Cef1p Is a Component of the Prp19p-associated Complex and Essential for Pre-mRNA Splicing*

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The Prp19p protein of the budding yeast Saccharomyces cerevisiae is an essential splicing factor and is associated with the spliceosome during the splicing reaction. We have previously shown that Prp19p is not tightly associated with small nuclear ribonucleoprotein particles but is associated with a protein complex consisting of at least eight protein components. By sequencing components of the affinity-purified complex, we have identified Cef1p as a component of the Prp19p-associated complex, Ntc85p. Cef1p could directly interact with Prp19p and was required for pre-mRNA splicing both in vivo and in vitro. The c-Myb DNA binding motif at the amino terminus of Cef1p was required for cellular growth but not for interaction of Cef1p with Prp19p or Cef1p self-interaction. We have identified a small region of 30 amino acid residues near the carboxyl terminus required for both cell viability and protein-protein interactions. Cef1p was associated with the spliceosome in the same manner as Prp19p, i.e. concomitant with or immediately after dissociation of U4. The anti-Cef1p antibody inhibited binding to the spliceosome of Cef1p, Prp19p, and at least three other components of the Prp19p-associated complex, suggesting that the Prp19p-associated complex is likely associated with the spliceosome and functions as an integral complex.

The eukaryotic spliceosome is a multicomponent ribonucleoprotein particle composed of five small nuclear RNAs, U1, U2, U4/U6, and U5, and a number of protein factors (for reviews, see Refs. 1–6). Spliceosome assembly is a stepwise process involving sequential binding of small nuclear RNAs and protein factors (7–13). During spliceosome assembly, U1 first binds to the 5′ splice site followed by binding of U2 to the branch site through base pair interactions between the small nuclear RNAs and the intron sequences. U4/U6 and U5 are then added to the spliceosome as a preformed three-small nuclear RNP particle. This triggers a conformational rearrangement of the spliceosome in which base pairing of U1 with the 5′ splice site is replaced by U6 and U2 (14–17). U1 and U4 thus become only loosely associated with the spliceosome, which is now activated and ready for catalytic reactions. It is believed that such structural rearrangements of the spliceosome are mediated by protein factors. Although several proteins containing the DEXD/H box motif have been shown to unwind RNAs (6, 18–21), no substrate specificity could be demonstrated. It remains a question what dictates the substrate specificity and mediates conformational rearrangement of the spliceosome during spliceosome assembly.

We have previously shown that the yeast Saccharomyces cerevisiae Prp19p protein is essential for pre-mRNA splicing and is required before the first step of the splicing reaction. Prp19p is not tightly associated with small nuclear RNAs but is associated with the spliceosome immediately after or concomitant with dissociation of U4 from the spliceosome, suggesting a possible role in mediating structural rearrangement of the spliceosome during U4 dissociation (22). In splicing extracts, a protein complex consisting of at least eight polypeptides was found associated with Prp19p, and at least one of these proteins is also essential for the in vitro splicing reaction (23). Genetic screening of synthetic lethal mutants to prp19 mutations has identified a novel splicing factor Snt309p as a component of the Prp19p-associated complex, Ntc25p (Ntc stands for PRP nineteen complex). Snt309p is encoded by a nonessential gene and also has no functional motif in its protein sequence. Snt309p is associated with the spliceosome in the same manner as Prp19p, suggesting a coordinate action of these two proteins (24).

By sequencing components of the purified Prp19p-associated complex, we show here that the CEF1 gene encodes another component, Ntc85p, of the Prp19p-associated complex. Cef1p belongs to a family of Myb-related proteins, which contain two copies of the c-Myb DNA binding motif at their amino termini and are highly homologous to the fission yeast Schizosaccharomyces pombe Cdc5p protein (25). We show that Cef1p is required for splicing both in vivo and in vitro. The c-Myb DNA binding motif was required for cellular growth but not for interaction of Cef1p with Prp19p or for Cef1p self-interaction. We have also identified a small region of the protein near the carboxyl terminus of Cef1p required both for cellular growth and for protein-protein interactions. Like Snt309p, Cef1p is also associated with the spliceosome in the same fashion as Prp19p. Moreover, anti-Cef1p antibody inhibited binding of all identified components of the Prp19p-associated complex to the spliceosome, which further suggests that the Prp19p-associated complex likely functions as an integral complex.

**EXPERIMENTAL PROCEDURES**

**Strains**—The strains used were as follows: YSCC1, Mata pep1 prb1 pep4 leu2 trpl ura3 prp19-HA; SEY6210.5, Mata/lacZ-leu2/leu2 ura3/ura3 his3/his3 trpl1 trpl1 suc2/suc2 lys2/lys2 ADE2/ade2; EGY48, Mata/ura3 his3/trpl1 LexAop-leu2 ade2; WT5141, Mata/ura3

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Cef1p Interacts with Prp19p and Is Essential for Splicing

Two-hybrid Assays—The Prp19p, CEF1, and CEF1 deletion genes were fused to the LexA DNA binding domain in plasmid pEG202 and the GAL4 activation domain in plasmid pACT2, and each pair of plasmids was transformed into yeast strain EGY48 together with the β-galactosidase reporter plasmid pHIS1-34. Two-hybrid assays were carried out according to the Matchmaker system by CLONTECH. At least three transformants from each pair of plasmid were assayed for β-galactosidase activity in liquid culture.

Puriﬁcation of the Prp19p-associated Complex—The Prp19p-associated complex was puriﬁed according to the procedure described by Cheng et al. (26). The pellet fraction (40P) was chromatography on an anti-HA antibody column (23). The complex, a yeast strain in which the Prp19p protein was tagged at amino acid residues 49–163 of CEF1 in pWT91; pBDN3, deletion of amino acid residues 490–570 of CEF1 in pWT86; pADC3, deletion of amino acid residues 163–281 of CEF1 in pWT86; pBDN2, deletion of amino acid residues 459–570 of CEF1 in pWT91; pBTC1, deletion of amino acid residues 490–570 of CEF1 in pWT91; pBDN1, deletion of amino acid residues 94–163 of CEF1 in pWT85; pBDN3, deletion of amino acid residues 163–281 of CEF1 in pWT86; pBTC3, deletion of amino acid residues 459–570 of CEF1 in pWT85; pBTC2, deletion of amino acid residues 459–570 of CEF1 in pWT91; pBTC1, deletion of amino acid residues 1–94 of CEF1 in pWT86; pBTC2, deletion of amino acid residues 94–163 of CEF1 in pWT86; pADC3, deletion of amino acid residues 163–281 of CEF1 in pWT86; pADC2, deletion of amino acid residues 459–570 of CEF1 in pWT86; pADC1, deletion of amino acid residues 490–570 of CEF1 in pWT86.

Puriﬁcation of the Prp19p-associated Complex—The Prp19p-associated complex was puriﬁed according to the procedure described by Tarn et al. (23) with some modiﬁcations for large scale preparations. 50 liters of yeast culture of strain YSC11-1 were grown in a 75-liter fermenter under the control of the TR promoter. Total lysates prepared from induced cells were centrifuged at 10,000 × g for 10 min. The supernatant was then used without further puriﬁcation for yeast two-hybrid assays.

Isolation of the Spliceosome by Precipitation with Streptavidin-Agarose Beads—Poly(A)+ RNA was isolated from large scale splicing reactions by precipitation with streptavidin-agarose beads according to the procedure of Brumme and Bond (23). The RNA was then puriﬁed by centrifugation through a 50% sucrose cushion in a Beckman 59Ti rotor at 20,000 × g for 30 min.

Identification of Cef1p as Ntc85p of the Prp19p-associated Complex—To identify the components of the Prp19p-associated complex, a yeast strain in which the Prp19p protein was tagged with the HA-epitope at its carboxyl terminus was puriﬁed in plasmid pACT1 for preparation of spliceosome extracts. The Prp19p-associated complex was puriﬁed from these extracts by afﬁnity chromatography on an anti-HA antibody column (23). The yeast strain was transformed with each deletion plasmid encoding HA-tagged Prp19p and CEF1 deletion genes, and each pair of plasmids was transformed into yeast strain EGY48 together with the β-galactosidase reporter plasmid pHIS1-34. Two-hybrid assays were carried out according to the Matchmaker system by CLONTECH. At least three transformants from each pair of plasmid were assayed for β-galactosidase activity in liquid culture.

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RESULTS

Identification of Cef1p as Ntc85p of the Prp19p-associated Complex—To identify the components of the Prp19p-associated complex, a yeast strain in which the Prp19p protein was tagged with the HA-epitope at its carboxyl terminus was puriﬁed in plasmid pACT1 for preparation of spliceosome extracts. The Prp19p-associated complex was puriﬁed from these extracts by afﬁnity chromatography on an anti-HA antibody column (23). The components of the complex were fractionated on SDS-polyacrylamide gel electrophoresis and electroblotted to polyvinylidene difluoride membranes for microsequencing. Sequences obtained were compared with the yeast genome data base. One peptide
sequence PVPIYVKGEVWTNV obtained from Ntc85p was identified to be encoded within the CEF1 gene (25).

We have previously shown that Prp19p interacts with Ntc85p on Far Western blots when Prp19p was used as a probe (23). To determine whether CEF1 indeed encodes Ntc85p, the interaction between Prp19p and Cef1p was examined by Far Western blotting using Cef1p as a probe. A DNA fragment containing CEF1 and 460 base pairs of its downstream sequence was retrieved by polymerase chain reaction using oligonucleotides 85-1 and 85-2 and cloned into plasmid pGEM-1 for in vitro transcription and translation. Components of the Prp19p-associated complex were fractionated on SDS-polyacrylamide gel electrophoresis and electroblotted to polyvinylidene difluoride membranes for Far Western blot analysis using in vitro translated 35S-labeled Cef1p or Prp19p as probes. As shown in Fig. 1A, when Cef1p was used as a probe, interaction of Cef1p with Prp19p was detected. While Prp19p could interact with five proteins, Ntc85p, Ntc40p, Ntc25p, Ntc20p, and itself, in the Prp19p-associated complex, Cef1p could only interact with Prp19p. No interaction with other components of the complex was detected in this analysis. The fact that Cef1p interacted with Prp19p and the in vitro translated Cef1p migrated as 85 kDa on SDS-polyacrylamide gel electrophoresis (data not shown) strongly suggested that Cef1p is Ntc85p of the Prp19p-associated complex.

To verify that Cef1p is Ntc85p of the Prp19p-associated complex, antibodies were raised against recombinant Cef1p for Western blot analysis of the Prp19p-associated complex. As shown in Fig. 1B, while preimmune serum did not react with any protein, the anti-Cef1p antibody indeed reacted with Ntc85p of the complex. Preincubation of the antibody with the recombinant Cef1p protein blocked its reaction with Ntc85p. This confirms that CEF1 indeed encodes Ntc85p of the Prp19p-associated complex.

The Amino-terminal Conserved Region and a Small Region Near the Carboxyl Terminus Are Required for Cef1p Function—Cef1p is a protein of 590 amino acids and belongs to a family of Myb-related proteins identified in yeast, Caenorhabditis elegans, Arabidopsis, and humans (25, 31–33). These proteins contain two copies of the c-Myb DNA binding motif at their amino termini (25). Cef1p is 48% identical to the fission yeast S. pombe Cdc5p protein over a region of 267 amino acid residues in its amino terminus (25, 33). The carboxyl-terminal halves of these proteins are less alike.

The CEF1 gene is essential for vegetative yeast growth. It has been shown that multiple point mutations of tryptophan residues located within the c-Myb DNA binding motif impair cellular growth, indicating that the c-Myb DNA binding domain is required for Cef1p function (25). To examine the importance of other regions of the protein, we performed deletion analysis for the in vivo function of Cef1p. The CEF1 gene under the control of its own promoter in pRS414 was deleted in different regions and transformed into strain WT85161, which contained a null allele of the CEF1 gene on the chromosome and a wild-type copy of the CEF1 gene under the control of its own promoter in plasmid pRS416. These transformants were plated on 5-fluoro-orotic acid plates for selection of loss of the wild-type CEF1-containing plasmid after growth in the absence of uracil selection.

As shown in Fig. 2, deletion of amino acid residues 8–93 (N1) within the c-Myb DNA binding motif failed to rescue lethality of CEF1 disruption, indicating that the Myb motif is essential for yeast growth. This is consistent with that reported by Ohi et al. (25). Deletion of other regions of the NH2-terminal half conserved sequence of the protein (N2 and N3) also resulted in cell lethality. The OOCH-terminal half of Cef1p, being less conserved among the proteins of this family, appeared to be less important, since deletion of most of the sequence (C1 or C3) did not affect growth. However, a small region, C2, at amino acid residues 460–490, is absolutely essential, since deletion of both C1 and C2 resulted in lethality. Thus, the NH2-terminal half of the conserved region and approximately 30 amino acids in the COOH-terminal half of the protein are important for the function of Cef1p in vivo.

Interaction of Cef1p with Prp19p and with Itself Requires the

FIG. 1. A, far Western blotting of the Prp19p-associated complex using Prp19p (lane 1) or Cef1p (lane 2) as probes. B, Western blotting of the Prp19p-associated complex using anti-Cef1p antibody as a probe. Lane 1 shows the complex stained by silver. Lanes 2–4 show Western blot. Pre, preimmune serum; Imm, immune serum.
Carboxyl-terminal Essential Region—Since Cef1p is a component of the Prp19p-associated complex and can directly interact with Prp19p, we asked whether its interaction with Prp19p correlated with its function. We used the yeast two-hybrid assay to examine the requirement of various regions of Cef1p for interaction between Cef1p and Prp19p. Prp19p and Cef1p, including its truncated versions, were fused to both LexA DNA binding domain (BD) and GAL4 activation domain (AD) individually. Interactions between Cef1p and Prp19p were assayed by measuring the β-galactosidase activity. Fig. 3A shows that interaction between Prp19p and Cef1p could be detected by the two-hybrid assay when Prp19p was fused to the binding domain and Cef1p was fused to the activation domain and vice versa. Combination of Cef1p-BD and Prp19p-AD seemed to give higher β-galactosidase activity than that of Cef1p-AD and Prp19p-BD. Furthermore, most Cef1p deletions fused to the activation domain affected interactions more than when fused to the LexA DNA binding domain. The cause of such differences is not clear. Therefore, evaluation of the effect of deletion was mostly based on deletions in Cef1p-BD except for dN1, whose fusion to the binding domain was not available.

Deletion analysis revealed that at least one region of the Cef1p protein is essential for interaction. In the NH₂-terminal conserved half of the protein, deletion of the c-Myb DNA binding domain (N1) or deletion of N3 did not strongly affect the level of β-galactosidase expression. Thus, the c-Myb DNA binding domain is not required for interaction of Cef1p with Prp19p. In contrast, deletion of approximately 70 amino acids behind the Myb domain (N2) drastically decreased the β-galactosidase activity. In the carboxyl half of the protein, while deletion of C1 alone only had a moderate effect, deletion of both C1 and C2 resulted in no β-galactosidase activity. Deletion of C3 also only partially affected the β-galactosidase expression. Failure in detecting the β-galactosidase activity in dN2 and dC1,2 may be attributed to loss of its ability to interact with Prp19p or instability of the fusion proteins. The possibility of protein instability for dC1,2 was ruled out by immunoblot analysis, which detected the fusion protein of dC1,2 in cell lysates at levels comparable with others (data not shown). Therefore, it is suggested that the C2 region is essential for Cef1p-Prp19p interaction. The dN2 fusion protein, however, was not present at detectable level in total yeast lysates (data not shown).
though this does not disprove the essential role of N2 in Cef1p-Prp19p interaction in vivo, the requirement of N2 for such interaction cannot be concluded.

In Fig. 1, Cef1p was detected to interact only with Prp19p by Far Western blotting. By two-hybrid assays, self-interaction of Cef1p was observed as shown in Fig. 3B. Interestingly, deletion of either N2 or C2 also abolished self-interaction of Cef1p. This indicates that the C2 region is responsible for both Cef1p-Prp19p interaction and Cef1p self-interaction. The requirement for N2 remains undetermined for the same reason of protein instability. Deletion of N3, however, seemed to weaken self-interaction of Cef1p considerably despite slight enhancement upon interaction between Prp19p and Cef1p. In contrast, C1 and C3 deletions, when fused to the DNA binding domain, enhanced self-interaction of Cef1p but weakened its interaction with Prp19p. This may indicate a competition between Cef1p-Prp19p interaction and Cef1p self-interaction, since both interactions involved the same region(s) of the Cef1p protein.

CEF1 Was Required for Pre-mRNA Splicing in Vivo—Since Cef1p is a component of the Prp19p-associated complex, it is likely that Cef1p is also a splicing factor. To examine whether Cef1p is required for pre-mRNA splicing, yeast strain WT8501, which contained a null allele of the CEF1 gene on the chromosome and a wild-type copy of the CEF1 gene under the control of the inducible GAL1 promoter on plasmid pRS414, was grown in galactose medium to mid-log phase and then either continuously grown in galactose or shifted to glucose medium to repress the CEF1 gene. RNA was isolated at different times after the shift and analyzed for accumulation of pre-mRNA by Northern blot analysis using actin as a probe. 10 μg of RNA was loaded in each lane.

RNA, IVS-E2, IVS, and the excised E1 were efficiently precipitated by the anti-Cef1p antibody, but only a small amount of the mature message was precipitated, indicating precipitation of the spliceosome (lane 4). No RNA was precipitated with the preimmune serum (lane 3) or when the antibody was preincubated with recombinant Cef1p (lane 5). These results indicate that the spliceosome was specifically precipitated by the anti-Cef1p antibody. Thus, Cef1p was also associated with the spliceosome during the splicing reaction and remained associated until the reaction was completed.

We have previously shown that Prp19p is associated with the spliceosome concurrently with or immediately after dissociation of U4 during spliceosome assembly (22). A previously identified component of the Prp19p-associated complex, Sn309p, was also shown to associate with the spliceosome in the same manner as Prp19p (24). This raises the possibility that the Prp19p-associated complex may function as an integral complex and all associated components bind to the spliceosome simultaneously. We have previously demonstrated that dissociation of U4 is highly sensitive to ATP concentrations (22). At lower concentrations of ATP, U4 dissociation is blocked, and the U4-containing splicing complex A2–1 accumulates in larger amounts as shown in Fig. 6A. When ATP concentration increases, transition from complex A2–1 to A1 is rapid, and only a small amount of complex A2–1 accumulates (22, 24). This can be revealed by immunoprecipitation of the spliceosome with the anti-Prp4p antibody, since Prp4p is tightly associated with U4 and therefore is only present in complex A2–1 (22). In contrast, Prp19p was detected in splicing complexes at higher ATP concentrations and to a much lesser extent at lower ATP concentrations (Fig. 6B; Ref. 24). To examine whether Cef1p is associated with the spliceosome in the same fashion as Prp19p, immunoprecipitation was performed with the anti-Cef1p antibody to reveal binding of Cef1p to the spliceosome. Splicing reactions were carried out at various ATP concentrations in the extract prepared from a strain in which Prp19p was tagged with the HA-epitope so that binding of Prp19p to the spliceosome could be monitored by the anti-HA antibody. Fig. 6B shows that immunoprecipitation of the spliceosome by the anti-HA and anti-Cef1p antibodies followed the same pattern in the course of ATP titration. The amount of the spliceosome precipitated by both antibodies decreased with decreasing ATP concentrations. These results indicate that like
Prp19p, Cef1p was associated with the spliceosome concomitant with or immediately after U4 dissociation.

Cef1p Was Essential for Pre-mRNA Splicing in Vitro and Was Required for Binding of the Prp19p-associated Components to the Spliceosome—Since CEF1 is essential for yeast growth and is required for splicing in vivo, it is possible that Cef1p plays an essential role in the pre-mRNA splicing reaction. We therefore examined the function of Cef1p in the in vitro splicing reaction by using the anti-Cef1p antibody. Fig. 7 shows that preincubation of the extract with the anti-Cef1p antibody inhibited the splicing activity. Increasing the amount of the antibody increased the level of inhibition (lanes 2–5). Preincubation of the extract with an unrelated antibody had no effect on the splicing reaction (lane 6). Furthermore, the inhibition was antagonized by preincubation of 150 ng of the antibody (as in lane 5) with 300 ng of the recombinant Cef1p protein prior to incubation with the extract (lane 7), suggesting that the inhibition was specific for Cef1p. These results indicate that Cef1p is essential for the splicing reaction in vitro.

To determine whether inhibition of the splicing reaction by the anti-Cef1p antibody was due to blocking the binding of Cef1p to the spliceosome or inhibition of the Cef1p function after its binding to the spliceosome, the spliceosome was formed on biotinylated pre-mRNA and then isolated by precipitation with streptavidin-agarose. The association of Cef1p with the spliceosome in the presence or absence of the anti-Cef1p antibody was examined by Western blotting. As shown in Fig. 8, Cef1p was detected in the spliceosome in the absence of the antibody when the pre-mRNA was biotinylated (lane 3). The amount of Cef1p associated with the spliceosome greatly diminished if the extract was preincubated with the anti-Cef1p antibody (lane 5). Control experiments using nonbiotinylated pre-mRNA did not precipitate any Cef1p (lanes 2 and 4). This indicates that binding of the antibody to Cef1p prevented binding of Cef1p to the spliceosome.

In Fig. 6 we show that Cef1p was associated with the spliceosome in the same manner as Prp19p during spliceosome assembly. At least three other components of the Prp19p-associated complex, Snt309p, Ntc20p and Ntc30p, were also found to be associated with the spliceosome in the same manner (24). This suggests a possibility that all components of the Prp19p-associated complex were added to the spliceosome as an integral complex. In such a case, failure of binding of Cef1p to the

Footnote:
2 W.-Y. Tsai, C.-H. Chen, and S.-C. Cheng, unpublished results.
Cef1p Interacts with Prp19p and Is Essential for Splicing

The Prp19p protein was previously shown to be essential for the in vitro splicing reaction (30). In splicing extracts, Prp19p is associated with at least seven proteins in a large complex, and its association with these components is important for its function (23). At least one of these associated components is also required for splicing (23). A genetic screen for synthetic lethal mutants at prp19 has identified a component of the Prp19p-associated complex, Snt309p, which can directly interact with Prp19p and bind to the spliceosome in the same manner as Prp19p (24). Snt309p, although not essential for vegetative yeast growth, appears to play a role in modulating interactions of Prp19p with its associated components. Two other components identified in the genetic screen, Ntc20p and Ntc30p, are also encoded by nonessential genes.

Distinct from those proteins identified by genetic screening, Cef1p was identified by biochemical sequencing of components of the Prp19p-associated complex. Cef1p is encoded by an essential gene and is required for splicing both in vitro and in vivo. The CEF1 gene did not complement any of the 15 synthetic lethal mutants isolated in our screening (data not shown), suggesting that CEF1 cannot be identified using this genetic method. Since CEF1 is essential for cellular growth, it is possible that cef1 mutants might have grown poorly and escaped selection during the isolation of synthetic lethal mutants. Biochemical methods to identify these components, although somewhat inefficient in isolating large amounts of proteins due to low abundance, appear to be necessary to define the full spectrum of the Prp19p-associated complex.

Cef1p contains the c-Myb DNA binding motif at its amino terminus and is highly homologous to the fission yeast Cdc5p protein in the amino half of the protein with 48% identity over a region of 267 amino acid residues. Homologues of Cdc5p have been identified in humans, Drosophila, C. elegans, and Arabidopsis (25, 31, 32). Although the recombinant Arabidopsis Cdc5p protein (AtCDC5) is shown to possess sequence-specific DNA binding activity and recognize the DNA sequence CT-CAGCG, which is distinct from the recognition sequence by Myb oncoproteins (32), high affinity DNA binding of specific sequences could not be demonstrated for Cef1p or its human homolog (25). Nevertheless, mutational analysis and our deletion analysis showed that the c-Myb DNA binding domain is essential for cellular growth (25). The role of the c-Myb DNA binding domain of Cef1p therefore is puzzling but intriguing. It is possible that Cef1p might bind DNA without stringent specificity and be involved in other biological functions in addition to splicing. Alternatively, as a splicing factor, the Myb motif may be involved in binding of RNA during spliceosome assembly.

**DISCUSSION**

spliceosome in the presence of the anti-Cef1p antibody may reflect a failure in binding of the entire Prp19p-associated complex. As shown in the Western blot of Fig. 8A, binding of Prp19p, Snt309p, Ntc20p, and Ntc30p to the spliceosome was also inhibited by the anti-Cef1p antibody (lane 5). To rule out the possibility that such inhibition was due to dissociation of the Prp19p-associated complex upon binding of the antibody to Cef1p, components precipitated by the anti-Cef1p antibody were examined by immunoblotting. Splicing extracts were preincubated with the affinity-purified anti-Cef1p antibody followed by precipitation with protein A-Sepharose. The precipitates were analyzed by immunoblotting using a mixture of antibodies against all identified components of the Prp19p-associated complex. As shown in Fig. 8B, antibodies added in the amount of 3 ng (lane 3) or 150 ng (lane 5) per μl of the splicing extract, the latter corresponding to the amount used for inhibition of splicing, did precipitate all identified components of the Prp19p-associated complex, suggesting that the Prp19p-associated complex remained intact upon binding of the anti-Cef1p antibody. This strengthens the notion that the Prp19p-associated complex is associated with the spliceosome as an integral complex and further suggests that Cef1p is required for binding of the complex to the spliceosome. Thus, Cef1p may play a role in promoting binding of the Prp19p-associated complex to the spliceosome.

**Fig. 8.** A, Western blotting of the affinity-purified spliceosome using mixtures of anti-Cef1p, anti-Prp19p, anti-Ntc30p, anti-Snt309p, and anti-Ntc20p antibodies as probes. Pre-mRNA was biotinylated (lanes 3 and 5) or nonbiotinylated (lanes 2 and 4). Splicing extracts were preincubated with (lanes 4 and 5) or without (lanes 2 and 3) the anti-Cef1p antibody. In each experiment, 800 μl of the splicing reaction mixture, containing 400 μl of the splicing extract, was precipitated with 250 μl of streptavidin-agarose. B, Western blotting of the components precipitated by the anti-Cef1p antibody. Splicing extracts were incubated with 3 ng (lanes 2 and 3) or 150 ng (lanes 4 and 5) of the antibody per μl of the extract prior to incubation with protein A-Sepharose. Lanes 2 and 4 are controls of incubation with no extracts. C, the Prp19p-associated complex; −E, without extract; +E, with extract.

3 H.-R. Chen, T. Y. Tsao, and S.-C. Cheng, manuscript in preparation.
4 W.-Y. Tsai and S.-C. Cheng, unpublished data.
bly. Our intensive efforts have failed to detect binding of Cef1p to the pre-mRNA by UV cross-linking (data not shown). Yet, the possibility of its binding to small nuclear RNAs during spliceosome assembly cannot be excluded. Whether the c-Myb DNA binding domain plays an important role in splicing is to be investigated.

It is interesting that the C2 region, encompassing only 31 amino acid residues and positioned in the less conserved C-terminus, is essential for both cellular growth and for protein-protein interaction. Examination of the sequence feature of this small region revealed the presence of four amino acids (EVES), a core sequence of the EVES motif present in the C terminus of c-Myb oncoproteins (34). The EVES motif was shown to be involved in interactions of Myb proteins with their interacting components and with themselves (34). Although the sequence surrounding EVES is not closely similar to the EVES consensus sequence, C2 may contain a variant of the mammalian EVES motif. The specificity of the EVES residues for viability and for protein-protein interactions remains to be studied. Interestingly, such EVES-like sequence is not present in other Myb-related proteins. In fact, the carboxyl half of the Cef1p protein is more divergent from those of the other Myb-related proteins. This may suggest that Cef1p and Cdc5p share partial homologues. It will be interesting to know whether other Myb-related proteins play a direct role in cell cycle progression.

In this report, we show that the C2 region is required both for cellular growth and for protein-protein interactions. Previously, we have also shown by Far Western blotting that a loss of function deletion of 91 amino acids from the N terminus of Prp19p also abolished its interactions with itself and with other Prp19p-associated components, including Cef1p (23). These results demonstrate a possible link between protein-protein interactions in the Prp19p-associated complex, at least for Cef1p and Prp19p, and cell viability, suggesting that interactions between these components are important for their functions.

The N3 region, although conserved and essential, is not required for Cef1p-Prp19p or Cef1p-Cef1p interaction. The functional role of N3 is not known. It was noted that deletion of N3 weakened self-interaction of Cef1p but enhanced its interaction with Prp19p. Conversely, deletion of C1 or C3 enhanced Cef1p self-interaction but weakened Cef1p-Prp19p interaction. Thus, except for the c-Myb DNA binding motif, whose deletion slightly weakened both Cef1p-Prp19p interaction and Cef1p self-interaction, other sequences not essential for protein-protein interaction affected Cef1p-Prp19p interaction and Cef1p self-interaction in an opposite way. This might indicate a competition between Cef1p-Prp19p interaction and Cef1p self-interaction, since both involved the same region(s) of the Cef1p protein. Such competition may be important in regulating formation of the Prp19p-associated complex, or binding of the complex to the spliceosome.

Like Snt309p, Cef1p is also associated with the spliceosome in the same manner as Prp19p. In fact, both Ntc20p and Ntc30p also bind to the spliceosome in a similar way. Furthermore, the anti-Cef1p antibody blocked binding of all known components of the Prp19p-associated complex to the spliceosome. These results strongly suggest that the Prp19p-associated complex is added to the spliceosome as an integral complex and that the associated components may function in a coordinate fashion, although a final conclusion cannot be drawn until all of the components of the Prp19p-associated complex are identified. Cef1p might play an important role in promoting binding of the Prp19p-associated complex to the spliceosome as the anti-Cef1p antibody prevented binding of the complex to the spliceosome. Since binding of the Prp19p-associated complex to the spliceosome requires interactions of its associated components with those already present in the spliceosome, Cef1p may be involved in mediating such interactions. Confirmation of whether Cef1p directly interacts with the pre-existing spliceosomal components awaits further study.

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