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A Single Base Pair Dominates over the Novel Identity of an *Escherichia coli* Tyrosine tRNA in *Saccharomyces cerevisiae*

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The *Escherichia coli su*$^+$*3* tyrosine tRNA was shown recently to be a leucine-specific tRNA in *Saccharomyces cerevisiae*. This finding raises the possibility that some determinants for tRNA identity in *E. coli* may be different in *S. cerevisiae*. To investigate whether the fungal system is sensitive to the major determinant for alanine acceptance in *E. coli*, a single G3 · U70 base pair was introduced into the acceptor helix of the *su*$^+$*3* tyrosine tRNA. This substitution converts the identity of the *E. coli* suppressor in *S. cerevisiae* from leucine to alanine. Thus, as in *E. coli*, G3 · U70 is a strong determinant for alanine acceptance that can dominate over other features in a tRNA that might be recognized by alternative charging enzymes.

In principle, the determinants for the identity of a tRNA can differ from organism to organism without compromising the conservation of the genetic code. The only requirement is that the anticodon triplet of each tRNA must specify the same amino acid from one organism to another. In the case of *Escherichia coli* alanine tRNA, a single G3 · U70 base pair is a major determinant for identity (9, 13). Substitution of this base pair with G · C, A · U, or U · G abolishes aminoacylation with alanine, and transfer of G3 · U70 into *E. coli* $tRNA^{Cys}$, $tRNA^{Phe}$, and $tRNA^{Tyr}$ can confer total or partial alanine acceptance on each (13, 14, 21, 25). Cytoplasmic *Bombyx mori* and human alanine tRNAs each encode a G3 · U70 base pair, and the aminoacylation of these tRNAs by their homologous enzymes is abolished when that base pair is changed to G · C or A · U. Moreover, expression of these eukaryotic tRNAs in *E. coli* results in charging with alanine and no other detectable amino acid (15). Thus, in the evolution of these alanine tRNAs, the G3 · U70 base pair has been conserved. Furthermore, nucleotide sequence differences in other parts of the *B. mori* and human cytoplasmic alanine tRNAs do not encode major determinants for any other *E. coli* aminoacyl-tRNA synthetase.

In contrast to the results obtained with alanine tRNAs, the identity of the *E. coli su*$^+$*3* tyrosine tRNA is changed in the yeast *Saccharomyces cerevisiae*. This amber suppressor inserts only tyrosine in *E. coli*, but in *S. cerevisiae* only leucine is inserted at the position of an amber codon (7). This change in specificity may correlate with a switch in the type class of cytoplasmic tyrosine tRNAs in comparisons of *E. coli* with *S. cerevisiae*. In *E. coli*, tyrosine, leucine, and serine tRNAs are type II tRNAs, which have three base pairs in the dihydrouridine stem and 13 to 22 nucleotides in the variable loop (also known as the extra arm). The remaining *E. coli* tRNAs are type I tRNAs which typically have four base pairs in the dihydrouridine stem and five nucleotides in the variable loop. While the cytoplasmic leucine and serine tRNAs are also type II structures in *S. cerevisiae*, the tyrosine tRNA is a type I molecule. Thus, the switch in identity of the *su*$^+$*3* tyrosine tRNA in *S. cerevisiae* may mean that, in the evolution of some tRNAs, molecules in the same type class may be more related than ones in different type classes that are specific for the same amino acid.

The mode of recognition in *S. cerevisiae* may also differ from that of *E. coli* for tRNAs other than tyrosine. To explore this issue, the wild-type U3 · A70 base pair in *E. coli su*$^+$*3* tyrosine tRNA was changed to G3 · U70, and the resulting tRNA (Fig. 1) was expressed and tested for function in *S. cerevisiae*. Unless G3 · U70 is specifically a negative determinant for the *S. cerevisiae* leucyl-tRNA synthetase, substitution of G3 · U70 should not affect recognition by the *S. cerevisiae* leucyl-tRNA synthetase because the two known cytoplasmic leucine tRNA isoacceptors (31) have either a U · A or a G · C base pair at the 3 · 70 position and therefore appear not to use the 3 · 70 position in determining leucine specificity. Thus, the experiments presented here test for conservation in yeast of a major determinant for alanine identity and for the possibility that recognition by the yeast alanine tRNA synthetase can overcome the leucine specificity of the *E. coli* tyrosine tRNA in *S. cerevisiae*.

For these studies, we constructed a deletion-disruption of the endogenous chitinase locus and an amber mutation in a cloned copy of chitinase. Expression of the amber mutant in the chitinase disruption strain together with a suppressor tRNA led to production of chitinase protein, which was then purified and sequenced to determine the identity of the amino acid inserted at the position of the amber codon. This system should be generally applicable for determining tRNA identity in yeast cells.

MATERIALS AND METHODS

Strains and genetic methods. Bacterial strain DH1 (18) was used for plasmid preparation, and strains TG1 (Amersham Corp.) and JM101 (18) were used for maintenance of M13-derived vectors. *S. cerevisiae* W303-1B (α *trp1-1 his3-11,15 leu2-3,112 ura3-1 ade2-1 can1-100*) and HEY301-129 (**a** *met8-1 trp1-1 his 4-580 leu2-3,112 ura3-1 ade1 can1-100*) were described previously (6).

Standard protocols for growth and maintenance of *S. cerevisiae* strains were used (30). Yeast were transformed by the method of Hinnen et al. (11) or Becker and Guarente (2).

Plasmid construction. A *Pvu*II-*Eco*RI fragment from pHET100am, which spans a *Sal*I fragment encoding the gene for the modified *su*$^+$*3 E. coli* amber suppressor tyrosine tRNA (6), was cloned into the *Hin*cII and *Eco*RI sites of

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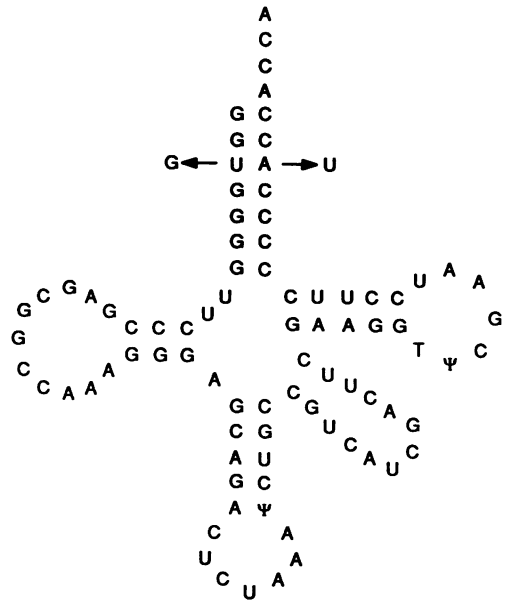

FIG. 1. *E. coli su*$^+$*3* tyrosine tRNA structure. The cloverleaf structure of the wild-type tRNA is shown, and the G · U substitutions at position 3 · 70 are indicated by arrows.

M13mp18. This construct was used as a template for oligonucleotide-directed mutagenesis (in vitro mutagenesis system, version 2.0; Amersham) with two 17-mers (5′-CTTCCC CCTCCACCATT-3′ and 5′-ACCCGTGGGGGGGTTCC-3′) so as to replace the U3 · A70 base pair of the encoded tRNA with G3 · U70. Mutants were identified by DNA sequencing (3, 27) with a 19-mer complementary to a region near the *Sal*I site of the insert.

A *Hin*dIII-*Eco*RI fragment encoding the mutated tRNA gene and the flanking vector sequences (derived from YEp21 [4]) was gel purified. Leu$^+$ transformants of yeast strain HEY301-129 were isolated after cotransformation of the pure fragment together with *Sal*I-digested plasmid YEp21. Homologous recombination of the fragment with the vector sequences in *S. cerevisiae* (17) essentially leads to insertion of the mutated tRNA gene into YEp21 at the *Sal*I site. The recombinant plasmid, pVTT21-GU, was recovered from transformants in which amber suppression of the *met8-1* marker was observed (12), and the presence of the insert was confirmed by restriction analysis. The *Sal*I fragment which contains the gene for the G3 · U70 mutant was isolated from pVTT21-GU and inserted into the *Sal*I site of pMP78-1 (8) to generate pVTT78-GU. The presence of the insert and the orientation were determined by restriction analysis. Plasmid pVTT78-GU was recovered from HEY301-129 transformants which showed suppression of three amber alleles. The *Sal*I fragment which encodes the tRNA was cloned into M13mp18 and sequenced by using a universal primer (New England BioLabs, Inc.).

Plasmid pCT28 (16a) encodes the *S. cerevisiae* structural gene for chitinase (*CTS1-1*) between the *Bam*HI and *Eco*RI sites of vector YEp352 (10). (Plasmids pCT28 and YEp352 were a gift from M. J. Kuranda, Repligen Corp.)

Plasmid pCTam encodes a version of the *CTS1-1* gene in which the codon for Asn-8 of the mature protein was converted to an amber codon. To generate this construct, the *Bam*HI-*Eco*RI fragment of pCT28 was inserted into the *Bam*HI and *Eco*RI sites of M13mp18 and was mutagenized with a 33-mer with the sequence 5′-AGTTCTGCGAACAC GTAGATTGCTGTTTATTGG-3′. The desired mutant was identified by DNA sequencing from a primer which corresponds to the sequence from −47 to −22 nucleotides upstream of the chitinase coding sequence (5′-CCAATACATT GAAATTTTAATTCAAA-3′). The *Bam*HI-*Eco*RI fragment with the amber mutation was reinserted into the *Bam*HI and *Eco*RI sites of YEp352 to make pCTam. The final structure of the plasmid was checked by restriction digestion, and the presence of the amber mutation in the construct was verified by loss of one of eight *Ssp*I sites.

A plasmid-encoded disruption of *CTS1-1* was also derived from pCT28. Plasmid pCT28 was linearized at the *Bgl*II and *Avr*II sites to remove nucleotides −177 through +294 of the structural gene, where +1 is the first nucleotide of the coding sequence. A 1.7-kbp *Bam*HI fragment, which encodes the yeast *HIS3* gene (32), was ligated to the *Bgl*II end of linearized pCT28. The free ends of this ligation product (*Bam*HI and *Avr*II) were made flush with Klenow enzyme (18) and then ligated together. Ampicillin-resistant colonies were selected after transformation of *E. coli* DH1. Plasmid pΔCT28::*HIS3*, which sustained the *Bgl*II-*Avr*II deletion and insertion of the *HIS3* fragment (in the same orientation as the *CTS1-1* gene), was identified by restriction analysis.

Disruption of *CTS1-1* in W303-1B. The 4.5-kbp *Sal*I-*Eco*RI fragment from plasmid pΔCT28::*HIS3* was gel purified, and 1, 2, or 4 μg of pure fragment was used to transform yeast strain W303-1B by electroporation (2). His$^+$ transformants were selected and assayed for stability of the His$^+$ phenotype after growth overnight in YPD medium (yeast extract, peptone, glucose [30]). Dilutions of the YPD culture were plated onto YPD plates and replica plated onto SD plates (minimal medium supplemented with required amino acids [30]) to score for each marker. The His$^+$ phenotype was stable (143 of 143 colonies for disruption W303∇*cts1* used in these studies), and all other auxotrophies of the parent were retained as expected. No chitinase could be isolated from this strain.

Southern and Northern (RNA) blot analysis. The structure of the gene disruption in W303∇*cts1* was confirmed by Southern blot analysis. Chromosomal DNA was isolated from yeast cells after growth in selective medium (6). Following restriction digestion, DNA fragments were separated on a 0.8% agarose gel. The gel was processed and transferred to a nitrocellulose filter (Schleicher & Schuell, Inc.) as described previously (18). A *Bgl*II-*Eco*RI fragment from pCT28 was radiolabeled by nick translation (18), and the probe was separated from unincorporated nucleotides by chromatography on a Sephadex G-100 column. Prehybridization and hybridization were performed overnight at 37°C in 50% formamide–5× SSPE (18)–5× Denhardt's solution–and 0.5% SDS (sodium dodecyl sulfate). Two final washes were performed in 0.1× SSPE–0.1% SDS at 65°C for 20 min each.

Northern blot analysis and quantitation of *E. coli* tRNA in yeast cells were carried out as described previously (6) except that the amount of *E. coli* suppressor tRNA was determined relative to the amount of yeast tyrosine tRNA from the same cell extract, instead of relative to the average amount of yeast tyrosine tRNA from several cell extracts.

Chitinase preparation and sequencing. Cultures of W303-1B or W303∇*cts1* containing various plasmids were grown to saturation at 30°C in 6 ml of SD medium supplemented with the relevant amino acids. Samples (3 ml) of these cultures were added to 300 ml of YPD medium and grown overnight at 30°C to a cell density of about 2 × 10^7

cells per ml of culture. Cell supernatants were collected by filtration through a Nalgene filter unit (0.45-μm pore size). A chitin suspension (250 μl) was added to the filtrate, and the mixture was agitated overnight at 4°C. To prepare the chitin suspension, 1 g of pure chitin (Sigma Chemical Co.) was boiled in 1% SDS–1% β-mercaptoethanol for 10 min and washed extensively in distilled water. The chitin was resuspended finally in 10 ml of distilled water and stored at 4°C. The chitin-chitinase complex was recovered as a precipitate from the mixture by filtration through a Nalgene filter unit (0.45-μm pore size) and washed with 50 to 100 ml of chitinase buffer (0.8% NaCl, 0.02% KCl, 0.12% Na_2HPO_4, 0.02% KH_2PO_4). The precipitate was resuspended in 200 μl of 2× SDS sample buffer for proteins (1) and boiled for 10 min. After centrifugation at room temperature (1 min), the supernatant (25 to 50 μl) was loaded onto 0.1% SDS–7% polyacrylamide gels for visualization. To obtain protein sequences from chitinase, the supernatant (150 μl) was subjected to electrophoresis as described above and transferred electrophoretically to 0.45-μm-pore-size Immobilon transfer membranes (Millipore Corp.) in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer (pH 10.6), with 10% methanol. Filters were processed as described by Matsudaira (20). After staining with Coomassie blue, the bands of interest (apparent molecular mass of 110 kDa) were excised from the blot and submitted to C. Burkins and P. Matsudaira (Whitehead Institute and Massachusetts Institute of Technology) for sequence analysis on either a Porton 2090 gas-phase sequenator or an Applied Biosystems model 475A protein sequencing system.

RESULTS

The G3 · U70 *su*$^+$*3* tyrosine tRNA variant has enhanced suppressor activity in yeast cells. A G3 · U70 variant of the *E. coli* suppressor tyrosine tRNA (wild-type U3 · A70) was obtained by oligonucleotide-directed mutagenesis. This mutant was inserted into the vector YEp21 (selectable marker *LEU2*) by using an in vivo recombination technique in an amber tester strain (HEY301-129) as described in Materials and Methods. Leu$^+$ transformants were isolated which presumably contained the desired construct (pVTT21-GU), and these transformants were screened directly for suppression of the *met8-1*, *trp1-1*, and *his4-580* amber alleles. Transformants were patched to YPD plates (30) and replica plated to minimal medium supplemented with various amino acids to score each nutritional marker. Over 90% of the Leu$^+$ transformants (139 of 149) were found to be Met$^+$ Trp$^-$ His$^-$, indicating suppression of the *met8-1* amber allele. Ten Leu$^+$ transformants were found to be Met$^-$, suggesting that these transformants did not incorporate the suppressor gene. Transformants with two copies of the wild-type tyrosine tRNA suppressor on pHET120am were unable to suppress any of the amber alleles, as found previously (6).

To confirm the structures of the constructs, plasmids were recovered from 10 Leu$^+$ Met$^+$ and one Leu$^+$ Met$^-$ yeast transformant and subjected to restriction analysis. The results were consistent with the integration of the tyrosine tRNA gene fragment into the *Sal*I site of vector YEp21 for all of 10 Leu$^+$ Met$^+$ isolates and indicated that no major rearrangement of the plasmid had occurred in yeast cells. The plasmid from the Leu$^+$ Met$^-$ transformant contained no tRNA gene insert as expected, consistent with the observation that YEp21 transformants show no suppression. (The Leu$^+$ Met$^-$ transformant may have been due to some undigested vector used in the transformation.) Two isolates of pVTT21-GU were used to back-transform amber tester strain HEY301-129, and all of 56 Leu$^+$ transformants were again found to be Met$^+$ Trp$^-$ His$^-$ (Fig. 2). Parallel transformants of the vector alone or pHET120am (two inserts) showed no suppression. These results confirm that the suppression of the *met8-1* amber allele is dependent on the presence of pVTT21-GU and the G3 · U70 variant tRNA gene that it encodes.

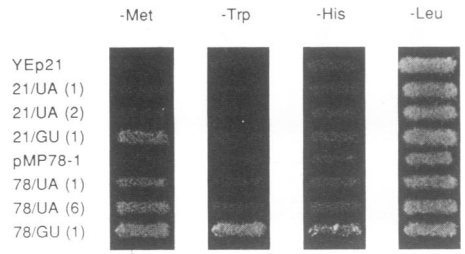

FIG. 2. Suppression of amber alleles in yeast cells by *E. coli* *su*$^+$*3* tyrosine tRNA and the G3 · U70 variant. Leu$^+$ transformants of HEY301-129 which carry plasmid YEp21, pHET100am, pHET120am, pVTT21-GU, pMP78-1, pHET78-1Aam, pHET78-6Bam, or pVTT78-GU were isolated, patched to YPD plates, and replica plated to minimal media supplemented with the relevant amino acids to score for each nutritional marker. The designation for each type of transformant is indicated on the left; the number indicates the parent vector (21 for YEp21 or 78 for pMP78-1), UA or GU indicates the wild-type or G · U variant suppressor gene, and the number of tRNA gene inserts is in parentheses. Suppression is indicated by growth in the absence of methionine (−Met), tryptophan (−Trp), or histidine (−His). The −Leu panel indicates the presence of plasmid in each transformant.

The 2-μm vector pMP78-1 is about 4.5-fold as abundant as typical 2-μm vectors like YEp21 (8). When the *E. coli* suppressor tyrosine tRNA (U3 · A70) gene is cloned into this vector, the level of suppressor tRNA is increased substantially (7), and weak suppression of the *met8-1* amber allele of the tester strain is observed. To determine whether increased expression could similarly enhance the range of suppression of the G3 · U70 variant, the *Sal*I fragment encoding this tRNA was cloned into the *Sal*I site of pMP78-1. The resulting plasmid, pVTT78-GU, has the same structure as pHET78-1Aam (7), which encodes the U3 · A70 suppressor tyrosine tRNA. Each plasmid contains one copy of the *E. coli* suppressor tRNA in the same orientation. Tester strain HEY301-129 was transformed with vectors pMP78-1, pVTT78-GU, pHET78-1Aam, and pHET78-6Bam. Leu$^+$ transformants were selected, plated on YPD plates, and replica plated onto minimal media supplemented with various amino acids to score for nutritional markers. As observed elsewhere (6), the vector alone had no suppressor activity and pHET78-1Aam transformants suppressed only the *met8-1* amber allele (Fig. 2). Even with six U3 · A70 tRNA gene inserts in pHET78-6Bam, only the *met8-1* allele was suppressed. In contrast, pVTT78-GU transformants were Met$^+$ Trp$^+$ His$^{+/-}$, indicating suppression of the *trp1-1* and *his4-580* amber alleles in addition to the *met8-1* amber allele (Fig. 2).

To demonstrate the plasmid linkage of suppression, pVTT78-GU transformants were grown nonselectively in rich medium to allow plasmid segregation. Dilutions of the culture were plated on rich plates and replica plated onto minimal medium supplemented with amino acids to score for suppression. All of the seven colonies that lost plasmid pVTT78-GU, as indicated by a Leu$^-$ phenotype, were found

to be Met$^-$ Trp$^-$ His$^-$. Of the remaining 135 Leu$^+$ colonies analyzed, 126 were Met$^+$ Trp$^+$ His$^{+/-}$, like the original transformant, 8 colonies were Met$^+$ Trp$^+$ His$^-$, and one colony was Met$^+$ Trp$^-$ His$^-$. Although the plasmid was lost at a low frequency (similar to that reported for the parent vector [8]), the results suggest that suppression depends on the presence of the plasmid. The progressive loss of suppression of the *trp1-1* and *his4-580* amber alleles might be a result of loss of suppressor function caused by toxicity (19). Consistent with the plasmid linkage of the suppression phenotypes, back-transformation of the tester strain with pVTT78-GU isolated from yeast cells exhibiting suppression (Met$^+$ Trp$^+$ His$^{+/-}$) led to an identical pattern of suppression in all of 68 transformants from four separate transformation experiments. The four vector controls again showed no suppression of amber alleles.

The enhanced spectrum of suppression of the G3 · U70 *su*$^+$*3* tyrosine tRNA variant correlates with the G3 · U70 base pair. To exclude the possibility that unexpected changes in tRNA sequence caused the improvement in suppression by the G3 · U70 *su*$^+$*3* tyrosine tRNA variant, the tRNA gene fragment was cloned into M13mp18 and sequenced. The fragment was obtained from a plasmid preparation of pVTT78-GU isolated from yeast cells, which consistently conferred a Met$^+$ Trp$^+$ His$^{+/-}$ phenotype in the amber tester strain. Other than the programmed G3 · U70 variation, no change was found.

The enhanced range of suppression of the G3 · U70 tRNA gene variant might be due to elevated expression of suppressor tRNA. Thus, the amount of *su*$^+$*3* tRNA produced by cells which carry plasmid pVTT78-GU was compared with that produced by cells which carry pHET78-1Aam, which differs only at the 3 · 70 base pair. Total RNA isolated from yeast cells transformed with vector pMP78-1, pVTT78-GU, or pHET78-1Aam was analyzed on Northern blots, using ^{32}P-labeled oligonucleotide probes specific for the *E. coli* tyrosine tRNA (Fig. 3A) or the yeast cytoplasmic tyrosine tRNA (Fig. 3B). A band comigrating with pure *E. coli* tyrosine tRNA was detected in extracts from cells which carried pVTT78-GU or pHET78-1Aam but not in extracts from cells which carried the parent vector pMP78-1 (Fig. 3A). In contrast, yeast tyrosine tRNA was evident in total RNA from each of these strains (Fig. 3B). To determine the amount of radioactivity in each band, the corresponding region of the filter was cut out and counted in a scintillation counter. In this experiment, the *su*$^+$*3* tyrosine tRNA (U3 · A70) was produced at a level comparable to 80% of total yeast tyrosine tRNA (eight chromosomal copies), while the G3 · U70 *su*$^+$*3* tyrosine tRNA variant was present at a level about 20% that of total yeast tyrosine tRNA. (When produced from YEp21-derived vectors, the G3 · U70 tRNA was also present in levels one-half to one-fourth that of the U3 · A70 suppressor.) Thus, the greater range of suppression of G3 · U70 *su*$^+$*3* tyrosine tRNA in *S. cerevisiae* appears to reflect a more efficient utilization of this tRNA than of *su*$^+$*3* tyrosine tRNA (U3 · A70).

The G3 · U70 base pair confers alanine identity on the *su*$^+$*3* tyrosine tRNA in yeast cells. Because the G3 · U70 base pair is known to be a major determinant for alanine identity in *E. coli*, the silkworm *B. mori*, and human cytoplasmic tRNAs, we investigated the possibility that this base pair conferred a new identity upon the *su*$^+$*3* tyrosine tRNA (U3 · A70) in *S. cerevisiae*. In *E. coli*, the amino acid inserted by various suppressor tRNAs is conveniently determined by direct sequence analysis of the N terminus of a plasmid-encoded version of dihydrofolate reductase which has an amber

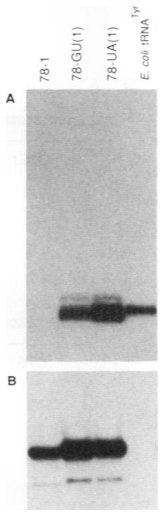

FIG. 3. Northern blot analysis of total tRNA from HEY301-129 transformants. Total RNA was isolated from HEY301-129 transformants which carry plasmid pMP78-1 (78-1), pVTT78-GU [78-GU (1)], or pHET78-1Aam [78-UA(1)]. Plasmid pMP78-1 is the parent vector of pVTT78-GU and pHET78-1Aam. Total RNA (15 μg per lane) was fractionated on 7 M urea–10% polyacrylamide gels. Pure *E. coli* tyrosine tRNA was loaded in the last lanes of each gel. Following electrophoretic transfer to nylon filters, blots were probed with ^{32}P-end-labeled 14-base oligonucleotide probes. (A) Northern blot probed with an oligonucleotide complementary to the *E. coli* tyrosine tRNA; (B) duplicate filter probed with an oligonucleotide complementary to the yeast cytoplasmic tyrosine tRNA. (Variation in the amount of host cell-encoded tyrosine tRNA may be due to experimental variation in the amount of RNA applied to the gel.)

codon at position 10 (24). We wished to develop an analogous system for *S. cerevisiae* with a test protein which meets the following requirements: (i) the gene and protein sequence of the test protein are known; (ii) the N terminus is not blocked; (iii) the protein is highly expressed to compensate for low efficiency of suppression; (iv) the protein is easily purified, allowing rapid preparation of many samples; and (v) the suppressed protein preparation is not contaminated by endogenous protein.

These criteria for a test protein were met by the secreted protein chitinase, a glycosidase specific for chitin, which is a minor component of the yeast cell wall. A chitinase gene called *CTS1-1* was isolated from a yeast library (16), and a 3.3-kbp *Bam*HI-*Eco*RI fragment of chromosomal DNA encompassing the structural gene and its promoter was subcloned into the 2-μm vector YEp352. The resulting plasmid, pCT28, directs expression of chitinase in yeast cells such that as much as 1 mg of protein can be purified from 1 liter of culture. The purification procedure can be completed in essentially one step by precipitation of chitinase with a suspension of chitin. For further analysis, the protein is subjected to SDS-polyacrylamide gel electrophoresis, and N-terminal protein sequence can be obtained after transfer to Immobilon membranes (16a).

Sequence data indicate that a leader sequence of 19 amino acids is removed during protein secretion by cleavage of an Ala-Phe bond (16a). Thus, nucleotides +82 through +84, which correspond to the asparagine at position 8 of the mature protein, were chosen as the site for an amber codon to determine the identity of the amino acids inserted by

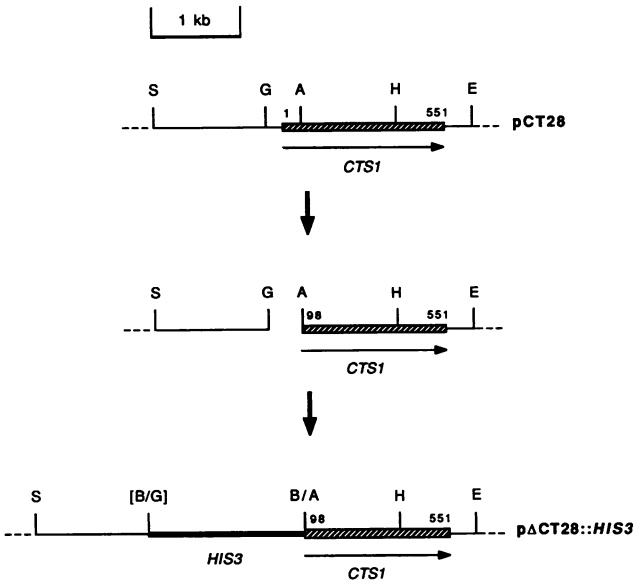

FIG. 4. Restriction maps of pCT28 and pΔCT28::*HIS3*. *CTS1* refers to the chitinase coding region (hatched box), the solid line represents 5′- and 3′-flanking sequences, and the dashed line represents the parent vector, YEp352. Plasmid pCT28 was digested with *Avr*II and *Bgl*II, and a 1.8-kbp *Bam*HI fragment which encodes the yeast *HIS3* gene (solid box [32]) was ligated to the *Bgl*II site at one end. After filling in of the free ends (*Bam*HI and *Avr*II) by treatment with Klenow enzyme (18), the plasmid was religated to yield pΔCT28::*HIS3*. The numbers 1, 98, and 551 refer to the first, 98th, and last codons of the *CTS1* gene product. Restriction sites: A, *Avr*II; B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; S, *Sal*I.

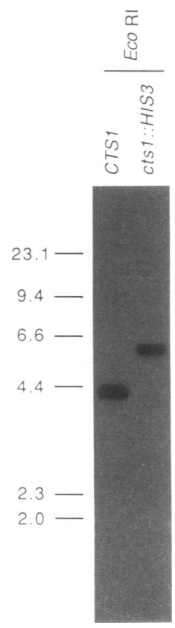

FIG. 5. Southern blot analysis of chromosomal DNA isolated from disruption strain W303∇*cts1* and the parent W303-1B. Chromosomal DNA was isolated from strains W303-1B (*CTS1*) and W303∇*cts1* (*cts1*::*HIS3*) and was digested with *Eco*RI. The digestion products were fractionated on a 0.8% agarose gel, transferred to a nitrocellulose filter, and probed with a ^{32}P-labeled 2.0-kbp *Bgl*II-*Eco*RI fragment from pCT28 which carries the chitinase structural gene. Locations in the gel and sizes of the molecular weight markers (*Hin*dIII digest of lambda DNA) are indicated at the left in kilobase pairs.

suppressor tRNAs in yeast cells. The plasmid coding for the *CTS1-1* amber mutant was called pCTam.

To avoid contamination by chromosomally encoded chitinase of the chitinase produced from suppression of the amber codon of pCTam, a disruption of the endogenous gene was constructed. Sequences of the chromosomal locus which overlap position 8 were deleted in the construct to prevent recombination between the plasmid-encoded gene and the chromosomal gene, which might yield spurious wild-type sequence. A plasmid-encoded disruption of the chitinase gene, pΔCT28::*HIS3* (Fig. 4), was derived from pCT28 via replacement of nucleotides −177 to +294 with a 1.7-kbp fragment encoding the *HIS3* structural gene (32). The 3.3-kbp *Sal*I-*Eco*RI fragment which contains the chitinase deletion-disruption fragment was then used to transform strain W303-1B (relevant genotype *his3-11,15*), and stable His$^+$ transformants were isolated. Cells from these strains do not separate after budding, a phenotype also consistent with disruption of the chitinase locus (16a).

Partial deletion and disruption of the chromosomal chitinase gene was assessed by Southern blot analysis as described in Materials and Methods. Chromosomal DNA was prepared from the parent W303-1B and the disruption strain W303∇*cts1*, digested with *Eco*RI, and analyzed with a 2.0-kbp *Bgl*II-*Eco*RI probe from pCT28 (Fig. 4). As *Eco*RI does not cut within the region detected by the probe, and as a result of the deletion of 471 bp coupled with an insertion of 1,823 bp, the fragment detected in the disruption strain should be 1,352 nucleotides larger than the fragment detected in the parent strain. The resulting blot shown in Fig. 5 reveals a ~4,200-bp fragment in parent W303-1B (*CTS1*) and a ~5,600-bp fragment in W303∇*cts1* (*cts1*::*HIS3*). The observed size increase of approximately 1,400 bp is in close agreement with the expected size difference of 1,352 bp. A similar analysis with *Hin*dIII or *Pst*I confirmed the structure of the disruption (data not shown).

Chitinase preparations were obtained from the untransformed parent strain W303-1B and from Ura$^+$ transformants of the disruption strain W303∇*cts1* which carry YEp352, pCT28, or pCTam. On SDS-polyacrylamide gels, chitinase from the parent strain W303-1B has an apparent molecular mass of around 130 kDa (Fig. 6, lane a). In contrast, no chitinase was evident in the preparation from the disruption which carries YEp352 (the vector control for pCT28 and pCTam; Fig. 6, lane b) or in the disruption strain which contains pCTam (Fig. 6, lane d). These findings are consistent with the disruption of the chitinase gene and also indicate that the amber mutation at position 8 of pCTam prevents synthesis of chitinase. Kuranda and Robbins (16a) also obtained a viable disruption of the chitinase locus, which produces no detectable chitinase activity or protein.

Chitinase isolated from the disruption strain transformed with the cloned chitinase gene on pCT28 has an apparent molecular mass of around 110 kDa (Fig. 6, lane c). The size discrepancy between the chitinase produced by W303-1B and that produced from pCT28 may be due to the existence of highly similar (>90% at the DNA level) alleles of chitinase particular to different strains (16a). In any case, disruption strain W303∇*cts1* secretes no detectable chitinase protein and, when transformed with pCT28, secretes the form of chitinase expected from this plasmid by both size and sequence (see below) criteria.

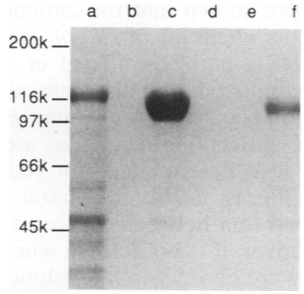

FIG. 6. Suppression of *cts1-82,84* amber allele in W303∇*cts1* by *E. coli su*$^+$*3* tyrosine tRNA (G3 · U70). The chitinase fractions from W303-1B (a) or from W303∇*cts1* transformed with YEp352 (b), pCT28 (c), pCTam (d), pCTam plus pVTT21-GU (e), or pCTam plus pVTT78-GU (f) were fractionated on a 0.1% SDS–7% polyacrylamide gel. The gel was stained with Coomassie brilliant blue G. The apparent molecular masses are 120 to 130 kDa for the endogenous chitinase of W303-1B and around 110 kDa for chitinase encoded by pCT28. Locations and sizes of the molecular weight markers are indicated at the left (rabbit skeletal muscle myosin, 200 kDa; *E. coli* β-galactosidase, 116 kDa; rabbit muscle phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; hen egg ovalbumin, 45 kDa [Bio-Rad Laboratories]).

su$^+$*3* tyrosine tRNA (G3 · U70) identity in *S. cerevisiae* was investigated by using the disruption strain and cloned chitinase amber mutant on pCTam. W303∇*cts1* cells (relevant genotype *leu2-3,112 ura3-1*) were cotransformed with pCTam (selectable marker *URA3*) and with YEp21, pVTT21-GU (selectable marker *LEU2*), pMP78-1, or pVTT78-GU (selectable marker *leu2*^{d}). Leu$^+$ Ura$^+$ transformants were selected, patched on YPD plates, and replica plated to minimal medium to score each marker. The pCTam transformants which carried vector YEp21 or pMP78-1 or the G3 · U70 *su*$^+$*3* tyrosine tRNA on pVTT21-GU (four each) were found to be His$^+$ Ade$^-$ Trp$^-$. In contrast, four pCTam-plus-pVTT78-GU transformants which were His$^+$ Ade$^-$ were found to be Trp$^+$ due to suppression of the *trp1-1* amber allele present in the disruption strain.

Two transformants of each type were then analyzed for chitinase production. No chitinase could be isolated from pCTam transformants which carried vector YEp21 or pMP78-1 (data not shown) or plasmid pVTT21-GU (Fig. 6, lane e). However, chitinase was clearly produced in pCTam transformants which carry plasmid pVTT78-GU (Fig. 6, lane f), an observation consistent with the increased range of suppression observed with this plasmid (Fig. 2).

The amount of chitinase produced from pCT28 (Fig. 6, lane c) and the amount of chitinase produced from pCTam by the suppressor encoded on plasmid pVTT78-GU (Fig. 6, lane f) can be compared because equal amounts of each preparation were loaded in these lanes. The data indicate that pCT28 produced 5 to 10 times more chitinase than was produced by suppression from pCTam by pVTT78-GU. Thus, the apparent suppression efficiency of the *E. coli* G3 · U70 tRNA^{Tyr} in *S. cerevisiae* is 10 to 20%. By comparison, the wild-type *E. coli* tRNA^{Ala} amber suppressor has an efficiency in *E. coli* of about 20% on two different amber alleles (13).

For amino acid analysis, larger volumes of the chitinase preparations (150 μl) from the disruption strain containing pCT28, pCTam plus pHET78-6Bam, or pCTam plus pVTT78-GU were separated on 0.1% SDS–7% polyacrylamide gels. Proteins were electrophoretically transferred to an Immobilon membrane, processed, and stained as described in Materials and Methods. The bands of interest were excised and submitted for amino acid sequence analysis. Sequences were determined from two separate experiments. In each case, the sequence of 11 N-terminal amino acids, including position 8, was determined to be certain that the sequence was that of chitinase.

Typical results from one sequence determination are shown in Fig. 7. The N-terminal sequence of chitinase encoded by pCT28 for amino acids 1 to 11 was found to be Phe-Asp-Ser-Ser-Ala-Asn-Thr-Asn-Ile-Ala-Val (Fig. 7A). This sequence agrees with previous determinations (16a). The sequence derived for chitinase produced by suppression of the amber allele of pCTam by the wild-type *su*$^+$*3* tyrosine tRNA (U3 · A70) was identical to the wild-type sequence determined above except at position 8 (specified by the amber codon), where leucine was inserted (Fig. 7B, independently confirming the result Edwards and Schimmel [6]). The sequence of chitinase produced by suppression of the amber allele of pCTam by the G3 · U70 variant of the *su*$^+$*3* tyrosine tRNA also differed only in the amino acid at position 8, where alanine was exclusively inserted (Fig. 7C). Asparagine was not present above background levels at this position. Again the low level of leucine detected in cycle 8 of this sequence was no higher than those found in every other

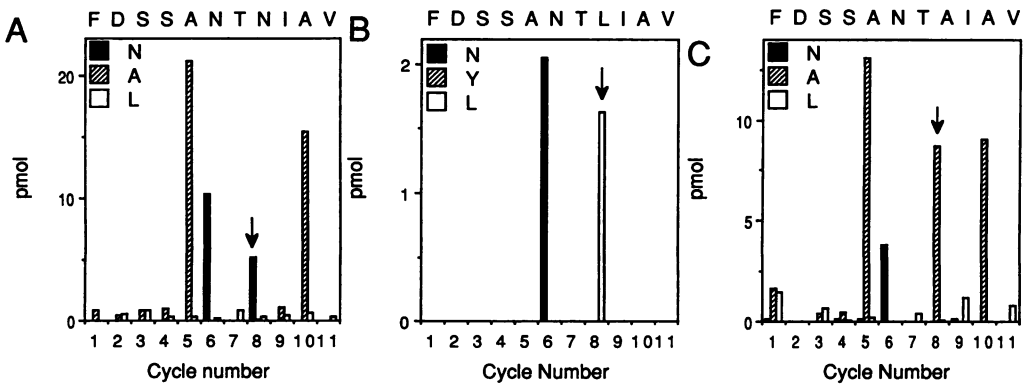

FIG. 7. Amino acid sequence analysis of the first 11 amino acids of chitinase encoded by pCT28 (A), by pCTam suppressed by *E. coli su*$^+$*3* tyrosine tRNA (U3 · A70) (B), or by pCTam suppressed by *E. coli su*$^+$*3* tyrosine tRNA (G3 · U70) (C). The sequence of CTS1 obtained in each case is given at the top of each panel. The arrows indicate position 8. The picomolar yields for asparagine, alanine, and leucine are plotted versus the residue number. At each cycle, the carryover from the preceding cycle for each of these amino acids has been subtracted.

cycle. Thus, as in *E. coli*, the G3 · U70 base pair appears to be a strong determinant for alanine acceptance in yeast cells, converting the *su*$^+$*3* tyrosine tRNA from a leucine to an alanine tRNA.

DISCUSSION

It is worth noting that, in addition to the wild-type protein with asparagine at position 8, chitinase variants that have either alanine, leucine, or tyrosine inserted by amber suppression of codon 8 have been collectively isolated and sequenced in this and another recent study (7). This suggests that the chitinase system will be broadly applicable for investigations of the amino acids inserted at an amber codon in *S. cerevisiae*.

Although Northern blot analysis indicates that the level of the G3 · U70 variant of the *E. coli su*$^+$*3* tyrosine tRNA is lower than that of its wild-type counterpart (U3 · A70), the G3 · U70 tRNA suppresses a wider range of amber mutations in yeast cells. Because the constructs encoding these two tRNAs differ only at the 3 · 70 position of the tRNA gene, the enhanced range of suppression associated with the G · U base pair correlates with the switch from leucine to alanine identity. Unless this base pair directly influences the efficiency in translation of the amber suppressor, or induces a posttranscriptional modification that in turn affects translational efficiency, the increased range of suppression may be a result of the new alanine identity. One possibility is that each of the amber alleles tested here is suppressed better by alanine than by leucine. Alternatively, it is possible that the charging in vivo of the G3 · U70 variant with alanine is much stronger than the charging of the U3 · A70 suppressor with leucine.

While the determinants for leucine identity are unknown, the 3 · 70 position is probably not essential because both U · A and G · C pairs are found in the cytoplasmic tRNAs aminoacylated with leucine in yeast cells (31). Thus, the conversion from leucine to alanine specificity by a G3 · U70 substitution in the *E. coli su*$^+$*3* tyrosine tRNA is unlikely to depend on interference by G3 · U70 with recognition by leucyl-tRNA synthetase.

The G3 · U70 variant of the *E. coli su*$^+$*3* tyrosine tRNA retains tyrosine identity in *E. coli*. The tRNA inserts 95% tyrosine and a trace of glutamine. However, the identity of the tRNA can be switched to alanine by elevating the level of alanine tRNA synthetase in vivo (14). Thus, in *E. coli* the tRNA possesses either of two identities, depending on which synthetase dominates in competition for the tRNA (33). In yeast cells, the identity of this tRNA may be easily switched from leucine to alanine because the leucine determinants are much weaker, allowing the interaction with alanine tRNA synthetase to dominate.

That a single G3 · U70 base pair is sufficient for alanine acceptance has been demonstrated in *E. coli*, and cytoplasmic silkworm *B. mori*, human, and now fungal systems. As in the case of the *E. coli* and cytoplasmic *B. mori* and human systems, the G3 · U70 pair is unique to alanine tRNAs, according to the most recent compilation of yeast cytoplasmic tRNA sequences (31). Thus, alanine tRNAs can in principle be distinguished from all others in *S. cerevisiae* on the basis of this single base pair.

Recently, the determinants for identity of a number of tRNAs have been established (23, 27a). Normanly and Abelson (23) estimate that about half of the *E. coli* tRNAs utilize nucleotides outside of the anticodon to determine amino acid specificity. On the other hand, Schulman and Pelka (28, 29) have shown that the anticodon is the major determinant for the identities of *E. coli* methionine, valine, and arginine tRNAs, and Muramatsu et al. (22) provided evidence that the anticodon is also critical for determining the efficiency and specificity of charging *E. coli* isoleucine tRNA. The three anticodon nucleotides are also among the five bases that act as determinants for yeast phenylalanine tRNA identity (26). In all but the latter case, for which significant conservation between the yeast and *E. coli* systems has been shown, it is not known whether the determinants for identity are conserved in evolution.

The idiosyncratic locations of the determinants for identity may reflect a dynamic process whereby the synthetases and tRNAs coevolve, making subtle adaptations in each organism that produce variations in the locations of the major sites for recognition and, additionally or alternatively, in the specific nucleotides at those locations. This process could produce a variation in recognition sites from specific tRNA to tRNA, as well as from organism to organism for tRNAs of the same specificity. However, were such a dynamic adaptation process to occur, then it is not uniformly applied to all of the tRNAs, with the novel leucine specificity of the *E. coli su*$^+$*3* tyrosine tRNA in yeast cells contrasting with the apparently widespread conservation of determinants for alanine tRNA identity among various organisms.

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