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The yeast gene YNL292w encodes a pseudouridine synthase (Pus4) catalyzing the formation of Ψ_{55} in both mitochondrial and cytoplasmic tRNAs

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ABSTRACT

The protein products of two yeast *Saccharomyces cerevisiae* genes (YNL292w and CBF5) display a remarkable sequence homology with *Escherichia coli* tRNA:pseudouridine-55 synthase (encoded by gene *truB*). The gene YNL292w coding for one of these proteins was cloned in an *E.coli* expression vector downstream of a His$_6$-tag. The resulting recombinant protein (Pus4) was expressed at high level and purified to homogeneity by metal affinity chromatography on Ni^{2+}–NTA–agarose, followed by ion-exchange chromatography on MonoQ. The purified Pus4p catalyzes the formation of pseudouridine-55 in T$_7$ *in vitro* transcripts of several yeast tRNA genes. In contrast to the known yeast pseudouridine synthase (Pus1) of broad specificity, no other uridines in tRNA molecules are modified by the cloned recombinant tRNA:Ψ_{55} synthase. The disruption of the corresponding gene YNL292w in yeast, which has no significant effect on the growth of yeast cells, leads to the complete disappearance of the Ψ_{55} formation activity in a cell-free extract. These results allow the formal identification of the protein encoded by the yeast ORF YNL292w as the only enzyme responsible for the formation of Ψ_{55} which is almost universally conserved in tRNAs. The substrate specificity of the purified YNL292w-encoded recombinant protein was shown to be similar to that of the native protein present in yeast cell extract. Chemical mapping of pseudouridine residues in both cytoplasmic and mitochondrial tRNAs from the yeast strain carrying the disrupted gene reveals that the same gene product is responsible for Ψ_{55} formation in tRNAs of both cellular compartments.

INTRODUCTION

Due to the success of large-scale genome sequencing projects (1–4), researchers are faced by the daunting task of having to assign biological functions to an ever-increasing number of newly identified (putative) proteins. One logical approach to this task is the systematic analysis of sequence homology between a novel polypeptide and protein(s) whose function has already been established experimentally. In many cases a novel polypeptide could be identified as a potential member of a functionally distinct protein family (5,6), thus providing guidance to experimental testing. Such a homology-based approach was recently employed to analyze the family of RNA:pseudouridine synthases with different substrate specificity (7) and for an iterative search for new RNA-modifying enzymes (RNA/DNA:methyltransferases and RNA:pseudouridine synthases) (8,9). As a result, 13 new genes encoding putative RNA-modification enzymes were identified in *Escherichia coli*. The activity and substrate specificity of some of the enzymes encoded by these genes were subsequently confirmed experimentally (8,10).

Four distinct families of homologous RNA:pseudouridine synthases were found based on homology with the four known *E.coli* enzymes designated *truA* (also known as *HisT*), *truB*, *rluA* and *rsuA*. The members of the first two groups specifically introduce Ψ in tRNA at positions 38, 39 and 40 (*truA*, 'region-specificity') (11) and position 55 (*truB*, 'site-specificity') (12), respectively. The enzyme encoded by the *rluA* gene catalyzes the formation of Ψ at position 32 of tRNA and at position 746 of *E.coli* 23S rRNA ('dual-specificity') (13). The last group (*rsuA*) appears to be specific to a unique Ψ_{516} in *E.coli* 16S rRNA complexed with 30S ribosomal proteins (14,15).

While the *truB* gene encoding tRNA:Ψ_{55} synthase is unique in the *E.coli* genome, the *Saccharomyces cerevisiae* genome contains two genes, CBF5 (16) and YNL292w (17) [SwissProt protein database entry ORF YNL292w (locus YN32_YEAST) accession number P48567, or Z71568 (EMBL protein database) was previously refered to as Ynl0480 (GeneBank accession number U23084) by Koonin (7)], that display significant sequence homology with *truB*. A similar situation exists for the tRNA:$\Psi_{38/39/40}$ synthase family, represented by a unique *truA* gene in prokaryotes (*E.coli*, *Bacillus subtilis*....), of which at least three homologs have been detected in yeast [*PUS1*, *PUS2* (18) and *DEG1* (7,18,19)]. However, the degree of homology between the prokaryotic and yeast genes in this case is lower than in the case of the *truB* family.

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Despite the sequence homology between the yeast *PUS/DEG* genes and *E.coli truA*, the substrate specificity of the various enzymes differs. Only the Deg1p (Pus3p) protein, which has the highest degree of homology with its prokaryotic counterpart, catalyzes pseudouridine formation at the same positions in tRNA (38/39) as the *E.coli* enzyme (20). Pus1p causes Ψ to be formed at positions 27 and 34/36 in intron-containing yeast tRNAs (18), while the specificity of the Pus2p enzyme remains to be established. These findings demonstrate that sequence homology can provide useful guidance in functional characterization of novel proteins but experimental verification remains necessary.

In this paper we present experimental evidence showing that the protein encoded by the yeast YNL292w gene is tRNA:Ψ_{55} synthase (Pus4). We have, therefore, designated this gene *PUS4*.

MATERIALS AND METHODS

Chemicals and enzymes

α-^{32}P-radiolabelled nucleotide triphosphates (400 Ci/mmol) were from Amersham (UK). Tris, DTT, nucleoside triphosphates, *Penicillium citricum* nuclease P1, *Aspergillus oryzae* RNAse T2, phenylmethylsulfonylfluoride (PMSF) and spermidine were from Sigma (USA), diisopropylfluorophosphate (DFP) from Boehringer-Mannheim (Germany), 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-*p*-toluenesulfonate (CMCT) from Aldrich (USA), bacteriophage T7 RNA polymerase, restriction enzymes and isopropyl-β-D-thiogalactoside (IPTG) from MBI Fermentas (Vilnius, Lithuania), RNasin and AMV reverse transcriptase from Promega (USA). Thin layer cellulose plates and nitrocellulose membranes BA85 were from Schleicher & Schuell (Germany), all other chemicals from Merck Biochemicals (Germany). Chemically synthesized deoxyoligonucleotides were purchased from MWG-Biotech (Germany). They were used without further purification.

Plasmids and *in vitro* transcription

The plasmids bearing various tRNA genes under the T_7-promoter of wild-type yeast $tRNA^{Phe}$ (GAA) (p67YF0) and its mutant PheY67 (p67YF92), bearing the mutations G30C, C40G, A31C and U39G in the anticodon stem (21,22), mutant yeast $tRNA^{Asp}$ (mutations U1G and A72C; 23), $tRNA^{Asp}$ with deleted D-stem–loop (24) and TΨ-stem–loop minihelices (25,26), yeast pre-$tRNA^{Ile}$ (27) and yeast $tRNA^{Val}$(CAU) (28) were described elsewhere. Yeast $tRNA^{Trp}$(CUA) was recloned by PCR amplification using the corresponding mutant genes (29,30) and inserted in pUC118 vector. The plasmids with the cloned domains of yeast ribosomal 25S RNA [domain 2, nucleotides 650–1453 (5′-nt = 1)] and domain 4, nucleotides 1869–2389, were provided by Prof. H.Raué (Vrije Universiteit, Amsterdam, The Netherlands).

T_7-polymerase transcription of tRNA genes using α-^{32}P-radio-labelled nucleotide triphosphates and purification of the resulting tRNA transcripts by urea gels were performed as described previously (31).

Yeast gene disruption

The gene YNL292w (initially designated YNL0480; 17) was disrupted in strain FY1679 (*Mat***a**/α; *ura3-52/ura3-52*; *leu2Δ1*/+; *trp1Δ63*/+; *his3Δ200*/+) by replacing the coding region with *kanMX4* by PCR-targeting of the *kanMX* marker cassette flanked by short regions homologous to the target locus (32). The marker cassette (in plasmid pFA6a-kanMX4; 32) is a hybrid of the coding sequence of the *kan*^{r} gene and transcriptional and translational control sequences from the *TEF* gene of the filamentous fungus *Ashbya gossypii*, and confers geneticin (G418) resistance on yeast.

The haploid strain FY73 (*Mat*α) with the deleted YNL292w gene was obtained by tetrad analysis of the heterozygous deletant. The strains were grown in YPD containing 200 µg/ml of geneticin.

PCR amplification and gene cloning

The gene YNL292w (17) was amplified from the pBluescript plasmid carrying this gene on a 4 kb *Eco*RI-generated fragment of yeast genomic DNA using two oligonucleotides, corresponding to the 5′-end (AAAACATATGaatggaatatttgctattg) and complementary to the 3′-end (AAAAAAGCTTttacacctgttcgatttttct) of the gene, respectively. The sequence of the part of the oligonucleotides corresponding to the gene YNL292w is shown in lower-case. The 5′-oligonucleotide contained the restriction site for *Nde*I (underlined), while the 3′ sequence contained a site for *Hin*dIII (underlined). The amplified PCR product was cleaved by the two restriction enzymes and inserted into the *Nde*I/*Hin*dIII sites of pET28b-plasmid downstream to the T_7-promoter (Stratagene, USA). The sequence of the insert was verified by DNA sequencing using the set of synthetic primers complementary to the gene YNL292w.

Expression of YNL292w-encoded recombinant protein in transformed *E.coli* BL21(DE3), bearing the T7 RNA-polymerase gene under control of the lac-UV5 promoter (33), was performed by IPTG induction in MM9 medium. *Escherichia coli* cells were grown at 37°C to an OD_{600nm} of ~0.7 and expression was induced by IPTG (1 mM) at 37°C for 2 h. All subsequent operations were performed at 4°C. Cells were disrupted by sonication (20 times 10 s each) in twice their weight of buffer A (50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10 mM β-mercaptoethanol, 0.1% Triton) plus 1 mM of PMSF and DFP. The homogenate was centrifuged at 10 000 *g* for 10 min. The resulting S10 was further fractionated into ribosomal pellet and supernatant S100 by centrifugation at 80 000 r.p.m. in a TL-100 ultracentrifuge (Beckman) (rotor TLA100.3) for 2 h. The supernatant was then directly applied onto a Ni^{2+}–NTA–agarose column (Qiagen, Germany) and the column was washed with buffer A without Triton, then with buffer A containing 20 mM imidazol. The recombinant protein was eluted with the same buffer A but containing 150 mM imidazol. Further purification to homogeneity was performed at room temperature by ion-exchange chromatography on MonoQ HR5/5 (Pharmacia, Sweden) in 25 mM Tris–HCl buffer, pH 8.8, 2 mM β-mercapto-ethanol, 10% glycerol, 1 mM $MgCl_2$, 2 mM EDTA (buffer B). Elution was performed by a linear gradient of NaCl from 0 to 0.3 M in buffer B in a total volume of 30 ml.

Enzymatic assay

The activity of yeast extracts (0.6 mg/ml) and of purified enzymatic fractions (0.1 µg/ml) was tested at 30°C (37°C for *E.coli* extract) in 100 mM Tris–HCl, pH 8.0, 100 mM ammonium acetate, 5 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA and 1–2 fmol of ^{32}P-radiolabelled T7-runoff transcripts as substrate. The reaction was terminated by phenol–chloroform extraction and

RNA samples were treated as described previously (31). Digestion by nuclease P1 and RNAse T2, two-dimensional thin-layer chromatography on cellulose plates and identification of modified nucleotides were performed according to previously published procedures (34). Radioactivity in the spots of modified 3′- and 5′-mononucleotides was quantified by means of a PhosphorImager (Molecular Dynamics, USA) using ImageQuant software.

Immunochemical assay

Electrophoresis under denaturing conditions was performed according to Laemmli (35). The separated proteins were electrophoretically transferred onto nitrocellulose membranes BA85. Blotting was performed in 25 mM Tris–192 mM glycine buffer for 1 h at 100 V. For immunochemical detection the membrane was saturated by blocking agent (low fat milk) and incubated with specific anti-His_6-tag rabbit antibodies (His-probe G18, Santa Cruz Biotechnology, USA) followed by incubation with the anti-rabbit IgG–alkaline phosphatase conjugate (Sigma, USA). Activity of alkaline phosphatase was revealed using color substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (Sigma, USA).

Chemical mapping of pseudouridine residues

Localization of pseudouridine residues in tRNA was performed as described (36,37) with the following modifications: 10 µg of total tRNA extracted from wild-type or mutant yeast strain was treated by 0.17 M 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMCT) in bicine–urea buffer pH 7.5 for 5 min at 42°C. Reverse transcription was done at 42°C for 30 min using 5′-labelled synthetic primer and CMCT-modified tRNA (1 µg when analyzing cytoplasmic tRNA or 3 µg for mitochondrial tRNA). Reverse transcription products were separated on 15% denaturing PAGE and radioautographed overnight using X-OMAT Kodak film.

Miscellaneous

Analysis of protein sequences for the presence of cellular targeting signals was performed using PSORT software (38) (available at http://psort.nibb.ac.jp/).

RESULTS

Overexpression and affinity purification of the recombinant protein encoded by yeast ORF YNL292w

Sequence analysis of a 30 kb DNA segment from yeast chromosome XIV (17) revealed the presence of an open reading frame (ORF N0480, later designed YNL292w) having a high degree of sequence homology with the *E.coli truB* gene encoding tRNA:Ψ_{55} synthase (12). The yeast gene was amplified by means of PCR and cloned into an *E.coli* expression vector under control of the phage T7 promoter as described in Materials and Methods. The resulting construct encodes the YNL292w protein preceded by 20 amino acids (MGSSHHHHHH SSGLVPRGSH) including a His_6-tag and a thrombin site (underlined) sequences.

Expression of the tagged, recombinant protein was achieved by induction of the T7 RNA polymerase gene in *E.coli* BL21(DE3) transformants using IPTG. Figure 1A shows a separation of total

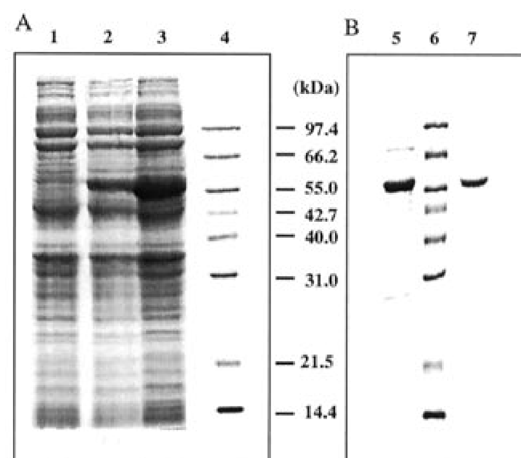

Figure 1. SDS–PAGE of protein samples from *E.coli* BL21(DE3) transformed by pET28b/YNL292w plasmid. (**A**) Expression of the recombinant protein (YNL292w) was induced by 1 mM IPTG for 0, 90 and 150 min (lanes 1, 2 and 3 respectively). (**B**) Aliquots of two subsequent steps of purification: Ni^{2+}–NTA–agarose column (lane 5) and Mono Q column (lane 7). Mid-range protein molecular weight markers (Promega) were applied to lanes 4 and 6. The electrophoresis was performed in 10% PAGE.

cellular proteins from non-induced and IPTG-induced *E.coli* cells on SDS–PAGE. A major protein band with apparent molecular mass of 55 kDa is visible in the extract from the induced cells. This value is in good agreement with the calculated molecular mass of the protein encoded by YNL292w (47.4 kDa, 423 amino acids including the His_6-tag and cleavage signal). The recombinant polypeptide remained soluble and could be efficiently purified from an S100 extract by metal-affinity chromatography on Ni^{2+}–NTA–agarose. This simple, one-step procedure led to a protein that was ~95% pure (Fig. 1B, lane 5). The yield was ~3–4 mg/l of induced *E.coli* culture.

Further purification was carried out by gradient anion-exchange chromatography on a MonoQ column, leading to a preparation that contained no more contaminants detectable by SDS–PAGE (Fig. 1B, lane 7). The tagged, recombinant protein remains tightly bound to the positively charged MonoQ matrix at pH 8.8 which, in accordance with its calculated pI value of 6.7. Expression of the recombinant protein in *E.coli* was verified by western blotting using polyclonal antibodies against the His_6-tag which detected a single 55 kDa band (data not shown).

The tagged, recombinant yeast protein has tRNA:Ψ_{55} synthase activity

On the basis of its sequence homology with *E.coli truB*, the product of the yeast YNL292w gene is expected to catalyze the formation of Ψ_{55} in yeast tRNA. We measured pseudouridine synthase activity using a synthetic wild-type yeast $tRNA^{Asp}$ and a mutant form of yeast $tRNA^{Phe}$ (PheY67) as the substrates. This $tRNA^{Phe}$ variant carries mutations at positions 31 and 39 and hence can only be pseudouridylated at position 55. Incubation of these tRNA transcripts with extracts from either yeast or untransformed *E.coli* cells in both cases led to the efficient formation of a single Ψ residue at position 55 as detected by thin layer chromatography of an RNase T2 or nuclease P1 digests (Fig. 2A and B).

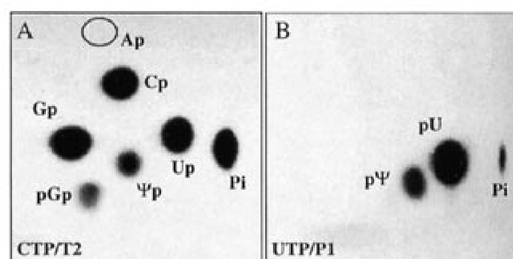

Figure 2. Autoradiography of two-dimensional chromatograms of 3′- and 5′-nucleotides on thin-layer cellulose plates. (**A**) The 3′-mononucleotides were obtained after T2-digestion of wild-type yeast $tRNA^{Asp}$ transcript labelled by [^{32}P]CTP and incubated with the recombinant protein (YNL292w) at 30°C as described in Materials and Methods (only 3′CMP, 3′UMP, 3′GMP, 3′ΨMP and inorganic phosphate are present). (**B**) The 5′-mononucleotides were formed by P1-digestion of yeast $tRNA^{Phe}$ mutant (PheY67) transcript labelled by [^{32}P]UTP and modified by incubation with the recombinant protein YNL292w at 30°C (only 5′UMP, 5′ΨMP and inorganic phosphate are present). The chromatographic system (N/N) has been described previously (55).

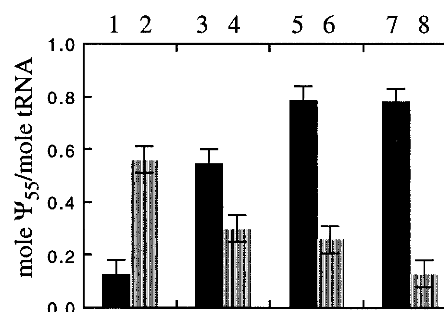

Figure 3. Activity of tRNA:Ψ_{55} synthase in various fractions of fractionated extract, prepared from wild-type and YNL292w-containing *E.coli* strains. Activity was measured using mutant (PheY67) $tRNA^{Phe}$ as a substrate. The amount of pseudouridine formed in the transcript upon incubation for 30 min at 30°C is indicated. Black bars represent the activity in the extract from transformed *E.coli* strain (2.5 mg/ml), while the shaded ones correspond to control extract from *E.coli* BL21 (DE3) (3.8 mg/ml). The extracts (~10 mg of total protein) were fractionated on 100 µl Ni^{2+}–NTA–agarose column. Unbound fraction (lanes 1 and 2), column wash with buffer A without Triton X-100 (lanes 3 and 4), fraction eluted at 20 mM imidazol (lanes 5 and 6) and fraction eluted at 150 mM imidazol (lanes 7 and 8).

Because in an extract prepared from transformed *E.coli* cells any tRNA:Ψ_{55} activity of the recombinant yeast protein would be obscured by the endogenous *E.coli* enzyme, we applied the same activity test to the material retained on a Ni^{2+}–NTA affinity-column after chromatography of an extract from transformed and control *E.coli* cells, respectively. When the column was eluted with a buffer containing 150 mM imidazol only the eluate from the transformed cell extract, which contains the almost pure recombinant yeast protein (Fig. 1B), was found to be able to catalyze Ψ_{55} formation (Fig. 3). Further activity tests using the homogeneous MonoQ-purified recombinant yeast protein allows us to rule out any possibility of this activity being due to contamination with the *E.coli* enzyme which has a molecular mass of 37 kDa (12) (data not shown). Therefore, we conclude that the YNL292w gene encodes *S.cerevisiae* tRNA:Ψ_{55} synthase. As this is the fourth yeast Ψ synthase identified so far, we propose to rename the gene in question *PUS4*.

Specificity of recombinant yeast tRNA:Ψ_{55} synthase

In vitro studies using S100 yeast extract have shown that yeast tRNA:Ψ_{55} synthase catalyzes the isomerization of U_{55} in a large variety of tRNA substrates, including several truncated tRNA molecules (26). We have tested the substrate specificity of the recombinant Pus4p protein using four different yeast $tRNA^{Asp}$ variants (Fig. 4A): $tRNA^{Asp}$ carrying a deletion of the D-stem–loop, TΨ-stem–loop minihelices bearing the amino acid acceptor branch or with shortened TΨ-stem–loop minihelices having 3 or 2 bp in the stem, respectively. As shown in Figure 4B, recombinant Pus4p is able to modify three of these substrates at 30°C but fails to recognize the substrate with the 2 bp long TΨ-stem–loop. However the reaction rate for the minisubstrates correlates with the length of the stem–loop. These results fully agree with those obtained for the tRNA:Ψ_{55} synthase activity present in yeast S100 extracts where we tested 43 yeast $tRNA^{Asp}$ variants as substrates (26).

We also tested recombinant Pus4p for a possible ‘dual-specificity’ function by incubation with synthetic transcripts corresponding to domains II and IV of yeast 25S rRNA. In natural 25S rRNA each of these domains contains 11 Ψ residues (39). Neither transcript was found to be modified, indicating that Pus4p is not involved in pseudouridylation of these domains of 25S rRNA.

Disruption of YNL292w abolishes Ψ_{55} formation in both cytoplasmic and mitochondrial tRNA

Previous experiments have shown that in several instances a single nuclear gene is responsible for modification of both cytoplasmic and mitochondrial tRNA (40–42). In order to determine whether a similar situation applies to the case of yeast tRNA:Ψ_{55} synthase, we studied the effects of disrupting the *PUS4* gene. Haploid yeast cells (FY73) carrying this disruption proved to be viable and to grow normally and, thus, can be assayed for the presence of tRNA:Ψ synthase activity.

S100 extracts prepared from this disrupted strain were tested using an array of synthetic tRNA transcripts as described previously (18). These transcripts correspond to variants of yeast $tRNA^{Asp}$, $tRNA^{Trp}$, $tRNA^{Val}$ and $tRNA^{Phe}$ (mutant PheY67). Altogether, nine potential sites in tRNA molecules were checked (positions 13, 26/27/28, 32, 34/36, 39 and 55) among 15 possible ones in yeast tRNAs. The results indicate that only the activity for pseudouridine-55 formation in tRNA is lacking in the extract from the disrupted strain, while the activities of other pseudouridine synthases remain unaffected. Time courses of Ψ_{55} formation in yeast $tRNA^{Phe}$ mutant (PheY67) in presence of S100 yeast extract containing or not Pus4p are shown in Figure 5. These results indicate that the disruption of the *PUS4* gene abolishes only the isomerization of U_{55} and leaves the enzymes responsible for modification at the remaining positions intact. Therefore, we conclude that the *PUS4* gene indeed encodes the only cytoplasmic tRNA-Ψ_{55} synthase present in *S.cerevisiae*.

In order to determine whether *in vivo* Pus4p also catalyzes formation of Ψ_{55} in mitochondrial tRNAs, we checked for the presence of Ψ_{55} in cytoplasmic as well as mitochondrial tRNA isolated from either wild-type yeast cells or the PUS4 disrupted strain. We used the reverse transcription approach of Bakin and

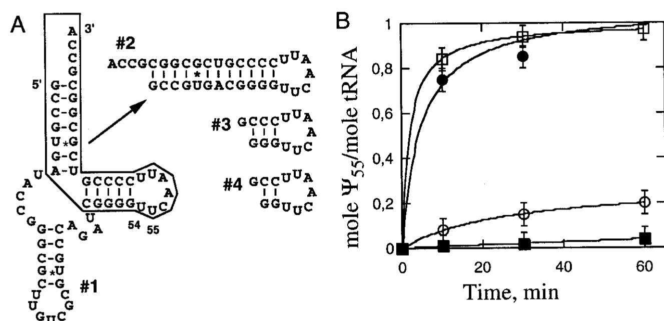

Figure 4. Sequences of four yeast tRNA^{Asp} mutants (**A**) tested for Ψ_{55} formation with the recombinant product from YNL292w (0.1 µg/ml). The corresponding time courses are shown in (**B**). tRNA^{Asp} #1(□), mut#2 (●), mut#3 (○), mut#4 (■).

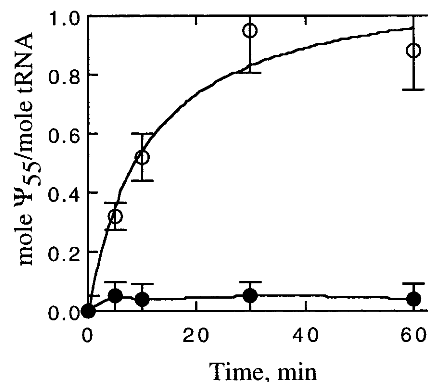

Figure 5. The activity of tRNA:Ψ_{55} synthase in wild-type strain and the YNL292w-disrupted haploid strain. Time courses of Ψ_{55} formation in yeast tRNA^{Phe} mutant (PheY67), labelled by [^{32}P]UTP incubated at 30°C in the presence of an S100 extract (0.6 mg/ml) from wild type yeast strain (○) and in presence of an S100 extract (0.6 mg/ml) from the haploid disruptant (●).

Ofengand (36,37) which takes advantage of the increased stability of CMC-Ψ derivative as compared to its CMC-U counterpart. Therefore, after alkaline treatment of the CMCT modified tRNA sample, the presence of modified CMC-Ψ can be detected by reverse transcription using appropriate DNA primer. Samples of total RNA extracted from wild-type or disruptant cells were analyzed using primers complementary to the 3′-terminal 18–20 nucleotides of cytoplasmic or mitochondrial tRNAs. Figure 6 shows the results of such analysis carried out on cytoplasmic and mitochondrial tRNAs^{Arg} (anticodons ICG and ACG respectively). In the wild-type strain (lanes 2 and 8) and in heterozygous diploid-disrupted strains (lanes 4 and 10) both tRNAs give rise to a strong stop corresponding to Ψ_{55}, indicated by the arrow. No such stop can be observed in either the cytoplasmic or the mitochondrial tRNAs^{Arg} present in the disrupted strains (lanes 6 and 12). Thus, Pus4p appears to modify position 55 in both cytoplasmic and mitochondrial tRNAs. In agreement with the *in vitro* experiments described above, Ψ formation at other positions within the tRNA^{Arg} molecules (positions 38, 39 and 40) is not affected by the disruption.

DISCUSSION

Our results clearly identify the product of the *S.cerevisiae* YNL292w (*PUS4*) gene as the enzyme that catalyzes the formation of Ψ_{55} in tRNA molecules (tRNA:Ψ_{55} synthase). The recombinant enzyme expressed in *E.coli* is active *in vitro* and displays the same substrate specificity towards minisubstrates as previously observed for the natural enzyme present in yeast extract (26). The results obtained with disrupted yeast strain allow to conclude that the gene YNL292w is one only gene for tRNA:Ψ_{55} synthase in yeast.

Pus4p has a high degree of sequence homology with its functional counterpart from *E.coli* encoded by the *truB* gene, which is in fact able to modify yeast tRNA (Fig. 3). A second yeast protein, CBF5, showing also a high homology to the *E.coli truB* product has been identified (7,16). This protein was studied extensively by genetic approach (43) and shown to bind *in vitro* to yeast centromeres and microtubules (16). Genetic depletion of CBF5 causes arrests of cell division in G_1/S phase (16). In contrast, cells carrying a disruption of the *PUS4* gene remain viable, appear to grow normally and have no specific phenotype under standard conditions, although they do not contain any detectable tRNA:Ψ_{55} synthase activity (Fig. 5). Our data also suggest that only one yeast homologue of *truB* (YNL292w) is implicated in the synthesis of pseudouridine residue at position 55 in all cellular tRNA molecules. Thus, CBF5, while expected to show pseudouridylation activity, must have a substrate specificity different from that of Pus4p. Experiments performed in the laboratory of Dr D.Tollervey (University of Edinburgh) have provided evidence that CBF5 may be involved in snoRNA-guided formation of most (if not all) Ψ residues present in yeast ribosomal RNA (44), a process taking place in the nucleolus (45,46; reviewed in 47).

Our results obtained *in vitro* and *in vivo* with the strain carrying a disruption of the *PUS4* gene allow the firm conclusion that the product of the *PUS4* gene is the only tRNA:Ψ_{55} synthase present in yeast and catalyzes Ψ_{55} formation in both cytoplasmic and mitochondrial tRNA. As the enzyme is the fourth one identified that catalyzes Ψ formation in yeast tRNA we have named it Pus4p.

The observation that Pus4p, as well as its *E.coli* counterpart (48,49), is dispensable for cell growth is somewhat surprising in view of the almost universal conservation of Ψ_{55} in tRNAs. Biochemical studies have shown, however, that the presence of Ψ_{55} is not required for efficient functioning of tRNA in protein synthesis. tRNA lacking Ψ_{55} is efficiently aminoacylated (50), forms the ternary complex with the *E.coli* elongation factor Tu with the same binding constant as fully matured tRNA (25) and efficiently supports poly-Phe synthesis in a polyU-dependent *in vitro* translation system (51). Modified nucleotides, however, including Ψ_{55}, may play a role in fine-tuning of selected steps in protein synthesis (discussed in 52).

Most mitochondrial tRNAs of *S.cerevisiae* are encoded in the mitochondrial genome, while the enzymes responsible for their modification and aminoacylation are derived from nuclear genes and, therefore, must be imported. In the case of yeast tRNA:$m^2_2G_{26}$-methylase a single nuclear gene gives rise to two protein products, one of which remains in the cytoplasm while the other is transported to the mitochondria (42). The same conclusion was reached in the case of tRNA-nucleotidyltransferase and tRNA-isoprenyltransferase, which are coded by single nuclear genes, but targeted to mitochondria in addition to the cytoplasm (40,41).

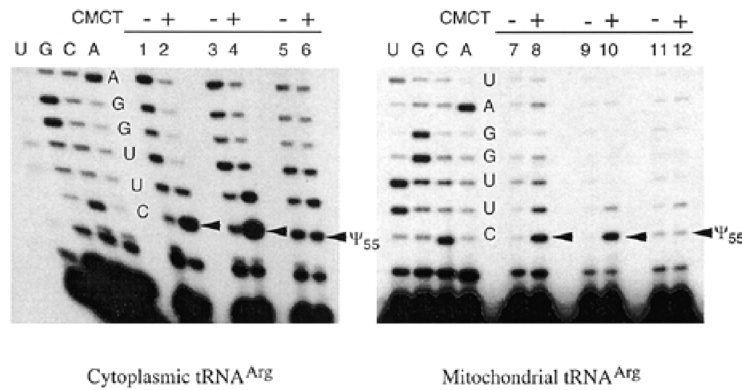

Figure 6. Chemical mapping of pseudouridine residues in cytoplasmic (left panel) and mitochondrial tRNAs^{Arg} (right panel) from wild-type (lanes 1 and 2, 7 and 8), YNL292w-heterozygous diploid-disrupted (lanes 3 and 4, 9 and 10) and YNL292w-disrupted (lanes 5 and 6, 11 and 12) yeast strains. Autoradiograms of reverse transcription products in 15% polyacrylamide–8 M urea gels are shown. The strong stops (shown by arrows) in the reverse transcription of tRNA correspond to pseudouridine residues. The corresponding tRNA sequencing is shown on the left of each gel. The samples of tRNA were treated with CMCT followed by Na-bicarbonate hydrolysis (lanes 2, 4, 6, 8, 10 and 12) and compared with control tRNA samples (lanes 1, 3, 5, 7, 9 and 11).

The absence of Ψ_{55} from both cytoplasmic and mitochondrial tRNAs in the strain carrying a disruption of the *PUS4* gene indicates that a similar situation exists in the case of tRNA-Ψ_{55} synthase. However, the manner in which a portion of the enzyme is imported into mitochondria is as yet unclear. Sequence analysis does not predict the presence of a typical mitochondria targeting signal in Pus4p. On the other hand, a sequence rich in lysine and arginine showing characteristic features of a nuclear import signal (RKQQYEKQTGKKASAKRK) is located close to its N-terminus. Predicted nuclear localization of tRNA:Ψ synthase is in agreement with the observation that Ψ_{55} is present in intron-containing tRNA precursors located in the nucleus (53,54). Studies on the distribution of yeast tRNA-Ψ_{55} synthase between the different cellular compartments are in progress.

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