Genetic and Biochemical Analysis of the Functional Domains of

Yeast tRNA Ligase

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ABSTRACT

Yeast tRNA ligase (Trl1) converts cleaved tRNA half-molecules into spliced tRNAs containing a 2'-PO$_4$, 3’-5’ phosphodiester at the splice junction. Trl1 performs three reactions: (i) the 2’,3’ cyclic phosphate of the proximal fragment is hydrolyzed to a 3’-OH, 2'-PO$_4$ by a cyclic phosphodiesterase (CPD); (ii) the 5’-OH of the distal fragment is phosphorylated by an NTP-dependent polynucleotide kinase; and (iii) the 3’-OH, 2'-PO$_4$ and 5’-PO$_4$ ends are sealed by an ATP-dependent RNA ligase. Trl1 consists of an N-terminal adenylyltransferase domain that resembles T4 RNA ligase 1, a central domain that resembles T4 polynucleotide kinase, and a C-terminal CPD domain that resembles the 2H phosphotransferase enzyme superfamily. Here we show that all three domains are essential in vivo, though they need not be linked in the same polypeptide. We identify five amino acids in the adenylyltransferase domain (Lys114, Glu266, Gly267, Lys284 and Lys286) that are essential for Trl1 activity and are located within motifs I (114KANG117), IV (266EGFV270), and V (282FFIK286) that comprise the active sites of DNA ligases, RNA capping enzymes and T4 RNA ligases 1 and 2. Mutations K404A and T405A in the P-loop (401GxGKT405) of the central kinase-like domain had no effect on Trl1 function in vivo. The K404A and T405A mutations eliminated ATP-dependent kinase activity, but preserved GTP-dependent kinase activity. A double-alanine mutant in the P-loop was lethal in vivo and abolished GTP-dependent kinase activity. These results suggest that GTP is the physiological substrate and that the Trl1 kinase has a single NTP binding site of which the P-loop is a component. Two other mutations in the central domain were lethal in vivo and either abolished (D425A) or severely reduced (R511A) GTP-dependent RNA kinase activity in vitro. Mutations of the signature histidines of the CPD domain were either lethal (H777A) or conferred a ts growth phenotype (H673A).
INTRODUCTION

Intron-containing tRNAs are widespread in the archaeal and eukaryal domains of the universal phylogenetic tree (1). The intron is usually located in the anticodon loop of the pre-tRNA and must be removed precisely for the tRNA to function in protein synthesis. tRNA splicing occurs in two stages: (i) intron excision and (ii) joining of the broken tRNA halves (2,3) (Fig. 1). Unlike pre-mRNA splicing in eukarya, which relies on ribonucleoprotein catalysts, all the reactions of the tRNA splicing pathway are performed by protein enzymes. The intron removal phase of tRNA splicing requires two incisions of the pre-tRNA at the exon-intron borders. The chemistry of the reaction entails breakage of the phosphodiester backbone by transesterification to yield 2’,3’ cyclic phosphate and 5’-OH termini at both incision sites (2). The breakage reactions are catalyzed by a tRNA splicing endonuclease that specifically recognizes the fold of the pre-tRNA (4-10). The specificity and fidelity of tRNA splicing are largely governed by the endonuclease component, which is conserved in structure and mechanism among archaea and lower and higher eukaryal species (4-10).

The joining phase of the tRNA splicing pathway has been studied most extensively in yeast, where a single multifunctional tRNA ligase enzyme (Trl1) catalyzes a series of chemical transformations at the ends of the broken tRNA half-molecules that eventuates in the formation of a ligated tRNA molecule containing a 2’-PO₄, 3’-5’ phosphodiester structure at the junction (3,11). Trl1 performs three reactions: (i) the 2’,3’ cyclic phosphate terminus is hydrolyzed to a 3’-OH, 2’-PO₄ terminus by a 2’,3’ cyclic phosphodiesterase (CPD) activity; (ii) the 5’-OH terminus is phosphorylated by an NTP-dependent polynucleotide kinase activity; and (iii) the resulting 3’-OH, 2’-PO₄ and 5’-PO₄ ends are sealed by an ATP-dependent RNA ligase activity (11-16). The mechanism of the ligase component of yeast tRNA ligase resembles that of bacteriophage T4 RNA ligase, whereby RNA joining entails three nucleotidyl transfer steps: (i) ligase reacts with ATP to form a covalent ligase-(lysyl-N)–AMP intermediate plus pyrophosphate; (ii) AMP is transferred from ligase-adenylate to the 5’-PO₄ RNA end to form an RNA-adenylate intermediate...
(AppRNA); and (iii) ligase catalyzes attack by an RNA 3'-OH on the RNA-adenylate to seal the two ends via a phosphodiester bond and release AMP (11,12,17-20). Trl1 is capable of modifying and/or ligating the ends of artificial RNA substrates such as oligo(A₁₆), oligo(U,G), 5'-(AUCUCG)₆AUCUCG, and 5'-GGGCGAAUU (11,14,15), which implies that Trl1 activity is not obligately linked to the processing of tRNAs. The final step in yeast tRNA splicing is the removal of the 2'-PO₄ at the splice junction by the essential 2'-phosphotransferase Tpt1, which catalyzes the transfer of the tRNA 2'-PO₄ to NAD⁺ to form ADP-ribose 1''-2'' cyclic phosphate (21-23) (Fig. 1).

We are interested in the end-remodeling and strand joining steps of tRNA splicing, which exemplify a general RNA repair pathway entailing the healing and sealing of broken RNA ends. Another well-characterized example of RNA repair is the tRNA restriction/repair phenomenon elicited by infection of E. coli prr strains with bacteriophage T4 (24,25). T4 infection activates a latent anticodon nuclease (PrrC) encoded by the host bacterium. PrrC specifically incises tRNA⁰⁰ at a single site in the anticodon loop. Depletion of tRNA⁰⁰ blocks phage protein synthesis and arrests the infection before it can spread. However, the T4 enzymes polynucleotide kinase and RNA ligase 1 repair the broken tRNAs and thereby thwart the host defense mechanism. The enzymatic steps in tRNA splicing and tRNA restriction/repair are generally similar. The incision steps in both cases result in the formation of 2',3' cyclic phosphate and 5'-OH termini (25). tRNA splicing requires two breaks in the backbone of the pre-tRNA to excise the intron, whereas tRNA restriction involves a single break in the mature tRNA. The healing and sealing steps of the phage-encoded tRNA repair pathway are performed by two separate enzymes (Pnk and Rnl1), whereas a single enzyme Trl1 performs these functions in yeast tRNA splicing. The structural basis for the T4 RNA repair reactions has been illuminated recently by extensive site-directed mutagenesis and X-ray crystallography (26-30). In contrast, the yeast Trl1 has received little attention during the past decade.
Renewed interest in yeast tRNA ligase is warranted for two reasons. First, the discovery that yeast tRNA ligase is responsible for non-spliceosomal splicing of mRNA in the unfolded protein response pathway \((31,32)\) extends the RNA repair paradigm to mRNA metabolism and holds out the prospect that there are other instances (yet to be discovered) in which RNA primary structure is altered by breakage and repair. Second, the phylogenetic distribution of Trl1-like proteins is surprisingly narrow, given the wide occurrence of tRNA introns. Trl1 homologs are found in several genera of fungi, including *Saccharomyces*, *Candida*, *Schizosaccharomyces*, and *Aspergillus* \((32)\) (Fig. 2). Trl1-like proteins are absent from the proteomes of archea and non-fungal eukarya. This may be because archaea and nonfungal eukarya use a different end-joining mechanism for tRNA splicing than do fungi \((34-36)\). Indeed, there is evidence that mammals have two different pathways of tRNA splicing, a yeast-like mechanism yielding a 2'-phosphate at the splice junction and a second pathway in which the 2',3' cyclic phosphate of the incised pre-tRNA is retained as the 3'-5' phosphodiester of the spliced tRNA product \((37)\). The latter pathway does not require phosphorylation of the 5'-OH of the cleaved tRNA \((34-36)\). Nonetheless, mammalian cells possess a nuclear 5'-OH polynucleotide kinase activity that could participate in the yeast-like pathway \((38)\). None of the specific proteins responsible for tRNA ligation in archaea or higher eukarya have been identified nor have their genes been cloned. A multifunctional Trl1-like enzyme has been purified to near-homogeneity from wheat germ \((39-43)\), but the gene encoding wheat germ RNA ligase has not been identified and there is no obvious Trl1 homolog in the proteome of the plant *Arabidopsis thaliana*. This scenario, in which the Trl1-like pathway in metazoa and plants is either redundant, or performed by enzymes without recognizable structural similarity to fungal tRNA ligases, recommends Trl1 as an excellent target for antifungal drug discovery.

As a trifunctional enzyme with an essential role \textit{in vivo}, Trl1 is ripe for structure-function analysis. Soon after the Trl1 gene was cloned, the Greer and Abelson labs demonstrated an autonomous CPD domain within the C-terminal portion of Trl1 and an autonomous
adenylyltransferase domain within the N-terminal portion of Trl1 (13,14). The site of covalent AMP attachment was mapped to Lys114 (13), which is located within a conserved sequence motif (Kx[D/N]G; motif I) that defines a superfamily of covalent nucleotidyl transferases that includes T4 RNA ligases 1 and 2, DNA ligases, and mRNA capping enzymes (44-47). Although the kinase function of Trl1 has not been assigned to an autonomous segment, the middle third of the Trl1 polypeptide bears some resemblance to the kinase domain of T4 Pnk (14), especially with respect to the presence of the Walker A-box motif GxGKT (also known as the “P-loop”) that comprises the NTP binding site of T4 Pnk and numerous other NTP-dependent phosphotransferases (26-29) (Fig. 2). The CPD domain of Trl1 resembles the so-called “2H” superfamily of phosphoesterases, which is defined by two copies of a histidine-containing motif HφTφ (where φ is a hydrophobic residue) (Fig. 2).

Here we address the following outstanding questions: (i) Does Trl1 activity in vivo depend on all three putative domains? (ii) Is the physical linkage of the domains within a single polypeptide essential for Trl1 function in vivo? (ii) Where is the boundary of the adenylyltransferase domain? (iii) Is the limited primary structure similarity between Trl1 and other covalent nucleotidyl transferases functionally important? (iv) Is the limited similarity between Trl1 and T4 Pnk functionally relevant? (v) Are the histidines of the 2H motifs relevant to Trl1 function in vivo? To answer these questions, we established a plasmid shuffle assay to gauge mutational effects on Trl1 function in vivo. Our studies show that all three domains are essential, though they need not be linked in the same polypeptide. We identify five individual residues in the adenylyltransferase domain that are essential for Trl1 activity and conserved in all members of the covalent nucleotidyl transferase family. In contrast, single mutations in the putative NTP binding P-loop of the kinase domain had no effect on Trl1 function in vivo. Our finding that single P-loop mutations severely decreased or eliminated ATP-dependent kinase activity, but preserved GTP-dependent kinase activity, supports the prior suggestion that GTP is the real substrate for the Trl1 kinase (15,16). However, a double-alanine mutant in the P-loop
was lethal \emph{in vivo} and abolished GTP-dependent kinase activity, suggesting that the Trl1 kinase
domain has a single NTP binding site of which the P-loop is a component. We report that
alanine mutations of the signature histidines of the CPD domain were either lethal (at His777) or
conferring a temperature-sensitive growth phenotype (at His673).
EXPERIMENTAL PROCEDURES

TRL1 Genomic Clone. A DNA fragment containing the TRL1 gene plus 550-bp of 5’ flanking DNA and 220-bp of 3’ flanking genomic DNA was amplified by PCR using total yeast genomic DNA as template and primers that introduced a SphI site at the 5’ end of the amplified fragment and a SacI site at the 3’ end. The amplified gene was cloned into pUC18 to generate pUC-TRL1. A BamHI site was introduced immediately 5’ of the start codon of the TRL1 open reading frame (ORF), which, together with an endogenous BglII site overlying codon 338 and an endogenous SacII site immediately 3’ of the TRL1 stop codon (TAGACCGCGG), facilitated the cloning of truncated and mutated versions of TRL1 under the control of the natural TRL1 promoter and flanked by the natural TRL1 3’ UTR.

Deletions and Alanine Mutations of TRL1. Deleted versions of TRL1 were constructed by PCR amplification using oligonucleotide primers that introduced either a start codon and a BamHI site or a stop codon and a SacI site at the desired positions in the TRL1 ORF. The PCR products were digested with BamHI and SacI and inserted into BamHI/SacI-cut pUC-TRL1. Alanine mutations and overlapping diagnostic restriction sites were introduced via the two-stage PCR overlap extension method (65). The mutated PCR products were digested with either BamHI and BglII or BglII and SacI (depending on the location of the mutation) and inserted into BamHI/BglII-cut or BglII/SacI-cut pUC-TRL1 in lieu of the wild-type gene fragment. The inserts were sequenced completely to confirm the presence of the desired mutations and to exclude the acquisition of unwanted coding changes during PCR amplification and cloning.

Plasmids for Expression of TRL1 in Yeast. Wild-type and mutated versions of the TRL1 gene plus 5’ and 3’ flanking genomic DNA were excised from the respective pUC-TRL1 plasmids with SphI and SacI and inserted into yeast shuttle vectors pSE360 (CEN URA3), pSE358 (CEN TRP1), or pSA360 (CEN ADE2). Selected TRL1 alleles were cloned into the PstI site of pRS413 (CEN HIS3).
Deletion of TRL1 in S. cerevisiae. TRL1 was disrupted using a kanMX cassette (61) flanked by 1-kbp tracts of yeast genomic DNA corresponding to the 5' and 3' sequences flanking the TRL1 ORF. The disruption was performed in the diploid strain W303 (MATa/MATα, ura3-1/ura3-1 ade2-1/ade2-1 trp1-1/trp1-1 his3-11,15/his3-11,15 leu2-3,11-2/leu2-3,11-2 can1-100/can1-100). Integrative recombination at one chromosomal locus deleted the entire TRL1 ORF. Correct insertion of the kanMX cassette into the TRL1 locus was confirmed by PCR analysis of G418-resistant transformants. A TRL1 trl1::kanMX diploid was sporulated. Tetrad analysis revealed a 2:0 segregation pattern for G418 sensitivity/resistance, consistent with the essentiality of TRL1 reported previously (62). The TRL1 trl1::kanMX diploid was transformed with pSE360-TRL1 (CEN URA3) and sporulated again. The resulting G418R/Ura+ haploid strain YRS1 (MATα ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,11 can1-100 trl1::kanMX p360-TRL1) was unable to grow on medium containing 5-fluoroorotic acid (5-FOA), a drug that selects against the URA3 gene on the pSE360-TRL1 plasmid. YRS1 was used to test the in vivo function of various mutant TRL1 alleles via the plasmid shuffle procedure.

Test of Trl1 Function by Plasmid Shuffle. YRS1 was transformed with CEN plasmids bearing wild-type or mutant alleles of TRL1. Transformants were selected on appropriate drop-out media. Two individual colonies were transferred to drop-out agar medium and cells from each isolate were then streaked on agar containing 0.75 mg/ml 5-FOA. The plates were incubated at 18°C, 25°C, 30°C and 37°C. Lethal mutations were those that did not allow formation of FOA-resistant colonies after 7 days at any of the temperatures tested. Other mutated alleles supported FOA-resistant colony formation within 4 days at one or more of the growth temperatures. Two individual colonies from each streak were picked from the FOA plate, transferred to yeast extract/peptone/dextrose (YPD) medium and then tested for growth on YPD agar at 18°C, 25°C, 30°C and 37°C.
Plasmids for Expression of Trl1 in Bacteria. T7-based expression vector pET28a-His<sub>10</sub> was constructed by replacing the MluI-NdeI fragment of pET28a (encoding a His<sub>6</sub> tag) with the MluI-NdeI fragment of pET16B (encoding a His<sub>10</sub> tag). The full-length TRL1 ORF was excised from pUC-TRL1 with BamHI and SacI and inserted into pET28a-His<sub>10</sub> to yield pET28a-His<sub>10</sub>Trl1. Full-length mutated genes <i>K404A</i>, <i>T405A</i>, <i>D425A</i> and <i>R511A</i> were similarly transferred from the respective pUC-TRL1-Ala plasmids into pET28a-His<sub>10</sub> to yield pET28a-His<sub>10</sub>Trl1-Ala plasmids. The ORF encoding the adenylyltransferase domain Trl1(1-388) was excised from pUC-Trl1(1-388) with BamHI and SacI and inserted into pET28a-His<sub>10</sub> to yield pET28a-His<sub>10</sub>Trl1(1-388). DNA fragments containing adenylyltransferase domain mutants <i>K114A</i>, <i>N116A</i>, <i>G152A</i>, <i>E153A</i>, <i>E266A</i>, <i>G267A</i>, <i>K284A</i> and <i>K286A</i> were amplified by PCR from the respective pUC-Trl1-Ala plasmids. The PCR products were digested with BamHI and SacI and inserted into pET28a-His<sub>10</sub> to yield pET28a-His<sub>10</sub>Trl1(1-388)-Ala derivatives.

Recombinant Trl1 Proteins. Cultures (600 ml) of <i>E. coli</i> BL21(DE3)/pET28-His<sub>10</sub>TRL1(wild-type or mutant) were grown at 37°C in Luria-Bertani medium containing 0.1 mg/ml ampicillin until the A<sub>600</sub> reached 0.5. The cultures were adjusted to 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation was continued at 17°C overnight. Cells were harvested by centrifugation and the pellet was stored at -80°C. All subsequent procedures were performed at 4°C. Thawed bacterial pellets were resuspended in 10 ml of buffer A (50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 10% sucrose) and left on ice for 10 min. The suspensions were then sonicated for 30 s. Lysozyme was added to a final concentration of 50 µg/ml and the suspensions were further incubated on ice for 30 min. Triton X-100 was added to a final concentration of 0.1% and sonication was repeated for one minute to reduce viscosity. Insoluble material was removed by centrifugation in a Sorvall SS34 rotor at 18,000 rpm for 45 min. The soluble extracts were applied to 1.5-ml columns of Ni-NTA agarose (Qiagen) equilibrated with buffer A containing 0.1% Triton X-100. The column was washed with the same buffer and then
eluted step-wise with buffer B (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 10% glycerol) containing 50, 100, 200, 500 and 1000 mM imidazole. The polypeptide compositions of the column fractions were monitored by SDS-polyacrylamide gel electrophoresis (PAGE). The recombinant Trl1 proteins were retained on the column and recovered in the 200 mM imidazole eluates. The 200 mM imidazole fraction was dialyzed against buffer C (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM DTT, 10% glycerol, 0.05% Triton X-100). The Trl1 preparations were stored at -80°C. Protein concentrations were determined using the Bio-Rad dye binding assay with bovine serum albumin as a standard.

**Adenylyltransferase Assay.** Reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 10 mM MgCl₂, 50 µM [α-³²P]ATP (3–5 Ci/mmol), and Trl1 as specified were incubated for 10 min at 30°C. The reactions were quenched with SDS and the products were analyzed by SDS-PAGE. The ligase-[³²P]AMP adduct was visualized by autoradiography of the dried gel and quantitated by scanning the gel with a Fujix BAS-2500 phosphorimager.

**Polynucleotide kinase assay.** Reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, [γ-³²P]ATP or [γ-³²P]GTP as specified, 80 pmol of a synthetic 5'-OH RNA oligonucleotide (5'-AUUCCGAUAGUGACUACA) and Trl1 as specified were incubated for 30 min at 30°C. The reactions were quenched by adding 6 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The products were analyzed by electrophoresis through a 15-cm 20% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris borate, 2.5 mM EDTA). The radiolabeled products were visualized by autoradiography of the gel and quantitated with a phosphorimager.
RESULTS

All three domains of Trl1 are required for cell viability, but they need not be linked in the same polypeptide

Trl1 consists of three modules: an N-terminal adenylyltransferase domain, a central kinase-like domain, and a C-terminal CPD domain (Fig. 3). Although the isolated domains have been characterized in vitro as DHFR fusion proteins (14), their individual functions in vivo have not been formally tested by site-directed mutagenesis, even though it is well established that TRL1 is an essential gene. Here we used the plasmid shuffle method to gauge the effects of structural alterations on Trl1 function in vivo. To establish the assay, TRL1 was disrupted in a diploid strain by replacement of the entire coding sequence with a kanMX cassette. The TRL1::kanMX diploid was transformed with a CEN URA3 plasmid containing TRL1 under the control of its own promoter. The diploid was sporulated and tetrads dissected to obtain G418R trl1 haploids. The trl1 strain was unable to grow on medium containing 5-FOA (a drug that selects against the URA3 TRL1 plasmid), but was able to grow on 5-FOA after being transformed with a CEN TRP1 TRL1 plasmid. FOA-resistant trl1 isolates containing the plasmid-borne TRL1 gene grew as well as the parental wild-type yeast strain on rich medium (YPD agar) at all temperatures tested (25, 30, and 37˚C; scored as +++ growth in Fig. 3).

The first question we asked was whether all three modules of Trl1 are required for yeast growth. Therefore, cDNAs encoding the adenylyltransferase domain (aa 1-388), the kinase-like domain (aa 389-561) and the CPD domain (aa 562-827) were cloned into CEN TRP1, CEN ADE2 and CEN HIS3 plasmids, respectively, under the control of the natural TRL1 promoter and then tested by plasmid shuffle for trl1 Δ complementation. None of the domains per se was able to support cell growth, i.e., no FOA-resistant transformants were recovered during selection at either 25 or 30˚C (scored as — in Fig. 3). To test if any two modules together sufficed for viability, we constructed a CEN TRP1 plasmid expressing the N-terminal adenylyltransferase-
kinase domain (aa 1-561) and a *CEN ADE2* plasmid expressing the C-terminal kinase-CPD domain (389-827). Neither construct was able to support growth on 5-FOA (Fig. 3). Cotransformation with plasmids expressing the adenylyltransferase domain on a *CEN TRP1* plasmid and the CPD domain on a *CEN HIS3* plasmid also failed to complement *trl1Δ* (Fig. 3).

The instructive finding was that complementation could be achieved by expressing the adenylyltransferase domain and the kinase-CPD domain as unlinked polypeptides by cotransformation with *CEN TRP1 TRL1(1-388)* and *CEN ADE2 TRL1(389-827)* plasmids (Fig. 4). The *TRL1(1-388) TRL1(389-827)* strain grew as well as wild-type *TRL1* on YPD agar at 25, 30, and 37˚C (scored as +++ growth in Fig. 4).

**Delineating the margins of the adenylyltransferase domain**

A series of truncated versions of the adenylyltransferase module were tested for their function *in vivo* when coexpressed with the separate kinase-CPD domain (Fig. 4). Carboxyl truncation mutants *TRL1(1-376)* and *TRL1(1-362)* complemented growth of the *trl1Δ TRL1(389-827)* strain on 5-FOA. However, the more extensive deletions *TRL1(1-320)* and *TRL1(1-312)* were lethal. Whereas cells expressing the Trl1(1-376) protein grew at 25, 30, and 37˚C, the strain expressing Trl1(1-362) grew at 25 and 30˚C, but not at 37˚C. Thus, the loss of the peptide segment from aa 363-376 resulted in a temperature-sensitive (ts) growth defect.

Amino terminal deletions *TRL1(21-388)*, *TRL1(41-388)* and *TRL1(59-388)* were unable to support growth of the *trl1Δ TRL1(389-827)* strain on 5-FOA. We conclude that the N-terminus of Trl1 is essential for activity *in vivo* and that the Trl1 segment from aa 1 to 362 comprises a minimal functional adenylyltransferase domain *in vivo*.

The biologically active adenylyltransferase domains Trl1(1-388) and Trl1(1-376) and the non-complementing N-terminal truncations Trl1(21-388), Trl1(41-388) and Trl1(59-388) were produced in bacteria as His<sub>10</sub>-tagged fusion proteins and then purified from soluble bacterial lysates by Ni-agarose chromatography. SDS-PAGE analysis showed that the preparations were
highly enriched with respect to the adenylyltransferase polypeptides and that the extents of purification were similar in each case (Fig. 5A). The sizes of the recombinant proteins and the electrophoretic mobility differences between the truncated versions were consistent with the values calculated by translation of the open reading frames. (His$_{10}$-tagged proteolytic fragments of the adenylyltransferase domain were also detected in each of the affinity-purified protein preparations.) The C-terminal truncation mutants Trl1(1-362) and Trl1(1-320) were extensively proteolyzed when expressed in *E. coli* and we were unable we to purify the intact His-tagged recombinant polypeptides (not shown). Thus, these two deletion mutants were not available for biochemical characterization.

The adenylyltransferase activity of recombinant Trl1(1-388) was evinced by label transfer from 50 µM [$\alpha$-$^{32}$P]ATP to the respective polypeptides to form a covalent enzyme-adenylate adduct (Fig. 5B). The reaction was divalent cation-dependent and optimal at 5 to 20 mM MgCl$_2$ (not shown). The divalent cation requirement was also satisfied by either 10 mM manganese or calcium; we detected no activity with 10 mM copper or zinc (not shown). The yield of Trl1(1-388)-[${}^{32}$P]AMP was proportional to input enzyme and optimal at pH 8.0 in 50 mM Tris-HCl buffer (not shown). The C-terminal truncated derivative Trl1(1-376) was active in autoadenylylation, but the N-terminal truncations Trl1(21-388), Trl1(41-388) and Trl1(59-388) were apparently inert (Fig. 5B). We conclude that the N-terminal peptide segment of Trl1 is essential for both *in vivo* function and adenylyltransferase catalytic activity.

**Identification of individual essential amino acids in the adenylyltransferase domain**

Xu *et al.* (13) mapped the AMP attachment site of Trl1 to Lys114, but there has been no report of the effect of mutating this residue on Trl1 function. The lysine nucleophile of Trl1 is located within a conserved sequence element referred to as motif I ($^{114}$Kx[D/N]G$^{117}$ in Fig. 2) that defines a superfamily of covalent nucleotidyl transferases, which includes DNA ligases, RNA ligases and mRNA capping enzymes (44-49). DNA ligases and capping enzymes have a
common tertiary structure composed of five peptide motifs (I, III, IIIa, IV, and V) that contain essential amino acids responsible for nucleotide binding and catalysis (46-54). It has been suggested that DNA ligases and capping enzymes evolved from a common ancestral nucleotidyl transferase (55), possibly from an ancient RNA strand-joining enzyme. This model remains speculative because there is no atomic structure available for a nucleotide-dependent RNA ligase. However, a recent mutational analysis of T4 RNA ligase 2 underscored the conservation of motifs I, III, IIIa, IV and V and the essentiality of the conserved side chains within these motifs for RNA ligation \textit{in vitro} (56,57). The adenylyltransferase domain of fungal tRNA ligase has scant similarity to T4 Rnl2, DNA ligases, or mRNA capping enzymes outside of motif I. However, perusal of the aligned primary structures of tRNA ligases from multiple fungal species highlights potential counterparts in Trl1 of nucleotidyl transferase motifs IV (266EG270) and V (282φKφK286, where φ is a hydrophobic side chain). In order to delineate if any of the conserved amino acids are functionally relevant, we performed an alanine scan of the 8 positions of the Trl1 adenylyltransferase domain indicated by \textverticalbar\ in Fig. 2. The targeted residues included: Lys114 and Asn116 in motif I; Glu266 and Gly267 in motif IV; and Lys284 and Lys284 in motif V. We also mutated Gly152 and Glu153, which are located between motifs I and IV and are conserved among fungal tRNA ligases (Fig. 2).

\textit{TRL1-Ala} alleles were cloned into a \textit{CEN TRP1} plasmid under the control of the native \textit{TRL1} promoter and transformed into the \textit{trl1A} plasmid shuffle strain. The \textit{K114A}, \textit{E266A}, \textit{G267A}, \textit{K284A} and \textit{K286A} transformants failed to give rise to FOA-resistant colonies at 18, 25, 30, or 37°C; thus these five alanine mutations were lethal \textit{in vivo}. The \textit{N116A}, \textit{G152A} and \textit{E153A} alleles supported growth on 5-FOA and the resulting \textit{TRL1-Ala} strains grew \textit{TRL1} cells on YPD agar at all temperatures (Table 1). These results show that that amino acids within putative equivalents of nucleotidyl transferase motifs I, IV and V are essential for tRNA ligase function \textit{in vivo}. 
The wild-type adenylyltransferase domain Trl1(1-388), and the K114A, N116A, G152A, E153A, E266A, G267A, K284A and K286A mutants thereof, were expressed in bacteria as His$_{10}$-tagged fusion proteins and then purified from soluble bacterial lysates by Ni-agarose chromatography. SDS-PAGE analysis showed that the preparations were highly enriched with respect to the ~48 kDa His-Trl1(1-388) polypeptide and the extents of purification were similar in each case (Fig. 6A). Whereas recombinant wild-type Trl1(1-388) reacted with [$\alpha$-$^{32}$P]ATP to form a covalent enzyme-adenylate adduct, the K114A mutant was inert in ligase adenylation (Fig. 6B), consistent with the assignment of this lysine as the active site nucleophile for AMP transfer (13). The concordant loss of adenylyltransferase activity in vitro and Trl1 function in vivo provides evidence that catalysis of the adenylyltransferase reaction is an essential part of Trl1’s repertoire.

Mutants E266A and K284A were severely defective in ligase adenylation (4% and 7% of wild-type activity, respectively), thereby explaining their inability to sustain yeast growth. In contrast, the N116A, G152A, and E153A mutants retained substantial adenylyltransferase activity in vitro (78%, 130%, and 33% of wild-type activity, respectively), consistent with their apparently normal growth phenotypes in vivo. It was noteworthy that the G267A and K286A mutations had little effect on ligase adenylation (Fig. 6B), despite being lethal in vivo. These results hint that the G267A and K286A changes may affect a downstream component of the three-step RNA ligase pathway.

Effects of mutations in the putative kinase domain

The central domain of the Trl1 polypeptide resembles the kinase domain of T4 Pnk with respect to the presence of a Walker A-box motif $^{401}$GCGKT$^{405}$ (the P-loop) (Fig. 2). The A-box motif of T4 Pnk ($^{12}$GSGKS$^{16}$) comprises part of the NTP-binding site, together with a second peptide motif $^{122}$RNSKR$^{126}$ located 106-aa downstream of the A-box (28,29). Yeast Trl1 has a similar element $^{507}$RVIKR$^{511}$ placed 102-aa downstream of its A-box. The lysine and serine of
the A-box and the second arginine of the RxxxR motif are each essential for the T4 polynucleotide kinase reaction (26,27). Here we queried by alanine-scanning whether the corresponding side chains in the central kinase domain of Trl1 (Lys404, Thr405 and Arg511) are relevant to Trl1 function in vivo or kinase activity in vitro. We also mutated Asp425 and Asp454, which are located between the A-box and the RxxxR motif and are conserved among fungal tRNA ligases (Fig. 2).

The five TRL1-Ala alleles were cloned into a CEN TRP1 plasmid under the control of the native TRL1 promoter and tested for trl1Δ complementation. The D425A and R511A transformants failed to give rise to FOA-resistant colonies at 18, 25, 30, or 37˚C; thus these two alanine mutations were lethal in vivo (Table I). The K404A, T405A and D454A alleles supported growth on 5-FOA and the resulting TRL1-Ala strains grew as well as TRL1 cells on YPD agar at all temperatures (+++ growth in Table 1). These results show that the defining Lys and Thr residues of the A-box motif are dispensable for tRNA ligase function in vivo. This initially surprising result was verified by recovering the TRP1 plasmid from FOA-selected K404A and T405A yeast strains, amplifying them in vivo by transformation in E. coli, and then sequencing the TRL1 genes of the clonal isolates, which revealed that the plasmid alleles retained the K404A and T405A mutations.

The wild-type Trl1 protein and the K404A, T405A, D425A, and R511A mutants were expressed in bacteria as His10-tagged fusion proteins and then isolated from soluble bacterial lysates by Ni-agarose chromatography. SDS-PAGE analysis showed that the material eluted from Ni-agarose with imidazole consisted of a mixture of full-length 90 kDa His-Trl1 (indicated by the arrow in Fig. 7A) plus a collection of smaller His-tagged N-terminal fragments of Trl1, presumably arising via proteolysis during recombinant protein production in E. coli, as described previously by Abelson and colleagues (11,13). The polypeptide compositions of the affinity-purified His-Trl1-Ala mutants were essentially identical to that of wild-type Trl1 (Fig. 7A). The adenylyltransferase activity of the full-length Trl1 polypeptide was evinced by label transfer from
[α-32P]ATP to form a ~90 kDa covalent Trl1-adenylate adduct (Fig. 7B). A ~65 kDa N-terminal fragment of Trl1 seen in the Coomassie-blue stained gel (Fig. 7A) was also labeled with [32P]AMP (Fig. 7B). The full-length mutant enzymes K404A, T405A, D425A, and R511A retained adenylyltransferase activity (Fig. 7B), suggesting that these mutations in the central kinase domain did not result in global unfolding of Trl1.

The 5’ polynucleotide kinase activity of wild-type Trl1 was assayed by the transfer of 32P_i from 100 µM [γ-32P]ATP to the 5’-OH terminus of an 18-mer RNA oligonucleotide to form a 5’ 32P-labeled RNA product that was resolved from free ATP by polyacrylamide gel electrophoresis (Fig. 7C). The extent of label transfer from ATP to the 5’-OH RNA acceptor was reduced to <10% of the wild-type value by the K404A mutation in the Walker A-box. Kinase activity was abolished by the T405A mutation in the A-box and by the D425A and R511A changes (Fig. 7C). The loss of in vitro kinase activity elicited by the single mutations in the NTP-binding P-loop motif was paradoxical in light of the finding that the K404A and T405A mutations had no apparent effects on yeast cell growth (Table I). Prior studies by the Greer and Abelson labs (15,16) had shown that Trl1 is capable of using GTP as the phosphate donor in the polynucleotide kinase reaction; indeed their work suggested that GTP is the preferred substrate for the Trl1-associated kinase activity in vitro. We assayed the ability of wild-type and mutated versions of Trl1 to transfer 32P_i from 100 µM [γ-32P]GTP to the 5’-OH terminus of an 18-mer RNA oligonucleotide. The instructive findings were that the K404A and T405A mutants, which were active in vivo, retained substantial GTP-dependent kinase activity in vitro (35% and 72% of wild-type Trl1, respectively). Thus the lysine and threonine side chains of the P-loop are critical for ATP-dependent kinase activity, but not for the GTP-dependent reaction. The R511A mutant, which was lethal in vivo and inactive as an ATP-dependent kinase, was ~14% as active as wild-type Trl1 in GTP-dependent RNA phosphorylation (Fig. 7C). Conceivably, the low residual GTP-dependent activity of R511A did not suffice for Trl1 function in vivo. The lethal D425A mutation abolished GTP-dependent RNA kinase activity, just as it abolished the ATP-dependent kinase.
These results suggest that GTP rather than ATP is the physiological substrate for the Trl1 kinase activity in vivo and that the central domain of Trl1 contributes to the kinase active site.

The role of the central region of Trl1 in polynucleotide kinase function was investigated further by purifying the Trl1 domains and testing them for GTP-dependent RNA kinase activity in vitro. The polypeptide compositions of the full-length Trl1 (aa 1-827), the adenylyltransferase domain (aa 1-388), the kinase-CPD domain (aa 389-827) and the CPD domain (562-827) are shown in Fig. 8A. Whereas the full-length Trl1 protein and the kinase-CPD domain readily catalyzed phosphoryl transfer from GTP to the 18-mer 5'-OH RNA oligonucleotide, the isolated adenylyltransferase and CPD domains were inert with respect to kinase activity (Fig. 8B). We conclude from the deletion analysis that the adenylyltransferase domain plays no significant role in the kinase function of Trl1 and that the central domain is essential for kinase function. The latter inference is consistent with the observed inactivation of the kinase by the D425A mutation in the central domain. We were unable to produce the isolated central domain (aa 389-561) as an intact soluble protein in bacteria; thus, its biochemical properties could not be evaluated.

The findings that single mutations K404A and T405A in the P-loop signature GxGKT did not eliminate Trl1 function in vivo or GTP-dependent kinase activity in vitro raised the prospect that ATP and GTP might bind to separate sites on the enzyme, with ATP utilizing the P-loop and GTP binding elsewhere. Alternatively, the Trl1 kinase might have a single NTP site that includes the P-loop, at which ATP binding is more acutely dependent on contacts with the lysine and threonine side chains of the P-loop than is GTP binding. To explore the latter scenario, we constructed a double-mutant of the P-loop, K404A-T405A, in which both side chains were replaced by alanine. We found that the K404A-T405A mutation was lethal in vivo (Table I). The purified recombinant His-Trl1-(K404A-T405A) protein was inert in catalysis of phosphoryl transfer from [γ-32P]GTP to the 5'-OH terminus of the 18-mer RNA oligonucleotide acceptor (not shown). We infer from these results that utilization of GTP as the physiological substrate for the
Trl1 kinase depends on the P-loop element within the central kinase-like domain and that the lysine and threonine mutations synergize to inactivate the GTP-dependent Trl1 kinase.

**Delineating the margins of the CPD domain and essential amino acids therein**

Complementation of trl1Δ could also be achieved by coexpressing the adenylyltransferase-kinase domain Trl1(1-561) and the CPD domain Trl1(562-827) as unlinked polypeptides (Fig. 9). The \textit{TRL1(1-561) TRL1(562-827)} strain grew as well as wild-type \textit{TRL1} on YPD agar at 25 and 30°C (+++ growth at these temperatures), but formed only pinpoint-sized colonies at 37°C (scored as + growth). Thus, the kinase module can function \textit{in vivo} when linked either to the adenylyltransferase domain or the CPD domain.

Truncated versions of the CPD module were tested for their activity \textit{in vivo} when coexpressed with the separate adenylyltransferase-kinase domain (Fig. 9). Amino terminal deletions \textit{TRL1(597-827), TRL1(622-827) and TRL1(668-827)} and carboxyl terminal deletion \textit{TRL1(562-800)} were unable to support growth of the \textit{trl1} TRL1(1-561) strain on 5-FOA. We conclude that the polypeptide segments from 562 to 595 and 801-827 are essential for CPD activity \textit{in vivo}.

The CPD domain of yeast tRNA ligase belongs to the so-called “2H” superfamily of phosphoesterases, which is defined by the presence of two copies of the histidine-containing motif, HφTφ, spaced 70 to 110-aa apart in the primary structure (58,59). The crystal structure of a plant CPD enzyme, which hydrolyzes both nucleoside 2',3' cyclic phosphates and ADP-ribose 1',2'-cyclic phosphate, revealed that the defining histidines are located close together in the tertiary structure and together comprise the active site (60). Here we replaced the individual histidines of the 673HITL and 777HITL motifs of Trl1 with alanine. The H673A and H777A changes were made in the context of the full-length Trl1 protein and the mutant alleles were tested by plasmid shuffle for complementation of \textit{trl1Δ}. We found that the H777A mutation was lethal (Table I). The H673A allele supported growth of \textit{trl1Δ} on 5-FOA; the resulting FOA-resistant
TRL1-H673A strain grew as well as wild-type yeast on YPD agar at 25 and 30°C, but was unable to form any colonies at 37°C (Table I). The finding that elimination of the distal and proximal histidines of the putative CPD active site resulted in constitutive and conditional lethality, respectively, is consistent with CPD catalytic activity being important for Trl1 function in vivo.
DISCUSSION

Three enzymatic activities of the yeast tRNA ligase are organized in a modular fashion within a single 827-aa polypeptide. The physical order of the active sites within the primary structure of Trl1 – H$_2$N-adenylyltransferase-kinase-CPD-COOH – is conserved in other fungal tRNA ligase orthologs. Compaction of three RNA end-processing enzymes within a single protein echoes the case of the poxvirus mRNA capping enzyme, in which the RNA triphosphatase, RNA guanylyltransferase and RNA (guanine-N7)-methyltransferase active sites are arranged in a modular fashion within an 844-aa polypeptide (63). The emergence of such multifunctional enzymes likely occurred via gene duplication and fusion events, in which the individual modules first acquired their biological specificity for tRNA splicing (or capping) via gene duplication and divergence from an ancestral catalytic domain and then later fused to form a single unit dedicated to a particular RNA processing pathway. Gene fusion is the most plausible scenario for tRNA ligase, given the existence in other cellular and viral niches of free-standing monofunctional adenylyltransferase/ligase and CPD enzymes that resemble the Trl1 domains. The present study sheds light on the relationships of the three Trl1 domains to structurally related catalytic modules in other systems and the issue of whether the covalent connections between the Trl1 domains are critical for \textit{in vivo} function.

We show here that all three domains and enzyme activities are essential for yeast viability. The essentiality of all three enzymes had not been demonstrated previously and was not a foregone conclusion. For example, Phizicky et al. (62) reported that conditional repression of Trl1 production \textit{in vivo} resulted in the accumulation of unligated tRNA half-molecules that contained processed 5'-PO$_4$ ends on the distal half-fragment and decyclized monophosphate ends on the proximal half-fragment. Their results provided strong evidence that the ligase function of Trl1 was essential, but raised the possibility that other cellular enzymes might be able to perform the kinase and CPD reactions and thereby be redundant to these two functions of Trl1. The present findings that point mutations in the central kinase domain of Trl1 abolished
kinase activity in vitro and were lethal in vivo argue against the existence of a functionally redundant RNA kinase activity in budding yeast. The lethal effects of deleting the CPD domain, or of a point mutation within the CPD domain, suggest that there is no backup enzyme available to convert the cyclic phosphate end to the 3'-OH/2'-PO_4 terminus required for sealing by the RNA ligase component. Thus, the earlier findings concerning conditional repression may be attributable to residual levels of Trl1 kinase and CPD activity.

The N-terminal adenylyltransferase and C-terminal CDP fragment comprise autonomous catalytic domains in vitro (14). Here we show that they are functionally autonomous in vivo as well, i.e., they can complement trl1Δ when separated genetically from the rest of the Trl1 protein. The in vivo function of the isolated domains was evident without resorting to increased gene dosage or the use of non-native promoter to drive expression of the isolated domains. These results suggest that either there is no need for physical interactions between the N- and C-terminal portions of Trl1 or the separately expressed N- and C- domains are able to interact in trans. The kinase domain was functional in vivo whether fused to the adenylyltransferase domain or to the CPD domain. The recombinant kinase-CPD protein was catalytically active in RNA phosphorylation. We were unable to produce a recombinant version of the isolated central kinase domain, which suggests that fusion to one of the flanking domains might be critical for proper folding or activity. In the case of bacteriophage T4 Pnk, where the N-terminal kinase domain is fused to a C-terminal 3' phosphatase domain, severing the covalent connection between the two domains results in a 10-fold decrement in kinase activity for the isolated kinase domain compared to the full-length enzyme (26,28). We have not tested whether coexpression of the three isolated Trl1 domains in yeast could support cell growth. Little is known at present about the quaternary structure of yeast Trl1, its component domains, or its repertoire of protein-protein interactions; these will be the subjects of future studies.

The N-terminal segment Trl1(1-376) represents a minimal adenylyltransferase domain that suffices for yeast growth at all temperatures and catalytic activity in vitro. Apostol et al. (14)
reported that an internally deleted version of Trl1 of residues constructed by eliminating residues 379-396 and using the flanking segments together was inert in adenylytransferase activity. Thus, they suggested that amino acids 378-396 were required for adenylyltransferase activity. The present study shows this is not the case. It is possible that the internal deletion analyzed by Apostol et al. was deleterious in its own right to the proper folding of the upstream adenylyltransferase domain.

To better understand the structural requirements for Trl1 adenylyltransferase activity, we initiated an alanine-scanning mutational analysis of selected residues of Trl1 that are conserved in other fungal tRNA ligases and in bacteriophage T4 RNA ligase 1, an enzyme dedicated to tRNA repair \textit{in vivo} and the closest homolog of fungal tRNA ligases. We found that residues in nucleotidyl transferase motifs I (Lys114), IV (Glu266 and Gly267) and V (Lys284 and Lys286) were essential for the Trl1 activity \textit{in vivo}, whereas mutations at three other conserved residues (Asn116, Gly152 and Glu153) did not affect cell growth. Mutations K114A, E266A and K284A either abolished or suppressed adenylyltransferase activity \textit{in vitro}. These effects are concordant with previous studies of the equivalent side chains of DNA ligases, mRNA capping enzymes, and bacteriophage T4 RNA ligases 1 and 2 (30,51-57,64). Our results indicate that the structural basis for nucleotidyl transfer is at least partially conserved among tRNA ligases, phage RNA ligases, DNA ligases, and mRNA capping enzymes. Such conservation is consistent with the speculation that RNA-joining enzymes that evolved during a primordial RNA/protein world are the ancestors of present-day DNA ligases and mRNA capping enzymes (55).

The primary structure of the central portion of yeast Trl1 resembles the kinase domain of T4 polynucleotide kinase. The crystal structures of T4 Pnk (28,29) guided our mutational analysis of the central domain of Trl1. Single mutations K404A and T405A in the putative NTP binding P-loop ($^{401}\text{GxGKT}^{405}$) had no effect on Trl1 function \textit{in vivo}. The K404A and T405A mutations eliminated ATP-dependent kinase activity, but preserved GTP-dependent kinase activity. A
double-alanine mutant in the P-loop was lethal \textit{in vivo} and abolished GTP-dependent kinase activity. These results suggest that GTP is the physiological substrate \textit{in vivo} (as suggested originally by the \textit{in vitro} studies of Abelson and Greer [15,16]) and that the Trl1 kinase has a single NTP binding site of which the P-loop is a component. Two other mutations in the central domain were lethal \textit{in vivo} and either abolished (D425A) or severely reduced (R511A) GTP-dependent RNA kinase activity \textit{in vitro}.

We can make reasonable predictions about the roles of Trl1 residues Lys404, Thr405, Asp425 and Arg511 based on the crystal structures of T4 Pnk, which have been solved with either sulfate or ADP occupying the NTP binding site (28,29). Lys404, Thr405 and Arg511 are likely to coordinate the $\beta$ phosphate of the NTP substrate. The differential effect of single alanine mutations on the ATP- versus GTP-dependent RNA kinase activities of Trl1 suggests that there are specific contacts between Trl1 and GTP that do not apply to ATP, and that such GTP contacts compensate for the loss of either one of the P-loop side chains Lys404 or Thr405, but not for the simultaneous elimination of both P-loop residues. This model is consistent with available biochemical evidence that the apparent $K_m$ of the Trl1 kinase for ATP is 800-fold greater than the $K_m$ for GTP (15,16). Asp425 of Trl1 is the putative equivalent of the essential Asp35 residue of T4 Pnk. It is proposed that this aspartate is located near the 5'-OH of the RNA acceptor and that it either serves as a general base catalyst to promote attack of the 5'-OH on the $\gamma$ phosphorus of the NTP substrate (28) or comprises part of a divalent cation binding site (29).

Given that the biochemical similarities between tRNA splicing and tRNA restriction-repair, and the apparent mechanistic and structural similarities between the adenylyltransferase and kinase components of the yeast and phage T4 repair systems, we propose that Trl1 and T4 Rnl1/Pnk have a shared evolutionary history whereby they descend from ancestral ligases and kinases devoted to repairing broken tRNAs. Where the two systems differ is the covalent connection (yeast) or lack thereof (T4) between the adenylyltransferase and kinase modules.
and the characteristics of the enzymes that process the 3’ end of the tRNA break. It has been proposed that the CPD domain of Trl1 belongs to the 2H family of phosphotransferases \((58,59)\). Our findings that the two signature histidines that define this family are either essential or important for Trl1 function \textit{in vivo} supports this proposal. The 3’ phosphatase domain of T4 Pnk belongs to the DxD family of phosphotransferases \((26-29)\), which are structurally and mechanistically unrelated to the 2H enzymes.
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Legend to Table I

Effect of alanine mutations on Trl1 activity in vivo. YRS1 was transformed with CEN TRP1 plasmids encoding full-length Trl1 proteins with the indicated alanine substitutions in either the N-terminal adenylyltransferase (A) domain, the central kinase (K) domain, or the C-terminal CPD (C) domain. trl1Δ complementation was assayed by plasmid shuffle. Lethal mutations were those that did not allow formation of FOA-resistant colonies after 7 days 18°C, 25°C, 30°C or 37°C. The other alleles supported FOA-resistant colony formation within 4 days at one or more of the growth temperatures. Individual FOA-resistant colonies were tested for growth on YPD agar at 18°C, 25°C, 30°C and 37°C. Strains that formed wild-type sized colonies after 2 to 4 days at all temperatures were scored as ++++. Temperature sensitive (ts) mutants grew at all temperatures except 37°C.
FIGURE LEGENDS

Fig. 1. Yeast tRNA splicing pathway. The pre-tRNA is cleaved at the exon-intron junctions in the anticodon loop by a tRNA splicing endonuclease, which leaves a 2',3' cyclic phosphate end on the proximal half-molecule and a 5'-OH on the distal half-molecule. The ends are then remodeled and sealed by tRNA ligase (Trl1), a multifunctional protein with 2',3' cyclic phosphodiesterase (CPD), 5' kinase, and ligase activities. The residual 2'-PO₄ at the splice junction is then removed by the NAD-dependent 2'-phosphotransferase Tpt1.

Fig. 2. Fungal tRNA ligases. The amino acid sequence of S. cerevisiae (Sce) Trl1 from residues 51-798 is aligned to the sequences of the homologous proteins of Saccharomyces bayanus (Sba), Candida albicans (Cal), Neurospora crassa (Ncr), Aspergillus nidulans (Ani) and Schizosaccharomyces pombe (Spo). Positions of side chain identity or similarity in all six proteins are denoted by ●. Residues of Trl1 that were subjected to alanine scanning are indicated by |. Nucleotidyl transferase motifs I (KANG), IV (EGFVI) and V (FFKYK), kinase motifs GxGKT and RxxxR, and 2H motifs HITL and HITL in the CPD domain are highlighted in shaded boxes. The sites of C-terminal truncation of the adenylyltransferase domains are specified by arrows pointing left; sites of N-terminal truncation of the CPD domain are denoted by arrows pointing right.

Fig. 3. All three domains of Trl1 are required for activity in vivo. The N-terminal adenylyltransferase domain, central kinase-like domain, and C-terminal CPD domain of Trl1 are depicted as horizontal bars. Full-length Trl1 and truncated versions thereof were tested by plasmid shuffle for complementation of the trl1Δ mutant.

Fig. 4. Delineating the margins of the autonomous adenylyltransferase domain. trl1Δ was cotransformed with CEN TRP1 plasmids bearing the indicated adenylyltransferase domain ORF and a CEN ADE2 plasmid encoding the kinase-CPD domain.
Fig. 5. Adenylyltransferase activity in vitro. (A) Aliquots (4 µg) of recombinant His-tagged adenylyltransferase domain fragments were analyzed by SDS-PAGE. The Coomassie Blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (B) Adenylyltransferase reaction mixtures containing 25-28 pmol of the indicated enzymes were incubated for 10 min at 30°C. The products were resolved by SDS-PAGE and visualized by autoradiography.

Fig. 6. Effects of alanine mutations on adenylyltransferase activity. (A) Aliquots (4 µg) of recombinant wild-type and mutant His-Trl1(1-388) proteins were analyzed by SDS-PAGE. The Coomassie Blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (B) Reaction mixtures (20 µl) containing 42 pmol of the indicated enzymes were incubated for 10 min at 30°C. The products were resolved by SDS-PAGE and visualized by autoradiography.

Fig. 7. Effects of alanine mutations on polynucleotide kinase activity. (A) Aliquots (4 µg) of recombinant wild-type and mutant His-Trl1 proteins were analyzed by SDS-PAGE. The Coomassie Blue-stained gel is shown. The polypeptide corresponding to full-length Trl1 is indicated by the arrow on the right. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (B) Adenylyltransferase reaction mixtures containing the indicated enzymes (1.2 µg of the full-length Trl1 polypeptide) were incubated for 10 min at 30°C. The products were resolved by SDS-PAGE and visualized by autoradiography. (C) Polynucleotide kinase reactions mixtures containing 100 µM [γ-32P]ATP or [γ-32P]GTP (1.1 Ci/mmol), 80 pmol of an 18-mer 5'-OH RNA oligonucleotide and the indicated enzymes (1.2 µg of the full-length Trl1 polypeptide) were incubated for 30 min at 30°C. The products were resolved by PAGE and visualized by autoradiography. The 5'-32P-labeled 18-mer RNA product is indicated by the arrow on the right.
Fig. 8. Polynucleotide kinase activity of truncated Trl1 proteins. (A) Aliquots (3 µg) of recombinant full-length (1-827) and truncated His-Trl1 proteins were analyzed by SDS-PAGE. The Coomassie Blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (B) Kinase reactions mixtures containing 100 µM [γ-32P]GTP (1.2 Ci/mmol), 80 pmol of an 18-mer 5'-OH RNA oligonucleotide and 1.2 µg of the indicated enzymes were incubated for 30 min at 30°C. The products were resolved by PAGE and visualized by autoradiography.

Fig. 9. Delineating the margins of the autonomous CPD domain. trl1Δ was cotransformed with CEN HIS3 plasmids encoding the indicated CPD domain and a CEN TRP1 plasmid encoding the adenylyltransferase–kinase domain Tr1(1-561).
Table I

Effect of Alanine Mutations on Trl1 Activity In Vivo

| Domain | Mutation       | trl1Δ complementation |
|--------|----------------|-----------------------|
| A      | K114A          | lethal                |
|        | N116A          | +++                   |
|        | G152A          | +++                   |
|        | E153A          | +++                   |
|        | E266A          | lethal                |
|        | G267A          | lethal                |
|        | K284A          | lethal                |
|        | K286A          | lethal                |
| K      | K404A          | +++                   |
|        | T405A          | +++                   |
|        | K404A-T405A    | lethal                |
|        | D425A          | lethal                |
|        | D454A          | +++                   |
|        | R511A          | lethal                |
| C      | H673A          | ts                    |
|        | H777A          | lethal                |
Intron

tRNA splicing endonuclease

5' OH 3'

CPD
5' kinase ligase

2' phosphotransferase

5' 3'
| Adenylyltransferase | Kinase | CPD |
|---------------------|--------|-----|
| 1                   | 389    | 827 |
| 1                   | 389    | 827 |
| 1                   | 389    | 827 |
| 1                   | 389    | 827 |
| 1                   | 389    | 827 |
| 1                   | 389    | 827 |
| 1                   | 389    | 827 |
| 21                  | 389    | 827 |
| 41                  | 389    | 827 |
| 59                  | 389    | 827 |
