Genetic Interactions With *CLF1* Identify Additional Pre-mRNA Splicing Factors and a Link Between Activators of Yeast Vesicular Transport and Splicing

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ABSTRACT

Clf1 is a conserved spliceosome assembly factor composed predominately of TPR repeats. Here we show that the TPR elements are not functionally equivalent, with the amino terminus of Clf1 being especially sensitive to change. Deletion and add-back experiments reveal that the splicing defect associated with TPR removal results from the loss of TPR-specific sequence information. Twelve mutants were found that show synthetic growth defects when combined with an allele that lacks TPR2 (*i.e.*, *clf1Δ2*). The identified genes encode the Mud2, Ntc20, Prp16, Prp17, Prp19, Prp22, and Syf2 splicing factors and four proteins without established contribution to splicing (Bud13, Cet1, Cwc2, and Rds3). Each **s**ynthetic **l**ethal with ***c**lf1Δ2* (*slc*) mutant is splicing defective in a wild-type *CLF1* background. In addition to the splicing factors, *SSD1*, *BTS1*, and *BET4* were identified as dosage suppressors of *clf1Δ2* or selected *slc* mutants. These results support Clf1 function through multiple stages of the spliceosome cycle, identify additional genes that promote cellular mRNA maturation, and reveal a link between Rab/Ras GTPase activation and the process of pre-mRNA splicing.

THE spliceosome is composed of five small nuclear ribonucleoprotein (snRNP) particles and an undetermined number of non-snRNP splicing factors (Collins and Guthrie 2000; reviewed in Brow 2002). Each snRNP contains small nuclear RNA (snRNA) and an associated set of proteins. SnRNP particles interact dynamically with one another and with the pre-mRNA to configure an active splicing enzyme. The U2 and U6 snRNAs are believed to function at the catalytic center of the enzyme while U5 snRNA provides a key substrate alignment role. The U1 and U4 snRNAs act in earlier steps of spliceosome assembly but are not essential for catalysis.

Proteins serve to promote, stabilize, and resolve RNA-based interactions within the spliceosome. A number of crosslinking experiments place selected spliceosomal proteins at or near the active site of the splicing enzyme (*e.g.*, see Ismaili *et al.* 2001 and references in Reed and Chiara 1999) although it is unclear whether proteins take part in catalysis. Clf1 is an essential yeast splicing factor that resides in at least two distinct complexes, the Clf1-NTC and the Clf1-RNP (Wang *et al.* 2003). The Clf1-NTC is similar to the previously reported "nineteen complex" (NTC) implicated in a late stage of spliceosome maturation (Tarn *et al.* 1993a,b; Chen *et al.* 2002). The Clf1-RNP is reminiscent of a late-stage spliceosome and contains most Clf1-NTC proteins, the U2, U5, and U6 snRNAs, and a subset of the known snRNP proteins. While the functional relationship between the Clf1-NTC and Clf1-RNP complexes is still speculative, Clf1-based contacts are critical for completion of the spliceosome cycle *in vitro*. In the absence of Clf1 activity the U4/U6.U5 tri-snRNP particle is either no longer recruited to the assembled prespliceosome or, if recruited, no longer configured into an active state (Chung *et al.* 1999; Wang *et al.* 2003).

Clf1 is composed almost entirely of direct iterations of the 34-amino-acid TPR protein-binding motif. This repetitive structure and the abortive spliceosome assembly observed in Clf1-defective extracts led to speculation that Clf1 acts as a scaffold in spliceosome assembly (Wang *et al.* 2003). The Mud2, Prp40, Cef1, Isy1, Ntc20, Syf1, Syf2, Prp22, and Prp46 spliceosomal proteins have been shown to interact with Clf1 in two-hybrid or solution-binding assays (Chung *et al.* 1999; Ben-Yehuda *et al.* 2000; Chen *et al.* 2002; Ohi and Gould 2002). While identification of interacting proteins is consistent with a scaffold function, the selectivity of the poly-TPR platform is called into question by interactions that encompass identical or overlapping regions of Clf1.

Here a deletion approach is used to assay the Cfl1 TPR repeats for biological function and synthetic lethal and dosage suppressor screens are used to identify *trans*-acting factors that influence Clf1 activity. The results support the view that TPR-specific contacts promote Clf1 function, provide evidence for the involvement of several additional genes in the splicing pathway, and reveal a link between pre-mRNA splicing and Rab/Ras-GTPase activation in vesicular transport.

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TABLE 1

Oligonucleotides used in this study

Oligo name	DNA sequence (5′-3′)
TPR D1-1 UP	TCTTGCTTTTTGCCCCTTCTT
TPR D1-2	ATTGACCCGGGGATATGGGACAATGGATTCGTTATG
TPR D2-1 UP	ATCCAGTCGGTTTCTCTTCAA
TPR D2-2	TTCCCGGGGCTTTGGATACGATACATTGAT
TPR D3-1 UP	GAATGAACTATCTACTAATAATGC
TPR D3-2	CACCCGGGGACAAGTTATGGTACAAGTA
TPR D5-1 UP	AACCCCTGGCTCCAAGGAGCA
TPR D5-2	CACCCGGGATGCAGACATGGCTAAAGTGGGTG
TPR D6-1 UP	TTGCGGGTGAGCCATGACGTA
TPR D6-2	CTCCCGGGCAAAATTTACAGATTTGGTCTGAT
TPR D7-1 UP	ATTTTGGAGGTTAGCTACTGT
TPR D7-2	CACCCGGGATTGCTATAGAAAAATGGCCCTCC
TPR D8-1 UP	AGCAATTTGATAAAGAGCACT
TPR D8-2	AACCCGGGAGGAAAATGGAGTATGAAACAATA
TPR D10-1 UP	TTTTGATAGTTCTTTTGGTCG
TPR D10-2	AGCCCGGGTTAATTGACGACATCATCCCTCAT
TPR DCTD-1 UP	GTCTTCATCCACATTTTCGCT
TPR DCTD-2	AACCCGGGGAATTCGTGGATTACATTTTTCCT
AB1-1	GGGGGAGAGGATCGACTAATATTGATATA
AB1-2	ATCCAGTCGGTTTCTCTTCAAATA
AB2-1	GGGATGGGACAATGGATTACTTAT
AB2-2	GAACTATCTACTAATAATGC
AB3-1	GGGATCCCCCTTTGGATACGATAC
AB3-2	TACTCTGGGCAACGTACTAATAGC
TPR Dall-1	TCTTGCTTTTTGCCCCTTCTT
TPR Dall-2	GACCCGGGGTTTGAAATTACCGATGAGAA
RDS1-1	CCCGGGATTCATGTCCCGCCATCAGTTTGAT
RDS1-2	CCCGGGATCCAGTAGTTACGACGAATATAG

MATERIALS AND METHODS

Plasmid and strain constructions: The *clf1*Δ TPR deletion mutants were made by inverse PCR with the paired oligonucleotides listed in Table 1 [TPR D1-1 (upstream), TPR D1-2 (downstream), etc.] and a 3-kb *Xba*I-*Sph*I DNA fragment containing a TAP-tagged *CLF1* allele inserted in pTZ19U (USB). The primers introduce a *Sma*I recognition site at the deletion endpoint and the PCR fragments were cleaved with *Sma*I prior to ligation. After confirmation of the DNA sequence, the deletion constructs were transferred into the yeast shuttle vector, YCpLa22 (Gietz and Sugino 1988). The YCplac22 *clf1*Δ plasmids were transformed into the previously described yeast strain SY101 (*a ade2-101 clf1::HIS3 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1*Δ*1 ura3-52* pBM150 [*URA3 GAL1::clf1(697)*]; Chung *et al.* 1999). For the *clf1*Δ*2* add-back experiments, PCR fragments composed of TPR1 (oligonucleotides AB1-1 and AB1-2), TPR2 (oligonucleotides AB2-1 and AB2-2), or TPR3 (oligonucleotides AB3-1 and AB3-2) were inserted at the *Sma*I deletion endpoint site.

Clf1::LEU2 was made by blunt-end ligation of a 1.6-kbp *LEU2* restriction fragment from YdLEU2 (Berben *et al.* 1991) into an *Sma*I-cleaved *clf1* derivative in which all TPR coding sequences were removed by inverse PCR with oligonucleotides TPR Dall-1 and TPR Dall-1. The *clf1::LEU2* allele was excised from the vector and used to replace the endogenous *CLF1* gene in strain YCH125 (*a ade2 ade3 ura3 leu2 trp1*) transformed with p102 (YCplac22 *clf1*Δ*2*). The resulting strain was transformed with plasmid p101 (YCp50 containing *URA3, ADE3*, and *CLF1*) to create BRY556.

A diploid yeast strain heterozygous for the *rds3::Kan*^{R} disruption was obtained from the ATCC. The *GAL1::RDS3* fusion was prepared by insertion of a *Bam*HI-digested PCR fragment from yeast genomic DNA (strain MGD35346D; α *cyh*^{R} *leu2-3,113 his trp1-289 ura3-52*; primersRDS1-1 and RDS1-2) into the *Bam*HI site of pBM150 (Johnston and Davis 1984). Yeast that exclusively express the *GAL1* fusion gene were obtained from the meiotic offspring of diploid transformant. The *GAL1::rds3-1* strain was prepared similarly with DNA isolated from the *slc6-1* mutant.

Isolation of *slc* mutants and dosage suppressors: Strain BRY556 (*a ade2 ade3 ura3 leu2 trp1 clf1::LEU2* YCplac22 [*clf1*Δ*2 TRP1*] *p2965* [*CLF1 URA3*]) was mutagenized with ethyl methanesulfate (EMS) to 40% viability as previously described (Blanton *et al.* 1992). The *clf1*Δ*2* allele used for synthetic lethal selection was not marked with a TAP or HA epitope. Approximately 100,000 yeast colonies were plated on CSM-tryptophan plates and incubated 4–5 days at 23° or 30°. Nonsectoring (*i.e.*, solid red) colonies were scored for growth on 5-fluoroorotic acid (5-FOA) medium at 23° and for temperature sensitivity on YPD medium at 37°. The temperature-sensitive (ts) strains were backcrossed to the wild-type strain, MGD 35346D, and *slc* mutants isolated from the meiotic offspring free of the *clf1::LEU2* knockout and plasmids present in the parent. A YCp50-based yeast genomic library (Rose *et al.* 1987) was used to obtain the wild-type alleles of the *slc* mutants by complementation at 37°. The complementing genes were identified by subclone and linkage analyses.

Synthetic lethality between the *slc* mutants and alternative *clf1* deletion alleles was tested after transformation of the YC-

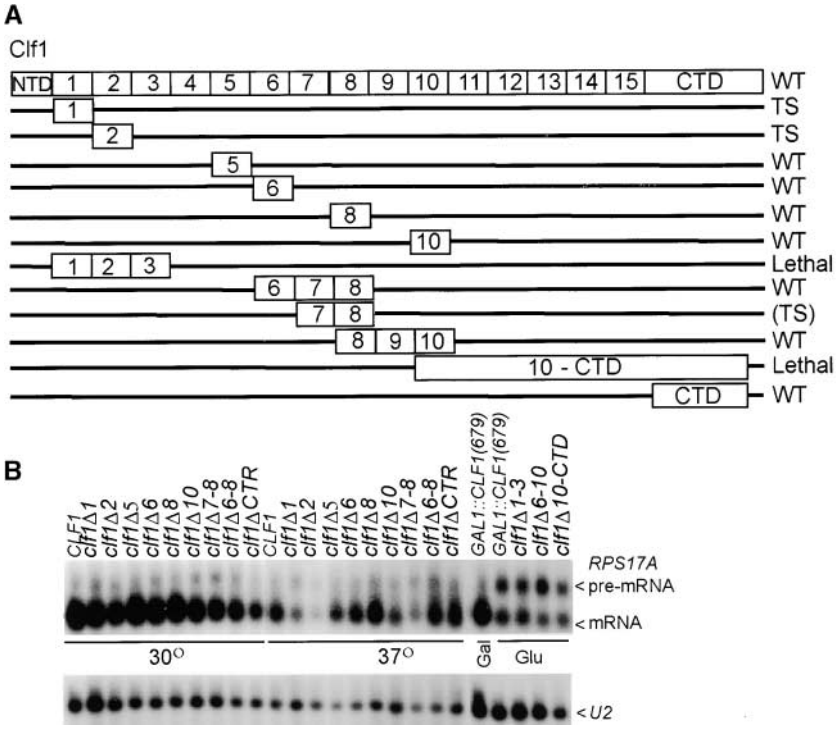

FIGURE 1.—Clf1 TPR deletion mutants vary in splicing impairment. (A) Individual or adjacent TPR elements were deleted from the *CLF1* gene (boxes) and the resultant mutations were assayed for function at 30° and 37°. NTD and CTD refer to the non-TPR amino (N) and carboxyl (C) terminal domains, respectively. Growth was considered wild type (WT) if colony diameter was within 50% of that found with the full-length construct at both temperatures. Temperature-sensitive (TS) colonies showed little or no growth at 37° after 4 days of incubation. (B) Northern analysis of pre-mRNA splicing. RNA was extracted from the wild-type strain (*CLF1*) and various deletion mutants were grown continuously at 30° or after a 2-hr shift to 37° and resolved on a 1% agarose formaldehyde gel. Hybridization probes were prepared from the intron-containing *RPS17A* gene and the intronless *SNR20* (U2 snRNA) gene.

plac22-based *clf1* deletion derivative into strain BRY555 (*a ade2 ade3 ura3 leu2 trp1 p2965* [*CLF1 URA3*]). Gene knockout mutants obtained from the ATCC were first mated with BRY556 followed by selection of diploid strains that lost plasmid *p2965* (*CLF1 URA3*) on 5-FOA medium. The offspring from at least 40 tetrads were then assayed for the kan^R gene on G418 medium and on selective plates for the nutritional markers present in the *clf1::LEU2* knockout and the YCplac22 (*TRP1*, *clf1Δ2*) plasmid. A heterozygous *PRP19/prp19-1, CLF1/clf1Δ2* diploid was generated as a cross between the *clf1Δ2* mutant and JM796 (α *ade2 his3Δ ura3 prp19-1 tyr1*). The meiotic offspring were scored by crossing all ts isolates back to the mutant parents and assaying for complementation at 37°.

High-copy-number dosage suppressors were isolated by transformation of YKH101 (*a clf1::HIS3 trp1-289 leu2-3,112 ura3-52*YCplac33 [*clf1Δ2 URA3*]) with a YEp13-based genomic DNA library (ATCC stock 37323) and screening for enhanced colony size at 34° on CSM-leucine medium (KAISER *et al.* 1994). To rule out library recovery of *CLF1*, colonies were counter-screened on 5-FOA medium, which selects against the YCplac33-*clf1Δ2* plasmid. Plasmids were recovered from yeast that were 5-FOA^- and showed enhanced growth at 34°. The suppressor genes were identified by subclone analysis with vector YEplac118 (GIETZ and SUGINO 1988). Tests of dosage suppression by *SSD1, BTS1*, and *BET4* were conducted on YPD medium at the semipermissive growth temperature of 35° (*slc1-1, slc2-1, slc2-2, slc4-1*, and *clf1Δ2*) or at 37° (*slc3-1, slc5-1, slc6-1*, and *slc7-1*). For segregation analysis, the yeast *URA3* gene was placed directly upstream of *SSD1* integrative transformation using a 2.6-kb *Eco*RI fragment of yeast DNA blunt end ligated into the *Hin*dIII and *Eco*RI sites of YIp211 (GIETZ and SUGINO 1988). This plasmid was cleaved with *Bgl*II prior to yeast transformation.

Analysis of pre-mRNA splicing: Total cellular RNA extracted from yeast cultures was resolved on a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and hybridized as previously described (BLANTON *et al.* 1992). Splicing efficiency was evaluated using a Typhoon phosphoimager (Molecular Dynamics, Sunnyvale, CA) to estimate the mRNA to pre-mRNA ratio, a more reliable indicator of splicing impairment than absolute changes of mRNA or pre-mRNA since mutants can show decreased pre-mRNA stability (RYMOND *et al.* 1990).

RESULTS

Clf1 is differentially sensitive to TPR motif deletions: Clf1 contains 15 direct iterations of the TPR motif flanked by 30- and 109-amino-acid non-TPR segments at the amino and carboxyl ends, respectively. To investigate this domain organization, individual or multiple TPR elements were deleted and the resulting constructs were assayed for biological activity in yeast on single-copy plasmids expressed by the natural *CLF1* promoter. The host strain contains a chromosomal gene disruption complemented by the functional but nutritionally regulated *GAL1::clf1(679)* allele (CHUNG *et al.* 1999). Downstream characterizations were facilitated by the insertion of the TAP affinity tag (PUIG *et al.* 2001) into the nonessential C-terminal coding sequence. No differences in growth rate or splicing efficiency were observed when the wild-type *CLF1* allele was modified in this way (CHUNG *et al.* 1999; WANG *et al.* 2003).

The *clf1Δ* mutant transformants were scored for colony formation on glucose-based medium [to repress *GAL1::clf1(679)* transcription] at 30° and 37°. The results show that while *CLF1* is quite tolerant of mutation, not all TPR elements function equivalently (Figure 1A; see CHUNG *et al.* 1999; WANG *et al.* 2003). The amino terminus of Clf1 is particularly sensitive to deletion, as removal of either TPR1 or TPR2 imparts a tight ts growth defect while deletion of elements TPR1–TPR3 is lethal. In contrast, deletion of individual or grouped TPR ele-

ments through the central TPR domain or a 76-amino-acid segment of the non-TPR carboxyl terminal domain (CTD; amino acids 579–653) has little or no impact on Clf1 function. The exception is a deletion of TPR elements 7-8, which causes a leaky ts growth phenotype. Improper protein folding may account for the *clf1Δ7-8* defect, as a larger deletion (*i.e.*, *clf1Δ6-8*) supports growth at near wild-type levels. We previously showed that the terminal 40% of Clf1 is nonessential, as yeast harboring a frameshift mutation at TPR11 are viable albeit growth and splicing impaired (Chung *et al.* 1999). In contrast, N-terminal expansion of this deletion from TPR10 (beginning at amino acid 357) through amino acid 653 within the CTD is lethal. The Clf1Δ10-CTD protein is stable in yeast (data not shown) and individual deletions of TPR10, the CTD segment (Figure 1A), or the removal of all coding sequence downstream of TPR12 (Chung *et al.* 1999) do not impair growth.

RNA isolated from wild-type and *clf1Δ* mutant cultures was probed with the intron-bearing *RPS17A* gene to assay for splicing inhibition (Figure 1B). U2 snRNA was used as a control for RNA loading and transfer efficiencies. For all nonlethal mutations, the *GAL1::clf1(679)*-bearing plasmid was removed by 5-FOA selection (Boeke *et al.* 1987) prior to assay. At 30° the functional mutant alleles of *CLF1* showed mRNA/pre-mRNA ratios within 2.5-fold of that of the wild-type allele (Figure 1B; mRNA/pre-mRNA ratio for wild type is ~20; Rymond *et al.* 1990). At 37°, however, splicing is greatly impaired in the three ts deletion mutants (*clf1Δ1*, *clf1Δ2*, and *clf1Δ7-8*; mRNA/pre-mRNA values of 1–2) and reproducibly less efficient in the *clf1Δ10* strain (mRNA/pre-mRNA value of 3.5). The remaining nonlethal deletions showed more modest splicing defects or were indistinguishable from wild type. The lethal mutant constructs were assayed 6 hr after *GAL1::clf1(679)* repression in glucose-based medium. The mRNA/pre-mRNA ratio for each lethal mutant was indistinguishable from that of the untransformed *GAL1::clf1(679)* strain (*i.e.*, 0.75–1), indicating that the products are nonfunctional. Primer extension with *RPS17A* and *ACT1* exon II oligonucleotides established a step 1 splicing block for each mutant (data not shown). Together these data show that the Clf1 TPR elements are not functionally equivalent in splicing and implicate the N-terminal region as contributing critical intra- or intermolecular contacts.

TPR elements are not interchangeable: TPR elements are often clustered and likely function in groups to support ligand association (reviewed in Blatch and Lassle 1999). In principle, deletion of a TPR element might impair function by the reduction in number of functionally equivalent repeats or by the loss of TPR-specific sequence information. At a superficial level the deletion analysis supports the second model since Clf1 activity is impaired only after removal of certain repeats. It is possible, however, that sensitive elements reside within in a spatially restricted domain (delimited by an uncharacterized feature of the Clf1 structure) and therefore deletions elsewhere in the protein are irrelevant with respect to the mutant phenotype. To address this, we assayed the activity of add-back constructs in which PCR products encoding TPR1, TPR2, or TPR3 are inserted at the site of the *clf1Δ2* deletion (Figure 2). The original *clf1Δ2* allele and each add-back construct support growth at 30°, showing that none of the reintroduced TPRs create a dominant-negative mutation. At 37°, however, the *clf1Δ2* strain produces no colonies while the reconstituted TPR2 construct displays wild-type growth. Neither the TPR1 nor the TPR3 add-back construct showed any rescue at 37°. Similar results were obtained when TPR4 was inserted into *clf1Δ2* (data not shown), leading us to conclude that TPR2 contains sequence-specific information critical for Clf1 activity.

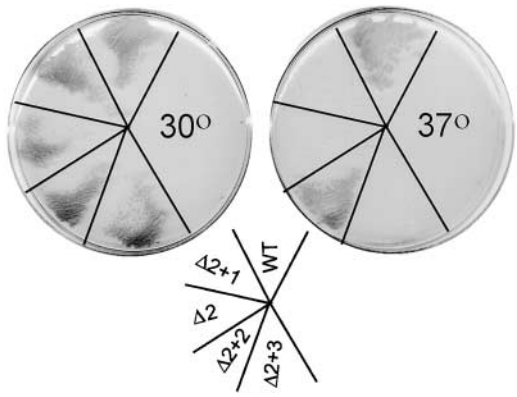

Figure 2.—TPR2 contains unique sequence information. Yeast strains that express a wild-type *CLF1* gene (WT), the *clf1ΔTPR2* mutation (Δ2), and the *clf1ΔTPR2* mutation modified by the insertion of TPR1 (Δ2+1), TPR2 (Δ2+2), or TPR3 (Δ2+3) were assayed for growth on YPD medium at 30° and 37°.

Isolation of mutants synthetically lethal with *clf1Δ2*: The *clf1Δ2* mutation provides a sensitized background with which to identify genes that act in support of Clf1 function. Using the red/white sectoring (Kranz and Holm 1990) and 5-FOA sensitivity (Boeke *et al.* 1987) plasmid-loss assays, we identified synthetic lethal mutants from an EMS-mutagenized culture as colonies that are inviable in the absence of *CLF1*. The putative synthetic lethal mutants were then screened for those that are growth impaired at 37° in the presence of the wild-type *CLF1*-bearing plasmid. Eight ts *slc* mutants were identified that also met additional screen criteria (see below). The mutants were backcrossed to obtain *slc* progeny in a *CLF1* chromosomal background. Each *slc* mutation segregated as a single Mendelian trait unlinked to *CLF1*. The complementation pattern obtained from crosses within this mutant set showed that two *slc* mutations are tightly linked and likely reside in the same gene (*slc2-1* and *slc2-2*) while the rest reside in unique genes.

In comparison to the wild-type strain and a ts but

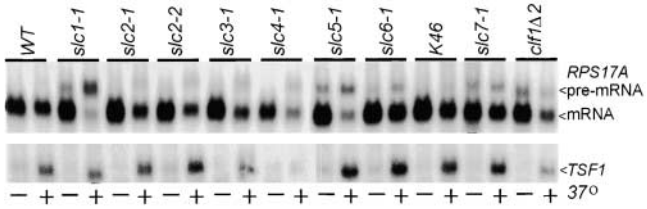

FIGURE 3.—*Slc* mutant inactivation inhibits pre-mRNA splicing. RNA was isolated from *CLF1* (WT) and the indicated mutant yeast strains were grown continuously at 23° (−) and after a 2-hr shift to the restrictive temperature of 37° (+). A Northern transfer of this RNA was hybridized with probes from the intron-containing *RPS17A* gene and from the intronless (but heat-inducible) *TSF1* gene. K46 is a ts mutant without an obvious splicing defect.

splicing competent control strain (K46), splicing in the *clf1Δ2* and *slc* backgrounds is 3- to 30-fold less efficient at the restrictive temperature and, at least for *slc1-1*, *slc5-1*, *slc7-1*, and the *clf1Δ2* strains, somewhat impaired at the permissive temperature (Figure 3). The ts growth and splicing defects cosegregated in eight of eight offspring tested from each *slc* mutant backcross. Primer extension showed most of the intron-bearing RNA that accumulates in the *slc1-1* mutant is lariat intermediate (data not shown). All other mutants show predominantly enhanced pre-mRNA levels relative to the wild-type control. With one exception, no reproducible differences in mRNA length or abundance were detected with the intronless, heat-shock-inducible *TSF1* gene probe (Figure 3). For *slc4-1*, however, the induced *TSF1*mRNA levels were always much lower than those of the wild-type control. Lower levels of the intronless yet constitutively expressed *ADE3* mRNA were also observed with *slc4-1* at the restrictive temperature while no differences were observed in the levels of rRNA or spliceosomal snRNAs (data not shown). These results indicate that the ts *slc1-slc3* and *slc5-slc7* mutations reside within genes that support pre-mRNA splicing while the *slc4-1* mutation influences splicing as well as the levels of certain intronless pol II RNAs.

Identification of the *SLC* genes: A yeast genomic DNA library assembled on a single-copy plasmid vector was used to recover genes that complement the *slc* mutations. DNA sequence analyses showed that all plasmids recovered for a given *slc* mutant defined identical or overlapping regions of the yeast genome. Subsequent subcloning experiments determined the identities of the effector genes. *SLC1* encodes Prp16, a DExD/H-box ATPase originally identified as a suppressor of mutant branchpoint sequences (COUTO *et al.* 1987) and subsequently shown to induce an ATP-dependent conformational change within the spliceosome required for the second RNA cleavage/ligation event in splicing (SCHWER and GUTHRIE 1992). *SLC2* encodes Prp22, a related DExD/H-box protein that is required for the ATP-dependent mRNA release from the spliceosome (COMPANY *et al.* 1991) and, at least *in vitro*, for a less well-defined ATP-independent step prior to exon ligation (SCHWER and GROSS 1998). The *slc2-1* and *slc2-2* alleles of *PRP22* were isolated and sequenced (Figure 4A). *slc2-1* contains two mutations that result in a V505A substitution at an often conserved residue immediately upstream of the DExD/H-box motif I (or Walker A box) and a G692S substitution within the core NTPase domain. The *slc22-2* allele also contains a mutation at codon 692, resulting in a G692D substitution. Both Prp16 and Prp22 copurify with Clf1-TAP complexes isolated from cell extracts through two rounds of affinity enrichment (Figure 4B; see WANG *et al.* 2003). Neither protein is recovered with a control extract that lacks a TAP-tagged protein. Thus, two enzymatic effectors of the spliceosome cycle, Prp16 and Prp22, interact genetically and physically with the Clf1-bearing complexes.

SLC3 encodes Cwc2, an essential yeast protein with C3H1 zinc finger (amino acids 67–94) and RRM (amino acids 136–210) motifs shared with a number of likely homologs [*Homo sapiens* (gi|8922328), *Drosophila melano-*

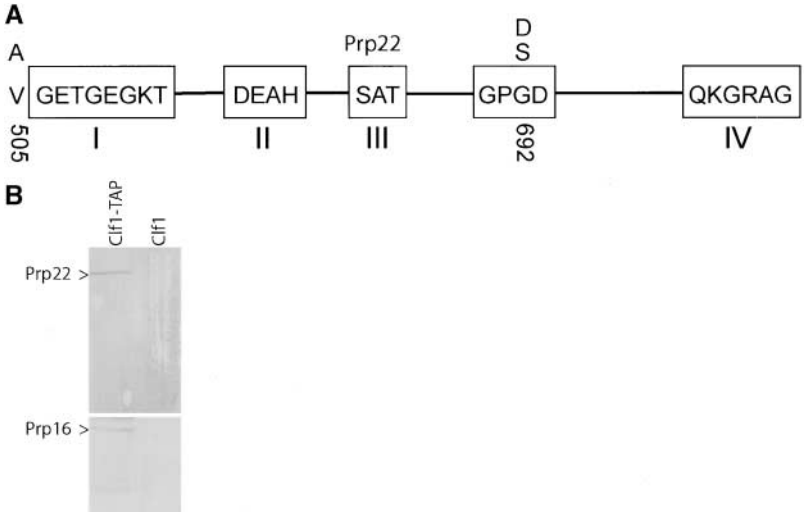

FIGURE 4.—Two DExD/H-box proteins genetically interact with *clf1ΔTPR2*. (A) A schematic of Prp22 domain organization. The conserved DEAD-box motifs I–IV are shown with the relevant Prp22-specific indicated. The protein changes caused by the *slc2-1* mutation (V505A and G692S) and from the *slc2-2* mutation (G692D) are presented above the schematic. (B) Western blot of Clf1-TAP complexes isolated from sequential affinity purification on protein A agarose and calmodulin Sepharose (Clf1-TAP). An equivalent sample prepared from an extract that lacks the TAP epitope is presented as a negative control (Clf1). The blot was incubated with polyclonal antibodies specific for Prp22 and Prp16.

gaster (gi|16769690), *Arabidopsis thaliana* (gi|15227567), and *Schizosaccharomyces pombe* (gi|19114249)]. When likely homologs are excluded, Cwc2 sequences best match with the N terminus of the yeast Hsh49 U2 snRNP protein (amino acids 10–175), showing 44% sequence similarity with Cwc2 residues 175–328. Although likely an RNA-binding protein, Cwc2 was not reported to copurify with yeast snRNP particles (Brow 2002). The *slc3-1* allele contains a single nucleotide change resulting in a glycine-to-aspartic-acid substitution at amino acid 79, a conserved position within the zinc finger domain. Cwc2 copurifies with Clf1 complexes (Ohi *et al.* 2002; Wang *et al.* 2003).

SLC4 encodes the Cet1 RNA triphosphatase, an integral component of the yeast mRNA capping enzyme (Tsukamoto *et al.* 1997 and references within). Cet1, the Ceg1 guanylyltransferase, and the Abd1 methyltransferase serve to replace the initiating 5′ triphosphate with the N^7 methyl cap structure common to pol II transcripts. The *cet1-1* allele contains two mutations that result in D422N and L495 amino acid substitutions within the catalytic domain (Lehman *et al.* 1999; Schwer *et al.* 2001). Although snRNA levels often decrease after splicing factor inactivation (Blanton *et al.* 1992), snRNA remains constant in the *slc4-1* mutant (and all other *slc* mutants) for at least 2 hr after temperature shift. Consequently, improper pre-mRNP organization rather than decreased snRNA stability may account for the rapid splicing impairment noted with s*lc4-1* inactivation (see discussion).

SSD1 was found to relieve the ts growth defect of the *slc5-1* mutant in two independent experiments. However, unlike the other *SLC* genes, *SSD1* incompletely abated the *slc5-1* phenotype. For instance, as shown in Figure 5, ectopically expressed *SLC1/PRP16* restores splicing and growth at 37° to wild-type levels in the *slc1-1* mutant whereas *SSD1* expression enhanced *slc5-1* splicing only weakly (≤1.5-fold). The enhanced growth with *SSD1* expression appears more than proportionate to the improvement in *RPS17A* splicing (2- to 3-fold *vs.* 1.5-fold), suggesting that the processing of this transcript is not rate limiting under these conditions. The comparatively weak effect of *SSD1* expression might reflect semidominance by the *slc5-1* mutation or *SSD1* function as a low-copy suppressor. Semidominance was ruled out by the observation that a heterozygous *slc5-1/SLC5* diploid grows as well at 37° as an otherwise isogenic wild-type control (data not shown).

Ssd1 has been shown to bind RNA (Uesono *et al.* 1997), suppress certain splicing mutants (Luukkonen and Seraphin 1999), and in someway function as a general post-transcriptional regulator of gene activity (Kaeberlein and Guarente 2002 and references within). If *SSD1* functions as a dosage suppressor of *slc5-1*, one should be able to genetically separate the *SSD1* and *SLC5* loci. To test this, the *SSD1* locus in the *slc5-1* mutant was marked with *URA3* (see materials and methods), this

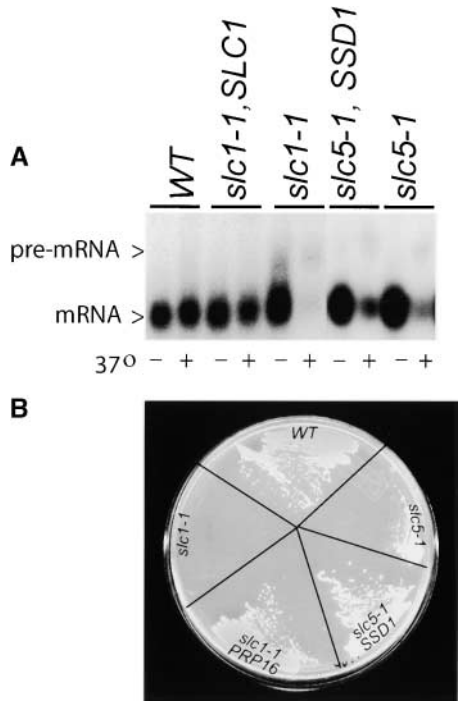

Figure 5.—Incomplete recovery of the *slc5-1* splicing defect with *SSD1* overexpression. (A) Northern analysis with the intron-bearing *RPS17A* gene on RNA from yeast grown continuously at 23° (−) or after a 2-hr shift to 37° (+). The cultures included yeast wild type for splicing related genes (WT) and the *slc1-1* and *slc5-1* mutants with and without plasmids containing genes that fully relieve (*PRP16*) or partially relieve (*SSD1*) the ts growth and splicing defects. (B) Growth of the same strains incubated at 37° for 3 days on YPD medium.

strain was backcrossed, and the genetic linkage of *URA3* and *SLC5* was scored. One-half of the offspring from 40 tetrads were temperature sensitive (due to *slc5-1*) and these were evenly divided between ura+ and ura− strains. Thus, *SSD1* is unlinked to *SLC5* and acts as a single-copy dosage suppressor of *slc5-1*. Repeated attempts to isolate *SLC5* proved unsuccessful.

SLC6 is defined by open reading frame YPR094W, named *RDS3* (for *r*egulators of *d*rug *s*ensitivity) in a recent study of zinc cluster protein function (Akache *et al.* 2001; Akache and Turcotte 2002). A *RDS3* knockout was reported to show increased sensitivity to ketoconazole and cyclohexamide and reductions in *PDR5* and *SNQ2* (drug transporter gene) mRNA, leading to the suggestion that Rds3 is a transcription factor in the drug transport pathway. Slc6/Rds3 is a 12-kD protein with five CxxC zinc fingers and a strongly basic carboxyl terminus (Figure 6). This is an exceptionally highly conserved protein with the fly and human homologs sharing 95% identity and the yeast/human homologs sharing 56% identity. The *slc6-1* mutation causes an aspartic acid for glycine substitution at position 20, a conserved residue three amino acids upstream of the first zinc finger.

RDS3 has been reported as an essential gene (Giaever

et al. 2002) and as a nonessential gene (Akache *et al.* 2001) in yeast. We obtained the heterozygous diploid strain used in both studies and confirmed that at 30° on rich agar medium the *rds3::Kan*^{R} knockout is lethal. All four meiotic offspring germinate, but two of the four haploid strains arrest as tiny colonies, which cannot be propagated on fresh medium (Figure 7A and data not shown). None of the large colonies are resistant to the antibiotic G418, showing that all encode the wild-type *RDS3* allele. The *rds3::Kan*^{R} knockout is complemented by ectopic expression of a *GAL1::RDS3* or a *GAL1::rds3-1* promoter fusion gene on a *URA3*-marked plasmid (Figure 7B). The *GAL1::rds3-1* strain exhibits temperature-sensitive, galactose-dependent growth. The *GAL1::RDS3* strain is not temperature sensitive and forms colonies on glucose-based medium, presumably due to residual transcription of the fusion gene (data not shown). The *GAL1::RDS3* and *GAL1::rds3-1* strains do not form colonies on 5-FOA glucose (or galactose) medium (Figure 7B), confirming the Rds3 requirement for mitotic growth.

When *GAL1::rds3-1* transcription is repressed at 37°, pre-mRNA levels elevate within 2 hr and mRNA levels drop to near minimal levels 8 hr later (Figure 7C). In contrast to the 10- to 20-fold reduction in splicing efficiency observed after 10 hr with the *GAL1::rds3-1* culture, only a minor splicing impairment (~2-fold; characteristic wild-type cultures) is observed with the *RDS3* strain. *GAL1::rds3-1* culture growth largely ceases after 10 hr of incubation under these conditions. This time course of splicing and growth impairment is very similar to what has been reported for the depletion of other essential pre-mRNA splicing factors (*e.g.*, see Brown and Beggs 1992; Lockhart and Rymond 1994). Splicing inhibition is also seen if the *GAL1::rds3-1* culture is grown on galactose at 37° or on glucose at 30° although in both cases the extent of inhibition is less complete, presumably due to residual Rds3 activity.

SLC7 encodes Bud13, a 30.5-kD basic protein recently identified in a screen for mutants defective in bud site selection (Ni and Snyder 2001). Diploid null mutants of *BUD13* display a unipolar budding pattern with bud scars restricted to a region near the birth scar at the proximate pole. Homologs of Bud13 are apparent in many organisms [*e.g.*, *H. sapiens* (gi|14249338), *Anopheles gambiae* (gi|21301692), *D. melanogaster* (gi|21355513), and *S. pombe* (gi|19075968)] and show considerable length variation with the highest level of primary sequence conservation present in the carboxyl half of the protein. All Bud13 homologs have 5–7 consecutive lysine or arginine residues present within the highly charged amino terminus and the human and fly homologs have lengthy, highly basic internal insertions. *Slc7-1* introduces a nonsense codon within the conserved carboxyl segment (codon 232).

Allele specificity and directed tests for synthetic lethality: A plasmid shuffle approach was used to score

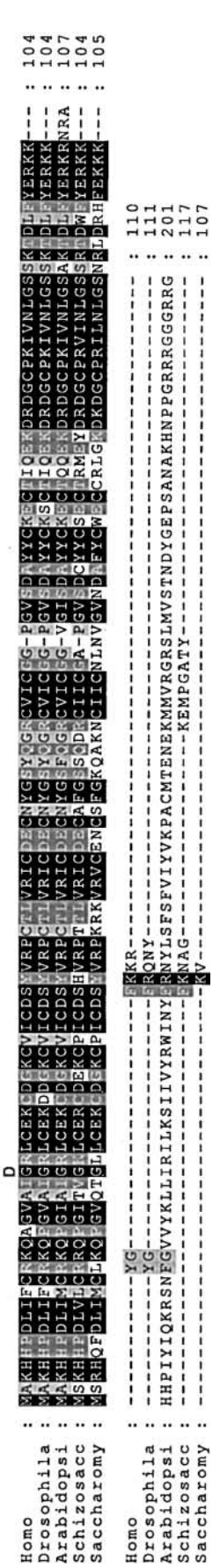

Figure 6.—Multiple sequence alignment of Rds3. ClustI-WP alignment of putative Rds3 homologs highlighted with the Blosum scoring matrix with 100, 80, and 60% sequence similarity presented in boxes of decreasing darkness. The sequences are from *H. sapiens* (gi|14249398), *D. melanogaster* (gi|21711783), *A. thaliana* (gi|25406958), *S. pombe* (gi|19114704), and *S. cerevisiae* (gi|6325351). The G20D amino acid substitution encoded by *slc6-1* (*rds3*) is shown above the alignment.

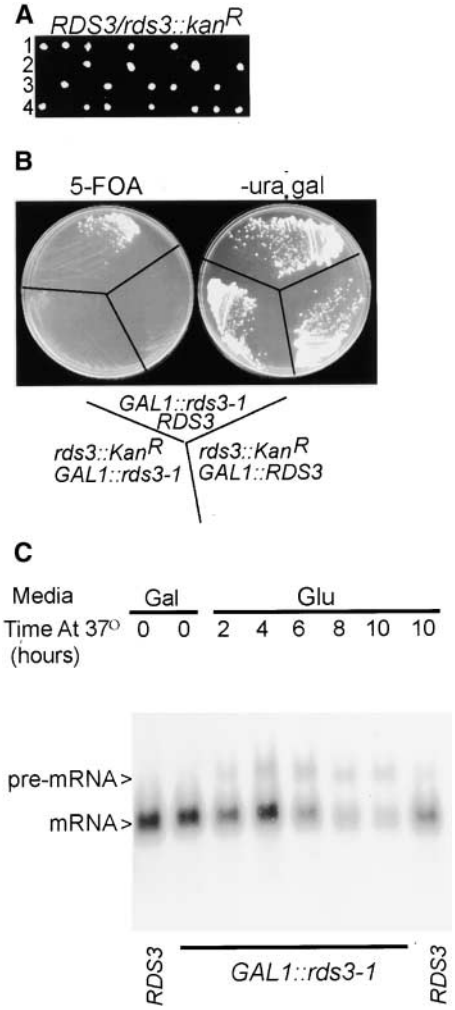

FIGURE 7.—RDS3 encodes an essential pre-mRNA splicing factor. (A) Tetrad dissection of a diploid strain heterozygous for the *rds3::Kan*^{R} mutation. The spores were arrayed on YPD agar at 30° for 5 days. All large colonies were confirmed to contain the wild-type *RDS3* allele. (B) Growth of wild-type yeast (*RDS3*) and the *rds3::Kan*^{R} mutant transformed with the *GAL1::RDS3* gene or the *GAL1::rds3-1* gene. The cultures were streaked on 5-FOA medium and complete medium that lacks uracil with galactose as a sugar source (−ura galactose) and incubated for 4 days at 30°. (C) Pre-mRNA splicing efficiency was monitored in wild-type yeast transformed with the *GAL1::rds3-1* gene (*RDS3*) and in the *rds3::Kan*^{R} mutant transformed with the *GAL1::rds3-1* gene (*GAL1::rds3-1*). RNA was isolated from yeast growing continuously on galactose (Gal) at 30° and after the indicated time in glucose (Glu)-based broth at 37°. *RPS17A* DNA was used as a probe.

four alternative *clf1* alleles for synthetic interactions with the *slc* mutants (Table 2). *clf1*Δ*1* shows a pattern of interaction equivalent to that of *clf1*Δ*2*. This is understandable given the close proximity of TPR1 and TPR2 repeats and the similar Clf1 complex defects observed with both mutants (WANG *et al.* 2003). Deletion of TPR7-8 imparts a ts growth defect and shows strong synthetic interaction with *slc1-1* (*prp16*), *slc3-1* (*cwc2*), *slc4-1* (*cet1*), *slc5-1*, and *slc7-1* (*bud13*). Synthetic lethality is also observed between the weak splicing mutant *clf1*Δ*6-8* and *slc7-1* (*bud13*) and greatly impaired growth is observed with double mutants of *clf1*Δ*6-8* and *slc1-1* (*prp16*), *slc3-1* (*cwc2*), and *slc5-1*. The *clf1*Δ*CTD* mutant supports near normal pre-mRNA splicing but shows a modest synthetic growth defect with *slc7-1* (*bud13*), *slc3-1* (*cwc2*), and *slc4-1* (*cet1*). By this assay, the *slc2-1, 2-2* (*prp22*), and *slc6-1* (*rds3*) interactions display the greatest allele specificity, producing strong synthetic growth defects only with TPR1 or TPR2 deletions.

Prp19-1 and several viable knockout mutants with genetic or biochemical links to Clf1 were also scored for synthetic interactions. Synthetic lethality is observed when *clf1*Δ*2* is combined with *prp19-1*, *ntc20::kan*^{R}, *prp17::kan*^{R}, or *syf2::kan*^{R}. In addition, *clf1*Δ*2, mud2::kan*^{R} double mutants show greatly impaired growth (Figure 8). No synthetic defects were observed between *clf1*Δ*2* and a randomly selected gene knockout (*fus1::kan*^{R}) or with a knockout of a gene unrelated to splicing that nevertheless interacts with Clf1 in the two-hybrid assay (*gpx2::kan*^{R}; UETZ *et al.* 2000).

Dosage suppressors link splicing and GTPase activation in vesicular transport: Dosage suppression was used as a complementary approach to identify genes that interact with *clf1*Δ*2*. From ~150,000 yeast transformants, 16 plasmids were recovered that enhanced growth at the semirestrictive temperature of 34° but which did not contain *CLF1*. Two of the plasmids contained overlapping sequences from the left arm of chromosome XVI (group 1) while 14 contained identical or overlapping regions of the left arm of chromosome X (group 2). Neither suppressor type supports growth of a *clf1::HIS3* null mutant.

The group 1 suppressor, *BTS1*, encodes the yeast geranylgeranyl diphosphate synthase (JIANG *et al.* 1995) while the group 2 suppressor, *BET4*, encodes the α-subunit of the Rab/Ypt-protein geranylgeranyltransferase (GGTase; JIANG *et al.* 1993). All known or predicted GGTase substrates are nucleotide-binding proteins involved in signal transduction events (see DISCUSSION). We believe that the *BTS1/BET4* dosage suppressors act downstream of the primary *clf1*Δ*2* pre-mRNA splicing defect since growth is enhanced without detectable improvement in pre-mRNA splicing (tested for *ACT1*, *SNR17*, and *RPS17A*; data not shown). In addition, a viable *bts1::Kan*^{R} knockout mutant shows no obvious splicing defect (Q. WANG and B. C. RYMOND, unpublished observations).

To learn how general the dosage suppression patterns are, *clf1*Δ*2* and the *slc* mutants were transformed with plasmids bearing *SSD1*, *BTS1*, and *BET4* and assayed for enhanced growth (Table 3). At the semirestrictive temperature used, all mutants are viable but colony size is reduced compared with wild type. The growth of four mutants, *slc4-1* (*cet1*), *slc2-2* (*prp22*), *slc6-1* (*rds3*), and *slc5-1* improved with *SSD1* overexpression. Suppression is not correlated simply with the tightness of the mutant allele as *slc4-1* (*cet1*) is ts lethal at 37° but suppressed by *SSD1* while *slc2-1* (*prp22*), *slc3-1* (*cwc2*), *slc7-1* (*bud13*),

TABLE 2

Allele specificity of *clf1* mutant interactions

	CLF1	*clf1Δ1*	*clf1Δ2*	*clf1Δ6-8*	*clf1Δ7-8*	*clf1ΔCTD*
SLC	+++	++	++	+++	+++	+++
slc1-1 (*prp16*)	++	−	−	+	±	++
slc2-1 (*prp22*)	+++	−	−	+++	+++	+++
slc2-2 (*prp22*)	+++	−	−	++	++	+++
slc3-1 (*cwc2*)	+++	±	−	±	±	++
slc4-1 (*cet1*)	+++	−	−	+	−	++
slc5-1	+++	−	−	±	−	+++
slc6-1 (*rds3*)	+++	−	−	+++	+++	+++
slc7-1 (*bud13*)	+++	−	−	−	−	++

Colony formation assayed at 30° after 3 days of growth on 5-FOA medium. Approximate colony diameters: −, no growth; ±, visible but <0.25 mm; +, 0.5 mm; ++, 1.0 mm; +++, 2.0 mm).

and the uncharacterized K46 mutant are slow growing at 37° but not suppressed with elevated *SSD1* expression.

BTS1 and *BET4* showed an identical suppression pattern, consistent with joint contribution to the pathway of protein geranylgeranylation. In addition to *clf1Δ2*, the *slc6-1* (*rds3*) mutant is enhanced by *BTS1/BET4* overexpression. Of the nine ts yeast strains assayed, only *slc6-1* (*rds3*) was suppressed by all three genes. The distinct *SSD1* and *BTS1/BET4* suppression patterns suggest that at least two distinct pathways exist to reduce splicing-related growth defects.

DISCUSSION

Clf1 interactions: The poly-TPR structure of Clf1 presents a potential docking surface for multiple splicing factors. The deletion analysis presented here supports a scaffold function for Clf1 by showing that the individual Clf1 TPR elements are biologically functional and differ in contribution to splicing. This and other recent studies have identified interactions between Clf1 and factors that act from the earliest stages of spliceosome assembly through product release (Table 4). For instance, U1-Prp40 and Mud2 bind Clf1 and promote U1 snRNP recruitment in the commitment complex while U2 snRNA, Hsh155, and Rse1 are present in Clf1 complexes and snRNP components of prespliceosome. Rds3 interacts genetically with Clf1 and, as described below, is also associated with U2 snRNP proteins. Such "early factor" associations are reinforced by the observation that wild-type *RPS17A* (*RP51A*) prespliceosomes fail to mature into stable snRNP-complete complexes in the absence of Clf1 (Chung *et al.* 1999).

The NTC appears to contain a dozen or fewer proteins that act after prespliceosome formation (Tarn *et al.* 1994; Wang *et al.* 2003). *Clf1*Δ2 shows synthetic lethal interactions with NTC members Prp19, Ntc20, Syf2, and Cwc2 and interacts with Ntc20, Syf2, and other NTC components in two-hybrid and protein-binding assays. Although it is attractive to imagine the NTC as a multisubunit splicing factor, there is no evidence to support a requirement for NTC preassembly in splicing. Nevertheless, Clf1 is an essential NTC protein that is implicated with other members of this structure in events that occur during and after the prespliceosome to spliceosome transition.

The NTC proteins also reside in RNP structures that contain additional proteins and the U2, U5, and U6 snRNAs (the Clf1-RNP and related complexes; Ohi *et al.* 2002; Wang *et al.* 2003) or the U1, U2, U4, U5, and U6 snRNAs (the penta-snRNP; Stevens *et al.* 2002). A number of the RNP-specific proteins interact with Clf1. For instance, Snu114, a U5 snRNP-associated protein that contributes to dissociation of the U4/U6 helices (Bartels *et al.* 2002), shows conditional association with ClfΔ2 (Wang *et al.* 2003) while overexpression of U2 snRNA partially suppresses certain *CFL1* (also called *SYF3*) mutations (Ben-Yehuda *et al.* 2000). Several second-step splicing factors are present in the U2, U5, and U6 snRNA-bearing complexes (but not in the penta-snRNP). Of these, Prp16, Prp17, and Prp22 were found as synthetic lethal mutants with *clf*Δ*2*. Like the *slc2* mutants, most other *PRP22* helicase domain mutants show a step 1 block to splicing *in vivo*. Interestingly, the Prp22 G692D mutant described here was also identified as an intragenic suppressor of an SAT → AAT motif III change that uncouples the Prp22 ATPase activity from the RNA helicase and mRNA release activities (Schwer and Meszaros 2000; Campodonico and Schwer 2002). Prp22 copurifies with Clf1 complexes and interacts with the Clf1 in the two-hybrid assay—although perhaps at a binding site well removed from TPR2 (Ben-Yehuda *et al.* 2000). Splicing inhibition by either *slc2* inactivation or *clf1Δ2* coexpression is consistent with joint Prp22/Clf1 contribution to a late (and likely ATP-dependent) step in the spliceosome cycle.

Newly defined splicing-relevant genes: Mutations within *BUD13*, *CET1*, *CWC2*, *RDS3*, and *SLC5* were found to be synthetically lethal with *clf1Δ2* and splicing defective when expressed in a wild-type *CLF1* background. Cet1 phosphatase activity is required for cap formation on pol II RNAs, including pre-mRNA and snRNA. The

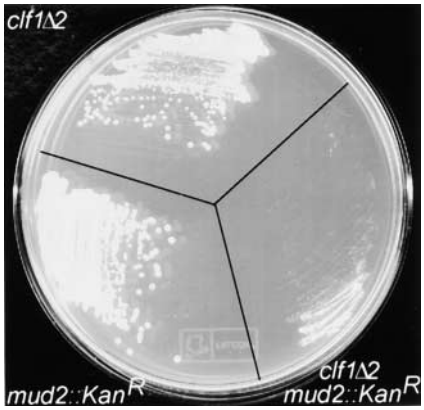

FIGURE 8.—Synthetic growth defect of the *mud2::kan*^{R}, *clf1Δ2* double mutant. Yeast colonies containing the *mud2::kan*^{R}, *clf1Δ2*, or both mutations are shown after 4 days of growth on YPD agar at 23°.

TABLE 3

Enhanced colony growth with dosage-suppressor expression

Mutant	*SSD1*	*BTS1*	*BET1*	Vector
slc1-1	−	−	−	−
slc2-1	−	−	−	−
slc2-2	+	−	−	−
slc3-1	−	−	−	−
slc4-1	+	−	−	−
slc5-1	+	−	−	−
slc6-1	+	+	+	−
slc7-1	−	−	−	−
clf1Δ2	−	+	+	−
K46	−	−	−	−
CLF1	−	−	−	−

Colony formation assayed at the restrictive temperature after 2 days of incubation. Approximate colony diameters: −, colony size identical to the untransformed strain; +, colony size >1.5 times the size of the untransformed mutant.

slc4-1 (*cet1*) mutations create amino acid substitutions at sites believed important for enzyme structure (residue 422) and activity (amino acid 495; PEI *et al.* 1999; BISAILLON and SHUMAN 2001). That the *slc4-1* (*cet1*) splicing defect is due principally to improper cap formation is reinforced by the observation that *CEG1* guanylyltransferase mutants are also splicing impaired (SCHWER and SHUMAN 1996). Cap structure enhances snRNA stability and, at least in mammals, facilitates nuclear import of snRNP particles (WILL and LUHRMANN 2001). *In vitro*, the nuclear cap-binding complex promotes the initial interaction between the U1 snRNP and the splicing substrate (COLOT *et al.* 1996; LEWIS *et al.* 1996) and functions during 5′ splice site rearrangements that lead to the displacement of U1 snRNA by U6 snRNA (O'MULLANE and EPERON 1998). While the *clf1Δ2/slc4-1* (*cet1*) arrest point is unknown, we note the cap-sensitive U6 for U1 snRNP swap occurs at or near the time of Clf1 (and Prp19) function in the spliceosome cycle.

Cwc2 is one of >30 proteins present in Clf1-containing complexes (OHI *et al.* 2002; WANG *et al.* 2003). While this work was in progress the Gould lab provided evidence that targeted degradation of Cwc2 blocks pre-mRNA splicing (OHI and GOULD 2002). The mutational data presented concur and extend this observation to show that the zinc finger domain of Cwc2 is essential for biological activity. Cwc2 interacts through its N-terminal region with Prp19 but does not appear to bind Clf1 directly (OHI and GOULD 2002). Cef1 binds both Clf1 and Prp19. Under restrictive conditions, the *clf1Δ2* mutation causes the release of Prp19 and Cef1 from Clf1 complexes and blocks recruitment of Prp19 to the spliceosome (WANG *et al.* 2003). As the *slc3-1* (*cwc2*) mutation resides within the proposed Prp19-binding domain (OHI and GOULD 2002), the *clf1Δ2/slc3-1* (*cwc2*) lethality most likely results from disruption of the Clf1/Cef1/Prp19/Cwc2 organization within the splicing complex.

The bud site defect of the *bud13* (*slc7-1*) (NI and SNYDER 2001) is most likely an indirect consequence of impaired pre-mRNA splicing as disruption of numerous biological pathways (*e.g.*, lipid metabolism, RNA transport and processing, translation, and vesicular transport) impairs the budding process. Consistent with this, mutations in the genes encoding the NTC splicing factor Isy1, the U2 snRNP protein Ist3/Snu17, and the U6 core snRNP protein Lsm6 also result in bud defects (NI and SNYDER 2001). Although little is known of Bud13 function in other organisms, RNAi experiments show that it is required for *Caenorhabditis elegans* embryogenesis (JIANG *et al.* 2001), consistent with a conserved role in pre-mRNA splicing.

Rds3 rivals the core histone proteins in its exceptional level of sequence conservation. Given the apparent stringent constraints on its sequence, it is not surprising that *RDS3* is essential for yeast viability. Turcotte and colleagues previously reported *RDS3* as a nonessential gene (AKACHE *et al.* 2001; AKACHE and TURCOTTE 2002). A possible explanation for this discrepancy is provided by the observation that when *rds3::Kan*^{R} yeast are cultured at 23° (rather than at 30°), cell division occurs but at a very slow rate (*i.e.*, a doubling time >12 hr). Rare bypass suppressors occasionally arise under these conditions and rapidly overgrow the culture. It is possible that such a bypass mutant was isolated by the random spore selection used to acquire the *rds3::Kan*^{R} strain for the drug sensitivity study. The decreased expression of the *PDR5* and *SNQ2* drug transporter genes reported in the putative *rds3::Kan*^{R} background might be an indirect consequence of splicing impairment although we note that neither gene contains an intron. Q. WANG and B. C. RYMOND (unpublished observations) found that Rds3 is a stable component of the spliceosome and acts, *in vitro*, to promote splicing. Although functional data are not available, the human homolog of Rds3 was recently found to be associated with the 17S U2 snRNP particle

TABLE 4

Summary of Clf1 genetic and biochemical interactions

Two-hybrid interaction	Direct protein interaction	Multisubunit complex	Synthetic growth impairment	clfΔ2 complex sensitive	Dosage suppressor
Mud2	+ (6) + (6)		+ (4)		
Prp40	+ (6) + (6)	+ (8)			
Hsh155		+ (2, 3, 8)		+ (3)	
Rse1		+ (2, 3, 8)		+ (3)	
U2 snRNA		+ (2, 3, 8)			+ (7)
Snu114		+ (2, 3, 8)		+ (3)	
Prp19	− (1, 5)	+ (1, 2, 3, 8)	+ (4)	+ (3)	
Cef1	+ (1, 5, 7)	+ (2, 3, 8)		+ (3)	
Isy1	+ (1, 5, 7) + (7)	+ (1, 2, 3, 8)			
Ntc20	+ (1, 5, 7)	+ (1, 2, 3, 8)	+ (4)		
Snt309	− (1, 5)	+ (1, 2, 3, 8)		− (3)	
Syf1	+ (1, 5)	+ (1, 2, 3, 8)		− (3)	
Syf2	+ (1, 7)	+ (1, 2, 3, 8)	+ (4)		
Cwc2	− (5)	+ (2, 3, 8)	+ (4)	− (3)	
Prp16		+ (3, 4)	+ (4)		
Prp17	− (7)	+ (2)	+ (4, 7)		
Prp22	+ (7)	+ (2, 3, 4)	+ (4)		
Prp46	+ (5)	+ (2, 3, 8)		− (3)	
Rds3			+ (4)		
Bud13			+ (4)		
Slc5			+ (4)		
Cet1			+ (4)		
Bts1					+ (4)
Bet4					+ (4)

(1) Chen *et al.* (2002) and references within; (2) Ohi *et al.* (2002); (3) Wang *et al.* (2003); (4) this study; (5) Ohi and Gould (2002); (6) Chung *et al.* (1999); (7) Ben-Yehuda *et al.* (2000); and (8) Stevens *et al.* (2002).

(Will *et al.* 2002). Yeast Rds3 also interacts with several U2 snRNP proteins, including Clf1-RNP components, providing possible targets for the synthetic lethal interaction (Q. Wang and B. C. Rymond, unpublished observations).

Suppression of pre-mRNA splicing defects: Mutations in the *PRP2* and *RSE1* splicing factor genes were shown previously to cause vesicular transport defects (Chen *et al.* 1998) that could be suppressed by enhanced expression of the *SAR1* GTPase that promotes ER to Golgi sorting. Thus, under some but perhaps not all (Biggins *et al.* 2001; Burns *et al.* 2002) conditions, splicing inhibition renders vesicular transport limiting for yeast cell growth. The substrate for Bet4 modification is not clear, however, since Sar1 lacks the consensus site for geranylgeranylation and none of the predicted Bet4 substrates contain introns. In the absence of GGTase activity, the small Ypt1 and Sec4 GTPases are not lipid modified, their membrane localization is impaired, and vesicular transport is disrupted (Jiang and Ferro-Novick 1994). Importantly, other known or suspected substrates for C-terminal geranylgeranyl modification are also rab-GTPases or ras-like proteins and casein kinase (*i.e.*, Cdc42, Rho1, Rho2, Rho3, Rho4, Rsr1, Sec4, Vps21, Yck1, Yck2, Ypt1, Ypt6, and Ypt7; Costanzo *et al.* 2001) that function in signal transduction pathways involving vesicular transport or related membrane-associated events. Although direct involvement of certain secretory proteins in splicing has been suggested (Awasthi *et al.* 2001), it seems likely that the *BTS1/BET4* dosage suppression works by increasing the pool of modified and hence active, membrane-bound signal transduction molecules, thus driving downstream events in the secretory pathway.

A recent analysis of global gene expression suggests differences in the set of pre-mRNAs most affected by the inactivation of specific splicing factors (Burns *et al.* 2002). Presumably dosage suppression depends on which RNAs become limiting for cellular growth. In the case of the RNA-binding protein, Ssd1, suppression is likely RNA based (for instance, through enhanced stabilization of pre-mRNA, mRNA, or snRNA). In contrast, for *BTS1*, *BET4* suppression most likely acts post-translationally to improve the efficiency of splicing-dependent biological pathways, most critically vesicular transport, which involves the activity of numerous intron-bearing genes (*e.g.*, *BET1*, *BOS1*, *ERV41*, *GOT1*, *MSB4*, *NYV1*, *SFT1, SNC1*, and others).

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