Cloning and characterization of the gene for the yeast cytoplasmic threonyl-tRNA synthetase

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ABSTRACT
A fragment of DNA from the yeast nuclear gene MST1 that codes for the mitochondrial tRNA\textsuperscript{Thr}\_1 synthetase was used as a probe to screen for other yeast threonyl-tRNA synthetase genes. At low stringency, the MST1 probe hybridizes strongly to a 6.6 kb EcoRI fragment of yeast genomic DNA with the homologous gene and in addition hybridizes more weakly to a smaller 3.6 kb EcoRI fragment with a second threonyl-tRNA synthetase gene (THS1). To clone THS1, a library was constructed by ligation to pUC18 of size selected (3-4.5 kb) EcoRI fragments of genomic DNA. Several clones containing the 3.6 kb EcoRI fragment were isolated. A 2,202 nucleotide long open reading frame corresponding to THS1 has been identified in the cloned fragment of DNA. The predicted protein encoded by THS1 is 38% identical to the E. coli threonyl-tRNA synthetase over the latter's length (642 amino acids) and is 42% identical to the predicted MST1 product over its 462 residues. \textit{In situ} disruption of the chromosomal copy of THS1 is lethal to the cell, indicating that this gene codes for the cytoplasmic threonyl-tRNA synthetase.

INTRODUCTION
At present there are only a few reported instances of yeast mutants with defective cytoplasmic tRNA synthetases (1-5). As a result only a small number of the genes have been cloned by transformation, and there is relatively little known about the extent to which the eucaryotic synthetases have diverged from the better studied procaryotic enzymes. In the present study we demonstrate that it may be feasible to isolate the genes for the yeast cytoplasmic aminoacyl-tRNA synthetases by using yeast mitochondrial synthetase probes.

We have recently reported the cloning of the yeast nuclear gene MST1 (6). This gene was shown to code for a mitochondrial aminoacyl-tRNA synthetase that charges the tRNA\textsuperscript{Thr}\_1 with the anomalous 3'-GAU-5' anticodon (6). Mutations in this synthetase gene were found to abolish charging of tRNA\textsuperscript{Thr}\_1 but had no effect on charging of tRNA\textsuperscript{Thr}\_2 whose anticodon recognizes the conventional ACN family of codons (6). Due to a high degree of homology between the MST1 gene and the gene coding for the yeast cytoplasmic threonyl-tRNA synthetase (here referred to as THS1), we were able to use an MST1 probe to select bacterial clones harboring recombinant plasmids with the THS1 gene. Further characterization of this gene has revealed that of the two known yeast threonyl-tRNA synthetases, the cytoplasmic species is more closely related to the procaryotic enzyme, both in size and primary sequence.
MATERIALS AND METHODS

Strains and media: The strains of Saccharomyces cerevisiae and the composition of media have been described previously (6).

Preparative and analytical procedures: Procedures for the preparation of plasmid DNA, yeast genomic DNA and their subsequent analyses are identical to those described previously (6,7).

Construction and screening of the yeast genomic library: Chromosomal DNA purified from S. cerevisiae D273-10B was digested to completion with EcoRI. The digestion products were separated on a preparative 1% agarose gel (8), and fragments ranging from 3 to 4.5 kb were recovered by electrophoresis. The DNA was extracted twice with water-saturated phenol, four times with ether, and was precipitated with alcohol from 2 M ammonium acetate. Following ligation to the EcoRI site of pUC18 (9), the mixture was used to transform E. coli RR1. Transformants were selected on 1.75% Antibiotic Medium #3 (Difco) containing 40 μg/ml ampicillin. Approximately 3,000 ampicillin resistant colonies were screened with a nick-translated fragment of DNA (BglII-HinTn) from the MST1 gene coding for residues 45-163 of mitochondrial tRNA^Thr synthetase (6). The transformant colonies were transferred to two separate nitrocellulose circles prewetted on an ampicillin plate (10). These were incubated face up at 37° C on fresh ampicillin medium. The colonies on the nitrocellulose filters were lysed and washed as described by Grunstein and Hogness (10). Hybridizations were done at 37° C for 36 hours in 43% formamide, 6x SSC, 0.1% sodium dodecyl sulfate (SDS), 5X Denhardt's, 50 mM NaPO₄ pH 6.5, 100 μg/ml salmon sperm DNA (11) and in duplicate at 30% formamide. Approximately 10⁷ cpm was added to 6 ml of the hybridization solution. The filters were washed two times at room temperature with 2x SSC, 0.1% SDS, and an additional two times at 50° C for 15 min in 2x SSC, 0.1% SDS (11). Colonies that gave positive signals under both hybridization conditions were purified and screened a second time as above except that Whatman 541 paper (12) was used instead of nitrocellulose.

Southern hybridizations: Yeast genomic DNA was separated electrophoretically on 1% agarose after digestion with restriction endonucleases and processed by the method of Southern (13). High stringency hybridizations were done either at 65° in 6x SSC, 0.01 M EDTA, 0.5% SDS, 5x Denhardt's, 100 μg/ml salmon sperm DNA or at 45° in the same buffer with 50% formamide. For low stringency hybridizations, the temperature was reduced to 37° with either 43% or 30% formamide added to the standard buffer.

DNA sequence analysis: The sequence of the THS1 gene was derived from the insert of pUC18/THS1. The 1.5 kb EcoRI-HindIII, 1.2 kb BglII-SalI, 750 bp BamHI-HindIII, and 650 bp SalI-BamHI fragments were isolated from preparative agarose gels. These were subjected to secondary cleavages with other restriction endonucleases. The digestion products were 5'-end labeled with γ-[³²P]-ATP (5,000 Ci/mmol, ICN) in the presence of T4 polynucleotide kinase. Following denaturation, single strands were separated on 4% polyacrylamide and sequenced by the method of Maxam and Gilbert (14). Most of the sequence was confirmed on both strands.

Extraction of cytoplasmic tRNAs and aminoacyl-tRNA synthetases: Cytoplasmic tRNAs were isolated from D273-10B p⁶ as described (15). S. cerevisiae αW303-1B and an αW303-1B transformant containing YEp351/THS1 were grown to late logarithmic phase in minimal glucose media. The cells were harvested by centrifugation, washed in MTE (0.25 M mannitol, 20 mM Tris-Cl pH 7.5, 1 mM EDTA) and resuspended in the same buffer at a concentration of 8 g. wet weight cells/30 ml. After cell disrup-
tion in a Braun glass bead homogenizer, the cell debris and mitochondria were pelleted at 15,000 rpm for 30 min at 4°C. The supernatant was applied to a DEAE column (Whatman DE52) equilibrated in 0.4 M NaCl, 20 mM Tris-Cl pH 7.5, after which the column was washed in 1 column volume of the same buffer. The flow-through and wash were collected, and dialysed 2 hr at 4°C vs. 10 mM Tris-Cl pH 7.5, 0.1 mM EDTA, 1 mM DTT (Dithiothreitol) and used as the source of yeast aminoacyl-tRNA synthetases. Protein concentration was determined by the Lowry method (16). In vitro aminoacylation reactions were performed as described by Maciño and Tzagoloff (15), except the reaction mix was 50 μl and contained 0.2 mM DTT and 50 μg of cytoplasmic tRNAs.

RESULTS AND DISCUSSION

Hybridization analysis of yeast genomic DNA with an MST1 probe under low stringency conditions

One of the yeast mitochondrial threonyl-tRNA synthetase genes (MST1) has been cloned and shown to code for an enzyme capable of acylating tRNA\textsubscript{Thr} but not tRNA\textsubscript{Val} (6). In order to determine whether an MST1 probe could be used to screen for one or both of the other threonyl-tRNA synthetase genes, chromosomal DNA of wild-type yeast was examined by Southern hybridization analysis with a probe containing the entire coding region of MST1. At high stringency the probe detects only a 6.6 kb EcoRI fragment of genomic DNA which was previously found to encompass the entire MST1 gene. As

![Fig. 1. Low stringency hybridization of wild-type yeast genomic DNA with an MST1 probe. DNA purified from the wild-type strain 41-8D was digested to completion with EcoRI and separated on a 1% agarose gel. The DNA was blotted to nitrocellulose and hybridized with pUC18/MST1, a clone of the 1.8 kb Aval fragment containing the MST1 gene in pUC18 (6). The hybridizations were done in 6x SSC, 0.01 M EDTA, 0.5% SDS, 5x Denhardt's, 100 μg/ml salmon sperm DNA at the following temperatures and concentrations of formamide. Lane 1: 30% formamide, 42°C, lane 2: 30% formamide, 30°C, lane 3: 50% formamide, 30°C, lane 4: no formamide, 4x SSC, 42°C. The positions and sizes of the EcoRI fragments containing the MST1 gene and the homologous THS1 gene are marked in the margin.](image-url)
shown in Fig. 1, a second EcoRI fragment of 3.6 kb is detected by the same probe when hybridizations are done at lower stringency. The signal of this smaller fragment is weaker but is reproducibly observed under the more relaxed conditions of hybridization and in several wild-type strains including 41-8D, W303 and D273-10B.

Cloning of the 3.6 kb EcoRI fragment

The cross-hybridization of the MSTI probe to a 3.6 kb fragment of yeast nuclear DNA suggested

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**Fig. 2.** Partial restriction map of pUC18/THS1. The positions of the HindIII, BglII, and SalI sites in the 3.6 kb EcoRI fragment are shown in the upper part of the figure. The fragment was ligated to the EcoRI site of the multiple cloning sequence (solid arc) of the pUC18 vector.

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**Fig. 3.** Restriction sites used to sequence part of the 3.6 kb EcoRI insert in pUC18/THS1. The region sequenced starts 260 bp upstream of the unique BamHI site in the insert and extends to the extreme right EcoRI site (see Fig. 2). The restriction sites used for 5'-end labeling were TaqI (Δ), HinfI (▲), BamHI (◇), DdeI (○), RsaI (●), BstN1 (□), HaeIII (◇), HindIII (◇), and BglII (◇). The arrows indicate the directions and approximate lengths of sequences obtained. The location of the open reading frame is shown by the open bar in the upper part of the figure.
that it might contain part or the whole gene for one of the other threonyl-tRNA synthetases. The gene will henceforth be referred to as \textit{THS1} (threonyl-tRNA synthetase). The following strategy was adopted to clone the gene. Chromosomal DNA purified from the wild-type strain D273-10B was digested with \textit{EcoRI}, and the products were separated on a preparative 1% agarose gel. DNA fragments in the range of 3 to 4.5 kb were eluted from the gel and ligated to the unique \textit{EcoRI} site of pUC18 (9). Approximately 3,000 ampicillin resistant transformants harboring the products of the ligation were screened at low stringency with a \textit{BglII-HindIII} fragment coding for residues 45-163 of tRNA\textsuperscript{T\textsubscript{rr}} synthetase. This probe hybridizes to the 3.6 kb \textit{EcoRI} fragment under low stringency (data not shown). Five clones each having plasmids with a 3.6 kb insert were obtained from the screen. A partial restriction map of the plasmid with the 3.6 kb \textit{EcoRI} fragment (pUC18/THS1) is presented in Fig. 2.

**Southern analyses of pUC18/THS1** confirmed the presence of a homologous gene in the 3.6 kb insert and further indicated that its 5' end was proximal to the \textit{SalI} site (Fig. 2).

**Sequence of part of the 3.6 kb insert in pUC18/THS1**

The insert of pUC18/THS1 was sequenced by the strategy outlined in Fig. 3. The sequence was obtained from the 650 bp \textit{SalI-BamHI}, 750 bp \textit{BamHI-HindIII} and 1,500 bp \textit{HindIII-EcoRI} fragments. The \textit{BamHI} and \textit{HindIII} sites were crossed from preparative \textit{SalI-BglII} and \textit{BglII-EcoRI} fragments. Most of the sequence was confirmed from the complementary strands.

The nucleotide sequence revealed a long continuous reading frame starting with the ATG codon at +1 and ending with an ochre termination codon at +2203 (Fig. 4). The predicted protein encoded in this gene is 734 amino acid residues in length with a molecular weight of 84,380. It has a hydrophilic composition with a net charge of -5. There is a moderate bias in codon usage. Noticeably lacking are the CGC, CGA, and CGG codons for arginine (Table I).

**In situ disruption of THS1**

In order to determine whether \textit{THS1} codes for a cytoplasmic or mitochondrial threonyl-tRNA synthetase, the chromosomal copy of the gene was disrupted by gene substitution (17). The \textit{THS1} gene was isolated on a 3.4 kb \textit{EcoRI-BglII} fragment and ligated at compatible sites in the multiple cloning region of pUC8 (18). This construct was used to delete a 1.16 kb segment of the coding region between the \textit{BamHI} and \textit{XbaI} sites (Fig. 5). The deleted region of the gene was substituted with a 1.7 kb fragment of yeast nuclear DNA with the \textit{HIS3} gene (19). The isogenic haploid and diploid respiratory competent strains W303 \(\alpha\), \(\alpha\), and \(\alpha/\alpha\) with a \textit{his3} marker were each transformed with the linear \textit{EcoRI-SalI} fragment containing the disrupted \textit{THS1} gene. Transformants selected for loss of the histidine auxotrophy were collected and further checked for the presence of the disrupted copy of \textit{THS1} in chromosomal DNA. Southern analysis of numerous haploid transformants indicated that none had acquired the disrupted \textit{THS1} gene. Several diploid transformants complemented for the histidine auxotrophy, however, had one altered copy of \textit{THS1} in addition to one wild-type copy. The results shown in Fig. 5 confirm the presence of the disrupted copy of \textit{THS1} in one such diploid clone. Genomic DNAs from the parental W303 diploid and from the histidine independent transformant were digested with \textit{EcoRI} alone and with a combination of \textit{EcoRI} and \textit{PstI}. Following separation on agarose and transfer to nitrocellulose, the Southern blot was hybridized with a nick-translated 1.3 kb \textit{EcoRI-BamHI} fragment containing 5' flanking and 127 bp from the coding region of the gene. As seen in lanes 1 and 2, the \textit{THS1} gene is present on a 3.6 kb \textit{EcoRI} fragment in both the haploid and diploid W303 strains. Although this band is also present in the diploid transformant, there is a second hybridizing band.
Fig. 4. Nucleotide sequence of the pUC18/THSl insert. The sequence shown is that of the sense strand. The amino acid sequence of the reading frame from nucleotides +1 to +2202 is shown above the DNA sequence.

approximately 550 bp larger. The second larger EcoRI fragment is consistent with the replacement of one wild-type copy of THS1 with the disrupted gene. This was confirmed by the results of the EcoRI plus PsA digest. Since PsA cleaves the HIS3 but not the THS1 gene, the larger 4.2 kb EcoRI fragment in the transformant should be reduced to a 2.5 kb EcoRI-PstI fragment. Lane 5 shows that genomic DNA from the transformant has the expected 3.6 kb EcoRI fragment and in addition the smaller 2.5 kb EcoRI-PstI fragment.

The diploid strain W303Vths1 with the disrupted threonyl-tRNA synthetase gene was sporulated and tetrads were dissected. If THS1 codes for a cytoplasmic synthetase, each tetrad should have only two viable spores, both being auxotrophic for histidine. If, on the other hand, the gene product is a mitochondrial aminocacyl-tRNA synthetase, all four meiotic spore progeny should be viable, but the two prototrophic for histidine would be expected to be respiratory deficient. Out of 8 tetrads dissected, 5 had 2 viable spores. The other 3 tetrads yielded only one viable spore each. All the spores, however,
TABLE I
Codon Usage in THSI

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<td>10</td>
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</table>

were auxotrophic for histidine and were respiratory competent. These results and our inability to
demonstrate disruption of THSI in the haploid strains of W303 indicate that the product of THSI is
essential for cell viability and therefore functions in cytoplasmic protein synthesis. Whether this synthetase
also functions in charging of the mitochondrial tRNA$^{Thr}$ is unclear at present. All the currently
known genes coding for mitochondrial tRNA synthetases (those coding for the threonyl-, tryptophanyl-, aspartyl-, α subunit of phenylalanyl-, and tyrosyl-tRNA synthetases) are unique and,
when inactivated, result in a subsequent loss of the corresponding mitochondrial synthetase alone (6,20;
A. Gampel, J. Hill, T. J. Koerner, A. M. Myers, and A. Tzagoloff, unpublished data).

Homology of the THSI product to the mitochondrial and E. coli threonyl-tRNA synthetases

The sequence of the protein encoded in THSI is homologous to both E. coli threonyl-tRNA synthetase (21) and to the mitochondrial tRNA$^{Thr}$ synthetase (6). This is illustrated in the two dot matrices
shown in Fig. 6. The homology with the bacterial enzyme extends over almost the entire lengths of the
two polypeptide chains. The two proteins share 246 identical residues with a total of only 8 gaps in the alignment. This represents 38% homology. The yeast protein, however, has an extra 49 amino terminal
residues for which there is no match in the sequence of the E. coli synthetase. It also has an extra
12 amino acids at the carboxyl terminal end. The yeast methionyl-tRNA synthetase also has additional
residues at its amino terminus not present in the E. coli counterpart; 191 amino acids in this case (22).

Previous studies indicated that the tRNA$^{Thr}$ of yeast mitochondria is considerably shorter than the E. coli threonyl-tRNA synthetase (6). The discrepancy in the sizes of the two proteins is due mainly to
the absence in the mitochondrial synthetase of some 200 amino terminal residues. This is also evident
in the dot matrix of the two yeast proteins. Even though there is a clear line of homology, the tRNA$^{Thr}$ synthetase is 300 residues shorter at the amino terminal end. An alignment of the two sequences shows
that the first unambiguous homology starts at residue 46 of tRNA$^{Thr}$ synthetase and residue 337 of the
THSI product (Fig. 7).

A more informative comparison of the three protein sequences is presented in Fig. 7 where the
Fig. 5. Southern hybridization analysis of genomic DNA from the wild-type a/α and aW303 strains and from the a/α W303Vths1 transformant. Purified chromosomal DNAs from the three strains digested with EcoRI alone and with EcoRI plus PstI were separated electrophoretically on a 1% agarose gel. Following transfer to nitrocellulose (13), the Southern blot was hybridized with a nick-translated 1.3 kb EcoRI-BamHI fragment that included 5' non-coding sequences of the THS1 gene and 127 bp of coding sequences. The hybridization was carried out overnight at 65°C in 6x SSC, 1% sodium lauroyl sarcosyl, 0.1 mg/ml salmon sperm DNA. The blot was washed once with 2x SSC, 0.1% SDS and three times with 5 mM Tris-Cl pH 8 at room temperature. Lane 1: a/α W303 digested with EcoRI; lane 2: aW303 digested with EcoRI; lane 3: a/α W303Vths1 digested with EcoRI; lane 4: aW303 digested with EcoRI and PstI; lane 5: a/αW303Vths1 digested with EcoRI and PstI. The sizes of the EcoRI and EcoRI-PstI fragments containing the THS1 gene are marked in the margin.

The lower half of the figure illustrates the constructed Vths1 gene. The THS1 gene and flanking regions are drawn with a solid line. The inserted fragment of DNA with the HIS3 gene is depicted by a broken line. The direction of transcription of the genes is indicated by the arrows. The probe used for the hybridization was the 5' EcoRI-BamHI fragment.

Alignment has been optimized based on identical residues. A visual inspection of the aligned sequences indicates that the most conserved regions occur in the central part of the proteins where there are numerous blocks of identical residues. In order to minimize errors introduced by faulty alignments, the analysis of the protein sequences has been confined to the regions spanning residues 251 to 544 of the
Fig. 6. Comparison of the THS1 product with the E. coli threonyl-tRNA synthetase and with the mitochondrial tRNA\textsuperscript{Thr} synthetase encoded in MST1. The matrix program used for the analysis scores a dot for every two consecutive identities. The residue numbers are shown on the abscissa and ordinate.

E. coli threonyl-tRNA synthetase, residues 46 to 352 of the yeast tRNA\textsuperscript{Thr} synthetase and residues 337 to 633 of the THS1 product. As can be seen in Fig. 7 these are the most homologous domains of the synthetases requiring the fewest deletions/insertions for alignment. The number of identities amongst the three proteins is summarized in Table II. Of the two yeast synthetases, the product of THS1 is more homologous to the bacterial synthetase (47.6% identities) than the tRNA\textsuperscript{Thr} synthetase (44.2% identities). The THS1 product and the bacterial synthetase share 140 identical amino acids and differ in length by only two residues. In the same region the mitochondrial tRNA\textsuperscript{Thr} synthetase had 130 identical amino acids with the E. coli synthetase. In addition, the mitochondrial protein is 13 residues longer necessitating 4 gaps in the alignment of the two sequences. An even more striking homology (52.2%) exists between the two yeast proteins.

The greater divergence of the tRNA\textsuperscript{Thr} synthetase is not unexpected. The tRNA\textsuperscript{Thr} has several unconventional features not seen in other threonine tRNAs. In addition to its leucine anticodon, it also has an extra nucleotide in the anticodon loop. Since the anticodon is one of the important factors in determining the specificity with which synthetases recognize their tRNA substrates (24,25), part of the domain involved in binding of tRNA\textsuperscript{Thr} must be different in this synthetase. A comparison of the three sequences reveals a number of regions that are unique to the tRNA\textsuperscript{Thr} synthetase. In the absence of information about the tRNA binding domains of synthetases in general, their significance is no clear.

In vitro aminoacylation of cytoplasmic tRNAs with wild-type and transformant extracts

Further confirmation that THS1 codes for the cytoplasmic threonyl-tRNA synthetase was obtained by measuring the in vitro acylating activity of wild type extract and of an extract obtained from a
Fig. 7. Protein sequence homologies of the two yeast threonyl-tRNA synthetases and of *E. coli* threonyl-tRNA synthetase. The alignments of the three proteins are based on the MFALGO alignment program (23). Identical residues in the synthetases encoded by the two yeast genes (*MST1, THS1*), and by the *E. coli* gene (*thrS*) have been boxed.
The data in this table are based on alignments of the sequences included between residues 251-544 of *E. coli* threonyl-tRNA synthetase, residues 337-534 of the *THS1* encoded synthetase and residues 46-352 of the synthetase encoded in *MST1*.

The data in Table II are based on alignments of the sequences included between residues 251-544 of *E. coli* threonyl-tRNA synthetase, residues 337-534 of the *THS1* encoded synthetase and residues 46-352 of the synthetase encoded in *MST1*.

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<th>Comparison</th>
<th>No. of Identities</th>
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<td><em>E. coli</em>/MST1/THS1</td>
<td>108</td>
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The strain with the *THS1* gene on an autonomously replicating plasmid. The 3.6 kb EcoRI fragment containing the *THS1* gene was transferred into the vector YEp351 (J. Hill, T. J. Koerner and A. Tzagoloff, personal communication). This construct, designated YEp351/THS1, was introduced into a wild-type strain auxotrophic for leucine, aW303-1B. A leucine independent transformant verified to harbor YEp351 was used to prepare total cytoplasmic aminoacyl-tRNA synthetases. A similar synthetase preparation was obtained from the parental strain, aW303-1B. Cytoplasmic tRNAs, isolated from a p° strain and hence devoid of any mitochondrial tRNAs, were acylated in the presence of L-[3H] threonine with varying concentrations of the wild-type aW303-1B extract and with the comparative extract obtained from the transformant with the *THS1* gene on the multiple copy vector YEp351. At low concentrations (1-3 μg protein per 50 μl reaction), the extract obtained from the transformant was consistently more active in acylating threonine tRNA than the wild-type extract (Table III). Saturating

<table>
<thead>
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<th>µg Protein</th>
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<th>Ratio of T1/wild-type</th>
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**TABLE III**

*W303/T1 designates the αW303-1B transformant harboring the multi-copy plasmid YEp351/THS1.*

After 30 min at 30°C in a 50 μl reaction mix consisting of 0.1 M Tris-Cl, pH 7.5, 5 mM ATP, 15 mM MgCl2, 0.2 mM DTT, 50 μg cytoplasmic tRNAs, 0-50 μg protein, and 2.5 μCi [3H] threonine (ICN, 20 Ci/mmol), the tRNAs were precipitated in 6% TCA, washed in 5% TCA and 80% ethanol, and counted. Table reports cpm recovered in the TCA precipitation.
levels of the cytoplasmic threonyl-tRNA synthetase were reached at a lower protein concentration in the transformant. These results lend further support for the assignment of THS1 as the gene that codes for the cytoplasmic threonyl-tRNA synthetase.

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