J. Biochem. 123, 853-858 (1998)

Effect of *B. subtilis* $tRNA^{Trp}$ on Readthrough Rate at an Opal UGA Codon

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Received for publication, November 10, 1997

Bacillus subtilis **has been thought to have a high readthrough rate at the UGA stop codon because no opal suppressor tRNA has been isolated so far [Lovett *et al.* (1991) *J. Bacteriol.* 173, 1810-1812]. To examine whether a $tRNA^{Trp}$ which we have characterized [Matsugi *et al.* (1992) *Nucleic Acids Res.* 20, 3514] has the ability to read the UGA codon, *in vitro* translation was performed with a synthetic mRNA containing a test codon, UGA, UAG, UAA, or UGG, in a reading frame. Addition of Trp-$tRNA^{Trp}$ to the system significantly increased the readthrough rate only in the case of UGA. This suggests that this $tRNA^{Trp}$ has a dual recognition pattern in *B. subtilis, i.e.*, for the canonical tryptophan codon and for readthrough at the UGA stop codon.**

Key words: ***Bacillus subtilis*****, readthrough, suppressor tRNA, translation, UGA codon.**

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In *Bacillus subtilis*, it has been suggested that the UGA termination codon is decoded to a certain extent, because no opal suppressor has been identified so far. In support of this hypothesis, Lovett *et al.* have revealed that the UGA codon introduced into the coding frame of a *cat-86* mRNA was translated as tryptophan *in vivo* (*1*). Their result suggested the participation of a tryptophan tRNA in the readthrough event. In *B. subtilis*, a single gene for tryptophan tRNA has been identified up to now, and the anticodon should be CCA (*3*). If *B. subtilis* has no other tryptophan tRNA gene, this tRNA has to decode the opal UGA as well as the original tryptophan codon. In this case, a C-A pair or other anticodon-codon interaction such as a two-out-of-three rule may be employed (*4, 5*), otherwise a modification which can switch the base recognition is required (*6-11*). To characterize this $tRNA^{Trp}$, we elucidated the RNA sequence at the modification level and found that the entire sequence was consistent with the sequence predicted from the gene. But, unexpectedly, the first letter of the anticodon was unmodified cytidine (Fig. 1) (*2*). It is noteworthy that we have not detected any other tryptophan tRNA species in the total tRNA. Therefore, it seemed probable that this tRNA reads the UGA termination codon.

Next, our attention was focused on whether this $tRNA^{Trp}$ actually has the ability to decode the UGA codon or not. To obtain clear evidence, we planned a cell-free translation experiment using this $tRNA^{Trp}$. The system consisted of a synthetic mRNA and *B. subtilis* S30 extract, and we added the purified natural $tRNA^{Trp}$ to the system as an aminoacylated form. Four kinds of mRNAs were designed to have a test codon, UAA (ochre), UAG (amber), UGA (opal), or UGG (Trp) in the coding sequence. If readthrough occurred at the test codon, labeled amino acid residues would be incorporated in the peptide and we would be able to estimate the readthrough at the test codon (*12*). Furthermore, if the addition of the $tRNA^{Trp}$ leads to an increase of the readthrough rate, this would also support the hypothesis that $tRNA^{Trp}$ has the ability to read the opal UGA as well as the tryptophan codon.

In *Escherichia coli*, it is well-known that tryptophan tRNA having unmodified cytidine acts as an opal suppressor (*13, 14*). However, the fact that no opal suppressor has been obtained in *B. subtilis* suggests that the readthrough rate at the UGA codon may be considerably higher than that of *E. coli* $tRNA^{Trp}$. Therefore, we are interested in the structural features of the $tRNA^{Trp}$ in *B. subtilis*.

Here we report that addition of Trp-$tRNA^{Trp}$ to the cell-free translation system increased the readthrough rate, but adding other tRNAs such as $tRNA^{Ser}$ did not cause such an increase in readthrough. This is strong evidence that the natural $tRNA^{Trp}$ with the CCA anticodon reads an opal codon *in vivo*.

MATERIALS AND METHODS

General—*B. subtilis* W168 S30 fraction was prepared according to the procedure of Dick and Matzura (*15*). The protein concentration of dialyzed S30 was 10 mg/ml. Oligodeoxyribonucleotides for the gene construction were synthesized by the phosphoramidite method (*16*). [^{14}C]Ile (315.5 mCi/mmol) and [^{3}H]Tyr (22.6 Ci/mmol) were purchased from NEN and [γ-^{32}P]dCTP (400 Ci/mmol) was from Amersham. pBluescript II (KS−) was purchased from Stratagene. Restriction endonucleases (*Xba*I, *Pst*I, and *Hin*dIII), T4 polynucleotide kinase, T4 DNA ligase, T7 RNA polymerase, and 7-Deaza-sequencing kit were purchased from Toyobo.

Genes and Plasmid Construction—The gene for the mRNA was prepared by ligation reaction using deoxyribo-oligomers and T4 DNA ligase. Each oligomer (0.2 OD), except for two 5′-terminal oligomers of the gene, was

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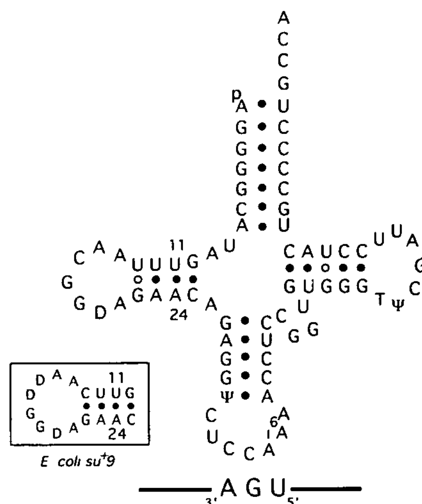

Fig. 1. **Secondary structure of *B. subtilis* tryptophan tRNA** (*2*). The nucleotide sequence data appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D10981. In the inset, the D-arm of *E. coli su*$^+$9 suppressor tRNA is shown and its wild type tRNA has G24 instead of A24.

phosphorylated by 2 mM ATP and T4 polynucleotide kinase (22 U) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 1 mM spermine. The reaction mixture was heated at 90°C for 5 min and all the phosphorylated oligomers and the two 5′-terminal oligomers were combined. The mixture was heated at 90°C for 3 min, and left to stand at room temperature for 10 min for annealing and then cooled to 15°C. This ligation reaction was done according to the supplier's recommendation. After the reaction, the product was purified on a low melting agarose gel (NuSieve GTG, 4%, EtBr stained) and the desired band was excised. The gel slice was melted at 70°C and the DNA was extracted. The 5′-terminus of the recovered gene was phosphorylated and used for the next ligation with the digested plasmid, which was prepared from pBluescript II (KS−) using *Xba*I and *Pst*I. After the ligation, the reaction mixture was used directly for transformation of *E. coli* JM109 [*recA1*, *Δ*(*lac-proAB*), *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *relA1*, *supE44*, (F′ *traD36*, *proAB*, *lacI*^{q}*Z* *Δ*M15)] according to Hanahan's method (*17*) and transformed cells were selected from the plate containing ampicillin, IPTG and X-gal. Plasmid DNA was prepared from transformants by the alkaline lysis method (*18*) and the sequence of the inserted DNA was confirmed by the dideoxy method (*19, 20*).

Preparation of Transcript—Prior to the transcription reaction, the plasmid was linearized by *Hin*dIII and then 2-10 μg of linearized plasmid was transcribed by T7 RNA polymerase (*18*). The reaction mixture was treated with DNase I at 37°C for 15 min and washed with chloroform-phenol and chloroform. The solution was transferred to a Centricon 10 (Amicon) to remove nucleotides and other low molecular weight substances. Chain length and purity of the transcripts were analyzed by 15% PAGE containing 7 M urea and transcripts were used without further purification.

In Vitro Translation Using B. subtilis S30 Fraction—The $tRNA^{Trp}$ (0-5 μl of 1 OD/ml for tRNA-dependent reaction) was aminoacylated using cold tryptophan (cold serine for $tRNA^{Ser}$) and the *B. subtilis* S30 fraction at 37°C for 15 min. The translation reaction was started by the addition of labeled amino acids (2.5 μl of 0.1 μCi/μl [^{14}C]Ile and 0.5 μl of 1 μCi/μl [^{3}H]Tyr) in 72 mM Tris/HCl (pH 7.5), 72 mM NH_4Cl, 12 mM $Mg(OAc)_2$, 0.6 mM MgK_2EDTA, 2 mM ATP, 0.3 mM GTP, 0.1 mM methionine, 4.8 mM DTT, 0.08 mM calcium folinate, 10 mM phosphoenolpyruvate, 2.5 U pyruvate kinase, 5 μl of S30 fraction, and synthetic mRNA (the RNA concentration was 12.5 OD/ml and 5 μl was used for the tRNA-dependent reaction) at 37°C for 20 min (total volume was adjusted to 55 μl) (*15*). A reaction without the mRNA was done as a control experiment and basal incorporation due to the endogenous tRNAs and mRNAs was subtracted from the data. After the translation, 30 μl of 1 M NaOH was added and the whole was gently mixed. The reaction mixtures were kept at 37°C for 15 min for deacylation. An aliquot sample of the total mixture (60 μl) was spotted on a Whatman 3MM filter disc, soaked with 10% trichloroacetic acid and gently shaken at 0°C for 10 min. The disc was washed twice with 10% trichloroacetic acid at 0°C and transferred to ethanol/diethyl ether (1 : 1 v/v) and diethyl ether, and then dried under a hood. The ^{14}C and ^{3}H radioactivities were measured in a liquid scintillation counter.

RESULTS

Plasmid Construction and Transcription—Each gene construct was designed to have the test codon just after an isoleucine codon repeat (Fig. 2) (*12*). After the test codon, a tyrosine codon repeats six times until a UAA double stop appears. The genes, Z-1, Z-2, Z-3, and Z-4 carry UGA, UGG, UAA, and UAG as the test codon, respectively. The genes were prepared by the ligation of oligodeoxyribonucleotides using T4 DNA ligase. The ligation products were analyzed by agarose gel electrophoresis and size of the construct was confirmed to be about 80 bp dsDNA. After phosphorylation, the gene was inserted downstream of a T7 promoter in linearized pBluescript II. Transformation was performed with competent cells derived from *E. coli* JM109 and the newly constructed plasmid as shown in Fig. 2. After cloning, we confirmed the plasmid sequence around the inserted gene by the dideoxy sequencing method. Next, the cloned plasmids, pZ-1, pZ-2, pZ-3, and pZ-4 were linearized by *Hin*dIII and transcribed by T7 RNA polymerase. The length of the transcript was confirmed on 15% PAGE containing 7 M urea; the mobility was consistent with that expected for a 130 bp fragment (data not shown).

Translation of the mRNA Using S30 Fraction—First, each mRNA was translated in the presence of [^{14}C]Ile and [^{3}H]Tyr, and their incorporations were measured according to the following procedures. (i) After the translation reaction, aminoacylated tRNA was hydrolyzed under an alkaline condition at 37°C. (ii) Acid-insoluble material was precipitated by adding trichloroacetic acid at 0°C and the radioactivities of [^{14}C]Ile and [^{3}H]Tyr trapped on the filter disc were measured simultaneously. All of the mRNAs showed effective and dose-dependent incorporation of [^{14}C]Ile (data not shown) and only the message with the UGG Trp as the test codon produced a high incorporation of [^{3}H]Tyr (Fig. 3). Thus, we concluded that the mRNAs functioned in this translation system as planned.

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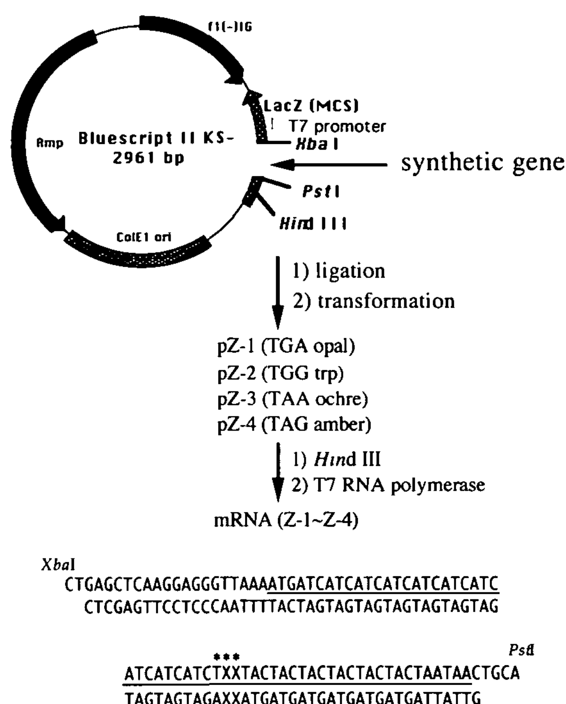

Fig. 2. **Construction of the vector for the synthetic mRNA.** pBluescript II (KS−) was digested by restriction endonucleases, *Xba*I and *Pst*I, and the gene coding for the message was inserted. In the gene sequence, TXX with asterisks represents TGA, TGG, TAA, or TAG for the test codon and the underlined sequence represents the coding region in the mRNA. For transcription reaction, the obtained plasmid was linearized by *Hind*III digestion.

It has been reported that the UGA codon inserted into an open reading frame in a *cat-86* mRNA was translated as tryptophan (*1*) and this implies that tryptophan tRNA plays a key role in the readthrough. We thought that if the tRNA^{Trp} really participates in the readthrough we would observe a correlation between the amount of the tRNA^{Trp} and the readthrough rate at the UGA codon. Thus, we first increased the amount of the Trp-tRNA^{Trp} while keeping the amount of mRNA constant. Figure 4A shows the case of the mRNA having the UGG tryptophan codon and the amount of the Trp-tRNA was raised from 0 to 4.5 pmol. We observed an increase of the [^{3}H]Tyr incorporation in a dose-dependent manner but beyond 1.5 pmol it reached a plateau level. This might imply that the relative concentrations of other tRNAs such as tRNA^{Ile} and/or tRNA^{Tyr} which participate in the peptide synthesis was lowered by the addition of the Trp-tRNA and thus the efficiency of the peptide synthesis was lowered as well. In Fig. 4, B-D, each solid line reveals the increment of the [^{3}H]Tyr incorporation when other mRNAs (containing UGA, UAA, or UAG test codon in Fig. 4, B, C, or D, respectively) were translated. Only the Z-1 mRNA with the UGA codon as a test codon showed a slight but significant increase of the readthrough against the addition of the Trp-tRNA^{Trp} (a solid line in Fig. 4B). On the other hand, UAG and UAA did not show such an increase (solid lines in Fig. 4, C and D). In order to confirm the reproducibility of this increment in the case of the UGA codon, we repeated the reaction and observed the same phenomenon. Consequently we concluded that the increase of the readthrough rate is significant only in the

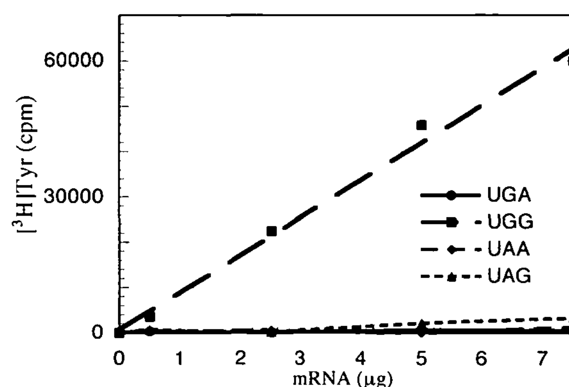

Fig. 3. **mRNA-dependent incorporation of [^{3}H]Tyr in cell-free translation system.** The different mRNAs are represented by symbols; circle represents the mRNA with the UGA (opal) test codon, square for UGG (Trp), diamond for UAA (ochre), and triangle for UAG (amber).

case of UGA. This indicates that this tRNA^{Trp} can promote the readthrough of the UGA codon.

To clarify whether this phenomenon is specific to the *B. subtilis* tRNA^{Trp} or not, we performed the same experiment using *B. subtilis* tRNA^{Ser}. This tRNA^{Ser} has mo^{5}UGA as an anticodon (*21*) and due to the middle letter G, this anticodon is not expected to match any of the test codons. In fact, the tRNA^{Ser} did not promote the incorporation of [^{3}H]Tyr in the presence of any of the stop codons (dotted lines in Fig. 4, B-D). Thus, this *B. subtilis* tRNA^{Trp} seemed to have a specific function for reading the UGA opal codon as well as the UGG tryptophan codon, and this result is consistent with the report that in *B. subtilis*, the UGA codon is read as tryptophan to a small extent *in vivo*.

DISCUSSION

In *B. subtilis*, no UGA suppressor tRNA has been identified so far and Lovett *et al.* reported that the UGA codon introduced in *cat-86* mRNA was translated as tryptophan to a small extent. This suggested that tryptophan tRNA is involved in the natural suppression. However, a single gene coding tryptophan tRNA has been identified and if it is responsible for the natural readthrough, then its unmodified CCA anticodon should read the UGA codon. In the translation system using the synthetic mRNA, the effect of the addition of the Trp-tRNA was studied. In Fig. 4A, the mRNA with the UGG tryptophan codon was translated and we observed the increased incorporation of [^{3}H]Tyr in the Trp-tRNA-dependent manner. However, beyond 1.5 pmol it gradually decreased rather than increased. We speculate that the relative concentrations of other tRNAs such as tRNA^{Met}, tRNA^{Ile}, and/or tRNA^{Tyr} which participate in the corresponding peptide synthesis were lowered by the addition of the Trp-tRNA and so efficiency of the peptide synthesis was lowered as well. On the other hand, in the UGA case, we observed an increase up to 4.5 pmol and the pattern of the increase was different from that in the UGG case. The reason for this might be that in the UGG case, the synthesized peptide amount is much larger than that of the UGA case, and thus the tRNAs other than the tryptophan tRNA function as a rate-limiting factor, but in the UGA

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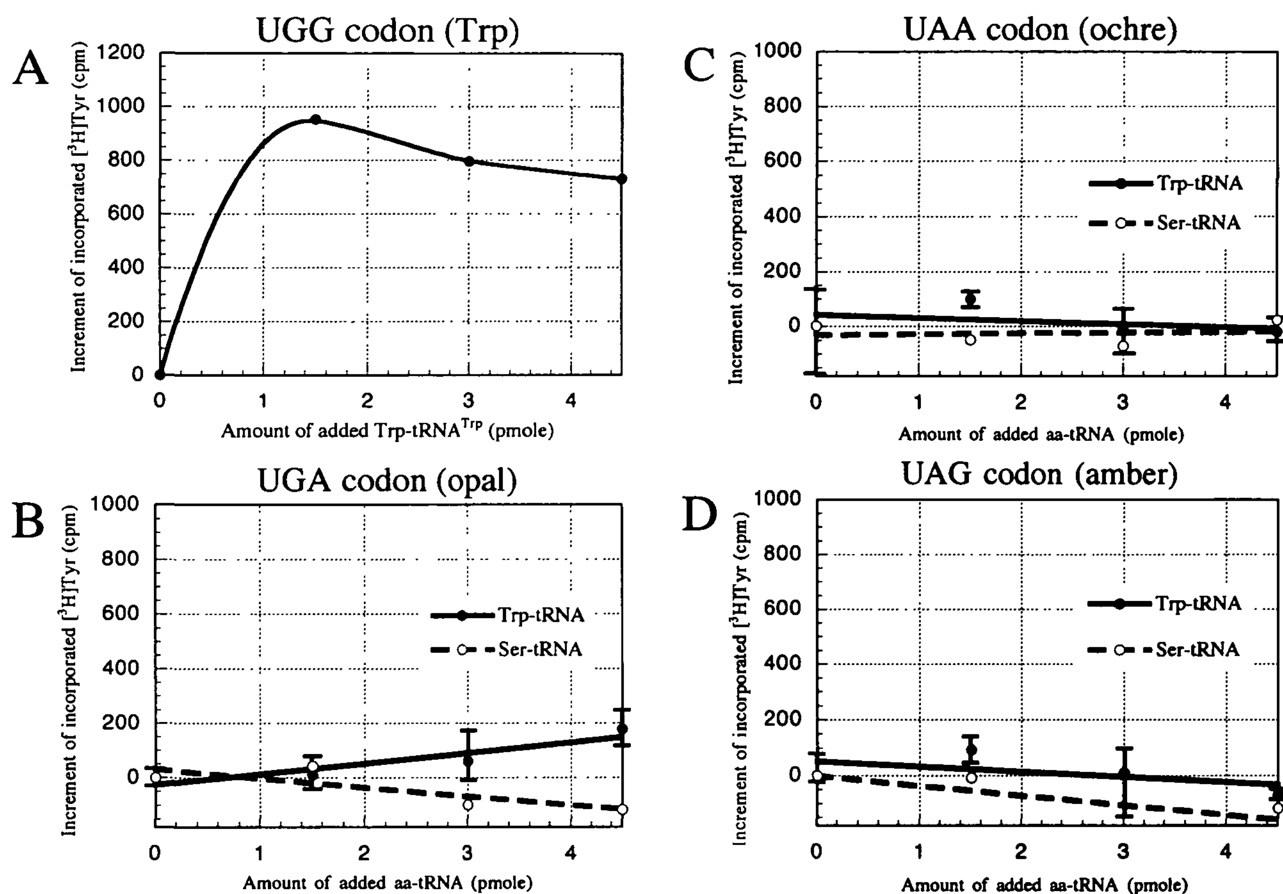

Fig. 4. **Aminoacyl-tRNA-dependent incorporation of [^{3}H]Tyr.** The left axis in each panel shows the increased [^{3}H]Tyr count after subtraction of the value in the control experiment (the mRNA was translated without the exogenous aminoacyl-tRNA). A: The mRNA containing UGG tryptophan codon as the test codon was translated. B-D: the mRNA containing UGA (panel B), UAA (panel C), or UAG (panel D) was translated with the Trp-tRNA^{Trp} (solid line with closed circle) or Ser-tRNA (dotted line with open circle). The data in panel B-D were tentatively fitted to a straight line using the software KaleidaGraph™ to clarify whether the incorporation is increasing or not. Error bars on the solid line in B, C, and D represent the dispersion of the cpm values in the repeated experiments.

case, the amount of the synthesized peptide is less. Consequently, tRNAs other than the tRNA^{Trp} were still abundant and the increase continued up to 4.5 pmol. As a result, only the UGA case showed an increase of the readthrough rate with increase of the Trp-tRNA amount. This increase in the UGA case does not tell us whether the readthrough mechanism depends on a two-out-of-three rule or on the formation of a C-A pair. However, we could, at least, make the following speculations. (i) The C-A wobble can be formed only when it is located in the first or third letter of the anticodon and it must be accompanied with two successive Watson-Crick base pairs, because the C-A wobble in the middle of the anticodon was prohibited, as shown in the case of UAG amber codon, even though it is sandwiched between two Watson-Crick pairs (Fig. 5). Our results do not rule out the possibility of the formation of the C-A pair in the third letter of the anticodon (*e.g.* CGG Arg codon with the CCA anticodon). But, if this is allowed, the genetic code would be too imprecise, and it seems most unlikely that such a mistranslation has a regulatory role. (ii) Two consecutive C-As might not form a stable pair. The lack of UAA codon readthrough by the tRNA^{Trp} suggests this instability. In a similar situation, Urban *et al.* have reported that UAG and UAA were not read by a plant natural suppressor tRNA^{Trp} with the CmCA (Cm=2′-*O*-methylcytidine) anticodon (*9*).

It is possible that the same tRNA^{Trp} species with a modification at the first cytidine of the anticodon, or a new tRNA^{Trp} species with an anticodon which forms a more stable interaction with the UGA codon exists, such as lysidine or 5-formylcytidine (*6, 10, 11*). However, we have to emphasize that no modification in the wobble position of the tRNA^{Trp} was detected in our study.

This kind of suppression is reminiscent of the suppression activity of *E. coli* tRNA^{Trp}. In *E. coli*, there is an opal suppressor tRNA (su^+9) with an unmodified cytidine in the first letter of the anticodon and its wild type tRNA^{Trp} also functions as a weak opal suppressor (*14*). The difference between the two *E. coli* tRNAs is a single nucleotide change from G24 (wt) to A24 and therefore it removes a G-U wobble pair from a wild type D-stem. As shown in Fig. 1, there is a sequence similarity between the D-stem of *B. subtilis* and *E. coli* tRNA^{Trp}. As for this structural similarity, Smith and Yarus suggested that the D-stem structure affects ribosome-tRNA interaction and consequently the rejection rate of a non-cognate tRNA at the stop codon is lowered (*22, 23*). As for the natural opal suppressor, the most striking point of the *B. subtilis* case is that no UGA suppressor has been obtained, and it may show a higher readthrough rate by tRNA^{Trp} than observed in *E. coli*. If so,

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a structural difference between the two $tRNA^{Trp}$s, especially around the D-arm, may influence the readthrough efficiency. But in order to obtain definitive evidence, a more systematic approach is necessary to relate the D-arm structure with the suppressor activity. In addition, an approach including the effects of the mRNA structure (*e.g.* the influence of context effect around the stop codon) may be necessary to study the biological function of this type of natural suppression (*9*).

It is well-known that in mitochondria and *Mycoplasma*, the UGA codon is assigned to tryptophan (*24, 25*). In *B. subtilis*, UGA is assigned to a stop signal, but if a high readthrough occurs, we have to consider its influence on the codon recognition. If the C-A wobble pair, which is formed between C in the anticodon side and A in the codon side, is permissive in the first letter of the anticodon, two possibilities arise. One is the case in which an isoleucine AUA is read as methionine by a $tRNA^{Met}$. In this case, a lethal amount of misincorporation may not occur since AUA is a rare codon and moreover, $tRNA^{Ile}$ having lysidine can decode it properly as isoleucine (*6*). Second is the case in which an amber suppressor with the CUA anticodon can read the ochre UAA codon. This also does not seem to be lethal to the normal cell. Thus, use of the C-A wobble pair will not seriously disrupt protein synthesis if the readthrough level at the UGA stop codon is not high. However, why does *B. subtilis* bring such leakiness to its own translation system? We speculate that UGA suppression could have some regulatory roles, because unnecessary readthrough would seem to be inefficient. An aspect of the *B. subtilis* a life cycle, such as sporulation, which *E. coli* does not have, might have some relation to the higher readthrough rate. In connection with the such a putative regulatory role, it is interesting that *Bacillus firmus* has a gene with an internal UGA stop signal in an ORF and its sequence resembles that of DNA topoisomerase (*26*). This could be a hint to the role of the natural suppression.

In addition to the readthrough by this $tRNA^{Trp}$, readthrough by selenocysteine tRNA is expected in *B. subtilis* because the $tRNA^{Sec}$ is believed to be widely distributed from bacteria to the animal kingdom (*27, 28*). Thus, we are also interested in the switching mechanism between the two kinds of readthrough at the UGA codon in *B. subtilis*.

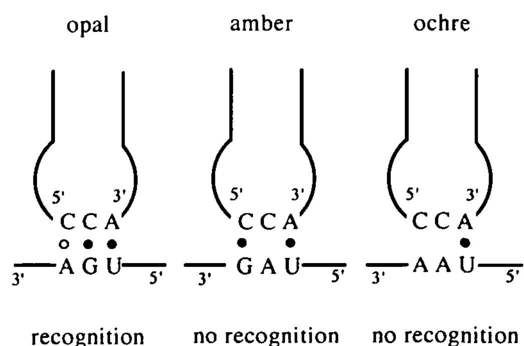

Fig. 5. **Possible anticodon-codon interactions when the C-A wobble is conjectured to be involved in the UGA readthrough.** Black and white circles represent a Watson-Crick and wobble base pair, respectively.

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