>95% pure, as judged from Coomassie brilliant blue staining of SDS gels, and was free of detectable deoxyribonuclease and RNase activity. Smearing of the major band suggests that there was some degree of proteolytic degradation. This preparation (1 mg/ml) was stored at −80°C in 50 mM tris-Cl (pH 7.5), 0.1% Triton X-100, 50 mM NaCl, 100 mM imidazole, 10 mM β-mercaptoethanol, and 20% glycerol (*15*). The concentration of protein required to enhance HH16 activity increased slowly with storage, which suggests that there was a loss of activity with time. Thus, the reported concentrations may overestimate the amount of fully active NC required for stimulation.

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16. In the absence of the NC, the rate-limiting step for the burst phase is the cleavage step (*4*). The cleavage step proceeds at the same rate in the presence and in the absence of the NC (Fig. 3B).
17. This NC protein was prepared from an *E. coli* overproducer in the laboratory of L. Henderson (National Cancer Institute, Frederick, MD). This 55–amino acid residue protein lacks 16 COOH-terminal residues as well as the NH_2-terminal $(His)_6$ tag present in our preparation (*8*) and is thought to correspond to the form found in the HIV virion [L. E. Henderson *et al.*, *J. Virol.* **66**, 1856 (1992)]. This preparation contained Zn^{2+} bound to the zinc fingers, whereas it was not known if Zn^{2+} was bound to the NC of the other preparation (*8*). Previous results have shown that binding and annealing activities occur in the absence of Zn^{2+}, with mutations in the zinc fingers and with a variety of shortened versions of the protein (*14*) [H. DeRocquigny *et al.*, *Nucleic Acids Res.* **21**, 823 (1993)]. Stimulation of both single turnover and multiple turnover reactions of HH16 was observed whether the 55–amino acid protein was incubated before reaction with EDTA, which is expected to remove the bound Zn^{2+} (*12*).
18. Additional control experiments are presented elsewhere (*19*).
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20. S (Fig. 1) is a 17-mer and is referred to as S_{17mer} for clarity; S'_{17mer} has the same sequence as S except for a change of G → U three residues from the 5′ end to give a mismatched duplex with HH16 (Fig. 1). In all cases, residues were removed from the 3′ end of S or S′ to give the shorter substrates.
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29. Before reaction, HH16 and S* were mixed and heated to 95°C for 2 min in the presence of 50 mM tris (pH 7.5) and 1 mM EDTA and cooled at room temperature to promote renaturation and annealing. Reactions were initiated by the addition of $MgCl_2$ directly after the addition of the NC. The order of addition had no effect.
30. All results were quantitated with a Phosphorimager (Molecular Dynamics). Rate constants were obtained for all reactions, from the initial rate formation of the 5′ end-labeled product (P*) (after the burst) in multiple turnover reactions and from the first-order disappearance of S* in single turnover reactions.
31. Before reaction, HH16 and S* were separately heated to 95°C for 2 min in the presence of 50 mM tris (pH 7.5) and 1 mM EDTA and cooled at room temperature. After cooling, $MgCl_2$ was added to HH16 and S* and we initiated the reaction by mixing together HH16 and S*. The NC or buffer was added 15 s after initiation. The order of addition had no effect.
32. The reactions whose results are shown in Fig. 4 were followed with a "kinetic ladder" (*7*). An alkaline ladder was generated with 5′ end-labeled S or S′. After this mixture was neutralized, reactions of all of the substrates in the ladder were followed simultaneously. It was important to maintain ribozyme in large excess of the RNA substrates so that the presence of one substrate did not affect the reaction of another substrate—for example, by competing for a limited number of binding sites. The rate constants obtained for several oligonucleotides were compared to those obtained in standard assays with the single purified oligonucleotide; these values were the same, which demonstrates the validity of this approach (*7*). Because we did not have a strong prediction for the length of oligonucleotide substrate that would be required to obtain NC-enhanced specificity and we wished to gather the most information about these reactions, we used the kinetic ladder approach to investigate the effect of the NC on specificity. Data for only S_{13mer} and S'_{13mer} are presented herein.
33. We thank K. Hertel, L. Henderson, and B. Pontius for generous gifts of materials, S. Muthani and M. Chada of CloneTec for oligonucleotides made by automated RNA synthesis, P. Brown for encouragement, and P. Brown, T. Cech, and I. R. Lehman for helpful comments on the manuscript. Supported by a grant from the Lucille P. Markey Charitable Trust to D.H. D.H. is a Lucille P. Markey Scholar in Biomedical Sciences. Z.T. was supported by the Howard Hughes Medical Institute.

27 May 1993; accepted 2 August 1993

Interaction of Mammalian Splicing Factor SF3a with U2 snRNP and Relation of Its 60-kD Subunit to Yeast PRP9

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In the assembly of a prespliceosome, U2 small nuclear ribonucleoprotein (snRNP) functions in pre–messenger RNA (mRNA) splicing together with splicing factors (SFs) 3a, SF3b, and several other proteins. The 17*S* but not the 12*S* form of U2 snRNP is active in splicing-complex formation. Here it is shown that the SF3a subunits correspond to three of the 17*S* U2 snRNP–specific polypeptides. SF3a interacts with U2 snRNP in the presence of SF3b to generate a structure similar to 17*S* U2 snRNP, which suggests a function for SF3a and SF3b in the incorporation of U2 snRNP into the spliceosome. Furthermore, the 60-kilodalton subunit of SF3a is related to the yeast splicing protein PRP9.

The active spliceosome, which is the site of two transesterification reactions that generate spliced mRNA, is assembled in a stepwise fashion by interactions between pre-mRNA, snRNPs, and a number of proteins (*1*). Components in five chromatographic fractions (SF1, SF3, U2AF, and U1 and U2 snRNPs) obtained from HeLa cell nuclear extracts function in the assembly of the adenosine triphosphate (ATP)–dependent presplicing A complex (*2*). SF3 has been separated into two components (SF3a and SF3b), both of which are required for A-complex formation (*3*). The component SF3a has been purified to homogeneity and consists of three subunits of 60, 66, and 120 kD ($SF3a^{60}$, $SF3a^{66}$, and $SF3a^{120}$, respectively); SF3b has been only partially purified.

As for U2 snRNP, which interacts with the pre-mRNA to form the presplicing complex (*4*), it exists in two forms, 12*S* or 17*S* (*5*, *6*). The 12*S* U2 snRNP contains the common snRNP proteins and the U2 snRNP–specific proteins A′ and B″ (*7*). The 17*S* U2 snRNP contains an additional set of polypeptides of 35, 53, 60, 66, 92, 110, 120, 150, and 160 kD (*6*). This 17*S* U2 particle unlike its 12*S* counterpart is active in spliceosome assembly and splicing

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(8), implying that at least some of the 17S U2 snRNP–specific proteins are required for splicing. The 60-kD polypeptide of the 17S U2 snRNP cross-reacts with an antibody to yeast PRP9 (anti-PRP9) (8); PRP9 functions in the assembly of the yeast prespliceosome complex (9). The presence of 60-, 66-, and 120-kD polypeptides in both SF3a and the 17S U2 snRNP along with the functioning of SF3a, 17S U2 snRNP, and PRP9 at the same stage of spliceosome assembly suggested that these factors may share structural features.

In Western blotting experiments (10) the anti-PRP9 specifically recognizes purified $SF3a^{60}$, and a single polypeptide of similar size was detected in a HeLa nuclear extract (Fig. 1A). No reaction with mammalian proteins was observed with preimmune serum (11). Anti-PRP9 also recognizes the 60-kD 17S U2–specific protein (Fig. 1B) (12). In the particular gel system used, staining of a doublet is sometimes observed (13). Moreover, a monoclonal antibody (mAb) directed against $SF3a^{66}$ (mAb66) (3) cross-reacts with the 17S U2 snRNP polypeptide of 66 kD. The direct comparison of SF3a and 17S U2 snRNP proteins by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1C) reveals that, in addition to the comigration of the 60- and 66-kD polypeptides, $SF3a^{120}$ comigrates with the 110-kD polypeptide of the 17S U2 snRNP. These results suggest that SF3a is part of the 17S U2 particle.

The 17S U2 snRNP–specific polypeptides dissociate from the particle at salt concentrations of 200 mM or higher (6). When nuclear extracts are fractionated in glycerol gradients at 150 mM KCl (14), SF3a (as detected with mAb66 and anti-PRP9) cosediments with U2 snRNP in the 17S region of the gradient (Fig. 2A). Some SF3a is found in the top fractions of the gradient, consistent with a sedimentation coefficient of 5.5S for purified SF3a (3). At 500 mM KCl, SF3a sediments as a free protein and U2 snRNP sediments at 12S (Fig. 2B). The concomitant decrease in the sedimentation rate of U2 snRNP and SF3a suggests that SF3a dissociates from the U2 particle in the presence of a high salt concentration. The similarity in the sedimentation behavior of the 17S U2 snRNP–specific polypeptides (6) and SF3a in response to increased salt concentrations is further evidence that this splicing factor is associated with U2 snRNP in nuclear extracts. In agreement with these data, U2 snRNA is the predominant RNA that is precipitated from nuclear extracts with mAb66 (11).

A reproducible heterogeneity in the snRNP populations has been observed after Mono Q chromatography (15). When analyzed by Western blotting with mAb66 or anti-PRP9, SF3a was detected (as expected) in Mono Q fractions (eluting between 200 and 280 mM KCl) that exhibited activity in presplicing-complex formation (11). Lower concentrations of SF3a were found in fractions enriched in U1 snRNP (350 mM KCl) which also contained the 17S U2 particle. Fractions highly enriched in the 12S U2 snRNP (450 mM KCl) were devoid of SF3a. Mono Q chromatography can separate specific proteins from snRNPs (16), and it is likely that SF3a dissociates from U2 snRNP as a result of the salt conditions used during chromatographic fractionation. A similar observation has been made in the case of the [U4/U6.U5]

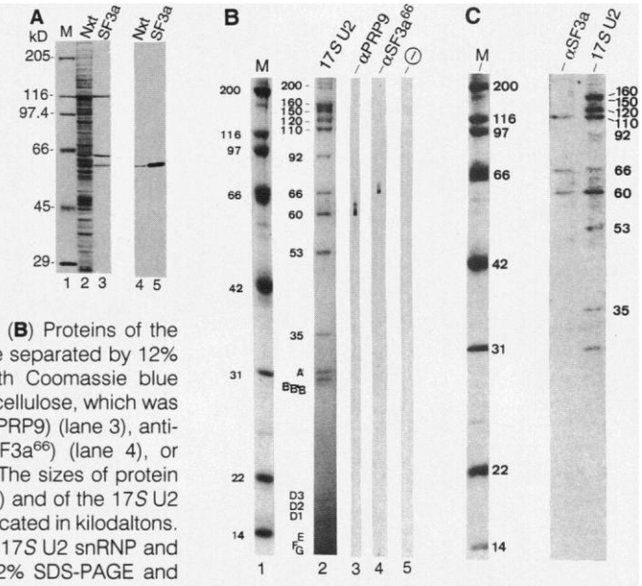

Fig. 1. Comparison of polypeptides associated with SF3a activity and the 17S U2 snRNP. (**A**) Nuclear extract (Nxt) and purified SF3a were separated by 7.5% SDS-PAGE and stained with silver (lanes 2 and 3) or blotted onto nitrocellulose and treated with anti-PRP9 (lanes 4 and 5) (10). The sizes of protein markers (Sigma) (M, lane 1) are indicated on the left. (**B**) Proteins of the purified 17S U2 snRNP were separated by 12% SDS-PAGE and stained with Coomassie blue (lane 2) or blotted onto nitrocellulose, which was incubated with anti-PRP9 (αPRP9) (lane 3), antibodies against $SF3a^{66}$ (αSF3a^{66}) (lane 4), or preimmune serum (lane 5). The sizes of protein markers (BioRad) (M, lane 1) and of the 17S U2 snRNP polypeptides are indicated in kilodaltons. (**C**) Polypeptides of purified 17S U2 snRNP and SF3a were separated by 12% SDS-PAGE and stained with Coomassie blue. The difference in size assigned to the 110-kD 17S U2 snRNP polypeptide and $SF3a^{120}$ is a result of the different gel systems used (10, 12). Molecular sizes are indicated in kilodaltons.

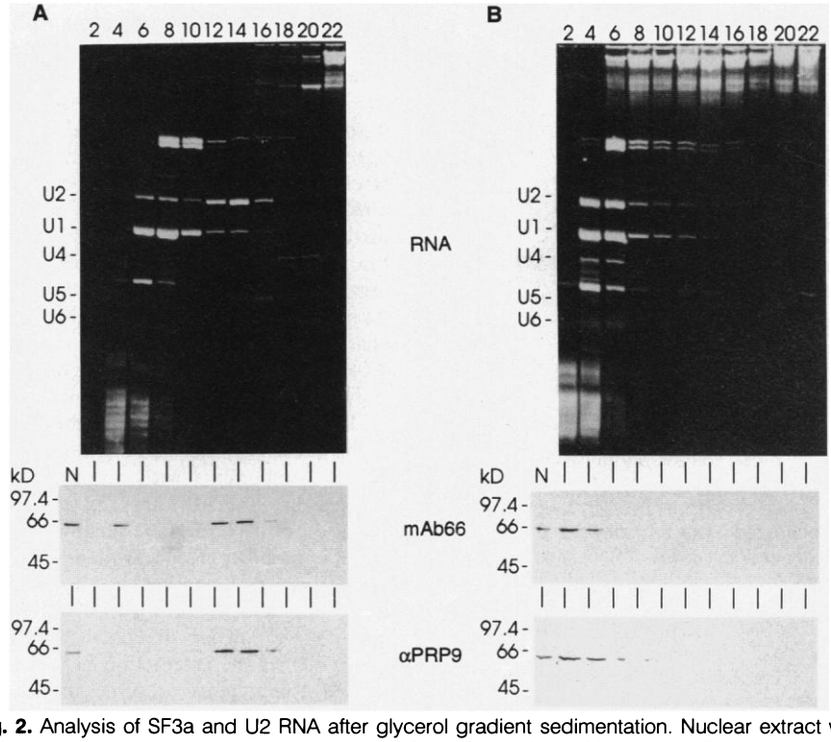

Fig. 2. Analysis of SF3a and U2 RNA after glycerol gradient sedimentation. Nuclear extract was sedimented in glycerol gradients containing 150 mM KCl (**A**) or 500 mM KCl (**B**) (14). Fractions (numbers above lanes) were analyzed for RNA content and cross-reactivity with the SF3a antibody (mAb66) and anti-PRP9 (αPRP9). N, nuclear extract.

triple snRNP–specific polypeptides (*17, 18*). These proteins are functionally equivalent to a splicing activity (heat shock labile splicing factor) that is inactivated during the heat shock of HeLa cells. Heat shock also affects U2 snRNP by converting it to a particle of fast electrophoretic mobility (*19*). Thus, the integration of the spliceosomal snRNPs into splicing complexes could be regulated by the association-dissociation of snRNP-specific polypeptides and splicing factors depending on the state of the cell. Both constitutive and alternative splicing could be influenced by the availability of such protein factors in different tissues or at different developmental stages of an organism.

We next tested whether purified SF3a could bind to U2 snRNP. Fractions containing SF3a or SF3b or both were incubated with U2 snRNP followed by the addition of a radiolabeled oligoribonucleotide complementary to the 5′ end of U2 RNA. This label allowed us to detect the RNA after native PAGE (*20*). A major and a minor form of U2 snRNP were detected in a nuclear extract in the upper third of the gel (Fig. 3) (*21, 22*). The 17S U2 snRNP present in the Mono Q–U1 fraction, used as a control, migrated slightly faster than the major form detected in the nuclear extract, whereas the U2 snRNP of the Mono Q–U2 fraction was detected at the bottom of the gel. When this fraction was incubated with SF3a, no change in electrophoretic mobility was apparent, indicating that SF3a by itself does not stably bind to U2 snRNP. However, when U2 snRNP was incubated with a fraction that contained SF3a and SF3b (Mono Q–SF3), two bands of low electrophoretic mobility appeared, suggesting that SF3b might be required for the SF3a–U2 snRNP interaction. When U2 snRNP was incubated with partially purified SF3b, U2 RNA comigrated with the lower complex formed in the presence of the Mono Q–SF3 fraction. Titration of SF3a into this reaction mixture converted the complex to a slower migrating form. The latter form of U2 snRNP comigrated with the major U2 complex detected in nuclear extract and with 17S U2 snRNP. The interactions of SF3b and SF3a with U2 snRNP appear to be specific for the U2 particle because no change in the electrophoretic mobility of U1 snRNP was observed in a similar experiment (*11*). Thus, a component or components present in the SF3b fraction prepare U2 snRNP for an interaction with SF3a, consistent with the finding that both splicing factors are required for presplicing-complex assembly (*3*). The finding that SF3b also participates in the formation of the 17S U2 snRNP suggests that this fraction contains some or all of the remaining 17S U2–specific proteins, although other possibilities cannot be ruled out. It should be possible to clarify this issue once SF3b is available in pure form. In any case, our results indicate that the assembly of the 17S U2 snRNP can be separated into at least two steps: the action of SF3b followed by the binding of SF3a.

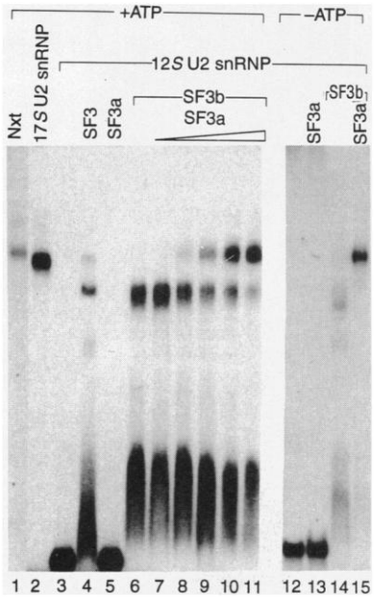

Fig. 3. Analysis of the interaction of SF3a and SF3b with 12*S* U2 snRNP. Nuclear extract and fractions containing U2 snRNP, SF3a, and SF3b as indicated above the figure were incubated with 0.5 pmol of a radiolabeled oligoribonucleotide complementary to the 5′ end of U2 RNA (*20*) in the presence (lanes 1 through 11) or absence of ATP (lanes 12 through 15). The fractions used were as follows: Nxt, 0.5 μl of nuclear extract; 17*S* U2 snRNP, 1 μl of a Mono Q fraction containing 17*S* U2 snRNP; 12*S* U2 snRNP, 1 μl of a Mono Q fraction containing 12*S* U2 snRNP (~15 pmol of U2 RNA); SF3, 4 μl of a Mono Q fraction containing both SF3a and SF3b; SF3a, Superose 12–purified SF3a (*3*) in amounts of 145 ng (lane 5) and 7, 35, 70, 110, and 145 ng (lanes 7 through 11, respectively); and SF3b, 1 μl of Mono S–purified SF3b. Reactions shown in lanes 13 and 15 contained 2.75 μl of Mono S–purified SF3a.

The association of SF3a and SF3b with the 12S U2 snRNP most likely corresponds to the previously described interaction of nuclear components with an in vitro–assembled U2 snRNP that results in the formation of a U2 particle of slow electrophoretic mobility (*23*). A difference between these two approaches, however, is the requirement for ATP to form the low-mobility U2 snRNP in nuclear extracts (*23*), whereas the interaction between the isolated components occurred in the absence of ATP (Fig. 3). A similar discrepancy has been observed for the assembly of the [U4/U6.U5] triple snRNP (*17*).

The ATP-independent formation of the active 17S U2 snRNP cannot account for the ATP requirement observed for presplicing-complex formation (*24*). Binding of U1 snRNP to the 5′ splice site as well as binding of U2AF to the polypyrimidine tract located upstream of the 3′ splice site occur in the absence of ATP (*5, 25*). Hence, the interactions of U1 snRNP and U2AF with the pre-mRNA and the assembly of the 17S U2 snRNP most likely precede the association of mammalian splicing factor SC35 with the prespliceosome, a reaction that is ATP-dependent (*26*).

The cross-reaction of anti-PRP9 with the 60-kD polypeptide of SF3a and the 17S U2 snRNP suggests that this polypeptide represents the human homolog of the yeast splicing protein PRP9. Both polypeptides also share significant homologies in their deduced amino acid sequences (*27*). An immunological relation to PRP9 has also been observed for SAP61 (*28*), a protein component of the mammalian presplicing complex (*29*). SAP61 corresponds to $SF3a^{60}$, as shown by co-electrophoresis of these polypeptides in 2D gels (*30*), and $SF3a^{66}$ and $SF3a^{120}$ are identical to SAP62 and SAP114, respectively. The protein SAP62 appears to be the human homolog of the yeast splicing protein PRP11 (*28*). Thus, SF3a activity can be correlated with two yeast proteins that are both required for the assembly of the ATP-dependent presplicing complex (*9, 31*).

Genetic and physical interactions between the yeast splicing proteins PRP5, PRP9, PRP11, and SPP91/PRP21 that function at the onset of the splicing reaction have recently been established (*32*). With the caveat in mind that more proteins have been identified in mammalian cells than in yeast that function in presplicing-complex formation, $SF3a^{120}$ could be related to either PRP5 or SPP91/PRP21. Cloning of the cDNA encoding the 120-kD polypeptide should clarify this issue. Given the structural relation between the mammalian and yeast splicing factors, it now becomes feasible to analyze whether this similarity extends to a conservation of their function during the splicing reaction.

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12. The 17*S* U2 snRNP was purified as described (*6*). Proteins were isolated by phenol-chloroform extraction and acetone precipitation and separated by SDS-PAGE [T. Lehmeier, K. Foulaki, R. Lührmann, *Nucleic Acids Res.* **18**, 6475 (1990)]. Western blotting with anti-PRP9 and mAb66 was performed as described (*8*).
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14. We adjusted nuclear extract (600 μl in buffer D) [J. D. Dignam, R. M. Lebovitz, R. G. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983)] to 15% (v/v) glycerol and 150 mM or 500 mM KCl by increasing the total sample volume to 1.2 ml with appropriate buffers; buffer D contains 20% (v/v) glycerol, 20 mM Hepes-KOH (pH 7.9), 100 mM KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol (*15*). The samples were loaded onto 12-ml gradients containing 15 to 40% (v/v) glycerol, 20 mM Hepes-KOH (pH 7.9), 150 (or 500) mM KCl, and 3 mM $MgCl_2$. Sedimentation was for 22 hours at 36,000 rpm at 4°C in a SW40 rotor (Beckman). Fractions of 500 μl were collected from top to bottom. Size standards were sedimented in parallel gradients. From every second fraction (400 μl), RNA was isolated by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. We then separated RNAs in denaturing 10% polyacrylamide gels and stained them with ethidium bromide. Proteins from 10 μl of every second fraction were analyzed by Western blotting (*10*).
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33. We thank P. Legrain for PRP9 antibodies, A. Lamond for synthetic oligoribonucleotides, and G. Bilbe for comments on the manuscript. We acknowledge communication of unpublished results by J. Abelson, P. Legrain, R. Reed, and S. Ruby. Supported by the Schweizerischer Nationalfonds and the Kantons of Basel-Stadt, Basel-Landschaft and Geneva (A.K.); the Deutsche Forschungsgemeinschaft (SFB 272/A3) and the Fonds der Chemischen Industrie (R.L.); and the Boehringer Ingelheim Fonds (K.G.).

19 April 1993; accepted 22 July 1993

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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