The *Saccharomyces cerevisiae TAN1* gene is required for N^4-acetylcytidine formation in tRNA

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ABSTRACT

The biogenesis of transfer RNA is a process that requires many different factors. In this study, we describe a genetic screen aimed to identify gene products participating in this process. By screening for mutations lethal in combination with a *sup61-T47:2C* allele, coding for a mutant form of $tRNA^{Ser}_{CGA}$, the nonessential *TAN1* gene was identified. We show that the *TAN1* gene product is required for formation of the modified nucleoside N^4-acetylcytidine (ac^4C) in tRNA. In *Saccharomyces cerevisiae*, ac^4C is present at position 12 in tRNAs specific for leucine and serine as well as in 18S ribosomal RNA. Analysis of RNA isolated from a *tan1*-null mutant revealed that ac^4C was absent in tRNA, but not rRNA. Although no tRNA acetyltransferase activity by a GST-Tan1 fusion protein was detected, a gel-shift assay revealed that Tan1p binds tRNA, suggesting a direct role in synthesis of ac^4C_{12}. The absence of the *TAN1* gene in the *sup61-T47:2C* mutant caused a decreased level of mature $tRNA^{Ser}_{CGA}$, indicating that ac^4C_{12} and/or Tan1p is important for tRNA stability.

Keywords: N^4-acetylcytidine; *YGL232w*; *sup61*; tRNA maturation; tRNA modification

INTRODUCTION

Eukaryotic transfer RNA genes are transcribed by RNA polymerase III generating precursor tRNAs that have to undergo a series of processing events to yield mature functional tRNAs (Hopper and Phizicky 2003). During the maturation of tRNA, a variety of different nucleoside modifications occur. Modified nucleosides are found in all phylogenetic domains and also in identical positions of the tRNA, suggesting a conserved function of some tRNA modifications (Björk 1986; Cermakian and Cedergren 1998; Björk et al. 2001). The modified nucleoside N^4-acetylcytidine (ac^4C) is found in tRNA from organisms within all three domains (Sprinzl et al. 1998). It is present at position 34 in some archaeal and bacterial tRNAs, and in vitro experiments using *Escherichia coli* $tRNA^{Met}_m$ suggested that ac^4C_{34} prevents misreading of AUA isoleucine codons during protein synthesis (Stern and Schulman 1978). In eukaryotes, ac^4C is found only at position 12 in a subset of tRNAs. The tRNAs in *Saccharomyces cerevisiae* containing ac^4C_{12} are the species for leucine and serine (Sprinzl et al. 1998). The modification of the cytidine at position 12 is an early step in the maturation of tRNAs in *S. cerevisiae*, as ac^4C_{12} is present in intron containing precursor tRNA (Etcheverry et al. 1979). To date, the biological function of ac^4C_{12} has not been elucidated.

The $sup61^+$ gene is a single copy and essential gene encoding $tRNA^{Ser}_{CGA}$, which is the only serine isoacceptor that decodes UCG codons (Etcheverry et al. 1982). We previously showed (Johansson and Byström 2002) that strains with a mutant *sup61* allele have a requirement for the genes encoding the modification enzymes tRNA(m^5U_{54}) methyltransferase (Trm2p; Nordlund et al. 2000), pseudouridine(Ψ_{55}) synthase (Pus4p; Becker et al. 1997), tRNA ($m^2_2G_{26}$) dimethyltransferase (Trm1p; Ellis et al. 1986) and the putative tRNA(Gm_{18}) 2′-O-ribose methyltransferase (Trm3p; Cavaille et al. 1999). The m^5U_{54}, Ψ_{55}, $m^2_2G_{26}$, and Gm_{18} nucleotides are all present in $tRNA^{Ser}_{CGA}$ (Etcheverry et al. 1979). Moreover, the *LHP1* gene product involved in 3′ end maturation of pre-tRNAs was important for growth of *sup61* mutants (Yoo and Wolin 1997; Johansson and Byström 2002). When null alleles of any of these genes were introduced into strains with mutant forms of $tRNA^{Ser}_{CGA}$, growth defects were obtained that correlated to a reduced level of $tRNA^{Ser}_{CGA}$ (Johansson and Byström 2002). These data implied that other genes encoding modification/maturation proteins interacting with $tRNA^{Ser}_{CGA}$ would be essential in the *sup61* mutants.

In this study, we report that a *sup61-T47:2C* mutant re-

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quires the open reading frame *YGL232w* for growth. We show that *YGL232w* is required for ac^4C formation in tRNA and denoted the gene *TAN1* for tRNA acetylation. The absence of the Tan1 protein in the *sup61-T47:2C* strain caused a decreased level of mature $tRNA^{Ser}_{CGA}$, suggesting that ac^4C_{12} and/or Tan1p is important for tRNA stability.

RESULTS

A strain with a mutant form of $tRNA^{Ser}_{CGA}$ requires the *YGL232w* open reading frame for growth

A yeast strain with the *sup61-T47:2C* allele (Fig. 1) requires, to various degrees, any of the *LHP1*, *PUS4*, *TRM1*, and *TRM2* genes for growth (Johansson and Byström 2002). This suggested that the *sup61-T47:2C* allele would cause a requirement for other proteins modifying or interacting with $tRNA^{Ser}_{CGA}$. To identify gene products involved in $tRNA^{Ser}_{CGA}$ biogenesis, a synthetic lethal screen was performed by using a red/white colony color assay (Kranz and Holm 1990; Bender and Pringle 1991). Briefly, a haploid *ura3 ade2 ade3 sup61-T47:2C* strain carrying a plasmid with the *ADE3*, *URA3*, and $sup61^+$ genes was mutagenized and colonies screened for the inability to lose the plasmid, which is scored as uniformly red (nonsectored) colonies. From ~20,000 colonies, 21 strains with a single recessive mutation were identified that required the plasmid borne $sup61^+$ gene for survival (see Materials and Methods). Complementation analysis of the $sup61^+$-dependent mutants defined 12 complementation groups. We expected to find *lhp1*, *pus4*, *trm1*, and *trm2* mutants among the isolates, and one complementation group consisted of two strains with a mutant *trm1* allele (data not shown). However, no *lhp1*, *pus4*, and *trm2* mutants were identified, showing that the screen was not saturated.

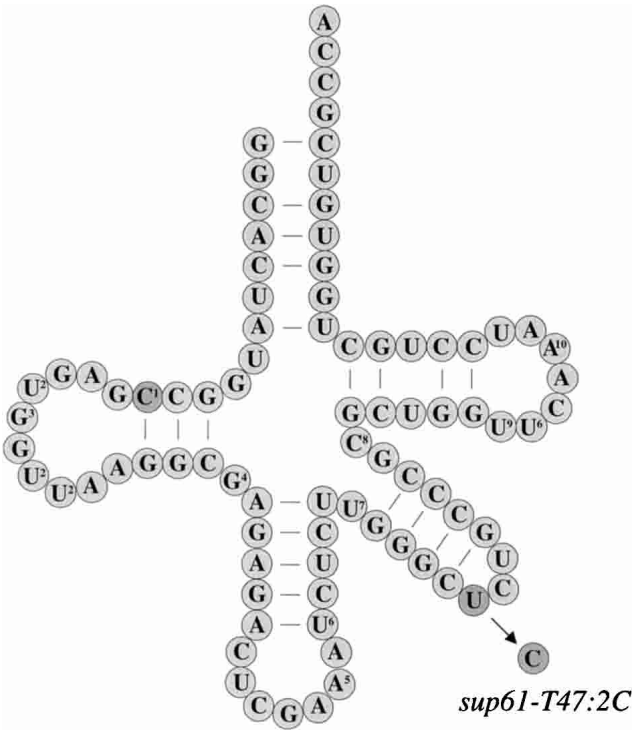

FIGURE 1. Cloverleaf structure of $tRNA^{Ser}_{CGA}$. The position of the *sup61-T47:2C* mutation and modified nucleosides are indicated where numbers within the circles represent the following modifications: 1, N^4-acetylcytidine (ac^4C); 2, dihydrouridine (D); 3: 2′-O-methylguanosine (Gm); 4, N^2,N^2-dimethylguanosine (m^2_2G); 5, N^6-isopentenyladenosine (i^6A); 6, pseudouridine (Ψ); 7, 2′-O-methyluridine (Um); 8, 5-methylcytidine (m^5C); 9, 5-methyluridine (m^5U); and 10, Etcheverry et al. (1979) detected a modification at position 57 or 58 that presumably corresponds to 1-methyladenosine (m^1A) at position 58.

In this study, we describe the identification and characterization of the gene mutated in a complementation group consisting of three mutants. By using a yeast genomic library, plasmids that complemented the nonsectoring phenotype were identified. Partial DNA sequencing and subsequent subcloning revealed that the *YGL232w* open reading frame restored sectoring in the three mutants. Genetic linkage between *YGL232w* and the original mutation was verified by targeted integration and tetrad analysis (see Materials and Methods). The *YGL232w* gene, predicted to encode a 33.6-kD protein, is not required for growth in a wild-type background (Giaever et al. 2002). To unambiguously demonstrate the synthetic interaction, the *sup61-T47:2C* mutation was combined with a *ygl232w*-null allele. The resulting *sup61-T47:2C ygl232w*Δ mutant was very slow growing at 25°C and nonviable at 30°C (Fig. 2A; data not shown). Thus, the *YGL232w* gene (hereafter referred to as *TAN1*, see below) is required for growth of a strain with a mutant form of $tRNA^{Ser}_{CGA}$.

Transfer RNA isolated from a *tan1*-null mutant lacks ac^4C

We previously showed that the genes encoding the modification enzymes catalyzing formation of $m^2_2G_{26}$, m^5U_{54}, and Ψ_{55} in tRNA were important for growth of the *sup61-T47:2C* strain (Johansson and Byström 2002). This suggested that the *TAN1* gene might encode a tRNA modification enzyme. To investigate this hypothesis, total tRNA from the wild-type strain (UMY2220), containing empty vector, and the *tan1*-null mutant (UMY2874), carrying either empty vector or plasmid bearing *TAN1*, was prepared. The tRNA was degraded to nucleosides and subjected to HPLC analysis. The analysis revealed that tRNA from the *tan1*-null mutant was missing one compound. This compound present in the wild-type and the null mutant carrying the *TAN1* plasmid has the retention time and spectrum of N^4-acetylcytidine (ac^4C; Fig. 3). To confirm the nature of the compound, synthetic ac^4C was added to a wild-type tRNA digest before HPLC analysis. The analysis revealed that the synthetic ac^4C comigrated with the compound that is absent in the *tan1*-null strain (Fig. 3). The ac^4C nucleoside is

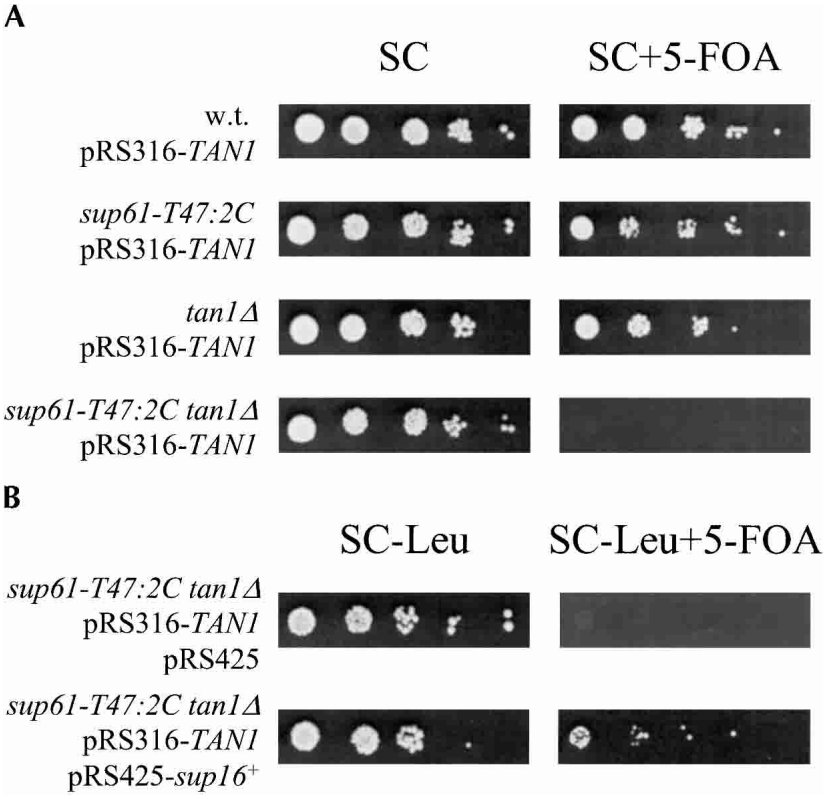

FIGURE 2. The *TAN1* gene is required for growth of a *sup61-T47:2C* strain. (*A*) Wild-type (UMY2220), *sup61-T47:2C* (UMY2256), *tan1*Δ (UMY2874), and *sup61-T47:2C tan1*Δ (derived from UMY2951) strains carrying the low copy *URA3* plasmid pRS316-*TAN1* were grown in synthetic complete medium (SC), serially diluted, spotted onto SC and SC+5-fluoro-orotic acid (5-FOA) plates, and incubated at 30°C for 2 d. Cells containing a *URA3* plasmid are unable to grow on 5-FOA–containing media (Boeke et al. 1984). (*B*) The *sup61-T47:2C tan1*Δ strain in A transformed with pRS425 or pRS425-*sup16*$^+$ (high copy *LEU2* plasmids) was grown in SC-Leu medium, serially diluted, spotted onto SC-Leu and SC-Leu+5-FOA plates, and incubated for 3 d at 30°C.

present at position 12 in tRNAs specific for leucine and serine (Sprinzl et al. 1998), including $tRNA^{Ser}_{CGA}$ (Etcheverry et al. 1979). In addition to tRNA, 18S rRNA contains ac^4C (Thomas et al. 1978). HPLC analysis of nucleosides derived from rRNA revealed that ac^4C was present in the *tan1*-null mutant (data not shown). Thus, the Tan1 protein influenced formation of ac^4C in tRNA but not rRNA. Based on these results, the *YGL232w* open reading frame was denoted *TAN1* for tRNA acetylation.

The Tan1 protein interacts with tRNA

To investigate whether the Tan1 protein catalyzed formation of ac^4C in tRNA, a GST-tagged version of the protein was expressed and purified from *E. coli* (Fig. 4A). Labeling experiments showed that both carbons of acetate were used to form the N^4-acetyl group of ac^4C, indicating that acetyl-coenzyme A (acetyl-CoA) is the donor in the modification reaction (Tumaitis and Lane 1970). The tagged protein was investigated for in vitro acetyltransferase activity by using T7 transcribed radiolabeled $tRNA^{Ser}_{CGA}$ as substrate and acetyl-CoA as the donor. The samples were degraded to nucleotides and subjected to two-dimensional thin-layer chromatography. However, no formation of pac^4C using the GST-Tan1 protein was detected (data not shown).

The Tan1 protein has been shown to contain a THUMP domain, a predicted RNA-binding motif shared by 4-thiouridine, pseudouridine synthases, and RNA methyltransferases (Aravind and Koonin 2001). To investigate whether Tan1p binds tRNA, a gel mobility shift assay was performed. Radiolabeled T7 transcribed $tRNA^{Ser}_{CGA}$ or $tRNA^{Glu}_{UUC}$ was mixed with increasing amounts of GST-Tan1 protein. The ac^4C nucleoside is normally present in $tRNA^{Ser}_{CGA}$ (Etcheverry et al. 1979), but not in $tRNA^{Glu}_{UUC}$, which has a G at position 12 (Kobayashi et al. 1974). As a control, purified GST protein was mixed with radiolabeled tRNA. Analysis by native PAGE revealed an ability of GST-Tan1p, but not GST alone, to shift the mobility of $tRNA^{Ser}_{CGA}$ (Fig. 4B; data not shown). Addition of an excess of total unlabeled tRNA prevented the mobility shift (data not shown). Although $tRNA^{Glu}_{UUC}$ does not contain ac^4C_{12}, the presence of GST-Tan1p changed the mobility of this tRNA (Fig. 4C). To verify that Tan1p and not a contaminant in the preparation was responsible for tRNA binding, a supershift experiment was performed in which radiolabeled $tRNA^{Ser}_{CGA}$ was incubated with GST-Tan1p in the presence of monoclonal anti-GST antibody. The anti-GST antibody caused a supershift of the $tRNA^{Ser}_{CGA}$/protein complex (Fig. 4B). These data show that Tan1p binds tRNA and indicate a direct role in synthesis of ac^4C_{12}.

The *TAN1* gene is required for stability of a mutant form of $tRNA^{Ser}_{CGA}$

To investigate the molecular mechanism for the synthetic interaction of the *tan1*Δ and *sup61-T47:2C* alleles, we intended to determine the relative amount of $tRNA^{Ser}_{CGA}$ in the double mutant. Although the *sup61-T47:2C tan1*Δ strain was viable at 25°C, it was extremely slow growing and difficult to handle. To circumvent this problem, we constructed a *sup61-T47:2C tan1*Δ strain rescued by a plasmid carrying *TAN1* and introduced a high copy plasmid carrying *sup16*$^+$, encoding $tRNA^{Ser}_{U^*GA}$. This tRNA contains an

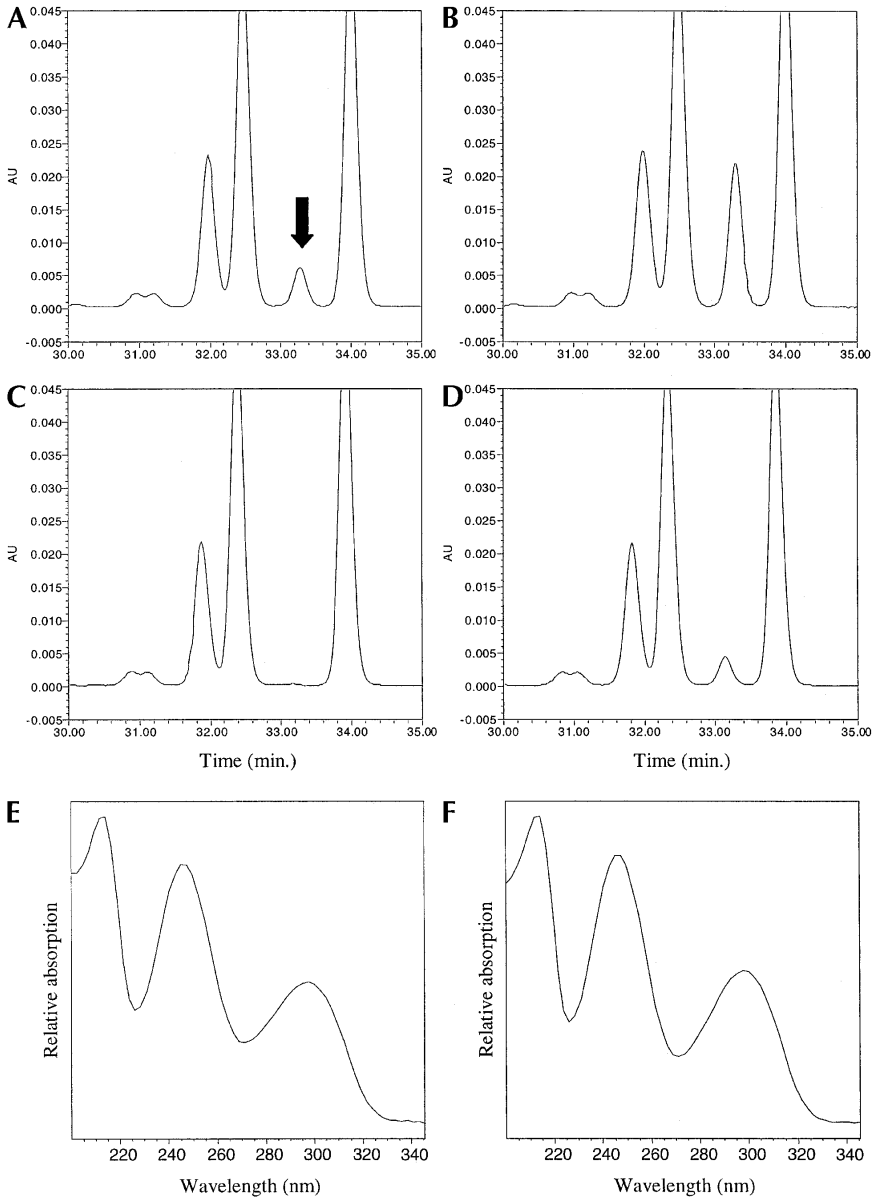

FIGURE 3. The *tan1*-null mutant lacks ac^4C in tRNA. HPLC analysis of nucleosides derived from total tRNA isolated from wild-type strain (UMY2220) carrying pRS316 (*A*); wild-type strain (UMY2220) carrying pRS316 with addition of 0.5 nmole synthetic ac^4C (*B*); *tan1*Δ strain (UMY2874) carrying pRS316 (*C*); and *tan1*Δ strain (UMY2874) carrying pRS316-*TAN1* (*D*). Only the portion of the chromatograms between retention times 30 and 35 min are shown. An arrow indicates the peak corresponding to ac^4C. (*E*) UV absorption spectrum of the peak indicated in *A*. (*F*) UV absorption spectrum of synthetic ac^4C.

uncharacterized modified uridine residue at position 34 that is likely to reduce wobble and thereby reading of UCG codons (Etcheverry et al. 1979, 1982). However, increased gene dosage of *sup16*$^+$ suppresses the lethality of a *sup61*Δ mutant (M.J.O. Johansson and A.S. Byström, unpubl.), possibly by an ability of a pool of hypomodified $tRNA^{Ser}_{U^*GA}$ to decode UCG codons. Alternatively, an increased pool of fully modified $tRNA^{Ser}_{U^*GA}$ might be sufficient to suppress the need for $tRNA^{Ser}_{CGA}$. Introduction of a high copy plasmid carrying *sup16*$^+$ alleviated the requirement for the *TAN1* plasmid in the *sup61-T47:2C tan1*Δ strain at 30°C (Fig. 2B).

Total RNA was prepared from exponentially growing cultures, at 30°C, of wild-type, *sup61-T47:2C*, *tan1*Δ, and *sup61-T47:2C tan1*Δ strains carrying the high copy *sup16*$^+$ plasmid. The relative amount of mature and precursor $tRNA^{Ser}_{CGA}$ was determined by using Northern blot analysis. The *sup61* gene contains an intron (Etcheverry et al. 1979) and two different ^{32}P-labeled oligonucleotides were used to detect mature and pre-$tRNA^{Ser}_{CGA}$. Signals from the mature and the different pre- $tRNA^{Ser}_{CGA}$ species (O'Connor and Peebles 1991; Yoo and Wolin 1997) were quantified and normalized to the initiator methionine tRNA ($tRNA^{Met}_i$) signal, which served as an internal control, as ac^4C is not present in $tRNA^{Met}_i$. The analysis revealed that the *tan1*Δ mutant showed essentially the same level of $tRNA^{Ser}_{CGA}$ as the wild-type strain (Fig. 5; Table 1). However, the *sup61-T47:2C tan1*Δ mutant showed a decreased level of mature $tRNA^{Ser}_{CGA}$ compared with the wild-type, *sup61-T47:2C*, and *tan1*Δ strains (Fig. 5; Table 1). The level of pre- $tRNA^{Ser}_{CGA}$ was similar in the wild-type, single mutant, and double mutant strains (Fig. 5; Table 1), indicating that ac^4C_{12} and/or Tan1p is required for stability of mature $tRNA^{Ser}_{CGA}$.

DISCUSSION

The modified nucleoside ac^4C is present at position 12 in a subset of eukaryotic tRNAs. In this study, we describe the identification and characterization of the *S. cerevisiae TAN1* gene encoding a protein required for formation of ac^4C_{12}. The gene was identified in a screen for mutations lethal in combination with a *sup61-T47:2C* allele, coding for a mutant form of $tRNA^{Ser}_{CGA}$. Analysis of RNA from a *tan1*-null strain revealed that the modified nucleoside ac^4C was absent in tRNA but not in rRNA (Fig. 3, data not shown). The absence of Tan1p in the *sup61-T47:2C* strain caused about a sixfold reduction in the level of mature $tRNA^{Ser}_{CGA}$, whereas the level of pre-$tRNA^{Ser}_{CGA}$ was relatively unaffected (Fig. 5, Table 1). This suggested that the acetylation of C_{12} and/or the Tan1p stabilized the mutant tRNA following removal of the 5′ leader, 3′ trailer, and intervening sequences in pre-$tRNA^{Ser}_{CGA}$.

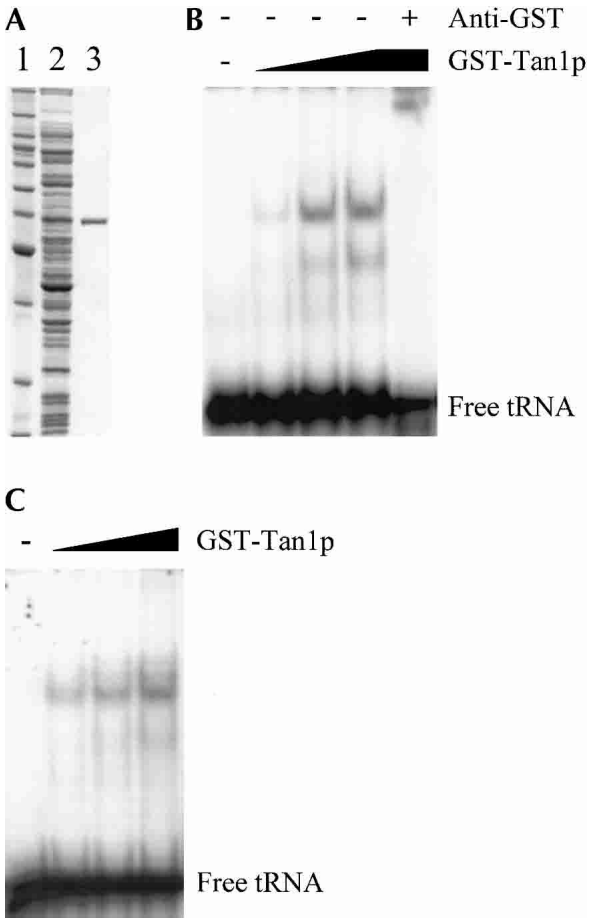

FIGURE 4. The Tan1 protein interacts with tRNA. (*A*) SDS-PAGE analysis of GST-Tan1 protein purified from *E. coli*. The gel was stained with Coomassie brilliant Blue R-250. (Lane *1*) Molecular weight standard (BenchMark protein ladder, Invitrogen). (Lane *2*) *E. coli* crude extract with expressed GST-Tan1 protein. (Lane *3*) Purified GST-Tan1 protein. (*B*) Gel mobility shift assay using ^{32}P-labeled T7-transcribed $tRNA^{Ser}_{CGA}$ and increasing amounts (0.15, 0.3, and 0.6 μg) of GST-Tan1 protein. The supershift was induced by monoclonal anti-GST antibody. (*C*) Gel mobility shift assay using ^{32}P-labeled T7-transcribed $tRNA^{Glu}_{UUC}$ and increasing amounts (0.15, 0.3, and 0.6 μg) of GST-Tan1 protein.

We were unable to detect any in vitro tRNA acetyltransferase activity by a GST-Tan1 fusion protein. It was unlikely that the GST moiety influenced the catalytic activity, as a GST-Tan1 protein expressed in yeast complemented the absence of ac^4C in the null mutant (data not shown). The Tan1 protein contains a THUMP domain proposed to be an RNA-binding motif shared by a number of modification enzymes (Aravind and Koonin 2001). By using a gel-shift assay, GST-Tan1p was found to interact with $tRNA^{Ser}_{CGA}$, suggesting that Tan1p is directly involved in synthesis of ac^4C_{12} (Fig. 4B). It is possible that Tan1p functions as the tRNA binding component of a modification enzyme consisting of more than one subunit. This would explain the absence of acetyltransferase activity by the purified GST-Tan1 protein. Consistent with this hypothesis, Tan1p does not show any apparent homology with known acetyltransferases. There are three examples in *S. cerevisiae* of tRNA modification enzymes consisting of two different subunits, Gcd10/Gcd14 (Anderson et al. 2000), Tad2/Tad3 (Gerber and Keller 1999), and Trm8/Trm82 (Alexandrov et al. 2002). An additional subunit(s) could be identified in the synthetic lethal screen, assuming that the mutant form of $tRNA^{Ser}_{CGA}$ requires presence of ac^4C_{12} and not the Tan1 protein per se. However, it cannot be excluded that the lack of in vitro acetyltransferase activity by GST-Tan1p was caused by unfavorable assay conditions. In addition to the $tRNA^{Ser}_{CGA}$ interaction, Gst-Tan1p was found to bind $tRNA^{Glu}_{UUC}$ (Fig. 4C), which has a G at position 12. It is not clear whether the interaction with $tRNA^{Glu}_{UUC}$ is relevant in vivo, that is, whether Tan1p is involved in maturation of tRNAs not containing ac^4C_{12}. Although only tRNAs specific for serine and leucine contain ac^4C_{12}, the Tan1 protein might recognize general tRNA structure. It has previously been shown that the *E. coli* $tRNA(m^1G_{37})$ methyltransferase in vitro binds tRNA species that are not methylated by the enzyme (Redlak et al. 1997). Alternatively, specificity in RNA binding by Tan1p might be provided by an additional subunit(s).

To date, six different gene products have been identified to be important for growth of strains with a mutant form of

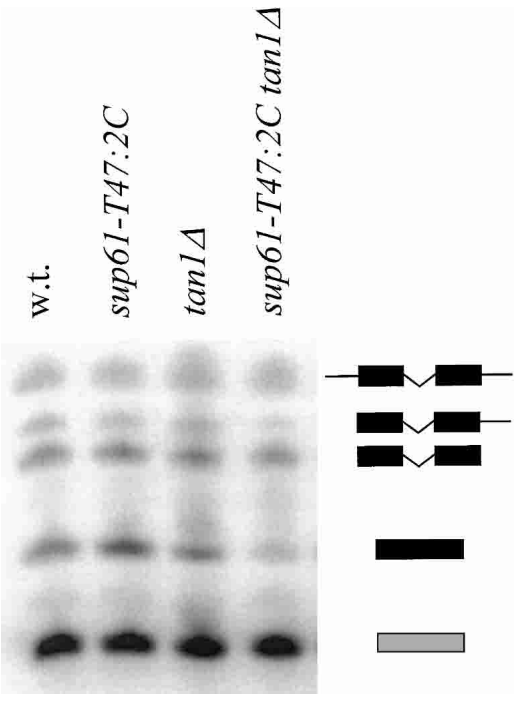

FIGURE 5. The *TAN1* gene product stabilizes the mutant form of $tRNA^{Ser}_{CGA}$. Northern blot analysis of total RNA isolated from wild-type (UMY2220), *sup61-T47:2C* (UMY2256), *tan1*Δ (UMY2874), and *sup61-T47:2C tan1*Δ (derived from UMY2951) strains, carrying pRS425-*sup16*$^+$, grown in SC-Leu medium at 30°C. The blot was probed simultaneously for pre- $tRNA^{Ser}_{CGA}$, $tRNA^{Ser}_{CGA}$, and $tRNA^{Met}_i$ (see Materials and Methods). The position and identity of $tRNA^{Ser}_{CGA}$ (black) and $tRNA^{Met}_i$ (gray) species are indicated on the *right*.

TABLE 1. Effects of the *tan1*-null allele on the relative level of $tRNA^{Ser}_{CGA}$

tRNA species	Wild type	*sup61-T47:2C*	*tan1Δ*	*sup61-T47:2C tan1Δ*
pre-$tRNA^{Ser}_{CGA}$	1	1.2 ± 0.1	1.2 ± 0.3	1.4 ± 0.5
$tRNA^{Ser}_{CGA}$	1	1.3 ± 0.4	0.8 ± 0.1	0.2 ± 0.1

Data from Fig. 5 and two additional independent experiments (data not shown) were averaged. The standard deviation is indicated. The level of pre-$tRNA^{Ser}_{CGA}$ is the combined signal of the three precursor forms normalized to mature $tRNA^{Met}_{i}$, relative to the corresponding value in the wild-type strain, which was set to 1. The normalized value for mature $tRNA^{Ser}_{CGA}$ was expressed relative to the corresponding value in the wild-type stain, which was set to one.

$tRNA^{Ser}_{CGA}$ (this study, Yoo and Wolin 1997; Johansson and Byström 2002). All of these gene products interact with and influence stability of mutant forms of $tRNA^{Ser}_{CGA}$. Thus, the strains in remaining 10 complementation groups identified in the screen are likely to carry mutations in genes coding for products that directly or indirectly influence $tRNA^{Ser}_{CGA}$ stability. In conclusion, the screen for mutations lethal in combination with a *sup61* allele is an excellent genetic method to identify gene products that participate in $tRNA^{Ser}_{CGA}$ maturation. It should be possible to identify gene products specific for other tRNA species using an analogous approach.

MATERIALS AND METHODS

Strains, media, and genetic procedures

The source and genotypes of yeast strains used in this study are listed in Table 2. *E. coli* strains used were DH5α (Bethesda Research Laboratories) and BL21 (Studier and Moffatt 1986). Yeast transformation (Gietz et al. 1992), media, and genetic procedures have been described (Burke et al. 2000). Strain UMY2704 was obtained from a tetrad in a cross between UMY2256, carrying pMJ1421, and UMY2220. Plasmid pRS304-*TAN1* was linearized with BglII and targeted (Orr-Weaver et al. 1981) to the *TAN1* locus in strain UMY2256 generating UMY2871. A *tan1*::*KanMX4* construct was PCR amplified from a homozygous *tan1*::*KanMX4* (*ygl232w*::*KanMX4*) diploid strain (34599, Research Genetics) and transformed into UMY2366, which is a diploid strain formed between UMY2219 and UMY2220. The generated heterozygous diploid was allowed to sporulate and the *tan1*-null mutant (UMY2874) obtained from a tetrad. Strain UMY2951 was obtained from a tetrad in a cross between UMY2256, carrying pMJ1422, and UMY2874. The pMJ1422 plasmid in UMY2951 was replaced with pRS316-*TAN1* generating the strain used in Figure 2.

Synthetic lethal screen and identification of the *TAN1* gene

The screen used to isolate mutants was based on colony sectoring as described previously (Kranz and Holm 1990; Bender and Pringle 1991). Strain UMY2704, carrying plasmid pMJ1421 bearing the $sup61^{+}$, *ADE3*, and *URA3* genes, was grown in selective medium to ~2×10^7 cells/mL. The cells were diluted with water, plated on YEPD plates, mutagenized with UV irradiation to ~20% survival, and incubated at 30°C. Uniformly red colonies were restreaked at least twice, and only those strains that continued to give nonsectored colonies were studied further. The candidate strains were transformed with pRS414-$sup61^{+}$ (p1229) and strains that regained the ability to sector were crossed to UMY2256 to test for dominance/recessiveness and for 2:2 segregation of the nonsectoring phenotype.

A low copy genomic library in YCp50 (Rose et al. 1987) was transformed into one of the mutants, carrying pMJ1422, and transformants selected on medium lacking uracil. Plasmids from transformants that regained the ability lose the $sup61^{+}$ vector were isolated and retransformed, and those that stayed positive were partially sequenced and subjected to homology searches against the *S. cerevisiae* genome. To investigate linkage between the *TAN1* gene and the mutation, UMY2871 was crossed to each mutant, and tetrad analysis revealed that the sectoring phenotype cosegregated with the integrated plasmid marker (*TRP1*).

TABLE 2. *S. cerevisiae* strains used in this study

Yeast strain	Genotype	Source or reference
UMY2219	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ade2-1 ade3::hisG*	this laboratory
UMY2220	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ade2-1 ade3::hisG*	this laboratory
UMY2366	*MATa/MATα ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 ade3::hisG/ade3::hisG*	this study
UMY2256	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ade2-1 ade3::hisG sup61-T47:2C*	(Johansson and Byström 2002)
UMY2704	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ade2-1 ade3::hisG sup61-T47:2C pMJ1421*	this study
UMY2871	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ade2-1 ade3::hisG sup61-T47:2C TAN1::pRS304-TAN1*	this study
UMY2874	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ade2-1 ade3::hisG tan1::KanMX4*	this study
UMY2951	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ade2-1 ade3::hisG sup61-T47:2C tan1::KanMX4 pMJ1422*	this study
34599	*MATa/MATα ura3 Δ0/ura3Δ0 leu2 Δ0/leu2Δ0 his3Δ1/his3Δ1 met15Δ0/MET15 lys2Δ0/LYS2 ygl232w::KanMX4/ygl232w::KanMX4*	Research Genetics

Plasmid constructions

DNA manipulations, plasmid preparations, and bacterial transformations were performed according to standard protocols. Plasmid pMJ1421 was constructed by cloning a SalI/SmaI *sup61*$^+$ fragment from pRS316-*sup61*$^+$ (Johansson and Byström 2002) into the SalI/NruI sites of plasmid c2013 (Cvrckova and Nasmyth 1993), removing the *spADH*::*CLN2* construct. The *TRP1* variant of the *ADE3 sup61*$^+$ plasmid (pMJ1422) was created by transforming a yeast strain carrying pMJ1421 with a *ura3*::*TRP1* construct. The plasmid was isolated from a Trp$^+$ Ura$^-$ strain and confirmed by restriction analysis. A low copy *TRP1* vector carrying the *sup61*$^+$ gene (p1229) was constructed by cloning a BglII/EcoRI *sup61*$^+$ fragment from pRS316-*sup61*$^+$ into the BamHI/EcoRI sites of pRS414 (Sikorski and Hieter 1989). Plasmids pRS304-*TAN1* (p1482) and pRS316-*TAN1* (p1483) were constructed by cloning an EcoRI/XhoI fragment, PCR amplified from the complementing library plasmid, into the corresponding sites of pRS304 and pRS316 (Sikorski and Hieter 1989). The oligonucleotides used were 5′-TTGAATTCTGTAGAAGGAGCTAACGGCGA-3′ and 5′-TTCTCGAGCAATGGGTCTCATTCCTAACG-3′. The *TAN1* gene contains an intron (Davis et al. 2000) and to express and purify the protein, the intron in plasmid pRS304-*TAN1* was removed by PCR mutagenesis, generating pRS304-*TAN1*Δ*intron* (p1547). The intronless gene was amplified and cloned as an EcoRI/XhoI fragment into pGEX-4T-2 (Amersham Biosciences) generating pGEX-4T-2-*TAN1*Δ*intron* (p1612). In this plasmid, transcription is under the *tac* promoter and the product is a GST-Tan1 fusion protein. The oligonucleotides used to amplify the intronless gene were 5′-AAGAATTCCCATGGGTGAAAAACGTAAC-3′ and 5′-TTCTC GAGCAATGGGTCTCATTCCTAACG-3′. An intronless *sup61*$^+$ gene under the T7 promoter was constructed by ligating three pairs of oligonucleotides into the EcoRI/BamHI sites of pUC18 (Roche Applied Science) generating pUC18-*T7sup61*$^+$Δ*IVS* (p1611). The oligonucleotides used were 5′-AATTGCTGCAGT AATACGAC-3′, 5′-TATAGTGAGTCGTATTACTGCAGC-3′, 5′-TCACTATAGGCACTATGGCCGAGTGGTTAAGGCGAGAGAC TCGAAATC-3′, 5′-GCCCAAGAGATTTCGAGTCTCTCGCCTT AACCACTCGGCCATAGTGCC-3′, 5′-TCTTGGGCTCTGCCCG CGCTGGTTCAAATCCTGCTGGTGTCGCCAG-3′, and 5′-GAT CCTGGCGACACCAGCAGGATTTGAACCAGCGCGGGCAGA-3′. Plasmids p1477, a pRS425 vector (Christianson et al. 1992) carrying the wild-type *sup16*$^+$ gene, and p1536, a pUC18 plasmid carrying a $tRNA^{Glu}_{UUC}$ gene under the T7 promoter, were obtained from Bo Huang (this laboratory). The $tRNA^{Glu}_{UUC}$ gene carried two mutations (G1-C72), to improve the efficiency of T7 transcription.

Purification of the GST-Tan1 protein

To purify the Tan1 protein, the bacterial strain BL21 (Studier and Moffatt 1986) was transformed with pGEX-4T-2-*TAN1*Δ*intron*. A total volume of 25 mL LB medium containing 50 μg/mL carbenicillin was inoculated and grown at 25°C to OD_{600} = 0.5 before IPTG was added to a final concentration of 0.1 mM. The cells were harvested after 2 h at 25°C, dissolved in PBS, and disrupted by sonication. The GST-Tan1 protein was batch-purified using glutathione Sepharose 4B according to manufacturer's instructions (Amersham Biosciences). Purification of GST protein was performed in an identical manner by using BL21 transformed with the pGEX-4T-2 vector.

RNA methods

Total RNA for Northern blots was prepared by using glass beads essentially as described (Ausubel et al. 2001). For Northern blot analysis ~10μg total RNA was separated on 8% polyacrylamide, 8 M urea gels and transferred to Zeta-Probe membranes (Bio-Rad). Oligonucleotides used to detect precursor and mature $tRNA^{Ser}_{CGA}$ were 5′- AGCCGAACTTTTTATTCCATTCG-3′ and 5′-GCCCA AGAGATTTCGAGTCTCT-3′, respectively. To detect $tRNA^{Met}_i$, the oligonucleotide 5′-GGACATCAGGGTTATGAGCC-3′ was used. Oligonucleotides were labeled by using 5′[γ^{32}P]ATP (5000 Ci/mmole, Amersham Biosciences) and polynucleotide kinase (Roche Applied Science). Northern blots were visualized and quantified by PhosphorImager analysis.

The T7-transcribed radiolabeled $tRNA^{Ser}_{CGA}$ and $tRNA^{Glu}_{UUC}$ was prepared by using MvaI linearized pUC18-*T7sup61*$^+$Δ*IVS* or p1536, 5′[α^{32}P]CTP or 5′[α^{32}P]UTP (400 Ci/mmole, Amersham Biosciences) and the Riboprobe in vitro transcription system (Promega). The radiolabeled transcripts were purified on an 8% polyacrylamide and 8 M urea gel. Transfer RNA was eluted from gel slices in 2 M ammonium acetate, 1% SDS followed by precipitation. The tRNA pellet was dissolved in water. Gel mobility shift assays was performed essentially as described previously (Kambampati and Lauhon 2000) by incubating ^{32}P-labeled $tRNA^{Ser}_{CGA}$ or $tRNA^{Glu}_{UUC}$ (1 to 2 ng) with varying amounts of GST or GST-Tan1 proteins for 10 min at 30°C before applying the samples to a native 8% polyacrylamide gel. The monoclonal anti-glutathione-s-transferase (GST) antibody used for the supershift experiment was purchased from Sigma.

Total tRNA for HPLC analysis was prepared essentially as described (Björk et al. 2001) followed by LiCl fractionation (Avital and Elson 1969). For HPLC analysis 50 μg tRNA was digested to nucleosides by using nuclease P1 and bacterial alkaline phosphatase (Gehrke et al. 1982), and the hydrolysate was analyzed by HPLC (Gehrke and Kuo 1990). N^4-acetylcytidine was purchased from Sigma.

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