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 N4-acetylcytidine (ac4C) in tRNA and that a lack of Pml1p reduces ac4C levels at elevated temperatures. The ac4C 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 ac4C. 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 ac4C 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 interacts 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
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<sup>GCG</sup>, induces a requirement for factors participating in modification and maturation of this particular tRNA (26). The absence of the tRNA modification/maturaton factors destabilized the altered tRNA<sup>GCG</sup>, 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 <i>TAN1</i> gene, encoding a tRNA-binding protein required for formation of N<sup>4</sup>-acyetylcytidine (ac<sup>C</sup>) at position 12 in serine and leucine isoacceptors, were identified in a genetic screen for mutations lethal in combination with the <i>sup61</i>-<i>T47:2C</i> 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<sup>C</sup> by promoting <i>TAN1</i> 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 <i>BUD13</i>, <i>SNU17</i>, <i>PML1</i>, <i>TAN1</i>, <i>GOT1</i> and <i>UPF1</i> 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 <i>bud13Δ</i> (MJY546 and MJY547), <i>snu17Δ</i> (MJY548 and MJY549), <i>pml1Δ</i> (MJY555 and MJY556), <i>tan1Δ</i> (MJY550), <i>got1Δ</i> (MJY648 and MJY649) and <i>upf1Δ</i> (MJY537, MJY538, MJY683, MJY684) strains obtained from tetrads. Double and triple mutants were obtained from crosses between the relevant strains. The <i>sup61</i>-<i>T47:2C</i> allele was combined with a <i>bud13Δ</i>, <i>snu17Δ</i> or <i>pml1Δ</i> mutation by crossing UMY2256 to MJY547, MJY549 or MJY536. The strains harboring the <i>rpl1</i>-<i>1</i> 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-kanMX<sub>6</sub> fragment amplified from pFA6a-3HA-kanMX<sub>6</sub> (30). The oligonucleotides used were 5′-ATCGAAAAATTCC GAAAGGATGAAGATAAGAGTGTAAAACAACGG ATCCCCGCGTTTAATTTA-3′ and 5′-ATACATTATA AAATATCTATTTGCATTATATGAGAATTCC GCTCGTTTAC-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 <i>TAN1</i> transcripts in which the intron was replaced with the corresponding sequence from <i>RPL25</i> or <i>GOT1</i>, we first replaced the <i>TAN1</i> intron in strain UMY2219 with a 3HA marker PCR amplified from pRS406 (31). The oligonucleotides used were 5′-CCCTAATGGTAAGGATGCCAAATCC CAAAAAGGAAAGAAATGATGGTAAACACGGT CAC-3′ and 5′-CAGCGCTACCCGGTGCTAAGAATCG CGAGAGTACTTTAATCTGCAGGATTTACCA CGG-3′. The generated strain (MJY573) was transformed with a DNA fragment harboring the intron sequence of interest and homologies to the exons of <i>TAN1</i>. Following selection for 5-FOA<sup>R</sup> 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 <i>GOT1</i> transcripts harboring the <i>TAN1</i> intron or hybrid <i>TAN1</i>/<i>GOT1</i> introns. For these strains, the <i>GOT1</i> intron in UMY2219 was replaced with the <i>URA3</i> 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 <i>sup61</i>-<i>T47:2C</i> 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-sector-
ing 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 SacI/SnaB1 BUD13 DNA
fragment, from a complementing YCp50 library plasmid
(32), into the SacI/SnaI 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 per-
formed 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
ac4C 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\textsubscript{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 modi-
fying enzymes (DUS2, MOD5 and TRM1) and one
defined strains with mutations in the TANI gene, which
we subsequently showed to be required for formation of
ac4C\textsubscript{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
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was required for growth of cells with the altered form of
tRNA\textsubscript{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\textsubscript{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\textsubscript{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 modi-
fication 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 ac4C levels (Figure 1D). Importantly,
no ac4C was detected in tRNA isolated from
tan1Δ cells (Figure 1D), showing that the small
amounts of ac4C detected in bud13Δ cells are not due to
contaminating ac4C-containing 18S rRNA. Introduction
of a plasmid harboring the wild-type BUD13 gene into the
bud13Δ mutant restored the ac4C levels, confirming that
the ac4C-deficiency is due to the lack of the BUD13 gene
product (Figure 1D). Thus, Bud13p promotes, but is not
essential for, formation of ac4C in tRNA.

Snu17p and Pml1p promote formation of ac4C 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 ac4C 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 ac4C, we analyzed the nucleoside com-
position in tRNA from snu17Δ and pml1Δ cells. The analyses
revealed that the ac4C levels in snu17Δ cells were compar-
able 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 ac4C
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
ac4C in tRNA.

The RES complex promotes splicing of TANI pre-mRNA

As there is no evidence to suggest a direct role of the RES
complex in tRNA modification, it seemed possible that the
ac4C-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 ac4C in tRNA (27), harbors a 58 nt intron at the 5' part of the ORF. To investigate if the RES complex controls ac4C 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 ac4C 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...
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 assess this possibility, we introduced an upf1A 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 bud13A, snu17A and pml1A cells (Figure 4A). Importantly, no accumulation of TAN1 pre-mRNA was observed in the upf1A 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 upf1A than in wild-type and in upf1A pml1A than in pml1A 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 2.** Effects of snu17A and pml1A alleles on the levels of ac4C in tRNA. (A) Effects of snu17A and pml1A alleles on growth of sup61-T47:2C cells. The wild-type (UMY2219), sup61-T47:2C (UMY2256), snu17A (MJY548), sup61-T47:2C snu17A (MJY554), pml1A (MJY555) and sup61-T47:2C pml1A (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), bud13A (MJY546), snu17A (MJY548) and pml1A (MJY553) cells grown in YEPD medium at 30°C. (C) Relative ac4C levels in tRNA. The peak area for ac4C was divided by the peak area for pseudouridine and this ratio was normalized to the ratio in wild-type cells, which was set to 100%. The ac4C levels in RES mutants grown at 30°C represent the average from the experiment shown in Figure 2B and two (bud13A and snu17A) or five (pml1A) additional independent experiments. The value for the pml1A strain at 37°C is based on three independent experiments. The standard deviation is indicated.

ac4C and not detectable in bud13A or snu17A cells (Figure 3B). Collectively, these results support a model in which the ac4C-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 tan1A bud13A cells restored wild-type levels of ac4C (Figure 3C). Small amounts of ac4C were observed when the tan1A bud13A cells were transformed with a plasmid carrying the wild-type intron-containing TAN1 gene, whereas no ac4C 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 ac4C in tRNA by enhancing TAN1 pre-mRNA splicing. Moreover, the growth defect induced by the combination of bud13A 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 bud13A single mutant (data not shown), suggesting that this phenotype is caused by reduced expression of other gene products.
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 GUAGU-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 pmn1Δ 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
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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 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^3C, which is normally present at position 12 in serine and leucine isoacceptors.

We show that the RES complex promotes formation of ac^3C 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^3C 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 C12 in the altered tRNA^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^2U^3a) methyltransferase activity of the Trm2 protein (26). The lack of the third RES subunit, Pml1p, affects the formation of ac^3C 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^3C levels in tRNA. These findings suggest that Tan1p is not, at least in exponentially growing cells, a limiting factor in formation of ac^3C.

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^3C 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 MATα1 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 MATα1 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 acC 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 acC in tRNA. Because acC 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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