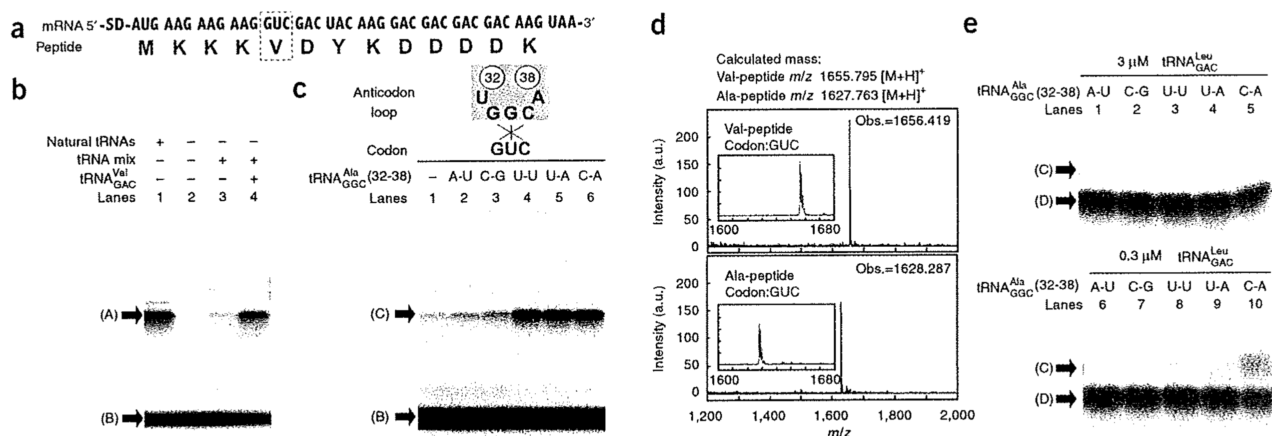

Figure 3 Influence of the sequence variation of the 32-38 pair in $tRNA^{Ala}_{GGC}$ on misreading of GUC codon. (**a**) Sequences of mRNA and peptide used in this study. The GUC (valine) codon was placed at the fifth position. (**b**) Tricine SDS-PAGE analysis of the peptide expressed in the presence of tRNA mix and the *in vitro* transcript of $tRNA^{Val}_{GAC}$ in the *w*PURE system. Other conditions were the same as **Figure 2b**. Arrows indicate Val-peptide (A) and [^{14}C]aspartate (B). (**c**) Tricine SDS-PAGE analysis of the peptide in the presence of tRNA mix and each $tRNA^{Ala}_{GGC}$ variant in the *w*PURE system. Arrows indicate Ala-peptide (C) (and Val-peptide in lane 1) and [^{14}C]aspartate (B). (**d**) MALDI-TOF analysis of the peptides expressed above. The Val-peptide (codon: GUC) was obtained from the expression sample in lane 4 in **Figure 3b** with aspartate instead of [^{14}C]aspartate. The Ala-peptide (codon: GUC) was obtained from the expression sample in lane 6 in **Figure 3c** with aspartate instead of [^{14}C]aspartate. Inset, expansion of the region between 1,600 and 1,680 *m/z* of the MS spectra. (**e**) Competitive decoding of the GUC codon by $tRNA^{Ala}_{GGC}$ variants and $tRNA^{Leu}_{GAC}$. In lanes 1–5, 3 μM $tRNA^{Leu}_{GAC}$ and 3 μM each $tRNA^{Ala}_{GGC}$ variant were used; in lanes 6–10, 0.3 μM $tRNA^{Leu}_{GAC}$ and 3 μM each $tRNA^{Ala}_{GGC}$ variant were used. Arrows indicate Ala-peptide (C), Leu-peptide (D).

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*w*PURE system was able to function like the ordinary PURE system[24] for the expression of a model peptide consisting of amino acids assigned by the above tRNAs, a 13-mer peptide, MKKK*ADYKDDDDK* (italicized residues indicate a Flag peptide sequence), was expressed from the corresponding mRNA (**Fig. 2a**) in the presence of wild-type $tRNA^{Ala}_{GGC}$ and [^{14}C]Asp in both systems. We determined the expression level of the peptide by the intensity of the radioactive band following tricine SDS-PAGE, showing that the *w*PURE system functioned like the ordinary PURE system for the expression of this peptide (**Fig. 2b**, lane 1 versus lane 4). Most importantly, the expression was $tRNA^{Ala}_{GGC}$ dependent (lanes 3 and 4). MALDI-TOF analysis of the peptide expressed in the *w*PURE system also confirmed the accuracy of expression (data not shown), indicating that correct reading of the GCC codon could be achieved by $tRNA^{Ala}_{GGC}$.

We then tested the $tRNA^{Ala}_{GGC}$ variants (**Fig. 1**) in the *w*PURE system for the decoding ability of the respective tRNAs to the GCC cognate codon. It should be noted that because *E. coli* alanyl-tRNA synthetase (AlaRS) does not recognize the anticodon loop[25–27], all the $tRNA^{Ala}_{GGC}$ variants were alanylated by AlaRS with virtually the same efficiency (**Supplementary Fig. 1** online). Thus, the observed translation efficiency is likely to reflect the intrinsic decoding ability of each $tRNA^{Ala}_{GGC}$ to the GCC codon. Unexpectedly, we observed no difference in incorporation efficiency (**Fig. 2c**).

To avoid exhausting the energy source of translation, we terminated the reaction described above after 15 min (**Supplementary Fig. 2** online); however, it was still possible that the difference in the decoding ability of each $tRNA^{Ala}_{GGC}$ was so small that the apparent translation efficiency was not sensitive enough to reflect to the actual value under such conditions. We therefore performed an additional experiment to rule out this possibility. Because *E. coli* leucinyl-tRNA synthetase (LeuRS) does not recognize the anticodon loop of $tRNA^{Leu}$ (refs. 28–30), LeuRS charged leucine on the engineered $tRNA^{Leu}$ carrying the anticodon loop sequence of *E. coli* wild-type $tRNA^{Ala}_{GGC}$ (**Supplementary Figs. 1** and **3** online). In fact, when we added $tRNA^{Leu}_{GGC}$ to the *w*PURE system instead of $tRNA^{Ala}_{GGC}$, translation of the same mRNA took place smoothly (**Fig. 2d**, lane 1). Notably, this leucine-containing peptide (Leu-peptide) appeared as a faster-migrating band than the alanine-containing peptide (Ala-peptide) band in tricine SDS-PAGE (**Fig. 2d**, lanes 1 and 2). MALDI-TOF analysis also revealed a molecular mass consistent with the Leu-peptide (data not shown), indicating that the single substitution of alanine to leucine in this peptide altered its migration properties. Thus, this feature allowed us to use tricine SDS-PAGE to conveniently assess the expression level of the individual peptides in competition assays between $tRNA^{Ala}_{GGC}$ and $tRNA^{Leu}_{GGC}$. We observed no appreciable difference in the intensities between the Ala- and Leu-peptides generated by any of $tRNA^{Ala}_{GGC}$ variants competing with $tRNA^{Leu}_{GGC}$ (lanes 3–7). These experiments clearly showed that the conserved and nonconserved $tRNA^{Ala}_{GGC}$ variants were able to decode the GCC cognate codon with similar efficiencies. We thus suspected that the evolutionary conservation of the 32-38 pair in $tRNA^{Ala}_{GGC}$ arose for a different reason(s).

The 32-38 pair controls misreading of GUC near-cognate codon

As sequence variation in the 32-38 pair did not affect decoding efficiency, we turned our investigation toward its decoding fidelity. The wobble pairing at the second G35 in $tRNA^{Ala}_{GGC}$ to a near-cognate valine codon, GUC, would be expected to alter the amino acid incorporation from valine to alanine. We therefore prepared another mRNA template based on the previously used mRNA in which the GCC codon was substituted with a GUC codon, and tested whether misreading by $tRNA^{Ala}_{GGC}$ would result in this substitution (**Fig. 3a**).

We first monitored the background incorporation of valine into the GUC codon in the *w*PURE system, which lacks the *in vitro* transcripts. In the absence of the tRNA mix, mRNA translation did not occur at all (**Fig. 3b**, lane 2); however, addition of the tRNA mix stimulated the expression of peptide (**Fig. 3b**, lane 3). Even though the isolated background-level peptide was present only in trace amounts, MALDI-TOF analysis revealed that it was consistent with the molecular mass

of the valine-containing peptide (Val-peptide) as a major peak (data not shown). This suggests that the background expression can be attributed to a trace amount of tRNA$^{Val}_{GAC}$ contaminating the *w*PURE system. On the other hand, addition of the *in vitro* transcript of tRNA$^{Val}_{GAC}$ to the *w*PURE system markedly elevated the expression level of peptide (**Fig. 3b**, lane 4).

We then tested whether alanine misincorporation at the GUC codon could be induced by addition of tRNA$^{Ala}_{GGC}$ variants to the *w*PURE system. The presence of wild-type or C32-G38 tRNA$^{Ala}_{GGC}$ slightly increased the background expression, presumably owing to misreading of the GUC codon resulting in alanine incorporation into the peptide chain (**Fig. 3c**, lanes 1–3). Unexpectedly, the presence of nonconserved tRNA$^{Ala}_{GGC}$ (U32-U38, U32-A38 and C32-A38) substantially increased the expression level (**Fig. 3c**, lanes 4–6, respectively). MALDI-TOF analysis of the isolated peptide showed a single major peak of molecular mass corresponding to the Ala-peptide (**Fig. 3d**). This result clearly shows that the background incorporation at the GUC codon by the contaminated tRNA$^{Val}_{GAC}$ was completely competed out by the nonconserved tRNA$^{Ala}_{GGC}$.

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Even though the nonconserved tRNA$^{Ala}_{GGC}$ misreads GUC effectively in the *w*PURE system, in *E. coli* the cognate tRNA$^{Val}_{GAC}$ coexists endogenously and thus competes out such a misreading event. Therefore, it was necessary to assess how effectively misreading occurred under the competitive conditions. Because the Val-peptide and the Ala-peptide had nearly the same migration pattern in tricine SDS-PAGE (**Fig. 3b,c**), it was difficult to quantitatively assess the competition. Instead, we engineered a tRNA^{Leu} containing the native anticodon loop sequence of *E. coli* tRNA$^{Val}_{GAC}$ (**Supplementary Fig. 3c**) and used it as a competitor against each tRNA$^{Ala}_{GGC}$ variant. As expected on the basis of previous experiments[28–30], LeuRS charged leucine onto the engineered tRNA$^{Leu}_{GAC}$ (**Supplementary Fig. 1**) and the resulting Leu-tRNA$^{Leu}_{GAC}$ decoded the mRNA GUC codon, yielding the Leu-peptide. Because the Leu-peptide migrated faster than the Ala-peptide in tricine-SDS-PAGE, we could readily visualize the degree of competition (**Fig. 3e**).

When we added an equal amount of each tRNA$^{Ala}_{GGC}$ variant and tRNA$^{Leu}_{GAC}$ to the *w*PURE system, only the Leu-peptide band was observed in all cases, suggesting that each Ala-tRNA$^{Ala}_{GGC}$ variant was completely competed out by Leu-tRNA$^{Leu}_{GAC}$ (**Fig. 3e**, lanes 1–5). However, when we reduced the concentration of the tRNA$^{Leu}_{GAC}$ to one-tenth that of tRNA$^{Ala}_{GGC}$, a faint but clearly visible Ala-peptide band appeared in the presence of the nonconserved tRNA$^{Ala}_{GGC}$ (**Fig. 3e**, lanes 6–10). Particularly, the frequency of misreading of GUC by Ala-tRNA$^{Ala}_{GGC}$ containing the C32-A38 pair reached approximately 30% (**Fig. 3e**, lane 10). This result clearly indicates that the 32-38 pair in tRNA$^{Ala}_{GGC}$ controls misreading of the near-cognate GUC codon.

Overexpression of the nonconserved tRNA$^{Ala}_{GGC}$ is toxic in *E. coli*

The above *in vitro* experiments clearly demonstrated that the nonconserved tRNA$^{Ala}_{GGC}$ misreads the near-cognate GUC codon involving the G35·U wobble pair. We wondered whether this misreading event could occur *in vivo*, so that the nonconserved tRNA$^{Ala}_{GGC}$ acts as a toxigenic tRNA. We transformed *E. coli* BL21 cells with a vector that could overexpress each conserved or nonconserved tRNA$^{Ala}_{GGC}$ variant under the control of an arabinose promoter (**Supplementary Fig. 4** online). The tranformed cells were grown individually on either 0.2% (w/v) glucose (negative control) or 0.2% (w/v) arabinose on LB agar plates at 42 °C. Before induction of tRNA expression, all cells appeared as healthy as the untransformed control cells (**Fig. 4a**). Upon induction, cells expressing the conserved tRNA$^{Ala}_{GGC}$ showed no change in growth, whereas those expressing the nonconserved tRNA$^{Ala}_{GGC}$ became

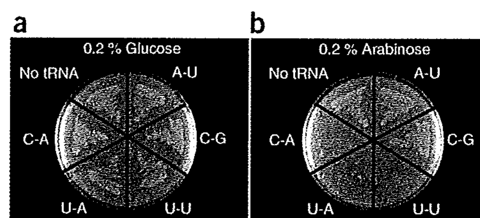

Figure 4 Overexpression of the conserved or nonconserved tRNA$^{Ala}_{GGC}$ in *E. coli* (BL21). Each tRNA$^{Ala}_{GGC}$ variant was cloned under the control of the arabinose promoter. LB plates contained 100 μg ml^{-1} ampicillin in the presence of 0.2% (w/v) glucose (**a**) or 0.2% (w/v) arabinose (**b**) and were incubated at 42 °C overnight.

unhealthy (**Fig. 4b**). Particularly, those expressing the nonconserved tRNA$^{Ala}_{GGC}$ with U32-A38 or C32-A38 were unable to grow. These U32-A38 and C32-A38 pairs were never found in the tRNA$^{Ala}_{GGC}$ sequence database, indicating that the sequence bias of the 32-38 pair in tRNA$^{Ala}_{GGC}$ probably appeared to avoid formation of toxigenic tRNAs *in vivo*.

It should be noted that at 37 °C most cells appeared to be healthy, with the exception of those cells expressing the nonconserved tRNA$^{Ala}_{GGC}$ with C32-A38, which grew slightly more slowly (data not shown). This temperature sensitivity may suggest that the frequency of the misreading of the GUC codon by tRNA$^{Ala}_{GGC}$ with the nonconserved C32-A38 pair is not marked because the codon is predominantly read correctly by the cognate tRNA$^{Val}_{GAC}$. However, in some proteins the resulting valine to alanine substitution would cause them to be less stable, resulting in loss of function at 42 °C. This probably led to the observed temperature-dependent cell growth. Nonetheless, our demonstration clearly shows that the nonconserved tRNA$^{Ala}_{GGC}$ is toxic *in vivo* and is therefore not conserved in the repertoire of functional tRNAs.

DISCUSSION

Here we provide *in vitro* evidence that the nonconserved tRNA$^{Ala}_{GGC}$ (**Fig. 1**) misreads its near-cognate valine codon, GUC, resulting in misincorporation of alanine into the valine site of the peptide chain (**Fig. 3**). In contrast, misreading of this codon by the conserved tRNA$^{Ala}_{GGC}$ (**Fig. 1**) is minimal and thus is readily competed out by the cognate tRNA$^{Leu}_{GAC}$ (**Fig. 3**). This observation is also valid *in vivo*, where overexpression of the nonconserved tRNA$^{Ala}_{GGC}$ is toxic, whereas that of the conserved tRNA$^{Ala}_{GGC}$ is not (**Fig. 4**). These results imply that the reason for the evolutionary force selecting the 32-38 pair in tRNA$^{Ala}_{GGC}$ is to secure the decoding fidelity.

Fidelity of aa-tRNA selection in the ribosome relies on two mechanistic steps, so-called initial selection and proofreading, which occur before and after GTP hydrolysis, respectively[31,32]. In the initial selection step, incorrect tRNA is rejected by rapid dissociation of the ternary complex of aa-tRNA–EF-Tu–GTP from the A site and the sluggish rate of GTP hydrolysis[33,34]. Even though GTP hydrolysis occasionally occurs for the incorrect aa-tRNA, in the next proofreading step the slow accommodation rate of the incorrect aa-tRNA to the peptidyl-transferase center results in its rejection, and therefore incorrect reading of the noncognate codon is avoided[34]. It is likely that the sequence variation of 32-38 pair in tRNA$^{Ala}_{GGC}$ also influences either or both steps of aa-tRNA selection. It was reported that the nonconserved tRNA$^{Ala}_{GGC}$(U32-U38 or U32-A38) binding to the cognate GCC codon has a slower dissociation rate from the A site than the conserved tRNA$^{Ala}_{GGC}$(A32-U38 or C32-G38)[22]. Therefore, an

explanation for the increase in the frequency of misreading of GUC by such nonconserved $tRNA^{Ala}_{GGC}$ is also due to their slow dissociation rate from the ribosome. Recently, various kinetics measurements were performed for misreading of near-cognate codons, including GUC, by the conserved $tRNA^{Ala}_{GGC}$(A32-U38, C32-G38) and nonconserved $tRNA^{Ala}_{GGC}$(U32-A38 or C32-A38)[35]. The apparent rate of peptide bond formation in misreading of the GUC codon by the two nonconserved $tRNA^{Ala}_{GGC}$(U32-A38 or C32-A38) is elevated to the level of that which occurs during reading of the cognate GCC codon. Clearly, this result is consistent with our finding that the nonconserved $tRNA^{Ala}_{GGC}$ tends to misread the near-cognate GUC codon.

Structures of the anticodon loop with various 32-38 pairs have been modeled *in silico* based on the available crystal structures[36]. The U32-A38 and C32-A38 pairs, belonging to the largest structural family I, form noncanonical structures involving bifurcated hydrogen bonds. In contrast, the U32-U38 pair, categorized in family II, forms a single, noncanonical hydrogen bond. Structures for the A32-U38 and C32-G38 pairs, in family III, cannot yet be predicted because of insufficient available structural information. It should be noted that families I and II combined constitute about 93% of bacterial tRNAs[36], implying that these base pairs evolved to maximize the decoding ability of tRNAs on the ribosome. In the present study, we have shown that, paradoxically, the family I $tRNA^{Ala}_{GGC}$ with U32-A38 or C32-A38 and the family II $tRNA^{Ala}_{GGC}$ with U32-U38 misread GUC codon. Consequently, the rare family III pairs, A32-U38 and C32-G38, are found in the naturally occurring $tRNA^{Ala}_{GGC}$. This suggests that the decoding fidelity of $tRNA^{Ala}_{GGC}$ is tuned by selecting uncommon 32-38 pairs during the evolution. Presumably, similar unique sequence biases that tune decoding fidelity can be found in many regions of the tRNA body sequence[37]. More extensive sequence analyses of tRNAs and biochemical studies on such evolutionarily biased variants will be important to reveal the mechanism of decoding fidelity in translation.

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METHODS

Materials. We prepared all of the tRNAs by *in vitro* run-off transcription using T7 RNA polymerase[38], and the DNA templates of mRNAs (5′-CGAAG CTAAT ACGAC TCACT ATAGG GCTTT AATAA GGAGA AAAAC ATGAA GAAGA AGNNN GACTA CAAGG ACGAC GACGA CAAGT AAGCT TCG -3′, where NNN indicates GCC or GUC, and the underlined sequence encodes the T7 promoter) by PCR using *Taq* DNA polymerase (**Supplementary Methods** online).

Translation. We performed batch translation using the PURE system without the tRNA mixture (*w*PURE system) according to described protocols[39–42]. The translation mixture contained 50 mM HEPES-K^+, pH 7.6, 20 mM creatine phosphate, 100 mM potassium glutamate, 14 mM magnesium acetate, 2 mM EDTA, 2 mM spermidine, 1 mM DTT, 2 mM ATP, 2 mM GTP, 1 mM UTP, 1 mM CTP and 10 μM 10-formyl-5,6,7,8-tetrahydrofolic acid. The translation was carried out with 0.02 μM DNA template of mRNA and a 200 μM concentration of 19 kinds of proteinogenic amino acids without aspartate and 50 μM [^{14}C]Asp. Natural tRNA extract (1.5 mg ml^{-1} at final concentration, Roche) was added in the control experiment. *In vitro* transcripts of $tRNA^{fMet}$, $tRNA^{Tyr}$, $tRNA^{Asp}$ (5 μM each tRNA at final concentration) and $tRNA^{Lys}$ (40 μM at final concentration) were added instead of natural tRNA extract in other all experiments. The concentrations of $tRNA^{Ala}_{GGC}$ variants, $tRNA^{Val}_{GAC}$, the engineered $tRNA^{Leu}_{GGC}$ and $tRNA^{Leu}_{GAC}$ are described in the figures. The reaction was carried out in a total volume of 2 μl at 37 °C for 15 min and the products were analyzed by tricine SDS-PAGE.

Mass spectroscopy measurements of peptides. For MS analysis, we performed the reactions (5 μl) with a 200 μM concentration of 20 proteinogenic amino acids. The products were precipitated with 50 μl of acetone, dissolved in 2.5 μl of water and then immobilized with 2.5 μl of Flag–M2 agarose (Sigma). After the resin was washed twice with 50 μl of W buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl), the immobilized peptides were eluted with 2.5 μl of 0.2% (v/v) trifluoroacetic acid (TFA), desalted with Zip tips C18 (Millipore) and eluted with 1.5 μl of a 50% (v/v) acetonitrile, 0.1% (v/v) TFA solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid. Mass measurements were performed using MALDI-TOF (Autoflex, Bruker) in the positive mode and externally calibrated with Substance P (average 1,348.66 Da), Bombesin (average 1,620.88 Da), ACTH clip 1–17 (average 2,094.46 Da) and Somatostatin 28 (average 3,149.61 Da) as standards.

Construction of plasmids. The DNA fragment was amplified by *Pyrobest* DNA polymerase (Takara) from pUC18 using primers (pUCHin.F33, 5′-GCAAG CTTGC TCTTC CGCTT CCTCG CTCAC TGA-3′, and pUCNotPst.R44, 5′-CCGCT GCAGA CGCGG CCGCG CCTGA TGCGG TATTT TCTCC TTAC-3′) and the product was digested with PstI and HindIII. The annealed DNA fragment (5′-GATCC TTAGC GAAAG CTAAG GATTT TTTTT A-3′ and 5′-AGCTT AAAAA AAATC CTTAG CTTTC GCTAA GGATC TGCA-3′) containing rrnC terminator was cloned in the PstI–HindIII site of the product DNA. The resulting plasmid was named pMUC. The DNA region that contains the *araC* gene and the P_{BAD} promoter of pBAD–GFPuv (BioRad) was amplified by PCR using primers (araNot.F35, 5′-ACGCG GCCGC GCATA ATGTG CCTGT CAAAT GGACG-3′, and araEcoPst.R43, 5′-CCGCT GCAGC AGAAT TCCCA AAAAA ACGGG TATGG AGAAA CAG-3′). After NotI-PstI digestion, the fragment was cloned into the NotI-PstI site of pMUC. The resulting plasmid was named pMUCA. Template DNA of $tRNA^{Ala}_{GGC}$ variants were amplified using primers (EcoT7.F26, 5′-GCGAA TTCTA ATACG ACTCA CTATA G-3′, and AlaPst.R35, 5′-GCGCT GCAGT GTTAT TGGTG GAGCT AAGCG GGATC-3′) from the corresponding PCR products described above and digested with EcoRI and PstI, and then cloned into EcoRI-PstI site in pMUCA. We confirmed the sequence between NotI-HindIII site by sequence analysis.

Overexpression of the $tRNA^{Ala}_{GGC}$ variant in *E. coli*. The plasmids were transformed into BL21 (Invitrogen) and spread on LB agar plates containing 100 mg ml^{-1} ampicillin and 4% (w/v) glucose. The plates were incubated at 37 °C overnight and the colonies were cultivated in LB medium containing 100 μg ml^{-1} ampicillin and 4% (w/v) glucose at 37 °C overnight. The cultures were diluted by 10× volume of LB medium and streaked on LB agar plates containing 100 μg ml^{-1} amplicillin and 0.2% (w/v) glucose or 0.2% (w/v) arabinose. The plates were incubated at 42 °C overnight.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

This study was designed by H.M., A.O. and H.S.; all of the experiments were performed by H.M.; the paper was written by H.M. and H.S.

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Messenger RNA-Programmed Incorporation of Multiple N-Methyl-Amino Acids into Linear and Cyclic Peptides

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SUMMARY

Natural peptide products often contain N-methylated backbones, and such a modification plays a crucial role in making natural peptides peptidase resistant and membrane permeable. Here, we demonstrate the ribosomal synthesis of N-methyl-peptides by means of genetic code reprogramming. Two key technologies, a ribozyme-based de novo tRNA acylation (flexizyme) system and an *E. coli* reconstituted cell-free translation (PURE) system, were used in order to reassign arbitrarily chosen codons to N^{α}-methylated amino acids (^{Me}aa). Using this combination, we determined the general structural requirement of "accessible" ^{Me}aa and demonstrated their multiple incorporations into the nascent peptide chain according to the assignments made on mRNA, giving linear and cyclic N-methyl-peptides in high purities. This platform technology offers a convenient tool for the construction of N-methyl-peptide libraries, potentially leading to the discovery of therapeutic peptides.

INTRODUCTION

Natural peptide products isolated from various organisms often contain N-methylated backbones (Billich and Zocher, 1990; Hornbogen and Zocher, 2005). Such a modification of peptide backbone alters the properties of the peptide bond, which confers their conformational rigidity (Sagan et al., 2004). This modification contributes to improvements in the biological properties of natural peptides, such as target affinity, proteolytic stability, and/or membrane permeability. Thus, N^{α}-methylated amino acids (^{Me}aa) are invaluable components for the synthesis of peptide libraries in screening for peptides with suitable drug-like properties for potential therapeutic use. The backbone N-methylation of these peptides are generally executed by one or more of enzymes in the multienzyme clusters, called nonribosomal peptide synthetases (NRPSs) (Billich and Zocher, 1990; Hornbogen and Zocher, 2005; Sieber and Marahiel, 2005; Walsh et al., 2001). This type of peptide synthesis machinery is known to be template independent, in contrast to the mRNA template-dependent ribosomal machinery. Unfortunately, their complexity demands an enormous effort to manipulate the systems, thereby making it difficult to generate desired peptide libraries (Baltz, 2006; Fischbach and Walsh, 2006; Hahn and Stachelhaus, 2006).

On the other hand, the translation machinery expresses peptides in an mRNA template-dependent manner, which makes this system exceptionally versatile and useful for the synthesis of peptides or proteins. Unlike NRPSs, the ordinary translation system strictly incorporates 20 proteinogenic amino acids into the nascent peptide chain. However, an appropriate manipulation of the translation apparatus enables us to incorporate nonproteinogenic amino acids into peptides (Hendrickson et al., 2004; Hohsaka and Sisido, 2002; Link et al., 2003; Wang and Schultz, 2004). A classical example is that when a nonproteinogenic amino acid is charged onto an orthogonal $tRNA_{CUA}$ (the subscript base sequence indicates the anticodon), this aminoacyl-$tRNA_{CUA}$ (aa-$tRNA_{CUA}$) is able to suppress UAG amber stop codon on mRNA; thereby, the amino acid can be incorporated into the nascent peptide at the designated site (Bain et al., 1989; Noren et al., 1989). Despite encouraging results from a number of successful examples for the incorporation of nonproteinogenic amino acids with various nonnatural side chains, it has been known that some ^{Me}aa are incorporated into a peptide chain with good or modest efficiencies, and some are not at all (Bain et al., 1991; Chung et al., 1993; Ellman et al., 1992; Gilmore et al., 1999; Karginov et al., 1997; Mendel et al., 1995; Murakami et al., 2006; Short et al., 2000). To the best of our knowledge in the literature, only three ^{Me}aa, ^{Me}Gly, ^{Me}Ala, and ^{Me}Phe, have been successfully incorporated into the nascent peptide chain by means of amber suppression. Moreover, neither incorporation of multiple ^{Me}aa nor a single ^{Me}aa with amino acids bearing noncanonical side chains has been thus far reported.

More recently, a new concept of genetic code reprogramming was introduced by Forster et al. and applied to the incorporation of nonproteinogenic amino acids into peptides (Forster et al., 2003). Genetic code reprogramming involves the reassignment of codons from proteinogenic amino acids to nonproteinogenic ones via multiple sense suppressions. Thus, this methodology enables us to simultaneously incorporate multiple nonproteinogenic amino acids into peptides, which represents a major advantage over the aforementioned amber suppression method (Forster et al., 2003; Josephson et al., 2005; Murakami et al., 2006; Ohta et al., 2007; Ohuchi et al., 2007; Tan et al., 2005).

In the context of ^{Me}aa using the sense suppressions, there were three examples in the literature (Frankel et al., 2003; Merryman and Green, 2004; Tan et al., 2004). Merryman and Green have reported that aa-tRNAs prepared by cognate aminoactyl-tRNA

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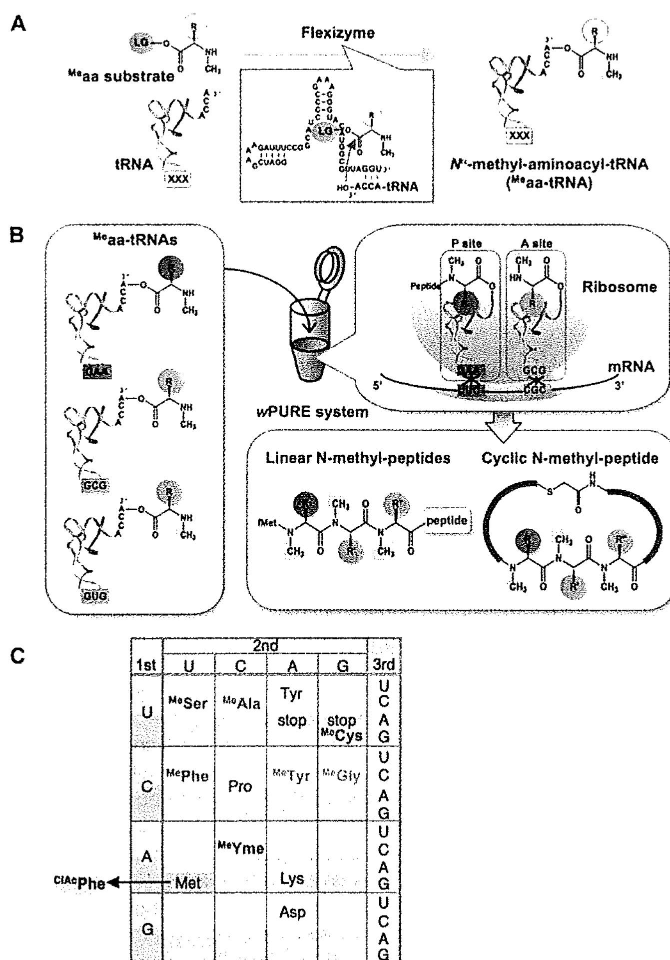

Figure 1. Messenger RNA-Programmed Synthesis of N-Methyl-Peptides by Genetic Code Reprogramming

(A) Synthesis of N^{α}-methyl-aminoacyl-tRNA (^{Me}aa-tRNA) by flexizyme system. Flexizyme recognizes leaving group (LG: highlighted in pink) on the ester bond of N^{α}-methyl-amino acid (^{Me}aa) substrate and conserved three bases on the 3′-terminus of tRNA, which allows for the aminoacylation of desired tRNA with any ^{Me}aa. N^{α}-methyl groups are highlighted in yellow.

(B) Ribosomal synthesis of N-methyl-peptide. ^{Me}aa-tRNAs bearing various combinations of ^{Me}aa and anticodon are added to the *w*PURE (*withdrawn* protein synthesis using recombinant elements) system for mRNA-programmed incorporation of ^{Me}aa into peptides. The *w*PURE system is an *E. coli* reconstituted cell-free translation system in which some components (amino acids and aminoacyl-tRNA synthases) are withdrawn from the ordinary translation system to reassign multiple sense codons to various ^{Me}aa.

(C) Reprogrammed genetic code table for the mRNA-programmed synthesis of N-methyl-peptides. Codons that are reassigned to various ^{Me}aa are shown in color letters. Initiation codon (AUG) highlighted in gray is reassigned from Met to N^{α}-(α-chloroacetyl)-Phe (^{ClAc}Phe) for the ribosomal synthesis of cyclic N-methyl-peptides.

synthetases (aaRSs) were converted to ^{Me}aa-tRNAs by the three-step procedure, where the α-amino group was alkylated by consecutive reductive amination with 2-nitrobenzaldehyde and formaldehyde, and then the 2-nitrobenzyl group was deprotected by UV irradiation (Merryman and Green, 2004). These ^{Me}aa-tRNAs derived from 20 proteinogenic amino acids were surveyed for the synthesis of a dipeptide, fMet-^{Me}aa, where thin-layer chromatographic electrophoresis was mainly used to discern the product of fMet-^{Me}aa from fMet (also possibly fMet-aa) and to determine the incorporation efficiency of each ^{Me}aa in a semiquantitative manner. In the second example, Tan and Cornish et al. prepared ^{Me}Ala-tRNA$^{AsnB}{}_{GAC}$ and ^{Me}Phe-tRNA$^{AsnB}{}_{GAC}$ by the chemoenzymatic aminoacylation procedure (Hecht et al., 1978; Robertson et al., 1991) and performed their single incorporation into a tripeptide (fMet-Xaa-Glu, where Xaa represents ^{Me}aa) upon the sense suppression of the Val codon (GUU) (Tan et al., 2004). Significantly, this work determined the incorporation efficiencies of these two ^{Me}aa into the peptide chain in a quantitative manner and also confirmed the product peptide by liquid chromatography based on its retention time by comparison with that of the corresponding synthetic authentic sample. Both examples above showed only a single incorporation of ^{Me}aa into a di- or tripeptide backbone, and therefore the concept of genetic code reprogramming has not yet been fully explored for the synthesis of N-methyl-peptides.

In the third example, Frankel and Roberts et al. have shown polymerization of ^{Me}Phe assigned to in repetition of two, five, or ten residues in an in vitro display system (Frankel et al., 2003). This work represents, to our knowledge, the first demonstration of consecutive incorporations of a single type of ^{Me}aa into a peptide stretch by using sense suppression. However, because the polymerization of ^{Me}Phe was only evidenced by observing the protease resistance of the respective peptide, it still remains unknown to what degree of the contamination of natural amino acids in the poly-^{Me}Phe chain occurred. In fact, the full-length peptide bearing multiple ^{Me}Phe was susceptible to protease to some extent, suggesting that competing incorporations of likely Val or possibly other proteinogenic amino acids occurred as Frankel et al. discussed in their report (Frankel et al., 2003). Taken together, although the above three examples clearly documented that the sense-suppression method could be used for the incorporation of ^{Me}aa, the proof-of-concept study on the genetic code reprogramming, i.e., performing multiple incorporations of two or more different ^{Me}aa with high fidelity control, remains to be demonstrated.

We report here incorporation of multiple ^{Me}aa into the peptide backbone with a nearly perfect control of sequences and lengths by using the concept of genetic code reprogramming. To reprogram the genetic code, we used two technologies, flexizyme and PURE (protein synthesis using recombinant elements) systems. Flexizyme system is a ribozyme-based de novo tRNA acylation system that is able to charge virtually any amino acids onto desired tRNAs with any body and anticodon sequences (Figure 1A) (Kourouklis et al., 2005; Murakami et al., 2003a, 2003b, 2006; Ohuchi et al., 2007; Saito et al., 2001). PURE system is a

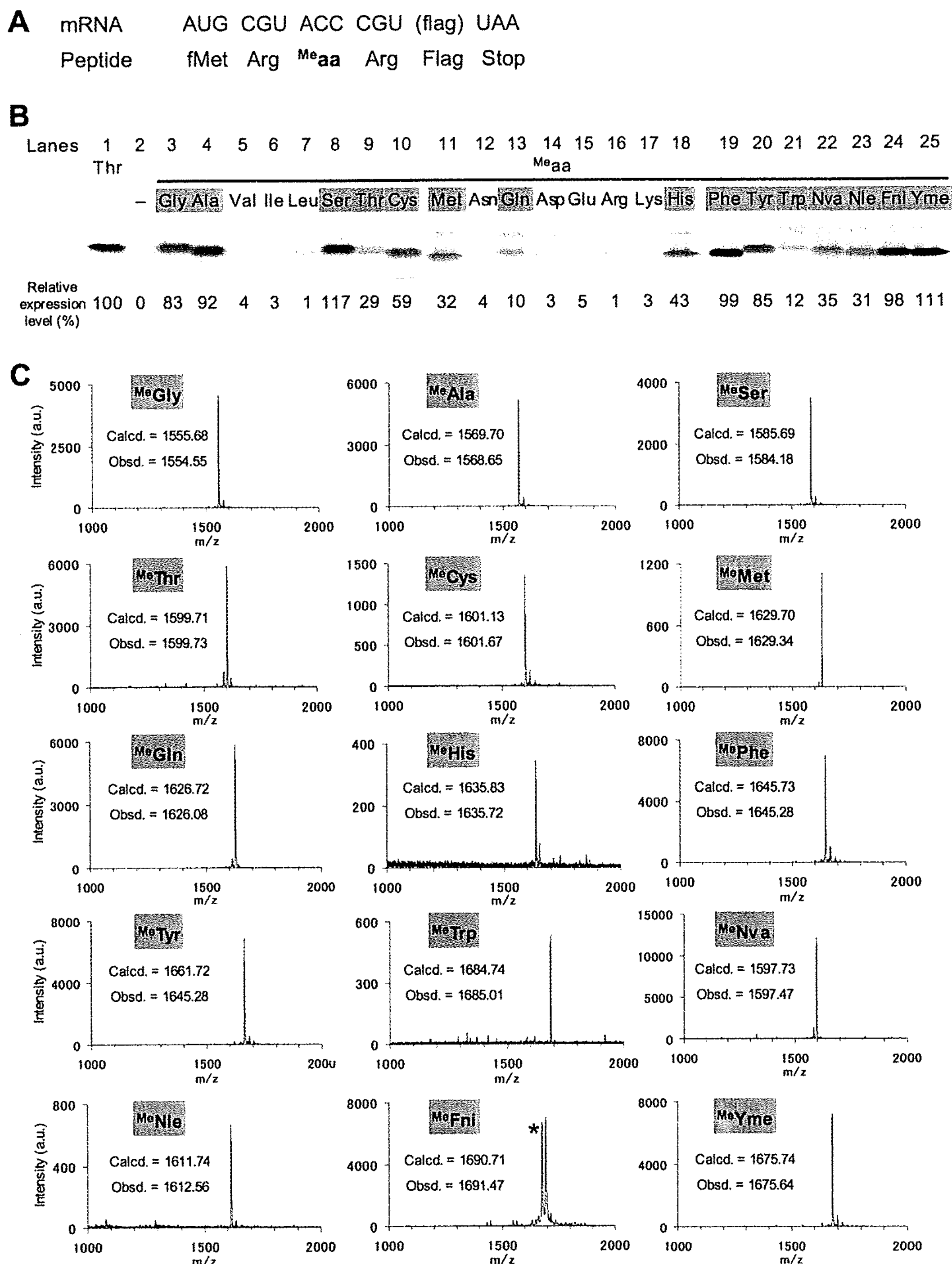

Figure 2. Single Incorporations of N^{α}-Methylated Amino Acids

(A) Sequences of mRNA and peptide. Flag in parentheses indicates the RNA sequence coding the Flag peptide (DYKDDDDK).

(B) Tricine-SDS-PAGE analysis of the expressed peptides labeled with [^{14}C]-Asp detected by autoradiography. Lane 1, the wild-type peptide expressed in the ordinary PURE system where ACC assigns Thr; lane 2, a negative control using *w*PURE system in the presence of uncharged $tRNA^{Asn-E1}_{GGU}$; lanes 3–25, expression of the peptide containing a single ^{Me}aa in the presence of designated $^{Me}aa\text{-}tRNA^{Asn-E1}_{GGU}$ prepared by the flexizyme system. Each expression level relative to wild-type was determined by a mean score of triplicates. The amino acids giving over 30% and 10% expression levels relative to the wild-type are highlighted in cyan and orange, respectively.

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reconstituted *Escherichia coli* cell-free translation system in which transcription and translation are coupled (Shimizu et al., 2001). The most important feature of this translation apparatus is that some components, such as amino acids and aminoacyl-tRNA synthases (aaRSs), can be withdrawn from the translation elements (Figure 1B). By means of such a *withdrawn* PURE system, named *w*PURE, we are able to vacant certain codons at our will. Combining these two systems enables us to readily reassign the vacant codons to ^{Me}aa and express peptides as designed on mRNA sequences according to the newly designated genetic table (Figure 1C).

In this report, we initially screened N^{α}-methylated proteinogenic and nonproteinogenic amino acids to evaluate the efficiency of their single incorporation into a peptide backbone. Then, we investigated the mRNA-programmed synthesis of peptides containing multiple ^{Me}aa, achieving the compositions of continuous stretches of up to ten ^{Me}aa residues. Finally, we investigated the synthesis of cyclic N-methyl-peptides closed by a physiologically stable thioether bond, showing its potential for the generation of in vivo compatible peptide libraries therapeutically targeting various proteins.

RESULTS

Single Incorporations of N^{α}-Methylated Amino Acids

To investigate the incorporation of multiple ^{Me}aa into a peptide backbone, various N^{α}-methylated proteinogenic and nonproteinogenic amino acids charged onto a tRNA by the flexizyme system were comprehensively evaluated for the efficiency of their single incorporation into a peptide backbone. As was discussed earlier, Merryman and Green performed a similar experiment with a dipeptide synthesis format (Merryman and Green, 2004). In contrast, we here incorporated ^{Me}aa into a 12-mer model peptide, thus involving the incorporation of ^{Me}aa into a specific site followed by elongation and termination of peptide synthesis as a format of normal peptide synthesis (Figure 2A, more details are discussed below).

For this study, we chose 23 ^{Me}aa substrates, 19 of which were derived from proteinogenic amino acids, and 4 of which were derived from nonproteinogenic amino acids (Figure S1, see the Supplemental Data available with this article online). The flexizyme-catalyzed aminoacylation efficiency for each ^{Me}aa was verified by our standard protocol (Figure S2), showing that the majority of ^{Me}aa could be charged onto tRNA with over 30% yields. Three amino acids, ^{Me}Val, ^{Me}Ile, and ^{Me}Asn, showed less than 30% yields. In the former two amino acids, their steric hindrance resulted from the combination of the β-branched side chain and the N^{α}-methyl-amino group presumably made these particular substrates much less reactive to flexizyme compared with other ^{Me}aa. In the case of ^{Me}Asn, based on our past experience working on Asn-tRNA, its tRNA-adduct might be labile due to the intramolecular attack of the amide side chain (Lee and Suga, 2001; Murakami et al., 2006); thereby the yield might appear to be low in PAGE analysis. However, we have previously observed that such aa-tRNAs prepared in similar ranges of quality (Val-, Ile-, and Asn-tRNAs in 13%–30% yield) could still be used for the incorporation into the nascent peptide chain with satisfactory yields (30%–70%) (Murakami et al., 2006). Thus, we pursued to survey all ^{Me}aa-tRNAs for the single incorporation into the model peptide.

The *w*PURE system used in our survey was composed of a limited member of amino acids and aaRSs. We designed an open-reading frame (ORF) in mRNA (Figure 2A) that expresses a short peptide consisting of fMet-Arg-^{Me}aa-Arg followed by a Flag peptide (DYKDDDDK; D, Asp; Y, Tyr; K, Lys). We selected a Thr codon (ACC) to assign ^{Me}aa and an orthogonal $tRNA^{Asn\text{-}E1}{}_{GGU}$ (Ohta et al., 2007) for its suppression. When the model peptide was expressed in the presence of uncharged $tRNA^{Asn\text{-}E1}{}_{GGU}$ in *w*PURE system, no product band appeared on tricine-SDS-PAGE (Figure 2B, lane 2), suggesting that $tRNA^{Asn\text{-}E1}{}_{GGU}$ could act as an orthogonal tRNA inert against the endogenous aaRSs added to the *w*PURE system.

We next tested the single incorporation of ^{Me}aa charged onto $tRNA^{Asn\text{-}E1}{}_{GGU}$ into the model peptide sequence. Eight out of 19 ^{Me}aa derived from proteinogenic amino acids were incorporated into the peptide with more than 30% efficiencies relative to wild-type expression (Figure 2B, lanes 1 versus those highlighted in cyan). The incorporation of the designated ^{Me}aa was also confirmed by MALDI-TOF analysis, giving the expected molecular mass as a sole peak in all cases (Figure 2C). Moreover, ^{Me}Thr, ^{Me}Gln, and ^{Me}Trp were modestly incorporated yet gave the single desired product (Figures 2B and 2C, those highlighted in orange). We called these ^{Me}aa molecules that could be incorporated into the peptide chain with more than 10% efficiencies as "accessible" ^{Me}aa. Interestingly, all of these "accessible" ^{Me}aa shared similar structural features of their side chains; either aromatic side chains (^{Me}Phe, ^{Me}Tyr, ^{Me}His, and ^{Me}Trp) or non-charged and nonbulky side chains (^{Me}Gly, ^{Me}Ala, ^{Me}Ser, ^{Me}Cys, ^{Me}Met, ^{Me}Thr, and ^{Me}Gln).

It should be noted that our results were mostly consistent with Merryman's observations, with some contradictions (Merryman and Green, 2004). In their experiments, ^{Me}Val, ^{Me}Ile, and ^{Me}Leu were ranked in the "efficient," or what we referred to as the "accessible," ^{Me}aa group. However, our experiments showed that ^{Me}Val, ^{Me}Ile, and ^{Me}Leu were grouped as rather "inaccessible" ^{Me}aa for incorporation (Figure 2B, lanes 5–7). The difference between the in vitro-transcribed $tRNA^{Asn\text{-}E1}$ and the native cognate tRNAs that Merryman et al. used as ^{Me}aa-carriers may have an effect on their incorporation efficiencies (Dale and Uhlenbeck, 2005; LaRiviere et al., 2001). However, it should be noted that in the Merryman's TLC electrophoresis assay, the mobility of these branched aliphatic ^{Me}aa were indistinguishable from that of the cognate natural ones. Therefore, it was not clearly defined if the observed product spot originated from fMet-^{Me}aa or fMet-aa. Because the reductive alkylation of some aa-tRNAs to generate ^{Me}aa-tRNAs possibly left a small amount of unreacted aa-tRNAs as Merryman et al. discussed (Merryman and Green, 2004), it could not be ruled out that such aa-tRNA contamitants competed out ^{Me}aa-tRNAs for incorporation into the nascent

(C) MALDI-TOF-MS spectra of the Flag-purified N-methyl-peptides. The calculated molecular mass (Calcd.) and observed molecular mass (Obsd.) for singly charged species, $[M+H]^{+1}$ are shown in each spectrum. In the spectrum of peptide with ^{Me}Fni, an additional peak (asterisk) was detected since the additional peak corresponds to N^{α}-methyl-*p*-nitroso-Phe (Calcd. = 1674.72, Obsd. = 1675.45) generated by photodecomposition of NO_2 to NO during the MALDI-TOF-MS analysis (Ho and Chow, 1996).

A

m4-I :	AUG	ACC	CUC	ACC	UUC	(kk-flag)	UAA
wt4-I :	fMet	Thr	Leu	Thr	Phe	KK-Flag	Stop
^{Me}P4-I :	fMet	^{Me}Yme	^{Me}Phe	^{Me}Yme	^{Me}Ser	KK-Flag	Stop
m4-II :	AUG	ACC	CUC	UUC	CUC	(kk-flag)	UAA
^{Me}P4-II :	fMet	^{Me}Yme	^{Me}Phe	^{Me}Ser	^{Me}Phe	KK-Flag	Stop
m4-III :	AUG	CUC	ACC	UUC	UUC	(kk-flag)	UAA
^{Me}P4-III :	fMet	^{Me}Phe	^{Me}Yme	^{Me}Ser	^{Me}Ser	KK-Flag	Stop
m4-IV :	AUG	UGG	CAC	UGG	CGC	(kk-flag)	UAA
^{Me}P4-IV :	fMet	^{Me}Cys	^{Me}Tyr	^{Me}Cys	^{Me}Gly	KK-Flag	Stop

B

Lanes	1	2	3	4	5
	wt4-I	^{Me}P4-I			
^{Me}Yme-tRNA$^{Asn-E1}_{GGU}$		–	+	+	+
^{Me}Phe-tRNA$^{Asn-E1}_{GAG}$		+	–	+	+
^{Me}Ser-tRNA$^{Asn-E1}_{GAA}$		+	+	–	+

C

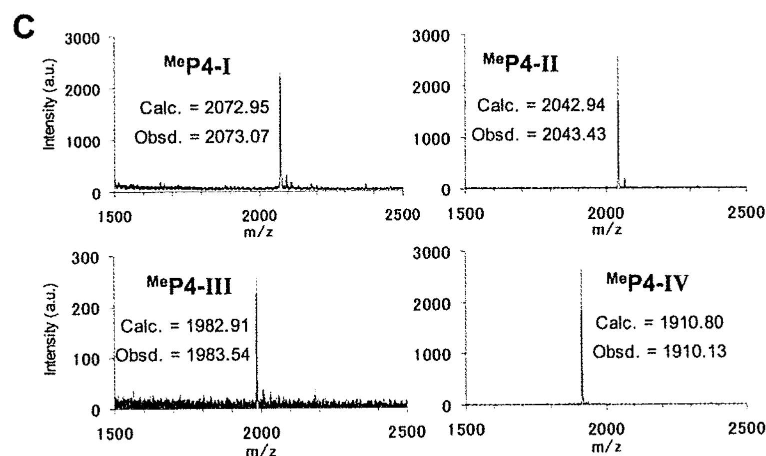

Figure 3. Four Successive Incorporations of Multiple N^{α}-Methylated Amino Acids

(A) Sequences of mRNA templates (m4-I–IV), tetra-N-methyl-peptides (^{Me}P4-I–IV), and the control wild-type peptide (wt4-I). The kk-flag in parentheses indicates the RNA sequence coding a KK-Flag peptide (KKDYKDDDDK). Arabic number denotes the number of N-methylated peptide bonds. The codons and amino acids are colored according to the reprogrammed genetic code table in Figure 1C.

(B) Tricine-SDS-PAGE analysis of the peptides expressed from m4-I labeled with [^{14}C]-Asp detected by autoradiography. Lane 1, the wild-type peptide expressed in the ordinary PURE system; lanes 2–4, negative controls in the presence of two of ^{Me}aa-tRNA^{Asn-E1} as shown; lane 5, in the presence of all three ^{Me}aa-tRNA^{Asn-E1}.

(C) MALDI-TOF-MS spectra of ^{Me}P4-I–IV. The calculated mass (Calcd.) and observed mass (Obsd.) are shown in each spectrum.

peptide chain. In contrast, in our studies, because the flexizyme system ensures the purity of each ^{Me}aa-tRNA, i.e., no contamination of aa-tRNA as confirmed by MALDI-TOF analysis (Figure 2C), we are able to avoid such issues and judge its intrinsic incorporation efficiency more reliably and quantitatively. To this end, we propose that ^{Me}Val, ^{Me}Ile, and ^{Me}Leu are inaccessible substrates due to the combination of bulkiness in the branched side chain and the N^{α}-methylated α-amino group.

Four ^{Me}aa derived from nonproteinogenic amino acids (Figure S1) were also incorporated into the corresponding peptide chain (Figure 2B, lanes 21–25), each of which the MALDI-TOF data was consistent with the expected molecular mass (Figure 2C). Again, the same trend for the incorporation efficiency seemed to apply to these nonproteinogenic ^{Me}aa, where the aromatic ones were incorporated more efficiently than the aliphatic ones. All data taken together, we concluded that ^{Me}aa having "aromatic" or "nonbulky and noncharged" side chains were preferable for the incorporation into the peptide backbone. This information is invaluable for us to select appropriate ^{Me}aa and design specific compositions of N-methylated peptides or peptide libraries while avoiding the risk of potential failure in the synthesis.

Four Successive Incorporations of Multiple N^{α}-Methylated Amino Acids

We next attempted the consecutive incorporations of multiple ^{Me}aa into the peptide backbone via genetic code reprogramming. Five ^{Me}aa derived from proteinogenic amino acids (^{Me}Ser, ^{Me}Phe, ^{Me}Tyr, ^{Me}Cys, ^{Me}Gly) and one derived from a nonproteinogenic amino acid (^{Me}Yme) were selected and assigned arbitrarily to six codons as shown in Figure 1C. The flexizyme system was utilized to charge the respective ^{Me}aa on tRNA^{Asn-E1} bearing the anticodon that reads the assigned codon. We also designed four mRNA templates containing ORFs (Figure 3A, m4-I–IV) that express the corresponding peptides comprised of three different ^{Me}aa in four successive residues (Figure 3A, ^{Me}P4-I–IV). The C terminus of the respective peptides was also designed to contain a modified Flag sequence (KK-Flag = KKDYKDDDDK; D, Asp; Y, Tyr; K, Lys) to facilitate its isolation as well as ionization upon MALDI-TOF analysis.

We first used m4-I to monitor the expression level of ^{Me}P4-I by tricine-SDS-PAGE with a series of appropriate controls (Figure 3B). ^{Me}P4-I was expressed only when all designated ^{Me}aa-tRNA^{Asn-E1}s were present in the *w*PURE system (lanes 2–5), suggesting that no competing background expression of undesignated peptides occurred. The expression level of ^{Me}P4-I was an approximately 20% relative to that of wild-type (wt4-I) expressed in the ordinary PURE system (lanes 1 versus 5). Despite the modest expression level of ^{Me}P4-I, MALDI-TOF analysis of the Flag-purified ^{Me}P4-I gave a single peak with the expected molecular mass, indicating that the assigned codons on mRNA were

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correctly read by these ^{Me}aa-tRNA^{Asn-E1}s and the designated tetra-N-methyl-peptide was expressed.

Likewise, ^{Me}P4-II–IV peptides were expressed at levels approximately 15%–20% of the corresponding wild-type peptides (data not shown). Again, MALDI-TOF data of ^{Me}P4-II–IV peptides were consistent with the expected mass (Figure 3C). Thus, all mRNA ORFs tested in this study could be correctly translated to the tetra-N-methyl-peptides according to the assigned codons with high fidelities. This study represents, to our knowledge, the first proof-of-concept experiment for the ribosomal synthesis of N-methyl-peptides containing multiple kinds of ^{Me}aa by the genetic code reprogramming.

Expression of Longer N-Methyl-Peptides

The achieved high fidelity control in the ribosomal synthesis of the tetra-N-methyl-peptides encouraged us to attempt the expression of longer N-methyl-peptides. We designed four mRNA templates that express N-methyl-peptides containing five, six, eight, or ten consecutive N-methyl-peptide bonds (Figure 4A) (m5/^{Me}P5, m6/^{Me}P6, m8/^{Me}P8, m10/^{Me}P10, respectively). These peptides consisted of three ^{Me}aa in a repetition of the sequence ^{Me}Yme, ^{Me}Phe, and ^{Me}Ser for the corresponding length. To compare the expression level of these N-methyl-peptides, the respective wild-type peptides (wt5–10) were also expressed by using the ordinary PURE system.

All mRNA templates successfully expressed the peptides in the presence of the corresponding ^{Me}aa-tRNA^{Asn-E1}s in *w*PURE system (Figure 4B, gel figure). The absolute expression level for each N-methyl-peptide, determined by its radioisotope intensity originating from the incorporation of five [^{14}C]-Asp residues in the Flag peptide, gradually decreased with increasing peptide length (Figure 4B, upper graph). However, since wild-type also exhibited an inverse-correlation between expression level and peptide length, the relative expression levels of N-methyl-peptides to the corresponding wild-types remained in a similar range (10%–20%), suggesting that the relative expression level was independent of length (Figure 4B, lower graph). Despite their modest yields, the respective N-methyl-peptide had a single peak with the expected molecular mass in MALDI-TOF analysis, indicating that all peptides were expressed with high fidelities according to the corresponding mRNA templates assigned with the reprogrammed genetic code (Figure 4C).

In conclusion, our work has clearly demonstrated up to ten successive incorporations of ^{Me}aa into the peptide backbone via genetic code reprogramming with high fidelity control of both the peptide sequence and length. This technology offers a new means for the sequence-controlled synthesis of peptides containing multiple N-methyl-modifications on their backbone.

Synthesis of Cyclic N-Methylated Peptides Closed by a Thioether Bond

With the above method in our hands, we next applied it to the synthesis of cyclic peptides closed by a physiologically stable thioether bond. The foundation of this peptide ring-closing method has been established during the course of our studies on initiation reprogramming (Goto et al., 2008). Briefly, we prepared a *w*PURE system where Met was depleted so that the initiation codon became vacant. This vacant initiation codon was then filled with initiator tRNA$^{fMet}_{CUA}$ charged with an arbitrarily chosen proteinogenic or nonproteinogenic amino acid by the flexizyme system, so that the translation starts from this newly assigned initiator amino acid. When N^{α}-(α-chloroacetyl)-amino acid (N^{α}-ClAc-aa) and Cys were assigned to the initiation and elongation codons, respectively, the expressed full-length linear peptide spontaneously cyclized via an intramolecular nucleophilic attack of the sulfhydryl group of the Cys side chain to the N-terminal α-carbon of ClAc group, resulting in the formation of a thioether bond. This technology has enabled us to synthesize various cyclic peptides with any sequences and ring sizes. We here attempted to combine initiation reprogramming with elongation reprogramming using ^{Me}aa, to enable us to devise a flexible and reliable methodology for the mRNA-programmed synthesis of cyclic N-methyl-peptides.

We designed three mRNA templates that express peptides containing multiple N-methylated peptide bonds (Figure 5A, mc-I–III). Six ^{Me}aa (^{Me}Ala, ^{Me}Yme, ^{Me}Tyr, ^{Me}Gly, ^{Me}Ser, ^{Me}Phe) and six proteinogenic amino acids (Phe, Lys, Tyr, Pro, Asp, Cys) were chosen as the components of the cyclic N-methyl-peptides in this study (Figures 5A and 5B, ^{Me}cP-I–III). The respective peptides ^{Me}cP-I–III have two, three, and four N-methylated peptide bonds, respectively, implanted by the newly assigned ^{Me}aa in the codon table shown in Figure 1C. It should also be noted that ^{Me}cP-I and ^{Me}cP-II contain Pro as a proteinogenic component that also gives an additional secondary amide bond. These peptides were expressed in the presence of initiator tRNA$^{fMet}_{CAU}$ charged with N^{α}-ClAc-Phe and the corresponding ^{Me}aa-tRNAs in the above *w*PURE system. The MALDI-TOF analysis of the Flag-purified ^{Me}cP-I–III showed that all peptides were spontaneously cyclized via a thioether bond upon completion of translation, giving only the programmed cyclic N-methyl-peptides (Figure 5C). This study indicates that this technology is applicable to a wide array of cyclic peptides with multiple N-methyl-peptide bonds.

DISCUSSION

In the first stage of our proof-of-concept studies, we evaluated 19 ^{Me}aa derived from proteinogenic amino acids and 4 ^{Me}aa derived from nonproteinogenic amino acids, to determine the accessible ^{Me}aa. This study has revealed that the ribosome is accessible to ^{Me}aa with aromatic or nonbulky and noncharged side chains for the incorporation into the growing peptide. Most likely the N^{α}-methyl group added a steric bulkiness to the already bulky structure of certain amino acids, thereby reducing their incorporation levels. It is not yet clear what is the most important filter for the selection of accessible or inaccessible ^{Me}aa, but either EF-Tu or ribosome (or possibly both) is very likely the determinant(s) for their selection (Dale and Uhlenbeck, 2005). Therefore, it is of importance in future experiments to define the selection filter and engineer it to increase the tolerance toward inaccessible ^{Me}aa (Dedkova et al., 2003, 2006).

In this study, we depleted both aaRSs and cognate amino acids from the translation components in order to create vacant codon boxes for ^{Me}aa assignments. In previous studies, the genetic code reprogramming for nonproteinogenic amino acids bearing nonstandard side chains could be achieved by using a PURE system in which only proteinogenic amino acids, not aaRSs, were withdrawn (Murakami et al., 2006). We found,

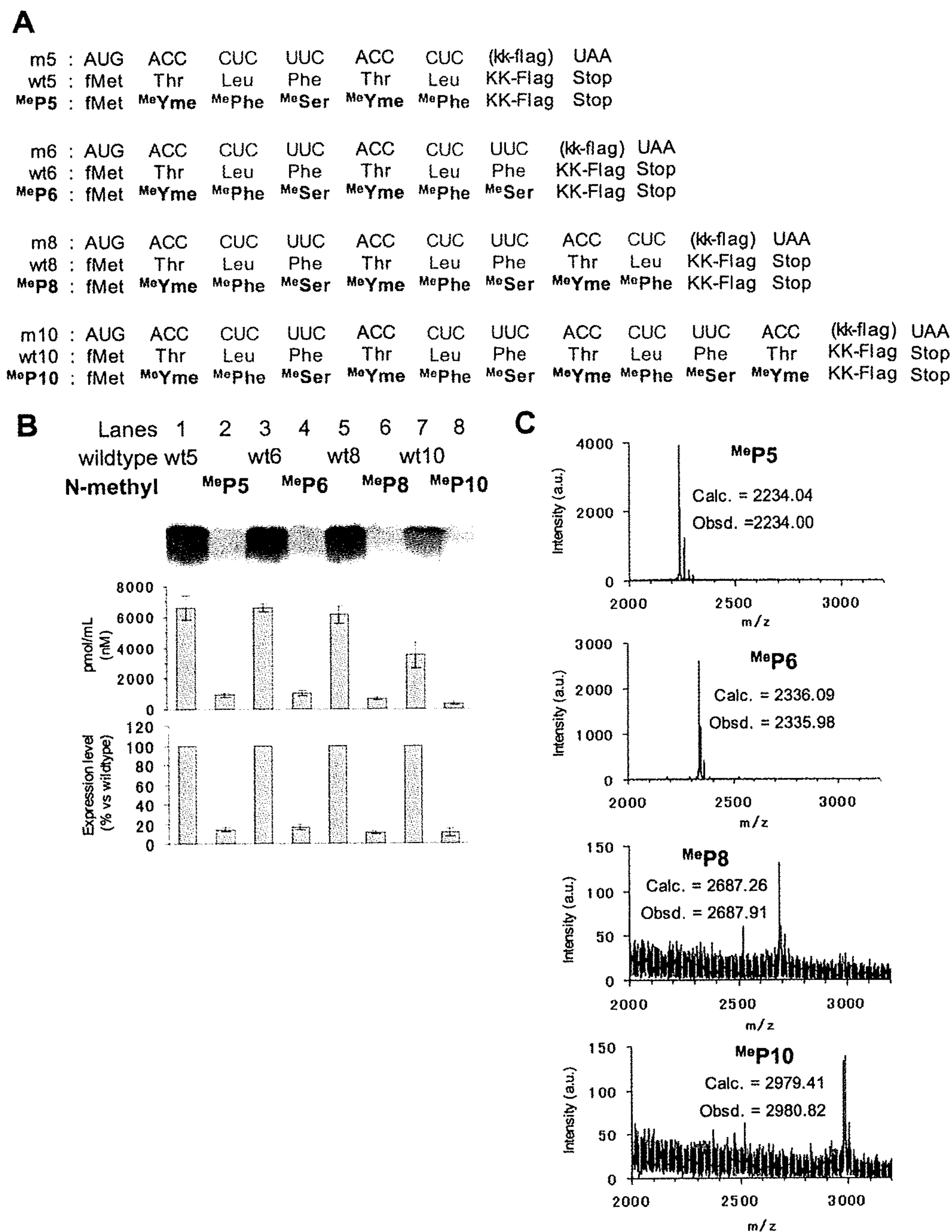

Figure 4. Expression of Longer N-Methyl-Peptides

(A) Sequences of mRNA templates (m5–10), control wild-type peptides (wt5–10), and peptides containing five, six, eight, or ten consecutive N-methyl-peptide bonds (^{Me}P5–10).

(B) Tricine-SDS-PAGE analysis of control wild-type peptides and N-methyl-peptides expressed from the respective mRNA. The peptides were labeled with [^{14}C]-Asp and detected by autoradiography. The wild-type peptides (lanes with odd numbers) were expressed in the ordinary PURE system, while N-methyl-peptides (lanes with even numbers) were expressed in the *w*PURE system containing ^{Me}Yme-tRNA$^{Asn-E1}_{GGU}$, ^{Me}Phe-tRNA$^{Asn-E1}_{GAG}$, and ^{Me}Ser-tRNA$^{Asn-E1}_{GAA}$. Absolute expression level of each peptide based on its observed radioisotope counts is shown in the upper graph, and relative expression level of each N-methyl-peptide against the corresponding wild-type peptide is shown in the lower graph. Each expression level was determined by a mean score of triplicates.

(C) MALDI-TOF-MS spectra of ^{Me}P5–10. The calculated mass (Calcd.) and observed mass (Obsd.) are shown in each spectrum.

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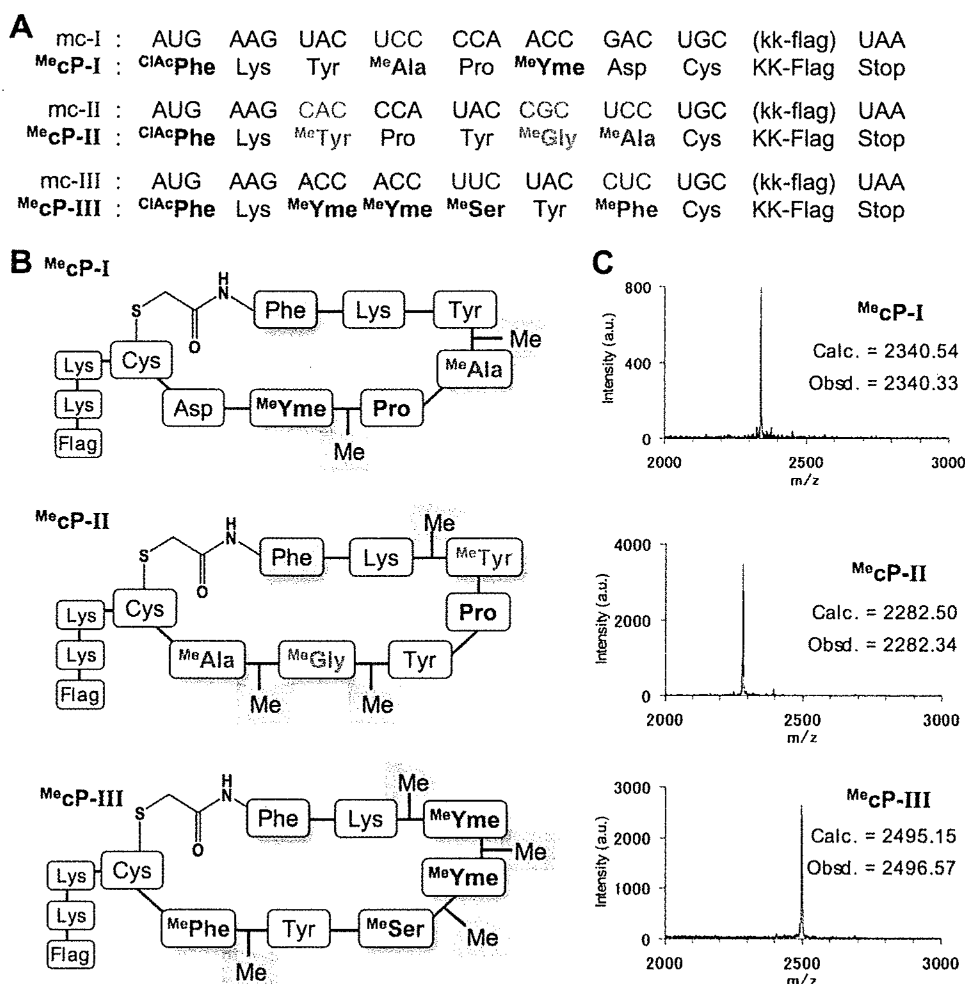

Figure 5. Synthesis of Cyclic N-Methyl-Peptides Closed by a Thioether Bond

(A) Sequences of mRNA templates (mc-I–III) and the precursor N-methyl-peptides (the linear version of ^{Me}cP-I–III). The codons and amino acids are colored according to reprogrammed genetic code table in Figure 1C.

(B) Structures of cyclic N-methyl-peptides. The locations of N-methyl groups on the peptide bond are shown in yellow.

(C) MALDI-TOF-MS spectra of cyclized ^{Me}cP-I–III. The calculated mass (Calcd.) and observed mass (Obsd.) are shown in each spectrum.

however, that in such a *w*PURE system, the incorporations of ^{Me}aa often suffered from contaminations caused by the competing incorporations of the proteinogenic amino acids to the assigned sites (data not shown). This was because that even though proteinogenic amino acids were supposedly depleted from the translation components, the recombinant translation factors and ribosome were often contaminated with small amounts of proteinogenic amino acids; therefore, they were charged onto the endogenous cognate tRNAs and readily competed out ^{Me}aa for the incorporation at the reprogrammed codon sites. Thus, it was critical to remove both aaRSs and proteinogenic amino acids from the translation components in order to repress the background incorporations of proteinogenic amino acids into the peptide. Moreover, we used orthogonal tRNAs as ^{Me}aa carriers aiming at eliminating potential mischarging events with proteinogenic amino acids catalyzed by aaRSs left in the *w*PURE system. Combining all of these controlled elements in the *w*PURE system, we were able to wipe out undesirable backgrounds and thus succeeded in synthesizing N-methyl-peptides as programmed by the mRNA.

Another key technology used in our proof-of-concept study is the flexizyme system. This system enables us to readily prepare a wide variety of ^{Me}aa-tRNAs with high purities, i.e., no contamination of the competing aa-tRNAs, so that we can ensure ^{Me}aa reassignments to the reprogrammed codons and thus determine the accessible ^{Me}aa in a quantitative manner. This technology has given us advantage over Merryman's method involving reductive alkylations on the precharged aa-tRNAs, where certain amounts of competing aa-tRNAs could remain unreacted and therefore contaminate the ^{Me}aa-tRNAs (Merryman and Green, 2004). As was discussed earlier, this might have led them to a different conclusion from ours on the accessible ^{Me}aa.

In a similar context, it would be difficult to perform ^{Me}aa assignments by the Szostak's method, where endogenous aaRSs or their mutants are used to mischarge nonproteinogenic amino acids onto endogenous tRNAs (Hartman et al., 2006; Josephson et al., 2005; Seebeck and Szostak, 2006). The major advantage of the Szostak's method is to in situ generate the mischarged aa-tRNAs and thus express the nonnatural peptides by simply adding nonproteinogenic amino acids to the *w*PURE system that lacks the corresponding proteinogenic amino acids. This system has been proven to be well suited for the incorporation of "highly efficient" nonproteinogenic amino acids into the target peptide, generally those structurally similar to proteinogenic amino acids that can readily compete out the background incorporations of amino acids originated by their contaminations (Seebeck and Szostak, 2006). Szostak et al. have also surveyed the aminoacylation ability of aaRSs toward ^{Me}aa, suggesting that ^{Me}Leu, ^{Me}Asp, ^{Me}Lys, ^{Me}His, and ^{Me}Trp could be charged onto the cognate tRNAs (Hartman et al., 2006). Unfortunately, among them only ^{Me}His and ^{Me}Trp were the "accessible" ^{Me}aa according to our experimental results shown in Figure 2C. As was discussed earlier, because of the intrinsic poor incorporation efficiency of ^{Me}aa, such background incorporations would give impure products containing certain or occasionally significant amounts of partially non-N-methylated peptides. In fact, by means of in situ aaRS's aminoacylation we found that ^{Me}His could be incorporated to the designated site of the same model peptide with competing incorporation of a smaller amount of His, whereas no incorporation of ^{Me}Trp, i.e., only incorporation of the competing Trp, was observed (T.K. and H.S., unpublished data).

Under the optimal conditions for the *w*PURE and flexizyme systems, we performed the mRNA-dependent synthesis of N-methyl-peptides containing more than four successive ^{Me}aa utilizing three different kinds of ^{Me}aa (Figures 3A and 4A). We monitored the expression level of each N-methyl-peptide by tricine-SDS-PAGE compared with the wild-type expression level based on their radioisotope intensities originating from the incorporation of [^{14}C]-Asp in the Flag peptide (Figures 3B and 4B). Using this strategy, we were able to directly compare the expression levels of the N-methyl-peptides in a quantitative manner.

Because we chose only accessible ^{Me}aa for the polymerization, we expected that the expression level would not be drastically reduced compared to the single incorporation. However, the expression of a tetra-N-methyl-peptide (^{Me}P4-I) gave only 20% of the wild-type expression level (Figure 3B, lanes 1 versus 5). Moreover, when longer N-methyl-peptides (from 5-mer to 10-mer) were expressed, their relative expression levels did not significantly change (Figure 4B). We wondered why the observed relative expression level of the tetra-N-methyl-peptides drastically dropped from that observed for the mono-N-methyl-peptide. To investigate this, we expressed mono-, di-, tri-, and tetra-N-methyl-peptides (Figure S3) (^{Me}P1-II, ^{Me}P2-II, ^{Me}P3-II, and ^{Me}P4-II) and compared their expression levels with those of the corresponding wild-type peptides (wt1-II, wt2-II, wt3-II, and wt4-II). Interestingly, the absolute expression levels of wt3-II and wt4-II were approximately three and four times higher, respectively, than that of wt1-II. On the other hand, the absolute expression levels of all N-methyl-peptides remained nearly the same. Thus, it became apparent that the relative expression levels of ^{Me}P3-II and ^{Me}P4-II to wt3-II and wt4-II appeared lower because the expression levels of wt3-II and wt4-II increased from wt1-II, whereas the absolute expression level of all N-methyl-peptides including 5- to 10-mers did not change significantly. Most importantly, MALDI-TOF analysis of the respective N-methyl-peptide gave the corresponding single peak with the correct molecular mass regardless of the length of the N-methyl-peptide. These findings led us to attempt the synthesis of cyclic-N-methyl-peptides.

In this application, we combined our technology for the ^{Me}aa incorporation into peptides with the method previously developed for the initiation reprogramming with N^{α}-ClAc-aa where a linear peptide containing Cys is expressed to afford the corresponding cyclic-peptide closed by a thioether bond between the N-terminus and the sulfhydryl group of the Cys. Because the thioether bond is nonreducible and thereby physiologically stable, it has been implanted into some therapeutic peptides in order to make them compatible for in vivo examination. Although such cyclic peptides were previously generated by only solid-phase chemistry, this method has enabled us to synthesize such cyclic peptides by means of the translation apparatus. Thus, the combination of the above two methods has offered us a platform technology for the synthesis of cyclic N-methyl-peptides by designing the corresponding mRNA templates. Remarkably, the expressed full-length cyclic N-methyl-peptides were highly pure in all three examples presented in this work. Thus, this technology is well suited for the preparation of libraries of cyclic N-methyl-peptides. Particularly, when this is coupled with in vitro display systems, such as ribosome (Hanes and Pluckthun, 1997) or mRNA displays (Nemoto et al., 1997; Roberts and Szostak, 1997), it becomes a very powerful means of screening large libraries and selecting in vivo-compatible cyclic N-methyl-peptides against therapeutic targets. Such experimental examples are shortly up coming from our laboratory.

SIGNIFICANCE

Reprogramming the genetic code allows for the simultaneous incorporation of multiple amino acid monomers with a wide variety of side chains into peptides through the ribosomal translation reaction. In this study, we achieved the mRNA-directed synthesis of various sequences and lengths of peptides containing multiple N-methylated amino acid residues upon reprogramming the genetic code by using the *w*PURE and flexizyme systems. Our demonstration shows the potential of this technology that leads us to a new strategy to construct libraries of linear and cyclic N-methyl-peptides simply by designing mRNA sequence libraries. Upon combining with appropriate screening strategies, this technology will yield a novel class of therapeutic peptides with protease resistance and cell permeability.

EXPERIMENTAL PROCEDURES

Materials and Synthesis of N^{α}-Methyl-Amino Acid Substrates

All N^{α}-methylated amino acids except N^{α}-methyl-L-cysteine and N^{α}-methyl-L-methionine were purchased from Watanabe Chemical, Japan. N^{α}-methyl-L-cysteine was synthesized by the procedure reported before from L-cysteine as a starting material (Park and Kim, 2002). N^{α}-tBoc-N^{α}-methyl-L-methionine was synthesized by the procedure reported before from N^{α}-tBoc-L-methionine as a starting material (Lee et al., 1990). N^{α}-methylated amino acids with the

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aromatic side-chain (^{Me}Phe, ^{Me}Tyr, ^{Me}Trp, ^{Me}Fni, and ^{Me}Yme) were converted to cyanomethyl esters (CMEs), and the others were converted to 3,5-dinitrobenzyl esters (DBEs) by the procedure reported elsewhere (Murakami et al., 2006).

Synthesis of N^{α}-Methyl-Aminoacyl-tRNA

Aminoacylation reactions were generally performed as follows: 12.5 μl of 20 μM tRNA in 0.2 M HEPES-KOH (pH 7.5) was heated at 95°C for 1 min and cooled to room temperature over 5 min. Five microliters of 0.1 M $MgCl_2$ and 2.5 μl of 0.1 mM dFx or 5 μl of 3 M $MgCl_2$ and 2.5 μl of 0.1 mM eFx were added to the solution. Five microliters of 25 mM N^{α}-methylated amino acid substrate (DBE or CME) in DMSO was then added to the mixture and incubated on ice for 2–24 hr. For ^{Me}Thr, ^{Me}Tyr, ^{Me}Fni, and ^{Me}Yme, 5 μl of 200 mM substrate was used. The acylation reaction was quenched by addition of 150 μl of 0.6 M sodium acetate (pH 5.0), and the tRNA was recovered by ethanol precipitation. The pellet was rinsed twice with 70% ethanol with 0.1 M sodium acetate (pH 5.0) and once with 70% ethanol.

Ribosomal Synthesis of Linear N-Methylated Peptides and Wild-Type Peptides

The following conditions were used for the peptide synthesis without [^{14}C]-labeling. For the synthesis of N-methylated peptides (^{Me}P4-I, ^{Me}P4-II, ^{Me}P4-III, ^{Me}P4-IV, ^{Me}P5, ^{Me}P6, ^{Me}P8, ^{Me}P10, ^{Me}P1-II, ^{Me}P2-II, and ^{Me}P3-II), translation reaction was carried out by using the *w*PURE system (see the Supplemental Data) in the presence of 40 nM DNA template (one of the following templates: m4-I, m4-II, m4-III, m4-IV, m5, m6, m8, m10, m1-II, m2-II, or m3-II), 0.1 mM of the respective ^{Me}aa-tRNA(s), and 0.2 mM each of Met, Asp, Tyr, and Lys. For the synthesis of wild-type peptides (wt4-I, wt5, wt6, wt8, wt10, wt1-II, wt2-II, wt3-II, and wt4-II), translation reaction was carried out by using the ordinary PURE system in the presence of 40 nM DNA template (one of the following templates: m4-I, m5, m6, m8, m10, m1-II, m2-II, m3-II, or m4-II) and 0.2 mM each of Met, Thr, Leu, Phe, Asp, Tyr, and Lys. The translation reactions were assembled on ice and started by incubating at 37°C for 3 hr. For the detection of the peptides using autoradiography, see the section of tricine-SDS-PAGE analysis.

Tricine-SDS-PAGE Analysis

For tricine-SDS-PAGE analysis, peptides were synthesized by using 50 μM [^{14}C]-Asp in the place of 0.2 mM Asp. The translation reaction (2.5 μl) was stopped by the addition of 2.5 μl loading buffer consisting of 0.9 M Tris-HCl (pH 8.5), 30% glycerol, and 8% SDS for loading to 15% tricine-SDS-PAGE. Separation gel contained 15% acrylamide (acrylamide: bisacrylamide = 19:1), 1 M Tris•HCl (pH 8.5), 0.1% SDS, and 13% Glycerol, while the stacking gel contained 4% acrylamide (acrylamide: bisacrylamide = 29:1), 0.75 M Tris•HCl (pH 8.5), 0.075% SDS. The anode running buffer was 200 mM Tris•HCl (pH 8.9), while the cathode running buffer contains 100 mM Tris, 100 mM tricine, 0.1% SDS (the resulting buffer was approximately pH 8.3). The products, labeled with [^{14}C]-Asp in the C terminus FLAG peptide, were quantified by autoradiography with an image analyzer, FLA-5100 (Fuji, Japan), and the specific activity of [^{14}C]-Asp.

Ribosomal Synthesis of Cyclic N-Methylated Peptides

For the synthesis of cyclic N-methylated peptide (^{Me}Pc1, ^{Me}Pc2, or ^{Me}Pc3), translation reaction was carried out by using the *w*PURE system with 40 nM DNA template (mc1, mc2, or mc3) in the presence of 0.1 mM N^{α}-ClAc-Phe-$tRNA^{fMet}_{CAU}$, 0.1 mM of the respective ^{Me}aa-tRNAs, and 0.2 mM each of Met, Lys, Tyr, Pro, Asp, and Cys. The translation reactions were assembled on ice and started by incubation at 37°C for 3 hr.

MALDI-TOF-MS Analysis

The respective peptide was incubated in prewashed Flag-M2 agarose (Sigma) for an hour. After the resin was washed with TBS (50 mM Tris-HCl [pH 8.0], 150 mM NaCl), the peptide was eluted with 0.2% TFA. Eluted peptide was desalted with C18 zip tips (Millipore) and eluted with 50% acetonitrile, 0.1% TFA solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid. MALDI-TOF-MS analysis was performed by using Autoflex TOF/TOF (Bruker Daltonics) operated in the linear positive mode and externally calibrated with Substance P (1348.66 Da), Bombesin (1620.88 Da), ACTH clip 1–17 (2094.46 Da), and Somatostatin 28 (3149.61 Da) standards.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures for RNA synthesis, tRNA aminoacylation, and preparation of the translation systems and are available at http://www.chembiol.com/cgi/content/full/15/1/32/DC1/.

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Reprogramming the Translation Initiation for the Synthesis of Physiologically Stable Cyclic Peptides

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ABSTRACT The initiation codon dictates that the translation initiation event exclusively begins with methionine. We report here a new technology to reprogram the initiation event, where various amino acids and those bearing N^α-acyl groups can be used as an initiator for peptide synthesis. The technology is built upon the concept of genetic code reprogramming, where methionine is depleted from the translation system and the initiation codon is reassigned to the desired amino acid. We have applied this technology to the synthesis of an antitumor cyclic peptide, G7−18NATE, closed by a physiologically stable bond, and it is also extended to the custom synthesis of its analogues with various ring sizes. Significantly, cyclization occurs spontaneously upon translation of the precursor linear peptides. To demonstrate the practicality of this methodology, we also prepared a small cyclic peptide library designated by 160 distinct mRNAs. Thus, this technology offers a new means to prepare a wide array of *in vivo* compatible cyclic peptide libraries for the discovery of peptidic drug candidates against various therapeutic targets.

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Polypeptide synthesis in prokaryotic translation is generally initiated with N^α-formyl methionine (fMet) (*1*). The initiation process involves methionylation of the initiator tRNA ($tRNA^{fMet}_{CAU}$) catalyzed by methionyl-tRNA synthetase (MetRS) followed by formylation of the α-amino group on Met by methionyl-tRNA formyltransferase (MTF); the resulting fMet-$tRNA^{fMet}_{CAU}$ subsequently binds to initiation factors (IFs) and the complex enters the peptidyl-tRNA site of the ribosome to initiate translation (*2, 3*). Although strict governance by the above mechanism controls the initiation event starting with fMet at the N-terminus exclusively, two methods have been devised to circumvent this requirement (*4–7*).

The first method involves engineering of the initiator tRNA where the CAU anticodon is altered to a triplet that assigns another amino acid (*4, 5*). Critical tRNA recognition elements in some aminoacyl-tRNA synthetases (aaRSs) reside in the anticodon interaction (*8*). Therefore, the transplantation of an appropriately chosen anticodon into $tRNA^{fMet}$ is able to deceive the recognition by aaRS, leading to a mischarging of the cognate amino acid onto the engineered initiator $tRNA^{fMet}_{XXX}$ (XXX indicates the implanted anticodon). When such a tRNA, together with a mRNA containing the countercodon to the tRNA anticodon, is overexpressed in *Escherichia coli* the translation starts with the mischarged N^α-formylated amino acid. Since not all aaRSs can be deceived by this type of tRNA engineering, only a limited number of aaRSs (Val, Phe, Gln, Ile, and Lys) were able to mischarge the cognate amino acids onto $tRNA^{fMet}_{XXX}$ and lead to initiation.

The second method also involves engineering of the initiator tRNA. The initiator tRNA anticodon is altered to CUA and the mRNA AUG codon is replaced with the UAG amber codon (*6, 7*), in a fashion similar to the incorpo-

ration of nonproteinogenic amino acids during the elongation event (9–11). When such a tRNA is *chemically* aminoacylated through multistep synthesis (12) and subjected to *in vitro* translation, the designated amino acid initiates peptide expression. Unfortunately, this approach suffers from the contamination of polypeptides initiated by other amino acids since the deacylated $tRNA^{fMet}_{CUA}$ can be recharged with the amino acids by cognate endogenous aaRSs (6, 7). This method has thus far demonstrated the initiation of translation with only a limited number of amino acids (Met, Val, and Lys) tagged with fluorescent or biotin groups for the purpose of expressing N-end-labeled polypeptides *in vitro*.

Taken together, the currently available technologies do not allow for the synthesis of polypeptides with a well-controlled N-terminal modification, and it remains unclear whether more diverse types of amino acids including those with various N^{α}-acyl groups are tolerated in the initiation event. We here have used the concept of genetic code reprogramming to engineer the initiation event. This new technology allows us to expand the repertoire of usable α-amino acids and their N^{α}-acyl groups for initiation.

As an application, we have demonstrated the ribosomal synthesis of an antitumor cyclic peptide, G7–18NATE, closed by a physiologically stable thioether bond. Significantly, the cyclization occurs spontaneously upon the translation of its precursor linear peptide so that no additional manipulation is necessary after translation. Moreover, this technology is applicable to the synthesis of cyclic peptides with a wide range of ring sizes, and thus it offers a novel tool to construct mRNA-programmed cyclic peptide libraries for the discovery of peptidic drug candidates with *in vivo* compatibility.

RESULTS AND DISCUSSION

Genetic Code Reprogramming for the Initiation Event. Reprogramming the genetic code is a technique in which the codons assigned to proteinogenic amino acids are reassigned to nonproteinogenic amino acids (13). This technique has been utilized for the reprogramming of the elongation event where multiple nonproteinogenic amino acids have been successfully incorporated into the nascent peptide chain (13–20). In principle, it is also applicable to the alteration of initiation, but it has not been yet extensively used for such an aim.

To perform reprogramming of the translation initiation, we have employed two key systems to facilitate reprogramming of the initiation event. The first one is an *Escherichia coli* reconstituted cell-free translation system, referred to as the PURE system (21). By *withdrawing* certain amino acids from the translation system (referred to as *w*PURE), vacant codons can be created in the genetic table. Met is depleted to make the initiation codon vacant, and therefore an alternative amino acid (Xaa) can be reassigned to initiation (Figure 1, panel a). The second key system is a *de novo* tRNA acylation system consisting of an artificially evolved ribozyme, termed flexizyme (Figure 1, panel b) (20, 22, 23). Flexizyme allows for virtually any amino acid to be charged onto tRNA. Thus, we expect that by filling the vacant start codon in *w*PURE system with Xaa-$tRNA^{fMet}_{CAU}$ prepared with the flexizyme system, *i.e.*, through the integration of these two systems, the initiator Met can be readily reassigned to any noncanonical amino acid (Figure 1, panel c).

To test whether these two systems could be integrated to reprogram the initiation event, a 14-mer peptide bearing a Flag tag sequence (24) at the C-terminus was expressed in the *w*PURE system lacking Met in the absence or presence of Met-$tRNA^{fMet}_{CAU}$ prepared by the flexizyme system (Figure 2, panel a). As a positive control, the wild-type peptide was expressed in the normal PURE system containing the full sets of amino acids (Figure 2, panel b, lane 1). As a negative control, when only the *w*PURE system was used, initiation did not occur and thereby no peptide was produced (Figure 2, panel b, lane 2). In contrast, when Met-$tRNA^{fMet}_{CAU}$ prepared by the flexizyme system was included in the *w*PURE system, initiation proceeded and yielded the expected band with the same intensity and mobility as the wild type (Figure 2, panel b, lane 3 *vs* lane 1). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis of the product gave the expected molecular mass of the peptide bearing fMet at its N-terminus (Figure 2, panel c, Met). Thus, Met-$tRNA^{fMet}_{CAU}$ synthesized by the flexizyme system was formylated by endogenous MTF and able to initiate translation as efficiently as the native initiation event in the normal PURE system. These results demonstrated that the concept of genetic code reprogramming would be applicable to engineering of the initiation event as well as the elongation event aiming to express peptides containing various amino acids at the N-terminus.

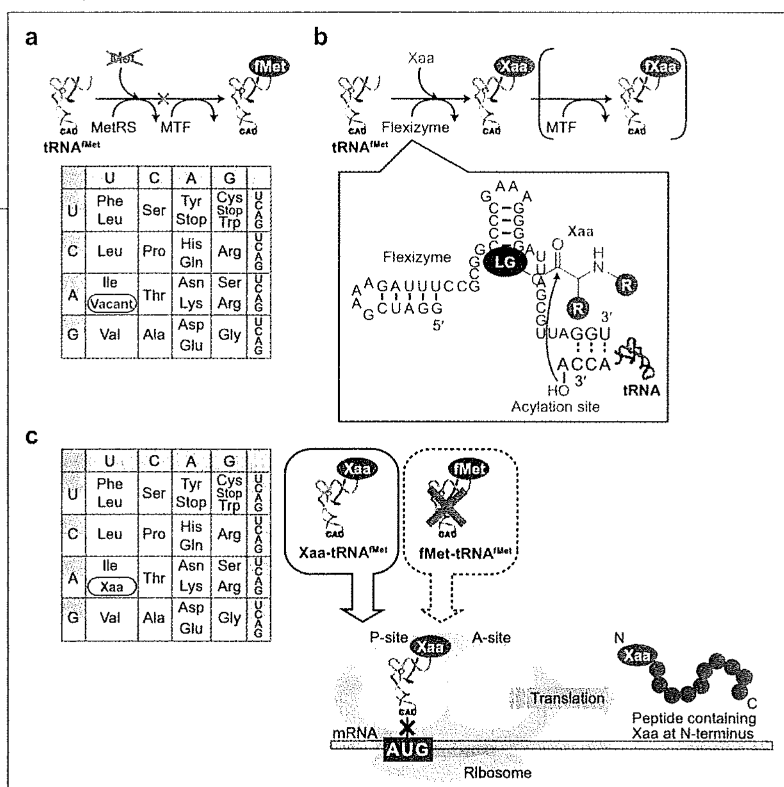

Figure 1. Engineering the initiation event by genetic code reprogramming. a) Codon table in the absence of Met. Upon removing Met from the translation mixture, $tRNA^{fMet}_{CAU}$ is no longer available for methionylation by MetRS, consequently making the start codon vacant, *i.e.*, *w*PURE system. b) Synthesis of Xaa-$tRNA^{fMet}_{CAU}$ by the flexizyme system. Xaa indicates various kinds of α-amino acid. The secondary structure of a representative flexizyme is shown in cyan letters. When Xaa-$tRNA^{fMet}_{CAU}$ is added to the translation apparatus, it can be formylated by endogenous MTF. c) Reassignment of the start codon from Met to Xaa. When Xaa-$tRNA^{fMet}_{CAU}$ prepared by the flexizyme system is added to the *w*PURE system, the translation is exclusively initiated with Xaa, expressing a peptide containing Xaa at the N-terminus.

Tolerance of Amino Acids in Initiation. We next tested initiation using each of the 19 proteinogenic amino acids by charging each amino acid to $tRNA^{fMet}_{CAU}$ with the flexizyme system. Tricine–SDS PAGE (soduim dodecyl sulfate polyacrylamide gel electrophoresis) analysis of the translation products showed that 11 amino acids out of 19 were able to initiate translation with greater than 50% efficiency relative to wild type (Figure 2, panel b, highlighted in orange). In addition, two amino acids exhibited the ability to initiate translation with approximately 30–50% efficiency relative to wild type (Figure 2, panel b, highlighted in pink). MALDI-TOF analysis of these translated peptides gave a single peak with the expected molecular mass of the N-terminal-formylated form initiated with the programmed amino acid (Figure 2, panel c, highlighted in the same color code as Figure 2, panel b). This indicates that the initiation occurs exclusively with the N^{α}-formylated amino acid as programmed by the flexizyme system.

The remaining six amino acids (Glu, Lys, Gly, Asp, Arg, and Pro) showed less than 30% translation efficiency compared to wild-type expression, with Arg and Pro producing yields in the single digits (Figure 2, panel b, highlighted in cyan). Despite their poor yields, the Flag-purified peptides initiated by Glu, Lys, Gly, and Asp gave the expected molecular mass of the formylated form, indicating that the full-length peptides were correctly initiated with the designated amino acids. Albeit with low efficiency, even Arg and Pro were able to initiate translation giving peaks corresponding to peptides initiated with programmed amino acid in the respective mass spectrum, although Arg gave a mixture of formylated and nonformylated peptides while Pro only produced the nonformylated peptide (Figure 2, panel c).

Interestingly, the majority of "good" initiators have hydrophobic side chains. On the other hand, six amino acids exhibited less than 30% efficiency in initiation (Figure 2, panel b, highlighted in cyan); among them, four have charged side chains (Lys, Glu, Asp, and Arg), one has no side chain (Gly), and the least efficient initiator Pro has the secondary amine. We assumed that inefficient initiation with these amino acids was caused by poor formylation of the α-amino group on the Xaa-$tRNA^{fMet}_{CAU}$ catalyzed by MTF. In the crystal structure of the complex between fMet-$tRNA^{fMet}_{CAU}$ and MTF, the side chain of Met fits into a binding pocket surrounded by hydrophobic amino acids (*25*). Presumably, this hydrophobic environment might dismiss the charged or hydrophilic side chains resulting in poor formylation on the α-amino group, while Pro, the only amino acid bearing the secondary amine, is likely enzymatically incompatible with MTF. Indeed, the two least efficient initiators, Arg and Pro, afforded nonformylated peptide in each mass spectrum of the product.

The above results indicated that the integration of the *w*PURE and flexizyme systems enabled us to readily reassign the AUG codon from Met to other amino acids and express the desired peptide initiated with the assigned amino acid. Although traces of undesirable peptides with slower or/and faster mobility were detected in the tricine-SDS PAGE analysis, these bands also appeared in the control experiment in the absence of aa-$tRNA^{fMet}_{CAU}$ (Figure 2, panel b, lane 2). This suggested that these bands appeared from aa-$tRNA^{fMet}_{CAU}$-independent expressions of the mRNA template, likely

due to in-frame mis-initiations. However, the expression levels of these peptides were the negligible background, and indeed we observed only a single desirable full-length product in the MALDI-TOF analysis. This was a sharp contrast to the previous method that suffered from contaminations of undesirable full-length peptides mis-initiated with undesignated amino acids.

We believe that the observed nearly exclusive expression of the desirable peptides has been achieved by two critical strategies we took for reprogramming the translation initiation event. First, depletion of Met in the *w*PURE system enabled us to suppress the background initiation reaction as low as possible. Second, the flexizyme system enabled us to use the natural initiator $tRNA^{fMet}_{CAU}$ as an aminoacyl-carrier for noncognate amino acids, and thereby we achieved a maximal efficiency for initiating the translation with each amino acid. By the combination of these two strategies, we were able to express the full-length peptide initiated with the designated noncanonical amino acid as a sole product.

Enhancing the Initiation Efficiency by Preacylation of α-Amino Group. As described above, an intriguing observation was made regarding the Arg-initiated peptide in which the N-terminus was not fully formylated (Figure 2, panel c, Arg). Likewise, the N-terminus of Pro-initiated peptide was not formylated at all (Figure 2, panel c, Pro). This result implies that these inefficient initiators are poor substrates for MTF (Figure 3, panel a). This observation led us to postulate that the efficiency of formylation on the α-amino group of Xaa-$tRNA^{fMet}_{CAU}$ by MTF might play an important role in determining the initiation efficiency, since the expression levels of these peptides were significantly lower than peptides initiated with other amino acids. RajBhandary and coworkers (*26*) have also proposed

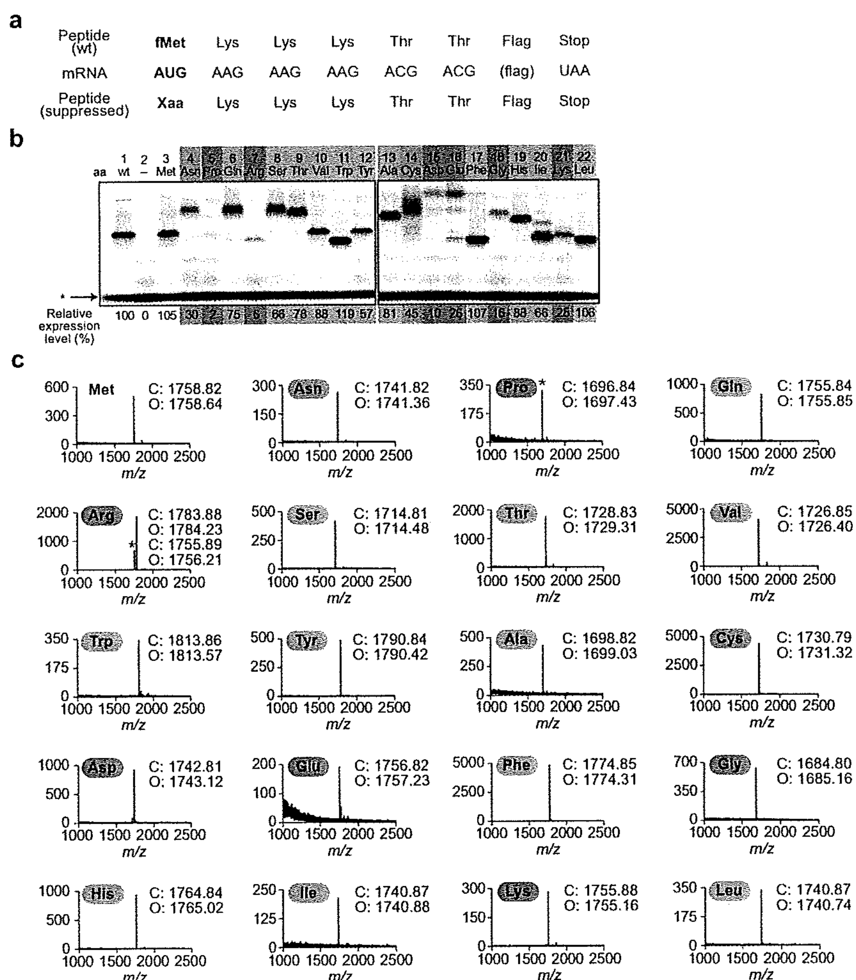

Figure 2. Tolerance of various amino acids in initiation. a) The mRNA sequence that expresses peptides initiated by various amino acids. Flag in parentheses indicates the RNA sequence encoding a Flag peptide (DYKDDDDK). b) Tricine-SDS PAGE analysis of the translation products. Lane 1, expression of wild type; lane 2, in the absence of Met; lane 3, initiated by Met-$tRNA^{fMet}_{CAU}$; lanes 4–22, initiated by Xaa-$tRNA^{fMet}_{CAU}$. Each expression level relative to wild type is determined by a mean score of triplicates or more. The band indicated by asterisk corresponds to the remaining [^{14}C]-Asp that was not incorporated into the peptide. The amino acids giving >50%, 30–50% and <30% of the wild-type expression level are highlighted in orange, pink, and cyan, respectively. Observed minor bands were likely attributed to peptides originated from the aa-$tRNA^{fMet}_{CAU}$-independent background expression, *i.e.*, random starts of translation of the RNA template. c) MALDI-TOF MS spectra of the translated peptides. The noncanonical amino acids are highlighted in the same color code as used in panel b. The red asterisk indicates a peak corresponding to the nonformylated peptide, while the unlabeled peak corresponds to the formylated peptides. The calculated mass (C) and observed mass (O) are shown in each spectrum.

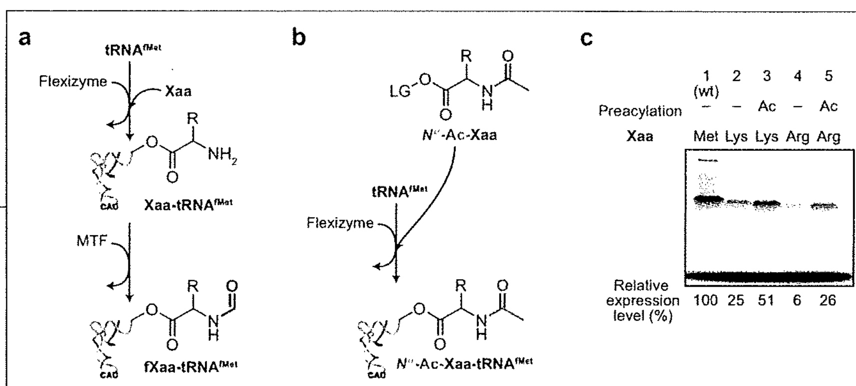

Figure 3. Increase in expression level by N^{α}-acylation. a) Formylation of Xaa-tRNA$^{fMet}_{CAU}$ in the *w*PURE system. When Xaa-tRNA$^{fMet}_{CAU}$ is used in the initiation, it is generally formylated. b) Aminoacylation of tRNA$^{fMet}_{CAU}$ with preacetylated amino acids by the flexizyme system. This method ensures N^{α}-acylation on the α-amino group. c) Tricine−SDS PAGE analysis of the translated peptide. Lane 1, expression of wild type; lane 2, initiated with Lys-tRNA$^{fMet}_{CAU}$; lane 3, initiated with N^{α}-Ac-Lys-tRNA$^{fMet}_{CAU}$; lane 4, initiated with Arg-tRNA$^{fMet}_{CAU}$; lane 5, initiated with N^{α}-Ac-Arg-tRNA$^{fMet}_{CAU}$. Each expression level relative to wild type is determined by a mean score of triplicates or more.

such a correlation based on experiments using an alternative codon for initiation. We therefore considered the possibility that preacylation on the α-amino group might increase the expression level of these peptides (Figure 3, panel b).

In order to verify this hypothesis, we chose two amino acids, Lys and Arg, both of which showed poor expression levels (25% and 6%, respectively) and prepared N^{α}-Ac-Lys-tRNA$^{fMet}_{CAU}$ and N^{α}-Ac-Arg-tRNA$^{fMet}_{CAU}$ by using the flexizyme system. It was investigated whether the expression level initiated with these preacylated amino acids increased compared with the corresponding α-amino-free amino acids. In both cases, peptide expression levels increased compared to those initiated with the corresponding α-amino-free amino acids (51% *vs* 25% for Lys and 26% *vs* 6% for Arg, Figure 3, panel c) and initiation proceeded exclusively with the designated N^{α}-Ac-amino acids (see Figure S1 for the MALDI-TOF molecular weight confirmation). It should be noted that even though the α-amino group on Arg was preacylated, the expression level of the peptide initiated with N^{α}-Ac-Arg did not exceed 50% efficiency compared with that of Met-initiated peptide. This suggests that other components in the *w*PURE system, such as IFs and ribosome, also play some roles in determining the initiation efficiency. Nonetheless, the preacylation on the α-amino group enhances the expression level for "modest" initiators.

Tolerance of N^{α}-Acyl Groups in Initiation. Biologically active peptides often have unique N-terminal-acyl groups. For instance, the N-terminus of some peptides synthesized by nonribosomal peptide synthetases are modified with fatty acids (*27*), while some peptide hormones have an acetyl or pyroglutamate group at their N-terminus (*28, 29*). Thus, it is of interest to see if the reprogrammed initiation in the *w*PURE system is able to tolerate amino acids with a variety of N^{α}-acyl groups. We chose Phe as an N^{α}-acyl carrier and expressed peptides initiated with Phe having alkyl or fatty acids (Ac, Hex, Mhe, Oct), pyroglutamate (pyE), and functionalities accessible to post-translational modification (Cba, Pen, Pyl, N_3Ac, oxP, ClAc; Figure 4, panel a).

Translation was initiated with N^{α}-acyl-Phe-tRNAs prepared by the flexizyme system, and the *N*-acyl-peptides were expressed in the *w*PURE system (Figure 4, panel b). Remarkably, the expression levels were generally higher than those of fMet- and fPhe-initiated control peptides; particularly the use of Phe modified with hydrophobic fatty acids gave significantly higher expression levels. MALDI-TOF analysis of these peptides agreed with the expected products without contamination of other misinitiated peptides (Figure 4, panel c). Thus, the results clearly indicated that a wide variety of N^{α}-acyl groups are tolerated in the initiation event. The N^{α}-acyl groups incorporated in this study involved various chemical functionalities such as fatty acid chains, pyroglutamate, and those that are applicable to post-translational modification. Hence, this approach would allow us to directly translate naturally occurring bioactive peptides containing unique N-terminal groups.

Ribosomal Synthesis of Cyclic Peptides *via* a Thioether Linkage. G7−18NATE is a potential peptidic anticancer agent, which has been shown to inhibit the interaction between the SH2 domain of Grb7 and receptor tyrosine kinases (*30−32*). The peptide consists of 11 natural amino acids and a non-natural cyclic structure with a nonreducible thioether bond linking the N-terminal acetyl group to the C-terminal Cys residue (see Figure S2a). Due to the fact that G7−18NATE contains a noncanonical bond, ordinary translation has been inapplicable to its synthesis; therefore this peptide could only be produced through chemical synthesis. However, we envisaged that our platform technology would enable us to perform the ribosomal synthesis of G7−18NATE.

We expected that reprogramming initiation with N^{α}-chloroacetyl (ClAc)-Trp would allow us to synthesize the linear N^{α}-ClAc-(G7−18NATE) precursor peptide, and subsequently the sulfhydryl group on the terminal Cys would spontaneously attack the α-carbon of the N^{α}-ClAc group, closing the ring (Figure 5, panel a) (*33−35*). We