Deciphering the reading of the genetic code by near-cognate tRNA

Sandra Blanchet1, David Cornu2, Isabelle Hatin2, Henri Grosjean2, Pierre Bertin1, and Olivier Namy1

1Institute for Integrative Biology of the Cell (I2BC), Commissariat à l’énergie atomique et aux énergies alternatives, CNRS, Université Paris-Sud, Université Paris-Saclay, 91198 Gif-sur-Yvette cedex, France
2Present address: Department of Physical Biochemistry, Max Planck Institute for Biophysical Chemistry, 37077 Goettingen, Germany.

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Some codons of the genetic code can be read not only by cognate, but also by near-cognate tRNAs. This flexibility is thought to be conferred mainly by a mismatch between the third base of the codon and the first of the anticodon (the so-called “wobble” position). However, this simplistic explanation underestimates the importance of nucleotide modifications in the decoding process. Using a system in which only near-cognate tRNAs can decode a specific codon, we investigated the role of six modifications of the anticodon, or adjacent nucleotides, of the tRNAs specific for Tyr, Gin, Lys, Trp, Cys, and Arg in Saccharomyces cerevisiae. Modifications almost systematically rendered these tRNAs able to act as near-cognate tRNAs at stop codons, even though they involve noncanonical base pairs, without markedly affecting their ability to decode cognate or near-cognate sense codons. These findings reveal an important effect of modifications to tRNA decoding with implications for understanding the flexibility of the genetic code.

Significance

Protein translation is a key cellular process in which each codon of mRNAs has to be accurately and efficiently recognized by cognate tRNAs of a large repertoire of noncognate tRNAs. A successful decoding process is largely dependent on the presence of modified nucleotides within the anticodon loop, especially of tRNAs having to read A/U-rich codons. In this latter case, their roles appear to stabilize the codon–anticodon interaction, allowing them to reach an optimal energetic value close to that of other interacting tRNAs involving G/C-rich anticodons. In this work we demonstrate that, while helping an efficient translation of A/U-rich codons, modified nucleotides also allow certain unconventional base pairing to occur, as evidenced in the case of stop codon suppression.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE108772).

1Present address: Department of Physical Biochemistry, Max Planck Institute for Biophysical Chemistry, 37077 Goettingen, Germany.
2To whom correspondence should be addressed. Email: olivier.namy@ibcp.paris-saclay.fr.

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on the decoding of cognate codons. We also showed that overall stop codon suppression efficiency appears as a poor indicator of the ability of individual tRNAs to decode stop codons, due to adjustments between the various competing near-cognate cellular tRNAs at reading the same stop codon. Taken together, our results highlight the flexibility of the genetic code and reveal an unexpected capacity of the Saccharomyces cerevisiae translation machinery to discriminate between sense and nonsense near-cognate codons. Although one cannot rule out that increased readthrough due to \( \Psi_{35} \) is a direct consequence of the increased stability of the codon:anticodon pairing, one appealing hypothesis is that genes coding for enzyme catalyzing \( \Psi_{35} \) have been conserved through evolution to regulate stop codon readthrough according to the cellular needs.

Results

Deletion of Genes Coding Enzymes Responsible for tRNA Modifications Have No Major Impact on Cell Growth and Polysomes. To study the impact of several yeast tRNA modifications on the decoding of near-cognate codons, we used a reporter system based on the stop codon readthrough-dependent expression of a gene encoding a GST protein. This system provides a precise quantitative analysis of the incorporation of natural suppressor tRNAs at stop codons. In a previous study, we identified tyrosine, glutamine, and lysine tRNAs as the cytoplasmic tRNAs incorporated at UAA and UAG codons, and tryptophan, cysteine, and arginine tRNAs as the tRNAs incorporated at UGA codons (Table S1) (16).

Here, we systematically deleted six genes encoding enzymes responsible for posttranscriptional modification of bases or hydroxyl-riboses in the anticodon loop of the above-identified suppressor tRNAs (Fig. L4). Three of these genes (Elp3p, Trm9p, and Tuc1p) are involved in conversion of U\(_{34}\) residue in the glutamine tRNA\(_{UAA}\), lysine tRNA\(_{UAA}\), and arginine tRNA\(_{UAA}\) into a hypermodified 5-methoxy-carbonylmethyl-2-thiouridine residue (mcm^3s^2U) (6). The deletion of ELP3 or TRM9 results in glutamine and lysine tRNAs carrying only s^2U\(_{34}\) or cm^3s^2U\(_{34}\), respectively (U\(_{34}\) and cm^3s^2U\(_{34}\) for arginine tRNA), whereas the deletion of TUC1 results in glutamine and lysine tRNAs with mcm^3U\(_{34}\) (17, 18). The anticodons of the unique tyrosine and tryptophan suppressor tRNAs are also modified by two other enzymes: Pus7p, which catalyses the pseudouridylation of U\(_{35}\) in the tyrosine tRNA and in U2 snRNA (19); and Trm7p, which methylates C\(_{34}\) and C\(_{32}\) in the tryptophan tRNA (19, 20). Another enzyme of interest is Mod5p, which converts the nucleotide at position 37, 3'-adjacent to the anticodon, to \( \Psi \)A in the tyrosine and cysteine tRNAs (21). We first confirmed that deletions of these genes have no severe effect on cell viability (Fig. S1). Doubling times were similar for the parental and deletion strains, except for the \( \Delta TRM9 \) and \( \Delta TRM7 \) strains, which grew more slowly, as reported in previous studies (20). Polysome profile analysis revealed that the proportions of 40S, 60S, and 80S ribosomes were unaffected in each strain carrying the above-selected deletion of modification enzyme genes (Fig. 1B).

U\(_{34}\) Modification Affects Stop Codon Recognition by the Gln, Lys, and Arg tRNAs. In yeast, most of the uridine residues in position 34 of tRNAs are hypermodified. We investigated the influence of different elements of mcm^3s^2U modification on stop codon decoding by natural suppressor tRNAs by deleting the ELP3, TRM9, and TUC1 genes separately in the parental strain. Because these enzymes modify U\(_{34}\) only in glutamine tRNA\(_{UAG}\), lysine tRNA\(_{UUG}\) (reading UAA), and arginine tRNA\(_{UUA}\) (reading UGA) (Fig. L4), we assessed their impact on these two stop codons. We first used a dual reporter system (22) to analyze the effect of each deletion on the levels of stop codon suppression. We found no significant effect of TRM9 deletion, but a slight decrease in readthrough efficiency in \( \Delta elp3 \) mutants, for the UAA and UGA codons only (Fig. 2A).

Next, we assessed the contributions of the glutamine tRNA\(_{UAG}\)-lysine tRNA\(_{UUG}\), and arginine tRNA\(_{UUA}\) to the levels of stop codon expression observed, by transforming the \( \Delta elp3 \), \( \Delta trm9 \), and \( \Delta tuc1 \) strains with the GST reporter system (16) and performing mass spectrometry on the GST proteins generated by stop codon suppression, to quantify the frequency of incorporation of the amino acids corresponding to the three tRNAs, as previously described (Fig. S2B) (16). Quantification of the proportions of amino acid present at UAA codon revealed that incorporation of the glutamine tRNA\(_{UAG}\) occurred less frequently in the three deletion strains than in the WT, this tRNA being replaced by the tyrosine tRNA, the incorporation of which was not affected by the deletions (Fig. 2B). We also found that deletions of ELP3 (resulting in mcm^3-lacking s^2U\(_{34}\)) and TUC1 (resulting in the

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**Fig. 1.** (A) A schematic diagram of the anticodon loops from the three near-cognate tRNAs inserted at the various stop codons. Modifications studied in this work are shown in red, with the name of the corresponding gene (= mcm^3s^2U). The position of the mismatch (or nonconventional base pairing) between the anticodon and the stop codon is highlighted in blue (or in purple if this position is also modified). Lysine tRNA\(_{UUG}\) and arginine tRNA\(_{UUA}\) also carry \( t^\Delta A \) modification that is not studied in this work because of the strong growth defect link to the absence of this modification. (B) The quantification of the various fractions of the polysome profiles obtained for each mutant. Three to five independent experiments were performed for each mutant.
Fig. 3 MOD5 genes were deleted. Blanchet et al. for decoding strain (had no effect on the ratios of the various amino acids, whereas the deletion of TRM9 strongly stimulated incorporation of arginine and also slightly increased cysteine incorporation, even though the corresponding cysteine tRNA is not thought to be modified by TRM9 (Fig. 2B). Clearly, 5-mc modification does not have the same effect on the ability of glutamine, lysine, and arginine tRNAs to read UAA and UGA stop codons.

Modification of the Nucleotides Adjacent to the Anticodon Plays an Important Role in Near-Cognate Decoding. Beside the arginine tRNA, the tryptophan and cysteine tRNAs, both of which carry nucleotide modifications. The tryptophan tRNA anticodon loop is modified at C32 and C34 into 2'-methyl cytosine (Cm) by Trm7p, while the Cys tRNA is modified at A37 into ψA37 by Mod5p (Fig. 1L). Both belong to the same four-codon family boxes. Suppression involved a mismatch base pair at position 34 of the anticodon and the third base (A) of the codon. As shown in Fig. S3A, efficiency of UGA readthrough is not affected by TRM7 deletion (Fig. S3B), which also had no effect on the proportions of tryptophan, cysteine, and arginine incorporated (Fig. S3B). In contrast, the absence of Mod5p affected only the relative proportions of suppressor tRNAs not the level of the UGA suppression. Indeed, cysteine was not incorporated anymore at the UGA codon in the ΔMOD5 strain (Fig. S3B).

Like the cysteine tRNA, the tyrosine tRNA is modified by Mod5p, resulting in a ψA37 residue at position 37. We analyzed the effect of the elimination of this modification from the tyrosine tRNA on the level of stop codon suppression. Cysteine tRNA harbors a G34-A3 mismatch when reading UAA and tyrosine tRNA harbors a G34-G3 mismatch when reading UGA). Quantitative analyses for the ΔMOD5 strain showed that stop codon suppression efficiency at both the UAA and UAG codons was much lower than in the parental strain (Fig. 3A). Contribution of the tyrosine tRNA to the global suppression observed in the ΔMOD5 strain was next investigated by quantifying the incorporation of suppressor tRNAs at UAA and UAG codons. Levels of tyrosine incorporation were slightly lower at the UAG codon and much lower at the UAA codon (Fig. 3B). Thus, the effect of the ψA37 modification of the tyrosine tRNA depends on the codon decoded.

The Presence of a Pseudouridine Residue at Position 35 of the Tyr tRNA Is Important for Stop Codon Suppression. In S. cerevisiae, there is only one cytosolic tyrosine tRNA, which acts as the major suppressor at the UAA and UAG stop codons, despite the creation of a G34-A3 and a G34-G3 mismatch, respectively. This tyrosine tRNA is unique because, in addition to being modified at position 37 (ψA), it is the only a suppressor in yeast that also has a pseudouridine (ψ) instead of a usual uridine at the center (position 35) of the anticodon. This modification is post-transcriptionally catalyzed by Pus7p (Fig. 1L).

The lack of this pseudouridine residue on suppression of the UAA and UAG stop codons was investigated as above for the other tRNA modifications. PUS7 deletion did not change tyrosine tRNA stability as measured by four-label clover (FL-quart-PCR) (23) (Fig. S5), but resulted in lowering the level of stop codon suppression at UAG with almost no effect on stop codon suppression at the UAA codon (Fig. 3D). However, a quantification of amino acids incorporated at UAA and UAG codons revealed that the frequency of tyrosine tRNA incorporation at UAG was strongly decreased at the benefit of the noncognate glutamine and lysine tRNAs (Fig. 3E), even more significantly than in the ΔMOD5 strain (resulting in ψ(A-lacking A37). While in the case of UAA suppression, the incorporation of tyrosine tRNA is now almost exclusively replaced by the noncognate glutamine tRNA (ψU34) (Fig. 3B). We next checked whether the observed phenotype was solely due to the absence of Pus7p by reexpressing either the WT protein or a catalytic mutant of Pus7p, Pus7-D256A [which has been reported to be inactive (19)] in the ΔPUS7 strain. Results indicate that reexpression of the WT protein restored parental amino acid levels, whereas expression of the single mutant did not (Fig. S4). Thus, both modifications of the tyrosine tRNA (ψA37 and ψU34) are important for stop codon suppression, the ψA37 modification being important only for UAA readthrough, whereas the ψU34 modification appears crucial mainly for the recognition of UAG codon.

We also tested the absence of both modifications by constructing a strain in which the PUS7 and MOD5 genes were deleted. Subsequent quantification of stop codon readthrough efficiency (Fig. 3A) and amino acid insertion at the UAA and UAG codons (Fig. 3B) demonstrated a significant additive effect on stop codon readthrough, especially on the UAG codon.

Absence of the Pseudouridine Residue at Position 35 in the Tyr tRNA Does Not Prevent the Decoding of Tyrosine Codons. In S. cerevisiae, synonymous tyrosine codons UAU and UAC are almost equally used in cytoplasmic mRNAs. Only one tyrosine tRNA isoacceptor containing a GΨA anticodon exists for reading each of these two codons. Because, tyrosine tRNA is dependent on ψU34 for decoding the two near-cognate stop codons (UAA and UAG, see above), it was of interest to determine how tyrosine tRNA lacking of ψU35 behaves for decoding the two tyrosine sense codons (UAU/C). We used a ribosome profiling technique that allows in vivo quantification of translation speed through calculation of the genomewide ribosome residence time at each codon (24). We first checked the periodicity of the signal to demonstrate that the signals obtained corresponded to translating ribosomes (Fig. 4A). Then, in both the parental and ΔPUS7 strains, we compared the
normalized ribosome density at each tyrosine and stop codons in the A site for each gene (Materials and Methods). We found that UAU and UAC were decoded with similar efficiency in both Δpus7 and parental strains (P = 0.4 and 0.6, respectively) (Fig. 4B) and that this efficiency was not different from that for all of the other codons. We concluded that Ψ35 in Tyr tRNA does not allow a better decoding of the near-cognate UAU codon, despite it playing an important role for decoding near-cognate UAA and UAG codons. Interestingly, we did not observe any significant difference in ribosome occupancy at stop codons (Fig. 4C), whereas a reduction in in-frame ribosome footprints downstream the stop codon is evident (P = 1.5 × 10−3) (Fig. 4D). This confirms that stop codon readthrough is impaired in the Δpus7 strain without accumulation of ribosomes at natural stop codons when tyrosine tRNA is lacking Ψ35.

**Discussion**

Decoding of mRNAs by tRNAs into polypeptides on the ribosome was considered for a long time as a relatively simple process, where formation of a set of H-bonds between three complementary base pairs of codons and anticodons within a minihelix configuration plays a major role. However, it is now clear that other elements of tRNA molecules, as well as of rRNA of the ribosome, are also important for accurate and efficient mRNA decoding (5, 8, 25, 26). The biochemical properties of tRNAs depend strongly on posttranscriptional modifications of nucleotides within the anticodon (positions 34 and 35) and the so-called “proximal anticodon loop” of tRNA (positions 37, 38, and/or 32) (Fig. S6) (6, 15). The main role of these nucleotide modifications is generally argued to notably fine-tune the accuracy of decoding: that is, to restrict the decoding of split box codons (2:2 or 3:1 decoding boxes) or to extend the decoding of nonsplit four-codon boxes. However, it is now clear that, together with other bases of the anticodon loop, their roles are to mainly achieve a uniform ribosome binding by stabilizing the codon–anticodon interaction of tRNAs (27). This is especially important for tRNA harboring an A/U-rich anticodon triplet that have to reach about the same optimal interaction energies than tRNAs harboring G/C-rich anticodon. Base or ribose modifications lead to various chemical and physical consequences, such as keto-enol tautomerism, base protonation, uridine isomerization to pseudouridine (Ψ), anti to syn base transconformation, freezing a 2′-O-glycosidyl bond into its 3′-endo configuration, improvement of base stacking with neighboring bases, or additional interactions with ribosomal elements. One corollary of these multiple stabilizing effects is to allow certain noncanonical base pairs, which are isosteric with standard Watson–Crick pairs, to occur during decoding (28, 29).

In a preceding work, we and others identified in yeast eight naturally occurring tRNAs that misread stop codons as sense codons under in vivo conditions (Fig. 1) (16, 30). Although we are using a [PSI+] strain, it has been previously shown that this does not impact tRNA ratios found at the stop codon during readthrough (30). Here, we investigated the consequences of the absence of a given modification normally present in the anticodon loops (at positions 34, 35, and/or 37) on their relative efficacy to readthrough stop codons. This is a different situation from studies of missense errors at sense codons during the elongation process (3), because the near-cognate tRNAs of a stop codon will never be in competition with cognate tRNA that normally does not exist.

**Fig. 3.** Consequences on stop codon suppression of deletion genes coding for enzymes catalyzing formation of Ψ35 and Ψ36 in various tRNAs. (A) Quantification of readthrough efficiency at UUA and UAG codons in the two mutants lacking Ψ35 and Ψ43 formation. At least five independent experiments were performed for each value. The box plot is done as in Fig. 2A. (B) Quantification of the relative proportions of the amino acids identified at the two stop codons as in Fig. 2B.
All tRNAs in Fig. S6 belong to the so-called "intrinsically weak, A/U-rich" interacting tRNAs (Lys, Tyr) or "intermediate" interacting tRNAs (Gln, Arg, Trp, Cys), which are supposed to be among the most prone for miscoding (for details, see ref. 25). First, in the glutamine and lysine tRNAs, uridine-34 is modified to mcm^5^U (symbolized as U^*^) and to mcm^-U (U^*) in the arinine tRNA_{GCC}. The chemical adduct at C of U^*^ has been shown to favor binding to G^_3_4_5_ over A^_3_4_5_ via enol-keto tautomerism, while the thiol (S^-) group, beside reinforcing base pairing mostly with A^_3_4_5_, also reinforces stacking, and thus stability and conformational rigidity of both base 34 and the neighboring base pair N^_3_5^-N^_3_2 (Fig. S6) (9, 10). Deletion (one by one) of each of the three genes responsible for U^*^ modification into mcm^5^U^*^ in S. cerevisiae (Eelp3, Trm9, and Tuc1) shows a slight growth defect for only the deletion of Trm9 (affecting only the last methylation step of cm^5^-U^*^) (Fig. S1). Deletion of these genes had only a slight impact on UAA readthrough efficiency, the most pronounced effect being observed for the ΔTuc3 mutant corresponding to the total lack of 5-mcm group on U^*^ (Fig. 2A). The interesting observation is that, while the efficacy is not much affected in both the ΔEelp3 (lacking 5-mcm but not 2-thio) and Δtrcu1 (lacking 2-thio but not 5-mcm) strains, glutamine tRNA_{UAG} was no longer able to compete with tyrosine tRNA, now becoming almost the only suppressor tRNA for UAA readthrough, whereas in the ΔTrm9 strain (resulting in the lack of the cm^5^-U^*^-U^*^ pair), 2-thio suppression persists between glutamine and tyrosine tRNAs (Fig. 2B). These results demonstrate that both the fully methylated 5-mcm and 2-thio adducts of U^*^ greatly improve the stability of the codon–anticodon interaction so that a noncanonical G^_3_5^-U can occur, allowing glutamine tRNA_{UAG} to be stably incorporated at UAA. Because all of the base pairs of the minihelix have to adopt a strict Watson–Crick geometry (5, 26), this G^_3_5^-U pair probably fulfills the requirement for a Watson–Crick base pair (28, 29). However, this conclusion is not simply achieved. First, the formation of the ribose, confines the H-bound formation, and increases hydrophobic surface, hence also stacking with neighboring bases (33). No significant effect of Trm7 deletion (lacking cm^5^-U^*) is found on either global stop codon readthrough efficiency (Fig. 3A) or the nature of the amino acids incorporated, attesting again that the strong central C^_3_5^-G^_3_2 pair rather than the 2'-O-methylation of G^_3_4_5^- and C^_3_4_5^- is probably the main stabilizing element (Fig. S3B). An isocysteic G^_3_5^- or Cm^5^-A^_3_2 opposition probably occurs through an amino ionization of either Cm^- or A^-; alternatively, Cm^-A can also occur in the absence of a hydrogen bond (28, 29).

In contrast, deletion of the isopentenyl group on A^_3_7^- of cysteine tRNA in the ΔMOD5 strain prevents incorporation of cysteine at UGA stop codon (Fig. S3B). The same trend was observed with ΔA^_3_7^- containing tyrosine tRNA, the suppression efficiency being more severely affected in the case of UAA than of UAG (Fig. 3B). Thus, lowering the stability of a A^_3_2^-U^_3^-Watson–Crick pair by the lack of an isopentenyl group on A^_3_7^- in cysteine or tyrosine tRNA affects the efficacy of a triplet pairing involving a G^_3_4_5^- mispair with A^_3^- of stop codon UGA and UAA, respectively, or with G^_3^- in the case of stop codon UAG. Formation of an isosteric base pair involving two purines requires that one of the two purines switches conformation from anti to syn, probably the one of the codon, to form a Watson–Crick/Hoogsteen base pair (28, 29). Notice that codon–anticodon involving noncanonical G^_3_4_5^-A or G^_3_4_5^-G have been demonstrated for several tRNAs involving G/C-rich anticodons and reading codons of the unsplitted four-encoding boxes. For a long time, this type of decoding process has been designated as a "two-out-of-three" decoding rule (34).

Tyrosine tRNA carries an unusual Ψ at the central position of the anticodon that is catalyzed by Pus7p. Psdouoridine enables engaging a strong base pairing (almost as strong as C-G), rigidifies the sugar-phosphate backbone, and improves stacking with a neighboring base pair, forcing a Ψ-A pair to adopt an A-form conformation, as in a genuine Watson–Crick helix (35, 36). In agreement with an earlier similar observation in a plant translation system (37), we found that tyrosine tRNA in a ΔPus7 strain (lacking Ψ_3_5^) is a less-efficient suppressor at both UAA and UAG stop codons, but mainly at UAA (Fig. 3B), although the tyrosine tRNA is equally stable in WT and ΔPus7 strains (Fig. S5B). Deletion of Ψ_3_5^-A in Pus7 strains (Fig. S5C) does not affect the trans-splicing of proximal extended anticodon (25), before generalizing any observation.

The opal UGA codon is decoded mostly by tryptophan tRNA, followed by cysteine tRNA, both belonging to the same four-codon box. In tryptophan tRNA, A^_3_7^- is unmethylated while in cysteine tRNA it is modified by Mod5p to Ψ^_3_7^-A. Conversely, in tryptophan tRNA, C^_3_4^- is modified to cm^5^-U (as well as cm^5^-A) by Trm7p, while G^_3_4^- in cysteine tRNA is not modified. The presence of a hydrophobic isopentenyl group on A^_3_7^- is known to reinforce the stacking power of the purine adenine on the adjacent base pair A^_3_5^-U^_3^- (both by lateral and interstrand stacking, thus with the first codon base) (32). A 2'-O-methyl group on the hydroxyl of C^_3_4^- is known to favor a more rigid 3'-endo conformation of the ribose, confines the H-bound formation, and increases hydrophobic surface, hence also stacking with neighboring bases (33).
pairs at the third anticodon position and the first codon position. The allowed base mispairs or base oppositions are, however, only those that would mold within a minihelix of the Watson–Crick type of geometry that is mandatory for the aminoacyl-tRNA to be accepted and finally captured by the mRNA-ribosome machinery (26). We observed that mismatch with a stop codon never occurred at the middle position of the anticodon, while such abilities have been demonstrated at sense locations (6, 7). The reason is that there is no naturally occurring tRNA that could sustain such base opposition (like U:G) within a Watson–Crick minihelix in the whole tRNA repertoire of S. cerevisiae.

Only a small subset of tRNAs have been analyzed and it is likely that studies of other tRNAs in other translation systems, as well as organisms, will turn up other surprises. In this work, we propose that the activities of certain tRNA modification enzymes can be a regulatory device for the production of certain functional “read-through” proteins. This work should help at elaborating synthetic or mutated tRNAs able to introduce nonproteinous amino acids at specific locations of a mRNA where a sense codon has been appropriately mutated into a stop codon (39).

Materials and Methods

Detailed information on materials and methods used in this study is provided in SI Materials and Methods.

Strains and Plasmids. All of the strains used in this study were derived from 74-D694 (mata ade1-14 [UGA] ura3-52 trp1-289 [UAG] his3A200 leu2-3,112 [PS])