The Clf1p Splicing Factor Promotes Spliceosome Assembly through N-terminal Tetratricopeptide Repeat Contacts*

Qiang Wang‡, Kathryn Hobbs‡, Bert Lynn§, and Brian C. Rymond‡¶

From the Departments of ‡Biology and §Chemistry, University of Kentucky, Lexington, Kentucky 40506-0225

Spliceosome assembly follows a well conserved pathway of subunit addition that includes both small nuclear ribonucleoprotein (snRNP) particles and non-snRNP splicing factors. Clf1p is an unusual splicing factor composed almost entirely of direct repeats of the tetratricopeptide repeat (TPR) protein-binding motif. Here we show that the Clf1p protein resides in at least two multisubunit protein complexes, a small nuclear RNA-free structure similar to what was reported as the Prp19p complex (nineteen complex; NTC) and an RNP structure that contains the U2, U5, and U6 small nuclear RNAs. Thirty Ccf (Clf1p complex factor) proteins have been identified by mass spectroscopy or immune detection as known or suspected components of the yeast spliceosome. Deletion of TPR1 or TPR2 from an epitope-tagged Clf1p protein (i.e., Clf1Δ2-TAP) destabilizes Clf1p complexes assembled in vitro, causing the release of the Ccf1p and Prp19p NTC factors and decreased association of the Rso1p, Snu114p, and Hsh155p snRNP proteins. In vitro, temperature inactivation of Clf1Δ2p impairs the prespliceosome to spliceosome transition and prevents Prp19p recruitment to the spliceosome complex. These and related data support the view that the poly-TPR Clf1p splicing factor promotes the functional integration of the U4/U6.U5 tri-snRNP particle into the U1-, U2-dependent prespliceosome.

The spliceosome is composed of five small nuclear RNAs (snRNAs) and over 70 distinct polypeptides (reviewed in Refs. 1 and 2; see references in Refs. 3 and 4). Numerous studies have demonstrated that in vitro spliceosome assembly progresses through an ordered sequence that is largely conserved from yeast to humans. By tracking assembly through small nuclear ribonucleoprotein (snRNP) association with pre-mRNA, it has been shown that the U1 snRNP binds independently other snRNP particles in an ATP-independent step said to commit the pre-mRNA to the splicing pathway. U2 snRNP is recruited next in an ATP-dependent step that forms the prespliceosome. Finally, the U4, U5, and U6 snRNAs are jointly recruited as the exceptionally large U4/U6.U5 tri-snRNP particle to produce the spliceosome. Together, the U1/U2 snRNP-dependent prespliceosome and the U4/U6.U5 tri-snRNP particle account for all of the snRNAs and the majority of proteins known to act in splicing. A more limited number of non-snRNP splicing factors bind directly to the pre-mRNA substrate to promote snRNP addition or promote conformational changes within the splice complex following snRNP addition (1–3).

Regulated pre-mRNA splicing events are driven by proteins that act to promote or block the recruitment of constitutive splicing factors (4, 5). The major snRNP addition steps are clear targets of this regulation. Whereas comparatively few pre-mRNAs appear regulated in yeast, this system does offer a valuable means to characterize the basic pathway for splicing factor association and hence to identify critical steps in assembly and possible targets for regulation. The addition of the U1 and U2 snRNPs to the spliceosome occurs in part through well characterized snRNA interactions at the pre-mRNA 5′ splice site and branch point regions, respectively, and is supported by a number of protein-based contacts, reviewed in Refs. 6 and 7. The U5 and U6 snRNAs also bind the splice substrate, although these interactions appear to occur subsequent to stable recruitment of the U4/U6.U5 tri-snRNP particle to the prespliceosome. In yeast, mutations that prevent stable U4/U6.U5 tri-snRNP assembly (e.g., prp6Δ; see Refs. 8 and 9) block spliceosome formation, yet little is known in yeast or in mammals of the contacts that actually promote U4/U6.U5 tri-snRNP particle/spliceosome association.

CLF1/SYF3 (henceforth referred to by the Stanford Genome Database standard name, CLF1) was identified as a putative Saccharomyces cerevisiae RNA-processing factor based on the presence of multiple crooked neck-like tetratricopeptide repeats (TPRs) (10). This protein binding motif was first recognized as a distinct TPR subtype in the characterization of the Drosophila melanogaster crooked neck (cnr) gene product (11) and appears restricted to RNA-processing proteins (10, 12). Crooked neck and its homologs, including the yeast Clf1p protein, are ~80 kDa in mass and composed largely of direct iterations of the TPR motif. Loss of function crooked neck mutations in Drosophila are lethal when homozygous (11) and influence alternative splice site selection when heterozygous (13). In humans, the crooked neck protein is a stable component of the spliceosome, recruited at or near the time of U4/U6.U5 tri-snRNP addition (14). In yeast, genetic and biochemical studies implicate Clf1p in a number of steps in spliceosome assembly and in both catalytic steps in splicing (10, 15–18). This broad spectrum of Clf1p interactions is consistent with the 15 TPR repeats of Clf1p serving as a platform for the recruitment of splicing factors during spliceosome assembly.

Clf1p is present in the nineteen complex (NTC), a structure that contains Prp19p and at least eight other proteins but no RNA (see Ref. 18 and references within) (19). Yeast extracts metabolically depleted of Clf1p block the prespliceosome to

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splicesome transition even in the presence of stable U4/U6.U5 tri-snRNP complexes (10), suggesting that Clf1p acts in the recruitment or retention of the U4/U6.U5 tri-snRNP particle. Curiously, whereas Clf1p functions prior to stable splicesome formation, the NTC is reported to bind the splicing complex particle later, during or after the U4 snRNA release from yeast, the pre-mRNA splicing was conducted as described (26). To deplete Clf1p allele were isolated from meiotic offspring.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—Details on the construction of the clf1::HIS3 yeast deletion mutant and the GAL1::clf1(H97), Ycplac22-CLF1-HA, and YEp22-clf1::IS1 plasmid constructs were previously published (10). Ycplac22-clf1::IS1 was prepared similarly by inverse PCR with oligonucleotides Δ1-1 and Δ1-2 (Table I). The Tap epitope was amplified from plasmid pBS1479 (22) with oligonucleotides TAP1 and TAP2 and blunt end-ligated into a Smal site created by PCR mutagenesis (with oligonucleotides SNA1 and SNA2) at the 3′-end of the CLF1 coding sequence. The Ycplac22-CLF1-TAP and deletion constructs were transformed into SY101 yeast (S288c, ade2-101, trp1A1, his3-200, leu2-Δ, clf1::HIS3, pSY1(GAL1::clf1(H679), URA3), selected on galactose-based complete medium without tryptophan (23). Subsequent assays for mutant gene activity were done on YPD medium made with 2% glucose or on complete medium with 1 μg/ml 5-fluoroorotic acid (24). Haploid strains deleted for IS1Y, SYF2, ECM2, and NTCl20 were obtained from the ATCC (Manassas, VA). CLF1-TAP was transformed into this mutant set as a XbaI-SphI DNA fragment cloned into the URA3-marked plasmid, Yclac33 (25).

**PRP46** was amplified from genomic DNA with oligonucleotides 46-1 and 46-2 and cloned into Ycplac111 (25). The TAP PCR fragment was inserted into a Smal site engineered in the 3′-end of the PRP46 coding sequence. The Ycplac111-PRP46-TAP plasmid was transformed into yeast heterozygous for a PRP46 deletion obtained from the ATCC. Haploid strains that exclusively expressed the plasmid-borne PRP46-TAP allele were isolated from meiotic offspring.

**Splicing and Spliceosome Assembly Assays**—The analysis of cellular pre-mRNA splicing was conducted as described (26). To deplete Clf1p yeast, the Ycplac22-clf1::IS1 strain was grown to early log phase in galactose-based YM medium (23), harvested by centrifugation, washed with 1 culture volume of sterile water, and then incubated in glucose-based YM medium for 6–8 h. Splicing extracts were prepared by grinding yeast cell pellets in liquid nitrogen as described (27). Pre-mRNA substrates were prepared by in vitro transcription of pSPPr51A (RP51A) (28) or PT722 (rp51A) (29) with T7 RNA polymerase

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**Table I**

| Oligonucleotide used in this study | Sequence |
|-----------------------------------|----------|
| Δ1–1 | 5′-GGGGAGAGGATCGACTAATTAGTATAA-3′ |
| Δ1–2 | 5′-GGCAGTCCGTTTCCCTTCAATAA-3′ |
| TAP1 | 5′-TACCGTATCGTACCCCGGAGATT-3′ |
| TAP2 | 5′-TCAGTTGACTTTCTCGGCTCGGT-3′ |
| SNA1 | 5′-8AGCTTGAACTGATATGAGGAAA-3′ |
| SNA2 | 5′-GCCAAAAATGGAACAGGACAGG-3′ |
| 46–1 | 5′-GAGTTCCGGATGATATGAGGAAA-3′ |
| 46–2 | 5′-GAGATCCCGGTCTTTTGGCTCGGT-3′ |

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reaction. Splicesome assembly was assayed by native gel electrophoresis (NEGE) and pre-mRNA splicing was assayed by degrading gel electrophoresis as described (31). Clf1Δ-TAP extracts were temperature-inactivated by incubation at 37 °C for 5 min or at 34 °C for 30 min and used without additional manipulation. Complementation experiments were performed by incubation of 50–100,000 rpm of labeled pre-mRNA substrate with the temperature-inactivated extract for 5 min under splicing conditions in a volume of 8.5 μl followed by the addition of 1.5 μl of Clf1p complex (approximately 3 ng of protein) together with a 50-fold molar excess of unlabeled pre-mRNA. Clf1-TAP, clf1Δ-TAP, clf1Δ2-TAP, and Prp46-TAP Purifications—To isolate preparative quantities of the Clf1-TAP complex, 50 liters of yeast were harvested by centrifugation at an A600 of 2–3. The cell pellets were washed twice with 2 liters of sterile water and resuspended in 500 ml of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 20 mM NaCl, 0.5 mM dithiobis(2-nitrophenyl) fluoride, 2 mM benzamidine, 0.5% Nonidet P-40). The yeast were frozen in liquid nitrogen and then broken in a Waring blender as described (32). The lysate was cleared by centrifugation at 20,500 rpm in a Beckman Ti45 rotor for 50 min after 34 min by 33,500 rpm in a Beckman Ti45 rotor. The cleared lysate was then dialyzed against buffer D (10 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM dithiobis(2-nitrophenyl) fluoride, 0.5 mM phenylmethylsulfonyl fluoride, 2% glycerol). The lyophilized yeast was adjusted to 200 mM NaCl, 0.1% Nonidet P-40 immediately prior to TAP affinity purification, performed as described (22), except that 2 ml of rabbit IgG agarose (Sigma) was used for 500 ml of lysate. Analytical scale preparation of 3S-labeled TAP complexes was performed by adding 1 ml of Trans-35S (ICN) to the equivalent of 10 ml of culture at an A600 of 1.0 for 4–5 h at 30 °C as described (33). For temperature shifts, cells were grown to early log phase in buffer D, and the culture was adjusted to 200 mM NaCl, 0.1% Nonidet P-40 and incubated at 37 °C or 37 °C. The final 1 h of labeling. The cell pellets were collected by centrifugation and washed once with water, and proteins were extracted by vortexing the pellet for 4 min with glass beads in buffer A. TAP purification was then performed as described (22), scaled down for use with the reduced volumes. Where indicated, NaCl was increased to 300 or 450 mM before cell resuspension. RNase-treated samples were incubated at room temperature for 15 min with 22 μg/ml RNase A and 444 units/ml of RNase T1 (Ambion) prior to TAP purification. The recovered protein samples were precipitated with 6% trichloroacetic acid, resolved on a 5–10% gradient (or 5% nongradient) SDS-polyacrylamide gel with Benchmark™ molecular weight markers (In vitro) and visualized with a Typhoon PhosphorImager (Amersham Biosciences). Alternatively, the samples were fractionated on a 15–40% glycerol gradient prepared in 50 mM Tris-HCl, pH 7.4, 20 (or 200) mM NaCl, and 5 mM MgCl2 prior to protein and RNA analyses.

**Mass Spectrometry**—The purified Clf1-TAP complex sample was resolved on a 5–10% gradient SDS-polyacrylamide gel in a Tris-Tricine buffer system (34). After electrophoresis, the gel was stained with silver (35), and the protein-containing bands were excised and digested with trypsin (36). The tryptic peptides were fractionated using 3D by HPLC (37). The fractions were pooled and freeze-dried (1 × 50 mm, 3-μm particle size column) (Phenomenex, Torrance, CA) using an acetonitrile/water (0.1% formic acid) gradient at a flow rate of 35 μl/min. The LC column was connected to the electrospray interface of an LCQ Classic mass spectrometer (ThermoFinnigan, San Jose, CA). Mass spectra were acquired using data-dependent analysis (full scan automatically followed by tandem mass spectrometry of the most intense ion detected by the data system). Alternatively, the Clf1-TAP complexes were assayed by DALPC without gel fractionation using strong cation exchange and C18 (Whatman) packing material on a LCQ Deca (ThermoFinnigan, San Jose, CA). In all cases, the tandem mass spectra were converted to mass/Intensity lists and searched against the nonredundant OWL database using SEQUEST and the nonredundant NCBI database using Mascot. The number of unique peptides identified for each protein was as follows: Clf1p, 26; Prp5p, 21; Brp2p, 10; Rse1p, 3; Snu14p, 5; Hsh155p, 4; Syf1p, 22; Cef1p, 26; Prp29p, 26; Prp19p, 14; Prp46p, 6; Prp45p, 6; Cwc2p, 3; Cwc23p, 4; Ec23p, 4; Isy1p, 5; Smb1p, 2; Syf2p, 2; Snt309p, 8; Smd1p, 3; Ntc20p, 4; Smd2p, 7; Smd3p, 3; Lsm2p, 1; Sme1p, 1; Smx3p, 1; Prp22p, 13; Cus2p, 2; Proteins without confirmed links to splicing that co-purified with Clf1-TAP in one or more preparations include Aad15p, Prp22p, 13; Cus2p, 2. Proteins without confirmed links to splicing that co-purified with Clf1-TAP in one or more preparations include Aad15p, Prp22p, 13; Cus2p, 2. Proteins without confirmed links to splicing that co-purified with Clf1-TAP in one or more preparations include Aad15p, Prp22p, 13; Cus2p, 2. Proteins without confirmed links to splicing that co-purified with Clf1-TAP in one or more preparations include Aad15p, Prp22p, 13; Cus2p, 2.
Clf1p Resides in at Least Two Distinct Multisubunit Complexes—To learn more of Clf1p function in spliceosome assembly, proteins associating with this essential splicing factor were purified from yeast. As a first step, Clf1p was modified to include a TAP affinity cassette (22) at the nonessential C terminus (10). The CLF1-TAP strain and a negative control strain that expresses a hemagglutinin (HA)-tagged protein that does not bind the TAP affinity resin were labeled with [35S]methionine and cysteine for 4 h prior to tandem affinity purification. PAGE analysis reveals numerous proteins present in the Clf1p-HA control sample (Fig. 1A). Thirty of the Clf1-HA proteins were identified as specific proteins and through the use of gene-specific deletion mutants or when an alternatively tagged strain is used for purification (see below). Clf1p co-migrates with Clf1p at ~84 kDa (due to the presence of residual TAP sequence) but can be resolved with the use of Clf1p TPR deletion mutants or when an alternatively tagged strain is used for purification (see below). Clf1p co-migrates with Clf1p at ~84 kDa (due to the presence of residual TAP sequence) but can be resolved with the use of Clf1p TPR deletion mutants or when an alternatively tagged strain is used for purification (see below). Clf1p co-migrates with Clf1p at ~84 kDa (due to the presence of residual TAP sequence) but can be resolved with the use of Clf1p TPR deletion mutants or when an alternatively tagged strain is used for purification (see below).

Results

In order to investigate the subunit diversity of the Clf1p complex(es), the [35S]-labeled affinity-purified proteins were resolved on a 15–40% glycerol gradient, and alternate fractions were compared (Fig. 1B; note, for clarity, where multiple proteins are present in a single band, only one is labeled). Fraction 1 contains mostly background proteins, a pattern similar to that observed with the control extract in Fig. 1A. Superimposed on this pattern in fraction 3 and extending through fraction 11 are Clf1p, Syf1p and Syf2p, and Isy1p (but presumably not the co-migrating Lea1p protein; see snRNA analysis, discussed below). Clf1p co-migrates with Clf1p at ~84 kDa (due to the presence of residual TAP sequence) but can be resolved with the use of Clf1p TPR deletion mutants or when an alternatively tagged strain is used for purification (see below). Other than the undetectable levels of certain low molecular weight proteins that label poorly (e.g. Syf2p and Snt309p), this banding pattern closely resembles that of the NTC and will be referred to as the Clf1p-NTP to designate the protein used for purification. Two unidentified proteins, Ccf8p, which may be equivalent to Ntc81p (37), and a previously unreported protein of ~220 kDa (Ccf25p) are also present in these fractions. A small amount of Prp8p and Brp22p are reproducibly observed in this region of the gradient as well. The Sm/Lsm core snRNP proteins are absent in fractions 1–11.

With the intriguing exception of Ccf8p, the Clf1p-NTP complex proteins become markedly enriched in fractions 13–15. Here too are the U5-snRNP-associated proteins Prp28p and Snu114p (as well as Prp8p and Brp22p), Prp46p, the co-migrating Prp45p/Slu7p and Cwc2p/Cwc23p/Ecm2p protein sets, the uncharacterized protein Ccf14p, the Sm/Lsm proteins, and four
protein bands not observed prior to gradient separation (Ccf25p–Ccf28p; see Table II). Ccf26p has been identified by Western blot as Prp22p, a protein that interacts genetically with Clf1p and other components of the Clf1p-RNP complex (16, 38). In addition to the mentioned proteins, fractions 17–23 also contain the U2 snRNP-specific proteins Rse1p and Hsh155p, presumably Lea1p, the Ccf29p band (Cus2p), and factors found in the lighter snRNP-enriched gradient fractions. Of the proteins identified by mass analysis, only Prp16p was not observed as an obvious gel band. The Ccf1p profile from whole cell extract (Fig. 1C) is similar to that of the purified complex with discreet pools in the lower and upper regions of the gradient, although the high concentration of total protein in fractions 3–5 partially inhibits Clf1p transfer (see Fig. 2E for a related image). The presence of Clf1-TAP in fractions 7–11 of the whole cell extract argues against the Clf1p-NTC arising from dissociation of the larger complex during the lengthy affinity purification steps. The Clf1-TAP peak enriched for U2 protein (Fig. 1, B and C, roughly fractions 17–19) overlaps the position of the U4/U5.U6 snRNP particle (Fig. 1D) and corresponds to a complex of ∼~40 S. The Clf1p-RNP Complex—A subset of the known U2 and U5 snRNAs co-purify with the Clf1p complex. Recently, Cef1p-bearing multi-snRNP complexes were reported to be RNase-resistant (39). In contrast, treatment of the Clf1-TAP extract with RNase A and T1 prior to purification causes release of Lsm2p, Smb1p, Smd2p, Sme1p, and Smx3p core snRNPs and a reduction in the Smd1p/Ntc20p band intensity (Fig. 2A). Multiple other proteins, including the U2- and U5-specific snRNP proteins, remain Clf1p-TAP-associated. The RNase treatment regime used degrades all snRNA detectable by Northern blot (data not shown). Consequently, it appears that snRNA contacts stabilize core snRNP protein association with the Clf1p complex, whereas the bulk of the remaining components associate through protein-based contacts. It is possible, however, that small RNA fragments persist in the Clf1p complex and contribute to this stabilization. Consistent with the snRNP protein composition, the U2, U5, and U6 snRNAs (but not U1 or U4 snRNA) are enriched in the affinity-purified Clf1-TAP complexes (Fig. 2B). Nonspecific binding to the TAP affinity resin is very low, since no detectable snRNAs are recovered with a control extract that lacks a TAP-tagged protein (i.e. Clf1-HA). The bulky TAP epitope enhances snRNA recovery, since previous experiments performed with alternative CLF1 alleles failed to recover snRNA (10) or recovered no U2 snRNA and nearly background levels of U5 and U6 snRNA (15). The amount of snRNA recovered with Clf1-TAP is less than what is commonly observed with snRNP-specific proteins, however. Soluble Clf1-TAP protein recovery is typically 30–70% (Fig. 2C), and only 2–5% of the U2, U5, and U6 snRNAs co-purify.

As shown above, the Clf1p present in whole cell lysates distributes broadly across a 15–40% glycerol gradient. When these gradient fractions are used for immune precipitation, the majority of Clf1-TAP-associated snRNA is recovered from the poly-snRNP fraction (fractions 15–19) with no detectable free U6 snRNA (Fig. 2D, fractions 5–7; see Fig. 1D) and little if any U4/U6 di-snRNA (fractions 9–11), or free U2 snRNA or U5 snRNA (fractions 7–13) precipitating. This recovery pattern indicates that Clf1p does not bind individual snRNP complexes but resides in a more complex RNP structure. Curiously, whereas Prp19p is reported as predominately RNA-free in extracts (see Ref. 18 and references within) (19), both Clf1-TAP and Prp19p are recovered from the RNP fractions with antibodies against the TAP epitope (Fig. 2E). This apparent discrepancy was resolved when representative gradient fractions were reassayed with the anti-Prp19p antibody. In this case, Prp19p is efficiently recovered from gradient fractions with little snRNA (other than U6) is present (e.g. Fig. 2F, fractions 4 and 7; see Fig. 1D) but poorly from the snRNP-containing fractions (e.g. fractions 10 and 17). As with the earlier reports, no snRNA was recovered with the anti-Prp19p antibody (data not shown). Thus, whereas Clf1p and Prp19p reside within RNA-free and RNP complexes, the RNP structure inhibits Prp19p recovery by immune precipitation.

### Table II

| Ccf   | Mass*  | Proposed identity | Ccf   | Mass*  | Proposed identity |
|-------|--------|-------------------|-------|--------|-------------------|
| Ccf1p | 279.5  | Prp8p1,2          | Ccf2p | 246.1  | Brz2p1            |
| Ccf3p | 153.8  | Recl              | Ccf4p | 114.0  | Snu114p1          |
| Ccf5p | 110.0  | Hsh155p          | Ccf6p | 100.2  | Syf1p1            |
| Ccf7p | 67.7   | Ccf1p            | Ccf8p | 74.0   | ND                |
| Ccf9p | 66.6   | Prp29p           | Ccf10p| 56.6   | Prp19p1,2         |
| Ccf11p| 50.7   | Prp46p           | Ccf12p| 44.6   | Sla7p2            |
|       |        |                   |       |        | Prp45p1           |
| Ccf13p| 40.9   | Cwc23p1          | Ccf14p| 30.0   | ND                |
|       | 40.9   |                  | Ccf16p| 22.4   | Smb1p1           |
| Ccf15p| 28.0   | Isy1p            | Ccf18p| 20.7   | Sm309p1          |
|       | 27.2   |                  | Ccf20p| 12.9   | Sm2dp1           |
|       | 38.4   |                  | Ccf22p| 11.2   | Lsm6p1          |
|       |        |                  | Ccf24p| 9.7    | Smx3p1           |
| Ccf17p| 24.8   | Syf2p1,2         | Ccf19p| 16.3   | Cm1p1            |
|       |        |                   | Ccf26p| 10.0   | Lsm3p3           |
|       |        |                   |       |        | Lsm5p3          |
|       |        |                   |       |        | Lsm6p1          |
| Ccf21p| 11.2   | Smd9p1          | Ccf27p| 10.4   | ND                |
|       | 12.1   |                  | Ccf28p| 19.0   | ND                |
|       | 10.4   |                  | Ccf29p| 32.3   | ND                |

* Molecular mass in kDa based on protein sequence (identifying factors) or estimated by band mobility (unidentified factors).

* Indicated by mass analysis of gel slice (1), immune detection (2), deletion analysis (3), mobility consistent with protein predicted by electrospray ionization mass analysis (4). Proteins in parentheses are nuclease-sensitive and consistent with the predicted sizes of the indicated core snRNP proteins. The number of tryptic fragments of each fragment observed by mass analysis are presented under “Experimental Procedures.” ND, the protein identity has not been determined.
Influence of TPR Structure on Clf1p Complex Integrity—Yeast with deletions of the first or second Clf1p TPR repeat have growth and splicing defects that become more pronounced at elevated temperatures, presumably due to changes in Clf1p stability or activity (10) (data not shown). To investigate protein abundance, lysates from $\text{CLF1-TAP}$, $\text{clf1}/\text{H9004}_1\text{-TAP}$, and $\text{clf1}/\text{H9004}_2\text{-TAP}$ cultures were assayed by Western blot before and after shift to the restrictive temperature. Clf1-TAP and the deletion derivatives co-migrate on this minigel system and are found at similar levels (Fig. 3A). In addition to the full-length proteins, a protein 20 kDa smaller is present in each sample but absent in an untagged extract (Fig. 3A and data not shown). The levels of this putative decay intermediate vary between extract preparations but are reproducibly greater with the TPR deletion strains. Nevertheless, as the full-length Clf1p (or TPR deletion derivative) levels decrease only slightly with temperature shift, it is unlikely that splicing inhibition observed in the $\text{clf1}\Delta 1\text{-TAP}$ or $\text{clf1}\Delta 2\text{-TAP}$ cultures results from lower Clf1p abundance. Furthermore, $\text{clf1}\Delta 1\text{-TAP}$ and $\text{clf1}\Delta 2\text{-TAP}$ are recessive mutations and do not act as dominant negative inhibitors of splicing. By this measure, it appears that the N-terminal deletion derivatives of Clf1p are stable but less active in splicing.

TAP affinity purification was repeated at different salt concentrations to learn whether the N-terminal TPR deletions described above destabilize the Clf1p complexes (Fig. 3B). For the wild type Clf1-TAP complex, the recovered protein set is equivalent at 200 and 300 mM NaCl and shows reduced levels of selected proteins at 450 mM NaCl. With the $\text{Clf1-HA}$ complex, the Ce1p and Prp19p proteins are reproducibly more sensitive to dissociation at 300 and 450 mM NaCl. Since Ce1p co-migrates with Clf1p, Ce1p release was monitored with complexes isolated using a Prp46-TAP construct (Fig. 3D). The pattern of recovered proteins is nearly identical with Clf1-TAP.
and Prp46-TAP, but when Prp46-TAP is used Clf1p and Cef1p are well resolved. Cef1p remains stably bound at 450 mM NaCl in the wild type complex but fully dissociates from the Clf1Δ2-TAP complex at 300 mM NaCl.

Rse1p and Hsh155p are sensitive to dissociation at 450 mM NaCl in the wild type extracts (Fig. 3B) and missing from gradient fractions 13–15 of the Clf1p-RNP even under low salt conditions (see Fig. 1B). Even so, Rse1p, Hsh155p, and Snu114p appear more sensitive to dissociation in the Clf1Δ2-TAP complex (Fig. 3B). Rse1p, Hsh155p, Cef1p, Prp19p, and to a lesser degree Snu114p are also more readily dissociated from the Clf1Δ1-TAP complex isolated from yeast shifted to the restrictive temperature of 37 °C prior to harvest (Fig. 3C). Therefore, the splicing defects resulting from the N-terminal TPR deletions are correlated with the selective loss of both snRNP and non-snRNP components from Clf1p complexes. Importantly, all of the Clf1Δ1-TAP/Clf1Δ2-TAP-sensitized proteins are essential; the failure to properly assimilate any one into the spliceosome would probably block pre-mRNA splicing.

**Clf1Δ2-TAP Inactivation Inhibits the Productive Addition of the U4/U6/U5 to the Prespliceosome**—To investigate the nature of the Clf1Δ2-TAP splicing defect, extracts were prepared from Clf1Δ2-TAP yeast and assayed for spliceosome assembly and splicing in vitro. Extract prepared at the permissive temperature supports spliceosome assembly on 32P-labeled RP51A pre-mRNA through the previously described prespliceosome (complex III), precatalytic spliceosome (complex I), and spliceosome (complex II) states (31) (Fig. 4A). In contrast, when this extract is briefly preincubated at the restrictive temperature, spliceosome assembly stops at the prespliceosome stage. As expected, the untreated sample supports pre-mRNA splicing (lane 3), whereas the temperature-inactivated sample does not (lanes 1 and 2). Splicing is restored by the addition of purified Clf1p complex in the presence of saturating amounts of unlabeled pre-mRNA substrate. This amount of cold competitor blocks de novo assembly on labeled pre-mRNA (lane 5). Consequently, the splicing complexes formed in the Clf1Δ2-TAP inactive extract, while incomplete, are functional and can be chased through the splicing pathway. The Clf1p complex itself does not support pre-mRNA splicing (lane 4), and the inactivation conditions do not impair splicing or spliceosome assembly in wild type extracts (e.g. Refs. 30 and 40; data not shown). Accordingly, conditions that destabilize the Clf1Δ1-TAP complex

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**Fig. 3. N-terminal TPR deletions destabilize Clf1p complexes.** A. Western analysis of yeast proteins from CLF1-TAP, clf1Δ1-TAP, and clf1Δ2-TAP cultures grown continuously at the permissive temperature (30 °C) and after a 2-h shift to the restrictive temperature (37 °C). The positions of the putative N-terminal degradation products are indicated with an asterisk. B. 35S-labeled Clf1p complexes were isolated in 200, 300, and 450 mM NaCl and resolved on a 5–10% polyacrylamide/SDS gel. Proteins that show enhanced salt sensitivity due to the Clf1Δ2-TAP mutation are indicated by bars at the right. The asterisks indicate where two or more Ccf bands co-migrate. The overall darker band intensities in the lower salt TAP complexes isolated from cultures shifted to 37 °C reflect minor experimental variation in the labeling efficiency. C. 35S-labeled proteins from CLF1-TAP and Clf1Δ1-TAP complexes isolated from cultures shifted to 37 °C for 1 h prior to harvest. The positions of the Clf1Δ1p and the Clf1Δ1p degradation products are indicated on the right, and proteins that show enhanced temperature sensitivity with the Clf1Δ1-TAP mutation are indicated by an asterisk. D. 35S-labeled proteins from a wild type Prp46-TAP culture isolated at 200, 300, and 450 mM NaCl. The positions of Prp46p, the temperature-sensitive proteins, and the stably bound co-migrating Clf1p/Ccf8p proteins are indicated at the right.

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**Fig. 4. Temperature inactivation of Clf1Δ2-TAP blocks splicing and impairs spliceosome assembly.** A, time course of spliceosome assembly on 32P-labeled RP51A pre-mRNA incubated in Clf1Δ2-TAP extract maintained at 23 °C (–) or shifted to 37 °C (+) for 5 min prior to substrate addition. The positions corresponding to the prespliceosome (I), the snRNP complete spliceosome (II), and the spliceosome after U4 snRNA dissociation (II) are indicated at the left. B, assay of RP51A pre-mRNA splicing in the Clf1Δ2-TAP extract before heat inactivation (lane 1) and after heat inactivation in the presence (lane 3) and absence (lane 2) of purified Clf1p complex. The bands corresponding to the prespliceosome (I), the snRNP complete spliceosome (II), and the spliceosome after U4 snRNA dissociation (II) are indicated at the left. B, assay of RP51A pre-mRNA splicing in the Clf1Δ2-TAP extract before heat inactivation (lane 1) and after heat inactivation in the presence (lane 3) and absence (lane 2) of purified Clf1p complex. Complementation was performed by preincubating the labeled pre-mRNA with the heat-denatured extract for 5 min followed by the addition of cold RNA. Lane 4 contains purified complex incubated with pre-mRNA under standard splicing conditions. In lane 5, the labeled and unlabeled RNAs were premixed and added directly to the Clf1Δ2-TAP extract. The positions of the lariat intermediate (II), excised intron (I), pre-mRNA (P), mRNA (M), and released 5′ exon (5′ E) are indicated. In order to resolve the very small 5′ exon, the samples were run a second time on a higher percentage polyacrylamide gel, imaged, and inserted below the solid bar at the bottom.
FIG. 5. Temperature inactivation of Clf1Δ2-TAP activity blocks Prp19p recruitment to the spliceosome but not its own association. A. Western blot of immune precipitated TAP complex (lanes 1 and 11) and spliceosomes recovered with biotin-labeled pre-mRNA (lanes 2, 3, 5–10, 12, and 13) or with a no-biotin control substrate (lane 4). Splicing complexes were assembled in wild type extract (lanes 2–4 and 12), extract metabolically depleted of Clf1p (lanes 5, 6, and 13), and Clf1Δ2-TAP extract before (lanes 7 and 8) and after (lanes 9 and 10) temperature inactivation. NS, a nonspecific band enriched by streptavidin chromatography and bound by the anti-Prp19p antibody. Samples present in lanes 1, 2, and 5 were run in lanes 11–13 and probed with mouse protein A followed by an alkaline phosphatase-conjugated goat anti-mouse antibody that does not detect the background band. B, in vitro splicing reactions were assembled before or after temperature shift (23 and 37 °C, respectively) on radiolabeled RP51A pre-mRNA and then immune precipitated with mouse IgG agarose. This antibody binds the TAP epitope present in samples 1–8 but missing in the untagged control (lanes 9 and 10). T, total unfractionated RNA; IP, immune pellet; LI, lariat intermediate; I, circular excised intron; P, pre-mRNA; M, mRNA; 5'E, upstream exon. The asterisk marks the position of the linear excised intron that can be seen in several lanes.

structure inhibit the productive recruitment of the U4/U6.U5 tri-snRNP particle to the prespliceosome.

Clf1p Is Required for Prp19p Recruitment to the Spliceosome—The results presented above show that brief heat treatment destabilizes the Clf1Δ2-TAP complex and impairs spliceosome formation. It was not clear, however, whether proteins released from the Clf1Δ2-TAP complex could be independently bound by the splicing complex. To address this, splicing complexes were assembled in vitro with or without active Clf1p and then assayed for the presence of the essential Prp19p maturation factor. Biotin-substituted RP51A pre-mRNA was incubated in extract for 30 min under splicing conditions, and then the assembled complexes were recovered by streptavidin chromatography. Bands corresponding to Clf1-TAP and Prp19p were observed in complexes assembled with the splicing-competent Clf1-TAP extract (Fig. 5A, lanes 1 and 2). The spliceosome association of Prp19p requires Clf1p function, however, since metabolic depletion of Clf1p by transcriptional repression of the GAL1-clf1(697) fusion gene (lane 5; see “Experimental Procedures”) blocks Prp19p recovery. Unfortunately, a nonspecific protein enriched on the streptavidin matrix co-migrates with Clf1-TAP and is bound by the Prp19p antibody. This band is prominent in all samples including controls where the pre-mRNA lacks biotin (lane 4) and where no TAP-tagged protein is present (lane 5). To circumvent this problem, Clf1p association with the spliceosome was tested by probing the recovered protein with a TAP-specific antibody (lanes 11–13) and through the use of the Clf1Δ2-TAP derivative, which migrates ahead of the background band (lanes 7–10). Similar to what was observed with the Clf1p-depleted samples, temperature inactivation of Clf1Δ2-TAP blocks Prp19p association. Equivalent results were observed with an alternate splicing-competent splicing substrate, rp51Δ2 (lanes 3, 6, 8, and 10; see “Discussion”). Curiously, under conditions where Prp19p recruitment to the spliceosome is blocked, Clf1Δ2-TAP still associates with the splicing complex (compare lanes 7 and 8 with lanes 9 and 10).

When radiolabeled pre-mRNA is used for in vitro splicing, lariat intermediate, excised intron, and low levels of pre-mRNA are recovered with the anti-TAP antibody from wild type extracts with or without heat treatment (Fig. 5B). The presence of pre-mRNA in the complex shows that Clf1p resides in the precatalytic spliceosome, whereas the absence of mRNA shows that Clf1p persists at least through the Prp22p-dependent step of mRNA release. The same RNA species are recovered with the Clf1Δ2-TAP extract when maintained at 23 °C (albeit at lower levels). Heat inactivation of Clf1Δ2-TAP prevents splicing but, as noted above, does prevent this protein from associating with splicing complexes. Consistent with this, the amount of RP51A pre-mRNA present in the heat-treated Clf1Δ2-TAP complex (lane 8) is well above the background (lane 10) and equal to or greater than that found in the splicing
competent extracts (lanes 2, 4, and 6). Together these data show that the Clf1p is a stable constituent of the spliceosome and demonstrate that the N-terminal TPR domain of Clf1p is required for recruitment of the essential Prp19p splicing factor to the yeast splicing apparatus.

**DISCUSSION**

Promessenger RNA splicing is an amazingly faithful process given the limited information content of splice site consensus sequences. Stepwise spliceosome assembly may offset the limitations of restricted sequence conservation, since this process advances only when previously bound factors are present in the correct position, orientation, and spacing. Whereas the recent identification of unexpected poly-snRNP complexes and interactions (e.g. see Refs. 41–44) suggests that the details of spliceosome assembly may be less rigidly constrained than formerly envisioned, the basic view that substrate selection is context-dependent and occurs through a dynamic process of splicing factor association is very likely correct. Here we show that the poly-TPR protein, Clf1p, plays a critical role in the union of the two large RNP “halves” of the spliceosome, namely the U1/U2-dependent prespliceosome and the U4/U6.U5 tri-snRNP particle. Whereas inactivation of the yeast U4/U6.U5 tri-snRNP protein, Prp31p (45), or the removal of the mammalian SR-like U4/U5.U6 tri-snRNP protein sp110/START1 or p65 (46) blocks spliceosome formation, to our knowledge this is the first example of a non-snRNP yeast splicing factor influencing the prespliceosome to spliceosome transition.

The Clf1p-NTC contains proteins previously reported in the Prp19p-NTC (see Ref. 18 and references within) (19), uncharacterized proteins of ~74 kDa (Ccf8p) and 220 kDa (Ccf25p), and, surprisingly, low levels of the normally snRNP-associated Prp8p and Brr2p. In addition to residing in the NTC state, Clf1p and Prp19p are also present in the Clf1-RNP. The presence of Prp19p in this RNP complex provides explanation for earlier observations that U6 snRNA levels decrease and “free” U4 snRNA levels increase after prp19-1p inactivation, characteristics tightly correlated with the perturbation of U6-bearing snRNP complexes (26, 47). The Clf1p-RNP is refractory to precipitation with an antibody against Prp19p, consistent with the inability of this antibody to deplete Prp19p activity from extracts (19) and accounting for the earlier suggestion that Prp19p exists largely in an RNA-free state in cell extracts (19, 20). Prp19p does not appear to bind Clf1p directly, and of the many protein-protein interactions identified among Clf1p-NTC components only Cef1 binds both Prp19p and Clf1p (18). Since Prp19p and Cef1p interact in two-hybrid and far Western assays (37) and, as shown here, dissociate in parallel from Clf1L1-TAP or Clf1Δ2-TAP inactivated complexes, Cef1p is a prime candidate for a protein that tethers Prp19p to Clf1-NTC.

In contrast to what was recently reported for related Cef1p/Cdc5p RNP complexes (21), we find that the Clf1-RNP is RNase-sensitive, since all detectable core snRNP proteins are released by this treatment. Many factors do remain bound to Clf1p after RNase digestion, however, showing that the Sm and Lam proteins do not provide extensive stabilizing contacts. In addition to the majority of proteins present in Cef1p/Cdc5p RNP complexes, we observe the Prp16p and Prp28p DExH/D-box proteins, Sme1p, and the Lsm2p core snRNP protein in the Clf1p-RNP. The relative Clf1p-NTC/Clf1p-RNP abundance is highly reproducible in *S. cerevisiae*, and we have observed nothing to indicate that the Clf1p-RNP complex can be dissociated into the RNA-free state by simple manipulation of salt or temperature. Curiously, no equivalent to the RNA-free NTC was found in fission yeast Cdc5p complexes (39). Given the observation of the spliceosome assembly pathway, we think it unlikely that Clf1p-NTC exists in budding yeast but not in fission yeast. Rather, the steady state abundance of this RNA-free NTC complex may be lower in the fission yeast, or Cdc5p may preferentially associate with the RNP form of this complex in *S. pombe*.

Pre-mRNA, splicing intermediates, and excised intron co-precipitate with Clf1p-TAP, supporting genetic and biochemical studies that suggest Clf1p-associated proteins act from the earliest stages of spliceosome assembly (e.g. Mud2p and Prp40p) through the final step in splicing and product release (e.g. Prp16p, Prp17p, and Prp22p) (see Refs. 10, 15–18, and 38 and references within). Cheng and co-workers (20) report that Prp19p binds spliceosomes with or soon after the release of U4 snRNA. This conclusion appears inconsistent with the observations that Prp19p requires Clf1p to bind the spliceosome and that the removal of Clf1p activity impairs stable association of the U4/U5.U6 tri-snRNP particle. A possible trivial explanation for this discrepancy is that Prp19p is present in earlier complexes but, similar to what was found with the Clf1p-RNP, is not antibody-accessible. We do find, however, that whereas assembly is arrested at the prespliceosome stage with *RP51A* pre-mRNA, certain other pre-mRNAs, including the *rp51Δ2* deletion derivative used here assemble snRNP-complete but catalytically inactive splice complexes in the absence of Clf1p. This shows that the prespliceosome and U4/U5.U6 tri-snRNP can interact, albeit nonproductively, in the absence of Clf1p. And whereas the molecular basis of pre-mRNA substrate discrimination is unknown, the *rp51Δ2* arrest point shows that Clf1p is also needed after the stable addition of the U4/U6.U5 tri-snRNP addition, a time when Prp19p is proposed to function. We note that Prp19p fails to stably bind to the spliceosome when Clf1Δ2-TAP is inactivated even with substrates that permit spliceosome formation, for example, *rp51Δ2* (see Fig. 5A). Consequently, the *rp51Δ2* arrest point reflects, at least in part, a defect in Prp19p recruitment.

Aebischer and colleagues (42) have reported the isolation from yeast of a complex containing all five snRNAs. The “penta-snRNP” protein constitution is evocative of a splicing complex prior to substrate association. The A (prespliceosome), B (spliceosome), and C (splicing intermediate bearing) splicing complexes characterized in mammalian extracts have likewise been analyzed (48–50). Whereas differences in sample origin and data base completeness complicate the direct comparison of these structures, the reported subunit compositions are generally consistent with the proposed time of complex function. For instance, early acting proteins, such as U1 snRNP factors and many U4/U6.U5 proteins, are present in the yeast penta-snRNP and in the A/B complexes but reduced or absent in the complex C and the Clf1p-RNP structures. Late acting factors, such as the second step proteins Slu7p, Prp16p, and Prp17p and the mRNA release protein, Prp22p, are absent from the earlier complexes but found in Clf1p RNP complex C and/or mammalian C complexes.

The protein and snRNA composition of the Clf1p-RNP is consistent with this complex being an endogenous late stage or postcatalytic spliceosome. The inhibition of pre-mRNA splicing by temperature inactivation of several early acting splicing factors blocks the formation of the Clf1p-RNP, consistent with a “splicing-dependent” origin and arguing against this structure arising as an artifact of protein isolation. If truly a late stage spliceosome, transition from complex C to the Clf1p-RNP would be accompanied minimally by the release of the cap binding proteins and the remaining U4/U6.U5-specific proteins. Whereas substrate RNAs would probably be in low abundance or missing altogether, it might be possible to trap such

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molecules through the use of mutants defective in mRNA or intron release (51, 52). What of the Clf1p-NTC? With the possible exception of Ccf6p, all Clf1p-NTC factors are present in the more complex Clf1p-RNP state. Given this situation, the Clf1p-NTC probably represents either a precursor of the Clf1p-RNP or a splicing complex at the terminal stage of dissociation. Whereas a careful kinetic analysis of complex assembly is needed to distinguish between these alternative origins of the Clf1p-NTC, some support for the latter model is provided by the presence of the stable snRNP proteins Brf2p and Prp5p in the snRNA-free Clf1p-NTC structure.

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