**Saccharomyces cerevisiae** NineTeen Complex (NTC)-associated Factor Bud31/Ycr063w Assembles on Precatalytic Spliceosomes and Improves First and Second Step Pre-mRNA Splicing Efficiency

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**Background:** Some yeast splicing factors, e.g. Bud31 in Cef1p subcomplex, are nonessential for cell viability.

**Results:** Bud31 occurs in precatalytic (B) and catalytic spliceosomes and stabilizes protein interactions with the pre-mRNA; its absence affects the first and second step of splicing.

**Conclusion:** Bud31 aids both catalytic steps through its spliceosome interactions.

**Significance:** Nonessential factors have auxiliary roles in multiple steps of pre-mRNA splicing.

Pre-mRNA splicing occurs in spliceosomes whose assembly and activation are critical for splice site selection and catalysis. The highly conserved NineTeen complex protein complex stabilizes various snRNA and protein interactions early in the spliceosome assembly pathway. Among several NineTeen complex-associated proteins is the nonessential protein Bud31/Ycr063w, which is also a component of the Cef1p subcomplex. A role for Bud31 in pre-mRNA splicing is implicated by virtue of its association with splicing factors, but its specific functions and spliceosome interactions are uncharacterized. Here, using in vitro splicing assays with extracts from a strain lacking Bud31, we illustrate its role in efficient progression to the first catalytic step and its requirement for the second catalytic step in reactions at higher temperatures. Immunoprecipitation of functional epitope-tagged Bud31 from in vitro reactions showed that its earliest association is with precatalytic B complex and that the interaction continues in catalytically active complexes with stably bound U2, U5, and U6 small nuclear ribonucleoproteins. In complementary experiments, wherein precatalytic spliceosomes are selected from splicing reactions, we detect the occurrence of Bud31. Cross-linking of proteins to pre-mRNAs with a site-specific 4-thio uridine residue at the -3 position of exon 1 was tested in reactions with WT and bud31 null extracts. The data suggest an altered interaction between a ~25-kDa protein and this exonic residue of pre-mRNAs in the arrested bud31 null spliceosomes. These results demonstrate the early spliceosomal association of Bud31 and provide plausible functions for this factor in stabilizing protein interactions with the pre-mRNA.

The spliceosome is a dynamic complex that assembles in a stepwise manner on the pre-mRNA with several conformational transitions for its activation, catalysis, and recycling. In vitro splicing assays with budding yeast cell-free extracts show that a critical and early step in spliceosome activation is the dissociation of the U1 and U4 snRNAs from the precatalytic B spliceosome that contains all five spliceosomal U-rich snRNAs. Concomitant with this transition, the Prp19 complex (NTC) stably joins the assembly pathway to form B act spliceosomes (1, 2). NTC stabilizes the interactions of U5 and U6 snRNAs with key cis splice site elements in the pre-mRNA. The core NTC consists of 10 proteins, associated with Prp19, identified by a combination of genetic and biochemical methods (3–7). Additional proteins join the core NTC complex as the spliceosome cycle progresses. These proteins have diverse functions spanning the various steps of the splicing pathway: spliceosome activation, catalysis, and recycling (reviewed in Ref. 8).

Factors of the NTC are evolutionarily conserved with **Saccharomyces cerevisiae**, *Schizosaccharomyces pombe*, and human complexes having similar protein compositions as determined by proteomic analyses. The human CDC5 complex contains homologs of four identified *S. pombe* Cwf proteins (Cwf1p, Cwf7p, Cwf8p, and Cwf9p) and Prp19, a principal *S. cerevisiae* NTC component (9, 10). In independent proteomic studies, several of the NTC proteins have also been isolated in a subcomplex with Cef1p along with other splicing factors (11). The latter have been classified as NTC-associated proteins. Bud31/Ycr063w is a nonessential, NTC-associated protein identified as a constituent of the Cef1p complex through proteomic analyses (11, 12). The predicted protein has 55% identity with its *S. pombe* homolog Cwf14, 43% identity with the human edg-2, and 51% identity with G10 protein in *Xenopus laevis* (13, 14). Other than a putative nuclear localization signal and C-terminal zinc finger motif, no other domains are annotated in this protein. Other studies have reported phys-
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Splicing defects seen at nonoptimal reaction temperatures. We demonstrate that the earliest association of Bud31 with spliceosomes is with the B precatalytic complex and that it continues its association with activated, catalytic, and recycling spliceosomes that are formed later. These inferences are based on our data from pulldown of the epitope-tagged protein and also through investigations on the presence of Bud31 protein in pre-mRNA substrate affinity-purified spliceosomes. We show that Bud31 is required for efficient cross-linking of a ~25-kDa protein with exon sequences near the 5’ splice site of the pre-mRNA substrate, particularly in reactions at higher temperatures. In the inefficiently assembled and arrested spliceosomes that lack Bud31, the absence of this ~25-kDa factor is possibly an underlying reason for the splicing defect seen at nonoptimal reaction temperatures. Taken together, our data illustrate an initial precatalytic interaction of Bud31 with the spliceosome and its sustained presence in complexes of later steps of the splicing cycle, define its functions through the catalytic steps of pre-mRNA splicing, and suggest a role in stabilizing protein-pre-mRNA interactions.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Splicing extracts were prepared from the haploid strains: ycr063w::KANMX4 (bud31::KANMX4), ycr063w::KANMX4 transformed with the expression construct pCHP425YCR063w/BUD31, WT BY4743, YCWC2TAP, YPRP21TAP, and YCR063wTAP (BUD31TAP). The haploid ycr063w::KANMX4 and the BY4743 strains were generated by random sporulation from the diploid ycr063w::KANMX4/ycr063w::KANMX4 and the BY4743 strains, respectively, which, in turn, were obtained from EUROSCARF, whereas YCR063wTAP/BUD31TAP was from Open Biosystems. The strains YCWC2TAP and YPRP21TAP were kindly provided by R. T. O’Keefe (17). pCHP425YCR063w/BUD31 was created by cloning the YCR063w::BUD31 open reading frame under its own promoter as a BamHI fragment into the BamHI site of the C-terminal epitope-tagging vector, pCHP425. Bud31/Ycr063w expressed from this construct is translationally fused at its C terminus with poly-histidine and polyoma middle T-antigen tags.

Yeast Splicing Extracts, in Vitro Transcription, and pre-mRNA Splicing Reactions—Yeast splicing extracts were prepared as in Ref. 19. In vitro transcription and splicing reactions were performed as in Ref. 20. [α-32P]UTP-labeled WT pre-mRNA transcript was prepared using EcoRI-linearized pSP65 actin plasmid (21) as the template using SP6 polymerase (GE Healthcare). Biotinylated 3’ splice site mutant C303/305 and a truncated pre-mRNA lacking the 3’ splice site Ac/Cla (34) actin were transcribed using HindIII and ClaI linearized pSP65C303/305 and pBS Actin plasmid templates, respectively, with the reaction being supplemented with 25 μM biotin-11-UTP (PerkinElmer Life Sciences) (5). The transcripts were gel-purified. DNA templates for transcription of CYH2 3’-RNA were prepared by polymerase chain reaction from r243 plasmid (22). CYH2 pre-mRNA containing 4-thio uridine (4-thio-U) at −3 E1 were synthesized by RNA ligation of a custom-synthesized 5’-oligonucleotide (Dharmacon) to in vitro transcribed 3’-CYH2 RNA (23, 24).

All splicing reactions were resolved on 7% (19:1) denaturing polyacrylamide gels. Reactions with 5 mM EDTA or with varying ATP concentrations were carried out as described previously (25, 26). The pre-mRNA substrate, splicing intermediates, and products were visualized by phosphorimaging and quantitated using MULTIGAUGE software.

Immunoprecipitation and Northern Analysis of snRNPs from Splicing Extracts and in Vitro Assembled Spliceosomes—Immunoprecipitation of Bud31 from in vitro assembled spliceosomes or native splicing extracts was carried out either with anti-polyoma monoclonal antibodies (27) immobilized on protein-G-Sepharose (GE Healthcare) for BUD31 fused to the polyoma tag by using IgG-agarose to pull down TAP epitope tagged Bud31 protein. For immunoprecipitation of snRNAs from S100 extracts (20) from the TAP-tagged strains, 30 μl of the S100 extract was added to 30 μl of IgG-agarose (Sigma) and used for bead binding in NET buffer (composition 10 mM Tris-Cl, 0.5% Sigma Nonidet P-40 1 mM EDTA) supplemented with 50, 150, 250, or 500 mM NaCl. All immunoprecipitations were carried out as in Ref. 28. The immunoprecipitated snRNAs were analyzed by Northern blot as described in Ref. 25. Radiolabeled U1, U2, U4, U5, and U6 DNA fragments of equal specific activity were used as probes.

Immunoprecipitation of pre-mRNA Substrate, Intermediates, and Products from in Vitro Reactions—40 μl of bead immobilized anti-polyoma antisera was added to 40 μl of the splicing reaction aliquot. Binding was carried out for 1.5 h at 4 °C in 1× NET-150 buffer followed by three washes with 1× NET-150. The beads were recovered and deproteinized with stop mix (1 mg/ml protease K, 5 mM EDTA, and 1% SDS) for 30 min. The RNA species associated with the beads were extracted and analyzed on 7% urea PAGE gels.

Affinity Purification of Spliceosomes, Western Blot Analysis of Proteins, and Primer Extension of snRNAs—For analysis of proteins in spliceosomes with biotinylated substrate RNA, splicing reactions containing 320 μl of splicing extracts were assembled on 500 ng of biotinylated C303/305 mutant actin pre-mRNA. As a control, the same substrate RNA was prepared in nonbiotinylated form. The reaction mixtures were incubated at 22 °C for 15 min prior to the addition of the specified precursor sub-
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strate pre-mRNA. After 30 min at 25 °C, 80 µl of streptavidin-Sepharose beads (GE Healthcare) in 800 µl of NET-150 buffer was added. Binding was allowed for 1.5 h at 4 °C, and bead-bound spliceosomes were washed extensively with NET-150 buffer. A 100-µl aliquot of the bead-bound spliceosomes was used for snRNP analysis, whereas the rest of the 700 µl of bead-bound spliceosomes was taken for protein analyses. The bead-bound proteins were extracted and analyzed by Western blot analysis using anti-TAP antibodies (Pierce) at 1:2000 dilution.

For primer extensions analysis of U snRNAs, 5’ end labeling of U1, U2, U4, U5, and U6 primers (17) was carried out with T4 polynucleotide kinase (New England Biolabs). The labeled primers were annealed in a 20-µl reaction with 4 µg of total RNA by heating to 65 °C for 10 min. The reaction mix was snap-chilled, and primer extension was carried out by adding 25 units of RNasin (New England Biolabs) and 50 units of MMLV reverse transcriptase (New England Biolabs) with incubation at 37 °C for 30 min. The reaction was stopped by heating to 70 °C for 10 min. The extension products were precipitated and analyzed on a 6% (19:1 acrylamide:bisacrylamide), 1 × Tris-borate-EDTA, 8 M urea, pH 8.3, gel, dried, and exposed to a phosphorimaging screen.

**UV Cross-linking of Proteins with 4-thioU (CYH2) Pre-mRNA—**In vitro splicing of mutant actin Ac/Cla mini-transcripts was carried out in 10-µl reactions with S100 extracts from TAP-tagged strains. These reactions were UV cross-linked (250 nm and 4111 µJ/cm² energy) on ice. The RNA in the cross-linked reaction was pulled down by streptavidin beads (Dynal, Dynabeads M-280 streptavidin) incubated for 45 min at room temperature. The paramagnetic particles were then captured in a Dynal magnetic rack and washed three times with 1 × binding buffer. The beads were resuspended in elution buffer (10 mM Tris-Cl, 1 mM EDTA) and RNase mixture (Ambion) and incubated at 37 °C for 40 min. The substrate-associated proteins were precipitated with 100% TCA with 0.4% sodium deoxycholate on ice for 20 min. The proteins were pelleted, washed with ice cold acetone, and air-dried. The samples were denatured and electrophoresed on a 12% SDS-PAGE gel. The proteins were taken for Western blot analysis with α-TAP antibody (Pierce) at 1:2000 dilution.

**UV Cross-linking of Proteins with 4-thioU (CYH2) Pre-mRNA Substrate—**10-µl splicing reactions were set up with 250,000 dpm of (−3) 4-thioU containing CYH2 pre-mRNA or 250,000 dpm of pre-mRNA substrate without 4-thioU as described in Ref. 24. The splicing reactions were carried out at 23 or 37 °C for 20 min. Subsequently the reaction samples were irradiated with a Blak-Ray B-100AP ultraviolet lamp at 365-nm filter (UV Products). All reactions were then treated with 1000 units of RNase T1 (Roche Applied Science) for 1 h at 37 °C. The cross-linked proteins were analyzed on a 12% SDS-PAGE gel, visualized, and quantitated by autoradiography.

**RESULTS**

**Bud31 Interacts with U5 and U6 snRNAs in Splicing Extracts—**Analysis of the common and unique spliceosome/snRNA interactions displayed by members of the NTC complex has provided insights on their individual contributions. For instance, factors such as Prp17 and Cef1p associate with U2, U5, and U6 snRNAs (10, 28), whereas Cwc2 associates with U2 and U6 snRNAs in *in vitro* assembled spliceosomes (17). Here, we investigated the snRNA association profile of Bud31. Epitope-tagged Bud31-TAP was immunoprecipitated from splicing extracts, and the precipitate was probed for the presence of U1, U2, U4, U5, and U6 snRNAs by Northern blotting. The strength of the interactions was tested by varying salt (50–500 mM NaCl) in the immunoprecipitation buffer. All five snRNA species are readily detectable in the input lane containing RNAs extracted from the splicing extract (Fig. 2, *lane 1*). In the TAP immunoprecipitation reactions, however, significant amounts of U5 and U6 are detected at 50 mM salt concentration, U2 association is nonoptimal or extreme temperatures, i.e. 37 °C (Fig. 1A) and 18 °C (data not shown). This temperature-sensitive phenotype is similar to that noted for deletion or missense mutants in a number of other nonessential splicing factors such as Prp17, Prp18, Snt309, and Lea1 (4, 29–33). Given previous studies on the proteomic association of Bud31 with splicing factors (10, 11) and its requirement for robust growth of yeast cells at 37 °C, we examined the *in vitro* splicing functions of Bud31. *In vitro* transcribed ACT1 mini-transcripts were spliced using cell-free extracts prepared from haploid bud31Δ and isogenic WT BY4743 cells. Splicing reactions were carried out at 23 and 37 °C, and reaction kinetics were determined by assessing and quantifying the reaction intermediates and products in a time series (5, 15, and 25 min). Extracts from WT cells splice the ACT1 mini pre-mRNA efficiently at 23 °C. Both catalytic reactions are efficient as significant amounts of products from the second step, i.e. the intron and mRNA, are detected at all time points (Fig. 1B, *lanes 1–3*, supplemental Fig. S1A for normalized first step and second step catalyzed RNA species). At 37 °C, overall splicing efficiency is slightly lower, even in WT extract reactions, but importantly no arrest was observed at either splicing step (Fig. 1B, *lanes 4–6*, supplemental Fig. S1A). In reactions with the bud31Δ extract, we observe reduced efficiency of the second step even at 23 °C as compared with reactions at 23 °C with WT extracts (Fig. 1B, *lanes 7–9*, supplemental Fig. S1A). In reactions at 37 °C, by 25 min, both first step and second step products are nearly 4-fold reduced as compared with that in reactions with WT extract (Fig. 1B, *lanes 10–12*, supplemental Fig. S1A). The ratios of the first and second step products are depicted in Fig. 1C to reflect splicing efficiency. Although the kinetics of the first step itself are reduced in reactions with bud31Δ extracts, the inefficiency of the second step, detectable at 23 °C, is exacerbated at elevated temperatures (Fig. 1C and supplemental Fig. S1A). These data are supported by *in vitro* splicing reactions performed with extracts from a diploid strain homozygous for bud31 null allele and a control WT diploid (supplemental Fig. S1, *B and C*). The *in vitro* accumulation of first step products at 37 °C in the absence of Bud31 correlate with the arrested growth of bud31Δ cells at 37 °C and implicate roles for this factor for splicing progression through the second catalytic step. **Splicing Functions and Spliceosome Associations of Bud31**

**Bud31 Improves First and Second Catalytic Steps of Splicing—**To study the growth phenotype of cells with a bud31Δ::kanMX4 (ycr063w::kanMX4) homozygous diploid. The haploid bud31Δ strain showed retarded growth at
only marginal, and no U1 and U4 snRNAs are pulled down. The association of Bud31 with U5 and U6 snRNPs was salt-stable and persisted even in immunoprecipitations performed with 500 mM NaCl (Fig. 2, lanes 2–5). These experiments clearly show that Bud31 interacts with the U5 and U6 snRNPs, hinting at its association with catalytic spliceosomes.

**Bud31 Associates with Spliceosomes Containing the Pre-mRNA, Intermediates, and Products**—Our data on the kinetics of splicing indicate that Bud31 improves progression to the first catalytic step of splicing and is required for the second step. To map the association of Bud31 with various spliceosome complexes, we created a functional C-terminal double epitope (polyoma middle T-antigen and poly-His) fusion to Bud31. The stable expression of the epitope-tagged protein, in this strain, was first confirmed by Western blot analysis of the extract (data not shown), and the functionality of the protein was tested in vivo (Fig. 3A) and in vitro (supplemental Fig. S2). In cells transformed with the plasmid, we observe a complete rescue of the temperature sensitivity at 37 °C, whereas the null strain transformed with the empty vector failed to grow at this temperature. In vitro, the splicing arrest for the second step seen in reactions with null extract is not observed in extracts with the plasmid-expressed epitope-tagged Bud31-polyoma-His protein. The ACT1 intron is spliced efficiently at all temperatures, showing that the Bud31-polyoma-poly-His fusion protein is fully functional (supplemental Fig. S2).

We next used these extracts for in vitro splicing reactions in varying conditions (Fig. 3B) followed by immunoprecipitation of the tagged Bud31 protein and analysis of the associated substrate RNA species. In reactions supplemented with 2 mM ATP that allow for efficient and complete splicing, we find that Bud31 associates with spliceosomes containing the substrate (pre-mRNA) as well with complexes with the splicing RNA intermediates and spliced RNA products (Fig. 3C, lanes 1–3). The co-precipitation of significant amounts of pre-mRNA with the protein hints at the presence of Bud31 in assembling spli-
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Bud31 Can Be Detected in Affinity-purified B and B\textsuperscript{act} Spliceosomes—To substantiate our data suggesting the association of Bud31 with the B and B\textsuperscript{act} spliceosomes, we assembled these spliceosome complexes on biotinylated actin pre-mRNAs, with a 3′ splice site mutation (CAG/AG to CAC/AC) in the actin intron (21). Although this pre-mRNA 3′ splice site mutation prevents the second catalytic step, it does not affect spliceosome assembly or activation (34). Large scale (800 μl) in vitro reactions were performed with Bud31-TAP extracts and, as a control, Prp21-TAP extracts, under conditions that cause arrest at B or at B\textsuperscript{act} complexes. To begin with, the status of the stalled complexes was ensured by assessing their snRNA content. For this purpose, the snRNA species in 50-μl aliquots of the large scale reactions with the respective spliceosomes were analyzed by primer extension. In splicing reactions carried out with 0.05 mM ATP and extracts from Bud31-TAP or the Prp21-TAP strains, the B complexes contained all five RNA species, as expected (lanes 8 and 9 in Fig. 4A and in supplemental Fig. S4, left). Subsequent conformational changes led to the dissociation of the U1 and U4 snRNAs; therefore, B\textsuperscript{act} spliceosomes show stable binding of U2, U5, and U6 snRNAs. The snRNA profile of our arrested B\textsuperscript{act} spliceosomes matches this expectation, as shown in lanes 12 and 13 in Fig. 4A and in supplemental Fig. S4, left. The control nonbiotinylated substrate showed no nonspecific association of snRNAs with the streptavidin beads (lanes 3, 4, 7, and 8 in Fig. 4B and in supplemental Fig. S4, right).

Having ensured that the large scale reactions with the biotinylated substrate are arrested at the B and B\textsuperscript{act} complexes, the presence of Bud31-TAP protein was examined, in the remainder of the splicing reaction, after pulldown of the substrate with streptavidin beads. Nonbiotinylated C303/C305 substrate in parallel splicing reactions was taken for “mock pulldown” to analyze the nonspecific stickiness of protein with the streptavidin-agarose beads. The proteins associated with the streptavidin bead-bound biotinylated pre-mRNA were analyzed by Western blot analysis using anti-TAP antibodies (Fig. 5). Both Bud31 and Prp21 are enriched in the B and B\textsuperscript{act} complexes over and above the nonspecific sticking with the beads, as deciphered from reactions with the pulled down nonbiotinylated substrate. These data demonstrate the presence of Bud31 in the reactions with stalled B complexes (Fig. 3C, lanes 7–9), indicating its coupling with the spliceosome at this stage. However, in reactions with no ATP, i.e. with stalled commitment complex, the RNA pulled down is nearly equivalent to the random stickiness of RNA to beads alone (Fig. 3C, lanes 10–12), hence suggesting that the Bud31 protein is not in commitment complexes. All pulldown experiments were replicated 3–4 times, and the normalized levels of the pulldown RNAs were quantitated and compared (Fig. 3D). To rule out experimental artifacts that may arise from the extracts having the tagged protein that is overexpressed, we performed independent experiments (supplemental Fig. S3) with extracts prepared from a strain where TAP-tagged Bud31 is expressed from its normal chromosomal locus. The results obtained from these experiments were quantitated, and they corroborate the data from the plasmid-expressed Bud31-Py-tagged extracts. These experiments clearly show a precatalytic association of Bud31, more specifically at the B step.

FIGURE 2. Bud31 stably associates with U5 and U6 snRNPs in splicing extracts. Bud31-TAP protein was immunoprecipitated from splicing extracts under increasing salt concentrations (NaCl conc., lanes 2–5). The co-immunoprecipitated U-rich snRNAs were detected by Northern blot of a denaturing urea PAGE gel. RNA from one-fifth of the extract used for immunoprecipitation was loaded as input. IgG-agarose pulldown from BY4743 extracts in 50 mM NaCl was loaded as control (C, lane 6).

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precatalytic spliceosomes, as early as the B complex, and complement the data from pulldown of substrate by immunoprecipitation of the protein.

Association of Bud31 with Pre-mRNA in Precataylhetic B^act Spliceosome Is Not Direct—We next asked whether Bud31 interacts directly with the pre-mRNA in the precataylhetic spliceosomes or whether the pulldown of the pre-mRNA reflects an indirect association. For these experiments, we assembled spliceosomes on a biotinylated actin substrate (Ac/Cla) lacking the 3’ splice site, which creates arrest before the first catalytic step but allows for NTC association (34). The reactions were carried out with extracts containing the TAP-tagged Bud31 protein, under normal splicing conditions. Parallel experiments were carried out with the same substrate and extracts from a Cwc2-TAP strain that served as a positive control as recent experiments have suggested that Cwc2 directly interacts with substrate RNA in spliceosomes.3 The pre-mRNA substrate and the proteins in the splicing reaction were UV cross-linked, and the proteins cross-linked to the biotinylated pre-mRNA were pulled down using streptavidin-coated magnetic beads. The substrate was digested with RNase, and the proteins were taken for detection by Western blot analysis. Both Bud31 and Cwc2p proteins are detected in the splicing extracts (Fig. 6, lanes 5 and 6). As expected, Cwc2p directly associated with the Ac/Cla substrate because the protein could be detected in the Western analysis of the UV cross-linked and pulled down spliceosomes (Fig. 6, lanes 3 and 4). However, Bud31 could not be detected in the Western analysis of proteins cross-linked to the substrate RNA (Fig. 6, lanes 1 and 2). These data, together with previous results, strongly suggest that although Bud31 interacts with

FIGURE 3. Bud31-polyoma middle T-antigen His-tagged protein is functional in vivo and associates with spliceosomes in vitro. A, growth profile of the bud31 transformed with pCHP425 plasmid expressing dual tagged Bud31 or after transformation with the empty vector at 23 or 37 °C. B, schematic representation of spliceosome assembly and the conditions that stall at B^act, B, and E complexes. U6snRNA, U-rich snRNA. C, in vitro reactions at 23 °C with 2 mM ATP (lanes 1–3), with 2 mM ATP and 5 mM EDTA to enrich B^act complex (lanes 4–6), with 0.05 mM ATP to obtain B complex (lanes 7–9), and with no ATP to arrest at E complex (lanes 10–12). The substrate RNA species in anti-polyoma-based pulldown from Bud31-associated spliceosomes is indicated in lanes marked as Ip. The nonspecific substrate association with beads is shown in lanes marked as B. RNA in one-fourth of the total reaction was loaded as input (I). D, the pre-mRNA in each lane was quantified and normalized to the input pre-mRNA, and the data from 3–5 experiments were plotted with error bars indicating S.E..
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Biotinylated C303/305 pre-mRNA

Non-biotinylated control C303/305 pre-mRNA

FIGURE 4. Affinity-purified B and Bex spliceosomes analyzed for their snRNA content. A and B, splicing reactions performed with biotinylated mutant C303/C305 actin pre-mRNAs (A) or control nonbiotinylated pre-mRNAs (B). Reactions with 0.05 mM ATP and with 2 mM ATP supplemented with 5 mM EDTA were done with Bud31-TAP extracts. The substrate-associated spliceosomes were pulled down, and snRNA content was assessed by primer extension. Reactions with U1 and U4 snRNA primers were coupled (lanes 6, 8, 10, and 12 in panel A and lanes 1, 3, 5, and 7 in panel B), whereas those with U2, U5, and U6 snRNA primers were multiplexed (lanes 7, 9, 11, and 13 in panel A and lanes 2, 4, 6, and 8 in panel B). The reaction products were separated on denaturing PAGE. From each reaction condition, snRNAs in a direct load of an aliquot of the reactions (lanes 6, 7, 10, and 11 in panel A and lanes 1, 2, 5, and 6 in panel B) were compared with those in the affinity pulled down spliceosome (lanes 8, 9, 12, and 13 in panel A and lanes 3, 4, 7, and 8 in panel B). To accurately assign each U-rich snRNA, parallel primer extension reactions were done with total RNA from splicing extracts (panel A, lanes 1–5).

Bud31-TAP

Prp21-TAP

FIGURE 5. Affinity-purified spliceosomes assembled on biotinylated and control pre-mRNAs probed for Bud31 and Prp21 proteins. Splicing reactions were performed with biotinylated mutant C303/C305 actin pre-mRNAs (lanes 3 and 5) or control nonbiotinylated pre-mRNAs (lanes 2 and 4) and extracts containing Bud31-TAP (upper panel) or Prp21-TAP (lower panel). The reaction conditions, as shown in Fig. 4, were enriched for B (lanes 2 and 3) and Bex complexes (lanes 4 and 5) as indicated above each lane. The proteins present in affinity-purified spliceosomes were probed by Western analyses with anti-TAP antibodies. The input lane had a direct load of proteins in the splicing extract (lane 1).

Precausal spliceosomes containing the pre-mRNA, this association is indirect.

Bud31 Affects Interaction of a ~25-kDa Protein to the Exonic 5' Splice Site in Splicing Reactions at Elevated Temperature—A plausible underlying reason for the kinetic defects of spliceosomes lacking Bud31 is that they have an altered conformation or that they exhibit poor conformational transitions due to missing or altered RNA-RNA or pre-mRNA-protein interactions. The latter are critical for spliceosome assembly progression and catalysis.

To address the spliceosomal status in WT and bud31A splicing reactions, we employed a modified CYH2 pre-mRNA substrate containing a 4-thioU residue at the −3 position in the 5’ exon. In addition, the substrate had a single radiolabeled phosphodiester bond between the −1 and −2 residue of the 5’ exon.

This 32P-radiolabeled 4-thioU CYH2 pre-mRNA substrate was used in in vitro reactions with extracts from bud31 null and WT cells. A similar single radiolabel containing CYH2 substrate but that without the 4-thioU residue at the −3 position served as an experimental control. The reactions were incubated at permissive (23 °C) and nonpermissive (37 °C) temperatures following which proteins were cross-linked with the pre-mRNA substrate by UV irradiation at 365 nm. Subsequently, the pre-RNA substrate was digested using RNase T1, and the profile of the radiolabeled RNA-cross-linked proteins was examined. No major radiolabeled proteins were seen in the control reactions performed with the radiolabeled substrate but with no 4-thioU residue in the substrate (Fig. 7, top, lanes 1–4). In reactions
containing the radiolabeled substrate with a −3 (E1) 4-thioU CYH2, we did not find any significant differences in the profile of cross-linked proteins in splicing reactions with WT and bud31 null extracts when these reactions are carried out at 23 °C, the permissive temperature (Fig. 7, top, lanes 1–4). Importantly, for reactions carried out at 37 °C, a protein corresponding to molecular mass ~25 kDa was not detectable in the profile of cross-linked proteins in spliceosomes lacking Bud31. This contrasts with reactions carried out with the WT extracts at 37 °C, where the ~25 kDa protein interacts with the substrate and is cross-linked with this exonic residue (Fig. 7, top, lanes 6 and 8). None of the other major cross-links to the ~3 residue seem altered in reactions with WT and bud31 null extracts. To account for differences that may arise from protein loading, the signal from the radiolabeled 25-kDa protein was normalized with that from the ~37-kDa band in each lane (Fig. 7, bottom). This quantitation shows that the association of the ~25-kDa protein in the bud31 null spliceosomes is significantly reduced in comparison with the WT spliceosomes in reactions at higher temperatures. We infer that Bud31 stabilizes the association of this ~25-kDa protein to the −3 residue of exon, perhaps to facilitate splicing efficiency. In the absence of Bud31 at nonpermissive temperature, this interaction is compromised and could be a likely reason for the inefficient splicing kinetics of the bud31 null extracts at elevated temperatures.

**DISCUSSION**

Bud31 is a nonessential splicing factor required for viability of *S. cerevisiae* cells at growth temperatures >34 °C. As is known for a number of other nonessential pre-mRNA splicing factors required for growth in specific conditions, Bud31 functions can be hypothesized to increase the efficiency of selected splicing steps. Such roles could be performed either directly or as auxiliary functions for the other essential factors. For instance, nonessential Mud2 interacts with Prp11 during the recruitment of U2 snRNP to the pre-mRNA, whereas Prp18, during the second catalytic step, acts in concert with Slu7 (35, 36). In this study, we show that the *in vitro* splicing kinetics of extracts from a bud31 null strain are temperature-dependent, and this corroborates with the temperature-sensitive growth of these cells. In reactions at 37 °C, Bud31 facilitates efficient first step splicing and is required for the second reaction. Our data show precatalytic spliceosome interactions for Bud31. As extracts lacking Bud31 have slower first step kinetics, our data indicate that these early spliceosomal interactions are functionally important for progression to the first catalytic step of splicing. These data on the interaction of Bud31 with early B pre-spliceosomes are corroborated by proteomic studies that detected Bud31 in the early acting Cef1p complex as well as with the B complex-specific SF3b (11, 15). Protein factors of the Cef1p complex display some shared and some distinct interactions with the assembling spliceosomes, indicating that members of the complex can have unique and dynamic functions. Using assays where we examined the pulldown of the pre-mRNA substrate, splicing intermediates, and products from *in vitro* reactions with fully active spliceosomes, we find that early precatalytic B complex interactions of Bud31 continue in catalytic spliceosomes containing exon 1, lariat intermediate, lariat intron, and mRNA. Unlike the stable association of Bud31 with B complex (Fig. 3C), some members of Cef1p complex (e.g. Prp17) show only a weak interaction with the assembling pre-spliceosome (B) before its catalytic activation (28). Most factors of Cef1p complex display stable interactions with the spliceosome concomitant to its activation that are marked by the establishment of U6-5′ splice site base pairing interaction (5, 7, 37). However, NTC77/Clf1p is an exception because it joins the spliceosome prior to the U4/U6.U5 tri-snRNP association and it aids in the transition to the B complex (38). Although we show that Bud31 occurs in the B complex, and in activated spliceosomes formed later, we do not have evidence to determine whether it aids in the progression to B complex. The association of Bud31 with the lariat intron species indicates that it is also part of the post-splicing complexes. In agreement with the presence of Bud31 in spliceosomes and in post-splicing complexes, we find that the association of

**FIGURE 7.** Bud31 mediates interaction of an ~25-kDa protein to ~3 residue in exon 1 of CYH2 pre-mRNA. Top, *in vitro* splicing reactions with control CYH2 pre-mRNA with no 4-thioU (lanes 1–4) and with a CYH2 pre-mRNA with 4-thioU at the −3 position of the 5′ exon (lanes 5–8). A single radiolabeled phosphodiester bond was introduced two residues downstream to the −3 position in both control and 4-thioU substrate. Using splicing extracts from WT and bud31 null strains, the reactions were performed for 15 minutes at 23 or 37 °C. All reactions were UV cross-linked at 365 nm at 4 °C and then treated with RNase T1. The cross-linked proteins were separated on a SDS-PAGE gel and visualized by autoradiography. Bottom, the quantitation of the levels of the 25-kDa protein normalized to the 37-kDa protein detected in various reactions.
Bud31 with U5 and U6 snRNPs is salt-stable. Taken together, these observations show the interaction of Bud31 with various assembling and active complexes and suggest functions for Bud31 in spliceosome progression for first and second step splicing catalysis.

UV cross-linking studies have been very useful to elucidate dynamic contacts between splicing factors, pre-mRNA, and snRNAs in active spliceosomes (22, 39). To assess interactions in the spliceosome that fail to occur or that are inefficiently established in the absence of Bud31, we undertook site-specific cross-linking of proteins to the −3 position of the 5′ exonic residue. This residue was chosen as most of the protein-RNA interactions in this substrate region describe early events in spliceosome assembly and activation steps. The failure to detect a cross-link between a ~25-kDa protein and the exon 1 nucleotide at the −3 position in reactions with bud31 null extracts at 37 °C suggests that Bud31 stabilizes the interaction of this protein with the pre-mRNA. The NTC is implicated in mediating specific and stable interactions of the U5 and U6 snRNAs with the pre-mRNA 5′ splice site (40). Cwc2, the only essential NTC protein with an RNA binding domain, cross-links to the U6 snRNA, providing a link between the NTC and the active site of the spliceosome (17). NTC-mediated protein association with the pre-mRNA is less understood. By virtue of their molecular weight, Snt309, Hsh49, Cwc25, Isy1, Spp2, and Lea1 are candidate proteins that we hypothesize would cross-link to the exon 1 −3 position in spliceosomes containing Bud31. Of these, Hsh49 and Lea1 are components of the U2 snRNP. Interestingly, U2 snRNA is known to cross-link to the 5′ exonic residue at the −6 position (24), consistent with our suggestion of the close proximity of these U2 proteins to the 5′ splice site. Thus, these U2 snRNP proteins may be involved in establishing or maintaining U1 snRNA-5′ splice site interactions and/or U2 snRNP interactions at the −3 position as well. Isy1 and Snt309 are NTC complex members, whereas Cwc25 (a member of the Cef1p subcomplex) and Spp2 act with Prp2 to facilitate the first catalytic step (41). All these factors join the spliceosomes prior to or immediately after dissociation of U4 in the same manner as Prp19p. Mol. Cell Biol. 18, 2196–2204.

To summarize, we have found that the nonessential Bud31 is required for efficient progression to the first catalytic step and for the second catalytic step of splicing in reactions at higher temperatures while identifying functions for this nonessential factor in stabilizing pre-mRNA-protein interactions that may facilitate efficient splicing.

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