLNVHPSQNYFCAQSMDNRIYSPSLKP
            340         350           360
REPEAT 6
            510         520           530
hPrp17   FKGHMVAGYACQVDFSPDMSYVISGDGNGKLNIWD
         FKGH..AGY:..:FS.D..Y:.SGD:::L..WD
Prp17    FKGHSSAGYGISLTFSGDGRYICSGDSKSRLFTWD
            380         390         400          410
REPEAT 7
            550         560           570
hPrp17   KAHDKVCIGAV-WHPHETSKVITCGWDGLIKLWD
         K.:..I..V.WHP:ETSKVI..G.G.I..:D
Prp17    KIPGNKPITQVDWHPQETSKVICSGAAGKIYVCD
            430         440           450
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Fig. 2. (**A** and **B**) Amino acid alignment of yeast and human Prp16 and Prp17 homologs. Alignments were done by the Clustal method (DNASTAR Inc). Identical residues are shown in white on black. An alignment of hPrp16 with yeast Prps 2, 22 and 43 was done by the same method (data not shown). The overall identity to hPrp16 for these proteins is 37, 33 and 34%, respectively, and the C-terminal identity is 47, 48 and 36%, respectively. (**C**) Alignment of the seven WD repeats (GH-X$_{27-35}$-WD) conserved between hPrp17 and Prp17 using the Lipman–Pearson method (DNASTAR Inc). Identical residues are indicated, and similar residues are shown by dots. GH and WD residues are underlined.

conserved in hPrp16/Prp16 and in the three DEAH box splicing factors, Prps 2, 22 and 43 (Burgess *et al.*, 1990; Chen and Lin, 1990; Company *et al.*, 1991; Ono *et al.*, 1994; Arenas and Abelson, 1997). All of the motifs indicated in the boxes are identical among these different proteins (Figure 1A and data not shown). The overall identity between hPrp16 and Prp16 is 41%, and hPrp16 is more related to yeast Prp16 than to the other members of the DEAH box family (Figure 2A and legend). The C-terminal 400 amino acids of hPrp16 are also highly conserved in Prp16 (identity 51%) and the other members of the DEAH box splicing factor family, implying a generally important function for this domain (Figure 2A and legend). hPrp16 diverges from its yeast counterpart in the N-terminus, where hPrp16 contains a region rich in the amino acids arginine, glutamate, aspartate and serine. These residues (REDS) account for 54% of the amino acids extending from 59 to 400 (shown in bold, Figure 1A). The N-terminus of hPrp16 is also enriched in TP and SP dipeptides which, like RS dipeptides, are potential phosphorylation sites. A likely *Caenorhabditis elegans* homolog of Prp16, which is 55% identical to hPrp16, is also present in the database (DDBJ/EMBL/GenBank accession No. P34498).

The closest relative to hPrp17 in the database is yeast Prp17, with the identity being 36% (Figure 2B). Yeast Prp17 is identical to Cdc40, a protein that plays a role in DNA replication (Vaisman *et al.*, 1995). How the functions of Prp17 and Cdc40 are related is not known. Homology between hPrp17 and Prp17 is the strongest in the C-terminal two-thirds of the proteins (41% identity, 67% similarity), which contains seven potential WD repeats (Seshadri *et al.*, 1996) (shown in bold in Figure 1C and aligned in Figure 2C). WD repeats are found in a wide variety of proteins and are ~40 amino acids long, beginning with a conserved GH and terminating with a conserved WD (Neer *et al.*, 1994). These repeats function in protein–protein interactions (see Discussion).

Complementation of a yeast Prp16 knockout strain

Four DEAH box splicing factors are highly related to one another (Prps 2, 16, 22 and 43) (Burgess *et al.*, 1990; Chen and Lin, 1990; Company *et al.*, 1991; Ono *et al.*, 1994; Arenas and Abelson, 1997; Gee *et al.*, 1997). To determine whether the hPrp16 cDNA that we have isolated is the functional homolog of yeast Prp16, we attempted to complement a yeast Prp16 knockout strain (kindly provided by C.Guthrie) using plasmid shuffling (Sikorski and Boeke, 1987) (see Materials and methods). The constructs that were used for the complementation are shown in Figure 3A. As expected, the wild-type yeast strain grows on plates containing selective media [5-fluoro-orotic acid (5-FOA)] as does the yeast Prp16 knockout strain transformed with yeast Prp16 (Figure 3B). In contrast, the vector alone, full-length hPrp16 and C- or N-terminal deletions of hPrp16 do not rescue the yeast Prp16 knockout (Figure 3A and B). Significantly, however, a chimeric construct containing the N-terminus of yeast Prp16 (amino acids 1–298) fused to the remainder of hPrp16 (251–1227) does rescue the knockout (Figure 3A and B). A chimera lacking the C-terminus (ChimeraΔC) or a construct containing the yeast N-terminus alone
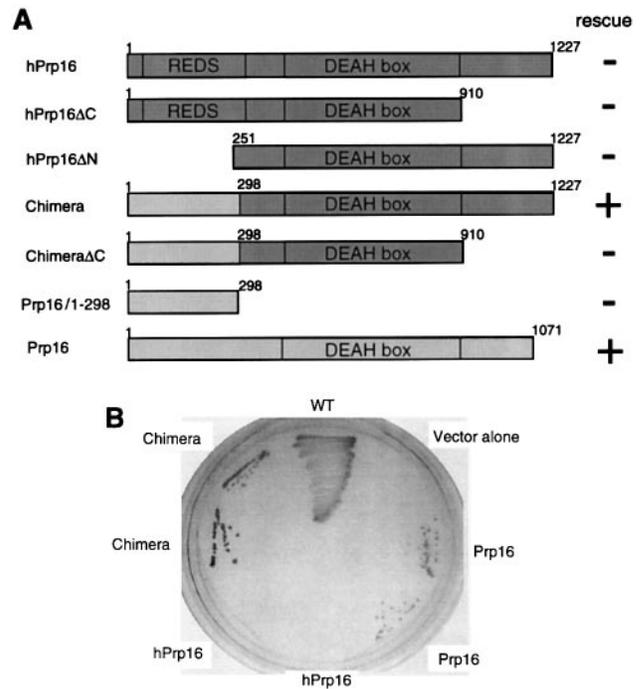
Fig. 3. Complementation of yeast Prp16 knockout strain with a human–yeast chimeric gene. (**A**) Schematic representation of Prp16 and hPrp16 derivatives used for transformation into the yeast Prp16 knockout strain. The yeast and human Prp16 are denoted by light or dark shading, respectively. The REDS and DEAH domains are indicated. The numbers indicate the amino acid positions in each construct. The constructs that rescue (+) or do not rescue (–) the Prp16 knockout are indicated. (**B**) Growth comparison on a 5-FOA plate of yeast cells transformed with Prp16, vector alone, hPrp16 and the chimera.

(Prp16/1–298) were non-functional in the assay (Figure 3A).

The observation that the chimeric construct of hPrp16, which contains 80% of the hPrp16 gene, complements the yeast Prp16 knockout indicates that our hPrp16 cDNA is the likely functional homolog of yeast Prp16. Additional studies supporting this conclusion are presented below (Figures 4–6). Our data also indicate that the N-terminus of yeast Prp16 contains a domain essential for function in yeast, and that the N-terminus of hPrp16, which contains the REDS-rich region, cannot provide this function. In addition, amino acids 911–1227 of hPrp16, which are highly conserved in DEAH box splicing factors (Figure 2A and data not shown), are essential for complementation, indicating that this region contains an important functional domain.

hPrp16 and hPrp17 join the spliceosome late in the splicing pathway

To obtain further evidence that hPrps 16 and 17 are splicing factors, we asked whether these proteins are present in purified human spliceosomes. A splicing time course was carried out, and spliceosomal complexes from each time point were isolated (Figure 4). At the 10 min time point, only unspliced pre-mRNA is detected (Figure 4A). The splicing intermediates and products are first detected at 25 min and increase in abundance at 40 and 60 min (Figure 4A). Western analysis of spliceosomal complexes isolated at each of these times shows that the
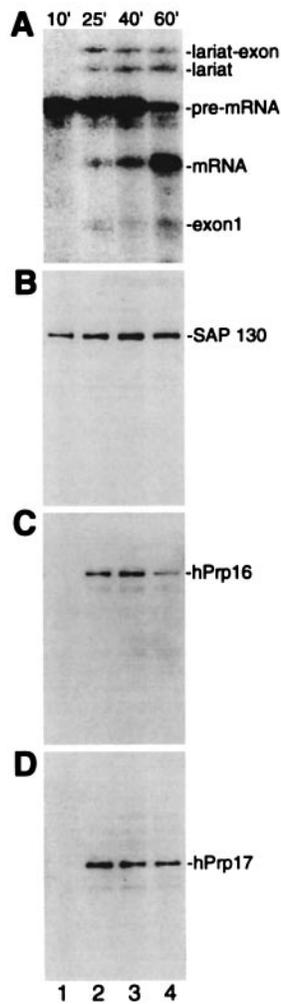
Fig. 4. hPrp16 and hPrp17 associate with the spliceosome late in the splicing pathway. (**A**) AdML pre-mRNA (1.92 μg) containing biotin was incubated under splicing conditions (2.4 ml reaction) for the times indicated. Complexes were fractionated by gel filtration. Total RNA from the complexes was prepared and separated on a 15% denaturing polyacrylamide gel. The bands corresponding to pre-mRNA, intermediates and products are indicated. (**B–D**) Gel filtration-isolated complexes in (A) were affinity-purified by binding to avidin–agarose. Western analysis of total protein isolated from the purified complexes was then carried out. The same Western blot was probed with a rabbit polyclonal antibody to the U2 snRNP protein SAP 130 (**B**), to hPrp16 (**C**) or to hPrp17 (**D**). Note that the 10 min time point is underloaded. On long exposures, no hPrp16 or hPrp17 was detected at this time point (data not shown).

U2 snRNP protein, SAP 130, is present at all of the time points (Figure 4B). This result is consistent with previous work showing that U2 snRNP first binds stably to the spliceosome in the A complex and remains bound throughout spliceosome assembly (for review, see Krämer, 1996). In contrast, hPrp16 and hPrp17 join the spliceosome late in the splicing pathway and concomitant with the transesterification reactions (Figure 4C and D). Interestingly, less hPrp16 is detected at 60 min than at 25 and 40 min, whereas hPrp17 is present at about the same levels at all three time points. We conclude that both hPrp16 and 17 associate with the spliceosome concomitant with the catalytic steps. However, hPrp16 appears to be destabilized from the spliceosome late in the splicing pathway whereas hPrp17 remains tightly bound. It is difficult to determine
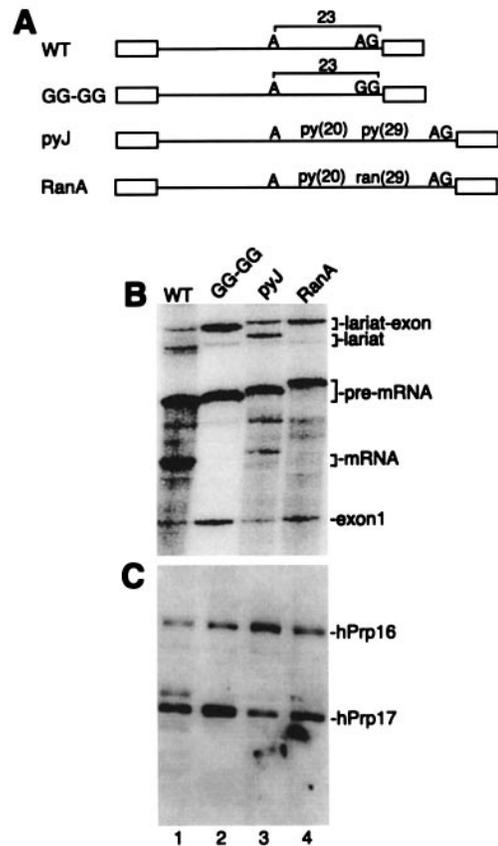
Fig. 5. Association of hPrp16 or hPrp17 with the spliceosome precedes recognition of the 3′ splice site during step II. (**A**) Schematics of AdML and mutant derivatives. The BPS–AG distance for wild-type (WT) and GG-GG is 23 nucleotides. In pyJ and RanA, py20 refers to the length of the pyrimidine tract which is isogenic with WT. Py(29) is a 29 nucleotide insertion of pyrimidines. Ran(29) is an insertion of 29 nucleotides of random sequence (Chiara *et al.*, 1997). (**B**) The indicated ^{32}P-labeled pre-mRNAs were incubated under splicing conditions for 40 min, and then total RNA was fractionated on a 15% denaturing polyacrylamide gel. Splicing intermediates and products are indicated. (**C**) Western assay of the purified complexes assembled on the pre-mRNA substrates indicated. The blot was probed with hPrp16 and hPrp17 antibodies, and the positions of hPrps 16 and 17 are indicated.

the exact point at which hPrp16 is destabilized because the complexes at any given time point are heterogeneous. However, if the release occurs at the same stage as in yeast, then it would occur concomitant with step II (Schwer and Guthrie, 1991).

A similar time course experiment was performed using different pre-mRNA substrates, α-tropomyosin and Fushi tarazu (ftz). Again, both hPrps 16 and 17 associated with these spliceosomes late in the splicing pathway, concomitant with the catalytic steps (data not shown). The observation that both hPrp16 and hPrp17 join the spliceosome late in the splicing pathway suggests that these proteins, like their yeast counterparts, function in catalytic step II of the splicing reaction.

Association of Prps 16 and 17 with the spliceosome precedes functional recognition of the 3′ splice site for step II

Previous studies showed that the presence of pyrimidines next to the AG at the 3′ splice site plays a critical role in
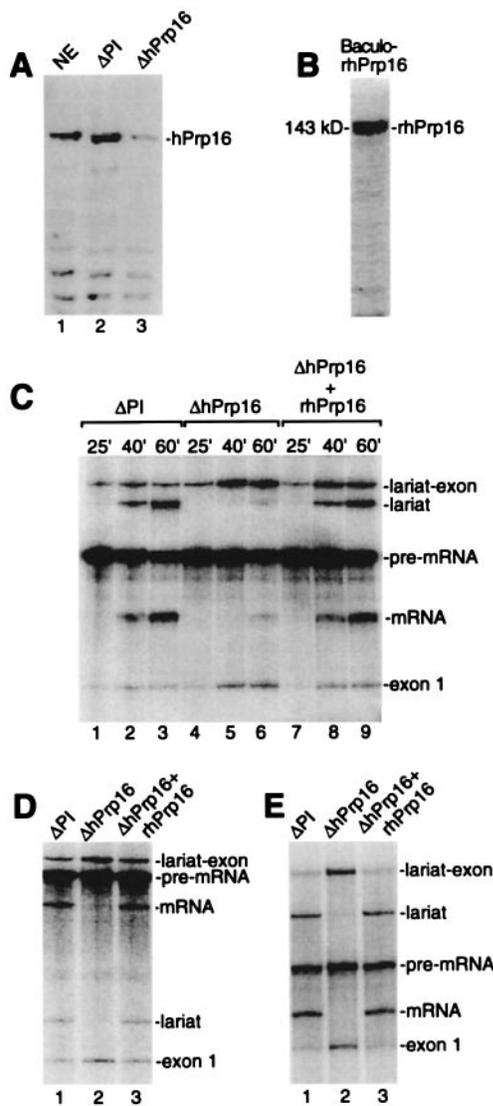
Fig. 6. hPrp16 is required for catalytic step II of the splicing reaction. (**A**) A Western blot probed with hPrp16 antibodies showing normal nuclear extract (lane 1) and the same extract depleted with pre-immune serum (ΔPI) (lane 2) or with hPrp16 antibodies (ΔhPrp16) (lane 3). The position of hPrp16 is indicated. (**B**) Coomassie blue staining of the recombinant hPrp16 protein from baculovirus (2 μg of the protein was loaded) which was used for complementation. rhPrp16 migrates slightly larger than hPrp16 because of the His tag. (**C**) Time course of pre-mRNA splicing in extracts mock depleted using pre-immune sera (ΔPI) or hPrp16 depleted with hPrp16 sera (ΔhPrp16). AdML pre-mRNA was incubated under standard splicing conditions using ΔPI nuclear extract (lanes 1–3) or ΔhPrp16 extract (lanes 4–6). For lanes 7–9, rhPrp16 was added to the ΔhPrp16 extract. The reactions were incubated for the times indicated. (D and E) Splicing of β-globin (**D**) or ftz (**E**) pre-mRNAs in ΔPI extract (lane 1), ΔhPrp16 extract (lane 2) or ΔhPrp16 extract containing rhPrp16 (lane 3). β-globin and ftz splicing products were separated on 9 or 12% denaturing polyacrylamide gels, respectively. Splicing intermediates and products are indicated.

catalytic step II in mammals (Chiara *et al.*, 1997). In addition, the distance between the branch point sequence (BPS) and AG also plays an important role in step II (Chiara *et al.*, 1997). To determine whether either of these parameters affects the association of hPrps 16 and 17 with the spliceosome, complexes were assembled on mutant pre-mRNAs and Western analyses were performed (Figure 5). The structures of the AdML pre-mRNAs are shown

in Figure 5A. GG-GG pre-mRNA contains GG substitutions of the AG at the 3′ splice site and the AG six nucleotides downstream. Catalytic step II is completely blocked with this pre-mRNA (Figure 5B, lane 2) (Chiara *et al.*, 1997). PyJ is the same as wild-type except for the insertion of 29 pyrimidines downstream of the normal pyrimidine tract. The BPS–AG distance is increased from 23 nucleotides in wild-type to 49 nucleotides in pyJ. This long BPS–AG distance results in an inefficient catalytic step II (Chiara *et al.*, 1997) (Figure 5B, lane 3). Finally, RanA contains random sequences adjacent to the AG at the 3′ splice site, and catalytic step II is abolished (Chiara *et al.*, 1997) (Figure 5B, lane 4).

As shown in Figure 5C, hPrp16 and hPrp17 are detected in complexes assembled on all of these pre-mRNAs (note that we could not establish whether the differences in the levels of these proteins are significant due to the differences in splicing kinetics of each pre-mRNA and the inherent variability in the complexes). The observation that both hPrp16 and hPrp17 are present in spliceosomes assembled on GG-GG pre-mRNA and RanA pre-mRNA, both of which cannot undergo step II, indicates that these proteins associate with the spliceosome prior to catalytic step II. Moreover, a specific BPS–AG sequence and distance are not required for the binding of hPrps 16 and 17 to the spliceosome. The AG itself is also not required for their binding. Thus, all three of these mutations impair catalytic step II at a stage that is subsequent to the binding of hPrp16 and hPrp17 to the spliceosome.

hPrp16 is essential for catalytic step II in humans

To determine directly whether hPrps 16 and 17 are required for splicing, we attempted to immunodeplete each protein from nuclear extracts (Figure 6A). Only 50% of the hPrp16 was depleted under normal splicing conditions, and little effect on splicing was observed with these extracts (data not shown). However, when the salt concentration of the nuclear extract was raised to 750 mM, hPrp16 was depleted efficiently (Figure 6A, lane 3). No hPrp16 was depleted with pre-immune sera (Figure 6A, lane 2), and no co-depletion of hPrp17 was detected (data not shown). Although hPrp17 antibodies specifically immunoprecipitate IVT-hPrp17 from reticulocyte lysates, we were not able to find conditions for immunodepleting this protein from splicing extracts (data not shown).

To determine whether splicing is affected in the hPrp16-depleted extracts, AdML pre-mRNA was incubated under normal splicing conditions in hPrp16-depleted (ΔhPrp16) extracts or extracts mock-depleted with pre-immune sera (ΔPI). A splicing time course using these extracts is shown in Figure 6C. The kinetics and efficiency of catalytic step I are the same in ΔPI and ΔhPrp16 extracts (e.g. compare 25 min time points, lanes 1 and 4). In contrast, catalytic step II is severely impaired in ΔhPrp16 extracts, relative to ΔPI extracts (Figure 6C, compare lanes 2 and 3 with 5 and 6). The low levels of step II activity in the ΔhPrp16 extract are most probably due to the incomplete depletion of hPrp16 (Figure 6A, and data not shown). To determine whether the step II activity depleted from ΔhPrp16 extracts can be reconstituted, we produced histidine-tagged recombinant hPrp16 in baculovirus (rhPrp16, see Materials and methods, Figure 6B) and added it to ΔhPrp16 extracts before incubation of splicing reactions for 25, 40 and 60

min (Figure 6C, lanes 7–9). Step I was unaffected by the addition of rhPrp16 (e.g. compare lanes 4 and 7). In contrast, step II was fully reconstituted by rhPrp16 (compare lanes 2 and 3 with 8 and 9).

To determine whether hPrp16 is a general step II splicing factor, immunodepletion/add-back studies were performed using different pre-mRNA substrates. For both β-globin (Figure 6D) and (ftz) (Figure 6E) pre-mRNAs, step II was severely impaired by depletion of hPrp16 and was restored by addition of rhPrp16. As shown in Figure 7A, the restoration of step II by rhPrp16 is also dose dependent. Quantitation reveals a linear increase in activity with increasing rhPrp16, and maximal activity was obtained at 100 ng (Figure 7A and data not shown). Together, these studies indicate that hPrp16 is a general splicing factor essential for catalytic step II.

To investigate the kinetics of step II when hPrp16 is added to ΔhPrp16 spliceosomes, AdML pre-mRNA was incubated for 40 min in ΔhPrp16 extracts to accumulate the splicing intermediates (Figure 7B, lane 2), and then rhPrp16 or buffer alone was added, and incubation was continued for 5, 15 or 30 min (Figure 7B, lanes 3–8). With buffer alone, the intermediates continued to accumulate (Figure 7B, lanes 3–5). When rhPrp16 was added, a low level of step II products appeared by 5 min of incubation and accumulated to much higher levels by 30 min (Figure 7B, lanes 6–8). Quantitation of the data (Figure 7C) shows that the intermediates are converted into products after addition of hPrp16. Thus, we conclude that the ΔhPrp16 spliceosome is a functional intermediate in the splicing pathway. As yet, we have not found suitable conditions for detecting the ΔhPrp16 spliceosome on native gels or for purifying this complex.

To obtain additional evidence that the complementation of ΔhPrp16 extracts is due to rhPrp16 (and not a contaminant in the preparation), we asked whether rhPrp16 is incorporated into functional spliceosomes. To do this, rhPrp16 was added to ΔhPrp16 extracts, and spliceosomes were assembled for 10 or 40 min on biotinylated AdML pre-mRNA (Figure 8). Complexes were then isolated by gel filtration and affinity-purified by binding to avidin–agarose. Western analysis of the complexes was performed using an antibody against the histidine tag in rhPrp16. As shown in Figure 8A, this antibody efficiently detects purified rhPrp16 alone (lane 3). Significantly, rhPrp16 is detected in the 40 min spliceosomal complex, but not in the 10 min complex (compare lanes 1 and 2). (Note that it was not possible to affinity-purify the spliceosome by binding to nickel resin, indicating that the His tag is not exposed in the native spliceosome.) The same blot was reprobed with antibodies to the U2 snRNP protein, SAP 130, which is present throughout spliceosome assembly (Figure 8B). This protein is detected in both the 10 and 40 min complexes. Together, these data show that rhPrp16 is indeed incorporated into the spliceosome. Furthermore, the association of rhPrp16 is specific as it is only detected in the 40 min spliceosome, consistent with the observation that native hPrp16 is present only in late spliceosomal complexes (Figure 4).

Discussion

Until recently, very little was known about the proteins involved in catalytic step II of the splicing reaction.
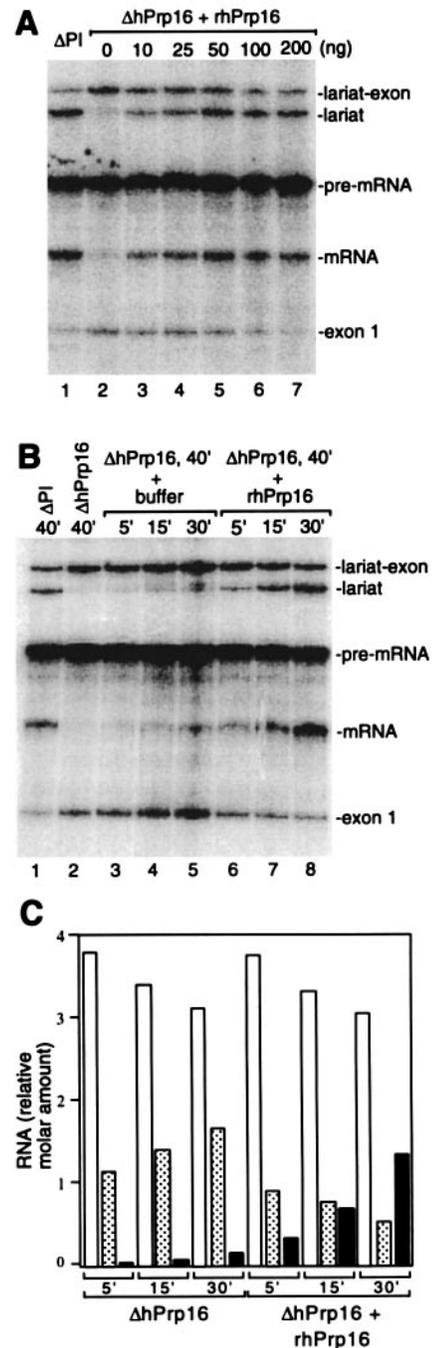

Fig. 7. (**A**) Reconstitution of splicing activity in ΔhPrp16 extracts by rhPrp16 is dose dependent. AdML pre-mRNA was incubated under splicing conditions in ΔPI extract (lane 1) or ΔhPrp16 extract (lane 2). rhPrp16 was added at 10, 25, 50, 100 or 200 ng, respectively, to ΔhPrp16 extract in a 25 μl standard splicing reaction (lanes 3–7). Splicing was carried out for 40 min. Splicing intermediates and products are indicated. (**B**) AdML pre-mRNA was spliced in ΔPI extract (lane 1) or ΔhPrp16 extract (lanes 2–8). Splicing was carried out for 40 min followed by addition of buffer alone or rhPrp16, and incubation was continued for 5 (lanes 3 and 6), 15 (lanes 4 and 7) or 30 min (lanes 5 and 8). Splicing intermediates and products are indicated. (**C**) Quantitation of the data shown in (B) (lanes 3–8) by phosphorimager analysis. Each of the RNA species was normalized against the total counts at the start of the chase. Pre-mRNA is shown in white, intermediates in stippled boxes, and products in black. The time points are indicated.

However, this picture has begun to change dramatically through a combination of genetic and biochemical studies in yeast and mammals. The known or putative step II proteins in humans that have yeast counterparts are summarized in Table I. So far, four step II proteins, Prps 16, 17 and 18 and Slu7, have been identified in yeast (for review, see Umen and Guthrie, 1995c). We now report the identification of human homologs of Prps 16 and 17. The human homologs of Prp18 and Slu7 have also been identified (Horowitz and Krainer, 1997; K.Chua and R.Reed, in preparation). All four of these proteins are known or are likely (hPrp17) to function exclusively in

Fig. 8. Recombinant hPrp16 is present in purified reconstituted spliceosomal complexes. (**A**) Biotinylated AdML pre-mRNA was incubated for 10 or 40 min in ΔhPrp16 extracts containing rhPrp16. Splicing reactions were fractionated by gel filtration, and spliceosomal complexes were affinity-purified by binding to avidin–agarose. A Western blot of the purified complexes was probed with antibodies to the His tag on rhPrp16. rhPrp16 alone (0.5 µg) was loaded in lane 3. (**B**) The same blot was reprobed with antibodies to the U2 snRNP protein, SAP 130.

step II in both humans and yeast. In yeast, Prps 16, 17, 18 and Slu7 all interact genetically with U5 snRNP (Frank et al., 1992). However, none of these proteins are actual U5 snRNP components, except Prp18, which is weakly associated (Horowitz and Abelson, 1993a,b; for review, see Umen and Guthrie, 1995c). In humans, Prp18 is not associated with U5 snRNP (Horowitz and Krainer, 1997), and it is not yet known whether hSlu7, hPrp16 and hPrp17 are U5 snRNP components (Table I).

In addition to the proteins that function exclusively in step II, some proteins may have roles in both catalytic steps. One clear example of this is the U5 snRNP protein, Prp8, which was first identified as an essential factor for spliceosome assembly (Whittaker et al., 1990; Brown and Beggs, 1992) and then was found, in both genetic and UV cross-linking studies, to function in recognition of the 3′ splice site for catalytic step II (Teigelkamp et al., 1995a,b; Umen and Guthrie, 1995a,b, 1996). Likewise, the human homolog of Prp8, $U5^{220}$, as well as two other U5 snRNP proteins ($U5^{100}$ and $U5^{116}$), UV cross-link to the 3′ splice site prior to step II (Chiara et al., 1997; Liu et al., 1997). In addition, antibody inhibition studies suggest a role for $U5^{116}$ in step II (Fabrizio et al., 1997). Essential yeast homologs of $U5^{100}$ and $U5^{116}$ (Snu114 and Prp28, respectively) have also been identified recently (Strauss and Guthrie, 1991, 1994; Fabrizio et al., 1997; Teigelkamp et al., 1997). These proteins have not yet been tested for a function in step II. Thus, although many gaps remain to be filled, all of the data point to a high level of conservation in the factors involved in step II and a key role for U5 snRNP in step II in both yeast and humans (Table I).
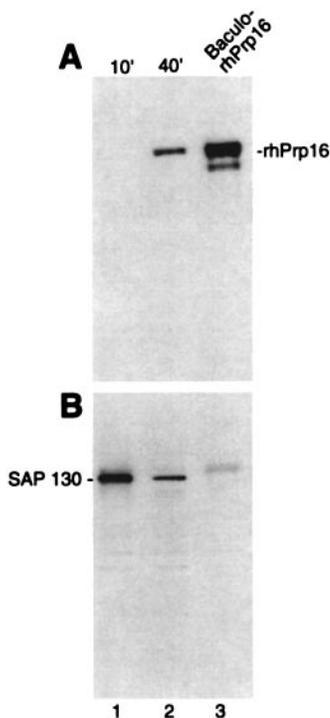
hPrp16 is an essential step II protein in humans

Our data indicate that hPrp16 is both structurally and functionally conserved. Prp16 is a member of the DEAH box family of splicing factors (Burgess et al., 1990). This family, which includes Prps 2, 22 and 43, is characterized by the presence of several motifs found in the prototypic family member, eIF4α, an ATP-dependent RNA helicase (for review, see Umen and Guthrie, 1995c). Prps 2 and 16 are indeed ATPases, but helicase activity for these proteins has yet to be reported (Schwer and Guthrie, 1991; Kim and Lin, 1996). hPrp16 resembles yeast Prp16 (41% identity) more closely than any of the other DEAH family

Table I. Known or putative human step II proteins that are conserved in yeast

Human	Yeast	Conserved structural domains	Step II activity	U5 snRNP component (human)	U5 snRNP interaction (yeast)
hPrp16	Prp16	DEAH box motifs	3′ ss xlink, ATPase, induces protection of 3′ ss (Y)	ND	synthetic lethal
hPrp17	Prp17	WD motifs		ND	synthetic lethal
hPrp18	Prp18	none identified		no	synthetic lethal and weakly associated
hSlu7	Slu7	Zn knuckle	3′ ss xlink (Y)	ND	synthetic lethal
$U5^{220}$	Prp8	none identified	3′ ss xlink (Y,H)	yes	associated
$U5^{100a}$	Prp28	DEAD box motifs	3′ ss xlink (H)	yes	genetic interaction
$U5^{116}$	Snu114	G domain	3′ ss xlink (H)	yes	ND

The structural domains that are conserved between corresponding yeast and human proteins are indicated. Step II activity indicates the main function identified for each protein related specifically to step II. Cross-linking to the 3′ splice site in the spliceosome prior to step II is designated as 3′ ss xlink. Y and H indicate that the activity has been shown in yeast or humans, respectively. ND: not determined. See text for references.
[a]$U5^{100}$ previously was named $U5^{110}$ (Bennett et al., 1992; Chiara et al., 1997) but we will now refer to it as $U5^{100}$ described in Teigelkamp et al. (1997) because the two proteins are the same (Bennett et al., 1992).

members. In addition, we show that a chimeric protein consisting of the C-terminal 977 amino acids of hPrp16 and the N-terminal 298 amino acids of yeast Prp16 can rescue a yeast Prp16 knockout strain. Due to the high similarity between Prp16 and the other DEAH family members, this rescue alone does not demonstrate that hPrp16 is the Prp16 homolog. However, this conclusion is supported by the observation that hPrp16 is essential for step II, like its yeast counterpart, whereas the other DEAH box splicing proteins are not. Another functional similarity between hPrp16 and Prp16 is that both proteins associate with spliceosome late in the splicing pathway (Schwer and Guthrie, 1991; this study). In addition, hPrp16 is destabilized from the splicesome late in the splicing pathway, which is most likely related to the ATP-dependent release of Prp16 from the yeast spliceosome (Schwer and Guthrie, 1992a,b). Finally, hPrp16, like its yeast counterpart, associates with spliceosomes assembled on pre-mRNAs containing an AG to GG mutation at the 3′ splice site, a mutation that blocks catalytic step II (Umen and Guthrie, 1995b; this study). Together, these data provide compelling evidence that hPrp16 is the functional homolog of Prp16.

hPrp16, unlike Prp16, contains a 340 amino acid domain highly enriched in the residues, REDS. Rescue of the yeast Prp16 knockout strain with hPrp16 required the replacement of this domain with the yeast N-terminus. Thus, the REDS domain probably plays a role specific to metazoans and the N-terminus of Prp16 contains a domain essential for function in yeast. A REDS domain similar to that in hPrp16 is found in the N-terminus of another human homolog of a DEAH box family member, Prp22, which is required for the release of spliced mRNA from the spliceosome (Company et al., 1991; Ono et al., 1994; Ohno and Shimura, 1996). This domain in hPrp22 interacts with members of the SR family of splicing factors and also functions as a nuclear localization signal (Ono et al., 1994; Ohno and Shimura, 1996). The observation that hPrp16 contains a REDS domain suggests that it also interacts with SR proteins, and that SR proteins may have a role in catalytic step II.

Evidence that hPrp17 is a step II protein in humans

hPrp17 is 36% identical to yeast Prp17, and the highest level of conservation is in the carboxy-terminus which contains seven WD motifs (Seshadri et al., 1996; M.Company and J.Abelson, unpublished; this study). These motifs are present in a number of proteins involved in RNA processing, signal transduction, DNA replication, cytoskeletal assembly and cell cycle control (for review, see Neer et al., 1994). Recently, the crystal structure of the prototypic WD family member, the β subunit of a heterotrimeric G protein, was solved (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996). This protein contains seven WD repeats and forms a seven-bladed β-propeller structure. Seven WD repeats are also present in the polyadenylation factor CstF (Takagaki and Manley, 1992) and the splicing factor, Prp4, and its human homolog (Banroques and Abelson, 1989; Bjorn et al., 1989; Dalrymple et al., 1989; Lauber et al., 1997). These WD domains and Prp17/hPRP17 presumably fold into a seven-bladed β-propeller structure and, like other WD proteins,

the WD motifs in Prp17/hPrp17 may function in protein–protein interactions (Neer et al., 1994; Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996). Consistent with the observation that the WD repeats are conserved between Prp17 and hPrp17, the repeats in Prp17 are essential for function in yeast (Seshadri et al., 1996).

Studies in yeast showed that Prp16 and Prp17 interact genetically and function at the same stage during step II (Jones et al., 1995; Umen and Guthrie, 1995b; Seshadri et al., 1996). These observations suggest that Prps 16 and 17 may physically interact. However, we have not obtained evidence for an interaction between the human counterparts. hPrp16 and hPrp17 antibodies specifically immunoprecipitate the corresponding in vitro translated proteins (data not shown). However, these proteins do not interact in co-immunoprecipitation, GST pull-down or far Western assays (data not shown).

In addition to the structural similarity between hPrp17 and Prp17, functional similarities between the proteins support the conclusion that they are functional homologs. Although we were unable to immunodeplete hPrp17 from nuclear extracts to demonstrate directly its role in step II, we have shown that hPrp17 is present in the spliceosome and joins it late in the splicing pathway. These observations are consistent with the step II role of Prp17 in yeast. Our data also show that hPrp17 is present in spliceosomes assembled on pre-mRNAs containing mutations that block or impair step II. Moreover, hPrp16 joins the spliceosome at about the same time as hPrp17 and is present in the step II-blocked spliceosomes. These results parallel functional studies in yeast showing that Prps 16 and 17 act at a similar stage of step II (Jones et al., 1995; Umen and Guthrie, 1995b). Thus, together, the data support the conclusion that hPrp17 is a step II protein in humans and is the functional homolog of yeast Prp17.

In yeast, Prp16 and 17 can be immunodepleted from extracts under splicing conditions (Schwer and Guthrie, 1991; Jones et al., 1995). In constrast, immunodepletion of hPrp16 requires high salt, and we were unable to immunodeplete hPrp17 even in high salt. Thus, it is possibile that the human proteins are present in a complex. Further work is necessary to test this possibility and to explain the differences between the yeast and human data.

Other known or putative step II proteins that have been identified in humans include U5[200] (Lauber et al., 1996), PSF (Patton et al., 1993; Gozani et al., 1994), Urp (Tronchere et al., 1997), AG[75] (Chiara et al., 1996) and p70 (Wu and Green, 1997). U5[200] is a human DEAD box family member that has an essential yeast homolog (Snu246/Brr2/Slt22) (Lauber et al., 1996; Noble and Guthrie, 1996; Xu et al., 1996). Interestingly, the ATPase activity of the yeast homolog is stimulated by U2 and U6 snRNAs, and the protein plays a role in association of U5 snRNP with the spliceosome (Xu et al., 1996). Moreover, antibodies to U5[200] inhibit catalytic step II in humans (Lauber et al., 1996). Thus, together, these data are consistent with a role for U2–U5 snRNP interactions in step II (see below). PSF and Urp do not have apparent yeast homologs, and it is not yet known whether these factors are general step II splicing factors. Consistent with a general role for PSF, it recently was detected in purified U4/U5/U6 snRNP (Teigelkamp et al., 1997). Urp contains an RS domain and interacts with U2AF[65] and other RS

domain-containing proteins, such as SR proteins (Tronchere *et al.*, 1997). Thus, it is possible that Urp has a uniquely metazoan role in step II because it interacts with an RS-type domain, such as that found in hPrp16. AG[75] and p70 both cross-link to the AG prior to step II, and their biochemical characteristics suggest that they are the same protein (Chiara *et al.*, 1996; Wu and Green, 1997). Cloning of these proteins is necessary to address this issue and determine whether they are functional step II proteins.

Remodeling the spliceosome for catalytic step II

Previous studies have identified several parameters that are important for the second catalytic step of splicing in humans. These include the AG dinucleotide at the 3′ splice site, the distance between the branch site and AG, and the presence of a pyrimidine tract immediately upstream of the 3′ splice site (Reed, 1989; Smith *et al.*, 1989, 1993; Chiara *et al.*, 1997). Prior to catalytic step II, U5 snRNP proteins span the distance between the BPS and the AG, replacing U2AF which binds early in spliceosome assembly (Chiara *et al.*, 1997). Thus, it is possible that the BPS–AG sequence constraint is due to U5 snRNP recognizing the pyrimidine tract for step II. The distance constraint may be due to U2 snRNP bound at the branch site, positioning U5 snRNP on the region between the BPS and AG. In this study, we have shown that the BPS–AG sequence and distance have no effect on the association of hPrps 16 and 17 with the spliceosome. In yeast, Prp16 cross-links to the 3′ splice site and then is replaced by Prp8 and Slu7 (Umen and Guthrie, 1995b). Prp16 also induces an alteration in the spliceosome that results in protection of the 3′ splice site from nuclease activity (Schwer and Guthrie, 1992a,b). Thus, putting these yeast and human data together, it is possible that hPrp16/Prp16 and hPrp17/Prp17 function in the remodeling of the spliceosome that results in recruitment of U5 snRNP to the 3′ splice site. Significantly, Prp17, Slu7 and Prp8 are synthetically lethal with mutations in U2 snRNA (Xu *et al.*, 1996). This may be explained by the proposed role for U2 snRNP in positioning U5 snRNP at the 3′ splice site for step II (Chiara *et al.*, 1997).

Studies of the *cis*-acting sequences required for step II have suggested that there could be significant differences in the mechanism for catalytic step II in yeast versus mammals (for review, see Umen and Guthrie, 1995c). In both organisms, the first AG downstream of the branch site usually functions as the 3′ splice site, and pyrimidines adjacent to the 3′ splice site are important for this recognition (Reed, 1989; Smith *et al.*, 1989; Patterson and Guthrie, 1991). In yeast, a downstream 3′ splice site can compete with an upstream 3′ splice site in artificial constructs (Patterson and Guthrie, 1991). This competition occurs when the downstream AG is preceded by pyrimidines and the upstream AG by purines. In contrast, in mammals, there are no examples in which a downstream AG can compete with an upstream AG (when the AG is located at a minimal distance from the branch site). The first AG is also selected in a *trans* splicing assay for step II (Anderson and Moore, 1997). This strict use of the first AG in humans has led to the proposal that the AG is selected by a scanning or threading type of mechanism (Smith *et al.*, 1989, 1993; Anderson and Moore, 1997).

In yeast, the competition results argue against such a model. Our studies of hPrps 16 and 17, together with the recent studies on hPrps 18 and hSlu7 (Horowitz and Krainer, 1997; K.Chua and R.Reed, in preparation), indicate that step II factors in yeast are conserved in humans. Given these results, it is highly likely that the mechanism for step II is also conserved, and the apparent distinctions between yeast and humans suggested by the *cis*-acting sequence studies may reflect only subtle differences, rather than fundamentally different mechanisms. Thus, studies in both organisms should expedite our understanding of the basic mechanism for the second catalytic step of splicing.

Materials and methods

Isolation of hPrp16 and hPrp17 cDNAs

The BLAST program was used to search the DDBJ/EMBL/GenBank database for sequences that resemble the yeast Prp16 sequence (Burgess *et al.*, 1990). A human cDNA isolated from a male bone marrow myeloblast KG-1 cDNA library (DDBJ/EMBL/GenBank accession No. D86977) was identified as the most similar to yeast Prp16. Oligonucleotides were designed that encompassed the ORF and were used to isolate an hPrp16 cDNA by PCR of HeLa cell cDNA. (The 3703 bp cDNA sequence of the hPrp16 gene has been deposited in DDBJ/EMBL/GenBank under accession No. AF038391.) The Prp17 sequence (kindly provided by M.Company and J.Abelson) was used to perform a BLAST search of the database which identified two short expressed sequence tags (ESTs, DDBJ/EMBL/GenBank accession Nos AA057404 and Z19190). To isolate the 5′ end of the hPrp17 cDNA, a primer was designed to the EST clone AA057404 isolated from a colonic epithelial cell cDNA library (obtained from Genome Systems Inc). This gene-specific primer (5′-GGCTGCACTGAGGAACTGTGTTCC-3′) and a nested gene-specific primer (5′-GTTCTCAGACAGCGCCGTTCTCC-3′) were used for 5′ RACE with Marathon RACE-ready cDNA (Clontech). The full-length clone, which contains the 579 amino acid ORF followed by a poly(A) signal, was generated by fusing the 5′ RACE product to the EST clone AA057404. (The 1979 bp cDNA sequence of the hPrp17 gene has been deposited in DDBJ/EMBL/GenBank under accession No. AF038392.)

hPrp16 immunodepletion and reconstitution

The hPrp16 N-terminus (amino acids 1–554) or C-terminus (amino acids 948–1227) were fused with GST in the vector pGEX2TK (Pharmacia) and expressed in *Escherichia coli*. Rabbit polyclonal antibodies were raised against the mixture of the N- and C-terminal fusion proteins. hPrp17 antibodies were raised against the N-terminal domain of hPrp17 (amino acids 1–223) expressed in *E.coli* as a GST fusion protein. For immunodepletions of hPrp16, protein A–Sepharose CL-4B (Pharmacia) was coupled with the hPrp16 serum at the ratio of 1 vol. of beads to 4 vols of sera and rotated at 4°C overnight. After extensive washing, the beads were added to nuclear extract containing 750 mM KCl at a ratio of 1 vol. of beads to 4 vols of extract. The mixture was rotated for 1.5 h at 4°C. The supernatant was transferred to a new tube, and the procedure was repeated twice more. The depleted extract was dialyzed against 20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM dithiothreitol (DTT). Recombinant His-tagged hPrp16 was produced using the Bac-to-Bac baculovirus expression system (Gibco/BRL). After 48 h of infection with the recombinant baculovirus, Sf9 cells were harvested and lysed in 50 mM HEPES, pH 7.9, 10 mM 2-mercaptoethanol, 1 mM PMSF and 1% Triton X-100 at 4°C. The expressed protein was present in inclusion bodies which, after washing, yielded a highly pure preparation of hPrp16 (see Figure 6). The inclusion bodies were solubilized in 6 M urea, and then the protein was renatured by dialysis into 50 mM HEPES, pH 7.9, 100 mM KCl, 10 mM β-mercaptoethanol, 1 mM PMSF. Except where indicated, 50 ng of rhPrp16 were added to 7.5 µl of hPrp16-depleted extracts in a 25 µl reaction to reconstitute splicing activity.

Complementation of Prp16 knockout

The haploid yeast strain YTdL19 (kindly provided by C.Guthrie) contains a disruption of the Prp16 gene, and the temperature-sensitive Prp16-1

gene is carried on a plasmid containing the *URA3* marker gene (Burgess *et al.*, 1990; Schwer and Guthrie, 1991). The full-length hPrp16 cDNA or the indicated derivatives were cloned downstream of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter in the 2 µm plasmid pG1 (containing a *TRP* marker gene). pG1-Prp16 and the indicated hPrp16 constructs were transformed into the YTdL19 strain and selected at 30°C in medium lacking tryptophan. Expression of hPrp16 genes was verified by Western blotting. Colonies were then patched on 5-FOA plates to select for cells lacking the URA3 plasmid. Those constructs that allow growth are able to rescue the yeast Prp16 knockout.

Analysis of spliceosomal complexes

Wild-type AdML is encoded by pAdML (Michaud and Reed, 1993), and the plasmids encoding GG-GG, PyJ and RanA pre-mRNAs were described in Chiara *et al.* (1997). Fushi tarazu (ftz) pre-mRNA was described in Rio (1988) and α-tropomyosin pre-mRNA was described in Smith and Nadal-Ginard (1989). pAdML and tropomyosin were linearized with *Bam*HI, and ftz was linearized with *Xho*I. AdML and ftz DNAs were transcribed with T7 RNA polymerase and tropomyosin was transcribed with SP6. The spliceosomal complexes were assembled by incubating 1.92 µg of ^{32}P-labeled biotinylated pre-mRNAs in a 2.4 ml reaction for the times indicated. Complexes were isolated by gel filtration followed by avidin affinity chromatography, and then RNA or protein were analyzed as described (Reed, 1990; Bennett *et al.*, 1992). Purified spliceosomal complexes containing rhPrp16 were assembled by incubating biotinylated AdML pre-mRNA in hPrp16-depleted extract containing 4.8 µg of rhPrp16 per 2.4 ml of splicing reaction.

Acknowledgements

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