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The pre-mRNA retention and splicing complex controls tRNA maturation by promoting *TAN1* expression

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ABSTRACT

The conserved pre-mRNA retention and splicing (RES) complex, which in yeast consists of Bud13p, Snu17p and Pml1p, is thought to promote nuclear retention of unspliced pre-mRNAs and enhance splicing of a subset of transcripts. Here, we find that the absence of Bud13p or Snu17p causes greatly reduced levels of the modified nucleoside N^4-acetylcytidine (ac^4C) in tRNA and that a lack of Pml1p reduces ac^4C levels at elevated temperatures. The ac^4C nucleoside is normally found at position 12 in the tRNA species specific for serine and leucine. We show that the tRNA modification defect in RES-deficient cells is attributable to inefficient splicing of *TAN1* pre-mRNA and the effects of reduced Tan1p levels on formation of ac^4C. Analyses of *cis*-acting elements in *TAN1* pre-mRNA showed that the intron sequence between the 5′ splice site and branchpoint is necessary and sufficient to mediate RES dependency. We also show that in RES-deficient cells, the *TAN1* pre-mRNA is targeted for degradation by the cytoplasmic nonsense-mediated mRNA decay pathway, indicating that poor nuclear retention may contribute to the tRNA modification defect. Our results demonstrate that *TAN1* pre-mRNA processing has an unprecedented requirement for RES factors and that the complex controls the formation of ac^4C in tRNA.

INTRODUCTION

The maturation of RNA polymerase II–transcribed pre-mRNA molecules involves several processing steps, including the removal of introns. The removal of introns in pre-mRNAs is catalyzed by the spliceosome, consisting of five small nuclear ribonucleoprotein particles (snRNPs) and numerous accessory proteins (1). Pre-mRNA splicing occurs by two successive transesterification reactions and depends on three *cis*-acting sequences in the intron: the 5′ splice site, the branchpoint and the 3′ splice site. In the budding yeast *Saccharomyces cerevisiae*, ~5% of protein-coding genes are intron-containing (2,3). The vast majority of these genes harbor a single intron and the splice site sequences generally conform to a strict consensus (2,3).

The pre-mRNA retention and splicing (RES) complex, consisting of Bud13p, Snu17p and Pml1p, was identified as a *S. cerevisiae* non-snRNP complex that associates with the spliceosome, probably with U2 snRNP, before the first transesterification step (4–7). The RES complex is organized around Snu17p, which independently binds Bud13 and Pml1p (8–10). Orthologues of RES subunits are found in humans (4), and hRES also associates with the spliceosome before the first catalytic step of splicing (11,12). Yeast cells deleted for any of the three RES subunits are viable, demonstrating that the complex is not essential for splicing (4). However, the lack of a RES factor induces growth defects, especially at elevated temperatures, and these phenotypes are stronger in cells lacking Bud13p or Snu17p than in those lacking Pml1p (4). The growth phenotypes of the individual mutants correlate to the importance of the respective factor in pre-mRNA splicing, i.e. splicing defects are stronger in *bud13Δ* and *snu17Δ* than in *pml1Δ* cells (4). Although genome-wide studies have suggested that the absence of Bud13p or Snu17p leads to increased accumulation of many intron-containing pre-mRNAs (13,14), direct tests have shown that the RES complex is particularly important for introns in which the 5′ splice site does not conform to the consensus sequence (4,15,16). In addition to influencing splicing, the lack of any of the three RES subunits leads to export of unspliced pre-mRNAs to the

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cytoplasm (4). In fact, the absence of Pml1p can, under some conditions, induce pre-mRNA leakage without any obvious defect in splicing, suggesting that the primary function of Pml1p may be in nuclear retention of unspliced transcripts (4).

Unspliced pre-mRNAs that enter the cytoplasm are usually targeted for degradation by the nonsense-mediated mRNA decay (NMD) pathway, as the intron sequence typically leads to inclusion or generation of a premature translation termination codon (17–20). The degradation of transcripts encompassing premature translation termination codons requires their translation and a distinct set of *trans*-acting factors, including the conserved Upf1, Upf2 and Upf3 proteins (21). By eliminating nonsense-containing transcripts, such as unspliced pre-mRNAs, the NMD pathway ensures that aberrant transcripts do not accumulate as substrates for the translational machinery (21).

Nucleus-encoded tRNA genes are transcribed by RNA polymerase III generating pre-tRNAs that undergo a series of processing steps to yield the mature tRNAs that are used in translation (22). A general feature of tRNA maturation is that a subset of the nucleosides undergoes post-transcriptional modification. Some modified nucleosides are found in essentially all tRNAs, while others are present in one or a few tRNA species (22,23). The physiological roles of modified nucleosides vary (22,23) but those in the anticodon region are usually important for modulating anticodon–codon interactions, whereas those outside this region are often important for maintaining tRNA structure and/or stability. Some nucleoside modifications, at various positions, also modulate interactions with different proteins. Of the 50 different modified nucleosides found in eukaryotic tRNAs, 25 are present in cytoplasmic tRNAs from *S. cerevisiae* (24,25).

We previously found that any of several different point mutations in the *sup61* gene, encoding $tRNA^{Ser}_{CGA}$, induces a requirement for factors participating in modification and maturation of this particular tRNA (26). The absence of the tRNA modification/maturation factors destabilized the altered $tRNA^{Ser}_{CGA}$, reducing its abundance to levels unable to sustain growth (23,26). These findings suggested that cells harboring *sup61* mutant alleles represent a sensitized genetic system that could be used to identify novel factors important for tRNA modification/maturation. Accordingly, strains with mutations in the *TAN1* gene, encoding a tRNA-binding protein required for formation of N^4-acetylcytidine (ac^4C) at position 12 in serine and leucine isoacceptors, were identified in a genetic screen for mutations lethal in combination with a *sup61-T47:2C* mutation (27). Here, we provide a detailed analysis of another mutant identified in the screen and we show that the RES complex controls formation of ac^4C by promoting *TAN1* pre-mRNA processing.

MATERIALS AND METHODS

Strains, media and genetic procedures

Yeast strains used in this study are listed in Supplementary Table S1. Yeast transformations, media and genetic procedures have been described (28). One copy of the *BUD13*, *SNU17*, *PML1*, *TAN1*, *GOT1* and *UPF1* genes were independently deleted in the diploid strain UMY2366 by using a polymerase chain reaction (PCR)-mediated strategy (29,30). The individual deletions were confirmed by PCR using primers that annealed outside of sequences present in the transformed DNA fragment. The generated heterozygous diploids were allowed to sporulate and the *bud13Δ* (MJY546 and MJY547), *snu17Δ* (MJY548 and MJY549), *pml1Δ* (MJY535 and MJY536), *tan1Δ* (MJY550), *got1Δ* (MJY648 and MJY649) and *upf1Δ* (MJY537, MJY538, MJY683, MJY684) strains obtained from tetrads. Double and triple mutants were obtained from crosses between the relevant strains. The *sup61-T47:2C* allele was combined with a *bud13Δ*, *snu17Δ* or *pml1Δ* mutation by crossing UMY2256 to MJY547, MJY549 or MJY536. The strains harboring the *rpb1-1* allele (MJY652, MJY653, MJY654 and MJY655) were obtained from a cross between MJY96 and MJY546.

A strain expressing a C-terminal 3HA-tagged version of Tan1p from the normal chromosomal location was constructed by transforming strain UMY2219 with a *3HA-kanMX6* fragment amplified from pFA6a-*3HA-kanMX6* (30). The oligonucleotides used were 5′-ATCGAAATTCC GAAAGGATGAAGATAAGAGTGTAAAACAACGG ATCCCCGGGTTAATTAA-3′ and 5′-ATACTTATATA AAATATCTATTCTGTTTCATATATGAGAATTCGA GCTCGTTTAAAC-3′. Following confirmation by PCR and subsequent DNA sequencing, the generated strain was crossed to MJY547, MJY549 or MJY536, and strains MJY664, MJY666, MJY668 and MJY670 obtained from tetrads.

To construct strains expressing *TAN1* transcripts in which the intron was replaced with the corresponding sequence from *RPL25* or *GOT1*, we first replaced the *TAN1* intron in strain UMY2219 with a *URA3* marker PCR amplified from pRS406 (31). The oligonucleotides used were 5′-CCGTAATGGTAAGGATGCAAATTCC CAAAACAGGAAGAAAAGATTGTACTGAGAGTG CAC-3′ and 5′-CAGACGTACCAGGGTCTAAGAATC CGGAAGATACTTTAAACTGTGCGGTATTTCACA CCG-3′. The generated strain (MJY573) was transformed with a DNA fragment harboring the intron sequence of interest and homologies to the exons of *TAN1*. Following selection for 5-FOA^R colonies, individual clones were screened for integration of the relevant DNA fragment by PCR and DNA sequencing. The generated strains were crossed to MJY694 and strains MJY701, MJY702, MJY703, MJY704, MJY717, MJY718, MJY719 and MJY720 were obtained from tetrads. A similar approach was used to construct strains expressing *GOT1* transcripts harboring the *TAN1* intron or hybrid *TAN1*/*GOT1* introns. For these strains, the *GOT1* intron in UMY2219 was replaced with the *URA3* marker and the generated strain (MJY574) was used for transformation with the appropriate DNA fragments. The resulting strains were crossed to MJY696 and MJY705-MJY716 obtained from tetrads.

The screen for mutations lethal in combination with the *sup61-T47:2C* allele and the identification of plasmids that complement the synthetic lethal phenotype has been

described (27). To investigate linkage between *BUD13* and the original mutation, we integrated a plasmid marker (*TRP1*) at the *BUD13* locus in strain UMY2256. The generated strain was crossed to the potential *bud13* mutant, and tetrad analyses revealed that the non-sectoring phenotype never co-segregated with the *TRP1* marker, i.e. the mutation was linked to the *BUD13* locus.

Plasmid constructions

A low-copy plasmid harboring the *BUD13* gene (pMJ51) was constructed by cloning a *Sac*I/*Sna*BI *BUD13* DNA fragment, from a complementing YCp50 library plasmid (32), into the *Sac*I/*Sma*I sites of pRS316 (31). Plasmid pRS316-*TAN1Δi* (pMJ43) was constructed by cloning a SpeI/XhoI DNA fragment from pRS304-*TAN1Δi* (27) into the corresponding sites of pRS316. Plasmid pRS316-*TAN1* (p1483) has been described (27).

RNA and protein methods

Total RNA for northern blotting experiments was prepared using the hot phenol method essentially as described (33). Procedures for determining steady-state tRNA levels have been described (26,27). Determinations mRNA abundance and mRNA decay rates were performed as described (33) with the difference that the RNA samples were separated on formaldehyde-containing 2% agarose gels followed by capillary transfer to the Zeta-probe (Bio-Rad) membrane. HPLC analyses of nucleosides from total tRNA were performed as previously described (34). Cell extracts for western blotting were obtained (34) and analyzed (35) as described with the difference that the secondary antibody was horseradish peroxidase-linked sheep anti-mouse IgG (GE Healthcare).

RESULTS

Mutations in the *BUD13* gene cause reduced levels of ac^4C in tRNA

To identify factors promoting maturation of tRNA, we previously performed a screen for genes required for growth of cells harboring a *sup61-T47:2C* allele, which codes for a $tRNA^{Ser}_{CGA}$ species with an alteration in the variable arm (Figure 1A) (27). The screen identified mutants representing 12 different complementation groups of which three groups consisted of strains with mutations in genes for already characterized tRNA modifying enzymes (*DUS2*, *MOD5* and *TRM1*) and one defined strains with mutations in the *TAN1* gene, which we subsequently showed to be required for formation of ac^4C_{12} in tRNA (Figure 1A) (23,27). By using a yeast genomic library, we found that a mutant in one of the remaining eight groups was complemented by the *BUD13* gene, which codes for a subunit of the RES complex. To demonstrate unambiguously that Bud13p is required for growth of cells with the altered form of $tRNA^{Ser}_{CGA}$, we combined the *sup61-T47:2C* mutation with a *bud13Δ* allele. The resulting *sup61-T47:2C bud13Δ* double mutants were slow-growing at 25°C and inviable at 30°C and 37°C (Figure 1B). To investigate the mechanism by which Bud13p influences viability of *sup61-T47:2C* cells, we used northern blotting to determine the levels of $tRNA^{Ser}_{CGA}$ in wild-type, *sup61-T47:2C*, *bud13Δ* and *bud13Δ sup61-T47:2C* cells. The analyses revealed that the combination of *bud13Δ* and *sup61-T47:2C* alleles caused a synergistic reduction in $tRNA^{Ser}_{CGA}$ levels (Figure 1C), indicating that Bud13p may be important for tRNA maturation and/or stability.

The finding that the phenotypes caused by the lack of Bud13p in *sup61-T47:2C* cells are similar to those caused by the absence of any of several tRNA modifying enzymes (23,26,27) suggested that Bud13p may influence the modification status of tRNA. To assess this possibility, we used HPLC to analyze the nucleoside composition in total tRNA from wild-type and *bud13Δ* cells. Interestingly, cells deleted for *BUD13* showed considerably reduced, although still detectable ac^4C levels (Figure 1D). Importantly, no ac^4C was detected in tRNA isolated from *tan1Δ* cells (Figure 1D), showing that the small amounts of ac^4C detected in *bud13Δ* cells are not due to contaminating ac^4C-containing 18S rRNA. Introduction of a plasmid harboring the wild-type *BUD13* gene into the *bud13Δ* mutant restored the ac^4C levels, confirming that the ac^4C-deficiency is due to the lack of the *BUD13* gene product (Figure 1D). Thus, Bud13p promotes, but is not essential for, formation of ac^4C in tRNA.

Snu17p and Pml1p promote formation of ac^4C in tRNA

As Bud13p is part of the RES complex, it seemed possible that the other two subunits of the complex may also influence the formation of ac^4C in tRNA and consequently the viability of *sup61-T47:2C* cells. Although none of the remaining complementation groups consisted of *snu17* or *pml1* mutants (data not shown), the screen was not saturated (27), which means that such mutants may have escaped detection. To investigate the role of Snu17p and Pml1p in tRNA maturation, we independently combined *snu17Δ* and *pml1Δ* alleles with the *sup61-T47:2C* mutation. The *snu17Δ sup61-T47:2C* double mutant behaved like the *bud13Δ sup61-T47:2C* strain, i.e. it was slow-growing at 25°C and inviable at 30°C and 37°C (Figure 2A). In contrast, *pml1Δ sup61-T47:2C* cells grew like the *sup61-T47:2C* single mutant at 25°C and 30°C (Figure 2A). However, a slight synergistic growth defect was observed at 37°C, implying that Pml1p may influence tRNA modification at elevated temperatures.

To directly assess the effect of Snu17p and Pml1p on formation of ac^4C, we analyzed the nucleoside composition in tRNA from *snu17Δ* and *pml1Δ* cells. The analyses revealed that the ac^4C levels in *snu17Δ* cells were comparable with those in *bud13Δ* cells, i.e. ~5% of the wild-type levels (Figure 2B and C). As predicted from the growth assays, cells deleted for *PML1* showed wild-type ac^4C levels at 30°C and a reduction to ~65% at 37°C (Figure 2B and C). Collectively, these results show that all three subunits of the RES complex influence formation of ac^4C in tRNA.

The RES complex promotes splicing of *TAN1* pre-mRNA

As there is no evidence to suggest a direct role of the RES complex in tRNA modification, it seemed possible that the

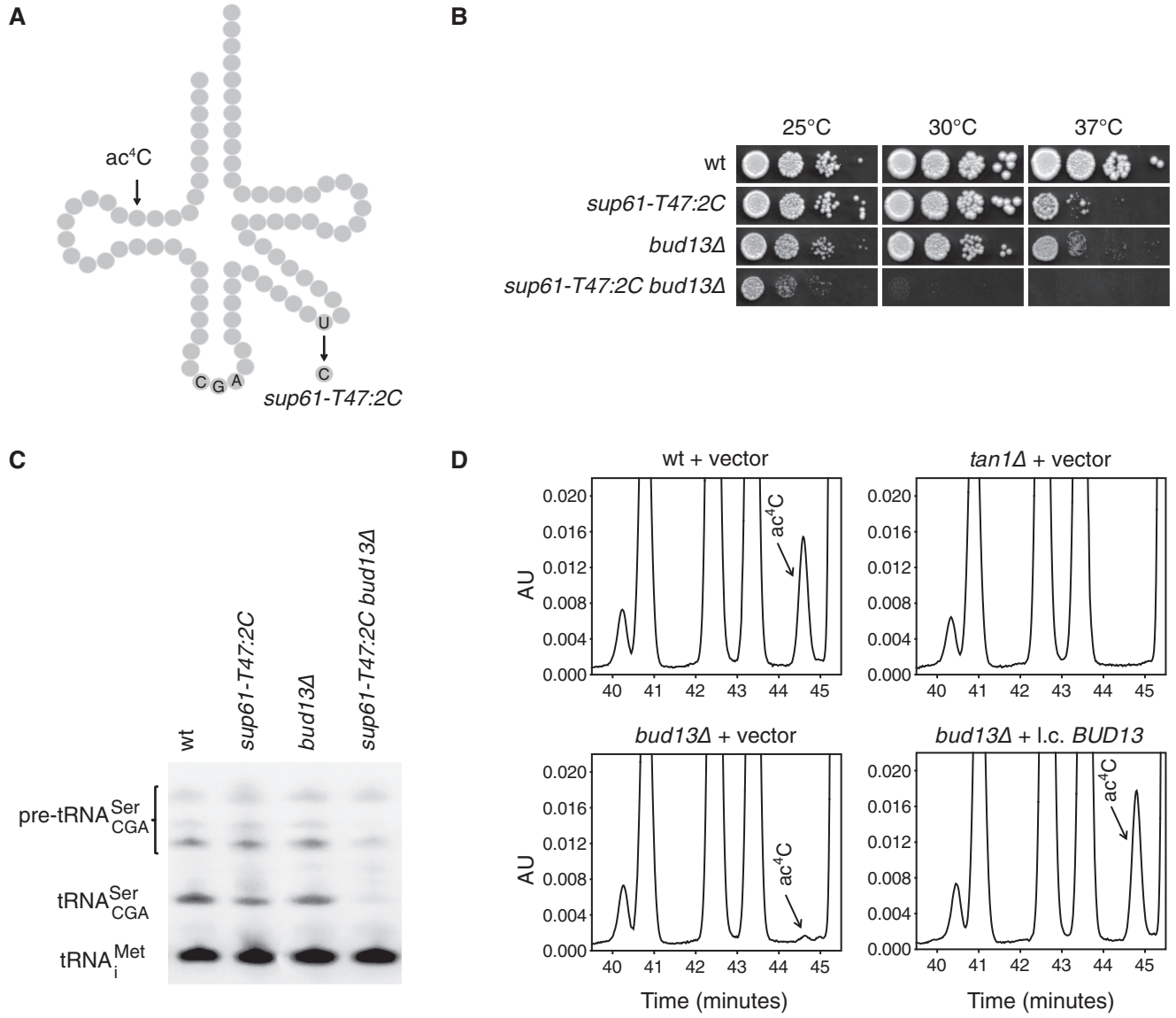

Figure 1. Mutations in the *BUD13* gene reduce the levels of ac^{4}C in tRNA. (**A**) Schematic secondary structure of tRNA$^{Ser}_{CGA}$. The alteration caused by the *sup61-T47:2C* allele and the position of ac^{4}C are indicated. (**B**) Growth of strains with *sup61-T47:2C* and/or *bud13Δ* alleles. The wild-type (UMY2219), *sup61-T47:2C* (UMY2256), *bud13Δ* (MJY546) and *sup61-T47:2C bud13Δ* (MJY553) strains were grown overnight in YEPD medium, serially diluted, spotted on YEPD plates and incubated at 25°C, 30°C or 37°C for 2 days. (**C**) Northern analysis of total RNA isolated from the strains described in B grown in YEPD medium at 25°C. The blot was probed for pre-tRNA$^{Ser}_{CGA}$, tRNA$^{Ser}_{CGA}$ and tRNA$^{Met}_{i}$ using oligonucleotide probes. tRNA$^{Met}_{i}$ serves as a loading control. (**D**) HPLC analyses of tRNA-derived nucleosides from wild-type (UMY2219), *tan1Δ* (MJY550) and *bud13Δ* (MJY546) cells carrying the indicated low-copy (l.c.) *URA3* plasmids. Cells were grown at 30°C in synthetic complete medium lacking uracil (SC-ura).

ac^{4}C-deficiency in *bud13Δ*, *snu17Δ* and *pml1Δ* mutants might be a consequence of defects in pre-mRNA splicing and/or nuclear retention. In fact, the tRNA modification defect of the individual mutants correlates to the importance of respective factor in splicing, i.e. the lack of Bud13p or Snu17p generates stronger splicing defects than a lack of Pml1p (4). Interestingly, the *TAN1* gene, which is the only, to date, identified gene required for formation of ac^{4}C in tRNA (27), harbors a 58 nt intron at the 5′ part of the ORF. To investigate if the RES complex controls ac^{4}C levels in tRNA by promoting *TAN1* pre-mRNA splicing, we used northern blotting to analyze *TAN1* transcripts in wild-type, *bud13Δ*, *snu17Δ* and *pml1Δ* cells. The blots were also probed for the intron-less *PGK1* mRNA and 18S rRNA, which served as a loading control. The analyses revealed an accumulation of unspliced *TAN1* pre-mRNA in all three mutants of which the *bud13Δ* and *snu17Δ* strains showed spliced *TAN1* mRNA levels below the level of detection (Figure 3A). The *TAN1* pre-mRNA splicing defect in *pml1Δ* cells was enhanced at 37°C (data not shown), explaining the reduced abundance of ac^{4}C at the elevated temperature. Western blot analyses of strains in which the DNA sequence for three tandem influenza virus hemagglutinin epitopes (3HA) was fused to the endogenous *TAN1* ORF showed that the Tan1 protein levels were reduced in

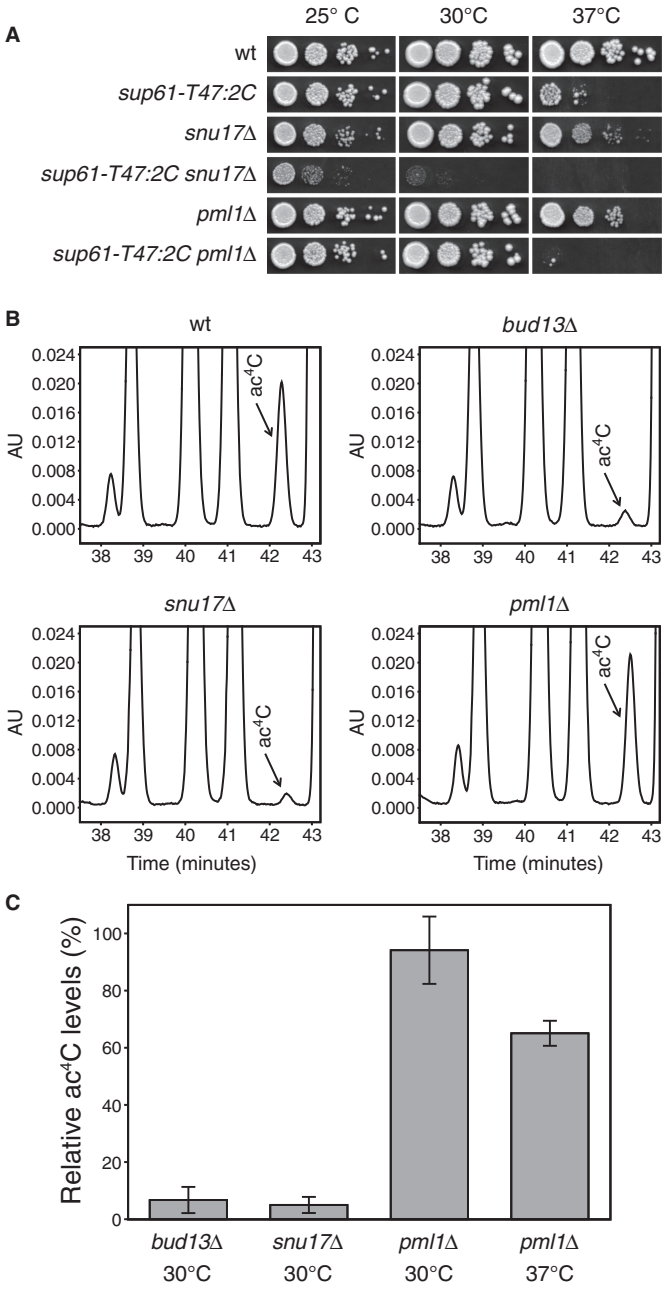

Figure 2. Effects of *snu17Δ* and *pml1Δ* alleles on the levels of ac^4C in tRNA. (**A**) Effects of *snu17Δ* and *pml1Δ* alleles on growth of *sup61-T47:2C* cells. The wild-type (UMY2219), *sup61-T47:2C* (UMY2256), *snu17Δ* (MJY548), *sup61-T47:2C snu17Δ* (MJY554), *pml1Δ* (MJY535) and *sup61-T47:2C pml1Δ* (MJY564) strains were grown overnight in YEPD medium, serially diluted, spotted on YEPD plates and incubated at 25°C, 30°C or 37°C for 2 days. (**B**) HPLC analyses of nucleosides derived from total tRNA isolated from wild-type (UMY2219), *bud13Δ* (MJY546), *snu17Δ* (MJY548) and *pml1Δ* (MJY535) cells grown in YEPD medium at 30°C. (**C**) Relative ac^4C levels in tRNA. The peak area for ac^4C was divided to the peak area for pseudouridine and this ratio was normalized to the ratio in wild-type cells, which was set to 100%. The ac^4C levels in RES mutants grown at 30°C represent the average from the experiment shown in Figure 2B and two (*bud13Δ* and *snu17Δ*) or five (*pml1Δ*) additional independent experiments. The value for the *pml1Δ* strain at 37°C is based on three independent experiments. The standard deviation is indicated.

pml1Δ and not detectable in *bud13Δ* or *snu17Δ* cells (Figure 3B). Collectively, these results support a model in which the ac^4C-deficiency in RES mutants is caused by inefficient splicing of *TAN1* pre-mRNA.

If inefficient *TAN1* pre-mRNA splicing is the explanation to the tRNA modification defect in RES-deficient cells, then the modification levels should be restored by expressing a *TAN1* allele that lacks the intron. To test this prediction, we removed the sequence for the intron in *TAN1* generating an allele (*TAN1Δi*) that generates splicing-independent expression of Tan1p. Introduction of a plasmid harboring this *TAN1Δi* allele into *tan1Δ bud13Δ* cells restored wild-type levels of ac^4C (Figure 3C). Small amounts of ac^4C were observed when the *tan1Δ bud13Δ* cells were transformed with a plasmid carrying the wild-type intron-containing *TAN1* gene, whereas no ac^4C was detected when the cells carried an empty vector or a plasmid harboring the *BUD13* gene (Figure 3C). These findings strongly support the notion that the RES complex promotes formation of ac^4C in tRNA by enhancing *TAN1* pre-mRNA splicing. Moreover, the growth defect induced by the combination of *bud13Δ* and *sup61-T47:2C* alleles was suppressed by introduction of the *TAN1Δi* plasmid (data not shown), indicating that the effect of the RES complex on viability of *sup61-T47:2C* cells is restricted to its role in *TAN1* pre-mRNA processing. The *TAN1Δi* allele did not, however, suppress the growth defect of the *bud13Δ* single mutant (data not shown), suggesting that this phenotype is caused by reduced expression of other gene products.

RES-deficiency induces degradation of *TAN1* pre-mRNA by the NMD pathway

In our analysis of RES-deficient cells, we observed that the total abundance of *TAN1* transcripts was reduced compared with the wild type (Figure 3A). This observation suggested that the RES complex also influences the synthesis or degradation of *TAN1* transcripts. Because the RES complex is thought to promote nuclear retention of pre-mRNAs (4), it seemed possible that the reduced abundance of *TAN1* transcripts in the RES mutants may be caused by cytoplasmic degradation of the pre-mRNA by the NMD pathway. To asses this possibility, we introduced a *upf1Δ* allele into cells deleted for *BUD13*, *SNU17* or *PML1* and analyzed the effects on abundance of *TAN1* transcripts. The analyses revealed that the unspliced *TAN1* pre-mRNA accumulated in NMD-deficient *bud13Δ*, *snu17Δ* and *pml1Δ* cells (Figure 4A). Importantly, no accumulation of *TAN1* pre-mRNA was observed in the *upf1Δ* single mutant, suggesting that the pre-mRNA is efficiently retained when cells harbor a functional RES complex. The levels of spliced *TAN1* mRNA were consistently slightly higher in *upf1Δ* than in wild-type and in *upf1Δ pml1Δ* than in *pml1Δ* cells (Figure 4A), indicating that NMD also controls the abundance of the processed transcript. To determine which of the *TAN1* transcripts that are targeted for degradation by NMD, we measured their decay rate following inhibition of RNA polymerase II transcription in strains harboring the temperature-sensitive *rpb1-1* allele. The analyses

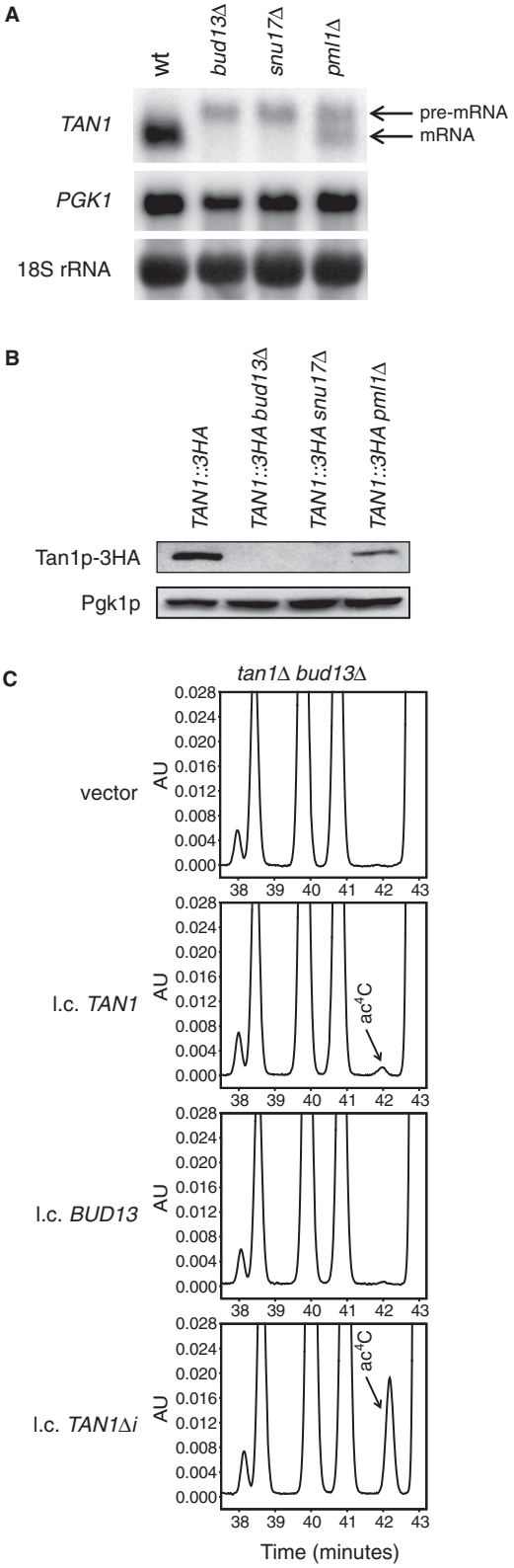

Figure 3. The RES complex promotes splicing of *TAN1* pre-mRNA. (**A**) Northern analysis of total RNA isolated from wild-type (UMY2219), *bud13Δ* (MJY546), *snu17Δ* (MJY548) and *pml1Δ* (MJY535) cells grown in YEPD medium at 30°C. The blot was probed for *TAN1* and *PGK1* transcripts using randomly labeled DNA fragments. 18S rRNA was detected using an oligonucleotide probe. (**B**) Western analysis of the indicated strains (MJY664, MJY666, MJY668 and MJY670) grown in YEPD medium at 30°C. Monoclonal antibodies against HA or Pgk1p were used to detect the indicated proteins. Control experiments showed that the sequence for the 3HA-tag did not alter the efficiency of which the *TAN1* pre-mRNA is spliced in either wild-type or RES-deficient cells (data not shown). (**C**) HPLC analysis of nucleosides of total tRNA isolated from *bud13Δ tan1Δ* (MJY568) cells carrying an empty low-copy (l.c.) *URA3* vector or the same plasmid containing the *BUD13*, *TAN1* or *TAN1Δi* gene. Cells were grown in SC-ura medium at 30°C.

revealed that the half-life of *TAN1* pre-mRNA was longer in *bud13Δ upf1Δ* than in *bud13Δ* cells, confirming that it is a *bona fide* NMD substrate (Figure 4B). These findings demonstrate that the absence of the RES complex not only causes inefficient splicing of *TAN1* pre-mRNA, but also poor nuclear retention and consequent degradation of the transcript by the NMD pathway. Consistent with the observation that NMD-deficient cells show a small increase in the levels of spliced *TAN1* mRNA, its half-life was slightly longer in *upf1Δ* than in $UPF1^{+}$ cells (Figure 4B).

It was previously suggested that the RES complex is particularly important for splicing of pre-mRNAs containing weak 5′ splice sites (4,15,16). However, the 58 nt intron in *TAN1* harbor the canonical GUAUGU-5′ splice site, suggesting that some other feature(s) causes the requirement for the RES complex. To assess the influence of the RES complex on other intron-containing transcripts, we probed the blots for *CYH2*, *GOT1* and *RPL25* transcripts (Figure 4A). These transcripts were selected, as they harbor canonical 5′ splice sites and the introns are of different lengths, 511, 82 and 414 nt, respectively. Moreover, the levels of *RPL25* and *GOT1* pre-mRNAs have been shown to be unaffected by the lack of the Upf1p (20), whereas the *CYH2* pre-mRNA is a well-characterized NMD substrate (17). The analyses revealed that the *CYH2* and *GOT1* pre-mRNA levels were largely unaffected by RES inactivation (Figure 4A). An accumulation of *RPL25* pre-mRNAs was observed in *bud13Δ*, *snu17Δ* and *pml1Δ* cells, but the effect was much less pronounced than that observed on *TAN1* transcripts (Figure 4A). The simultaneous lack of a RES subunit and Upf1p did not generate higher *CYH2*, *GOT1* and *RPL25* pre-mRNA levels than those observed in either single mutant, suggesting that the RES complex may not significantly affect retention of these transcripts. Collectively, these findings indicate that the *TAN1* pre-mRNA encompass some feature(s) that induces a requirement for the RES complex.

The *TAN1* intron promotes RES dependency

To get an initial assessment of the feature in *TAN1* pre-mRNA that mediates its RES dependency, we independently replaced the endogenous *TAN1* intron with the introns from the *GOT1* or *RPL25* gene. Analyses of wild-type, *upf1Δ*, *bud13Δ* and *upf1Δ bud13Δ* cells harboring these alleles revealed that *TAN1* pre-mRNA encompassing the *GOT1* or *RPL25* intron was spliced efficiently compared with the wild-type *TAN1* transcript (Figure 5A). To determine if the *TAN1* intron is sufficient to mediate RES dependency, we replaced the intron in the

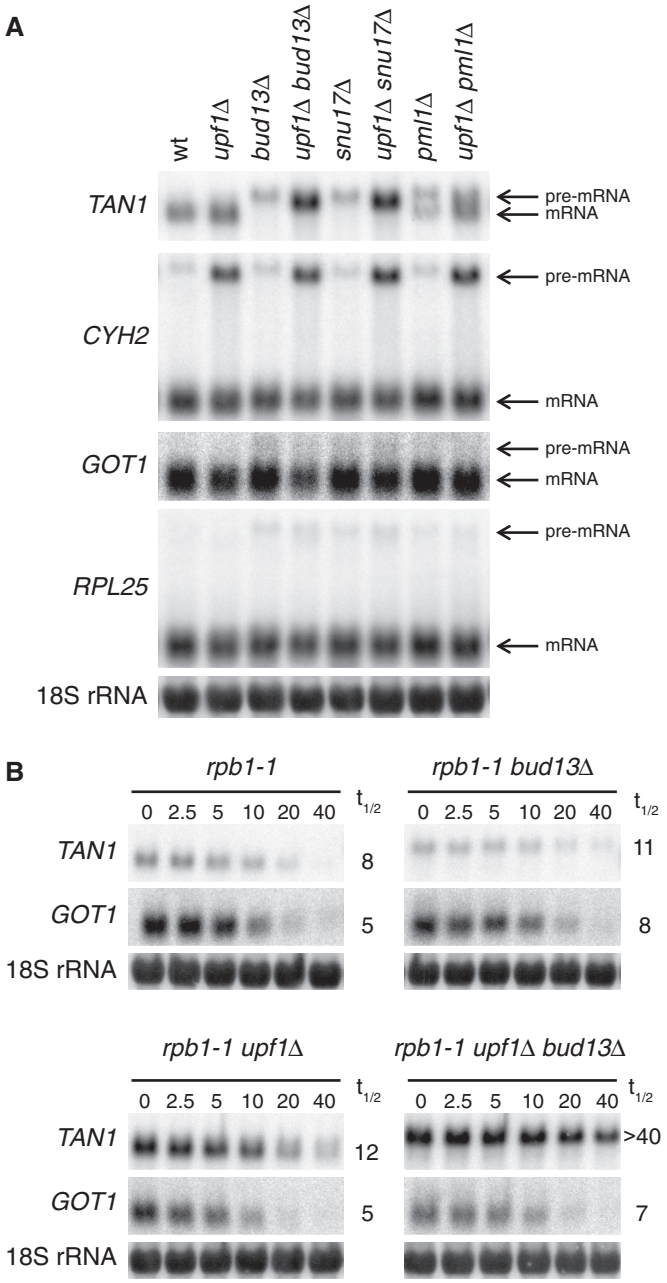

Figure 4. *TAN1* pre-mRNA is a NMD substrate in cells lacking a RES factor. (**A**) Northern analysis of total RNA isolated from wild-type (UMY2219), *upf1Δ* (MJY537), *bud13Δ* (MJY546), *upf1Δ bud13Δ* (MJY555), *snu17Δ* (MJY548), *upf1Δ snu17Δ* (MJY557), *pml1Δ* (MJY535) and *upf1Δ pml1Δ* (MJY559) cells grown in YEPD medium at 30°C. The blot was probed for *TAN1*, *CYH2*, *GOT1* and *RPL25* transcripts using randomly labeled DNA fragments. 18S rRNA was detected using an oligonucleotide probe. (**B**) mRNA decay rates in *rpb1-1* (MJY652), *rpb1-1 bud13Δ* (MJY653), *rpb1-1 upf1Δ* (MJY654) and *rpb1-1 upf1Δ bud13Δ* (MJY655) cells. The cells were grown in YEPD medium at 25°C followed by inhibition of RNA polymerase II transcription by a shift to 37°C. Time points (minutes) after the shift are indicated above the lanes. The signal in each lane was quantified and normalized to the corresponding 18S rRNA signal and the half-life ($t_{1/2}$, in minutes) determined from the initial slope of the curve.

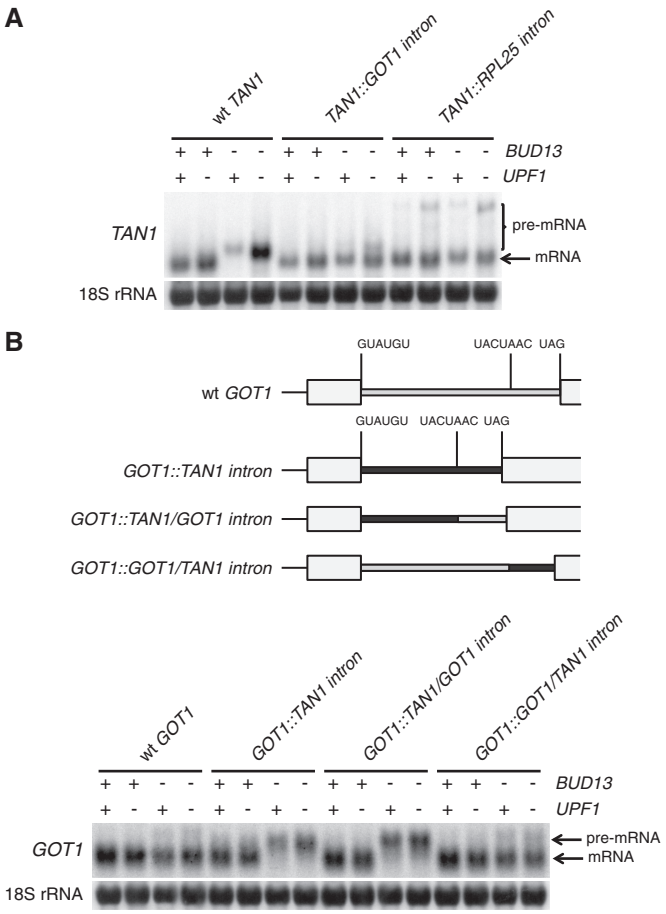

Figure 5. The *TAN1* intron is necessary and sufficient to mediate RES dependency. (**A**) Northern analysis of total RNA isolated from the indicated strains (UMY2219, MJY537, MJY546, MJY555, MJY717, MJY718, MJY719, MJY720, MJY701, MJY702, MJY703 and MJY704) grown in YEPD medium at 30°C. The blot was probed for *TAN1* transcripts using randomly labeled DNA fragments and for 18S rRNA using an oligonucleotide probe. (**B**) Northern analysis of total RNA isolated from the indicated strains (UMY2219, MJY537, MJY546, MJY555, MJY705, MJY706, MJY707 MJY708, MJY709, MJY710, MJY711, MJY712, MJY713, MJY714, MJY715 and MJY716) grown in YEPD medium at 30°C. A schematic representation of the different *GOT1* alleles is shown above the blot. The blot was probed for *GOT1* transcripts using randomly labeled DNA fragments and for 18S rRNA using an oligonucleotide probe.

endogenous *GOT1* gene with the intron from *TAN1*. The analyses revealed that *GOT1* transcripts harboring the *TAN1* intron behaved similar to the endogenous *TAN1* transcript, i.e. it was inefficiently spliced in *bud13Δ* cells and the pre-mRNA accumulated in *bud13Δ upf1Δ* cells (Figure 5B). Thus, the *TAN1* intron is necessary and sufficient to mediate RES-dependent splicing.

Because the *GOT1* and *TAN1* introns are both small (82 and 58 nt) and have identical sequences for the 5′ splice site, branchpoint and 3′ splice site, we argued that hybrid introns of the two could be used to further dissect the *cis*-acting element(s) that cause RES dependency. Accordingly, *GOT1* transcripts harboring an intron in which the sequence between the 5′ splice site and branchpoint was replaced with the corresponding sequence from the *TAN1* intron showed RES-dependent

splicing (Figure 5B). Further, the abundance of the transcript was higher in *bud13Δ upf1Δ* than *bud13Δ* cells, indicating that it is exported to the cytoplasm and degraded by the NMD pathway. Importantly, no RES dependency was observed when the *GOT1* transcripts harbored an intron where the sequence from the branchpoint to the 3′ splice site was replaced with the corresponding sequence from the *TAN1* intron (Figure 5B). Thus, the 30 nt sequence between the 5′ splice site and branchpoint in the *TAN1* intron is sufficient to induce RES-dependency.

DISCUSSION

Post-transcriptional modification of nucleoside residues is a general feature of the tRNA maturation process. Although the vast majority of factors directly involved in biosynthesis of the 25 modified nucleosides present in *S. cerevisiae* tRNAs have been identified (22), the cellular mechanisms that control tRNA modifications are poorly understood. In this study, we find that inactivation of the spliceosome-associated RES complex leads to reduced levels of the modified nucleoside ac^4C, which is normally present at position 12 in serine and leucine isoacceptors. We show that the RES complex promotes formation of ac^4C by enhancing splicing and nuclear retention of *TAN1* pre-mRNA, which codes for a tRNA modifying factor. Thus, our findings reveal a novel mechanism that controls the modification status of a subset of tRNA species.

We previously showed that a strain harboring a *sup61-T47:2C* allele requires the *TAN1* gene for proper growth (27). As expected from the finding that the levels of spliced *TAN1* mRNA and Tan1 protein are below the level of detection in *bud13Δ* or *snu17Δ* cells (Figure 3A and B), the *bud13Δ sup61-T47:2C* and *snu17Δ sup61-T47:2C* double mutants show growth phenotypes similar to those observed for *tan1Δ sup61-T47:2C* cells (27), i.e. they are viable but slow-growing at 25°C and inviable at 30 and 37°C (Figures 1B and 2A). However, *bud13Δ* and *snu17Δ* cells retain ~5% of ac^4C at 30°C (Figure 2B and C), suggesting that small amounts of Tan1p are being synthesized but that those are not sufficient to sustain growth of *sup61-T47:2C* cells. Mechanistically, the growth defect induced by RES inactivation in *sup61-T47:2C* cells could be caused either by reduced acetylation of C_{12} in the altered $tRNA^{Ser}_{CGA}$ or by the absence of a physical interaction between Tan1p and the tRNA. The latter possibility would be analogous to the requirement for the *TRM2* gene in a *sup61-T51C* mutant, which is not dependent on the tRNA (m^5U_{54}) methyltransferase activity of the Trm2 protein (26). The lack of the third RES subunit, Pml1p, affects the formation of ac^4C and viability of *sup61-T47:2C* cells at 37°C but not at 30°C (Figure 2), which is consistent with the observation that the *TAN1* pre-mRNA splicing defect is larger at the elevated temperature (data not shown). Apparently, the decreased abundance of spliced *TAN1* mRNA and Tan1p in *pml1Δ* cells at 30°C (Figure 3A and B) is not sufficient to reduce the ac^4C levels in tRNA. These findings suggest that Tan1p is not, at least in exponentially growing cells, a limiting factor in formation of ac^4C. This notion is further supported by the observation that the increased abundance of spliced *TAN1* mRNA in *upf1Δ* cells (Figure 4A) does not correlate with an increase in ac^4C levels (data not shown).

The RES complex was previously suggested to preferentially enhance splicing of transcripts that harbor introns with non-canonical 5′ splice sites (4). This notion was further supported by the finding that Snu17p and Bud13p are required for Mer1p-activated splicing of *AMA1* and *MER2* transcripts, which both encompass introns with weak 5′ splice sites (15,16). More recently it was shown that Bud13p and Snu17p are important for splicing of the *MATa1* transcript (36,37), which is one of only a few *S. cerevisiae* transcripts that harbor more than one intron (3,38). Although the two *MATa1* introns harbor canonical 5′ splice sites, the splicing of *MATa1* transcripts appears to be intrinsically inefficient (39). Moreover, spliced *MATa1* mRNA is readily detected in *bud13Δ* or *snu17Δ* cells (37), indicating that the splicing defect is smaller than that observed for *TAN1* pre-mRNA. Nevertheless, a feature shared between the *TAN1* intron and the two introns in *MATa1* is that they are all short (58, 52 and 54 nt, respectively) and it is possible that the RES complex is particularly important for splicing of pre-mRNAs with short introns. Consistent with this notion, the introns in the RES-controlled *AMA1* and *MER2* transcripts are also relatively short, 93 and 80 nt, respectively. However, the splicing of the *GOT1* pre-mRNA is largely unaffected in RES mutants even though it harbors an 82 nt intron (Figure 3A). Thus, the length of the introns may not, by itself, explain the RES dependency of target transcripts. Moreover, the finding that not only Bud13p and Snu17p, but also Pml1p, are important for *TAN1* pre-mRNA splicing (Figure 3A) suggests that this transcript has a unique requirement for the RES complex. Our analyses of *cis*-acting elements in *TAN1* show that the sequence between the 5′ splice site and branchpoint is necessary and sufficient to induce RES-dependent splicing (Figure 5). The mechanism by which this element triggers RES dependency remains to be determined.

It was previously shown that a lack of any of the three RES subunits leads to leakage of unspliced pre-mRNAs to the cytoplasm (4). In fact, many factors that act early in the splicing process promote nuclear retention of intron-containing pre-mRNAs, but this effect can often not be separated from their role in splicing (40,41). Some factors, including the RES subunit Pml1p, appear to promote nuclear pre-mRNA retention without significantly influencing splicing (4,19,42,43). Our finding that *TAN1* pre-mRNA is targeted for degradation by the cytoplasmic NMD pathway in RES-deficient but not in RES-proficient cells (Figure 3) supports a role of RES in pre-mRNA retention and suggests that poor nuclear retention may contribute to the tRNA modification defect. However, the observation that *TAN1* pre-mRNA accumulates in the *pml1Δ* single mutants indicates that it may be difficult, at least for this pre-mRNA, to differentiate between effects on splicing and retention.

Previous studies have shown that homozygous *bud13Δ* or *snu17Δ* diploid cells show a haploid-like bud-site selection pattern and an increased ability to mate as *MATα* cells (36,44). These phenotypes appear to be caused by inefficient splicing of *MATa1* pre-mRNA and the effects of reduced MATa1p levels on the ability to turn off haploid-specific genes and allow diploid gene expression (36,37). The reduced *MATa1* expression does not account for the growth defect of RES-deficient cells because the phenotype is also observed in haploid cells of both mating types. It was previously suggested that *tan1Δ* mutants grow slowly at 37°C (45) and it seemed possible that reduced Tan1p levels may contribute to the growth defect of RES-deficient cells. However, the growth defect of *bud13Δ* cells is not suppressed by the removal of the intron in *TAN1* even though it restores formation of ac^4C in tRNA (data not shown, Figure 3C). Moreover, inactivation of *TAN1* does not induce slow growth at 37°C in the genetic background used by us (data not shown). It is, therefore, likely that the growth defect of RES-deficient cells is caused by reduced expression of other gene products.

The relative abundance of a modified nucleoside in tRNA can be affected by growth rate, growth phase and stress conditions (46,47). Interestingly, the splicing efficiency of individual transcripts is also influenced by environmental signals (48). It is, therefore, feasible that *TAN1* pre-mRNA processing may, under some conditions, control the levels of ac^4C in tRNA. Because ac^4C is important for tRNA stability (27,45), such a regulatory mechanism could be important to fine-tune the abundance of serine and leucine isoacceptors.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary References [26,27,49].

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