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Correspondence Between a Mammalian Spliceosome Component and an Essential Yeast Splicing Factor

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None of the mammalian splicing factors that have been cloned corresponds to the yeast pre–messenger RNA splicing factors, the PRP proteins. Here, a generalizable strategy was used to isolate a complementary DNA encoding the mammalian spliceosome–associated protein (SAP) SAP 62. It is demonstrated that SAP 62 is the likely functional homolog of the yeast PRP11 protein. Both PRP11 and SAP 62 associate stably with the spliceosome, contain a single zinc finger, and display significant amino acid sequence similarity. Unlike PRP11, SAP 62 contains 22 proline-rich heptapeptide repeats at the carboxyl-terminus.

The mammalian spliceosomal protein SAP 62 first binds to pre-mRNA in the prespliceosomal complex A (*1*) and cross-links to the 3′ portion of the pre-mRNA in this complex (*2*). A functional 3′ splice site and adenosine triphosphate (ATP) are required for this interaction, indicating that SAP 62 may play an important role at the 3′ splice site (*1*). Originally SAP 62 was identified as one of more than 20 proteins that associate stably and in an ATP- and splice site–dependent manner with highly purified spliceosomes on two-dimensional (2D) gel electrophoresis (*1*). We have increased the scale of our spliceosome purification procedure to obtain sufficient amounts of individual proteins from 2D gels for sequence analysis (*3*). On the basis of the peptide sequences, we have isolated a complementary DNA (cDNA) clone encoding SAP 62 as well as cDNAs encoding other spliceosomal proteins (*4*). In the predicted amino acid sequence of SAP 62 (Fig. 1A) one zinc finger of the C_2H_2 class (*5*) is present. The presence of this zinc finger leads us to predict that SAP 62 is a nucleic acid binding protein, consistent with the observation that SAP 62 can be cross-linked by ultraviolet (UV) light to pre-mRNA in the spliceosome (*2*). In contrast with the essential splicing factors cloned so far in mammals, U2AF (*6*) and the serine-arginine–rich family (*7*), SAP 62 lacks a ribonucleoprotein (RNP) binding domain of the RNA recognition motif (RRM) type (*8*) and does not contain an arginine-serine (RS) domain.

The COOH-terminal third of SAP 62 is proline-rich (43%) and is organized into 22 tandem heptapeptide repeats of the sequence GVHPPAP (Fig. 1B). A similar number of heptapeptide repeats of the sequence YSPTSPS are present in the COOH-terminus of the large subunit of RNA polymerase II (RNAP II), and phosphorylation of serine and threonine residues in these repeats plays a critical role in RNAP II activity (*9*). A feature of the SAP 62 repeats is their proline richness, which suggests that the repeats may be involved in protein-protein interactions. Although proline richness is characteristic of several RNA and some DNA binding proteins (*10*, *11*), there is no precedent of conserved heptapeptide repeats in essential splicing factors or in other RNA binding proteins.

We detected by Northern (RNA) blot analysis of HeLa cell mRNA with the SAP 62 cDNA as a probe an mRNA of about 2 kb (Fig. 2) (*12*). The deduced SAP 62

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sequence of 464 amino acids is predicted to encode a protein of 49 kD. However, in vitro translation of mRNA generated from the SAP 62 cDNA yielded a protein that cofractionates by 2D gel electrophoresis with SAP 62 present in the spliceosome (Fig. 3, A and B). We conclude that SAP 62 fractionates anomalously by SDS–polyacrylamide gel electrophoresis (PAGE).

Comparison of SAP 62 to the GenBank database revealed a similarity to the yeast splicing factor PRP11 (*13*). Antibodies to two other yeast splicing factors PRP8 (*14*) and PRP9 (*15, 16*) have been found to recognize potential mammalian homologs, but similarities between these proteins have not been demonstrated at the amino acid sequence level. An alignment of SAP 62 and PRP11 amino acid sequences is shown (Fig. 1C). In PRP11 the COOH-terminus aligns with a region NH_2-terminal to the proline-rich repeats in SAP 62. Both SAP 62 and PRP11 have similarity in the zinc finger region (Fig. 1C), and the zinc fingers are located in comparable positions with respect to the NH_2-terminus of the two proteins. This conservation is consistent with studies indicating that the zinc finger motif is essential for PRP11 function in yeast (*17*). Other yeast splicing factors (PRP6 and PRP9) (*15*) and the mammalian U1 small nuclear ribonucleoprotein particle (snRNP)–specific protein C (*11*) also contain zinc finger motifs similar to that in PRP11 and SAP 62. With respect to amino acids 1 to 236 of SAP 62, which are in the region of similarity with PRP11, the overall resemblance is 39% and the identity is 25%. In addition, both this region of SAP 62 and the entire PRP11 protein are highly hydrophilic (*13*). Further evidence that SAP 62 and PRP11 are related is the observation that antibodies to PRP11 detect SAP 62 among the total affinity-purified spliceosomal proteins fractionated by 2D gel electrophoresis (Fig. 3D). Moreover, one band of the same molecular size as SAP 62 is detected in gel filtration–purified spliceosomes (Fig. 3D). Finally, only one additional band (~150 kD) is detected by PRP11 antibodies in total nuclear extracts; this extra band may result from the cross-reaction of the polyclonal antibody with a shared epitope (*18*).

In addition to SAP 62, seven other SAPs (155, 145, 130, 114, 61, 49, and 33 kD) and U2 snRNP bind to the 3′ portion of the pre-mRNA in the prespliceosome (*1*). Under low-salt conditions U2 snRNP was isolated and found to contain nine proteins (160, 150, 120, 110, 92, 66, 60, 53, and 35 kD) (*19*) in addition to the two U2 snRNP–specific proteins previously identified (B″, A′) (*20*). Significantly, antibodies to the essential yeast splicing factor PRP9 specifically recognize the 60-kD component of this 17S U2 snRNP particle (*16*). This observation, together with the observation that PRP9 is required for prespliceosome assembly in yeast (*15, 21*), prompted us to ask whether PRP9 antibodies detected any of the prespliceosome-associated SAPs. Among the total spliceosomal proteins, SAP 61 is recognized by PRP9 antibodies (Fig. 3C). Given this observation and the fact that the 17S U2 snRNP–specific proteins have molecular weights similar to those of the prespliceosome-associated SAPs, it seemed likely that the other prespliceosomal SAPs, including SAP 62, are also U2 snRNP proteins. Direct evidence for this was obtained by showing that the prespliceosomal SAPs cofractionate on a 2D gel with the set of proteins immunoprecipitated by U2 snRNP antisera from gel filtration fractions containing U2 snRNP (*22*). Together, these data indicate that PRPs 9 and 11 are the likely homologs of the U2 snRNP–associated prespliceosomal SAPs 61 and 62.

In addition to the pre-mRNA–protein interactions of SAP 62, we have also detected highly specific protein-protein interactions between SAP 62 and the prespliceosomal component SAP 114. In vitro translated SAP 62 detects only SAP 114 on a blot of affinity-purified spliceosomes fractionated by 2D gel electrophoresis (Fig. 4). Moreover, only the 114-kD band is detected by the SAP 62 probe in gel filtration–purified spliceosomes or in total nuclear extract (Fig. 4). Our observation that SAPs 62 and 114 interact with one another directly is consistent with the observation (*23*) that SAPs 61, 62, and 114 constitute the heterotrimeric splicing factor SF3a that is essential for prespliceosome assembly in mammals (*24*). In the accompanying report by Brosi *et al.* (*25*), the 60-kD subunit of SF3a is shown to be recognized by PRP9 antibodies, and all three SF3a subunits are detected among the proteins in the 17S U2 snRNP, consistent with our observations.

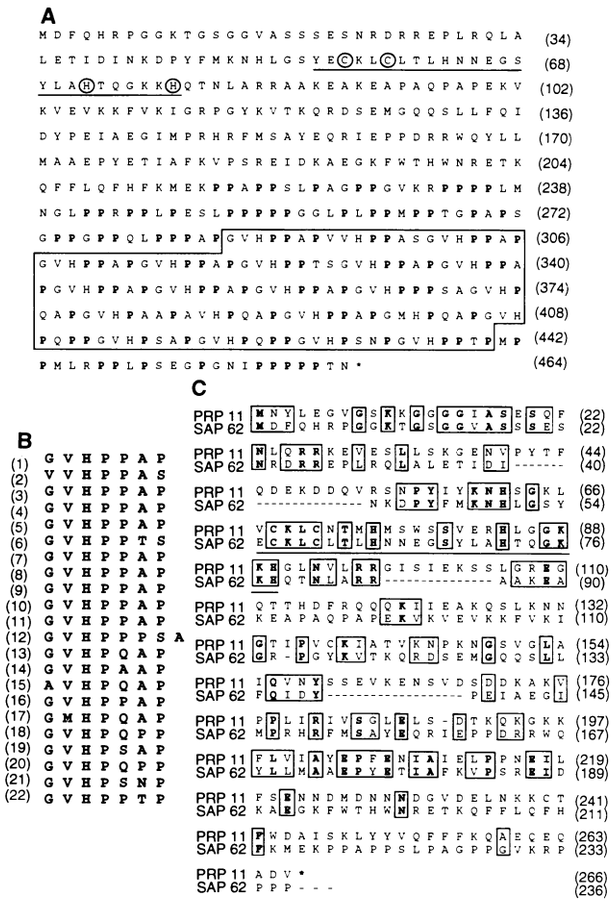

Fig. 1. Predicted amino acid sequence of the SAP 62 cDNA, COOH-terminal proline-rich repeats, and alignment with PRP11. (**A**) Amino acid sequence of SAP 62 (positions are shown on the right). The zinc finger motif is underlined, and the conserved histidine (H) and cysteine (C) residues are circled. The region containing the heptapeptide repeats is boxed and prolines (P) in this region are in bold. The cDNA sequence has been deposited in GenBank (accession number L21990). (**B**) The COOH-terminal proline-rich repeats beginning at amino acid 286 are numbered 1 to 22. (**C**) Alignment of the NH_2-terminal portion of SAP 62 with PRP11 according to the Lipman-Pearson protein algorithm (DNAStar, Inc.; gap penalty: 4, gap length: 12). Identical or conserved residues are boxed [amino acids are grouped as follows: (Y F), (L I V), (S T), (K R), (D E N Q), (A G)] (*9*). The identities are indicated in bold.

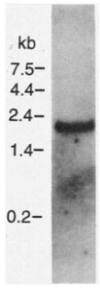

Fig. 2. Northern blot analysis. Poly(A) mRNA (1.8 μg) from HeLa cells was probed with the 435-bp PCR product encoding a portion of the SAP 62 cDNA (*12, 30*). The full-length cDNA probe detected one main band of the same size as that detected by the PCR product (*31*).

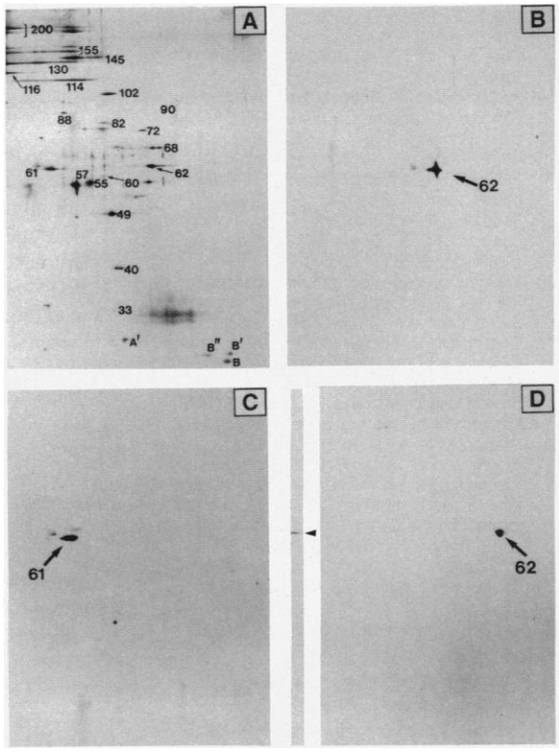

Fig. 3. Comigration of in vitro translated SAP 62 with HeLa cell SAP 62 and reactivity of SAP 61 with PRP9 and SAP 62 with PRP11 antisera. (**A**) Spliceosome-specific proteins were assembled on fushi tarazu pre-mRNA, fractionated by 2D gel electrophoresis, and silver stained (*1*). The abundant SAPs are designated according to the nomenclature of Bennett *et al.* (*1*). (**B**) The cDNA for SAP 62 was transcribed and translated in vitro in reticulocyte lysates as described (Promega TNT) incorporating ^{35}S-labeled methionine. Translation products were fractionated by 2D gel electrophoresis and analyzed by phosphorimage analysis (Molecular Dynamics). (**C**) Spliceosomes were fractionated by 2D gel electrophoresis, transferred to nitrocellulose, and probed with PRP9 antisera. SAP 61 is indicated. (**D**) Spliceosomes fractionated on a 2D gel and a gel filtration fraction containing spliceosomes fractionated on the second dimension of this 2D gel were transferred to nitrocellulose and probed with PRP11 antisera. SAP 62 is indicated by the arrow on the 2D blot and by the arrowhead next to the lane containing gel filtration–purified spliceosomes. Horseradish peroxidase–conjugated secondary antibodies were used, and detection was with the enhanced chemiluminescence system (ECL, Amersham).

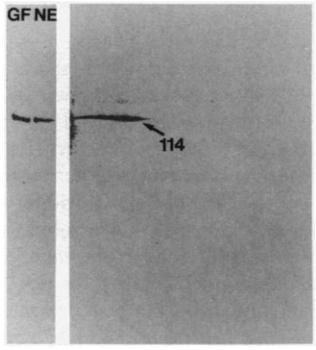

Fig. 4. Direct protein-protein interactions between SAP 62 and SAP 114. In vitro translated ^{35}S-labeled SAP 62 was used to probe a blot containing prespliceosomes (A complex) fractionated by 2D gel electrophoresis, total nuclear extract (NE), and the gel filtration fraction containing spliceosomes (GF); the latter two samples were fractionated on the second dimension of the 2D gel. As a control, the blot was probed with in vitro translated luciferase or SAP 49, neither of which detected SAP 114 (*18*). The blot was blocked and probed according to the methods of Zhang *et al.* (*32*).

The accompanying report by Legrain and Chapon (*26*) presents evidence for direct physical interactions between PRPs 9, 11, and 21 in yeast. These proteins are also known to interact with one another genetically (*27*) and are required for prespliceosome assembly in yeast (*15*, *21*, *26*, *28*). Thus, together these observations establish several key relations between the 17S U2 snRNP components, the prespliceosomal SAPs 61, 62, and 114, and the essential heterotrimeric splicing factor SF3a in mammals and PRPs 9, 11, and 21 in yeast.

The 17S U2 snRNP–specific proteins are thought to associate with the 5′ end of U2 snRNA in mammals (*19*). This region of U2 snRNA participates directly in several essential interactions in the spliceosome, including base pairing with the site of lariat formation in pre-mRNA, base-pairing with U6 snRNA, and the second catalytic step of the splicing reaction (*29*). Thus, the U2 snRNP–associated SAPs 61, 62, and 114, all of which bind directly to the 3′ portion of the pre-mRNA (*2*), may function in mediating these critical RNA-RNA interactions.

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4. Isolation of SAP 62 cDNA clone. Degenerate oligonucleotides based on the peptide 1 sequence (QLALETIDINKDPYFMK) were used as polymerase chain reaction (PCR) primers to obtain a 52-base pair (bp) cDNA. On the basis of this cDNA, PCR primers, together with degenerate oligonucleotides based on the peptide 2 sequence (QYLLMAAEPYE), were used to generate a 435-bp PCR probe. The probe was labeled by random-priming and used to screen a human fetal brain λZAPII bacteriophage library with standard protocols (*30*). Of 1 × 10^6 bacteriophage screened, 11 clones hybridized to the 435-bp probe. These were subcloned and sequenced. We found one clone to contain a 1950 nucleotide (nt) full-length cDNA encoding both peptides 1 and 2. A second clone was 1337 bp and encoded amino acids 56 to 465, whereas the other clones had other portions of the SAP 62 cDNA but then diverged into sequences that lacked open reading frames. There was no evidence for alternative splicing of SAP 62. For SAPs 145 and 49, cDNAs have also been isolated on the basis of peptide sequences obtained from the 2D gel–isolated proteins (*31*). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Interaction Between PRP11 and SPP91 Yeast Splicing Factors and Characterization of a PRP9-PRP11-SPP91 Complex

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Several proteins are involved in the early steps of the spliceosome assembly pathway. Protein-protein interactions have been identified between two *Saccharomyces cerevisiae* yeast splicing factors, PRP9 and SPP91. Here it is demonstrated that protein-protein interactions occur between SPP91 and PRP11. The combination of the *prp9-1* mutant and a truncated *prp11* mutant exhibits a synthetic lethal phenotype, suggestive of a common biochemical defect. The PRP9 and PRP11 proteins do not interact directly, but the PRP9 and PRP11 molecules can simultaneously bind SPP91 to form a three-molecule complex. Structurally and functionally related proteins are found in mammalian cells and are associated in a single biochemical fraction. This strongly suggests that the PRP9-SPP91-PRP11 complex is a key element of the splicing machinery.

Nuclear pre-mRNA splicing occurs in a complex called the spliceosome that contains U1, U2, and U4/U6.U5 small nuclear ribonucleoprotein particles (snRNPs) bound to the pre-mRNA (*1–3*). In yeast, more than 30 factors encoded by the *PRP* genes are implicated in splicing. The recognition of intron boundaries is a critical step in splicing and involves the U1 and U2 particles and additional protein factors. During spliceosome assembly, the U2 snRNP binds to the U1 snRNP–pre-mRNA complex to form the prespliceosome. Both the PRP11 and PRP9 yeast proteins are required for prespliceosome formation. Moreover, the PRP9 protein associates with the U2 snRNP particle in a salt-dependent fashion (*4–6*).

We identified an essential gene, *SPP91*, as a second-site suppressor of the *prp9-1* mutation (*7*). The *SPP91* gene is identical to the *PRP21* gene whose product is also required for the formation of the prespliceosome (*8*). In the absence of splicing complexes, neither the PRP9 protein nor the SPP91 protein is tightly associated with the U1 or the U2 snRNPs. In contrast, when splicing reactions are performed in vitro, both PRP9 and SPP91 are found associated with the prespliceosome (*6, 8*). These observations suggest that the PRP9 and SPP91 proteins act together to promote the interaction between the U2 snRNP particle and the U1 snRNP–pre-mRNA complex. Although it is likely that protein-protein interactions play a crucial role in spliceosome assembly, very little is known about these interactions. We have demonstrated that the PRP9 and SPP91 proteins interact (*9*). Here, we demonstrate that PRP11 also binds to SPP91, and we present genetic evidence that PRP9 and PRP11 are functionally related. Moreover, with a three-molecule binding assay, we identify a multimolecular complex formed by SPP91, PRP9, and PRP11.

The PRP9 and PRP11 proteins contain cysteine-histidine motifs (CH) that are loosely related to those found in C_2H_2 zinc finger proteins (Fig. 1) (*5*). When the *PRP11* allele deleted for the CH motif (*prp11ΔCH*) was expressed in a *prp11-1* strain, a dominant lethal phenotype was observed (Fig. 2). A *prp11* allele that contained only the region downstream of the CH motif still exhibited the dominant lethal phenotype (*10*). This dominant lethal phenotype suggests that the COOH-terminal region of PRP11 contains a binding site for a factor that is titrated by the truncated protein (*11*). Expression of *prp11ΔCH* also had a strong effect on *prp9-1* cell viability but not on the viability of wild-type isogenic cells (Fig. 2). This synthetic lethal phenotype suggests that there is a close functional relation between PRP9 and PRP11 proteins. Synergistic effects have been observed between the *prp5*, *prp9*, *prp11*, and *prp21* temperature-sensitive (ts) mutations (*12*).

The possible interactions between the PRP9 and PRP11 proteins were tested with the two-hybrid system which scores for in vivo interactions between two proteins overproduced in yeast (*13, 14*). In this assay, two proteins are fused to the DNA binding domain and to the activation domain of the yeast transcriptional factor GAL4, respectively. An interaction between these proteins brings together the two GAL4 domains, allowing the formation of a functional GAL4 complex that activates the expression of a *lacZ* reporter gene. When the *PRP9* coding region was fused to the GAL4 activation domain and the *PRP11* coding region was fused to the GAL4 DNA binding domain, very low amounts of β-galactosidase activity were detected. Similar results were observed for the reciprocal combination (Fig. 3A), suggesting that PRP9 and PRP11 proteins do not interact. In contrast, in an assay with the PRP11 and SPP91 GAL4 fusion proteins, high quantities of β-galactosidase activity were detected, revealing a strong interaction between these two proteins (Fig. 3A).

This interaction was characterized in greater detail with the PRP11-1 (*15*) and PRP11ΔCH mutant proteins (Fig. 1). We assayed the interaction of the PRP11-1 protein fused to either the GAL4 activation

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