The intron of tRNA-Trp_{CCA} is dispensable for growth and translation of *Saccharomyces cerevisiae*

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ABSTRACT

A part of eukaryotic tRNA genes harbor an intron at one nucleotide 3' to the anticodon, so that removal of the intron is an essential processing step for tRNA maturation. While some tRNA introns have important roles in modification of certain nucleotides, essentiality of the tRNA intron in eukaryotes has not been tested extensively. This is partly because most of the eukaryotic genomes have multiple genes encoding an isoacceptor tRNA. Here, we examined whether the intron of tRNA-Trp_{CCA} genes, six copies of which are scattered on the genome of yeast, *Saccharomyces cerevisiae*, is essential for growth or translation of the yeast *in vivo*. We devised a procedure to remove all of the tRNA introns from the yeast genome iteratively with marker cassettes containing both positive and negative markers. Using this procedure, we removed all the introns from the six tRNA-Trp_{CCA} genes, and found that the intronless strain grew normally and expressed tRNA-Trp_{CCA} in an amount similar to that of the wild-type genes. Neither incorporation of ³⁵S-labeled amino acids into a TCA-insoluble fraction nor the major protein pattern on SDS-PAGE/2D gel were affected by complete removal of the intron, while expression levels of some proteins were marginally affected. Therefore, the tRNA-Trp_{CCA} intron is dispensable for growth and bulk translation of the yeast. This raises the possibility that some mechanism other than selective pressure from translational efficiency maintains the tRNA intron on the yeast genome.

Keywords: tRNA; intron; tRNA splicing; tRNA processing; *Saccharomyces cerevisiae*

INTRODUCTION

tRNAs play an essential role in protein translation as adaptor molecules between a nucleic acid sequence and an amino acid sequence. During their maturation, tRNAs undergo complex but sophisticated processing and intracellular dynamics in eukaryotes (Yoshihisa 2006; Hopper et al. 2010; Phizicky and Hopper 2010). Primary transcripts of tRNAs receive 5' and 3' trimming catalyzed by RNase P and RNase Z, respectively. Then, a CCA sequence is added to their 3' end. Various positions of their bases and sugars are also modified. These modifications take place at appropriate time points and places in a cell.

Some tRNAs are encoded by genes with an intron. The canonical tRNA intron, in eukaryotes and archaea, is located at one nucleotide 3' to the anticodon (Abelson et al. 1998; Heinemann et al. 2010). In archaeal tRNA genes, typical splice sites consist of the bulge-helix-bulge motif that is

formed between the anticodon loop and intron. On the other hand, the eukaryotic intron does not have such a motif and is thought to be recognized by splicing machinery through the “ruler mechanism” to measure the distance between the cleavage sites and the body of mature tRNA (Abelson et al. 1998). Recently, noncanonical introns that interrupt other parts of tRNAs were discovered in various archaea and certain eukaryotes (for review, see Heinemann et al. 2010). In extreme cases, three introns are inserted in a tRNA-Pro^{UGG} gene of *Pyrobaculum islandicum*, and split tRNA transcripts are joined to form a mature tRNA by a splicing-like mechanism through base-pairing between their appended segments (Sugahara et al. 2007; Fujishima et al. 2009). To remove such tRNA introns, proteinaceous enzymes catalyze all of the splicing steps. tRNA splicing endonucleases, which govern cleavage of splice sites, are conserved in their amino acid sequences among eukaryotes and archaea, while their intracellular localization shows diversity in eukaryotic cells (Trotta et al. 1997; Yoshihisa et al. 2003; Paushkin et al. 2004; Englert et al. 2007; Yoshihisa et al. 2007). Furthermore, organisms have adopted several different reaction pathways to ligate tRNA exons (Filipowicz and Shatkin 1983; Phizicky et al. 1986; Culver

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et al. 1997; Englert and Beier 2005; Weitzer and Martinez 2007; Schwer et al. 2008; Englert et al. 2010; Popow et al. 2011). Despite the above diversity, intron removal itself is essential for functionalization of tRNAs in every organism.

The most well studied eukaryote in tRNA splicing is *Saccharomyces cerevisiae*, the genome of which harbors 61 intron-containing tRNA genes encoding 10 different isoacceptor tRNAs (Abelson et al. 1998; Lowe and Chan 2010). The introns vary in size from 14 nt to 60 nt, and all of them locate at the canonical position, one nucleotide 3' to the anticodon. It is of interest that all the genes encoding these isoacceptor tRNAs have introns, indicating that the yeast needs to maintain the tRNA introns of these genes. However, it is still not fully understood how the tRNA intron contributes to the life of yeast. The introns of some tRNAs are, indeed, required for their proper modifications. For example, among pseudouridylation at positions 25, 34, 36, and 67 in tRNA-Ile_{UAU} catalyzed by Pus1p, pseudouridylation at the positions 34 and 36 is applied only on the intron-containing precursor in vitro (Szweykowska-Kulinska et al. 1994). Similarly, Trm4p converts C34 of tRNA-Leu_{CAA} to 5-methylcytidine only when the nucleotide exists on the precursor (Strobel and Abelson 1986; Grosjean et al. 1997). Modification at the 34th nucleotide, the wobble position, is considered to affect decoding ability of tRNAs (Agris et al. 2007). Besides, class I aminoacyl-tRNA synthetase (ARS) recognizes the anticodon of its cognate tRNA, and the recognition may be affected by modifications of the anticodon (Senger et al. 1997). Despite these facts, tRNA introns are not considered as a critical element for tRNA biogenesis since deletion of these intron-dependent modification enzymes has little effect on yeast growth (Simos et al. 1996; Motorin and Grosjean 1999).

On the other hand, direct experiments to examine essentiality of eukaryotic tRNA introns have been hampered by the fact that most of the isoacceptor tRNAs are encoded by multiple genes. Even in the case of a simple organism like *S. cerevisiae*, intron-containing isoacceptor tRNAs are encoded by up to 10 degenerated genes. While suppressor tRNA mutation can be used to study functionality of intron-deleted tRNA genes expressed *in trans* (Strobel and Abelson 1986; Ho and Abelson 1988), this method cannot measure decoding activity of their original anticodons. The one exception is yeast tRNA-Ser_{CGA}, which is encoded by a single gene, *tS(CGA)C* or *SUP61*. The intron was proven to be nonessential by replacing chromosomal *tS(CGA)C* with an intronless version (Ho and Abelson 1988). However, the sequence of tRNA-Ser_{CGA} is quite similar to that of tRNA-Ser_{UGA} encoded by *tS(UGA)P* or *SUP16*; only three nucleotides are different between the two. Overproduction of *tS(UGA)P* can suppress lethality of *ts(cga)cΔ*, and an *elp3Δ* mutation, which causes a defect in ncm⁵U modification at many U34's and narrows decoding ability of U34-containing tRNAs like tRNA-Ser_{UGA}, abolishes this suppression of *ts(cga)Δ* lethality by *tS(UGA)P*

overproduction. These facts suggest functional redundancy between tRNA-Ser_{CGA} and ncm⁵U-modified tRNA-Ser_{UGA} (Johansson and Byström 2004; Johansson et al. 2008) and that marginal effects of intron removal from *tS(CAG)C* may be covered by this redundancy. Therefore, in the strictest sense, requirement of a tRNA intron in eukaryotic cells should be tested in the other isoacceptor tRNAs.

Here, we examined essentiality of the canonical intron in the tRNA-Trp_{CCA} genes of *S. cerevisiae*, as a model case to study essentiality of the tRNA introns on the yeast genome. tRNA-Trp_{CCA} is unique in that there is only one Trp codon, UGG, on the nuclear genome, so that only one cognate isoacceptor tRNA exists. Since tRNA-Trp_{CCA} is encoded by six genes with the same sequence including their intron (Atkin et al. 1992), we developed a systematic protocol to replace multiple tRNA genes on the yeast chromosomes with their intronless version. Using this technique, we succeeded in yielding a strain without any tRNA-Trp_{CCA} intron on its genome and demonstrated feasibility of this technique to examine necessity of the introns on the other isoacceptor tRNA genes. Surprisingly, we could not find any significant difference between the wild-type and tRNA-Trp_{CCA} intronless strains in growth and in protein synthesis. This indicates that the intron has little, if any, impact on functionality of tRNA-Trp_{CCA} in *S. cerevisiae*.

RESULTS

Strategy for sequential removal of tRNA-Trp_{CCA} introns from the yeast genome

In *S. cerevisiae*, six tRNA-Trp_{CCA} genes are not clustered but scattered around the genome; two genes exist on chromosome VII and the others on chromosomes X, XI, XIII, and XVI (Fig. 1A). All of the genes have the same sequence and are interrupted by a single 34-nt intron (Fig. 1B; intron sequences are enclosed by square). Conservation of the intron, which should be removed during tRNA maturation, is in great contrast to the variability of the 5'- and 3'-flanking sequences of the tRNA-Trp_{CCA} genes (Fig. 1B). It suggests that all the tRNA-Trp_{CCA} introns have likely been selected in the genome of *S. cerevisiae*. It is also true in the comparison between orthologous chromosomal regions corresponding to the tRNA-Trp_{CCA} locus, *tW(CCA)G2*, among closely related yeasts in a *Saccharomyces* genus (Fig. 1C). The intron is more conserved than the 5'- and 3'-flanking regions of the *tW(CCA)G2* locus.

To achieve sequential alterations of multiple tRNA-Trp_{CCA} genes for complete removal of the tRNA-Trp_{CCA} introns from the yeast genome, repetitive usage of selection markers is essential. For this purpose, we adopted marker cassettes devised by Akada et al. (2002). In these cassettes, an auxotrophic selection marker is accompanied by a negative selection marker, *GAL1p::GIN11M86*, which encodes a toxic protein under the control of the *GAL1* promoter.

The marker set is sandwiched by two *hisG* sequences, which enable pop-out recombination after its integration into the yeast genome. We also added a *Cam^r* marker to make the plasmid construction easy in *Escherichia coli*. Since we use *GIN11M86* as a negative selection marker for marker rescue, we can use a vector plasmid with a *URA3* marker, which can be used as another positive and negative selection marker, to complement defects derived from tRNA-Trp_{CCA} gene mutations if necessary (Boeke et al. 1984). Then, using these modified marker cassettes, we constructed a series of plasmids, each of which contained an intronless version of a tRNA-Trp_{CCA} gene and its flanking region divided by the marker cassette. A chromosomal tRNA-Trp_{CCA} gene was replaced with its corresponding intronless fragment by homologous recombination. Transformants with the targeted tRNA-Trp_{CCA} gene were selected through auxotro-

FIGURE 2. Confirmation of intron deletion. (A) Deletion of an intron of a tRNA-Trp_{CCA} gene in each integration procedure was confirmed by PCR and agarose-gel electrophoresis. DNA fragments amplified with probes marked with “a” in the bottom panel were analyzed on 2.5% agarose gels. Analyzed tRNA-Trp_{CCA} loci are shown on the left. (WT) Wild-type strain BY418, (1/6 Δ int) single intron deletion of *tW(CCA)G1* locus, (2/6 Δ int) double intron deletion of *tW(CCA)G1* and *tW(CCA)K* loci, (3/6 Δ int) triple intron deletion of *tW(CCA)G1*, *tW(CCA)K*, and *tW(CCA)M* loci, (4/6 Δ int) quadruple intron deletion of *tW(CCA)G1*, *tW(CCA)K*, *tW(CCA)M*, and *tW(CCA)P* loci, (5/6 Δ int) quintuple intron deletion of *tW(CCA)G1*, *tW(CCA)K*, *tW(CCA)M*, *tW(CCA)P*, and *tW(CCA)G2* loci, (6/6 Δ int) sextuple intron deletion of *tW(CCA)G1*, *tW(CCA)K*, *tW(CCA)M*, *tW(CCA)P*, *tW(CCA)G2*, and *tW(CCA)J* loci, (P) plasmid that harbors an intronless tRNA-Trp_{CCA} gene as a control. Assignment of the bands is shown schematically on the right. (B) The same samples were subjected to PCR with a single set of primers hybridized with the tRNA-Trp_{CCA} exons (primers “b” shown in gray arrows). Amplified fragments were analyzed on a 10% native polyacrylamide gel. (C) A diploid strain TYSC1361 [*tw(cca)g1* Δ ::*CgHIS3*/- *tw(cca)g2* Δ ::*LEU2*/- *tw(cca)j* Δ ::*kanMX*/+ *tw(cca)k* Δ ::*CgTRP1*/+ *tw(cca)m* Δ ::*hphMX*/+ *tw(cca)p* Δ ::*ADE2*/+] was sporulated, and the resulting tetrads were dissected on YPD (upper). A white circle indicates existence of a very tiny colony in it. The remaining tRNA-Trp_{CCA} genes estimated from marker phenotypes on each haploid descendant are shown in the lower part. Alphabets represent the chromosomes where each *tW(CCA)* locus locates. (D) The complete intronless strain, SMSC233 (6/6 Δ int), or its control wild-type strain, BY418 (WT), were crossed with a haploid strain TYSC1406 without any tRNA-Trp_{CCA} gene on its chromosomes but with pTYSC583 [*tW(CCA)K* 2 μ *URA3*] (6/6 Δ). Resulting diploids (6/6 Δ × WT, 6/6 Δ × 6/6 Δ int) and those parents with pTYSC583 were spotted onto an FOA plate to test whether those strains can lose the *URA3*-plasmid with *tW(CCA)K*. Dilution rates are shown above the panel.

fragment derived from the tRNA-Trp_{CCA} genes with the intron gradually decreased along with the progression of intron removal. While a considerable amount of the intron-containing DNA fragment was observed in the PCR sample of the quintuple intron deletion clone (one wild-type gene plus 5 intronless genes), no band with mobility of the intron-containing fragment was seen in the sample of the sextuple deletion clone. These results collectively indicate that, in the sextuple intron-deletion strain, the tRNA-Trp_{CCA} introns were completely removed from the yeast genome.

Third, we tested whether or not the tRNA-Trp_{CCA} genes we handled here are essential for the yeast, to negate a possibility that tRNA-Trp_{CCA} genes with unusual sequences compensate defects of the intronless tRNA-Trp_{CCA} genes. We disrupted *tW(CCA)* genes by replacing each gene with a different marker and left the marker at each *tW(CCA)* locus. Combination of iterative deletions and tetrad analyses revealed that the complete (sextuple) deletion mutant of tRNA-Trp_{CCA} is lethal, while mutants with only one of the six tRNA-Trp_{CCA} genes grow slowly (Fig. 2C, columns 2,3). Interestingly, the growth defects varied among different quintuple disruptants, probably reflecting the difference in transcription efficiency of the tRNA gene left on the chromosome. The growth defects of the quintuple and sextuple disruptants were complemented by introducing pTYSC583, a *URA3* plasmid with *tW(CCA)K*, and the strain TYSC1406, the sextuple disruptant with pTYSC583, could not be cured of the plasmid on the FOA medium (Fig. 2D, row 3), indicating that at least one tRNA-Trp_{CCA} gene is required for yeast viability, and other genes on the yeast genome cannot substitute for functionality of *tW(CCA)* loci (Fig. 2D).

Finally, we asked whether the intronless strain we constructed harbors any suppressor mutation(s) that compensates growth defects caused by the intron deletion, using the above strain. We crossed the complete intronless strain, SMSC233 (α cell), with TYSC1406 (a cell) and could obtain a diploid strain [*MATa*/ α *tw(cca)g1* Δ int/ Δ *tw(cca)g2* Δ int/ Δ *tw(cca)j* Δ int/ Δ *tw(cca)k* Δ int/ Δ *tw(cca)m* Δ int/ Δ *tw(cca)p* Δ int/ Δ] without pTYSC583. The growth of the diploid was essentially the same as that of a control heterozygote [*W(CCA)G1*/ Δ *tW(CCA)G2*/ Δ *tW(CCA)J*/ Δ *tW(CCA)K*/ Δ *tW(CCA)M*/ Δ *tW(CCA)P*/ Δ] (Fig. 2D, rows 4,5). Since the SMSC233 and TYSC1406 strains were constructed independently, it is unlikely that our intronless strain is viable because of a recessive suppressor mutation that specifically cancels growth defects of the intron deletion.

Functionality of the intronless tRNA-Trp_{CCA} genes

The fact that we could construct a yeast strain whose tRNA-Trp_{CCA} introns on the genome were completely removed indicates that the tRNA-Trp_{CCA} intron does not possess any essential role(s) on yeast growth. To extend this observation, first, we compared growth of the yeast strains with different numbers of tRNA-Trp_{CCA} genes converted to

the intronless version. We grew these strains on a rich medium (YPD) at 23°C, 30°C, and 37°C and found that all of these strains including the sextuple intronless strain showed quite similar growth to that of the wild-type strain (Fig. 3A).

Similar results were observed when these strains were grown on synthetic media, or on media with nonfermentable carbon sources like glycerol and lactate. The intronless strains also grew in the presence of 1 M NaCl or low pH stress (pH 5.5) (Fig. 3B; data not shown). We also tested sensitivity against an alkylating reagent, methyl methane-sulfonate (MMS). It was suggested that unspliced pre-tRNAs in the nucleus act as a signal to counteract against DNA damage (Ghavidel et al. 2007). We could not detect any difference in viability between the wild-type and complete intronless strains when the cells were treated with a sublethal concentration of MMS (Fig. 3C). Therefore, complete removal of the tRNA-Trp_{CCA} introns does not have a strong impact on yeast growth under any growth conditions examined so far.

Then, we examined the growth of the intronless strain more quantitatively. First, we measured growth rates of the wild-type and intronless strains in liquid YPD at 30°C. Their doubling times were 130 ± 4 min and 136 ± 6 min, respectively. Because the difference was minute and within the range of standard deviations, we further tried to detect a possible difference in their growth by competitive culturing in YPD. If there is a difference in their growth rates, one of the strains becomes dominant in population after long incubation in co-culture. The population of these strains at

each time point can be easily estimated by PCR of a certain *tW(CCA)* locus. As shown in Figure 4, quantitative analyses of the PCR products by chip electrophoresis revealed that the population of the intronless strain did not decrease but rather slightly increased during 50 generations of continuous growth in six independent experiments. These results, again, indicate that complete removal of the tRNA-Trp_{CCA} intron does not cause negative effects on yeast growth under the usual laboratory conditions.

Next, we analyzed expression and maturation of tRNA-Trp_{CCA} derived from the intronless genes *in vivo*. After its end-maturation, the intron-containing pre-tRNA-Trp_{CCA} is 109 nt in length and converted to the mature form with a length of 75 nt by splicing. When we analyzed tRNA-Trp_{CCA} by Northern blotting, similar amounts of tRNA-Trp_{CCA} were detected in the wild-type, quintuple, and sextuple intron-deletion strains (Fig. 5A, upper panel). This was not expected since distance between the internal promoter elements of tRNA genes, box A and box B, is known to affect transcriptional efficiency (Ciliberto et al. 1982; Fabrizio et al. 1987). The mobility of tRNA-Trp_{CCA} on the urea-PAGE in an alkaline buffer was not affected by the intron deletion, suggesting that tRNA-Trp_{CCA} in these strains received appropriate end-maturation and probably nucleotide modifications affecting electrophoretic mobility under the alkaline conditions. On the other hand, when the same samples were hybridized with a probe against the tRNA-Trp_{CCA} intron, weak but apparent signals derived from the primary transcript, 5'-processed, and end-matured pre-tRNA-Trp_{CCA} were observed in the lane of the quintuple intron-deletion strain, while no signal was seen in the lane of the sextuple deletion strain (Fig. 5A, middle panel). These results again indicate that no intron containing pre-tRNA-Trp_{CCA} is transcribed in the sextuple intron-deletion strain and, in addition, that tRNA-Trp_{CCA} is transcribed and end-matured from the intronless genes as the wild-type genes *in vivo*.

If tRNA-Trp_{CCA} from the intronless genes completes its maturation processes, it should be aminoacylated by Trp-RS. We thus measured how much of tRNA-Trp_{CCA} in the intron deletion strains are charged using an acidic gel. We prepared total tRNAs from the three strains shown above under acidic conditions (pH 4.5) to protect an aminoacyl group conjugated to the 3' end of tRNAs from hydrolysis. A portion of the RNA samples was treated with alkaline for deacylation, and both alkaline-treated and nontreated samples were subjected to Northern blotting. Large parts of tRNA-Trp_{CCA} from the quintuple and sextuple intron-deletion strains as well as that from the wild-type strain migrated more slowly in the absence of alkaline treatment than those treated with alkaline, indicating that most of the tRNA-Trp_{CCA} was aminoacylated (Fig. 5B). Levels of aminoacylation in the intronless strains are indistinguishable from that in the wild-type strain. Therefore, tRNA-Trp_{CCA} from the intronless genes is properly aminoacylated *in vivo*.

FIGURE 3. Growth of tRNA-Trp_{CCA} intronless yeasts. (A) Growth of tRNA-Trp_{CCA} intronless strains on YPD was compared with that of WT (BY418). The plates were cultured at 23°C, 30°C, and 37°C. The yeast strain streaked on each sector is shown in the lower right. (B) Growth of the wild-type (WT) and intronless (6/6Δint) strains was compared under several conditions described on the left by serial dilution. Cells were cultured at 30°C for 3 d on YPD and SD and for 4 d on YPGly and YPD with 1 M NaCl. (C) Viability of the wild-type and intronless cells treated with or without 0.12% MMS for 80 min at 30°C (Ghavidel et al. 2007) was analyzed by serial dilution on YPD.

FIGURE 4. Competitive growth between the wild-type and tRNA-Trp_{CCA} intronless yeasts. The wild-type (WT) and tRNA-Trp_{CCA} intronless (Δint) yeast cells were co-cultured in liquid YPD at 30°C. Six independent cultures (a–f) were initiated with a 1:1 mixture of these strains, and their logarithmical growth was kept by periodic dilution in the fresh medium. Samples taken at each time point were subjected to PCR amplification of the *tW(CCA)K* locus and analyzed by chip electrophoresis. (A) Electropherograms of the samples from the inoculants and from the 50th generation are shown with those of the wild-type (WT) and the intronless strains (Δint). Band assignment is shown on the left. (B) DNA amounts of PCR products from the wild-type *tW(CCA)* and *tw(cca)kΔint* alleles in each culture were quantified from the electropherograms, and their ratios, $[tw(cca)k\Delta int] / ([tw(cca)k\Delta int] + [tW(CCA)K])$, are plotted against culture length.

Finally, we examined whether tryptophan-ylated tRNA-Trp_{CCA} from the intronless genes normally acts in translation. We first examined the rates of bulk protein synthesis by measuring incorporation of ³⁵S-labeled amino acids (Met/Cys) into a trichloroacetic acid (TCA)-insoluble fraction. We could not observe any difference in the incorporation among the wild-type, quintuple, and sextuple intron-deletion strains as shown in Figure 6A, suggesting that tryptophan-ylated tRNA-Trp_{CCA} derived from intronless genes is utilized for bulk translation, as is that from the intron-containing genes in vivo. Second, we analyzed the effect of intron removal on individual protein synthesis by analyzing the protein composition. First, cell extracts prepared from the wild-type, quintuple, and sextuple intron-deletion cells in either log or stationary phases were subjected to SDS-PAGE with an 8%–14% polyacrylamide gradient gel. Essentially, the same protein patterns were observed in the extracts from the three strains (Fig. 6B), suggesting that there is no essential difference in protein compositions of these yeast cells under log or stationary culture conditions with this resolution. We further analyzed the difference in

protein compositions between the wild-type and sextuple intron-deletion strains by 2D gel electrophoresis. Protein extracts prepared from log phase cells were subjected to isoelectric focusing (IEF) with a pH gradient from 4 to 7 and then to SDS-PAGE with a 12% polyacrylamide gel (Fig. 6C). Comparison of over 500 spots on the gels showed that most of the proteins migrated with similar positions and similar amounts. On the other hand, we noticed that signal intensities of limited numbers of spots are apparently different between the two strains. Some examples are shown by pseudocolor expression of the part of the gel (Fig. 6C, right-most panel, circled spots). Taken together, we conclude that complete removal of the tRNA-Trp_{CCA} introns from the yeast genome has little, if any, effect on the growth and bulk translation of *S. cerevisiae*, while it causes minor effects in yeast protein composition.

DISCUSSION

Is the tRNA intron, which must be removed during maturation, essential for eukaryotic cells? The simplest way to answer this question is to examine whether an organism whose tRNA introns are removed from its genome can be genetically constructed. However, it is difficult to achieve this experiment since many eukaryotes have multiple isoacceptor tRNAs encoded by multiple intron-containing genes. Therefore, we, instead, asked whether the intron of a certain isoacceptor tRNA is essential for the life of a eukaryote, *S. cerevisiae*. To analyze this subject, we developed a systematic protocol to replace multiple tRNA genes on the yeast chromosomes with the intronless version. Although

FIGURE 5. tRNA-Trp_{CCA} from the intronless genes became matured and aminoacylated as efficiently as that from the wild-type genes. (A) Total RNAs were extracted from each strain shown on the top of the panel, and 3 μ g aliquots were subjected to Northern blotting with probes shown on the left. In the middle panel, bands corresponding to the primary transcript, 5'-processed precursor, and end-matured precursor were marked by the closed, gray, and open triangles, respectively. (B) Total RNAs were prepared from the indicated strains under acidic conditions to retain aminoacyl groups on the 3' end of tRNAs. Samples (3 μ g per lane) were pre-treated with (+ lanes) or without (– lanes) alkaline for deacylation, and were subjected to Northern blotting. Closed and open triangles represent the positions of aminoacylated and deacylated tRNA species, respectively.

FIGURE 6. Complete removal of tRNA-Trp_{CCA} intron from the yeast genome does not have a big impact on bulk translation and protein composition. (A) Protein synthesis was measured as ^{35}S -methionine/cysteine incorporation into a TCA-insoluble fraction. Averages of three independent experiments are shown with standard deviations. (B) Major protein contents in the wild-type (wt), quintuple (5/6 Δint), and sextuple (6/6 Δint) intron deletion strains were compared in SDS-PAGE/Coomassie Brilliant Blue staining. SDS-extracts from the above strains in log and stationary phases were analyzed on an 8%–14% polyacrylamide gel. For the log phase cell extracts, 0.10 OD₆₆₀ equivalents of yeast cells were loaded, while 0.20 OD₆₆₀ equivalents were loaded for the stationary cell extracts. (C) Protein compositions of wild-type cells (wt) and sextuple intron deletion cells (6/6 Δint) were compared in 2D electrophoresis. Fifty μg of total proteins were first analyzed by IEF (pH 4–7) and then subjected to 12% SDS-polyacrylamide gel. A part of the gel enclosed with a dashed rectangle of each panel is merged in the right-most panel (merged). In this panel, contrast was reversed, and signals were expressed with pseudocolor: (green) wild-type, (red) sextuple intron deletion.

removal of the intron from a certain tRNA gene was achieved previously in the yeast, that was only applied to a minor Ser isoacceptor, which is encoded by a single gene. In this report, we removed the introns from all of the six tRNA-Trp_{CCA} genes, whose gene product is the only isoacceptor tRNA decoding the Trp codon, as a model case to analyze essentiality of all the tRNA introns on the yeast genome. We could construct a yeast strain that has only intronless tRNA-Trp_{CCA} genes on its genome and could demonstrate feasibility of the procedure to modify heavily degenerated genes iteratively. We confirmed the complete loss of the tRNA-Trp_{CCA} intron by (1) PCR analysis of all the annotated tRNA-Trp_{CCA} loci, (2) PCR analysis with a set of primers amplifying a tRNA segment encompassing the anticodon loop, and (3) Northern blotting with a probe against the known tRNA-Trp_{CCA} intron.

One of our big surprises is that the intronless yeast cells seem to grow and translate proteins without any serious problems. The growth of the intronless strain was indistinguishable from that of the wild-type strain even in the co-culture analyses in a rich medium, YPD. The

intronless yeast also grows normally under various conditions, including those damaging chromosomal DNA, recovery from which correlates well with nuclear accumulation of certain intron-containing pre-tRNAs (Ghavidel et al. 2007). We could not detect any difference in ^{35}S -amino acids incorporation into protein fractions between the wild-type and completely intronless cells. The expression level and aminoacylation efficiency of tRNA-Trp_{CCA} derived from the intronless genes were comparable to those from the wild-type genes in vivo, suggesting that tRNA-Trp_{CCA} from the intronless genes undergoes the normal aminoacylation/peptidyl transfer cycle in addition to normal maturation. These results indicate that the intron of tRNA-Trp_{CCA} is dispensable for growth and bulk translation of *S. cerevisiae* under laboratory conditions. The effects of intron removal were further analyzed by comparing protein composition in SDS-PAGE/2D gel analyses. Again, we found that overall protein composition is not affected significantly by the complete removal of tRNA-Trp_{CCA} intron. However, expression of some proteins is altered by the intron removal (Fig. 6C). Identity of these proteins is of interest because it will reveal what kind of the codon sequences are the most susceptible to decoding by tRNA-Trp_{CCA}.

Another surprise is that the yeast cells with intronless tRNA genes can maintain a similar amount of tRNA-Trp_{CCA} to that of the wild-type genes. It is known that a promoter of a tRNA gene for RNA polymerase III consists of box A and box B, which are located in the tRNA coding region (Hall et al. 1982; Marck et al. 2006). The distance between the two elements affects transcription efficiency, so that removal of the intron alters expression levels of some tRNAs in *Xenopus* oocytes in vivo and in yeast extracts in vitro (Ciliberto et al. 1982; Fabrizio et al. 1987). Similarly, intron removal from a *SUP53/tL(CAA)C* gene, encoding tRNA-L_{CAA}, in yeast renders the gene less occupied by TFIIC in vivo (Donze and Kamakaka 2001). Furthermore, such an effect on TFIIC occupancy at the tRNA locus has a big impact on blocking expansion of heterochromatin across the tRNA gene. However, the present study showed that intron removal does not affect the amount of tRNA-Trp_{CCA} despite the fact that tRNA-Trp_{CCA} genes have a longer (34 bp) intron than *SUP53* (32 bp) and that neither box A- nor box B-like sequences are found in the tRNA-Trp_{CCA} intron. This suggests that the boxes A and B of

tRNA-Trp_{CCA} may have sufficient affinity for TFIIH proteins, irrespective of the distance between the two elements, to assemble the transcription initiation complex of RNA polymerase III. Alternatively, some mechanisms in the level of transcriptional elongation and in stability control of the tRNA may compensate reduction of promoter activity of the intronless tRNA-Trp_{CCA} genes.

The above results are in great contrast to the fact that *S. cerevisiae* maintains the intron in all the tRNA-Trp_{CCA} genes at the same position and with the same sequence (Fig. 1B). The sequences of the tRNA-Trp_{CCA} introns are rather conserved among yeasts over the *Saccharomyces* genus when compared with 5'- and 3'-flanking regions of the tRNA gene (Fig. 1C). On the other hand, among tRNA-Trp_{CCA} genes of the more distantly related yeasts, their intron sequences are more conserved in the same species when compared with those from other species (Fig. 7). When parts of genomic sequences including tRNA-Trp_{CCA} on six moderately related yeast genomes are analyzed with Clustal X, all the tRNA genes on the same genome are clustered. Among these, *Debaryomyces hansenii* harbors only tRNA-Trp_{CCA} genes without any intron, while more distantly related yeast *Yarrowia lipolytica* harbors intron-containing tRNA-Trp_{CCA} genes. These facts suggest that organisms avoid having intron-containing and intronless genes of an isoacceptor tRNA simultaneously on their genomes. If a certain gene of an isoacceptor tRNA acquires an intron, the sequence spreads throughout the genome, and maintenance of the intron on the genome does not seem to rely on functionality of the tRNA in translation. Alternatively, it may be possible that very minor differences in translational activity between tRNA-Trp_{CCA} from intron-containing genes and that from intronless genes, like the difference found in our 2D gel analyses (Fig. 6C), swings the balance to reach either of the two extreme cases.

tRNA-Trp_{CCA} of *S. cerevisiae* is the second but more strict case in which the tRNA intron is dispensable for growth in eukaryotic cells. The final answer will be obtained by extending our analysis to the rest of the isoacceptor tRNAs in *S. cerevisiae*. It is still too early to conclude that all the tRNA introns are dispensable and do not have any major roles on tRNA maturation and functionality. However, our results may lead to a new idea; the tRNA intron is maintained on the yeast genome not because of the essentiality in the tRNA function in translation, but because of some other cellular mechanisms, such as maintenance of genome integrity, chromatin organization, or others.

FIGURE 7. Comparison of tRNA-Trp_{CCA} genes in moderately related yeast genomes. Sequences of tRNA-Trp_{CCA} genes on six moderately related yeast genomes annotated in Genomic tRNA Database (<http://lowelab.ucsc.edu/GtRNAdb/>) are compared, and the results are expressed as a phylogram. The lower right inset shows the phylogenetic relation of the six yeast species according to Fischer et al. (2006). Clusters of tRNA genes from the same genome are shadowed. (*S. cer*) *Saccharomyces cerevisiae*, (*Y. lip*) *Yarrowia lipolytica*, (*D. han*) *Debaryomyces hansenii*, (*C. gla*) *Candida glabrata*, (*E. gos*) *Eremothecium gossypii*, (*K. lac*) *Kluyveromyces fragilis*.

MATERIALS AND METHODS

Yeast strains, primers, and plasmids

Yeast genetic techniques are essentially described in Guthrie and Fink (1991), and other molecular biological techniques are in Sambrook and Russell (2001). Yeast strains used and constructed in this study are summarized in Supplemental Table SI. Primers and plasmids are in Supplemental Tables SII and SIII, respectively. To construct pTYE379 and pTYE380, a 4.95 kb *Bgl*III-*Not*I fragment from pGG216 and a 6.03 kb *Bgl*III-*Not*I fragment from pGG217 (Akada et al. 2002) were inserted into the *Bam*HI-*Psp*OMI sites of a pDNR-1r (Clontech) derivative with a modified multicloning site, respectively.

Construction of tRNA-Trp_{CCA} intronless yeasts and growth analysis

A chromosomal region with a certain tRNA-Trp_{CCA} gene and its flanking region were amplified by PCR separately with ExTaq (Takara Bio) and cloned onto a vector, pTYE374, a derivative of pBluescript SK(-), which has a *loxP* site. The amplified regions are summarized in Supplemental Table SIV. To make an intronless version of a tRNA-Trp_{CCA} gene, we applied overlap extension mutagenesis with a set of four primers, tRNA-Trp_{CCA} gene specific forward and reverse primers, and tRNA-Trp_{CCA} exon primers (tWccaG2_ex1_3 and tWccaG2_ex2_5) that provided a joint of the two exons. A marker cassette derived from pTYE379 or pTYE380, which harbors an auxotrophic marker (*URA3* or *ADE2*) and a negative marker (*GAL1p::GIN11M86*) sandwiched with two *hisG* fragments (Akada et al. 2002), and *Cam*^r, was introduced

between the tRNA-Trp_{CCA} gene and its flanking region. All the sequences of the PCR fragments were confirmed after cloning.

For integration of the intronless tRNA-Trp_{CCA} gene to the yeast genome, the plasmid with the intronless gene was digested with appropriate restriction enzymes, and the resulting DNA fragments were introduced to an appropriate yeast strain. Transformants with an auxotrophic marker were selected on an SCD medium [0.67% w/v Difco Yeast Nitrogen Base without Amino Acids (BD Biosciences), 0.5% w/v Vitamin Assay Casamino Acids (BD Biosciences), and 2% w/v glucose] with appropriate supplements. Counter selection to obtain clones that lost the marker cassette was performed on YPGal with 2% w/v galactose to induce the toxic *GIN11M86* gene. The above set of procedures was repeated six times to remove all the introns of the tRNA-Trp_{CCA} genes in the yeast strain BY418. Integration of the DNA fragment in the corresponding chromosomal region was confirmed by PCR and electrophoresis on a 2.5% w/v agarose gel.

For analyzing population of the complete intronless cells in competitive growth with the wild-type cells, these strains were inoculated to YPD as a 1:1 mixture and incubated at 30°C. When OD₆₆₀ reached 0.4, a part of the culture was brought to 128-fold dilution in fresh YPD to allow continuous logarithmic growth, and the rest of the culture was further incubated for 12 h to achieve saturation of growth. These procedures were repeated seven times. DNA samples were prepared from the saturation cultures and were subjected to PCR as above. Amounts of PCR products derived from the wild-type and intronless alleles of *tW(CCA)K* or *tW(CCA)G1* loci were measured by chip electrophoresis with the MultiNA Microchip Electrophoresis System (Shimadzu).

Disruption of tRNA-Trp_{CCA} genes

Disruption of tRNA-Trp_{CCA} genes was performed by the PCR-based method using pCgHIS3 (*Candida glabrata* HIS3), pCgTRP1 (*C. glabrata* TRP1), pBS-kanMX (*kanMX4*), pBS-hygMX (*hphMX*), pJJ282 (*LEU2*) and pTYE512 (*ADE2*) as templates. First, we constructed double disruption strains TYSC1281 [*MATa tw(cca)g1Δ::CgHIS3 tw(cca)g2Δ::LEU2*] based on YCB436 and TYSC1283 [*MATα tw(cca)jΔ::kanMX tw(cca)kΔ::CgTRP1*] based on BY418, crossed these strains and dissected the resulting diploid to obtain a *MATα* strain with quadruple disruption TYSC1345. We also constructed another *MATa* quadruple disruption TYSC1349 [*tw(cca)g1Δ::CgHIS3 tw(cca)g2Δ::LEU2 tw(cca)mΔ::hphMX tw(cca)pΔ::ADE2*] from TYSC1281 by iterative disruption. These two haploids were crossed to obtain the diploid TYSC1361 [*tw(cca)g1Δ::CgHIS3/Δ tw(cca)g2Δ::LEU/Δ tw(cca)jΔ::kanMX/+ tw(cca)kΔ::CgTRP1/+ tw(cca)mΔ::hphMX/+ tw(cca)pΔ::ADE2/+*]. For complementing chromosomal disruption of tRNA-Trp_{CCA} genes, we constructed pTYSC583, in which the wild-type *tW(CCA)K* gene was inserted into pYO326, a 2μ plasmid with *URA3* as a marker. By introducing pTYSC583 into TYSC1361 and performing the tetrad analysis, we obtained TYSC1406, a sextuple *tW(CCA)*-deletion strain with pTYSC583.

RNA analysis

RNA extractions from yeast with a guanidine thiocyanate buffer and those under acidic conditions were performed as previously described in Yoshihisa et al. (2003) and in Sarkar et al. (1999), respectively. The former RNAs were kept in TE [10 mM Tris-HCl,

pH 7.5, 1 mM EDTA] and run on a 7.0 M urea/10% w/v polyacrylamide gel in TBE, while the latter RNAs were dissolved in AE [10 mM sodium acetate, pH 4.5, 1.0 mM EDTA] and run on a 10 M urea/10% w/v polyacrylamide gel in 0.10 M NaOAc, pH 5.0. Deacylation of tRNAs was performed in 50 mM Tris-HCl, pH 9.0 at 37°C for 1 h (Steiner-Mosonyi et al. 2003). After transfer to charged nylon membranes (Hybond N⁺, GE Healthcare Biosciences), a particular RNA was decorated with an appropriate digoxigenin-labeled oligonucleotide probe (Roche Diagnostics), and the signal was developed by ECF (GE Healthcare Biosciences) and read with a Storm 860 Image Analyzer (GE Healthcare Biosciences).

Protein analysis

Two-dimensional gel electrophoresis was performed with IEF as the first dimension and SDS-PAGE as the second dimension. To prepare protein samples for IEF, yeast cells suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA were treated with final 0.28 M NaOH and 6.2% v/v 2-mercaptoethanol on ice for 4 min, and proteins were precipitated by adding 10 volumes of ice-cold 10% w/v TCA. After collecting precipitates by centrifugation, the pellets were washed with ice-cold acetone and were dissolved in IEF Sample Buffer [7.0 M urea, 2.0 M thiourea, 4% w/v CHAPS, 2% v/v IPG Buffer, pH 4.0-7.0, 0.12% v/v DeStreak Reagent (GE Healthcare)]. The samples were analyzed with Immobiline Dry Strip, pH 4.0-7.0 and an IPGphor Electrophoresis System (GE Healthcare) according to the manufacturer's instructions, and developed strips were subjected to SDS-PAGE with 12% polyacrylamide gels. Gels were stained with SYPRO Red (Sigma-Aldrich) and analyzed with an Typhoon 9400 Image Analyzer (GE Healthcare).

³⁵S-amino acid-labeling to monitor protein synthesis

Protein synthesis was monitored by measuring ³⁵S methionine/cysteine incorporation into a TCA insoluble fraction. Briefly, 100 OD₆₀₀ cells cultured in an SD medium prepared from Difco Yeast Nitrogen Base without Amino Acids and Ammonium Sulfate (BD Biosciences) and supplemented with appropriate sulfate-free nutrients were labeled with 2.0 MBq of Tran³⁵S-Label (MP Biomedicals) for the indicated times. Three 200-μl aliquots were harvested in 1.0 mL of 15% w/v TCA, heated at 94°C for 5 min, and insoluble materials were recovered on a GF-75 glass filter (Advantec). After sequential washing with 15% w/v TCA and ethanol, the filter was dried, and the radioactivity trapped on the filter was measured with a liquid scintillation counter, LSC-5100 (Aloka).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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