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A conserved family of *Saccharomyces cerevisiae* synthases effects dihydrouridine modification of tRNA

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ABSTRACT

Dihydrouridine modification of tRNA is widely observed in prokaryotes and eukaryotes, as well as in some archaea. In *Saccharomyces cerevisiae* every sequenced tRNA has at least one such modification, and all but one have two or more. We have used a biochemical genomics approach to identify the gene encoding dihydrouridine synthase 1 (Dus1, ORF YML080w), using yeast pre-tRNA^{Phe} as a substrate. Dus1 is a member of a widespread family of conserved proteins, three other members of which are found in yeast: YNR015w, YLR405w, and YLR401c. We show that one of these proteins, Dus2, encoded by ORF YNR015w, has activity with two other substrates: yeast pre-tRNA^{Tyr} and pre-tRNA^{Leu}. Both Dus1 and Dus2 are active as a single subunit protein expressed and purified from *Escherichia coli*, and the activity of both is stimulated in the presence of flavin adenine dinucleotide. Dus1 modifies yeast pre-tRNA^{Phe} in vitro at U17, one of the two positions that are known to bear this modification in vivo. Yeast extract from a *dus1*-Δ strain is completely defective in modification of yeast pre-tRNA^{Phe}, and RNA isolated from *dus1*-Δ and *dus2*-Δ strains is significantly depleted in dihydrouridine content.

Keywords: biochemical genomics; *dus1*; tRNA processing; yeast

INTRODUCTION

tRNA molecules from all studied organisms bear a number of different modifications. Currently 22 different tRNA modifications have been found in the yeast *Saccharomyces cerevisiae* (Hopper & Martin, 1992), and more than 80 have been identified in total (Bjork, 1995). Dihydrouridine modification of tRNA (Fig. 1A), formed by reduction of the 5,6-double bond of the corresponding uridine residue (Madison & Holley, 1965), is a ubiquitous modification found widely in prokaryotes, eukaryotes, and in some archaea (Sprinzl et al., 1998). In *Saccharomyces cerevisiae*, all but one of the examined tRNAs ($tRNA^{Glu}_{GAA}$) have two or more dihydrouridines. Although most dihydrouridines are found in the D-loop of tRNAs, for which it is named, some dihydrouridines are also found in the extra loop. In addition, dihydrouridine is found in *Escherichia coli* rRNA at the invariant U2449 of the central loop of domain V of 23 S rRNA (Kowalak et al., 1995). This dihydrouridine residue is 2 nt from the site interacting with the C-terminus of tRNA at the ribosomal P-site, but the function of the dihydrouridine residue is not essential (O'Connor et al., 2001).

The role of dihydrouridine in tRNA is currently unknown. It is suggested from studies with tRNAs that dihydrouridine may lend a certain degree of conformational flexibility to RNA (Dalluge et al., 1996). Interestingly, an increase in the level of dihydrouridine was previously observed in tumor-specific tRNA^{Phe} purified from human malignant tissues (Kuchino & Borek, 1978).

To begin to explore the biochemistry and biology of dihydrouridine modification we sought to identify the dihydrouridine synthase and its corresponding gene. Previously, *S. cerevisiae mia* strains were shown to have a reduced extent of dihydrouridine modification of multiple isoacceptors of tRNA^{Phe} and tRNA^{Tyr} (Lo et al., 1982). Because there was no additional growth phenotype, the *MIA* gene has not been cloned. To identify the structural gene encoding dihydrouridine synthase, we screened a genomic library of *S. cerevisiae* GST-ORF proteins (Martzen et al., 1999) with a yeast

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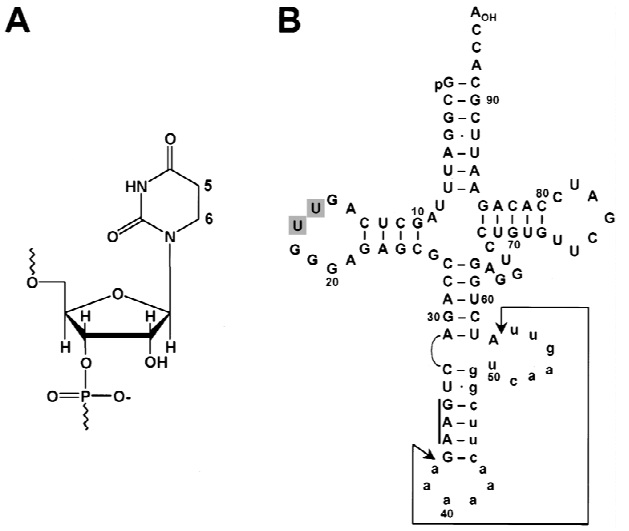

FIGURE 1. Dihydrouridine in tRNA. **A**: The structure of dihydrouridine in RNA. **B**: The secondary structure of unspliced but end-matured $tRNA^{Phe}_{GAA}$ from *S. cerevisiae*. The nucleotides are numbered, and bases in the intron are indicated by lower case letters. The uridines that are naturally modified to dihydrouridine are shaded, the anticodon GAA is designated by a thick bar, and the cleavage sites by tRNA splicing endonuclease are shown by arrows.

pre-$tRNA^{Phe}$ substrate that is normally modified at positions 16 and 17 in the D-loop, and identified a dihydrouridine synthase (Dus1) and its corresponding ORF, YML080w. We provide evidence that this protein catalyzes modification of pre-$tRNA^{Phe}$ at U17 in vitro, and is responsible in vivo for a portion of the dihydrouridine modification of tRNA. This ORF is part of a large diverse family of conserved proteins, three other members of which are found in *S. cerevisiae*. We find that at least one of these proteins, Dus2 (encoded by YNR015w), catalyzes dihydrouridine modification of pre-$tRNA^{Tyr}$ and pre-$tRNA^{Leu}$ in vitro and is responsible for a portion of dihydrouridine modification of tRNA in vivo. Dus2 was previously identified as *SMM1*, a multicopy suppressor of a mitochondrial $tRNA^{Asp}$ mutant defective in 3′ end maturation (Rinaldi et al., 1997).

RESULTS

Detection of dihydrouridine synthase activity

To assay dihydrouridine modification of tRNA, we used unspliced but end-matured yeast pre-$tRNA^{Phe}$ as a substrate (Fig. 1B). It is known from previous work in yeast that dihydrouridine modification of $tRNA^{Ser}$ normally occurs in vivo before the tRNA is spliced (Etcheverry et al., 1979), that dihydrouridine is present at both U16 and U17 in unspliced pre-$tRNA^{Phe}$ that accumulates in *rna1-1* mutants (Knapp et al., 1978), and that dihydrouridine formation in vitro is intron insensitive (Jiang et al., 1997). To detect dihydrouridine formation, the [α-^{32}P]UTP-labeled tRNA was incubated with yeast crude extract in the presence of a mixture of NADPH and NADH, the likely source of reducing power for the reaction. Following incubation, the RNA was treated with nuclease P1 and the resulting 5′-phosphorylated uridine-labeled nucleotides were separated by two-dimensional thin layer chromatography (Bochner & Ames, 1982). As shown in Figure 2A, we can easily detect formation of dihydrouridine 5′-monophosphate (pD), as determined by the production of material that migrates at the expected position of pD, and by stimulation of production of this material by NADH + NADPH. Quantitation of the labeled spots in this chromatogram (Table 1) indicates that pD formation is stimulated 11-fold in the presence of the coenzymes NADH + NADPH, relative to that observed in unsupplemented extract,

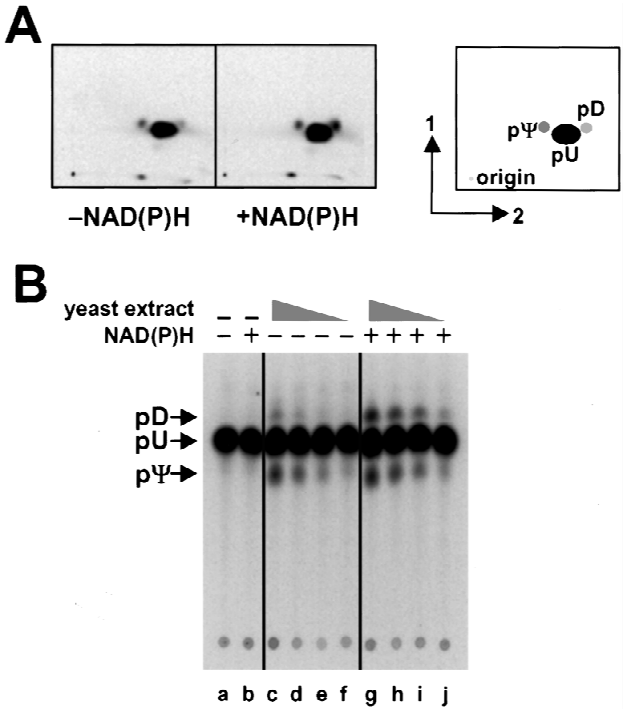

FIGURE 2. Formation and detection of dihydrouridine in extracts. **A**: Coenzyme dependence of modification of uridines in extracts. [α-^{32}P]UTP-labeled yeast pre-$tRNA^{Phe}$ was incubated with 15 μg of yeast crude extract in the presence or absence of 1 mM NADPH and NADH at 30 °C for 30 min, and then RNA was extracted with phenol/chloroform, precipitated, treated with P1 nuclease, and resolved on a two dimensional PEI-cellulose thin layer chromatography plate as described in Materials and Methods. The right panel shows a schematic of the positions of resolved nucleotides. **B**: Detection of dihydrouridine by a simplified thin layer chromatography system. The tRNA substrate was treated the same way as in **A** with buffer (lanes a and b) or extract (lanes c–f and lanes g–j, containing 30, 15, 7.5, and 3.8 μg respectively), and with 1 mM NADH and 1 mM NADPH (lanes b and g–j), and buffer (lanes a and c–f). Purified RNA was treated with P1 nuclease and nucleotides were applied to a cellulose thin layer plate and resolved by development in one dimension as described in Materials and Methods. pD: dihydrouridine 5′ monophosphate; pU: uridine 5′ monophosphate; pΨ: pseudouridine 5′ monophosphate.

TABLE 1. Dihydrouridine modification is coenzyme dependent.

	% nucleotides	
	− NADPH/NADH	+ NADPH/NADH
pU	98.71	96.60
pΨ	1.11	1.47
pD	0.18	1.93
total	100.00	100.00

whereas pΨ formation is virtually unchanged under these conditions (1.3-fold higher).

To facilitate the analysis of multiple samples, we used a simplified one-dimensional chromatography system for detection of dihydrouridine, using a different solvent system. Figure 2B shows a titration of yeast extract in this system in the presence and absence of cofactors. It is apparent that pD migrates above the main spot for pU, as it is much more prominent in the presence of coenzymes than in its absence, whereas pΨ is just below the main pU spot and is present in relatively constant amounts in the presence or absence of NADH and NADPH. The residual dihydrouridine activity that appears in the absence of added coenzymes is presumably due to the presence of endogenous NADH and NADPH in extracts.

The GST-ORF of YML080w copurifies with dihydrouridine synthase activity

We identified a gene whose product is associated with dihydrouridine synthase activity using a biochemical genomics approach for mapping activities to the corresponding *S. cerevisiae* genes (Martzen et al., 1999). This is accomplished with a set of 6,144 strains each expressing a different ORF fused to glutathione S-transferase gene (GST) under control of the P_{CUP1} promoter. By assay of 64 pools of purified GST-ORF fusion proteins, each derived from 96 corresponding strains housed in a microtiter plate, and by subsequent deconvolution of the positive pool, we could rapidly identify the strain and the GST-ORF associated with an activity.

We screened this genomic array of protein pools for dihydrouridine synthase activity using yeast pre-$tRNA^{Phe}$ as a substrate and the simplified one-dimensional thin layer chromatography system shown in Figure 2B. As shown in Figure 3A, dihydrouridine activity was detected in pool 30. To find the source strain and the GST-ORF responsible for dihydrouridine synthase activity, we purified and assayed the GST-ORFs from each of the 8 rows and 12 columns of strains in the corresponding plate. In this way, we mapped dihydrouridine synthase activity to the strain at row C and column 8 of Plate 30 (see Fig. 3B), which is supposed to harbor plasmids with the ORF YML080w insert. We confirmed that dihydrouridine synthase activity is due to YML080w by isolating the plasmid and sequencing the insert from the source strain, transforming the analyzed plasmid back into yeast, and then repurifying GST-YML080w protein and testing for activity. As shown in lanes c and d of Figure 3C, the observed activity is completely dependent on the coenzymes. Thus, the protein encoded by YML080w is responsible for dihydrouridine synthase activity.

DUS1 encodes the catalytic activity for dihydrouridine synthase

The GST-ORF of YML080w might copurify with dihydrouridine synthase activity because it is the corresponding enzyme, or because it copurifies with the enzyme due to a protein–protein interaction. Two lines of evidence indicate that the protein encoded by YML080w has dihydrouridine synthase catalytic activity. First, we demonstrated that overexpression of the GST-ORF results in overexpression of dihydrouridine synthase activity. Yeast extract isolated from the strain expressing GST-YML080w had 200-fold more activity than that from the control strain (see Table 2). This is the expected result if YML080w encodes the synthase activity. Second, we demonstrated that purification of the protein after expression in *E. coli* resulted in active protein. To this end, we cloned and expressed YML080w as a His6-ORF fusion in *E. coli*, and purified the protein by immobilized metal-ion affinity chromatography. Purified His6-YML080w yielded a specific activity of 22,200 U/mg protein, whereas the activity from a mock purification was not detectable (less than 300 U; see Table 2). The activity of His6-YML080w protein is further stimulated 25-fold by the addition of 250 μM FAD. Thus, the protein encoded by YML080w is dihydrouridine synthase, and the gene was named *DUS1*.

YNR015w encodes a second yeast dihydrouridine synthase

Database searches indicate that Dus1 protein is one member of a large family of similar proteins in yeast and other organisms. A BLAST search of the NCBI Database (Altschul et al., 1997), using Dus1 protein sequence as a query, yields a large number of protein sequences in different organisms that are very similar to Dus1. Figure 4 shows an alignment of Dus1 protein with some of these proteins from vertebrates, invertebrates, plants, fungi, and bacteria (with e values ranging from e^{-16} to e^{-104}), as well as with three other family members in *S. cerevisiae* (YLR405w, e^{-28}; YNR015w, e^{-9}; and YLR401c, e^{-6}). The alignment shows that these proteins share many of the same conserved re-

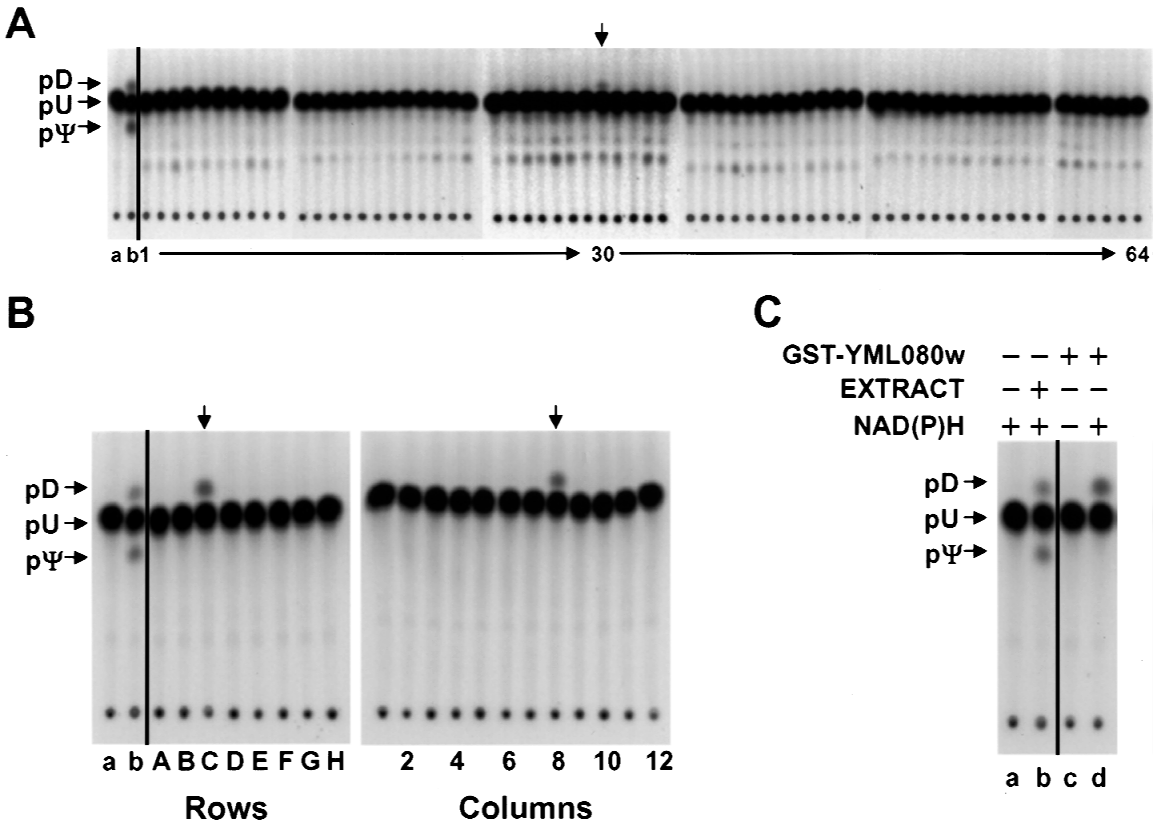

FIGURE 3. Identification of the GST-ORF copurifying with pre-tRNA^{Phe} dihydrouridine synthase activity. **A**: Assay of genomic pools of purified GST-ORF proteins for dihydrouridine synthase activity. [α-^{32}P]UTP-labeled pre-tRNA$^{Phe}_{GAA}$ substrate was incubated with 2 μL of each of 64 pools of purified GST-ORF proteins at 30 °C for 2 h in the presence of 1 mM of NADPH and NADH, and modified nucleotides were analyzed by P1 nuclease digestion and thin layer chromatography as described in Materials and Methods. Lane a: buffer control; lane b: 30 μg crude extract. **B**: Deconvolution of pool 30 to identify the GST-ORF associated with dihydrouridine synthase activity. Subpools of GST-ORF proteins prepared from each of 8 rows and 12 columns of the strains from microtiter plate 30 (comprising pool 30) were purified and assayed for dihydrouridine synthase activity as described above. Lane a: buffer control; lane b: 30 μg crude extract. **C**: Assay of individual GST-ORF implicated in dihydrouridine synthase activity. The GST-ORF of YML080w was purified from the strain in well C8 of microtiter plate 30, and assayed for dihydrouridine synthase as described above in the absence (lane c) or presence (lane d) of NADPH and NADH. Lane a: buffer control; lane b: 30 μg crude extract.

gions, and a search for conserved domain architecture (NCBI DART program) demonstrates that the proteins are members of the same uncharacterized protein family, UPF0034. Thus, the proteins in this family may have a similar activity.

To determine if any of the three other yeast proteins had dihydrouridine synthase activity, we purified the GST-fusion proteins from the corresponding yeast GST-ORF library strains, and assayed their activity with three different yeast tRNAs: pre-tRNA$^{Phe}_{GAA}$, pre-tRNA$^{Tyr}_{GUA}$,

TABLE 2. Expression and purification of Dus1 (Yml080) in yeast and *E. coli*.

	Activity U/μL	Activity U/μL (+ FAD)	Protein (mg/mL)	Specific activity (U/mg)	Total activity (− FAD)
Control yeast extract (EJ758)	5	N/D	50	100	7.5×10^3
FX-20 yeast extract (expressing GST-YML080)	700	N/D	35	20,000	1.05×10^6
Purified GST-YML080 (from FX-20)	20	N/D	1.18	16,700	1.18×10^4
Purified His6-YML080 (from *E. coli* EFX-08)	20	500	0.9	22,200	1.20×10^4
Mock purified (from *E. coli* parent)	<0.1	N/D	0.34	<300	<60

N/D: not determined.

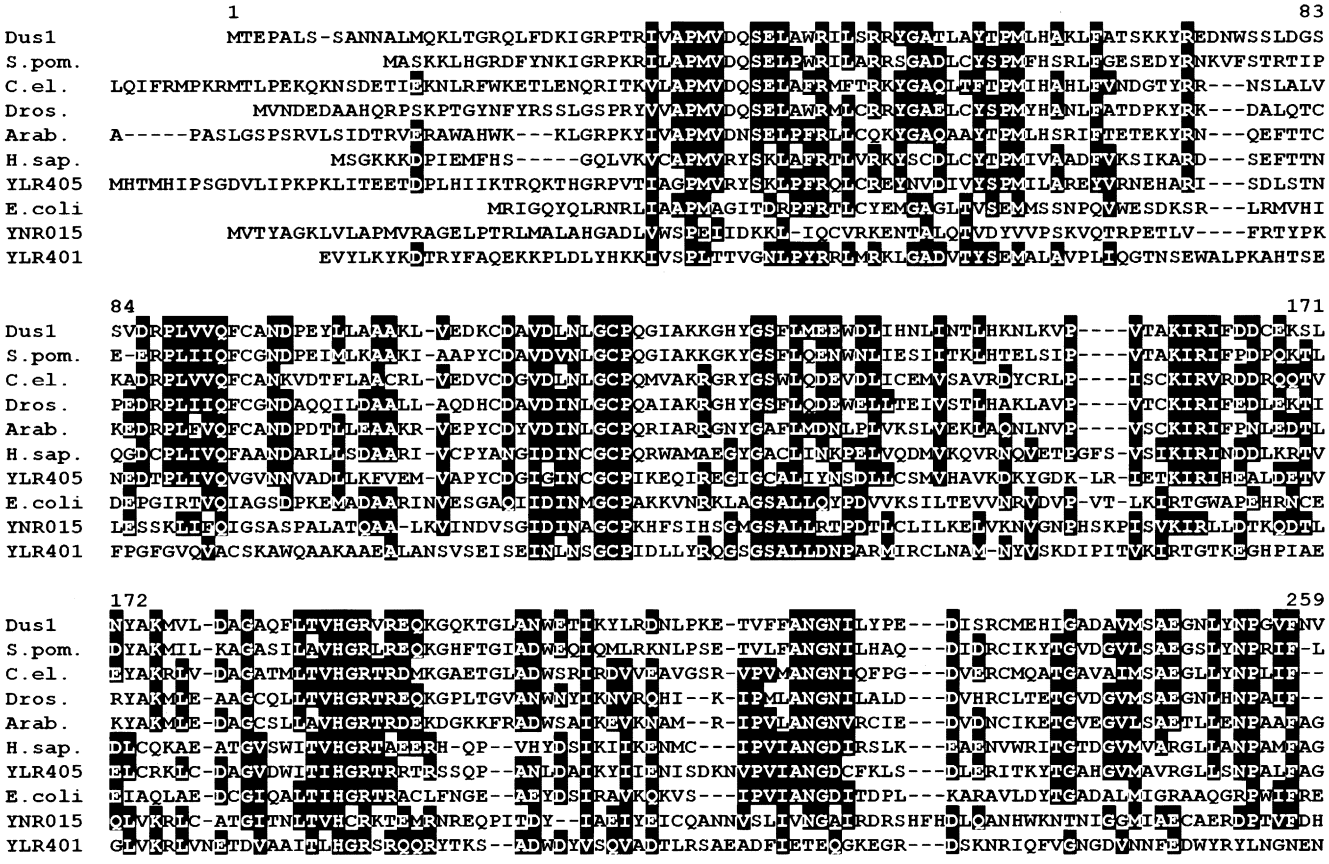

FIGURE 4. Alignment of protein sequences similar to Dus1. Members of the Dus1 protein family from *S. cerevisiae* and from other representative organisms were aligned using the multalin program (Corpet, 1988). *S. cerevisiae* (Dus1, NP_013631, 259/423 residues; YLR405w, NP_013509, $9e^{-28}$, 267/367 residues; YNR015w, NP_060273, $1e^{-09}$, 260/384 residues; YLR401c, NP_013505.1, $3e^{-6}$, 257/609 residues, starting with residue 271), *S. pombe* (SPBC36B7, CAC05725, e^{-104}, 245/399 residues), *C. elegans* (F36A2.2, T21830, $2e^{-68}$, 264/527 residues, starting with residue 35), *Drosophila* (CG3645, AAF51525, $2e^{-84}$, 251/358 residues), *Arabidopsis thaliana* (At5g67220, NP_201523, $7e^{-64}$, 256/423 residues, starting with residue 58), *Homo sapiens* (PP35, NP_008947, $4e^{-32}$, 242/307 residues), and *E. coli* (*yhdG*, NP_289828, $2e^{-16}$, 235/321 residues). Consensus levels: 50% or more.

and pre-tRNA$^{Leu}_{CAA}$. It is known that yeast tRNA$^{Tyr}_{GUA}$ is modified to dihydrouridine at multiple positions (U16, U17, U20, U20A, U20B, and U47) in vivo, and yeast tRNA$^{Leu}_{CAA}$ is modified in vivo at a different location (U20 and U20B) than tRNA^{Phe} (Sprinzl et al., 1998). As illustrated in Figure 5A, Dus1 has dihydrouridine synthase activity with both pre-tRNA^{Phe} and pre-tRNA^{Tyr}. Moreover, dihydrouridine synthase activity is also observed with the GST-YNR015w fusion (designated as Dus2), using both pre-tRNA^{Tyr} and pre-tRNA^{Leu}, and very weak activity is observed with YLR405w, acting on pre-tRNA^{Leu}. It is currently not clear if the trace activity copurifying with GST-YLR405w is real, but purification of Dus2 as a His6-Dus2 fusion protein in *E. coli* (like for Dus1) yielded comparably active protein that was similarly stimulated by FAD (data not shown). The *DUS2* gene was previously identified as *SMM1*, a weak multicopy suppressor of a mitochondrial tRNA^{Asp} mutant defective in 3′ end maturation (Rinaldi et al., 1997).

To further examine the activities of these proteins, we titrated yeast extracts isolated from strains deleted for each of the four Dus homologs, using the same three tRNA substrates. As illustrated in Figure 5B with pre-tRNA^{Phe} substrate, extracts from a *dus1*-Δ strain had little or no dihydrouridine synthase activity (see Fig. 5B with pre-tRNA^{Phe}), whereas extracts from each of the other three deletion strains (*dus2*-Δ, *ylr401c*-Δ, and *ylr405w*-Δ) showed full activity compared to the wild-type control strain. This result is consistent with the observation that dihydrouridine modification of pre-tRNA^{Phe} is observed only with purified Dus1 and not with the other purified homologous proteins (Fig. 5A), and suggests that Dus1 is responsible for the observed dihydrouridine modification of yeast tRNA^{Phe} in vitro. In contrast, deletion of any of the four *DUS* homologs does not yield extracts with a significant difference in dihydrouridine modification activity on tRNA^{Tyr} or tRNA^{Leu} (see Fig. 5B). This result is consistent with the observation that dihydrouridine modification of tRNA^{Tyr}, and possibly of tRNA^{Leu}, is effected by more than one purified Dus protein homolog (Fig. 5A).

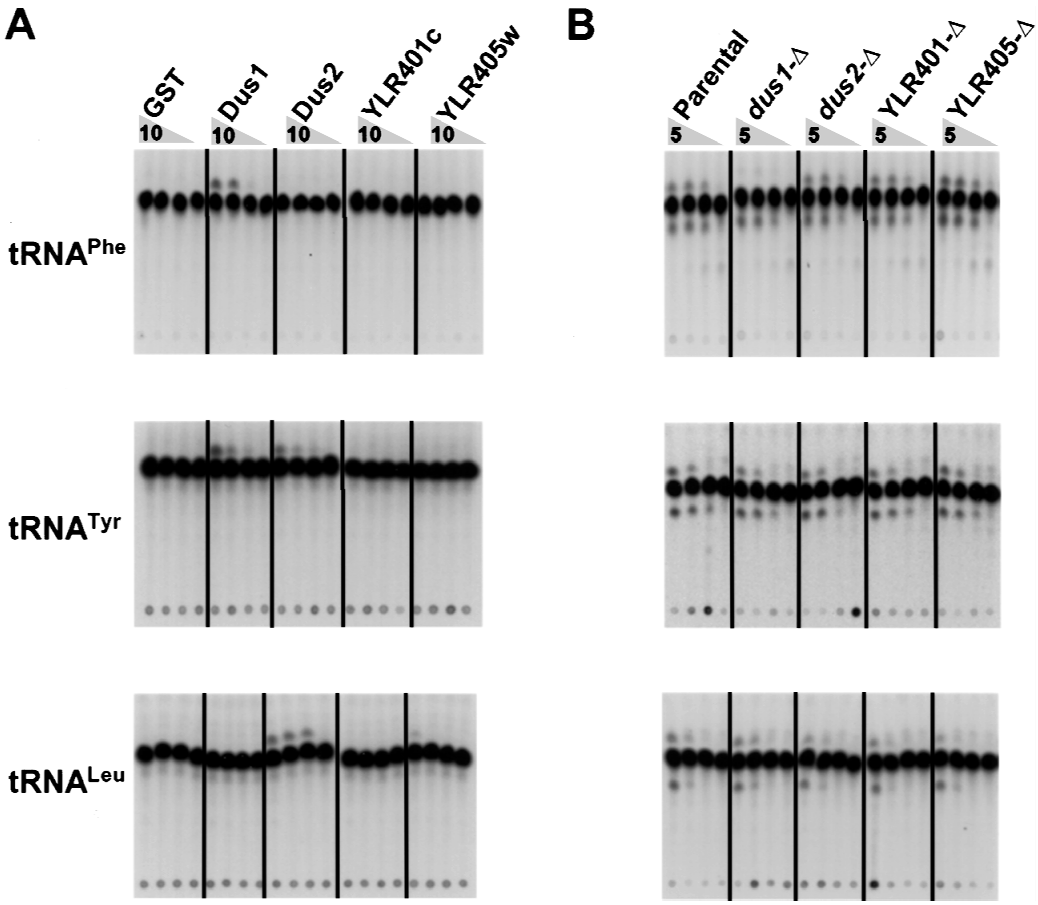

FIGURE 5. Comparison of dihydrouridine synthase activity of yeast Dus protein family members, using different tRNA substrates. **A**: Activity of purified dihydrouridine synthase family members from yeast. GST and GST-ORF fusion proteins of Dus1, Dus2, YLR401c, or YLR405w were compared for activity with pre-tRNA^{Phe}, pre-tRNA^{Tyr}, or pre-tRNA^{Leu}, in the presence of 1 mM NADPH and 1 mM NADH, 250 μM FAD, and ~250 ng or 10-fold serially diluted amounts of protein, as indicated. **B**: Dihydrouridine synthase activity of extracts from strains deleted for Dus family members. Crude extracts derived from the parental and corresponding *orf*-Δ strains were compared for activity with different pre-tRNA substrates, 1 mM NADPH and 1 mM NADH, 250 μM FAD, and ~15 μg or 5-fold serially diluted extract, as indicated.

Dihydrouridine formation by Dus1 in vitro occurs primarily at U17, one of the two positions that is modified in vivo

Because the data in Figure 5 suggested that only Dus1 could act on yeast pre-tRNA^{Phe} in vitro, we mapped the site of dihydrouridine modification of yeast pre-tRNA^{Phe} after treatment with Dus1. tRNA^{Phe} is naturally modified to dihydrouridine at U16 and U17 in the D-loop (Raj-Bhandary et al., 1966). To map the modification site, we first treated [α-^{32}P]UTP-labeled yeast pre-tRNA^{Phe} with GST-Dus1, and obtained 5.2% incorporation of dihydrouridine, indicating 1.14 modified nucleotides per pre-tRNA. Then we cleaved the pre-tRNA with tRNA splicing endonuclease and purified the 5′-half molecule, 3′-half molecule, and the intron (see Fig. 1B) after their separation on a 15% polyacrylamide gel. P1 nuclease analysis showed that pD was formed predominantly in the 5′-half molecule (see lanes c and d of Fig. 6A), accounting for 14.5% of the label (1.02 modified nucleotides per 5′-half molecule). The residual amount of pD observed in the 3′-half molecule and possibly the intron is presumably due to contamination of fragments during separation. The spot below pU may be uridine 5′ phosphate 1′,2′ cyclic phosphate (pU > p) formed from the terminal U residue of the intron during endonucleolytic cleavage, which yields ends bearing a 2′-3′ cyclic phosphate (Peebles et al., 1983).

To determine the exact modification positions, we further digested the 5′-half molecule with RNase T1, which hydrolyzes RNA after the 3′ phosphate of each guanosine residue. This treatment results in production of four ^{32}P-labeled oligonucleotides: a 3-mer ($U_{16}U_{17}G_{18}$), a 4-mer ($A_{31}C_{32}U_{33}G_{34}$), a 5-mer ($C_{11}U_{12}C_{13}A_{14}G_{15}$), and a 6-mer ($A_5U_6U_7U_8A_9G_{10}$). The labeled oligonucleotides were separated on a 22% polyacrylamide gel. Analysis of these oligonucleotides by nuclease P1 digestion and TLC resolution revealed a predominant dihydrouridine in the 3-mer, which corresponds to nt 16 to 18 in the D-loop (Fig. 6B). Because the phosphate

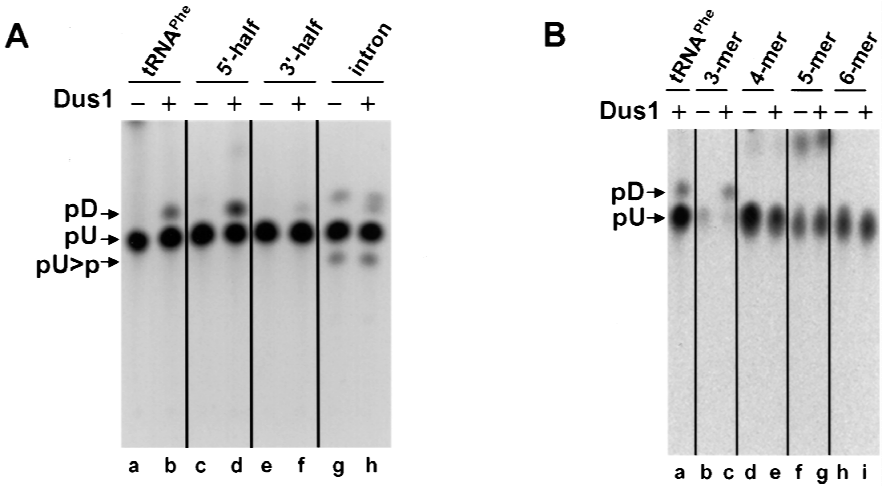

FIGURE 6. Mapping the site of dihydrouridine modification of yeast pre-tRNA^{Phe} by Dus1. **A**: Dihydrouridine modification is localized to the 5′-half molecule of pre-tRNA^{Phe}. Dus1-treated and mock-treated [α-^{32}P]UTP-labeled yeast pre-tRNA^{Phe} were cleaved with tRNA splicing endonuclease, and the resulting 5′-half molecule, 3′-half molecule, and the excised intron were separated on a 15% polyacrylamide gel, eluted, and analyzed for dihydrouridine modification by P1 nuclease treatment followed by thin layer chromatography, as described in Materials and Methods. pU > p: uridine 5′-monophosphate, 2′,3′-cyclic phosphate. **B**: Dihydrouridine modification is localized to U17. The Dus1-treated 5′-half molecule and its mock-treated control were incubated with 1 U RNase T1, and the four resulting ^{32}P-labeled oligonucleotides were purified after separation on a 22% polyacrylamide gel, and analyzed for dihydrouridine by P1 nuclease treatment followed by thin layer chromatography.

between U16 and U17 is the only one that is labeled in the 3-mer, the dihydrouridine observed in this analysis has to be D17. The dihydrouridine in this spot corresponded to >75% of the label, but could not be further quantitated accurately because of the low recovery after elution of the 3-mer. Corresponding RNase T2 analysis of the trimer, which should yield Dp if U16 is modified, gave only trace amounts of Dp (data not shown). Thus, Dus1 can modify U17 of pre-tRNA^{Phe} to dihydrouridine.

Dus1 and Dus2 are responsible for dihydrouridine modification of tRNA in vivo

To study the in vivo activity of Dus enzymes, we determined the dihydrouridine content of RNA samples purified from *dus*-Δ strains and from the corresponding parental strain. If, as indicated by our results in vitro, Dus1 and Dus2 are the two major dihydrouridine synthases, deletion of either one should strongly impact dihydrouridine modification in vivo. To test this, low molecular weight RNA was isolated from log phase cells (see Materials and Methods), and then samples were hydrolyzed to nucleosides and analyzed on a C18 reverse phase HPLC according to a slight modification of a published method (Gehrke & Kuo, 1989; see Materials and Methods). A typical chromatogram is shown in Figure 7A, monitored at 210 nm. The individual peaks were assigned based on spectra of the nucleosides, and also conformed with the published elution order.

The identity of the dihydrouridine peak was established based on two criteria. First, dihydrouridine absorbs at 210 nm, but not at 263 nm, whereas pseudouridine absorbs at both 210 nm and 263 nm (Gehrke & Kuo, 1989). Thus, of the two minor peaks corresponding to D and Ψ in the enlarged portion of the chromatogram shown in Figure 7B, the first peak (retention time 4.25 min) corresponds to D and the second peak (retention time of 4.58 min) corresponds to Ψ. Second, the assignment of dihydrouridine was independently determined by evaluation of the dihydrouridine content of the RNA from a *mia* strain which, as mentioned above, is deficient in dihydrouridine in some tRNAs. Our analysis, described below, indicates that the dihydrouridine content of RNA from the *mia* strain (Lo et al., 1982) is ~56% of that in the *MIA*$^+$ strain (Table 3).

Comparison of the chromatograms in Figure 7C with that in Figure 7B (which are at the same scale) shows a significant decrease of dihydrouridine content in *dus1*-Δ/*dus1*-Δ and *dus2*-Δ/*dus2*-Δ strains relative to that in the parent strain, but little change in relative pseudouridine levels. To confirm this qualitative observation, we calculated the integrated peak areas for six individual nucleosides (A, G, C, U, Ψ, and D) from a chromatogram (measured at 210 nm), normalized the areas of each nucleoside by expressing them as a percentage of the total area from the six nucleosides of the corresponding chromatogram, and compared the normalized areas for each nucleoside from each deletion strain with the corresponding value from the parent strain. The results are tabulated in Table 3. It is evident that the dihydrouridine content from the *dus1*-Δ/*dus1*-Δ and *dus2*-Δ/*dus2*-Δ strains are each significantly lower than in the corresponding parental strain (about 50% of normal). In contrast, the levels of the five other nucleosides in the *dus1*-Δ/*dus1*-Δ and *dus2*-Δ/*dus2*-Δ strains remained virtually the same as that in the control strain. The major nucleosides all varied less than 3%, and Ψ varied less than 8%. Thus, we conclude that Dus1 and

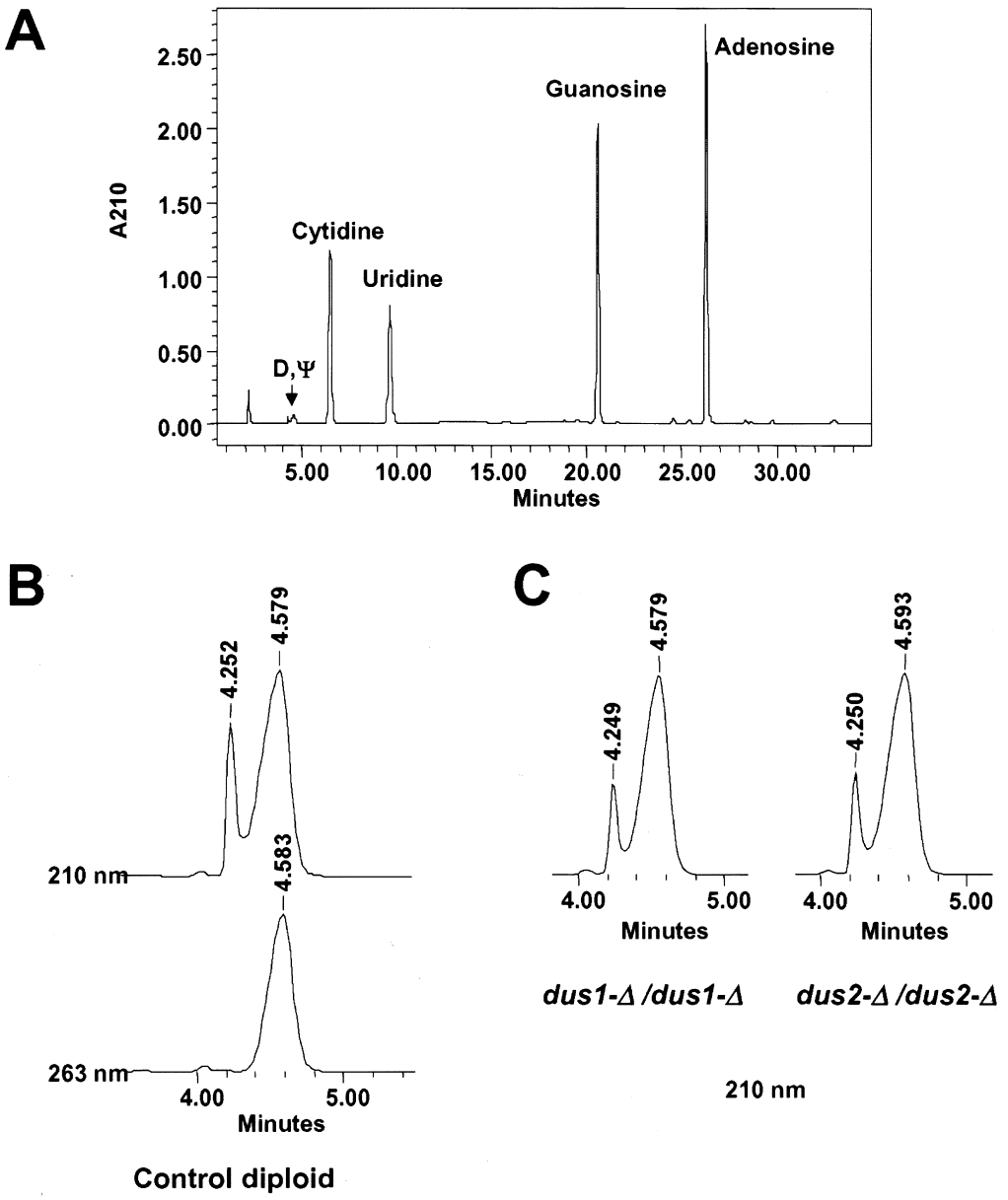

FIGURE 7. HPLC analysis of dihydrouridine content of RNA from strains deleted for *DUS* gene family members. **A**: Chromatogram of nucleosides derived from RNA of wild-type *S. cerevisiae*. RNA prepared from a wild-type yeast strain (BY4743) was treated with P1 nuclease and phosphatase to produce nucleosides, and then nucleosides were chromatographed on a C18 reverse phase HPLC column and elution monitored at 210 nm as described in Materials and Methods. Dihydrouridine (D) and pseudouridine (Ψ) were collectively indicated by an arrow. **B**: Identification of the dihydrouridine peak. An enlarged view is shown of a portion of the chromatogram in **A**, showing the region between 4 and 5 min, which corresponds to the pseudouridine/dihydrouridine elution region. Upper trace: monitored at 210 nm; lower trace: monitored at 263 nm. **C**: Evaluation of dihydrouridine content of strains lacking *DUS1* or *DUS2*. Similar amounts of RNA from *dus1*-Δ/*dus1*-Δ or from *dus2*-Δ/*dus2*-Δ isogenic strains were chromatographed, and an enlarged view of the elution pattern between 4 and 5 min is shown, at the same scale as in **B**.

Dus2 proteins are each responsible for a portion of the dihydrouridine modification in the cell. Similar analysis of *ylr401*-Δ/*ylr401*-Δ and *ylr405*-Δ/*ylr405*-Δ strains (Table 3) shows that they too have somewhat less dihydrouridine than in the wild-type strain (69–71%). The moderate decline of dihydrouridine observed in *ylr401*-Δ/*ylr401*-Δ and *ylr405*-Δ/*ylr405*-Δ strains may also implicate these two Dus homologs in dihydrouridine modification of RNA; however, we are not as certain of the significance of this modest decrease, because of the partial overlap of dihydrouridine and pseudouridine in the chromatogram, and because of the inherent error in measuring low amounts of dihydrouridine (Fig. 7A).

TABLE 3. HPLC analysis of dihydrouridine in strains deleted for members of the *DUS* gene family.

	% nucleosides in diploid homozygous deletion strains/parental strain[a]				
Nucleoside	*dus1*-Δ/parent	*dus2*-Δ/parent	*ylr401*-Δ/parent	*ylr405*-Δ/parent	% mia/MIA[a]
D	51 ± 2	51 ± 3	69 ± 5	71 ± 3	56 ± 4
Ψ	108 ± 2	99 ± 3	102 ± 4	107 ± 3	120 ± 2
C	101 ± 0	99 ± 1	101 ± 1	102 ± 0	104 ± 1
U	101 ± 0	103 ± 1	102 ± 1	100 ± 0	95 ± 3
G	100 ± 0	99 ± 1	100 ± 0	100 ± 0	103 ± 1
A	100 ± 0	101 ± 0	100 ± 1	99 ± 0	99 ± 1

[a]All data are an average of results from two independent reverse-phase HPLC analyses.

DISCUSSION

We have used a biochemical genomics approach to show that the GST-ORF of YML080w (*DUS1*) copurifies with dihydrouridine synthase activity on pre-tRNA^{Phe}. Because Dus1 protein is active when purified either as a GST-fusion from yeast or as a His6-fusion from *E. coli*, we conclude that Dus1 is the catalytic subunit rather than an interacting protein that copurifies with the activity from yeast cells. As inferred from its catalytic role, Dus1 activity requires NADPH/NADH. In addition, activity is stimulated 25-fold in the presence of FAD; presumably Dus1 retains some FAD during purification.

We have also shown that Dus1 is a member of a widely conserved family of proteins that likely have a similar function. Three other family members are found in *S. cerevisiae*, as well as in bacteria and a number of different eukaryotes, with E values ranging from e^{-104} to e^{-6}. Moreover, the aligned proteins show many of the same conserved amino acid residues (Fig. 4); thus it seems likely that these proteins have similar function. Indeed, at least one other member of the yeast family, Dus2 (e^{-9}), also has demonstrable dihydrouridine synthase activity either as a GST-fusion purified from yeast or as a His6-fusion purified from *E. coli*, and is similarly FAD-stimulated. We note that multiple homologs of the Dus family proteins are observed in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila*, and humans, as well as in *S. cerevisiae*.

It seems likely that the multiple Dus family members differ in their substrate specificity. This would explain why there are multiple proteins in this family, much as the different pseudouridine modifications of RNA are catalyzed by 10 distinct pseudouridine synthases in *E. coli* (see Del Campo et al., 2001), and by at least 6 different pseudouridine synthases in yeast (Becker et al., 1997; Lafontaine et al., 1998; Lecointe et al., 1998; Motorin et al., 1998; Ansmant et al., 2000, 2001). There are two observations that are consistent with this idea. First, substrate specificity is apparent in vitro. Thus, Dus1 is specific for modification of U17 of pre-tRNA^{Phe}, and none of the other yeast family members is observed to modify this pre-tRNA. Moreover, Dus1 can modify both pre-tRNA^{Phe} and pre-tRNA^{Tyr}, but not pre-tRNA^{Leu}, whereas Dus2 can modify pre-tRNA^{Tyr} and pre-tRNA^{Leu}, but not pre-tRNA^{Phe}. The observation that both Dus1 and Dus2 can modify pre-tRNA^{Tyr} may mean that each protein modifies a different set of the six characterized dihydrouridine modifications of this tRNA. Alternatively, their substrate specificity may be partially overlapping. Second, analysis of the dihydrouridine content of mutant strains suggests that individual Dus proteins are responsible for some, but not all, of the dihydrouridine modifications in vivo. Thus, *dus1*-Δ/*dus1*-Δ and *dus2*-Δ/*dus2*-Δ mutants are both substantially reduced in the dihydrouridine content of their RNAs relative to that observed in control strains.

This substrate specificity may also extend to the other two yeast Dus family members, as YLR405w appears to have marginal activity on tRNA^{Leu}, but no detectable activity with the other two tRNAs, and strains lacking either YLR405w or YLR401c are both somewhat defective in the dihydrouridine content of their low molecular weight RNAs. However, we note that the total loss of dihydrouridine content in the four strains deleted for individual Dus family members is closer to 1.6 than 1.0 (Table 3). This could arise because the absence of one Dus protein affects the activity of other Dus proteins; for example, modification of a tRNA by one Dus protein may be required for modification at a nearby uridine residue of the same tRNA by another Dus protein. Alternatively, the quantitation of dihydrouridine may be inaccurate, as noted above, because of the low values of dihydrouridine and its proximity in the chromatogram to pseudouridine. The relationship of the *DUS* gene family with the *mia* mutant (Lo et al., 1982), which also has substantially lowered dihydrouridine content, is not yet clear.

One source of different substrates for the yeast dihydrouridine synthases may be mitochondrial tRNAs, many of which also have dihydrouridine (Sprinzl et al., 1998). Although the cellular localization of the Dus protein family members is not known, use of a computational prediction program suggests that Dus1 has a 90% probability, and Dus2 a 29% probability, of having

a mitochondrial targeting motif, whereas the other two Dus family members have 0% probability of such a motif (Claros & Vincens, 1996). Indeed, Dus2 may have a role in mitochondria, because Dus2 was previously identified as *SMM1*, a weak multicopy suppressor of a mitochondrial $tRNA^{Asp}$ mutant defective in 3′ end maturation (Rinaldi et al., 1997). This mutant tRNA has a C-to-U transition at the invariant position 61 in the TΨC loop of mitochondrial $tRNA^{Asp}$ (Zennaro et al., 1989). The relationship between the dihydrouridine synthase activity of Dus2 and its role as a suppressor of the $tRNA^{Asp}$ mutant is not clear. However, a parsimonious explanation is that Dus2 catalyzes dihydrouridine modification of some tRNAs in mitochondria, and that overproduction of this activity rescues the $tRNA^{Asp}$ defect. The activity of Dus2 (or Dus1) on nuclear tRNAs, as shown in Figure 5, does not preclude its activity on mitochondrial tRNAs, as several tRNA modification enzymes (Mod5, catalyzing i^6A formation; Trm1, catalyzing m_2^2G formation; and Pus3, catalyzing Ψ formation) have roles in both compartments (Ellis et al., 1986; Gillman et al., 1991; Lecointe et al., 1998). Alternatively, Dus2 may have a second distinct role, as was previously shown for *E. coli* RluD, a Ψ synthase responsible for both pseudouridine modifications in 23S rRNA and an unrelated role in growth (Gutgsell et al., 2001).

Elucidation of the cellular role of dihydrouridine and of the Dus protein family members awaits further study. We note that yeast strains deleted for each individual Dus family member are viable and there is no obvious growth phenotype for any of these strains in rich medium containing glucose. Examination of other phenotypes, and determination of the role of overexpressed Dus2 in suppressing a mitochondrial $tRNA^{Asp}$ mutant, will shed light on these roles.

MATERIALS AND METHODS

Strains and plasmids

Strains used in this study are summarized in Table 4. Plasmids pEFX-08 and pEFX-04 were created by PCR amplification of *DUS1* and *DUS2* genes respectively from *S. cerevisiae* genomic DNA, treatment of the DNA with *Xho*I and *Bam*HI, and ligation of the resulting fragment into the *Xho*I/*Bam*HI site of pET-15b. The primers used were as follows: 5′ primer of *DUS1*, 5′-CTTCTCGAGATGACTGAACCTGCCCT GAG; 3′ primer of *DUS1*, 5′-CCTGGATCCTTAAGCTTTCA CGTCCTTCTTTTTATC; 5′ primer of *DUS2*, 5′-CTGCTCGA GATGGTTACATATGCTGGAAAACTGG; 3′ primer of DUS2, 5′-CCTTGGATCCTTATATATCTGTGGGGAGGGGTAC.

Purification of GST-ORF fusion protein from yeast

The 64 pools of purified yeast GST-ORF fusion proteins were each prepared from 250-mL cultures expressing 96 different GST-ORF strains from the genomic collection of strains, as outlined previously (Martzen et al., 1999), and described in detail elsewhere (Phizicky et al., 2002). Purified proteins were stored at −20 °C after dialysis in storage buffer, which contains 20 mM Tris-HCl, 2 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 55 mM NaCl, and 50% (v/v) glycerol. Subpools of yeast GST-ORF fusion proteins and individual GST-ORF fusion proteins were prepared in the same way. Typically, crude extracts contained 20–50 mg/mL protein, and purified preparations of GST-ORFs contained 150–350 μg/mL protein. Purified preparations of individual GST-ORFs from yeast were about 50% pure, with few visible contaminants.

Purification of His6-tagged Dus1 and Dus2 from *E. coli*

Dus1 and Dus2 were expressed as His6-Dus1 and His6-Dus2 fusion proteins in *E. coli* and were purified from 250 mL

TABLE 4. Strains used in this study.

Strain	Genotype	Source
EJ758	**MATa** *his3-Δ200*, *leu2-3, 112*, *ura3-52*, *pep4*::*HIS3*	Martzen et al., 1999
FX-20	EJ758 [pFX20- (pYEX4T-1rec with P_{CUP1} GST-YML080w)]	This study
FX-02	EJ758 [pFX02- (pYEX4T-1rec with P_{CUP1} GST-YNR015w)]	This study
BY4743	**Mata**/**Matα** *his3-Δ1/his3-Δ1*, *leu2-Δ0/leu2-Δ0*, *ura3-Δ0*/*ura3-Δ0*, *met15-Δ0*/+, *lys2-Δ0*/+	Research Genetics, Inc.
BY4743.30490	BY4743 *dus1*-Δ::*kanMX4/dus1*-Δ::*kanMX4*	Research Genetics, Inc.
BY4743.35390	BY4743 *dus2*-Δ::*kanMX4/dus2*-Δ::*kanMX4*	Research Genetics, Inc.
BY4743.35310	BY4743 ylr401c-Δ::*kanMX4*/ylr401c-Δ::*kanMX4*	Research Genetics, Inc.
BY4743.35314	BY4743 ylr405w-Δ::*kanMX4*/ylr405w-Δ::*kanMX4*	Research Genetics, Inc.
BY4741	**MATa** *his3-Δ1*, *leu2-Δ0*, *met15-Δ0*, *ura3-Δ0*	Research Genetics, Inc.
BY4741.5390	BY4741 *dus2*-Δ::*kanMX4*	Research Genetics, Inc.
BY4741.5310	BY4741 *ylr401c*-Δ::*kanMX4*	Research Genetics, Inc.
BY4741.5314	BY4741 *ylr405w*-Δ::*kanMX4*	Research Genetics, Inc.
BY4730.490	**MATa** *leu2-Δ0*, *met15-Δ0*, *ura3-Δ0*, *dus1*-Δ::*kanMX4*	Research Genetics, Inc.
RL1015B	**MATα** *trm1*, *mia*, *ade*$^-$, *leu2-1*	Anita Hopper
EFX-08	BL-21 [pEFX-08- (pET15b-P_{T7}YML080w)]	This study
EFX-04	BL-21 [pEFX-04- (pET15b-P_{T7}YNR015w)]	This study

of culture by immobilized metal ion affinity chromatography as described previously (Steiger et al., 2001). Purified proteins (0.9 mg/mL and 3.2 mg/mL, respectively) were of comparable purity, ~25%, and were stored at −20 °C after dialysis in storage buffer (described above).

In vitro transcription of tRNA genes

Plasmid-borne copies of end-matured yeast pre-tRNA$^{Phe}_{GAA}$ (Reyes & Abelson, 1987) and pre-tRNA$^{Leu}_{CAA}$ (obtained from Jane Jackman) and PCR amplified yeast pre-tRNA$^{Tyr}_{GUA}$ DNA containing the 5′-leader and the 3′-trailer sequences (Lee et al., 1997) were used as templates in transcription. The [α-^{32}P]UTP-labeled tRNA transcripts were synthesized using ~1 μg of the *Bst*NI linearized plasmids or ~50 ng of the PCR product in 10 μL of reaction mixture containing 20 U T7 RNA polymerase (Boehringer-Mannheim), 1× transcription buffer, 7.4 mM DDT, 51 μM [α-^{32}P]UTP (76 Ci/mmol), 1 mM each of the other three NTPs, and 20 U of RNasin, followed by purification of labeled RNA on a 10% polyacrylamide gel containing 4 M urea, and elution of the transcripts (Culver et al., 1997).

Assay of dihydrouridine synthase activity

Dihydrouridine synthase activity was assayed in 20 μL reaction mixtures containing 100 mM Tris-HCl, pH 8.0, 100 mM ammonium acetate, 5 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 1 mM NADPH, 1 mM NADH, 50,000 cpm of labeled transcript (6 fmol), and up to 30 μg of yeast or *E. coli* extracts, 300 ng of GST fusion proteins, or 1 μg of His6-tagged protein. In titration assays, extracts and purified proteins were diluted in buffer containing 50 mM Tris-HCl, pH 8.0, 250 μg/mL bovine serum albumin, and 2 mM DTT. In some assays, 250 μM of flavin adenine dinucleotide (FAD) was also included. Samples were incubated for 30 min at 30 °C and RNA was extracted with phenol/chloroform, precipitated with ethanol, dried, resuspended in 5 μL of buffer containing 30 mM NaAc, pH 5.3, 200 μM $ZnCl_2$, and treated with 1 μg P1 nuclease at 37 °C for 1 h. Then nucleotides were resolved by thin layer chromatography using either cellulose plates developed in one dimension with solvent containing ammonium sulfate (74 g/100 mL H_2O, pH 3.5):H_2O:isopropanol (80:18:2, v/v/v), or using PEI-cellulose plates developed in two dimensions with 1 M acetic acid, pH 3.5, for the first dimension, and in buffer containing 74 g ammonium sulfate/100 mL H_2O (adjusted to pH 3.5 with H_2SO_4) for the second dimension (Bochner & Ames, 1982). Radioactive signals were detected after exposure of the plates on a PhosphorImager screen (Molecular Dynamics, Sunnyvale, California), and were analyzed using the software ImageQuant. In titration assays, 1 U of activity is defined as the amount of protein required to convert half of the tRNA substrate to product (3 fmol).

Mapping of dihydrouridine modification site by Dus1 on pre-tRNA^{Phe}

The approach involves two steps. In the first step, 4 × 10^6 cpm of [α-^{32}P]UTP-labeled pre-tRNA^{Phe} were incubated with 1.2 μg of GST-Dus1 in 100 μL at 30 °C for 6 h in the presence or absence of 1 mM of NADPH and NADH, and were extracted with phenol/chloroform followed by ethanol precipitation. Recovered tRNA was then cleaved with tRNA splicing endonuclease in 100 μL (McCraith & Phizicky, 1990), and the resulting 5′-half molecule (37 nt), 3′-half molecule (39 nt), and intron (19 nt) were separated on a 15% polyacrylamide gel (acrylamide:bis/37.5:1) containing 4 M urea and TBE, eluted in 200 μL of 200 mM NH_4Ac containing 5 μg carrier RNA, ethanol precipitated, and resuspended in buffer containing 10 mM Tris-HCl, pH 8, and 1 mM EDTA. A small portion of the purified fragments was analyzed by P1 nuclease digestion and one-dimensional thin layer chromatography, and 100,000 cpm of the 5′-half molecule was further hydrolyzed in 5 μL with 1 U of RNase T1. The four ^{32}P-labeled oligonucleotides produced this way (see text) were separated on a 22% polyacrylamide gel, eluted in 200 μL of 200 mM NH_4Ac containing 5 μg carrier RNA, ethanol precipitated, dried, resuspended in water, and analyzed by P1 nuclease digestion and thin layer chromatography.

Preparation of RNA from *S. cerevisiae* strains

RNA was isolated from 125 mL log phase yeast cells by hot phenol extraction, as previously described (Ogden et al., 1979). This protocol yields approximately 6 mg of predominantly low-molecular-weight RNA from 125 mL as analyzed on a 1% agarose gel.

Reverse-phase HPLC analysis of dihydrouridine content in yeast cells

Twenty micrograms of RNA prepared from yeast were hydrolyzed to nucleosides in 100 μL buffer by digestion with 1 μg P1 nuclease at 37 °C overnight, followed by digestion with 1 U calf intestinal phosphatase for 3 h at 37 °C, and analyzed on a Waters 2690 HPLC by a reverse-phase C18 column (supelcosil LC-18-T, 25 cm × 4.6 mm, 5 μm; Supelco, Inc., Bellefonte, Pennsylvania) as described (Gehrke & Kuo, 1989) with a slight modification of the gradient procedure in which, during the first 6 min, solvent A is dropped from 100% to 95%, and solvent B is increased from 0 to 5% at a flow rate of 1.3 mL/min. Pseudouridine, cytidine, uridine, guanosine, and adenosine were identified by comparing their spectra and retention time with that of corresponding standards. Dihydrouridine is identified as described in the text, using absorbance at 210 nm and at 263 nm (Topp et al., 1993). Calculations of the relative amounts of each nucleoside are described in the text, using the chromatogram obtained at 210 nm.

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