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Characterization of Yeast Protein Deg1 as Pseudouridine Synthase (Pus3) Catalyzing the Formation of Ψ_{38} and Ψ_{39} in tRNA Anticodon Loop*

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François Lecointe‡, George Simos§, Anke Sauer§, Eduard C. Hurt§, Yuri Motorin‡¶, and Henri Grosjean‡

From the ‡Laboratoire d'Enzymologie et Biochimie Structurales, CNRS, Gif-sur-Yvette, France and the §Universität Heidelberg, Institut für Biochemie I, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany

The enzymatic activity of yeast gene product Deg1 was identified using both disrupted yeast strain and cloned recombinant protein expressed in yeast and in *Escherichia coli*. The results show that the *DEG1*-disrupted yeast strain lacks synthase activity for the formation of pseudouridines Ψ_{38} and Ψ_{39} in tRNA whereas the other activities, specific for Ψ formation at positions 13, 27, 28, 32, 34, 35, 36, and 55 in tRNA, remain unaffected. Also, the His_6-tagged recombinant yeast Deg1p expressed in *E. coli* as well as a protein fusion with protein A in yeast display the enzymatic activity only toward Ψ_{38} and Ψ_{39} formation in different tRNA substrates. Therefore, Deg1p is the third tRNA:pseudouridine synthase (Pus3p) characterized so far in yeast. Disruption of the *DEG1* gene is not lethal but reduces considerably the yeast growth rate, especially at an elevated temperature (37 °C). Deg1p localizes both in the nucleus and in the cytoplasm, as shown by immunofluorescence microscopy. Identification of the pseudouridine residues present (or absent) in selected naturally occurring cytoplasmic and mitochondrial tRNAs from *DEG1*-disrupted strain points out a common origin of Ψ_{38}- and Ψ_{39}-synthesizing activity in both of these two cellular compartments. The sensitivity of Pus3p (Deg1p) activity to overall three-dimensional tRNA architecture and to a few individual mutations in tRNA was also studied. The results indicate the existence of subtle differences in the tRNA recognition by yeast Pus3p and by its homologous tRNA:pseudouridine synthase *truA* from *E. coli* (initially called *hisT* or PSU-I gene product).

The modified nucleoside pseudouridine (5-(β-D-ribofuranosyl)uracil, abbreviated as Ψ),[1] is found very frequently in all kinds of RNA from eubacteria, archaea, and eukaryotes (1). It is present in all transfer RNAs (2), large and small subunits of ribosomal RNA (3, 4), most small nuclear RNAs (5, 6), and selected small nucleolar RNAs (7).

The numerous Ψ residues in RNA are produced by a family of enzymes (pseudouridine synthases, RNA:Ψ synthases). These enzymes act on specific uridine residues of the RNA molecules, but still very little is known about the number of these enzymes in a given cell as well as their mechanism and their RNA recognition mode.

In yeast tRNA, formation of pseudouridines at positions 13, 32, and 55 is catalyzed by three distinct enzymes (8), whereas in the same yeast cell one single enzyme (Pus1p) is responsible for Ψ formation at positions 27, 34, and 36 (9). Several distinct pseudouridine synthase activities acting on eukaryotic small nuclear RNAs were also detected in crude cell extracts (5); however, none of these enzymes has been identified so far. From recent works on rRNA maturation, it appears that most (if not all) of the Ψ in eukaryotic rRNA is probably synthesized by a single (or at least very few) rRNA:Ψ synthase(s); in this latter case the enzyme(s) is(are) guided to the different target uridines within the rRNA by a huge family of diverse small nucleolar ribonucleoproteins present in the nucleolus (10, 11; for review, see Ref. 12).

The first RNA:pseudouridine synthase that was purified and fully characterized came from *Salmonella typhimurium* (13) and later from *Escherichia coli* (14). It corresponds to a tRNA:Ψ synthase (PSU-I, previously called *hisT* gene product, recently renamed to *truA*). It catalyzes the formation of Ψ_{38}, Ψ_{39}, and/or Ψ_{40} in several cellular tRNAs. Only the gene for the *E. coli* enzyme (PSU-I) was cloned and sequenced (15). The *E. coli* PSU-Ip is a monomer with a molecular mass of 31 kDa (270 amino acids), whereas PSU-Ip from *S. typhimurium* has a subunit size of about 50 kDa and dimerizes in the presence of tRNA substrate (13). A higher eukaryotic homolog of PSU-I from calf thymus was highly enriched after five chromatographic steps (16); however, a homogeneous preparation was not obtained. All of these enzymes have similar properties: they do not require any cofactor, they catalyze the Ψ formation at contiguous sites (region specificity), and they demonstrate similar kinetic properties (13, 14, 16).

Recently three other *E. coli* RNA:Ψ synthases were identified and their corresponding genes cloned: *truB*, which is site-specific for Ψ at position 55 in tRNA (17); *rluA*, specific for Ψ at position 32 in tRNA and at position 746 in 23 S rRNA (dual specificity; Ref. 18); and *rsuA*, specific for U at position 516 in 16 S rRNA associated with ribosomal proteins within a ribosomal ribonucleoprotein complex (19).

The genes for two other tRNA:pseudouridine synthases (Pus1p and Pus2p) from yeast were also identified, one of them (*PUS1*) was cloned and overexpressed in *E. coli* (9). The recom-

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¶ Supported by the Medical Research Council of Canada Operating Grant MRC-MT-1226 awarded to Dr. B. Lane, University of Toronto, Canada. Correspondence should be addressed to Y. Motorin, Laboratoire d'Enzymologie et Biochimie Structurales, Avenue de la Terrasse, Bat. 34, CNRS, Gif-sur-Yvette, France. Tel.: 33-1-6982-3498; Fax: 33-1-6982-3129; E-mail: Yuri.Motorin@lebs.cnrs-gif.fr.

[1] The abbreviations used are: Ψ, pseudouridine (5-(β-D-ribofuranosyl)-uracil; CMCT, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate; PCR, polymerase chain reaction; ORF, open reading frame; GFP, green fluorescent protein; ProtA, *S. aureus* protein A; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

binant yeast Pus1p was shown to be specific for Ψ at position 27 in several yeast tRNAs and for Ψ at positions 34 and 36 in the intron-containing pre-tRNA^{Ile} (9; for review, see Ref. 20). This enzyme has a molecular mass of 62 kDa and was shown to be located essentially in the yeast nucleus (9). The target RNA and position(s) of uridine to be modified by Pus2p (a 42-kDa protein) have not been determined yet.

Another yeast protein has been reported to be homologous to *E. coli* and *S. typhimurium* pseudouridine synthases PSU-I (*hisT* gene product, truAp) (21). This protein (called Deg1 because disruption of the gene causes depressed growth; SacchDB accession number YFL001W, SwissProt P31115) also displays a significant homology to the yeast Pus1p (9). Based on this sequence homology with PSU-I, it was suggested (21) that Deg1 protein may possess the corresponding activity of *hisT* gene product, but this plausible hypothesis was never tested experimentally. Here we present experimental evidence that yeast protein Deg1 is indeed a new distinct yeast tRNA:Ψ synthase (Pus3p) with a slightly different specificity toward tRNA compared with *E. coli* enzyme truA since it catalyzes the formation of only Ψ_{38} and Ψ_{39} (not Ψ_{40}). The structural substrate requirements and the intracellular localization of Pus3p are also analyzed.

EXPERIMENTAL PROCEDURES

Chemicals, Enzymes, and Materials—α-^{32}P-Radiolabeled nucleotide triphosphates (400 Ci/mmol) were from Amersham (U. K.). Tris, dithioerythritol, dithiothreitol, nucleoside triphosphates, *Penicillium citricum* nuclease P1, *Aspergillus oryzae* RNase T2, and phenylmethylsulfonyl fluoride were from Sigma. Diisopropyl fluorophosphate and CMCT were from Aldrich. Bacteriophage T7 RNA polymerase, restriction enzymes, and isopropyl-1-thio-β-D-galactopyranoside were from from MBI Fermentas (Vilnius, Lithuania). RNasin and avian myeloblastosis virus reverse transcriptase were from Promega. Synthetic oligodeoxynucleotides used as primers for reverse transcription were purchased from MWG-Biotech (Germany) and used without further purification. Thin layer cellulose plates were from Schleicher & Schuell (Germany), and all other chemicals were from Merck Biochemicals (Germany).

Plasmids and Transcription of tRNA Genes—The plasmids carrying the synthetic genes of tRNAs used in this work were described previously tRNA^{Asp} (GUC) (22), pre-tRNA^{Ile} (UAU) and its mutant (anticodon UUA) (23), pre-tRNA^{Tyr} (mutant UUA) (24), and *E. coli* tRNA^{Ser} (GGA) (25). Plasmids with cloned genes of yeast tRNA^{Phe} (GAA) and its mutants PheY54 (p67YF1, mutation C56G) and PheY55 (p67YF2, mutations G19C and C56G) have been described (26). Plasmids carrying yeast tRNA^{His} (GUG), yeast tRNA^{Ser} (IGA), and mutant yeast tRNA^{Val} (harboring anticodon CAU instead of UAC) were gifts, respectively, of Dr. J. Rudinger (IBMC, Strasbourg), Dr. H. Himeno (Tokyo University, Japan), and Dr. F. Fasiolo (IBMC). The synthetic gene for yeast tRNA^{Ala} (anticodon IGC) was constructed by ligation of sets of complementary oligonucleotides as described (22).

The DNA template for T7 transcription of *E. coli* tRNA^{Leu} (CAG) and yeast intronless tRNA^{Trp} (bearing a mutated anticodon CUA) were prepared by PCR amplification of the corresponding sequence present in *E. coli* genomic DNA and in plasmid (pT7T3am2ΔIVS, provided by Dr. J. B. Bell, University of Alberta, Canada) using two complementary oligonucleotides to each gene sequence, one bearing a T7 promoter sequence and the other one half of an *Mva*I restriction site. The minisubstrate composed of the anticodon stem-loop of tRNA^{Phe} (with and without intron) was prepared by T7 transcription using synthetic double-stranded DNA template as described (27).

In vitro T7 RNA-polymerase transcription using α-^{32}P-radiolabeled nucleotide triphosphates and purification of the resulting T7 runoff tRNA transcripts by urea gels were performed as described (27, 28).

Yeast Strains, Media, and Microbiological Techniques—The wild type yeast strain used in this study was RS453 (*α/a, ade2/ade2, leu2/leu2, ura3/ura3, his3/his3, trp1/trp1*), and all mutant strains containing disrupted genes were derived from this one. Yeast cells were grown on minimal SDC and rich YPD medium (37), and sporulation of diploid cells on YPA plates and tetrad analysis were performed according to Ref. 29. Minimal SDC medium/plates were supplemented by all amino acids and nutrients except the ones used for the selection or, if indicated, contained 5-fluoroorotic acid (CSM medium, BIO 101, La Jolla). Genetic manipulations were performed as described (30).

Cloning and Expression of Yeast Deg1p in E. coli—Preparation of the construct for expression of Deg1 in yeast was done as follows. The *DEG1* gene was amplified by PCR from total yeast genomic DNA using two primers that created an *Xba*I restriction site in the 5′-untranslated region of the gene 151 nucleotides upstream of the start codon (tttttctagAATCAATGGGCTCAGCTC, complementary sequence in capital letters) and an *Sal*I restriction site (tttttgtcgacAAGAAATATAGTCTTCAAGG) in the 3′-untranslated region of the gene 50 nucleotides downstream of the stop codon. This allowed cloning of the gene into a pRS315 vector previously cut with *Xba*I/*Sal*I. This construct could complement the slow growing phenotype when transformed in the *deg1*$^-$ haploid cells.

The construct for expression of His$_6$-tagged recombinant Deg1 in *E. coli* was prepared by the following procedure. The *DEG1* ORF was amplified by PCR from total yeast genomic DNA using two primers that created an *Xho*I restriction site at the ATG start codon (aaaaactcgagcAGTAATTTCATTAGAAGGCTAG) and an *Mlu*I restriction site (aaaaaacgcgtAAGAAATATAGTCTTCAAGG) in the 3′-untranslated region of the gene. This manipulation allowed cloning of the ORF into a modified pET (pET-His$_6$/pET8c) vector previously cut with *Xho*I/*Mlu*I and created an in-frame fusion protein of 6 histidine residues joined by a spacer Ser-Ser dipeptide to the amino acid immediately after the start methionine. The vector containing the fusion gene was transformed into *E. coli* BL21(DE3) cells.

Gene Disruption of DEG1—Disruption of the *DEG1* gene was done by the one-step gene replacement method (31). In this study, the *DEG1* gene was disrupted by inserting a *Bam*HI fragment 0.9 kilobases long and containing the *HIS3* gene into the *Bam*HI site of the *DEG1*-ORF cloned in the pET8c vector. The disrupted gene was excised and the linear fragments used to transform the diploid strain RS453. *HIS*$^+$ transformants with the correct integration of the interrupted gene at the *DEG1* locus were verified by PCR analysis (data not shown). Correct integrants were sporulated, and tetrads were dissected. A 4:0 segregation for viability and a 2:2 segregation for the *HIS* marker were found for the *DEG1* gene disruption showing that this gene is not essential for cell growth. However, all spores carrying a disrupted *DEG1* gene grew slower giving rise to small colonies as reported previously (21).

Construction of Doubly Disrupted Mutants—To construct a haploid yeast strain in which the disrupted *PUS1* and *DEG1* genes are combined, a *PUS1*$^-$ mutant harboring pURA3-PUS1 was mated to the mutant *DEG1*$^-$. The resulting heterozygous diploids were sporulated, and tetrad analysis was performed. For complete tetrads in which the *HIS*$^+$*/his*$^-$ genotype segregates 2:2, one can predict that the two HIS$^+$ progeny are *deg1*::*HIS3*/*pus1*::*HIS3*. A complete tetrad showing this segregation pattern was analyzed in greater detail for the segregation of the *HIS3* and *URA3* markers by plating cells on SDC-his and SDC-ura plates, respectively. The HIS$^+$ progeny *deg1*::*HIS3*/*pus1*:*HIS3* also contained the plasmid pURA3-PUS1; we could shuffle out this plasmid and test whether the double mutant *pus1*$^-$*/deg1*$^-$ gives synthetic lethality by plating this strain on 5-fluoroorotic acid containing plates at 30 °C. The los1$^-$*/deg1*$^-$ strain was constructed in a similar way.

Construction of the Deg1p Fusion Protein Carrying Protein A or Green Fluorescent Protein (GFP) as a Tag—Epitope tagging of Deg1p was done by fusing two IgG binding units from *Staphyloccus aureus* protein A to the N-terminal end of Deg1p. For this gene fusion, a new *Pst*I restriction site was generated at the ATG codon of *DEG1* by PCR-mediated mutagenesis, and the ORF was subcloned into the plasmid pRS315 in-frame with the two IgG binding units under the control of the *NOP1* promoter (P$_{Nop1}$-ProtA cassette; see Ref. 32), creating the plasmid pNOP-ProtA-Deg1. Affinity purification of the ProtA fusion protein was done as described previously (32). Tagging with GFP was done in a similar way, but the IgG binding units were replaced by the ORF for GFP (P$_{Nop1}$-GFP cassette),[2] creating the plasmid pNOP-GFP-Deg1. The GFP used is a S65T/V163A variant exhibiting enhanced fluorescence properties (33, 34).

Cellular Localization of DEG1 Gene Product—Intracellular localization of the ProtA-Deg1p fusion protein was performed by indirect immunofluorescent microscopy as described in Ref. 9 using as first antibody rabbit anti-protein A (Sigma) and as second antibody Cy™3-conjugated AffiniPure donkey anti-rabbit IgG (Dianova). GFP-Deg1p was observed in living cells by direct fluorescent microscopy.

Pseudouridine Formation Assay in Vitro—Preparation of S100 extract from yeast as well as from *E. coli* was made as described elsewhere (25, 28). The activity of yeast extracts and purified yeast enzyme fractions was tested at 30 °C; 37 °C was used for testing *E. coli* extracts. The

[2] K. Hellmuth and E. C. Hurt, unpublished data.

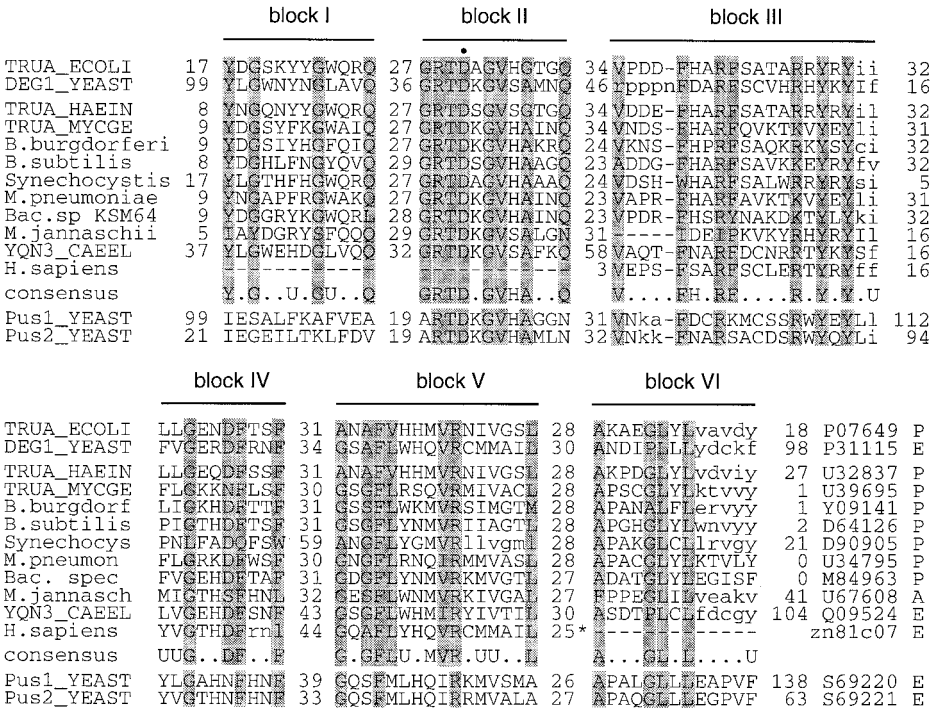

FIG. 1. **Conserved sequence motifs in a family of *truA*-like proteins.** Putative pseudouridine synthases I ($\Psi_{38/39/40}$) from *Haemophilus influenzae* (U32837), *Mycoplasma genitalium* (U39695), *B. burgdorferi* (Y09141), *Bacillus subtilis* (D64126), *Synechocystis* sp. (D90905), *Mycoplasma pneumoniae* (U34795, putative 28-kDa protein), *Bacillus* sp. (M84963, ORF 5′-adjacent to endo-1,4-β-glucanase gene), *Methanococcus jannaschii* (U67608), and *Caenorhabditis elegans* (Q09524) are aligned with tRNA:$\Psi_{38/39/40}$ synthase from *E. coli* (TRUA_ECOLI, P07649) and *Saccharomyces cerevisiae* (Deg1, P31115). Higher eukaryotes are presented by the partial nucleotide sequence of human (zn81c07.s1) Deg1-like protein. Yeast pseudouridine synthases Pus1 and Pus2 are presented on the *bottom*. The number of amino acid residues between blocks and from the protein termini are indicated by *numbers*. The *consensus line* shows the domains of amino acids that are conserved in the majority of sequences. Universally or highly conserved amino acid residues are *shaded*. *U* in the *consensus line* indicates a hydrophobic residue. The invariant aspartic acid residue (D) in block II is indicated by *dot*. The accession numbers of each sequence and their respective kingdom (*P* for prokaryotes, *A* for archaea, *E* for eukaryotes) are given at the end of each *line*. The abbreviated names of organisms corresponds to that in SwissProt.

incubation mixture contained 100 mM Tris-HCl, pH 8.0, 100 mM ammonium acetate, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, and 1–2 fmol of ^{32}P-radiolabeled T7 runoff transcripts as substrate. After incubation, the pseudouridine content in the radiolabeled transcripts was analyzed as described previously (27, 28). In brief, the RNA was first extracted with phenol-chloroform, precipitated in ethanol, and then hydrolyzed completely to 3′-nucleotide monophosphates by RNase T2. Each hydrolysate was chromatographed on two-dimensional thin layer chromatography plates, and the radioactivity in the ΨMP and UMP spots was evaluated after exposing the thin layer chromatography plates with a PhosphorImager screen. Taking into account the relative number of ΨMP and UMP in the tRNA molecule, the relative amount of Ψ over tRNA molecule (expressed in mol/mol of tRNA) can be evaluated. The accuracy of this method was found to be about ± 0.05 mol of Ψ/mol of tRNA.

Identification and Localization of Naturally Occurring Ψ Residues in tRNAs—Localization of pseudouridine residues in tRNA was performed as described (35, 36) with the following modifications. 10 μg of total tRNA extracted from wild type or mutant yeast strain was treated by 0.17 M CMCT in Bicine-urea buffer, pH 7.5, for 15 min at 42 °C. Reverse transcription was done at 42 °C for 45 min using about 1 μg of CMCT-modified cytoplasmic tRNA or 3 μg of CMCT-modified mitochondrial tRNA and 1–2 pmol of 5′-^{32}P-labeled synthetic oligodeoxynucleotide primer. Reverse transcription products were separated on 15% denaturing polyacrylamide gel. The oligodeoxynucleotide primers were chosen to be complementary to the 18 nucleotides at the 3′-end of cytoplasmic $tRNA^{Gly}$ and mitochondrial $tRNA^{Arg}$, respectively, because both of these tRNAs have a low content of modified nucleotides downstream from the pseudouridylation sites.

Miscellaneous—Isolation of total yeast DNA was done essentially as described in Ref. 37. DNA and plasmid manipulations (restriction analysis, end filling reactions, ligations, PCR amplifications, DNA fragment recovery, and small scale and large scale plasmid preparations) were done essentially according to standard procedures (38). The nucleotide sequence of tRNA inserts in the various plasmids used was verified systematically by the dideoxy sequencing technique. Evaluation of the protein concentration was done according to Bradford (39), and Western blotting was performed as described in Ref. 9.

Screening of the nonredundant GenBank data base was performed using BLAST algorithm, version 1.4.9; amino acid substitution matrix BLOSUM62 was used in all data base searches. Multiple sequence alignment was constructed using Macintosh version of MACAW software, version 2.0.5.

RESULTS

Yeast Protein Deg1 Displays Significant Sequence Homology with E. coli truA and Yeast Pus1—The accessibility of whole genome sequencing data for several prokaryotic and eukaryotic organisms allows the systematic screening for the corresponding enzymes based on sequence homology. BLAST search, using the *hisT*(*truA*) gene of *E. coli* (coding for tRNA:Ψ_{38-40} synthase; see Ref. 15) as a query sequence, allows detection of several homologous proteins in the GenBank. Partial sequence alignment of *hisT*(*truA*)-like proteins of different origin is presented on Fig. 1. These are mostly putative pseudouridine synthases I (PSU-I) from several bacteria and lower eukaryotes. Higher eukaryotes are presented by the partial nucleotide sequence of human (zn81c07.s1) Deg1-like protein. All of these putative proteins display significant sequence homology with *E. coli truA* (BLAST *p* value is less than 0.001). All proteins share common signature blocks of amino acid, which suggests the same or similar function in the cellular metabolism. Here it is noteworthy to remember that the yeast *PUS1* gene also shares high homology with the *truA*(*hisT*) gene, but the corresponding RNA:Ψ synthase has a different specificity and properties (9). Also, as already noted by Koonin (40), this group of *hisT*(*truA*)-like enzymes is rather distant from the pseudouridine synthases identified so far, specific, respectively, for Ψ_{55} (*E. coli truB*-like), for Ψ_{32} in tRNA and Ψ_{746} in 23 S rRNA (*E. coli rluA*-like), and for Ψ_{516} in *E. coli* 18 S rRNA.

From the above sequence homology one can expect that protein Deg1 has enzymatic activity similar to that of *E. coli* truA.

Disruption of Deg1 Gene Results in the Disappearance of tRNA:$\Psi_{38/39}$ Enzymatic Activity in the Yeast Cell Extract—To test the function of yeast Deg1 protein, we performed one-step gene disruption of the corresponding gene in yeast. The resulting yeast strain remains viable, but the deg1$^-$ mutant grows slower compared with parent wild type strain cells as was reported previously (21). Furthermore, we observed that the slow growth phenotype is particularly strong at 37 °C (Fig. 2). This thermosensitive phenotype and growth defect of the deg1$^-$ strain could be fully complemented by expressing the cloned *DEG1* gene or the fusion proteins ProtA-Deg1p and GFP-Deg1 (Fig. 2 and below).

The cell-free extracts (S100) obtained from *DEG1*-disrupted and from the wild type yeast strains (used as a control) were tested for pseudouridine synthase activity using several synthetic tRNA transcripts as substrates. Taking advantage of 11 different yeast tRNA transcripts labeled internally by appropriate α-^{32}P-NTPs (see "Experimental Procedures"), 10 potential pseudouridylation sites in tRNA molecules were tested (Table I).

The results indicate that activities for the formation of Ψ_{13}, Ψ_{27}, Ψ_{28}, Ψ_{32}, Ψ_{34}, Ψ_{35}, Ψ_{36}, and Ψ_{55} are present in both the wild type and the mutant yeast extracts. Only the pseudouridine synthase activity toward of U_{38} and U_{39} was lacking in the extract of the *DEG1*-disrupted strain, whereas the same uridine residues were modified quantitatively to pseudouridines when the extract of the wild type strain was used (Table I and Fig. 3). From these results, one can conclude that the disruption of the *DEG1* gene interferes with the enzymatic formation of both Ψ_{38} and Ψ_{39} in anticodon branch of tRNAs.

$\Psi_{38/39}$ Synthase Activity Resides in the Deg1 Protein—To show that Deg1p is solely responsible for the modifications missing in the *DEG1*-disrupted strain, the recombinant protein was expressed in *E. coli* and its activity tested *in vitro*. The results show that the activity of tRNA:$\Psi_{38/39}$ synthase is readily detected in the extract of *E. coli* expressing His_6-Deg1 and also upon the fractionation of the induced extract on Ni^{2+}-nitrilotriacetic acid-agarose. Activity of the expressed yeast pseudouridine synthase was retained considerably by metal affinity column, and activity toward Ψ_{38} in yeast tRNA^{Ala} was detected only in the case of induced *E. coli* extract. Similar results were obtained using purified by S-Sepharose FF, hydroxyapatite, and Ni^{2+}-nitrilotriacetic acid-agarose column recombinant Deg1p (more than 95% purity), which efficiently catalyzes the pseudouridine formation in the transcripts of yeast tRNA^{Phe} (Ψ_{39}) and tRNA^{Ala} (Ψ_{38}) (data not shown).

To confirm the above data obtained with Deg1p expressed in *E. coli*, we created a ProtA-Deg1p fusion protein by tagging to the N terminus of Deg1p with the IgG binding domain derived from *S. aureus* protein A. This fusion protein, which is functional since it can complement the slow growth phenotype of the disrupted strain (see Fig. 2), was expressed in *deg1*$^-$ cells, and total cell extract was passed through an IgG-Sepharose column. The only protein that bound to the column was ProtA-Deg1p as shown by elution at low pH (Fig. 4*A*). The suspension of IgG-Sepharose beads with bound ProtA-Deg1 has been used as a source of enzyme and tested under standard conditions using the transcripts of yeast tRNA^{Ala} (for Ψ_{38} formation) and yeast tRNA^{Phe} mutant (PheY55) (for Ψ_{39} formation). Two independently prepared suspensions were fully active in reactions leading to both Ψ_{38} and Ψ_{39} formation. This experiment

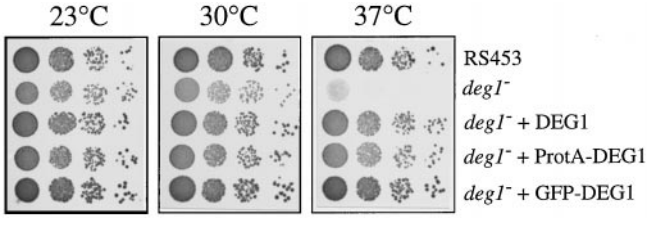

FIG. 2. **Disruption of *DEG1* leads to slow growth and a temperature-sensitive phenotype.** Serial dilutions of wild type strain (RS453), *deg1*$^-$ strain, and *deg1*$^-$ strain transformed by plasmids encoding for Deg1p or N-terminally tagged forms were spotted on YPD plates and incubated for 3 days at the indicated temperatures.

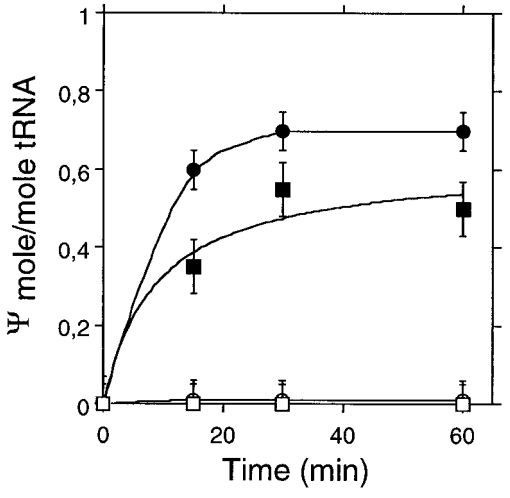

FIG. 3. **Time courses of Ψ_{38} and Ψ_{39} formation in the extract of wild type (*filled symbols*) and *DEG1*-disrupted yeast strains (*open symbols*).** Pseudouridine formation was measured using as substrates the transcript of yeast tRNA^{Ala} for Ψ_{38} formation (*squares*) and tRNA^{His} for Ψ_{39} formation (*circles*).

TABLE I
Pseudouridine formation in various tRNA transcripts incubated with extracts of wild type and DEG1-disrupted yeast strain

Position of Ψ	tRNA transcript	Labeling	mol Ψ/mol tRNA	
			Wild type	*DEG1*$^-$
Ψ_{13}	Yeast tRNA^{Asp}	ATP	0.30	0.30
Ψ_{26} + Ψ_{27}	Yeast tRNA^{Trp} mutant (CUA)	UTP	1.20	1.30
Ψ_{28} + **Ψ_{39}** + Ψ_{55}	Yeast tRNA^{Trp} mutant (CUA)	CTP	**2.15**	**1.55**
Ψ_{32}	Yeast tRNA^{Ser}	UTP	0.40	0.20
	Yeast tRNA^{Val} mutant (CAU)	UTP	0.20	0.15
	Yeast tRNA^{His}	UTP	0.85	0.85
Ψ_{34} + Ψ_{36}	Yeast pre-tRNA^{Ile}	ATP	1.75	1.80
Ψ_{35}	Yeast pre-tRNA^{Ile} mutant (UUA)	ATP	0.65	0.75
	Yeast pre-tRNA^{Tyr} mutant (UUA)	ATP	0.25	0.20
Ψ_{38}	Yeast tRNA^{Ala}	GTP	**0.50**	**<0.05**
Ψ_{39}	Yeast tRNA^{His}	GTP	**0.70**	**<0.05**
	Mut yeast tRNA^{Phe} (PheY55)	CTP	**0.85**	**<0.05**
Ψ_{39} + Ψ_{55}	Yeast tRNA^{Phe}	CTP	**1.70**	**1.00**
Ψ_{55}	Yeast tRNA^{Asp}	CTP	0.80	0.95

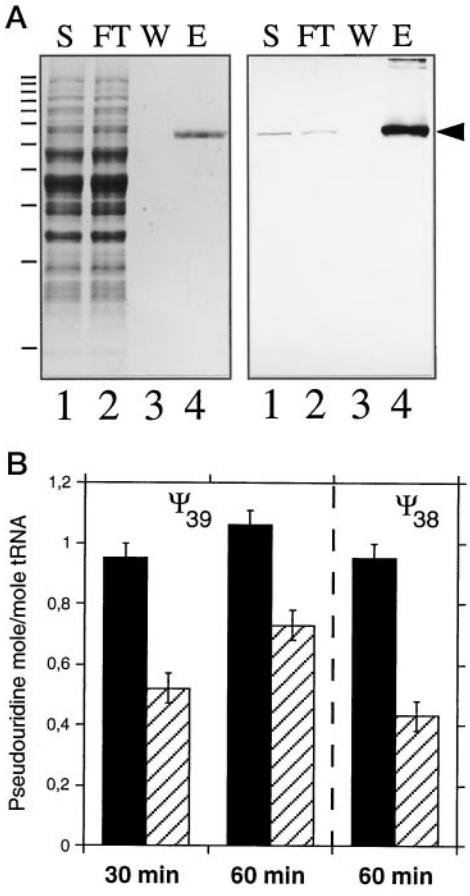

FIG. 4. *Panel A*, affinity purification of ProA-Deg1p by IgG-Sepharose chromatography. Fractions of the soluble total cell extracts (*S*), the column flow-through (*FT*), the pH 5 wash (*W*), and the pH 3.4 eluate (*E*) were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining (*left panel*) or immunoblotting with antibodies that react with the protein A moiety of the fusion protein (*right panel*). *Panel B*, Ψ_{39} and Ψ_{38} formation in mutant yeast tRNA^{Phe} (PheY55) and yeast tRNA^{Ala} using the suspension of IgG-Sepharose with bound ProtA-Deg1 fusion as the source of enzymatic activity. Time points at 30 and 60 min are presented for Ψ_{39} formation by two independent preparations of ProtA-Deg1 (shown as *filled* and *shaded bars*). Only the 60-min point was measured for Ψ_{38} formation.

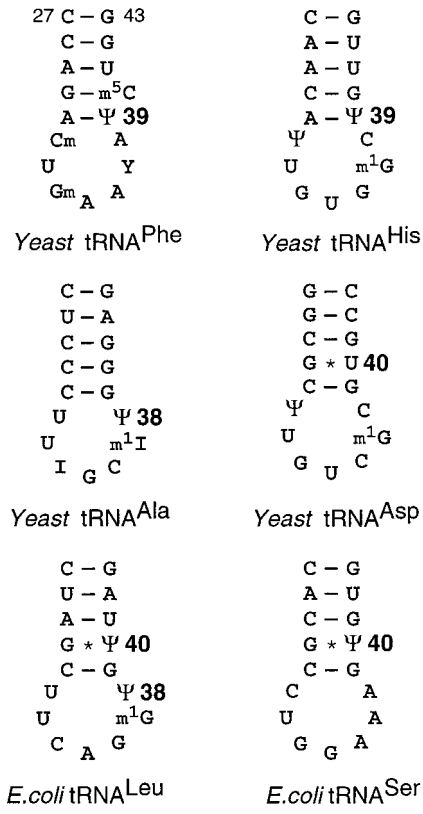

FIG. 5. **Anticodon stem-loops of naturally occurring yeast and *E. coli* tRNAs.** In this study we used as substrates the corresponding runoff transcripts, lacking all modified nucleotides.

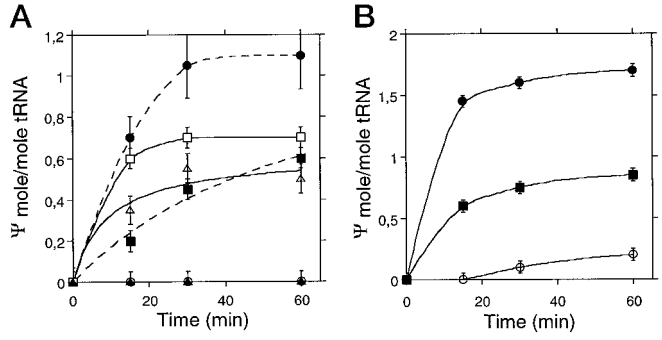

FIG. 6. *Panel A*, time courses of Ψ_{38}, Ψ_{39}, and Ψ_{40} formation in the transcripts of yeast tRNA^{Ala} (*triangles*), yeast tRNA^{His} (*squares*), and *E. coli* tRNA^{Ser} (*circles*) incubated, respectively, with yeast (*open symbols*, *solid lines*) and *E. coli* extracts (*filled symbols*, *dashed lines*). *Panel B*, time courses of pseudouridine Ψ_{39} formation in a wild type and mutants of yeast tRNA^{Phe}(GAA). Wild type yeast extract was used as the source of enzyme. Wild type tRNA^{Phe} (●) and mutants PheY54 (○) and PheY55 (■).

confirms that the activity of yeast tRNA:$\Psi_{38/39}$ synthase resides solely in Deg1 protein (Fig. 4*B*).

Specificity of Yeast and E. coli tRNA:Ψ$_{38-40}$ Synthases Is Different—We compared the specificity of the yeast and *E. coli* enzymes using the transcripts of various yeast and *E. coli* tRNAs. Fig. 5 shows the anticodon stem-loop regions of the naturally occurring tRNAs, the transcripts of which were tested for the formation of Ψ_{38}, Ψ_{39}, and Ψ_{40}. As shown in Fig. 6*A* (see also Table II), the transcript of yeast tRNA^{His} (anticodon GUG) is modified efficiently to pseudouridine at position 39 upon the incubation in yeast or *E. coli* extract.

Interestingly, the transcript of yeast tRNA^{Ala} remained completely unmodified while incubated with heterologous *E. coli* extract. However, the Ψ_{38}-forming activity was present in the extract as detected using the transcript of homologous *E. coli* tRNA^{Leu} (Table II). This clearly demonstrates the existence of species specificity for Ψ_{38} formation. The corresponding yeast enzyme is apparently less specific than the *E. coli* one, as a low but significant level of Ψ_{38} formation was detected upon the incubation of *E. coli* tRNA^{Leu} in heterologous yeast extract. Therefore, the use of yeast tRNA^{Ala} allows the reliable detection of yeast tRNA:Ψ_{38} synthase activity even in the presence of the corresponding enzyme from *E. coli*. Thus the enzymatic activity toward Ψ_{38} as detected in the case of expressed in *E. coli* His$_6$-tagged Deg1p (see above) undoubtedly belongs to the expressed yeast protein and not to endogenous *E. coli* truAp.

No activity for U_{40} modification was detected in the yeast extract using *E. coli* tRNA^{Ser}, naturally bearing Ψ_{40} (Fig. 6*A*). Interestingly, U_{40} in yeast tRNA^{Asp}, when incubated with *E. coli* extract, becomes converted to Ψ_{40}, albeit at low rate compared with the rate of U_{40} modification in the transcript of *E. coli* tRNA^{Ser} (Table II). These results provide the evidence that the substrate specificity of yeast and *E. coli* tRNA:$\Psi_{38/39/(40)}$ synthases is different.

Deg1p Activity Is Sensitive to tRNA Three-dimensional Structure—Yeast tRNA^{Phe} (anticodon GAA) is an excellent experimental model to study the influence of overall three-dimensional tRNA structure on the modification reaction. This tRNA naturally contains 14 modified nucleosides, among them two

TABLE II
Pseudouridine formation in various tRNA transcripts incubated with S100 extracts from yeast and E. coli

Ψ	tRNA transcript	Labeling	mol Ψ/mol tRNA	
			S. cerevisiae S100 extract	*E. coli* S100 extract
Ψ_{39} + Ψ_{55}	Yeast $tRNA^{Phe}$	CTP	1.70	ND[a]
Ψ_{39}	Mut $tRNA^{Phe}$ (PheY54)	CTP	0.20	ND
	Mut $tRNA^{Phe}$ (PheY55)	CTP	0.85	ND
Ψ_{39}	Yeast $tRNA^{His}$	GTP	0.70	0.60
Ψ_{38}	Yeast $tRNA^{Ala}$	GTP	0.50	<0.05
	E. coli $tRNA^{Leu}$	GTP	0.1[b]	0.55
Ψ_{40}	*E. coli* $tRNA^{Ser}$	GTP	<0.05	1.10
	Yeast $tRNA^{Asp}$	GTP	<0.05	0.40

[a] ND, not determined.
[b] A small but significant level of Ψ38 modification was detected.

pseudouridine residues (at positions 39 and 55). The set of $tRNA^{Phe}$ mutants with well defined disruptions in tertiary interactions was also available (26). As shown in Fig. 6*B* and Table II, almost 1.7 mol of Ψ/mol of $tRNA^{Phe}$ transcript was formed upon the incubation with the yeast extract.

When the transcript of $tRNA^{Phe}$ mutant (PheY54) bearing the mutation (C56G) in the TΨ loop was incubated with yeast extracts formation of Ψ_{39} could be detected only to a very low level (Fig. 6*B* and Table II). This low modification level is most probably the result of the disruption of tertiary interactions (G19 . . . C56) that stabilize the three-dimensional tRNA architecture (26). For this reason we tested another mutant of yeast $tRNA^{Phe}$ (PheY55) bearing a compensatory mutation at position 19 (G19C) which restores tertiary interactions and consequently the correct tRNA structure (26). The resulting tRNA transcript became an excellent substrate for yeast tRNA:Ψ_{39} synthase present in the extract (Fig. 6*B*).

In agreement with the observed sensitivity of Ψ_{39} formation to correct tRNA folding (see above), the results obtained with RNA minisubstrate consisting of $tRNA^{Phe}$ anticodon loop (19-mer) show the complete absence of Ψ_{39} formation (see also Ref. 27). Likewise, the same stem-loop minisubstrate but prolonged by a 19-nucleotide intron (36-mer) also remains unmodified (data not shown).

In summary, the yeast tRNA:Ψ_{39} synthase appears to be extremely sensitive to global tRNA structure and, in contrast, to some other tRNA:pseudouridine synthases (*e.g.* specific for Ψ_{32} and Ψ_{55}; Ref. 41), it does not recognize and modify the uridine residues within stem-loop minisubstrates.

Intracellular Location of Deg1p—The subcellular localization of ProtA-tagged Deg1p in yeast cells was analyzed by indirect immunofluorescence microscopy using tag-specific antibodies and by direct fluorescence microscopy using a GFP-tagged version of Deg1p which could also complement the *deg1*$^-$ strain. In both cases a specific intranuclear signal could be detected, but the cytoplasm was also diffusely stained (Fig. 7). These results show that Deg1p resides in both the nucleus and the cytoplasm.

Pseudouridine Residues Ψ_{38} and Ψ_{39} Are Absent in tRNA from Deg1$^-$ *Strain*—The cellular localization of Deg1p studied by immunofluorescence techniques reveals the presence of nuclear and cytoplasmic pools of the protein but leaves open the question about its presence in yeast mitochondria. To answer this question we performed the analysis of pseudouridine residues present (or absent) in tRNAs extracted from wild type and *DEG1*-disrupted strain. Chemical mapping of pseudouridines was done on total tRNA fraction by CMCT-reverse transcription technique, as described previously (35). The synthetic oligonucleotides, complementary to the last 18–20 3′-nucleotides in tRNA, were used for primer extension analysis using unmodified and CMCT-treated tRNA. The results of reverse

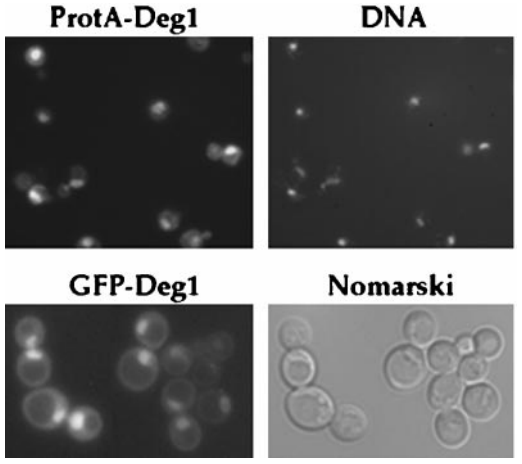

FIG. 7. **Cellular localization of Deg1.** ProtA-Deg1p was localized by indirect immunofluorescent microscopy. The same sample was also stained for DNA. GFP-Deg1p was observed in living cells by direct immunofluorescent microscopy. The same cells are also shown by Nomarski optics.

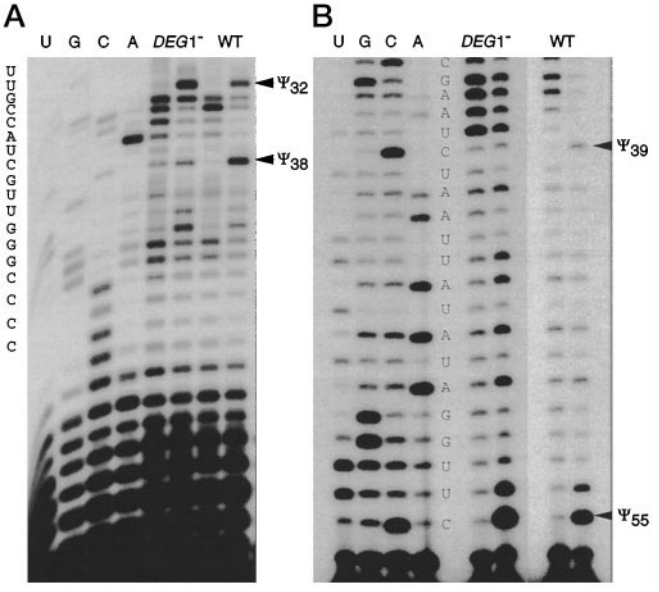

FIG. 8. **Chemical mapping of pseudouridine residues in cytoplasmic $tRNA^{Gly}$ (*panel A*) and mitochondrial $tRNA^{Arg}$ (*panel B*) from wild type and *DEG1*-disrupted yeast strains.** Autoradiographies of reverse transcription products in 15% polyacrylamide and 8 M urea gels are shown. The strong stops (shown by *arrows*) in reverse transcription of tRNA correspond to pseudouridine residues. The corresponding tRNA sequencing is shown on the *left* of each gel. Control tRNA samples and samples of tRNA treated by CMCT followed by sodium bicarbonate hydrolysis are presented side by side.

transcription for cytoplasmic $tRNA^{Gly}$ (anticodon GCC) and mitochondrial $tRNA^{Arg}$ (anticodon ACG) extracted from wild type and mutant strains are presented in Fig. 8, *A* and *B*. The strong reverse transcription stops corresponding to Ψ_{38} in cytoplasmic $tRNA^{Gly}$ and Ψ_{39} in mitochondrial $tRNA^{Arg}$ are totally absent in the mutant *DEG1*-disrupted strain (indicated by *arrows*). The formation of other pseudouridines naturally present in these two tRNAs (Ψ_{32} and Ψ_{55} in cytoplasmic and mitochondrial tRNA respectively) is not affected by *DEG1* gene disruption. These results confirm the absolute requirement of Deg1 protein for modification of the pool of cytoplasmic tRNAs and reveal that the product of the same gene *DEG1* participates also in pseudouridine formation in mitochondrial tRNAs.

Deg1p Is Not Linked Genetically to Pus1p or Los1p—We have shown previously that Pus1p interacts genetically with the

nuclear pore-associated proteins Nsp1p and Los1p (9), suggesting that tRNA modification may be linked to tRNA nuclear export. To test this possibility for Deg1p, we combined the *deg1* disruption with the *los1* or *pus1* disruption by mating the corresponding strains and performing tetrad analysis. The double-disrupted haploid strains *los1*$^-$*/deg1*$^-$ and *pus1*$^-$*/deg1*$^-$ were viable and did not exhibit any further growth defect than the single-disrupted *deg1*$^-$ strain (data not shown). Therefore, the functional interaction with the nuclear pore complex appears to be specific for Pus1p and does not occur in the case of Deg1p, suggesting that modification only at particular sites is important for the tRNA transport process. Furthermore, the viability of the *pus1*$^-$*/deg1*$^-$ strain shows that yeast cells can tolerate the absence of Ψ modification in positions 27, 34, 36, 38, and 39. Thus, despite the different specificity of Pus1p and Pus3p there is no obvious synergism between them.

DISCUSSION

Yeast Protein Deg1 Is tRNA:Ψ$_{38/39}$ Synthase—Direct evidence that the yeast Deg1 protein is a tRNA:pseudouridine synthase comes from two types of experiments: by detecting the corresponding enzymatic activity in S100 extracts of a transformed *E. coli* strain harboring the yeast *DEG1* gene and by measuring the enzymatic activity of a ProtA-Deg1 fusion protein purified from a *deg1*$^-$ yeast strain. In both cases, the protein catalyzes *in vitro* formation of pseudouridines at position 38 or 39 of several tRNA transcripts. Additional evidence came also from identification of the lacking pseudouridines at position 38 or 39 in cytoplasmic tRNA^{Gly} and in mitochondrial tRNA^{Arg} present in *DEG1*-disrupted yeast strain. As predicted previously (21), Deg1p is indeed the yeast homolog of *E. coli* tRNA:Ψ synthase I (PSU-I, also called truA, initially discovered as *hisT* gene product). Therefore, after Pus1 and Pus2 (9), this is the third tRNA:Ψ synthase (PseudoUridine Synthase, Pus3) so far characterized in yeast.

Yeast Pus3 and E. coli truA Display Different Substrate Specificity—Testing several tRNA substrates allowed us to reveal subtle differences in substrate specificity between yeast Deg1p and *E. coli* truAp. Indeed, although both enzymes display rather good cross-reactivity toward uridine 39 in two different tRNA transcripts (tRNA^{Phe} and tRNA^{His}), the situation for Ψ$_{38}$ and Ψ$_{40}$ formation was different. *E. coli* truA modified U$_{40}$ fairly well in both *E. coli* tRNA^{Ser} and yeast tRNA^{Asp}, whereas the yeast Deg1p did not modify U$_{40}$ at all in the same tRNA transcripts. This observation fits well with the fact that U$_{40}$ in naturally occurring yeast tRNA^{Asp} is not modified into Ψ, but in *E. coli* all U$_{40}$-containing tRNAs bear Ψ$_{40}$ (42). This absence of Ψ$_{40}$ is valid only for tRNAs from fungi and does not apply for tRNAs from higher eukaryotes, where few cases of Ψ$_{40}$-containing tRNAs were found (42). However, the above observation for U/Ψ$_{40}$ relationship in yeast tRNAs is valid for both yeast cytoplasmic and the mitochondrial tRNAs, as in cytoplasm, several naturally occurring yeast mitochondrial tRNAs contain Ψ$_{38}$ or Ψ$_{39}$ but not Ψ$_{40}$. This is consistent with the fact that the same gene product modifies all tRNA substrates in the different cellular compartments in yeast (nucleus, cytoplasm, and mitochondria; see below).

In transcripts of yeast tRNA^{Ala} and *E. coli* tRNA^{Leu}, U$_{38}$ was modified with different efficiency by yeast or *E. coli* enzymes. Yeast enzyme catalyzes the Ψ$_{38}$ formation in both transcripts, whereas the *E. coli* homolog is exclusively specific toward *E. coli* tRNA. Indeed, inspecting the tRNA sequence data bank (42) we notice that all four yeast U$_{38}$-containing tRNAs are fully modified to Ψ$_{38}$, but in *E. coli* only five out of eight U$_{38}$ are converted to Ψ$_{38}$. It is interesting to note that all five Ψ$_{38}$-containing *E. coli* tRNAs bear G$_{36}$ in the anticodon, whereas the other unmodified U$_{38}$-containing *E. coli* tRNA have a C$_{36}$. Therefore the presence of a G$_{36}$ (or C$_{36}$) could act as a positive (or negative) determinant for *E. coli* truAp.

Because all U$_{38}$- or U$_{39}$-containing cytoplasmic tRNAs in yeast (22 out of 34 sequenced so far (42)), despite very different nucleotide sequences, are modified to Ψ$_{38}$/Ψ$_{39}$, this suggests that there might be no other essential nucleotides (identity elements) needed for the recognition by Deg1 enzyme.

In contrast, the overall three-dimensional structure of tRNA molecule seems to be important for tRNA recognition. Indeed, the tRNA^{Phe} mutant with disrupted interaction between TΨ and D loops was a rather weak substrate compared with the wild type tRNA^{Phe}. The double mutant restoring the three-dimensional pairing became again a good substrate for the yeast Deg1p. Moreover, none of the fragmented or minimalist tRNA^{Phe} served as a substrate for the yeast Deg1p. Thus, the enzyme catalyzing the formation of Ψ$_{38}$ or Ψ$_{39}$ in yeast tRNAs clearly belongs to the group II of tRNA modification enzymes, which are sensitive to three-dimensional perturbation of the tRNA architecture (41).

A Single Nuclear Gene DEG1 Provides the Enzyme for Three Cellular Compartments—Immunochemical studies demonstrate that Deg1 protein is mostly located in the nucleus, but a significant part of it is also found in the yeast cytoplasm. Moreover, the analysis of the pseudouridine tRNA modification pattern in the *DEG1*-disrupted strain demonstrates clearly that a single gene *DEG1* is responsible for the enzymatic formation of Ψ$_{38/39}$ in both cytoplasmic and mitochondrial tRNAs.

A similar situation has been already ascribed to a few other tRNA modification enzymes in yeast. The yeast tRNA modification enzyme Mod5p, which catalyzes the formation of isopentenyladenosine in the anticodon loop of several yeast tRNAs, apparently behaves similarly, and its cellular location resembles the one observed for Deg1p (43, 44). In both cases, the enzymes were located mainly in the nucleus, but a significant amount was also detected in the cytoplasm. In contrast, yeast Trm1p, the enzyme catalyzing the formation of N^2,N^2-dimethylguanosine 26 in several yeast tRNAs, was shown to be located at the nuclear internal periphery, and no significant signal was detected in the cytoplasm (45). The reason why Mod5p and Deg1p are present in both the cytoplasm and the nucleus but Trm1p as well as Pus1p (9) are almost exclusively nuclear is probably related to the temporal events in which these enzymes have to function during the complex tRNA maturation process.

Deg1p Belongs to a truA-like Family That Is Distinct from All Other RNA:Ψ Synthases Sequenced So Far—Comparison of the amino acid sequences of 12 *truA*-like proteins identified so far from different organisms reveals the presence of highly conserved residues (shown in *gray* in Fig. 1) within six blocks that are common to *all* proteins of the family. Interestingly, almost the same signature is present in Pus1p and Pus2p but is not found in any other protein of the whole protein data bank (SwissProt and GenBank). Only the sequence GRTD*X*GVHXG (block II in Fig. 1), bearing a conserved aspartic acid residue (indicated by a *dot* in Fig. 1), is similar to amino acid sequence G*X*RD*XXX*G (also referred as block II by Koonin (40)) present in all other RNA:pseudouridine synthases. As already proposed by Koonin, this universally conserved aspartic acid residue may be implicated in the enzymatic catalysis. Recent cross-linking studies and site-directed mutagenesis performed on *E. coli* tRNA pseudouridine synthase I (prokaryotic homolog of Pus3) clearly demonstrate that Asp-60 residue is essential for enzymatic activity (46). Successful crystallization and resolution of the structure of *E. coli* tRNA pseudouridine synthase I have also been reported (47), which should shed light on the catalytic mechanism of the uridine isomerization.

The fact that Pus1p and Deg1p (Pus3p) belong to the same

family (truA-like family) may be related to the RNA recognition mode. Indeed, both Pus1p and Deg1p are multisite-specific enzymes that catalyze the formation of pseudouridines in 1, 2, or 3 positions within a given region of tRNA (positions 27 and 28 as well as positions 34, 35, and 36 for Pus1p (9) or positions 38 and 39 for Deg1p; this paper).

In agreement with the recent observation that none of the cysteine residues present in *E. coli* truAp is required for its enzymatic activity (48), no conserved cysteine is detected in the alignment of the various truA-like proteins.

Disruption of the DEG1 Gene Influences Yeast Cell Growth—In yeast, gene *DEG1* was discovered incidentally by transcriptional analysis of the centromere region of yeast chromosome VI. This gene is located very close to the centromere and is expressed at only a low level. Its disruption, although not lethal, causes a pronounced slow growth phenotype (DEpressed Growth) (21). In this work, we show that this slow growth phenotype, which is much more pronounced at 37 °C, is entirely the result of the absence of Deg1 protein as it can be complemented by the corresponding gene or by clones expressing N-terminally tagged proteins. The absence of the Deg1p correlates with the absence of Ψ at positions 38 and 39 of tRNA.

The strong relationship between the reduced growth rate of *E. coli* or *S. typhimurium* and the absence of pseudouridines 38/39/40 in anticodon stem-loop of several tRNAs was observed more than 2 decades ago (49). This reduced growth rate was correlated with the derepression of several amino acid operons by an attenuation type of mechanism as demonstrated in the case of the *his* operon (50). Because different kinds of nonsense, missense, or frameshift tRNA suppressors were also affected by the *hisT*$^-$ mutation (reviewed in Ref. 51), it is now evident that major function of the *hisT* gene product in prokaryotes is related to protein synthesis on the ribosome. Thus, pseudouridylation of the anticodon loop and its proximal stem in prokaryotic tRNAs has a central role in regulation of cellular metabolism.

Yeast cells lacking pseudouridine 38/39 synthase activity (*DEG1*-disrupted strain) display slow growth phenotype, similar to the one observed for *E. coli* and the *Salmonella hisT* mutant. Therefore, as in prokaryotes, the pseudouridines in tRNA anticodon stem-loop are also important for the modulation of the translation process in yeast. Attenuation type of gene regulation cannot occur in eukaryotic cells because of the different compartmentalization of the transcription and the translation machinery. However, the absence of $\Psi_{38/39}$ in yeast tRNA may affect the rate of mRNA decoding on the ribosome.

An alternative explanation could be that deletion of *DEG1* gene affects primarily the mitochondrial translation process by prokaryotic-type attenuation mechanism giving rise the phenotype similar to the one observed in the case of mitochondrial disorders such as mioclonic epilepsy and ragged red fibers syndrome (MERRF) or mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS) (52).

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Characterization of Yeast Protein Deg1 as Pseudouridine Synthase (Pus3) Catalyzing the Formation of Ψ_{38} and Ψ_{39} in tRNA Anticodon Loop

François Lecointe, George Simos, Anke Sauer, Eduard C. Hurt, Yuri Motorin and Henri Grosjean

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