

Wobble Inosine tRNA Modification Is Essential to Cell Cycle Progression in G₁/S and G₂/M Transitions in Fission Yeast*

Received for publication, August 17, 2007, and in revised form, September 17, 2007. Published, JBC Papers in Press, September 17, 2007, DOI 10.1074/jbc.M706869200

Satoshi Tsutsumi[‡], Reiko Sugiura[§], Yan Ma[‡], Hideki Tokuoka[‡], Kazuki Ohta[‡], Rieko Ohte[‡], Akiko Noma[¶], Tsutomu Suzuki[¶], and Takayoshi Kuno^{‡,1}

From the [‡]Division of Molecular Pharmacology and Pharmacogenomics, Department of Biochemistry and Molecular Biology, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan, [§]Laboratory of Molecular Pharmacogenomics, School of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi-Osaka, 577-8502, Japan, and the [¶]Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Inosine (I) at position 34 (wobble position) of tRNA is formed by the hydrolytic deamination of a genomically encoded adenosine (A). The enzyme catalyzing this reaction, termed tRNA A:34 deaminase, is the heterodimeric Tad2p/ADAT2-Tad3p/ADAT3 complex in eukaryotes. In budding yeast, deletion of each subunit is lethal, indicating that the wobble inosine tRNA modification is essential for viability; however, most of its physiological roles remain unknown. To identify novel cell cycle mutants in fission yeast, we isolated the *tad3-1* mutant that is allelic to the *tad3*⁺ gene encoding a homolog of budding yeast Tad3p. Interestingly, the *tad3-1* mutant cells principally exhibited cell cycle-specific phenotype, namely temperature-sensitive and irreversible cell cycle arrest both in G₁ and G₂. Further analyses revealed that in the *tad3-1* mutant cells, the S257N mutation that occurred in the catalytically inactive Tad3 subunit affected its association with catalytically active Tad2 subunit, leading to an impairment in the A to I conversion at position 34 of tRNA. In *tad3-1* mutant cells, the overexpression of the *tad3*⁺ gene completely suppressed the decreased tRNA inosine content. Notably, the overexpression of the *tad2*⁺ gene partially suppressed the temperature-sensitive phenotype and the decreased tRNA inosine content, indicating that the *tad3-1* mutant phenotype is because of the insufficient I₃₄ formation of tRNA. These results suggest that the wobble inosine tRNA modification is essential for cell cycle progression in the G₁/S and G₂/M transitions in fission yeast.

The transfer RNAs (tRNAs)² are adapter molecules, which decode mRNA into protein and thereby play a central role in

gene expression. There exist far fewer tRNA genes than the 61 codons specifying amino acids, hence many tRNAs must be capable of recognizing more than one codon. It has been known that inosine (I) occurs at position 34 (the first anticodon position or wobble position) of tRNA (1), and it is believed that when present at the wobble position, inosine can pair degenerately with U, C, or adenosine, enabling a single tRNA to recognize up to three different codons (2). This modification occurs in eight cytoplasmic tRNAs in higher eukaryotes (seven in budding yeast *Saccharomyces cerevisiae*) and in tRNA^{Arg} from prokaryotes and plant chloroplasts (3). Inosine at the wobble position is formed by the hydrolytic deamination of a genomically encoded adenosine (A), and the enzyme catalyzing this reaction is termed tRNA A:34 deaminase. In budding yeast, it has been shown that tRNA A:34 deaminase is a heterodimer formed by the sequence-related subunits Tad2p and Tad3p, both of which contain cytidine deaminase motifs (C/H)XEX_nPCXXC (where X denotes any amino acid, and n is any number of residues) (4). According to mechanistic and structural studies of bacterial and eukaryotic cytidine deaminases, the active site of these enzymes has a zinc ion tetrahedrally coordinated to either a histidine and two cysteine residues or three cysteine residues; the fourth ligand is a zinc-activated water. Proton shuttling during the hydrolytic deamination reaction is mediated by a conserved glutamate (5–7). The absence of the essential glutamate residue in the putative Tad3p deaminase domain suggests that Tad3p is catalytically inactive and that Tad2p is the catalytic subunit of the enzyme. Each subunit is encoded by an essential gene (*TAD2* and *TAD3*), indicating that I₃₄ of tRNAs is an essential base modification for viability. However, most of its physiological roles remain unknown.

In this study using fission yeast *Schizosaccharomyces pombe*, we screened for mutants that showed temperature-sensitive growth arrest but did not accumulate phloxin B (a red dye staining dead cells), and we isolated a *tad3-1* mutant that was allelic to the *tad3*⁺ gene encoding a homolog of budding yeast Tad3p. Surprisingly, the phenotypic characterizations of the *tad3-1* mutant indicated that cell cycle arrest in G₁ and G₂ was the principal outcome in the *tad3* mutation. Further analyses revealed that in the *tad3-1* mutant cells, the S257N mutation that occurred in Tad3 deaminase domain destabilized a Tad2-Tad3 enzyme complex, leading to a reduced activity of tRNA A:34 deaminase. These findings suggest that wobble

* This work was supported by the 21st Century Center of Excellence Program and research grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom correspondence should be addressed. Tel.: 81-78-382-5440; Fax: 81-78-382-5459; E-mail: tkuno@med.kobe-u.ac.jp.

² The abbreviations used are: tRNA, transfer RNA; Tad, tRNA adenosine deaminase; GFP, green fluorescent protein; GST, glutathione S-transferase; YPD, yeast extract/peptone/dextrose; EMM, Edinburgh minimal medium; RT, reverse transcription; PCR, polymerase chain reaction; LC/MS, liquid chromatography-mass spectrometry; FACS, fluorescence-activated cell sorting; SPBs, spindle pole bodies; CYH, cycloheximide; WT, wild-type; I₃₄, inosine at position 34; A₃₄, adenosine at position 34; G₃₄, guanosine at position 34; m¹₃₇, N¹-methylinosine at position 37.

TABLE 1

Fission yeast strains used in this study

Strain	Genotype	Reference
HM123	<i>h⁻ leu1-32</i>	Our stock
HM528	<i>h⁺ his2</i>	Our stock
KP186	<i>h⁻ leu1-32 tad3-1</i>	This study
KP207	<i>h⁺ his2 leu1-32</i>	Our stock
KP1248	<i>h⁻ leu1-32 ura4-294</i>	Our stock
KP2886	<i>h⁻ leu1-32 ura4-294 ura4-294::ura4⁺::nmt1-GFP-tad2⁺</i>	This study
KP3360	<i>h⁻ leu1-32 tad3-1 sad1⁺-GFP::LEU2</i>	This study
KP3361	<i>h⁻ leu1-32 sad1⁺-GFP::LEU2</i>	Our stock
KP3364	<i>h⁻ leu1-32 ura4 tad3-1 cds1::ura4⁺</i>	This study
KP3365	<i>h⁻ leu1-32 ura4 tad3-1 chk1::ura4⁺</i>	This study

inosine tRNA modification plays an important role in cell cycle progression.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic and Molecular Biology Methods—Fission yeast strains used in this study are listed in Table 1. The complete medium, yeast extract-peptone-dextrose (YPD) and the minimal medium, Edinburgh minimum medium (EMM), have been described (8, 9). Standard genetic and recombinant DNA methods (8) were used except where noted.

Bioinformatics—Data base searches were performed using the National Center for Biotechnology Information BLAST network service (www.ncbi.nlm.nih.gov), the Sanger Center *S. pombe* data base search service (www.sanger.ac.uk), and the genomic tRNA data base (rna.wustl.edu/GtRDB/Sp/).

Isolation of KP186 Strain—The KP186 strain was isolated in a screen of cells that had been mutagenized with nitrosoguanidine (8). Cells of HM123 strain were mutagenized with 300 μ M nitrosoguanidine (Sigma) for 60 min (~10% survival) as described previously. Mutants, in appropriate dilutions, were seeded onto YPD plates and incubated at 27 °C for 4 days. The plates were then replica-plated onto YPD plates containing 5 μ g/ml phloxin B red dye, which accumulated in dead cells (10) and incubated at 36 °C for 1–2 days. Mutants that showed temperature sensitivity but were not stained red by phloxin B were selected. The original mutants isolated were backcrossed three times to wild-type strains HM123 or HM528.

Gene Cloning—To clone the mutated gene, KP186 cells were transformed with a fission yeast genomic DNA library constructed in the pDB248 vector and were grown at 27 °C. The Leu⁺ transformants were replica-plated onto YPD plates at 36 °C, and the plasmid DNA was recovered from the transformants that showed a plasmid-dependent rescue. By DNA sequencing, the suppressing plasmids fell into a single class that contained the *tad3⁺* gene (SPAP27G11.04).

To investigate the genetic relationship between the mutated gene and the *tad3⁺* gene, linkage analysis was performed as follows. The *tad3⁺* gene was subcloned into the pUC-derived plasmid containing budding yeast *LEU2* gene. Using this construct, the *LEU2* gene was integrated by homologous recombination into the *tad3⁺* gene locus of the genome of KP207. The integrant was mated with KP186 strain, and the resultant diploid was sporulated. When the tetrads were dissected, only parental ditype tetrads were found, indicating that the mutated gene was tightly linked to the *tad3⁺* gene locus.

Microscopic Analysis—Techniques in fluorescence microscopy such as the localization of the GFP-tagged proteins were

performed as described previously (11). Calcofluor staining was performed as described previously (11).

FACS Analysis—Cells were collected by centrifugation and fixed in ethanol at 4 °C overnight. After washing with 1 ml of 50 mM sodium citrate, the fixed cells were resuspended in 1 ml of a solution containing 50 mM sodium citrate and 0.1 mg/ml RNase A and incubated at 37 °C for at least 2 h. Before analysis, the cells were briefly sonicated and stained with propidium iodide at a final concentration of 8 μ g/ml. Ten thousand cells were collected with a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed with Cell Quest 4.0 software.

Nitrogen source starvation was done as described previously (12). Briefly, cells were cultured in EMM medium to mid-log phase at 27 °C, washed four times with EMM without NH₄Cl, and then cultured in EMM without NH₄Cl at 27 °C for 36 h. The FACS analysis confirmed that the cells were arrested in the G₁ phase. Arrested cells were released from the G₁ block by switching them to YPD medium.

Sequence Analysis of tRNA^{Ala}—Total RNA was isolated from cultures of cells grown to mid-log phase at 27 °C by the method of Kohrer and Domdey (13). RNA was further treated with RNase-free DNase. RT was performed using 1 μ g of total RNA as a template with tRNA^{Ala}(AGC)-specific reverse primer (5'-CGC GGA TCC TGG ACA AGC CAG AAC TCG AAT C-3'). PCR was then performed using 1 μ l of the RT reaction as a template with tRNA^{Ala}(AGC)-specific forward (5'-CGC GGA TCC GGG CAT GTG GTG TAG ATG GTT A-3') and reverse primers. Controls included a negative control in which the RT reaction without RT was used as a template to test for DNA contamination in the RNA sample and a positive control for amplification in which the genomic DNA was used as a template. The PCR products were subcloned into pBluescript SK(+) (Stratagene), and the individual clones were sequenced.

Gene Expression—The full coding sequences of Tad2 and Tad3 were amplified by PCR using genomic DNA from wild-type strain as a template. The PCR primers used were as follows: Tad2F (5'-CGC GGA TCC ATG GCA GGA GAT AGT GTG AAA AG-3') and Tad2R (5'-CGC GGA TCC TTA TTT GAA ACG AGA TAA ATC CAA AC-3'); Tad3F (5'-CGC GGA TCC ATG GTG AAA ACT AAT ATT TCT AA-3') and Tad3R (5'-CGC GGA TCC TCA GAC ATG AAT ATT CTC CTT-3'). The Tad3^{S257N} coding sequence was also amplified by PCR using the genomic DNA from KP186 strain as a template. These PCR fragments were ligated to the C terminus of GFP or GST and subcloned into the pREP1 expression vector, containing a thiamine-repressible *nmt1* promoter (14). Expression was

induced by the incubation of the cells in EMM lacking thiamine.

To obtain cells expressing GFP-Tad2 from the chromosomally integrated gene, the fused gene was subcloned into the vector containing a *nmt1* promoter and a *ura4⁺* marker, and was integrated into the chromosome at the *ura4⁺* gene locus of KP1248 cells as described (11, 15).

GST Pull-down Assay—Cells expressing GFP-Tad2 from the chromosomally integrated gene were transformed with pREP1-GST, pREP1-GST-Tad3, or pREP1-GST-Tad3^{S257N}, and were cultured at 27 °C for 20 h in EMM without thiamine to induce the gene expression. Cells were resuspended in ice-cold homogenizing buffer, 50 mM Tris-HCl, pH 7.8, containing 2 mM EDTA, 1 mM dithiothreitol, and a mixture of the protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamide, 0.1 mM sodium metabisulfite, 0.1 μg/ml chymostatin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml phosphoramidon, and 0.5 μg/ml leupeptin). Glass beads were then added, and the cells were homogenized using a Mini-Beadbeater (Bio-Spec Products) at 5,000 rpm for 30 s, after which the tubes were placed on ice for 1 min. Homogenization and cooling were repeated three times, after which the glass beads and cellular debris were removed by centrifugation at 15,000 rpm for 5 min. GST-fused proteins in the protein extracts were purified with glutathione-Sepharose beads (Amersham Biosciences) and were subjected to immunoblot analysis with anti-GFP and -GST antibodies.

Mass Spectrometry—Crude tRNA was isolated from cultures of cells grown to mid-log phase at 27 °C as described previously (16). Briefly, cells were resuspended in 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂), and treated with 500 μl of neutral pH phenol/chloroform by shaking for 3 h at room temperature. RNA was recovered by ethanol precipitation from the aqueous phase and then dissolved in sterile water. 1-μg aliquot of the RNA sample was separated on a 10% polyacrylamide gel containing 7 M urea, and the gel staining with ethidium bromide confirmed that almost all of the RNA sample was from tRNA.

To analyze RNA nucleosides, a 20-μg aliquot of the crude tRNA obtained from each strain was digested to nucleosides with nuclease P1 (Yamasa) and bacterial alkaline phosphatase derived from *Escherichia coli* strain C75 (BAP.C75, Takara) for 3 h at 37 °C and was analyzed by LC/MS using ion-trap MS as described previously (17) with slight modifications. Nucleosides were separated by an ODS reverse-phase column (Intertsil ODS3 5 μm, 2.1 × 250 mm, GL Science) using an HP1100 liquid chromatography system (Agilent). The solvent consisted of 0.1% acetonitrile in 5 mM NH₄OAc (pH 5.3) (solvent A) and 60% acetonitrile in H₂O (solvent B) in the following gradients: 1–35% B in 0–35 min, 35–99% B in 35–40 min, 99% B in 40–50 min, 99–1% B in 50–50.1 min, and 1% B in 50.1–60 min. The chromatographic effluent was directly conducted to the ESI source to ionize the separated nucleosides, which were analyzed on an LCQ DUO ion-trap mass spectrometer (Thermo-Electron). The mass spectrometer was operated with a spray voltage of 5 kV and a capillary temperature of 245 °C. The sheath gas flow rate was 95 arb, and auxiliary gas flow rate was 5 arb. Positive ions were scanned over a *m/z* range of 100–900.

RESULTS

Isolation of KP186 Mutant—To identify novel temperature-sensitive cell cycle mutants in fission yeast, we screened for mutants that showed a temperature-sensitive growth arrest but did not accumulate phloxin B (a red dye that stains dead cells) at the restrictive temperature. An initial screen from 200,000 nitrosoguanidine-treated wild-type cells identified two complementation groups (data not shown). The mutant KP186, belonging to one of the two complementation groups, was selected to identify the mutated gene. KP186 mutant cells grew at 27 °C, but cells failed to grow at 33 °C or at a higher temperature (Fig. 1A).

KP186 Mutant Cells Exhibited Temperature-sensitive Cell Cycle Arrest in G₁ and G₂—Next, we examined the phenotypic changes in the cells that were subjected to a temperature upshift. Wild-type and KP186 cells expressing Sad1-GFP to visualize spindle pole bodies (SPBs) were grown to mid-log phase at 27 °C in liquid YPD medium, and then each culture was divided into two portions. One portion was maintained at 27 °C, whereas the remaining portion was shifted to 36 °C for 8 h. The cells were collected and stained with Calcofluor to visualize septa (Fig. 1B). Two lines of evidence suggest that the KP186 phenotype is associated with cell cycle. First, even at 27 °C, KP186 cells were slightly longer than wild-type cells, and this cell elongation was more evident when temperature was upshifted to 36 °C (Fig. 1, B and C). Second, upon temperature upshift to 36 °C in KP186 cells, the frequency of mitotic cells characterized by duplicated SPBs and/or septa significantly decreased, whereas in wild-type cells, the frequency of mitotic cells remained unchanged (Fig. 1, B and D).

The above-mentioned phenotype prompted us to check whether the KP186 mutant had a defect in cell cycle progression. FACS analysis showed that KP186 cells incubated at 36 °C in liquid YPD medium consistently showed post-replicative DNA content (Fig. 1E), confirming that KP186 cells arrested in the G₂ phase at the restrictive temperature. When first arrested in the G₁ phase at 27 °C by nitrogen starvation and then released from the G₁ block at 36 °C, KP186 cells, unlike wild-type cells, could not initiate the S phase and remained arrested in the G₁ phase (Fig. 1F). In addition, we found that the longer preincubated at the restrictive temperature of 36 °C, the more KP186 cells failed to grow when shifted back to 27 °C (Fig. 1G), suggesting that growth arrest induced by temperature upshift in KP186 cells was irreversible. These results showed that the temperature-sensitive phenotype of KP186 cells was because of the irreversible cell cycle arrest in G₁ and G₂ at the restrictive temperature.

KP186/*tad3-1* Is a Novel Allele of the *tad3⁺* Gene Which Encodes a Non-catalytic Subunit of tRNA A:34 Deaminase—To clarify the mechanism of cell cycle arrest in KP186 cells, the mutated gene in KP186 strain was cloned by complementation of the temperature-sensitive growth arrest (Fig. 2A). Nucleotide sequencing of the cloned DNA fragment revealed that the mutated gene was SPAP27G11.04, which encoded a 315-amino acid homolog of budding yeast Tad3p, a catalytically inactive subunit of tRNA A:34 deaminase. Linkage analysis also indicated that the mutated gene was tightly linked to the

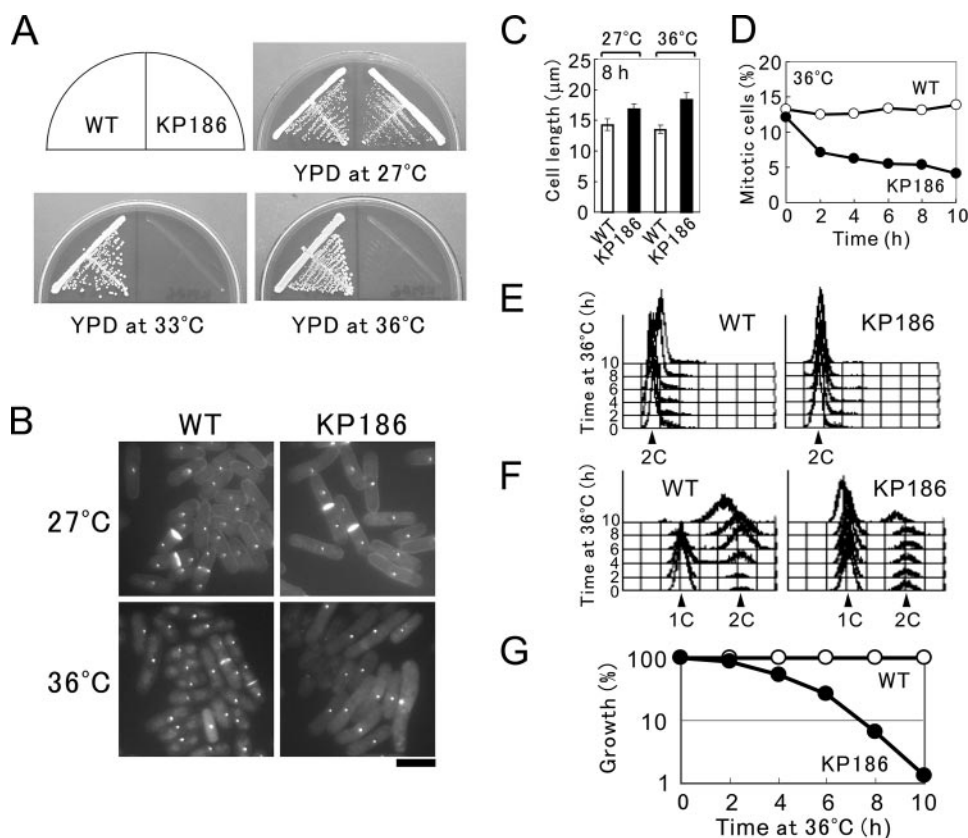


FIGURE 1. The phenotype of KP186 cells. *A*, temperature-sensitive phenotype of KP186 cells. WT and KP186 cells were streaked onto YPD plates containing phloxin B (a red dye staining dead cells) and then incubated at 27 °C for 4 days, 33 °C for 3 days, or 36 °C for 3 days, respectively. *B*, visualization of SPB and septum in wild-type and KP186 cells. Wild-type and KP186 cells expressing Sad1-GFP were grown to mid-log phase at 27 °C in liquid YPD medium, then shifted to 36 °C, and stained with Calcofluor as described under "Experimental Procedures." The bar indicates 10 μm. *C*, comparison of the cell length between KP186 and wild-type cells. The cell length before undergoing cell division with a medial septum position was measured in WT (open bars) and KP186 cells (closed bars) incubated at 27 or 36 °C for 8 h. Data are presented as the means ± S.D. *D*, the frequency of mitotic cells in WT (open circles) and KP186 cells (closed circles) upon temperature upshift to 36 °C. Percentage of mitotic cells distinguished by having duplicated SPBs, and/or septa was measured every two h. Note that the frequency of mitotic cells in KP186 cells significantly decreased. *E*, FACS analysis of WT and KP186 cells upon upshift to 36 °C. Cells grown to mid-log phase at 27 °C in liquid YPD medium were shifted to 36 °C at time intervals as indicated, and their DNA contents were measured using FACS. The DNA content and relative cell number were plotted along with *x* and *y* axes, respectively. *F*, FACS analysis of WT and KP186 cells released from the G₁ block. Cells were first arrested in the G₁ phase by nitrogen starvation at 27 °C and then shifted to the YPD medium at 36 °C. C indicates unreplicated DNA content, whereas Fig. 2C indicates post-replicative DNA content. *G*, cell growth rate (Growth %) of WT (open circles) and KP186 cells (closed circles) preincubated at 36 °C for 0–10 h. Cell growth rate was determined by plating ~500 cells per YPD plate. These plates were placed at 36 °C for 0–10 h and then shifted back to 27 °C for 4 days at which time colonies were counted on three plates for each time point. The cell growth rate data were averaged from two independent experiments.

SPAP27G11.04 locus (data not shown). Hence, we named this gene as *tad3*⁺ and the KP186 strain as the *tad3-1* mutant. Sequence analysis of the genomic DNA from the *tad3-1* mutant revealed that serine at 257 was mutated to asparagine by a G-to-A transition (S257N; AGT→AAT). This missense mutation is located in the evolutionarily conserved deaminase domain (Fig. 2B). The absence of the glutamate residue essential for catalysis in the Tad3 deaminase domain suggests that Tad3 is catalytically inactive. This raises the possibility that the mutation of Tad3 might indirectly affect the enzyme activity of tRNA A:34 deaminase.

Impaired Conversion of A₃₄ to I₃₄ in tRNA^{Ala} from *tad3-1* Mutant—Although fission yeast uses four alanine codons GCU, GCC, GCG, and GCA, its genome encodes only three

different tRNA^{Ala} genes with anticodons AGC, CGC, and TGC (Fig. 3A). This discrepancy implies that tRNA A:34 deaminase converts tRNA^{Ala} (AGC) into tRNA^{Ala} (IGC), which then pairs with and decodes an alanine codon GCC. Therefore we performed the sequence analysis of tRNA^{Ala} (AGC) as one of putative substrates of tRNA A:34 deaminase. With oligonucleotide primers specific for tRNA^{Ala} (AGC) (Fig. 3B), RT-PCR was performed on total RNA isolated from wild-type and *tad3-1* mutant cells grown at 27 °C. The resultant cDNAs were subcloned, and 15 different clones derived from *tad3-1* mutant cells as well as 15 clones from wild-type cells were sequenced. Because I pairs with C during reverse transcription, A to I deamination changes the sequence from A to G. All clones (100%) derived from wild-type cells contained guanosine at position 34 (G₃₄), demonstrating that the genomically encoded A at this position was deaminated to I. On the other hand, 1 out of 15 (7%) clones derived from *tad3-1* mutant cells carried guanosine at position 34, whereas the other 14 clones carried adenosine at position 34 (Fig. 3, C and D). These results indicate that the enzyme activity of tRNA A:34 deaminase is indeed impaired in the *tad3-1* mutant.

Notably, the genomically encoded A at position 37 was consistently changed to T in both RT-PCR products from wild-type and from *tad3-1* mutant cells (Fig. 3C). As has been described in budding yeast tRNA^{Ala} (18), this might be because

A at position 37 is edited to N¹-methylinosine (m¹I), which base pairs with reduced specificity in the RT reaction. This finding suggests that A₃₇ to m¹I₃₇ editing in tRNA^{Ala} is not impaired in the *tad3-1* mutant.

The *tad3-1* Mutant Is Hypersensitive to Cycloheximide—As insufficient I₃₄ formation of tRNA in the *tad3-1* mutant may affect its decoding system and impair translation step, we then examined the effect of a protein synthesis inhibitor cycloheximide (CYH) that mainly blocks the elongation process of translation (19) on *tad3-1* mutant (Fig. 3E). At a low concentration of CYH (10 μg/ml), the growth of *tad3-1* mutant was significantly inhibited as compared with that of wild-type cells. At a higher concentration of CYH (20 μg/ml), the growth inhibition of *tad3-1* mutant was further exacerbated. These results suggest

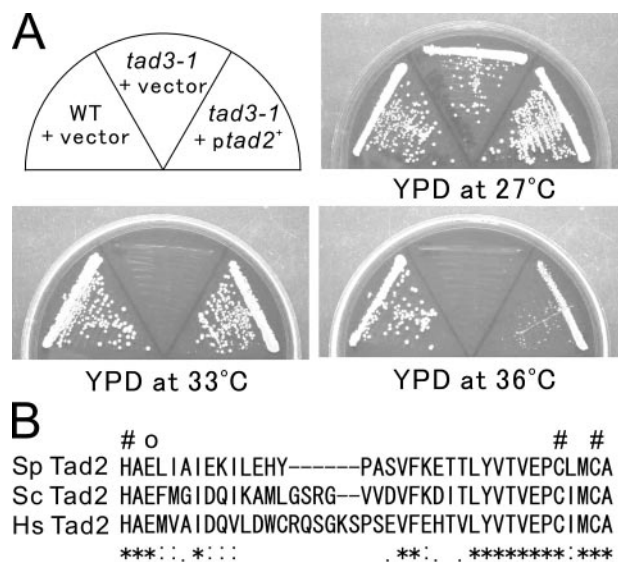


FIGURE 4. Cloning of *tad2*⁺ gene as a multicopy suppressor of the *tad3-1* mutant. A, cloning of the *tad2*⁺ gene. WT and *tad3-1* mutant cells transformed with a multicopy vector pDB248, or a vector containing the *tad2*⁺ gene were streaked onto YPD plates and then incubated as in Fig. 1A. B, ClustalW multiple sequence alignment of the deaminase domains of Tad2, the catalytic subunit of tRNA A:34 deaminase from fission yeast (*Sp*), budding yeast (*Sc*), and human (*Hs*). Identical (asterisks), strictly conserved (colons), and conserved (dots) regions are indicated. The *tad2* deaminase domains have a full active site consensus motif including the proton-transferring glutamate residue (o) as well as the Zn²⁺-chelating residues (#).

and Tad3. On the other hand, the pull-down for GST-Tad3^{S257N} consistently showed less amount of GFP-Tad2 protein than was seen in the pull-down for GST-Tad3 (Fig. 5B), demonstrating that Tad3^{S257N} was indeed defective in its association with Tad2. This result suggests that the overexpression of Tad2 in the *tad3-1* mutant cells may partially suppress the decreased level of the enzyme complex and result in the partial suppression of the temperature-sensitive phenotype. Consistently, the overexpression of GST-Tad3^{S257N} partially but significantly suppressed the temperature sensitivity of the *tad3-1* mutant (Fig. 5A). These results also suggest that serine at position 257 in the deaminase domain of Tad3 plays an important role in its interaction with Tad2.

Suppression of the *tad3-1* Mutant Phenotype Depends on the Recovery of the Wobble Inosine tRNA Modification—We next examined the relationship between the suppression of the *tad3-1* mutant phenotype (Figs. 2A and 4A) and the extent of the wobble inosine tRNA modification (Fig. 6). Crude tRNA extracted from each transformant grown at 27 °C was analyzed by LC/MS to measure the tRNA inosine content. Consistent with the decreased wobble inosine modification of tRNA^{Ala} (AGC), tRNA inosine content in *tad3-1* mutant cells was decreased to 36% as compared with that in wild-type cells. The overexpression of the *tad3*⁺ gene in *tad3-1* mutant cells completely suppressed the decreased tRNA inosine content. On the other hand, the overexpression of the *tad2*⁺ gene in *tad3-1* mutant cells partially suppressed the decreased tRNA inosine content. These findings suggest that the phenotype of *tad3-1* mutant cells is because of the insufficient I₃₄ formation of tRNA.

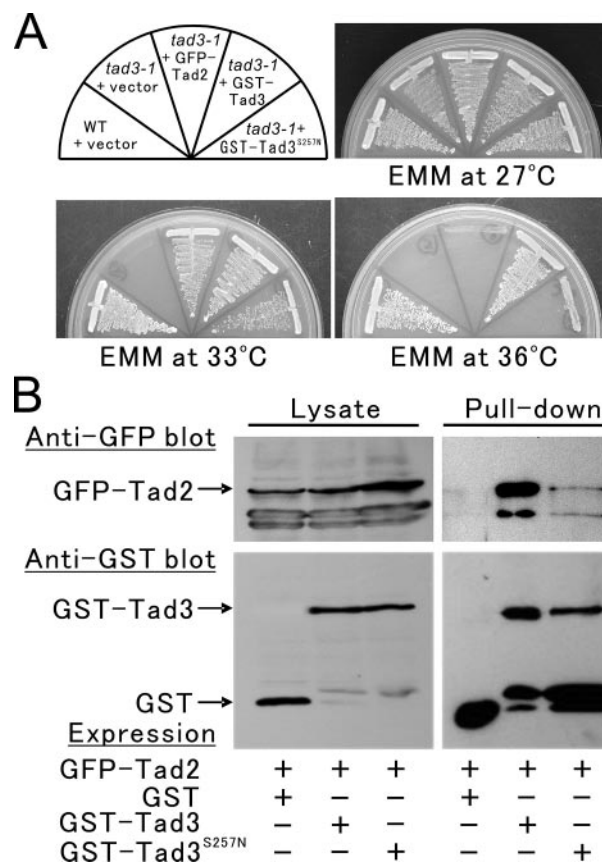


FIGURE 5. The S257N mutation of Tad3 destabilizes a Tad2-Tad3 complex. A, GFP-Tad2 and GST-Tad3 are fully functional, and the overexpression of GST-Tad3^{S257N} partially suppressed the temperature-sensitive phenotype of *tad3-1* mutant. The *tad3-1* mutant cells transformed with pREP1-GFP-Tad2, pREP1-GST-Tad3, and pREP1-GST-Tad3^{S257N} were streaked onto EMM plates lacking thiamine to induce gene expression and then incubated as shown in the legend to Fig. 1A. B, effect of the S257N mutation of Tad3 on Tad2-Tad3 interaction. In cells expressing GFP-Tad2 from the chromosomally integrated gene (KP2886), GST, GST-Tad3, or GST-Tad3^{S257N} was expressed from the harboring plasmid at 27 °C. GST-tagged protein was pulled down by glutathione beads, washed extensively, subjected to SDS-PAGE, and immunoblotted using anti-GFP or anti-GST antibodies.

DISCUSSION

In the present study, in an attempt to identify novel cell cycle mutants in fission yeast, we isolated the *tad3-1* mutant that exhibited temperature-sensitive growth arrest in G₁ and G₂. Further analyses revealed that in the *tad3-1* mutant cells, the S257N mutation that occurred in Tad3 deaminase domain destabilized a Tad2-Tad3 enzyme complex, leading to the reduced activity of tRNA A:34 deaminase. Furthermore, we confirmed that such phenotype of the *tad3-1* mutant was suppressed by the recovery of the wobble inosine tRNA modification. These findings demonstrate that the wobble inosine tRNA modification is essential for cell cycle progression in the G₁/S and G₂/M transitions. This is the first report to link the wobble inosine tRNA modification to cell cycle progression.

The tRNAs containing inosine at position 34 are major species, and all belong to the subset of amino acids represented by four codons (20). The architectural and functional significance of the base modification is not yet fully understood, however, it is believed that the modified nucleosides can influence the stability and structure of the tRNAs and thus improve the fidelity

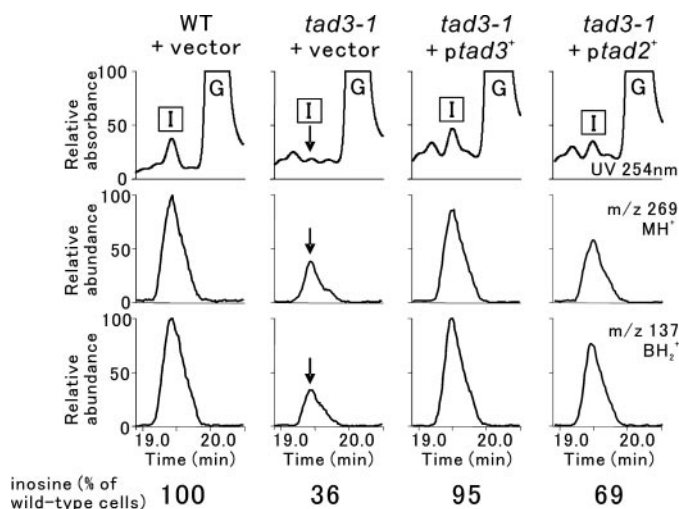


FIGURE 6. Suppression of *tad3-1* mutant phenotype depends on the recovery of the wobble inosine tRNA modification. Crude tRNA was extracted from each transformant grown to mid-log phase at 27 °C and was analyzed by LC/MS to measure the tRNA inosine content. The upper panel is the UV trace at 254 nm. The middle and lower panels are mass chromatograms detecting MH^+ of inosine (m/z 269) and BH_2^+ of inosine base (m/z 137), respectively. Arrows indicate the retention time for inosine (I). The relative amount of inosine in each strain was normalized by intensity of N^2, N^2 -dimethylguanosine (m/z G), and this is expressed as percentage relative to that in wild-type strain.

and efficiency of the tRNAs in decoding the genetic message (20, 21). According to Crick's wobble hypothesis(2), inosine at position 34 is believed to play a crucial role in protein synthesis by allowing alternative pairing with U, C, or A in the third position of appropriate codons in the mRNA. Moreover, inosine at position 34 in budding yeast tRNA^{Ile} has been shown to be a positive determinant for aminoacylation by isoleucyl-tRNA synthetase (22). Therefore, it is thought that a defect in the I_{34} formation of the tRNA should impair protein synthesis at different steps and produce a wide spectrum of phenotypes. Surprisingly, the phenotypic characterizations in this study indicate that cell cycle arrest in G_1 and G_2 is the principal outcome in the *tad3* mutation.

One possible explanation for this unexpected result is that the status of tRNA base modification might be monitored by a kind of cell cycle checkpoint, the activation of which might produce the cell cycle-specific phenotype of *tad3-1* mutant. But this checkpoint hypothesis is unlikely based on the following data. First, to examine whether the cell cycle arrest of *tad3-1* mutant is dependent on the DNA damage or replication checkpoint, we constructed *tad3-1Δcds1* and *tad3-1Δchk1* double mutants (KP3364 and KP3365, respectively). Neither of the double mutations broke the cell cycle block at the restrictive temperature, verifying that the *tad3-1* mutant cells arrested independently of the DNA damage and replication checkpoints (data not shown). Second, from 10,000 nitrosoguanidine-treated *tad3-1* mutant cells, we searched for mutants that grew at the restrictive temperature, but we failed to isolate such mutants (data not shown).

Hence based on the above findings, it is more likely that the cell cycle genes could not be efficiently translated in the *tad3-1* mutant cells at the restrictive temperature. As mentioned

above, the decoding system by inosine-bearing tRNAs contributes to decrease the number of isoacceptor tRNAs and thus enables the efficient translation. Translation is the most energetically expensive process occurring in the exponentially growing cells, and therefore its efficiency is under stringent selective pressure (23). Although no direct evidence has been presented, fission yeast might have in the process of evolution adopted the decoding system by inosine-bearing tRNAs toward cell cycle genes and have acquired the ability to grow faster by their efficient translation. The cell cycle-specific phenotype of *tad3-1* mutant might reflect such a condition. Further studies are needed to test this hypothesis and to better understand the cell cycle-specific phenotype of the *tad3-1* mutant.

Acknowledgments—We thank Takashi Toda and Mitsuhiro Yanagida for providing strains and plasmids, Susie O. Sio for critical reading of the manuscript, and Koei Okazaki for valuable discussion.

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