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Wide cross-species aminoacyl-tRNA synthetase replacement *in vivo*: Yeast cytoplasmic alanine enzyme replaced by human polymyositis serum antigen

(tRNA recognition/heterologous aminoacylation/RNA duplex substrates)

TRACY L. RIPMASTER*, KIYOTAKA SHIBA†, AND PAUL SCHIMMEL*

*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; and †Department of Cell Biology, Cancer Institute, Japanese Foundation for Cancer Research, Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan

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ABSTRACT **Because of variations in tRNA sequences in evolution, tRNA synthetases either do not acylate their cognate tRNAs from other organisms or execute misacylations which can be deleterious *in vivo*. We report here the cloning and primary sequence of a 958-aa *Saccharomyces cerevisiae* alanyl-tRNA synthetase. The enzyme is a close homologue of the human and *Escherichia coli* enzymes, particularly in the region of the primary structure needed for aminoacylation of RNA duplex substrates based on alanine tRNA acceptor stems with a G3·U70 base pair. An *ala1* disrupted allele demonstrated that the gene is essential and that, therefore, *ALA1* encodes an enzyme required for cytoplasmic protein synthesis. Growth of cells harboring the *ala1* disrupted allele was restored by a cDNA clone encoding human alanyl-tRNA synthetase, which is a serum antigen for many polymyositis-afflicted individuals. The human enzyme in extracts from rescued yeast was detected with autoimmune antibodies from a polymyositis patient. We conclude that, in spite of substantial differences between human and yeast tRNA sequences in evolution, strong conservation of the G3·U70 system of recognition is sufficient to yield accurate aminoacylation *in vivo* across wide species distances.**

Aminoacyl-tRNA synthetases must discriminate among a population of tRNA species to precisely aminoacylate their cognate tRNAs. Due to changes in tRNA sequences through evolution, the extent to which specific tRNA determinants are conserved from organism to organism is variable. For example, expression of human cytoplasmic $tRNA^{Ala}$ in *Escherichia coli* results in specific charging by alanyl-tRNA synthetase (1). In contrast, an *E. coli* tyrosine amber suppressor is mischarged with leucine in *Saccharomyces cerevisiae* (2). Failure to cross-aminoacylate their cognate tRNAs or misacylation of noncognate tRNA species may therefore limit the ability of an aminoacyl-tRNA synthetase to function heterologously.

Aminoacyl-tRNA synthetases are a diverse group of enzymes that vary in size and quaternary structure (3). In spite of their diversity, the enzymes can be grouped into two classes of 10 enzymes each, and this grouping is conserved through evolution (4, 5). Cognate enzymes from divergent organisms can have extensive sequence similarity as is the case for the alanine enzymes from *E. coli* and human (ref. 6 and unpublished results), can be limited as seen with the serine enzymes from *E. coli* and *S. cerevisiae* (8, 9), or lacking entirely as seen with the *E. coli* and human glycine enzymes (10–12). The ability of tRNA synthetases to cross-aminoacylate cognate tRNA substrates across wide species barriers has been investigated *in vitro*. Glycyl-tRNA synthetases from *E. coli* and human are unable to cross-aminoacylate human $tRNA^{Gly}$ and *E. coli* $tRNA^{Gly}$, respectively (11). Extracts of *E. coli* fail to aminoacylate mammalian tRNA with isoleucine (13). In contrast, human and *E. coli* alanyl-tRNA synthetases cross-aminoacylate their respective tRNAs, as well as RNA duplexes based on the acceptor helices of these tRNAs (unpublished results).

The ability of a heterologous tRNA synthetase to functionally replace an endogenous synthetase requires specificity of aminoacylation and levels of activity necessary to sustain cell growth. Expression of *Bacillus stearothermophilis* tyrosyl-tRNA synthetase in *E. coli* is toxic, apparently due to misaminoacylation of noncognate tRNAs (14). Misacylation of noncognate tRNAs due to relaxed tRNA discrimination of a mutant *E. coli* glutaminyl-tRNA synthetase is deleterious to cell growth (15). Cross-species replacement of a tRNA synthetase was observed by the ability of *E. coli* tyrosyl-tRNA synthetase to complement a defective allele of the mitochondrial enzyme in *S. cerevisiae* (16). The success of this experiment may be accounted for by the close similarity between *E. coli* and yeast mitochondrial tyrosine tRNAs (17). Additionally, both yeast cytoplasmic seryl- and isoleucyl-tRNA synthetases, which show considerable sequence homology with their bacterial counterparts, can recognize bacterial cognate tRNAs *in vivo* (18, 19). However, functional complementation in *E. coli* or yeast by heterologous expression of aminoacyl-tRNA synthetases widely separated in evolutionary time has not been demonstrated.

Recognition of $tRNA^{Ala}$ by the class II *E. coli* alanyl-tRNA synthetase is determined in large part by a single base pair, G3·U70, in the acceptor helix (20, 21). Aminoacylation of tRNA substrates in a G3·U70-dependent manner by *Bombyx mori* and human alanyl-tRNA synthetases suggests that this recognition has been conserved in evolution (1). The G3·U70 base pair is present in eukaryotic cytoplasmic alanine tRNAs and is unique to alanine tRNA in *E. coli*. The G3·U70 base pair is also unique to the cytoplasmic alanine tRNAs of *S. cerevisiae* and human. In *S. cerevisiae* the cytoplasmic alanyl-tRNA synthetase is able to charge an *E. coli* tyrosine tRNA containing the G3·U70 base pair with alanine (22). This observation indicates that the G3·U70 determinant is important for recognition by the yeast alanine enzyme.

Human aminoacyl-tRNA synthetases, including alanyl-tRNA synthetase, are frequent targets of autoantibodies in patients with polymyositis (23). These autoantibodies tend to bind to conserved epitopes critical to the function of these proteins (23–25). It is unknown whether the structural features of mammalian alanyl-tRNA synthetase that direct autoantibody synthesis will interfere with its function in a heterologous system.

To extend our understanding of structural relationships associated with G3·U70 recognition by alanyl-tRNA synthetases and to investigate the possibility of achieving wide cross-species tRNA synthetase replacement in a system where that possibility seemed plausible because of the retention of the

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G3·U70 base pair in alanine tRNAs in evolution, we cloned the alanyl-tRNA synthetase gene (*ALA1*) from the yeast *S. cerevisiae*.‡ The cloned gene provided the opportunity to investigate the primary structure of the yeast protein, investigate its relationship to the *E. coli* and human enzymes, and construct a yeast null strain into which a cDNA encoding the human polymyositis serum antigen could be introduced.

MATERIALS AND METHODS

Strains and Media. *S. cerevisiae* strain MM1401 (*MATa/α, ade2-101/+, can1/+, his3Δ200/his3Δ200, leu2Δ1/leu2Δ1, lys2-801/lys2-801, trp1Δ101/trp1Δ101, ura3-52/ura3-52*), obtained from John Woolford (Carnegie Mellon University, Pittsburgh) was the diploid strain used to disrupt the *ALA1* gene. Standard genetic techniques were employed (26). Yeast cells were grown in YEPD or defined synthetic medium supplemented with 2% dextrose as a carbon source.

Materials. Restriction endonucleases and yeast tRNA were obtained from Boehringer Mannheim. T4 DNA ligase was obtained from New England Biolabs. [α-^{32}P]dATP, [γ-^{32}P]ATP, and [α-[^{35}S]thio]dATP were obtained from Amersham.

Cross-Species Polymerase Chain Reaction (PCR). Total genomic DNA from strain MM1401 was used for cross-species PCR (10). The primers used were KY23 (5′-TTRCIAAYGCIGGIATGAAYCARTTYAAR-3′) and KY25 (5′-RTTICCCATCATYTCRAAIGTRTGRTG-3′) where Y = C or T, R = A or G, and I = deoxyinosine. PCR products were directly sequenced by use of a CircumVent kit (New England Biolabs).

DNA Manipulations and Southern Analyses. Recombinant DNA techniques, gel electrophoresis, Southern blotting, and hybridization of DNA were performed as described (27). Qiabrane nylon membrane (Qiagen, Chatsworth, CA) was used for DNA blotting. Subclones designed to be used in both *E. coli* and yeast were inserted into the pRS series of vectors (28).

DNA Sequence Determination and Analysis. Restriction fragments from *ALA1* were subcloned in pBluescript KS(+) and SK(+) (Stratagene). The sequences of both strands of the *ALA1* gene were determined by the dideoxy chain-termination method (29) using Sequenase version 2.0 (United States Biochemical). Gaps of sequence in either strand were completed by using synthetic oligonucleotide primers synthesized by the Biopolymers Laboratory at the Massachusetts Institute of Technology.

Immunoblot Analysis. Proteins from yeast cell extracts (26) were subjected to PAGE (27), electrophoretically transferred to Immobilon membrane (Millipore), and assayed by immunoblot analysis using alkaline phosphatase-conjugated anti-human antibodies (Promega) and nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt from GIBCO/BRL. Antibodies against human alanyl-tRNA synthetase were a generous gift of Michael Mathews (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Aminoacylation of RNA Duplexes. Ability of purified human alanyl-tRNA synthetase to aminoacylate duplex substrates was assayed with protocols described by Hill and Schimmel (30). Chemical synthesis and purification of RNA duplexes were carried out as described (31–33).

RESULTS

Identification of a *S. cerevisiae* Gene Encoding an Alanyl-tRNA Synthetase. Degenerate oligonucleotides designed to hybridize to DNA encoding a highly conserved motif found in the N-terminal domain of alanyl-tRNA synthetases from *E. coli, B. mori, Rhizobium leguminosarum*, and *Rhizobium meliloti* (7) were used to amplify the corresponding sequence from total yeast genomic DNA by PCR. One amplified sequence was identified and shown to encode a peptide with high similarity to alanyl-tRNA synthetase from *E. coli* and *B. mori*. A nondegenerate 59-mer oligonucleotide corresponding to the sequence of the yeast gene was radioactively labeled and used to screen a library of yeast genomic DNA cloned in plasmid YCp50 (34). Plasmids recovered from four cross-hybridizing colonies were shown to have overlapping inserts by restriction endonuclease mapping, and PCR amplification and sequencing confirmed that these plasmids contained the region of interest (data not shown). Southern analysis indicated that the oligonucleotide hybridized to a 715-bp *Xba* I–*Bgl* II fragment (Fig. 1 and data not shown).

Alanyl-tRNA Synthetase from Yeast Is Highly Conserved. The complete nucleotide sequence of the open reading frame including and surrounding the location where the 59-mer oligonucleotide hybridizes was determined (GenBank accession no. U18672). The last 300 nt of the *ALA1* gene were previously reported during the cloning of the *MRS2* gene (35), indicating that *ALA1* is directly adjacent to *MRS2* on chromosome XV. Comparison of the translated amino acid sequence of this open reading frame with proteins in GenBank and EMBL databases indicated extensive homology to alanyl-tRNA synthetases including those of *E. coli* (6), *B. mori* (36), *Arabidopsis thaliana* (GenBank/EMBL accession no. Z22673), and human (unpublished results). Alignment of the 958-aa polypeptide with *E. coli, B. mori, A. thaliana*, and human alanyl-tRNA synthetases indicates the close relatedness of all five proteins, particularly in the N-terminal domain which contains the three conserved motifs of the active-site region (Fig. 2). The yeast protein is 49.7% and 37.2% identical to the human and *E. coli* enzymes, respectively. A fragment containing the first 461 aa of the *E. coli* enzyme is a monomer and aminoacylates microhelix substrates with the same efficiency as the tetrameric full-length enzyme (41). Within this domain (corresponding to the N-terminal 483 aa of the yeast protein), the identity between the yeast and human sequences increases to 60%. On the basis of this high similarity we conclude that this open reading frame encodes *S. cerevisiae* alanyl-tRNA synthetase and designate the gene *ALA1*.

***ALA1* Is an Essential Gene.** An *ala1* disrupted allele was created by replacing the 510-bp *Bgl* II–*Sal* I restriction fragment of *ALA1* coding sequence with the *TRP1* gene (Fig. 1). Trp$^+$ colonies were selected after transformation of an *Xba* I–*Xho* I restriction fragment containing this *ala1Δ::TRP1* disruption into MM1401, which is homozygous for *trp1Δ101*. The expected transplacement was confirmed by genomic Southern blotting (data not shown). Diploids were sporulated and dissection of 27 tetrads indicated only two spores were viable in each tetrad, all of which were Trp$^-$. To verify that *ALA1* is required for mitotic growth, a YCp50 plasmid bearing *ALA1* and *URA3* (pSCAlaRS) was used to transform the *ala1::TRP1/ALA1* diploid and the transformant was sporulated. Trp$^+$ spores were recovered, all of which were Ura$^+$, indicating that *ALA1* is an essential gene. Due to the essential nature of the gene, we conclude that *ALA1* encodes an *S. cerevisiae* alanyl-tRNA synthetase required for cytoplasmic protein synthesis.

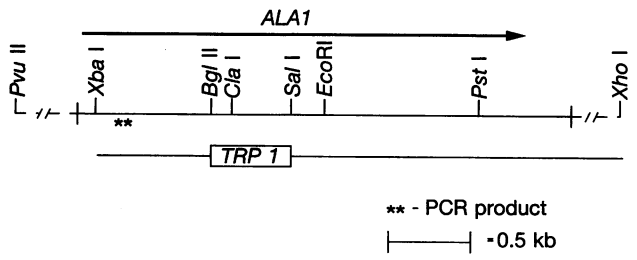

FIG. 1. Structure of the *ALA1* locus. A partial restriction map of the *ALA1* gene is depicted. A disrupted allele of *ALA1* was constructed by replacing the *Bgl* II–*Sal* I fragment encoding aa 259–429 with a *Bam*HI–*Sal* I fragment of the *TRP1* gene.

‡The sequence reported in this paper has been deposited in the GenBank database (accession no. U18672).

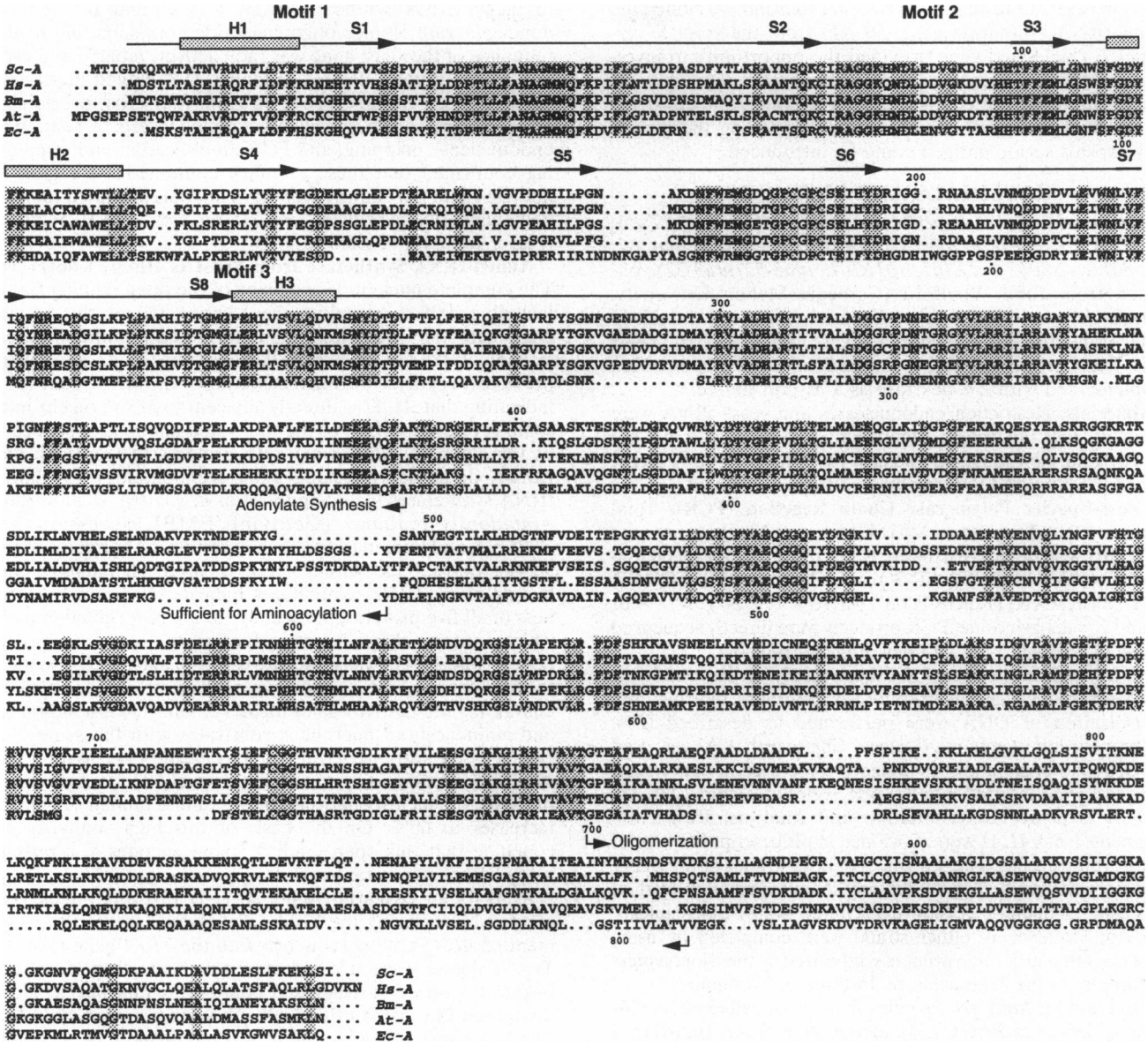

FIG. 2. Multiple sequence alignment of alanyl-tRNA synthetases. The PILEUP program (Genetics Computer Group, Madison, WI) was used to align alanyl-tRNA synthetases from *E. coli* (*Ec*), *B. mori* (*Bm*), *Homo sapiens* (*Hs*), *A. thaliana* (*At*), and *S. cerevisiae* (*Sc*). Residues conserved among all four sequences are shaded. Numbering at top corresponds to the yeast enzyme, and that at the bottom to the *E. coli* protein. The predicted secondary structure elements of the *E. coli* enzyme are shown across the top (7, 38, 39). Functional units of the *E. coli* enzyme for adenylate synthesis, aminoacylation, and oligomerization are also shown (40).

Human Alanyl-tRNA Synthetase Aminoacylates Duplexes Based on Yeast Cytoplasmic tRNA^{Ala} *in Vitro*. Recently, human alanyl-tRNA synthetase has been cloned and expressed in the yeast *Pichia pastoris* (unpublished results). The major determinant for aminoacylation with alanine of the *E. coli* tRNA^{Ala} is a G3·U70 base pair (20) that is conserved in the alanyl-tRNAs of *S. cerevisiae* (17). In addition, an *E. coli* tyrosine tRNA containing a G3·U70 base pair is charged with alanine *in vivo* in *S. cerevisiae* (22), indicating conservation of the critical determinant. We therefore tested whether purified human alanyl-tRNA synthetase could aminoacylate crude yeast tRNA and a chemically synthesized 9-bp RNA duplex substrate based on the acceptor helix of yeast cytoplasmic tRNA^{Ala} (Fig. 3*A*). The recombinant human enzyme was able to aminoacylate crude yeast tRNA as well as the "yeast" RNA duplex (Fig. 3*B*), indicating that the human enzyme can recognize the G3·U70 base pair in the context of a yeast cytoplasmic alanyl-tRNA which differs from the human counterpart at base pairs 4·69, 6·67, and 7·66. A single U70-to-C change eliminates aminoacylation of the "yeast" duplex by the human enzyme.

A Disrupted Allele of *ALA1* Is Rescued by Human Alanyl-tRNA Synthetase. To test whether the human enzyme could functionally complement a strain bearing an *ala1* disrupted allele, we cloned the coding sequence of human alanyl-tRNA synthetase into pDB20L*Bgl*II (42), a 2μ plasmid that contains the *LEU2* gene, creating plasmid pHsAlaRS. Expression of the human enzyme from plasmid pHsAlaRS is driven by the strong constitutive alcohol dehydrogenase promoter. Immunoblot analysis with autoimmune antibodies that crossreact with human alanyl-tRNA synthetase (23) detected expression of the human enzyme in yeast (Fig. 4*A*). After transformation of pHsAlaRS into a haploid strain bearing the *ala1* disrupted allele and pScAlaRS bearing *ALA1* and *URA3*, Leu$^+$ colonies were plated on solid medium containing 5-fluoroorotic acid to select for loss of the *URA3* plasmid (43). Only strains containing the human alanyl-tRNA synthetase gene in the "sense" orientation were able to grow on 5-fluoroorotic acid (Fig. 4*B*), indicating

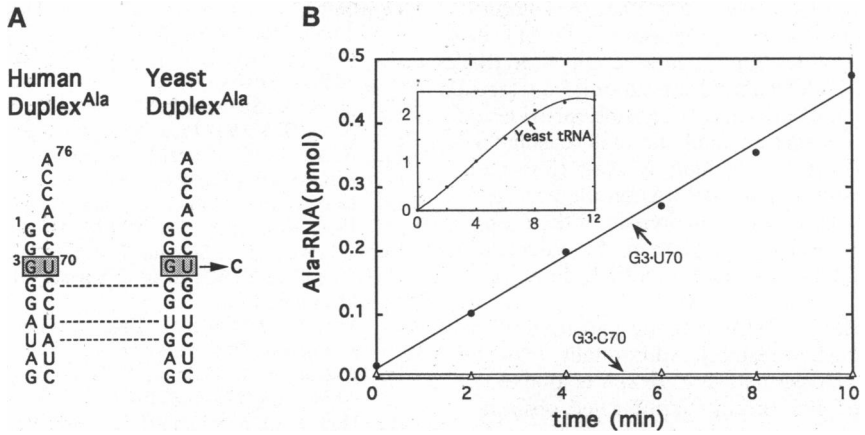

FIG. 3. (*A*) RNA duplex substrates based on the 7-bp acceptor stems of human cytoplasmic tRNA^{Ala} or *S. cerevisiae* cytoplasmic tRNA^{Ala} and the first two base pairs of the *E. coli* TΨC helix (17). The critical G3·U70 base pair is boxed and positions where substrates differ are indicated by dashed lines. (*B*) Aminoacylation of crude yeast tRNA and synthetic RNA duplexes by human alanyl-tRNA synthetase. Reactions were carried out with either crude *S. cerevisiae* tRNA (*Inset*) or the acceptor stems of *S. cerevisiae* cytoplasmic tRNA^{Ala} containing either G3·U70 or G3·C70. Enzyme concentration was 200 nM. RNA concentration was 5 μM for crude tRNA and 50 μM for the duplex substrates.

that human alanyl-tRNA synthetase can functionally replace the product of the *ALA1* gene. Colonies from the 5-fluoroorotate plate were subsequently confirmed to be Leu$^+$Ura$^-$ and to contain the *ala1* disrupted allele (data not shown).

DISCUSSION

With the exception of histidyl- and valyl-tRNA synthetases (44, 45), the cytoplasmic and mitochondrial aminoacyl-tRNA synthetases are encoded by separate nuclear genes in *S. cerevisiae.* Because the product of the *ALA1* gene is essential for mitotic growth, we postulate that it encodes cytoplasmic alanyl-tRNA synthetase. Southern blot analysis of the yeast genome has not unequivocally identified a second locus which cross-hybridizes to DNA probes representing the 5′ end of *ALA1* (unpublished results). Thus, it remains to be established whether a second locus encodes mitochondrial alanyl-tRNA synthetase.

The ability of human alanyl-tRNA synthetase to complement a disrupted allele of the yeast enzyme suggests a high degree of specificity of the human enzyme for yeast tRNA^{Ala}. It also indicates that little if any nonspecific aminoacylation by the human enzyme is occurring, as seen in other systems where heterologous aminoacylation of the cognate tRNA is accompanied by acylations of noncognate tRNAs (2). This observation is consistent with the G3·U70 base pair being unique to tRNA^{Ala} in the yeast cytoplasm (17). These incorrect aminoacylations can arise from serendipitous "identity elements" in heterologous noncognate tRNAs which are distinct from those found in the homologous cognate tRNA. An experimental demonstration of this possibility was provided by the isolation of a mutant of a normally inactive *E. coli* G3·C70 tRNA^{Ala} which had a second-site compensatory mutation that reestablished function (46).

The sequence-specific aminoacylation of RNA oligonucleotides based on tRNA acceptor stems suggests an operational RNA code for amino acids (47). Although many synthetases make contacts with the anticodon in addition to the acceptor stem, and the anticodon contacts may contribute to specificity and efficiency of aminoacylation in these instances, the alanine system is unusual in that at least the *E. coli* enzyme makes no contact with the anticodon trinucleotide of tRNA^{Ala} (48). Thus, the ability of the human enzyme to complement the yeast *ala1* disrupted allele is consistent with strong selective pressure throughout evolution to preserve the operational RNA code for alanine as the major source of specificity and efficiency of aminoacylation.

The class-defining domain and catalytic core of *E. coli* alanyl-tRNA synthetase is contained within the N-terminal 461 aa of the protein (40). It is within the analogous N-terminal regions that both yeast and human alanyl-tRNA synthetases have the highest similarity to the *E. coli* enzyme. This relatedness indicates a strong selective pressure to maintain the region of the protein implicated in recognition of the G3·U70 base pair as the RNA determinant for alanine.

In addition, strong conservation in the regions of predicted secondary structure provide support for the secondary structure model of the class-defining domain of the *E. coli* enzyme (38, 39). Complete divergence of the sequence of the yeast and *E. coli* enzymes in an internal portion of the C-terminal half that is required for tetramer formation by the *E. coli* enzyme (49) is consistent with the enzyme from *S. cerevisiae* behaving as a monomer (50).

Class II aminoacyl-tRNA synthetases are almost exclusively α_2 dimers. The dimeric quaternary structure places motif 1 of the class II proteins at the subunit interface in the crystal

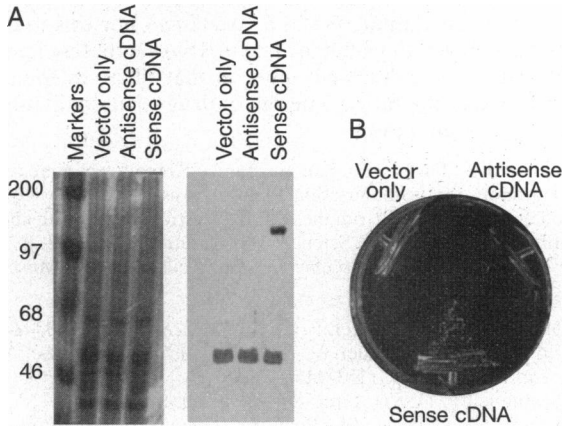

FIG. 4. Human alanyl-tRNA synthetase can functionally complement a deletion of the *ALA1* gene. (*A*) Protein extracts were prepared from strains bearing the *ala1* disrupted allele and a YCp50 plasmid with *ALA1* and *URA3*, containing either vector (pDB20L*Bgl*II), vector with human alanyl-tRNA synthetase cDNA in the antisense orientation, or vector with human alanyl-tRNA synthetase cDNA in the sense orientation. Duplicate samples were subjected to SDS/PAGE followed by either Coomassie blue staining (*Left*) or immunoblot analysis (*Right*) with antibodies (PL-12) known to recognize human alanyl-tRNA synthetase. (*B*) Strains bearing the *ala1* disrupted allele, a YCp50 plasmid with *ALA1* and *URA3*, and either vector (pDB20L*Bgl*II), vector containing human alanyl-tRNA synthetase cDNA in the antisense orientation, or vector containing human alanyl-tRNA synthetase cDNA in the sense orientation were streaked on medium containing 5-fluoroorotic acid.

structures of yeast aspartyl-tRNA synthetase and *E. coli* and *Thermus thermophilus* seryl-tRNA synthetases (7, 37, 51). In this location, motif 1 provides for an interaction with the acceptor end of bound $tRNA^{Asp}$ across the subunit interface. Thus, the structural design of the class II enzymes provides a rationale for the dimeric structure, and for that reason the monomeric structures of the *S. cerevisiae, B. mori* (36), and human (unpublished results) alanyl-tRNA synthetases are remarkable. Possibly the oligomerization domain of the *E. coli* enzyme is replaced in the eukaryotic proteins by sequences which, within a single subunit, re-create a motif 1, dimer-like "interface."

Recombinant human alanyl-tRNA synthetase purified from *E. coli* is inactive (unpublished results). Additionally, expression of human alanyl-tRNA synthetase in *E. coli* is unable to sustain growth of an *alaS* null strain (unpublished observation), making mutational analysis of the human enzyme difficult. The reason for inactivation of the human enzyme in *E. coli* is not known, but because the same enzyme is active when expressed in *P. pastoris* or *S. cerevisiae*, we imagine that a structural alteration has occurred in *E. coli*. The ability of human alanyl-tRNA synthetase to complement a disrupted allele of *ALA1* provides a system to assay variants of the human enzyme, and to test whether the results of mutagenesis, modeling, and domain structure of the *E. coli* enzyme can be used as a template for the human enzyme. Construction of C-terminal deletions in both the yeast and human alanyl-tRNA synthetases should allow us to determine whether, analogous to the fully active truncated *E. coli* enzyme (40), the N-terminal catalytic domains alone can support growth of the *ala1* disrupted strain.

Autoimmune antibodies found in patients with the degenerative muscle disorder polymyositis have been shown to crossreact with alanyl-tRNA synthetase. In this study, PL-12 antibodies, which recognize both the alanyl-tRNA synthetase and the alanyl-tRNA, were shown to specifically react with the human enzyme expressed in yeast. These same antibodies do not crossreact with endogenous yeast alanyl-tRNA synthetase (Fig. 4*A*). Thus, the structural features of human alanyl-tRNA synthetase that elicit an immune response do not interfere with its ability to function *in vivo* in *S. cerevisiae*. For this reason, yeast strains expressing human alanyl-tRNA synthetase may be useful in understanding the epitopes that illicit an immune response and in the rational design of drugs that could inhibit autoantibody generation.

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