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Yeast Nfs1p Is Involved in Thio-modification of Both Mitochondrial and Cytoplasmic tRNAs*

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The IscS protein is a pyridoxal phosphate-containing cysteine desulfurase involved in iron-sulfur cluster biogenesis. In prokaryotes, IscS is also involved in various metabolic functions, including thio-modification of tRNA. By contrast, the eukaryotic ortholog of IscS (Nfs1) has thus far been shown to be functional only in mitochondrial iron-sulfur cluster biogenesis. We demonstrate here that yeast Nfs1p is also required for the post-transcriptional thio-modification of both mitochondrial (mt) and cytoplasmic (cy) tRNAs *in vivo*. Depletion of Nfs1p resulted in an immediate impairment of the 2-thio-modification of 5-carboxymethylaminomethyl-2-thiouridine at the wobble positions of mt-tRNA$^{Lys}_{UUU}$ and mt-tRNA$^{Gln}_{UUG}$. In addition, we observed a severe reduction in the 2-thio-modification of 5-methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U) of cy-tRNA$^{Lys2}_{UUU}$ and cy-tRNA$^{Glu3}_{UUC}$, although the effect was somewhat delayed compared with that seen in mt-tRNAs. Mass spectrometry analysis revealed an increase in 5-methoxycarbonylmethyluridine concomitant with a decrease in mcm^{5}s^{2}U in cy-tRNAs that were prepared from Nfs1p-depleted cells. These results suggest that Nfs1p is involved in the 2-thio-modification of both 5-carboxymethylaminomethyl-2-thiouridine in mt-tRNAs and mcm^{5}s^{2}U in cy-tRNAs.

NifS is a pyridoxal phosphate-containing cysteine desulfurase that was first described in the biosynthesis of nitrogenase in diazotrophic bacteria such as *Azotobacter vinelandii*. Subsequently, it was identified as a supplier of the sulfur atom that is incorporated into the iron-sulfur cluster (ISC)[1] in nitrogenase (1, 2). IscS is a homolog of NifS that is found in a wide range of organisms, including non-nitrogen-fixing bacteria (3–5) and even eukaryotes (6–8), and it is now known to mobilize a sulfur atom from L-cysteine to a nascent ISC on a scaffold protein such as IscU or NifU (9–12).

In eukaryotes, the IscS homolog Nfs1 (or Nfs1p) is most frequently found in mitochondria (6–8), along with various iron-sulfur proteins that include essential electron carriers of the respiratory cascade. *Saccharomyces cerevisiae* Nfs1p, which is essential for cell viability, is also located mainly in mitochondria, where it serves as a sulfur supplier in ISC biogenesis (6, 7, 13–16). Nfs1p has also been localized to the nucleus, where it is thought to function in essential processes other than ISC biosynthesis (14).

Recently, *Escherichia coli* IscS was shown to mobilize a sulfur atom via pyridoxal phosphate-dependent formation of a disulfide intermediate on the enzyme (17). This unique sulfur-mobilizing action of IscS allows it to participate in the biosynthesis of other sulfur-containing cofactors or small molecules, as well as ISC formation. For example, an *E. coli* IscS deletion mutant showed a significant decrease in the production of nicotinic acid and branched chain amino acids (18). Serial deletions of the *E. coli iscS* gene region revealed that IscS is involved in amino acid metabolism (19). Furthermore, in *E. coli* and *Salmonella enterica* serovar Typhimurium, IscS contributes to thiamine biogenesis by catalyzing the transfer of sulfur in the pathway involved in the formation of thiazole rings (18, 20).

Further evidence of the sulfur-transferring activity of IscS is seen in the thio-modification of nucleotides in tRNA. *E. coli* IscS transfers a sulfur atom from the substrate cysteine to produce a 4-thiouridine at position 8 of tRNA *in vivo* (21) and *in vitro* (22). This reaction is partly shared with the thiamine biosynthesis pathway described above by cooperating with ThiI (18, 23). *E. coli* IscS is also involved in 2-thiouridine formation in tRNA *in vitro* (24). In this case, IscS works together with MnmA (24), and the resulting 2-thiouridine is hypermodified to 5-carboxymethylaminomethyl-2-thiouridine via the joint action of two other proteins (MnmE and MnmC) (25). Furthermore, in *E. coli* and *S. enterica*, the IscS protein is involved in the biosynthesis of at least five different thio-modified nucleotides *in vivo*, suggesting that IscS plays a critical role in thio-modification in bacterial cells (26, 27).

Modified nucleotides are found in various RNAs in virtually all living organisms (28). Although thio-modification has been found in eukaryotic tRNAs, the participation of Nfs1 (or Nfs1p) has yet to be proven. In eukaryotic cells, thio-modified tRNA molecules are found in both mitochondria and the cytoplasm (29–36), whereas Nfs1 is mainly localized in mitochondria. Therefore, the question remains as to whether Nfs1 is involved in sulfur donation during mitochondrial (mt) and/or cytoplasmic (cy) tRNA thio-modification. In this study, we used a yeast conditional mutant strain (in which the expression of Nfs1p was repressed) to investigate the participation of Nfs1p in the thio-modification of both cy- and mt-tRNAs.

EXPERIMENTAL PROCEDURES

Yeast Strains and Cell Growth Conditions—S. cerevisiae W303-1B (*MAT***a**, *ade2-1, his3-11,15, ura3-1, leu2-3,112, trp1-1, can1-100*) was used as the wild-type strain, along with its derivative YN101, in which

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[1] The abbreviations used are: ISC, iron-sulfur cluster; mt, mitochondrial; cy, cytoplasmic; APM, [(*N*-acryloylamino)phenyl]mercuric chloride; mcm^{5}U, 5-methoxycarbonylmethyluridine; mcm^{5}s^{2}U, 5-methoxycarbonylmethyl-2-thiouridine.

the Nfs1p gene (*NFS1*) is expressed under the control of the *GAL1* promoter (14). Cells were grown at 30 °C in galactose medium (2% D-galactose, 0.5% casamino acids, and 0.67% yeast nitrogen base without amino acids, plus adequate supplements for selection; BD Biosciences), lactate medium (2% lactate, 0.1% D-glucose, 0.5% casamino acids, and 0.67% yeast nitrogen base without amino acids, plus adequate supplements for selection), or glucose medium (2% D-glucose, 0.5% casamino acids, and 0.67% yeast nitrogen base without amino acids). For sulfate-limited growth, cells cultivated in normal medium were further incubated with medium prepared with yeast nitrogen base lacking ammonium sulfate (BD Biosciences).

Pulse Labeling of Yeast Cells with L-[^{35}S]Cysteine—Yeast cells incubated with the sulfur-lacking medium described above were harvested and further incubated with L-[^{35}S]cysteine (Amersham Biosciences) at a concentration of 10^7 cells/ml/1000 μCi for 30 min at 30 °C. Cells were then harvested and used to prepare the total tRNA.

Total tRNA Preparation, Followed by Electrophoresis for Detection of the Radiolabeled tRNA Fraction—Total tRNA from yeast cells was extracted with phenol, precipitated with isopropyl alcohol, and washed once with 70% ethanol. tRNAs were applied to 10% polyacrylamide gels containing 8 M urea. Following electrophoresis, the gel was wrapped entirely and exposed to a Fuji Film imaging plate for >30 h. The exposed imaging plate was then subjected to analysis by a Fuji Film BAS-2500 image analyzer to detect radioactivity in the tRNA fractions.

Detection of Thiomodified Uridine in tRNAs by [(N-Acryloylamino)-phenyl]mercuric Chloride (APM) Gel Electrophoresis, Followed by Northern Hybridization (APM/Northern)—The presence of thiouridine in the prepared tRNA was verified by the retardation of electrophoretic mobility on polyacrylamide gels containing 0.05 mg/ml APM (kindly provided by Naoki Shigi, University of Tokyo) (37) in a procedure originally developed by Igloi (38). Total RNA (0.05 A_{260} units) was separated by PAGE as described above and blotted onto Hybond N^+ membranes (Amersham Biosciences). Each tRNA fraction was detected with a specific ^{32}P-5′-labeled oligonucleotide probe. The following oligonucleotides were used: 5′-TGGTGAGAATAGCTGGAGTTGAAC-3′ for mt-tRNA$^{Lys}_{UUU}$, 5′-TGGTTGAATCGG TTTGATTCGAAC-3′ for mt-tRNA$^{Gln}_{UUG}$, 5′-TGGCTCCTCATAGGGGGCTCGAAC-3′ for cy-tRNA$^{Lys2}_{UUU}$, and 5′-TGGCTCCGATACGGGGAGTCGAAC-3′ for cy-tRNA$^{Glu3}_{UUC}$. Following hybridization, membranes were washed, dried, and then exposed to the imaging plate for 1 h to detect cy-tRNAs and for at least 24 h to detect mt-tRNAs. Radioactivity was detected with the BAS-2500 image analyzer.

Mass Spectrometry Analysis of RNA Modification in Yeast tRNAs—Total tRNAs (1.5 A_{260} units in each case) were digested with 10 μg/ml ribonuclease P1 (Seikagaku Kougyo) and 9 units/ml bacterial alkaline phosphatase (Takara Shuzo) at 37 °C for 3 h. The nucleosides were subjected to liquid chromatography/mass spectrometry analysis using an Agilent 1100 liquid chromatography system equipped with a ThermoFinnigan LCQ Duo ion-trap mass spectrometer as described previously (39).

Immunochemical Detection of Yeast Nfs1p—Yeast cells were harvested by centrifugation, and total proteins were extracted by an alkali-SDS method (14). Protein concentrations were determined by the BCA method (Pierce) with bovine serum albumin (Sigma) as a standard. A total of 2 μg of cellular proteins were analyzed immunochemically with anti-Nfs1p antibody to examine the expression levels of Nfs1p (14).

RESULTS

Thio-modification of tRNA Is Dependent on the Expression of Yeast Nfs1p—Considering that bacterial IscS proteins participate in the thio-modification of tRNA (18, 21, 22, 24, 26, 27), it is probable that homologous eukaryotic Nfs1 proteins are also involved in the similar post-transcriptional thio-modification of tRNA. To examine this possibility, we first investigated whether the expression of Nfs1p is necessary for the transfer of a sulfur atom from L-cysteine to tRNA in yeast cells. YN101 cells, in which the Nfs1p gene (*NFS1*) is expressed under the control of the *GAL1* promoter, grew normally in galactose medium (designated as YN-G cells), but their growth was inhibited in glucose medium (designated as YN-D cells) (14), in which the expression of *NFS1* was repressed (Fig. 1, *A* and *B*). YN-G and YN-D cells, together with wild-type cells grown under similar conditions (designated as WT-G and WT-D cells, respectively) and used as controls, were then separately subjected to *in vivo* pulse labeling experiments with L-[^{35}S]cysteine to see if its radioactive sulfur transfers to tRNAs. As shown in Fig. 1*B*, total tRNAs from both WT-G and WT-D cells efficiently incorporated the radioactive sulfur of L-[^{35}S]cysteine. In contrast, total tRNAs from YN-D cells were not radiolabeled with sulfur. As predicted, radiolabeling could be induced by the expression of *NFS1* following growth in galactose medium (YN-G cells) (Fig. 1*B*). These results clearly demonstrate that the efficient thio-modification of tRNAs *in vivo* depends on the presence of *NFS1*.

Nfs1p Depletion Results in a Lack of Thio-modification of Uridine in mt-tRNAs—As yeast Nfs1p is localized mainly in mitochondria (6, 7), we next investigated whether depletion of Nfs1p can affect the thio-modification of mt-tRNAs. The wobble modification in both mt-tRNA$^{Lys}_{UUU}$ and mt-tRNA$^{Gln}_{UUG}$ has recently been found to possess 5-carboxymethylaminomethyl-2-thiouridine-modified uridine.[2] We employed APM-PAGE/Northern analysis using a specific probe, to detect 2-thiouridine modification in each of these two tRNAs (see "Experimental Procedures") (37). Both mt-tRNAs from WT-G and WT-D cells showed a remarkably retarded migration on the APM gel (Fig. 2, *A* and *B*, *right panels*) compared with the migration on a gel without APM (*left panels*). However, mt-tRNAs from glucose-grown YN101 cells (YN-D) were not retarded on the APM gel, whereas mt-tRNAs from cells cultured in galactose medium (YN-G) were retarded because of the induction of the 2-thio modifications (Fig. 2, *A* and *B*, *right panels*). We also analyzed a 3-fold excess amount of tRNA prepared from YN-D cells (Fig. 2, *A* and *B*, *YN-Dx3*) to examine whether residual amounts of mt-tRNAs remained to be thio-modified. However, no thiouridine could be detected in both mt-tRNAs. Moreover, we carried out the experiment with YN101 cells under a different Nfs1p-depleted growth condition in which 2% lactate and 0.1% glucose were included instead of 2% glucose (designated as WT-L and YN-L cells) (Fig. 2). Under this non-fermentative growth condition, yeast mitochondria are known to develop well because of the lack of metabolic repression. However, as shown in Fig. 2 (*A* and *B*), both mt-tRNAs from YN-L cells displayed a similar absence of thiouridine compared with those from YN-D cells.

These results indicate that the thio-modification of uridine in both mt-tRNA$^{Lys}_{UUU}$ and mt-tRNA$^{Gln}_{UUG}$ requires the presence of Nfs1p. Because both mt-tRNAs are encoded by mitochondrial DNA, the thio-modification of uridine in these mt-tRNAs is most likely an intramitochondrial event that is facilitated by mitochondrial Nfs1p.

Deficiency in Thio-modification of Uridine in cy-tRNAs Is Caused by Nfs1p Depletion—We further investigated whether Nfs1p is involved in the 2-thio modification of uridine in cy-tRNAs. The 2-thio modification of cy-tRNA$^{Glu3}_{UUC}$ and cy-tRNA$^{Lys2}_{UUU}$ at the wobble positions has been reported previously (40). By APM-PAGE/Northern analysis, we found two yeast cy-tRNAs (cy-tRNA$^{Glu3}_{UUC}$ and cy-tRNA$^{Lys2}_{UUU}$) from wild-type cells that displayed retarded migration on the APM gel (Fig. 3, *right panels*), indicating the presence of thiouridine. This was the case regardless of the culture medium. However, in Nfs1p-depleted YN101 cells (YN-L and YN-D), the retarded bands with 2-thiouridine were less intense, and samples also displayed non-retarded bands (Fig. 3). These results demonstrate that Nfs1p depletion affects the 2-thio modification of uridine in these cy-tRNAs *in vivo* without completely abolishing it.

The most remarkable finding of the results shown in Fig. 3 is that significant fractions of these cy-tRNAs were still thio-modified despite the cells being cultured for 48 h in a medium that results in Nfs1p depletion. This is compared with the

[2] N. Umeda, T. Suzuki, and K. Watanabe, manuscript in preparation.

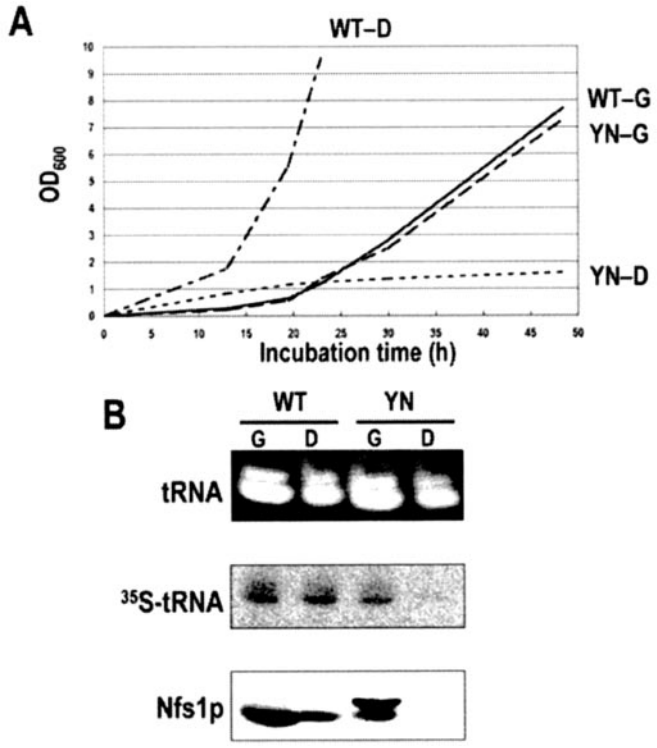

FIG. 1. **Incorporation of labeled sulfur derived from L-[^{35}S]cysteine into tRNA is affected by Nfs1p depletion *in vivo*.** *A*, shown are the growth curves of the *GAL1-NFS1* strain (YN101) and the wild-type strain. Cells precultured in galactose medium were grown in either galactose medium (YN-G and WT-G) or glucose medium (YN-D and WT-D). *B*, cells were incubated with L-[^{35}S]cysteine, and their total tRNAs were separated by electrophoresis on 8 M urea-containing polyacrylamide gels and then stained with ethidium bromide (*upper panel*). After electrophoresis, radioactive tRNA fractions were detected by autoradiography using an imaging analyzer (*middle panel*). Nfs1p proteins in these cells were immunochemically detected using anti-Nfs1p antibody (*lower panel*).

complete absence of 2-thiouridine formation in both mt-tRNA$^{Lys}_{UUU}$ and mt-tRNA$^{Gln}_{UUG}$ in the corresponding cells (Fig. 2). Furthermore, the remaining thio-modified cy-tRNA molecules were more abundant in the respiratory-grown cells (YN-L) compared with those in the glucose-utilizing cells grown under fermentative conditions (YN-D), in which mitochondrial development was repressed. Because *NFS1* expression seemed to be similarly repressed under both growth conditions, our findings suggest that mitochondrial activity directly contributes to the 2-thio modification of cy-tRNAs.

Thio-modification Deficiency in mt-tRNAs Precedes That in cy-tRNAs following Depletion of Nfs1p—According to the results shown in Figs. 2 and 3, impairment of the 2-thio modification of uridine in mt-tRNAs seemed to precede that in cy-tRNAs following depletion of Nfs1p. We further analyzed the levels of 2-thiouridine formation at 30 and 48 h after Nfs1p depletion (Fig. 4). YN101 cells could survive and grow slowly for some time in glucose medium, in which *NFS1* gene expression was repressed (Fig. 1*A*), and indeed, viable cells were still obtained after >30 h of growth under Nfs1p-depleted conditions, although Nfs1p levels were already markedly reduced (data not shown). We performed APM-PAGE/Northern analysis for both cy- and mt-tRNAs prepared from YN-D cells after 30 and 48 h of growth under Nfs1p-depleted conditions. Both mt-tRNAs were shown to lack 2-thio modification at uridine by the 30-h time point (Fig. 4*A*). On the other hand, more than half of the total cy-tRNA$^{Lys2}_{UUU}$ and cy-tRNA$^{Glu3}_{UUC}$ displayed 2-thiouridine modification at 30 h, and even after 48 h, a small but significant fraction of cy-tRNA was still found to be thio-modified (Fig. 4*B*). To examine whether cy-tRNAs can be thio-modified by an alternative Nfs1p-independent pathway utilizing inorganic sulfur as a source, YN101 cells were grown in medium with or without excess ammonium sulfate (0.5%). We observed no difference in the level of thio-modified cy-tRNA from the cells harvested 48 h after Nfs1p depletion, regardless of the presence or absence of excess ammonium sulfate (Fig. 5), indicating that excess inorganic sulfurs in the culture medium were not responsible for the delayed impairment of the thio-modification of cy-tRNAs.

Nfs1p Depletion Causes an Accumulation of 5-Methoxycarbonylmethyluridine (mcm^{5}U) and a Concomitant Decrease in 5-Methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U) in cy-tRNAs—We employed liquid chromatography/ion-trap mass spectrometry to analyze total nucleosides from both WT-D and YN-D cells. Because cy-tRNAs comprise a larger percentage of the total tRNA fraction compared with mt-tRNAs, practically all nucleosides detected in this analysis were regarded as being derived from cy-tRNAs. In wild-type cells, mcm^{5}s^{2}U was found to be a major constituent of 2-thiouridine-derived nucleosides, along with a smaller quantity of mcm^{5}U, a precursor form of mcm^{5}s^{2}U (Fig. 6*A*). In Nfs1p-depleted YN101 cells (YN-D), we saw a reduced amount of mcm^{5}s^{2}U and an increased amount of mcm^{5}U (Fig. 6*B*). The relative abundance of each nucleoside was determined by comparison with the amount of N^6-threonylcarbamoyladenosine, which was used as an internal standard (Fig. 6, *A* and *B*, *lower panels*). These results clearly indicate that Nfs1p depletion affects the thio-modification of cy-tRNAs at the level of sulfur incorporation into position 2 of mcm^{5}U to form mcm^{5}s^{2}U.

DISCUSSION

In prokaryotes, IscS-mediated sulfur mobilization is utilized in various processes, including the biosynthesis of ISC, thiamine, and thionucleosides in tRNAs (18, 20, 41, 42). From our results here, we conclude that the yeast IscS homolog, Nfs1p, is also required for the post-transcriptional modification of tRNAs, suggesting that, in addition to the well known essential contribution to ISC formation, eukaryotic Nfs1 also participates in multiple physiologically important reactions as a sulfur donor.

The main fraction of Nfs1p, together with other proteins required for ISC biogenesis, is located in mitochondria, with only a trace amount of Nfs1p being localized in nuclei (14). Mitochondrial Nfs1p is thought to donate a sulfur atom to Isu1p, the IscU homolog, on which a transient ISC is assembled (16, 43). We have shown in this study that Nfs1p depletion caused an immediate decrease in the levels of thio-modified tRNA$^{Lys}_{UUU}$ and tRNA$^{Gln}_{UUG}$ in mitochondria. In *E. coli*, MnmA has been shown to be involved in 2-thiouridine formation of 5-carboxymethylaminomethyl-2-thiouridine (24). YDL033 is a yeast homolog of MnmA and has been predicted to be located in mitochondria (*Saccharomyces* Genome Database).[3] Therefore, in mitochondria, it seems likely that Nfs1p cooperates with YDL033 to modify mt-tRNAs by incorporating a sulfur atom derived from L-cysteine.

Furthermore, we demonstrated that, in addition to the thio-modification of mt-tRNAs, Nfs1p is also involved in the thio-modification of cy-tRNAs. This phenomenon is quite intriguing, as Nfs1p has never been detected in the cytosolic fraction in yeast, so direct involvement of mitochondrial Nfs1p in the thio-modification of cy-tRNAs seems unlikely. From this point of view, it should be noted that impairment of the thio-modification of cy-tRNAs following depletion of Nfs1p seemed to be somehow delayed compared with the immediate defect in the

[3] Available at www.yeastgenome.org.

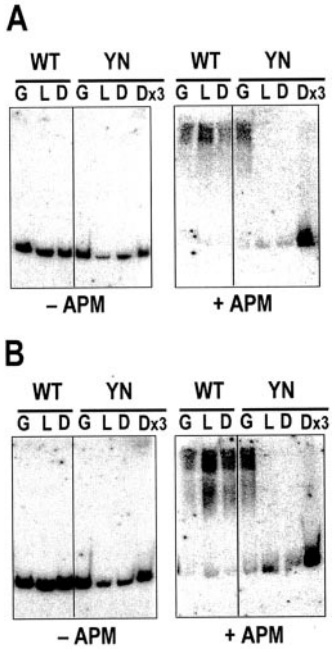

FIG. 2. **Nfs1p-dependent thio-modification of uridine occurs in mt-tRNA$^{Lys}_{UUU}$ and mt-tRNA$^{Gln}_{UUG}$.** Separate amounts (0.05 A_{260} units each) of the total tRNAs prepared from yeast cells were separated by electrophoresis on 8 M urea-containing polyacrylamide gels with (+) and without (−) 120 μM APM, blotted onto the membrane, and subjected to the Northern hybridization analysis with ^{32}P-labeled DNA probes. *A* and *B* show the results using the DNA probes specific for mt-tRNA$^{Lys}_{UUU}$ and mt-tRNA$^{Gln}_{UUG}$, respectively. *Dx3* indicates that a 3-fold increase in the amount of total tRNA (0.15 A_{260} units) from YN-D cells was applied.

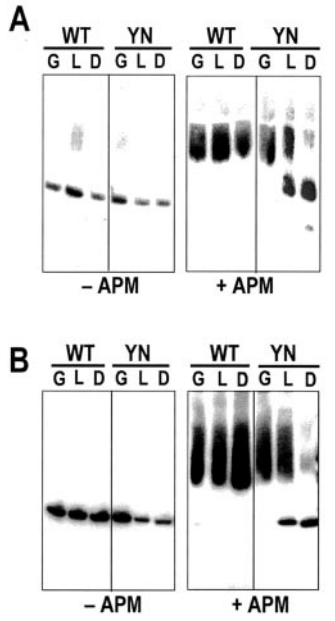

FIG. 3. **Thio-modification of uridine in cy-tRNA$^{Lys2}_{UUU}$ and cy-tRNA$^{Glu3}_{UUC}$ is also affected by the depletion of Nfs1p.** The total tRNAs prepared from cells were separated using urea-containing gel with (+) and without (−) 60 μM APM and then subjected to Northern analysis. *A* and *B* show the results with the DNA probes for cy-tRNA$^{Lys2}_{UUU}$ and cy-tRNA$^{Glu3}_{UUC}$, respectively.

thio-modification of mt-tRNAs. One possible explanation is that, compared with the thio-modified mt-tRNAs, thio-modified cy-tRNAs are more stable, and their turnover is quite slow *in vivo*, despite the fact that we could observe *de novo* synthesis of thio-modified tRNAs (mostly cy-tRNAs) as early as 30 min after addition of L-[^{35}S]cysteine. We previously showed that a trace amount of the nuclear version of Nfs1p is needed for an as yet unknown essential function besides ISC formation (14). Therefore, it is possible that nuclear Nfs1p might be involved in the thio-modification of cy-tRNA during the process of tRNA maturation in the nucleus. On the other hand, another pathway that incorporates inorganic sulfur compounds such as ammonium sulfate is unlikely, as the presence of excess sulfate in glucose medium did not influence the impairment of the

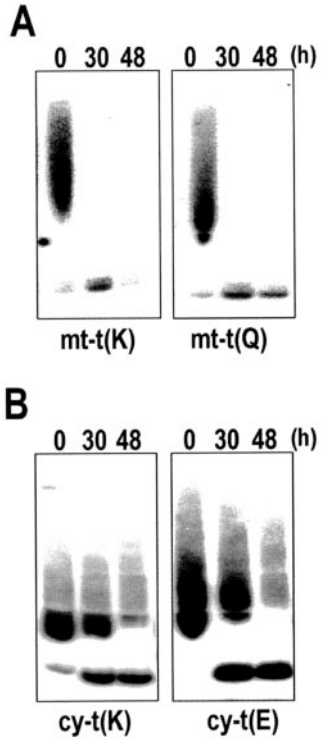

FIG. 4. **Impairment of the thio-modification of cy-tRNA$^{Lys2}_{UUU}$ and cy-tRNA$^{Glu3}_{UUC}$ following depletion of Nfs1p is delayed compared with the immediate defect found in mitochondrial thio-modification.** YN101 cells were cultured in glucose medium (Nfs1p-depleted conditions) for various incubation times (0, 30, and 48 h). Total tRNAs were prepared from these cells and subjected to APM-PAGE/Northern analysis (Fig. 3) using the probes for mt-tRNAs (*A*) and cy-tRNAs (*B*). *mt-t(K)* and *mt-t(Q)* indicate mt-RNA$^{Lys}_{UUU}$ and mt-tRNA$^{Gln}_{UUG}$, respectively, and *cy-t(K)* and *cy-t(E)* indicate tRNA$^{Lys2}_{UUU}$ and cy-tRNA$^{Glu3}_{UUC}$, respectively.

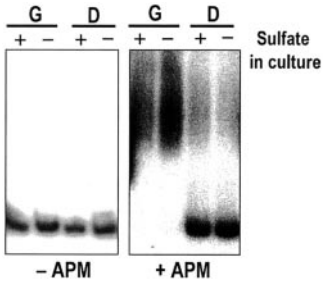

FIG. 5. **Cultivation under sulfate-limiting conditions does not affect the delayed impairment of the thio-modification of cy-tRNA$^{Lys2}_{UUU}$.** YN101 cells were cultured either in galactose medium (*G*) or glucose medium (*D*) in the presence of ammonium sulfate for 24 h and then harvested. These harvested cells were cultured for an additional 24 h in fresh galactose or glucose medium under sulfate-containing (+ *Sulfate in culture*) or sulfate-limiting (− *Sulfate in culture*) conditions. Note that, for both growth conditions, sulfur-containing amino acids such as cysteine and methionine were included in the medium. Cells were then analyzed by APM-PAGE/Northern analysis using the probe for cy-tRNA$^{Lys2}_{UUU}$ as described in the legend to Fig. 3.

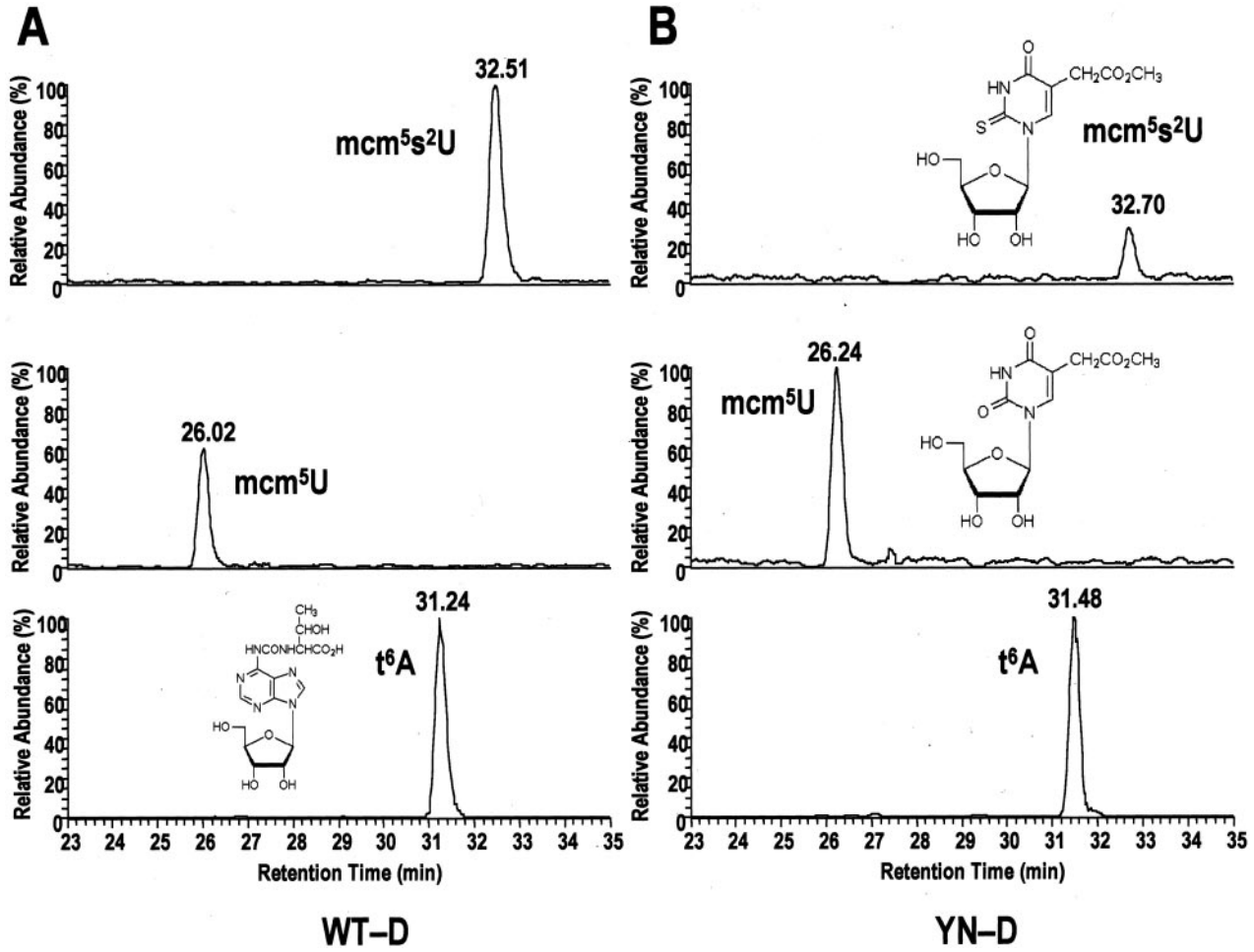

FIG. 6. **Liquid chromatography/mass spectrometry nucleoside analysis of total tRNAs prepared from wild-type and Nfs1p-depleted yeast cells.** Shown are chromatograms for total tRNAs prepared from WT-D (*A*) and YN-D (*B*) cells. *Upper panels*, chromatograms for modified uridines at a range of *m/z* 332.5 to 333.5 to detect ionized mcm^5s^2U. *Middle panels*, chromatograms at a range of *m/z* 316.5 to 317.5 to detect ionized mcm^5U. *Lower panels*, chromatograms at a range of *m/z* 412.5 to 413.5 to detect ionized N^6-threonylcarbamoyladenosine (t^6A). Relative amounts of each nucleoside were normalized with the content of N^6-threonylcarbamoyladenosine, which could be regarded as an internal standard. The chemical structures of nucleosides detected are shown in the chromatograms.

thio-modification of cy-$tRNA^{Lys2}_{UUU}$ under the Nfs1p-limiting condition (Fig. 5).

Alternatively, if a certain ISC protein is involved in the thio-modification of cy-tRNAs, and if mitochondrial Nfs1p is responsible for ISC synthesis, Nfs1p depletion may indirectly impair the thio-modification of cy-tRNAs. This would be similar to the case in *E. coli* for the iron-sulfur protein MiaB, which produces an IscS-dependent methylthio-modification of 2-methylthio-N^6-isopentanyladenosine at position 37 in *E. coli* tRNA (26). Although it has been reported that *iscS* deletion results in a defect in the methylthio-modification in *E. coli* (44), it is not known whether *E. coli* IscS is directly involved in the methylthio-modification or is just responsible for the biosynthesis of MiaB.

Another possibility is that sulfur atoms found in thionucleotides of cy-tRNAs are generated by Nfs1p-mediated cysteine desulfuration in mitochondria and are then transported outside via an as yet unknown pathway. We observed that the thio-modification of cy-tRNAs seemed to be prolonged in the Nfs1p-depleted cells grown under non-fermentative conditions, where mitochondria were well developed, suggesting that mitochondrial development might further increase such accumulation of any sulfur-containing metabolite. In the case of cytoplasmic iron-sulfur protein maturation, cluster-containing sulfur atoms are delivered via mitochondrial ISC biosynthesis machinery, and mitochondrial glutathione and at least two mitochondrial proteins, Atm1p and Erv1p, have been found to be required in this pathway (7, 45, 46). These mitochondrial factors, including some possible small sulfur-containing non-proteinaceous factors, might also be involved in the sulfur delivery system for cy-tRNAs. In addition, delayed impairment of the thio-modification of cy-tRNAs following Nfs1p depletion might be due to cytoplasmic accumulation of any sulfur-containing intermediate metabolite(s) for a pathway located downstream of the reaction of mitochondrial Nfs1p. Such accumulated metabolites could be used as sulfur donors in the absence of Nfs1p. Further study will be required to test these hypotheses and to identify any additional components required for these thio-modifications.

Bacterial IscS is involved in all thio-modifications of tRNAs, raising the possibility that yeast Nfs1p may also be involved in other thio-modifications of both cy- and mt-tRNAs. Future studies should investigate whether Nfs1p is indispensable in other thio-modifications. Yeast Nfs1p was first identified as a protein involved in tRNA splicing *in vivo* (47), but the relationship between Nfs1p function and tRNA splicing has not yet been elucidated. We hope that the actions of Nfs1p in tRNA thio-modification demonstrated in this study may shed some light on this problem, which we will address in the future.

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