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Synthesis of Polyester by Means of Genetic Code Reprogramming

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SUMMARY

Here we report the ribosomal polymerization of α-hydroxy acids by means of genetic code reprogramming. The flexizyme system, a ribozyme-based tRNA acylation tool, was used to reassign individual codons to seven types of α-hydroxy acids, and then polyesters were synthesized under controls of the reprogrammed genetic code using a reconstituted cell-free translation system. The sequence and length of the polyester segments were specified by the mRNA template, indicating that high-fidelity ribosome expression of polyesters was possible. This work opens a door for the mRNA-directed synthesis of backbone-altered biopolymers.

INTRODUCTION

The translation system polymerizes amino acids to polypeptides using the genetic information present in the form of trinucleotide codons in the mRNA sequences. In this manner, the ribosome machinery acts as a template-directed synthesizer of polymers consisting of amino acids. Each of the codons directs the incorporation of 1 of 20 proteinogenic α-amino acids into the polypeptide chain, and therefore the ribosome is generally used for the synthesis of polypeptides, not for other biopolymers. If the codons are reassigned to nonproteinogenic amino acids, and if the translation system is adaptable to such alterations, mRNA-directed synthesis of nonproteogenic polypeptides by means of genetic code reprogramming is possible [1–6]. However, we have not yet witnessed the ribosomal synthesis of biopolymers with a nonpeptide backbone by such a methodology.

An α-hydroxy acid is chemically analogous to an α-amino acid, where the hydroxy group acts as a nucleophile. Despite this atomic difference, the ribosome is able to accept an α-hydroxy acid as a substrate to form an ester bond when hydroxyacyl-tRNA (ha-tRNA) is supplied to the translation system [7–14]. Previously, the specific incorporation of a single α-hydroxy acid into the polypeptide chain using amber stop codon suppression has been reported [7, 8, 10, 13]. Unfortunately, this strategy allows for the assignment of only a single kind of α-hydroxy acid, and therefore it is not suitable for the synthesis of complex polyesters composed of several different α-hydroxy acids.

In another approach reported over 30 years ago, the ribosome polymerizes phenyllactic acid (F^{lac}) on polyuridylic acid (poly-U) via random initiation and termination upon the addition of F^{lac}-$tRNA^{Phe}$ [12]. However, due to the methodology for the preparation of F^{lac}-$tRNA^{Phe}$ where Phe-$tRNA^{Phe}$ was chemically deaminated by nitrous acid, it was difficult to deplete the Phe-$tRNA^{Phe}$ contaminant completely from the translation mixture; therefore, the resulting polyester was actually composed of a heterogeneous random mixture of F^{lac} and Phe with a ratio of approximately 7:3. Due to the fact that the poly-U template did not specify either a start or stop site, the ribosome could randomly initiate or terminate the synthesis of polyesters, and thus the length of the synthesized polyester could not be regulated.

Collectively, these previous findings indicate that the ribosome is capable of catalyzing the polymerization of α-hydroxy acids, but it remains unknown whether the mRNA-directed polymerization of several different α-hydroxy acids is possible. Here we report the synthesis of polyester composed of various α-hydroxy acids using a reprogrammed genetic code where the codons are reassigned from proteinogenic α-amino acids to α-hydroxy acids.

RESULTS

Genetic Code Reprogramming for the Incorporation of α-Hydroxy Acids

To demonstrate programmed synthesis of polyesters, we took advantage of two recently developed technologies (Figure 1). The first one is a de novo tRNA acylation system consisting of artificially evolved ribozymes, called flexizymes [14–17]. This system enables us to charge various hydroxy acids onto tRNAs bearing any desired anticodons and body sequences. The second one is a reconstituted *Escherichia coli* cell-free translation system, called the PURE (protein synthesis using recombinant elements) system [18, 19]. This translation system lets us create vacant codon boxes by withdrawing the corresponding aminoacyl-tRNA synthetases (ARSs) and amino acids from the reconstituted translation mixture (referred to as

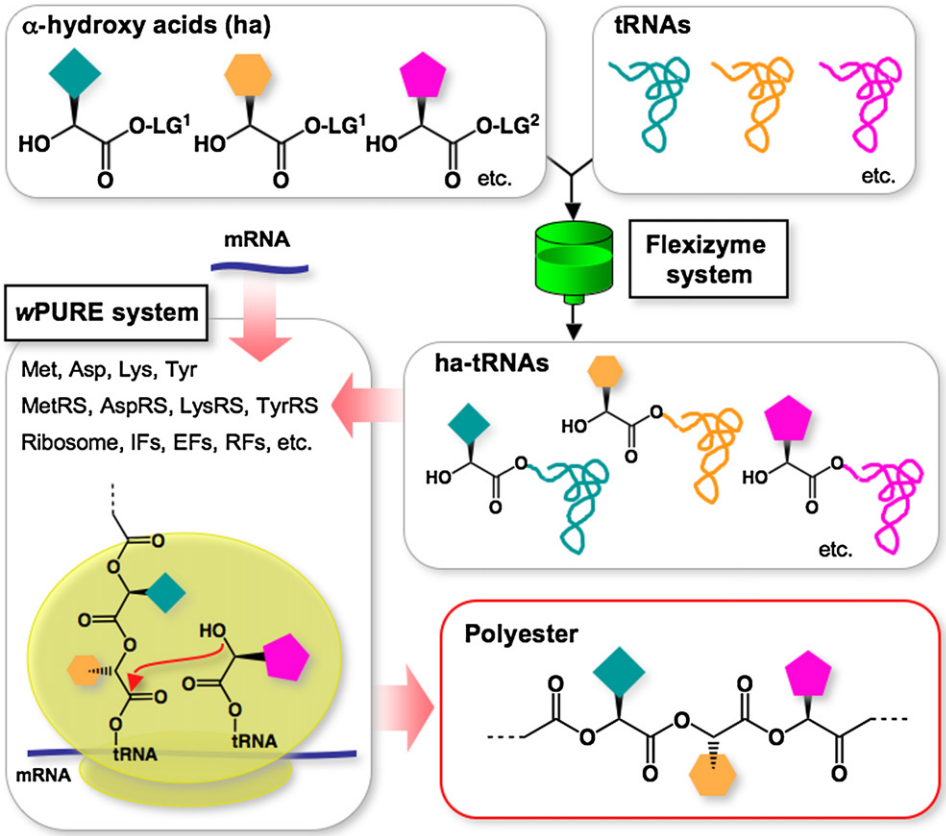

Figure 1. Ribosomal Polyester Synthesis by Means of the Flexizyme (eFlexiresin or dFlexiresin) and *w*PURE Systems

LG^1 and LG^2 indicate cyanomethyl and 3,5-dinitrobenzyl leaving groups, respectively (see Figure 2A). IFs, initiation factors; EFs, elongation factors; RFs, release factors.

the *w*PURE system). Vacant codons can then be reassigned to any desired α-hydroxy acids by using the flexizyme system to charge the α-hydroxy acids to the vacant codon's cognate tRNA. By combining these two technologies, the genetic code can be reprogrammed for the mRNA-directed synthesis of polyesters.

We chose seven α-hydroxy acids, of which four were phenyllactic acid derivatives (F^{lac}, mF^{lac}, and cF^{lac}, pF^{lac}) and three were nonaromatic α-hydroxy acids (G^{lac}, L^{lac}, and A^{lac}), to investigate mRNA template-directed ester polymerization (Figure 2A). We arbitrarily selected seven codons, and their corresponding anticodons were implanted into $tRNA^{Asn\text{-}E1}_{NNN}$ and $tRNA^{Asn\text{-}E2}_{NNN}$ sequences (NNN indicates anticodon sequence; Figures 3A and 3B), with each of the seven codons assigned to one of the seven α-hydroxy acids (Figure 2B). These tRNA sequences were chosen because we have previously shown that they could act as orthogonal tRNAs in the PURE system [14]. We next designed the mRNA template to initiate the polyester synthesis with fMet. For purification and detection purposes, we also incorporated a modified FLAG peptide (KKDYKDDDDK) at the C terminus (vide infra). Accordingly, the *w*PURE system for the polyester synthesis included only four amino acids (Met [M], Lys [K], Asp [D], and Tyr [Y]) and their cognate ARSs. It should be noted that although the translated polyesters would be conjugated with the above polypeptide sequence (i.e., polyester-polypeptide hybrids would be translated), the polypeptide segment was embedded in the polymers strictly for detection and purification purposes. Because the genetic code reprogramming was applied to the polyester segments only, we referred to such polymers as polyesters for the sake of simplicity of our experimental significance.

We first tested whether each tRNA bearing the designated anticodon retained its orthogonality and thereby could incorporate an α-hydroxy acid at a single template-directed position in a model polypeptide, without significant competition from other amino acids present in the translation mixture. Our conventional assay using a microhelix RNA indicated that the flexizyme system was able to efficiently charge all of the α-hydroxy acids onto either $tRNA^{Asn\text{-}E1}_{NNN}$ or $tRNA^{Asn\text{-}E2}_{NNN}$ (see Figure S1 in the Supplemental Data available with this article online). All of the α-hydroxy acid-charged tRNAs retained their orthogonality in the *w*PURE system, unfortunately with one exception: neither $tRNA^{Asn\text{-}E1}_{CUG}$ nor $tRNA^{Asn\text{-}E2}_{CUG}$ bearing a CUG anticodon assigning A^{lac} possessed the expected orthogonality, resulting in the minor incorporation of Lys into the targeted CAG position in the

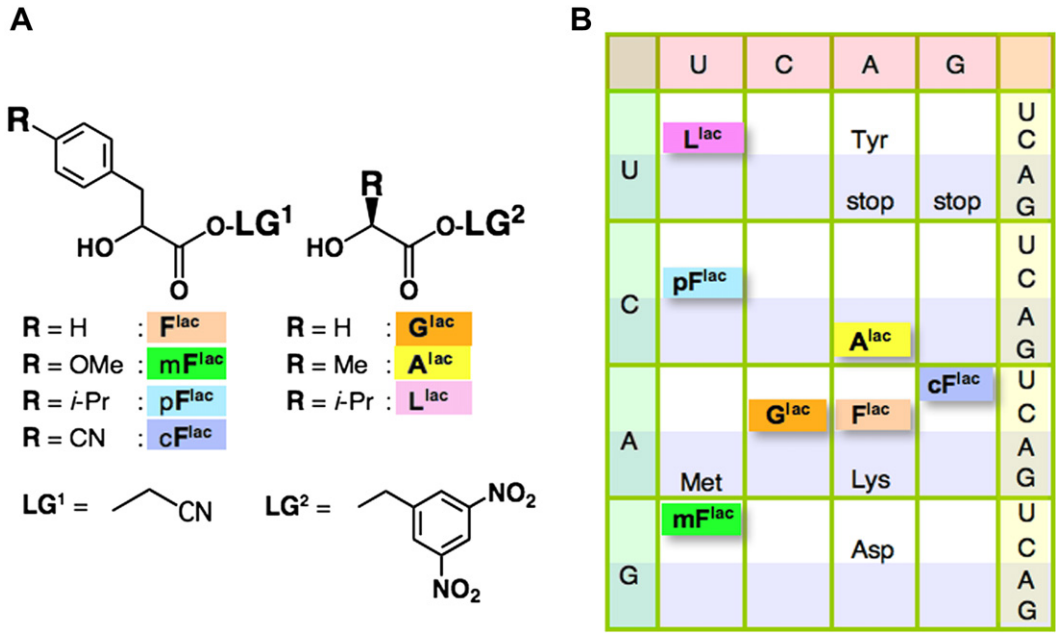

Figure 2. Genetic Code Reprogramming for mRNA-Directed Polyester Synthesis

(A) Chemical structure and abbreviation of α-hydroxy acids used in this study. F^{lac}, phenyllactic acid; mF^{lac}, *p*-methoxyphenyllactic acid; pF^{lac}, *p*-isopropylphenyllactic acid; cF^{lac}, *p*-cyanophenyllactic acid; G^{lac}, glycolic acid; A^{lac}, lactic acid; L^{lac}, isopropyllactic acid. The abbreviations used are based on structurally similar amino acids. The stereochemistry of pF^{lac}, mF^{lac}, and cF^{lac} is racemic, whereas that of other α-hydroxy acids is *S* configuration. All phenyllactic acid derivatives bear a cyanomethyl ester group (LG^1) and are charged onto tRNAs shown in Figure 3 using eFlexiresin, while others bear a 3,5-dinitrobenzyl ester group (LG^2) and are charged onto the tRNAs using dFlexiresin.

(B) The reprogrammed genetic code used in this study. α-hydroxy acids assigned to the respective triplets are color-coded as shown.

polyester-reading frame. Presumably, these tRNAs were susceptible to Lys misacylation by LysRS included in the *w*PURE system, so that the resulting Lys-$tRNA^{Asn\text{-}E1/E2}_{CUG}$ competed with the A^{lac}-$tRNA^{Asn\text{-}E1/E2}_{CUG}$ for incorporation into the polypeptide chain (data not shown). To circumvent this problem, we developed an orthogonal tRNA derived from a *Mycobacteriophage L5* $tRNA^{Asn}_{CUG}$ ($tRNA^{MLAsn}$ in Figure 3C). We found that $tRNA^{MLAsn}_{CUG}$ was not aminoacylated with Lys by LysRS, and thus the misincorporation of Lys was not observed. To this end, we engineered each of the seven anticodons, each assigned to a different α-hydroxy acid, into three different tRNA body sequences to explore the mRNA-directed expression of polyesters (Figures 2B and 3A–3C).

mRNA-Directed Synthesis of Polyesters Containing Four Consecutive Ester Linkages

We designed four nucleotide templates (hereafter referred to as T1–T4) to express polyesters (E1–E4) consisting of four consecutive ester bonds in the *w*PURE system (Figure 4A). As a control, these templates were also translated in the conventional PURE system, and thereby the expression level of polyesters could be compared with that of polypeptides (P1–P4). To detect the expressed products containing polyester, [^{14}C]Asp was included in the *w*PURE system. [^{14}C]Asp was incorporated into the Asp residues in the C-terminal FLAG peptide sequence, and the expressed polyester-[^{14}C]FLAG products were monitored by tricine-SDS-PAGE and autoradiography.

Tricine-SDS-PAGE analysis revealed that expression of E1 was observed only when all ha-tRNAs were present in the *w*PURE system and its expression level was comparable to that of the control peptide, P1 (Figure 4B, lanes 1–5). The molecular mass (ms) of E1 determined by MALDI-TOF analysis was consistent with the calculated ms of the full-length E1 (Figure 5A, E1). Likewise, the respective T2–T4 templates expressed the E2–E4 polyesters with the expected ms (Figure 4B, lanes 6–11; Figure 5A, E2–E4). The expression levels of P1, E1, E2, E3, and E4 were estimated by the incorporation of [^{14}C]Asp by tricine-SDS-PAGE autoradiography analysis considering that five aspartic acids were included in the polymers, giving approximately 6 pmol/5 μl, 9.5 pmol/5 μl, 15 pmol/5 μl, 12 pmol/5 μl, and 5.3 pmol/5 μl, respectively (data not shown). These results provide solid evidence that the length and sequence of the tetrapolyesters are strictly controlled by the mRNA template sequence.

mRNA-Directed Synthesis of Longer Polyesters

We next explored the capability of ribosomes for the synthesis of various lengths of polyesters. In addition to T1 expressing tetrapolyester E1, five templates T5–T9 were designed to express tri-, penta-, hexa-, octa-, and dodecapolyesters E5–E9 (Figure 4A). We were able to detect tripolyester E5 with the expected ms similar to that of tetrapolyester E1 (Figure 5B, E5 and E1), whereas the full-length peak of pentapolyester E6 was barely detectable exhibiting a significantly poor signal/noise ratio, while the

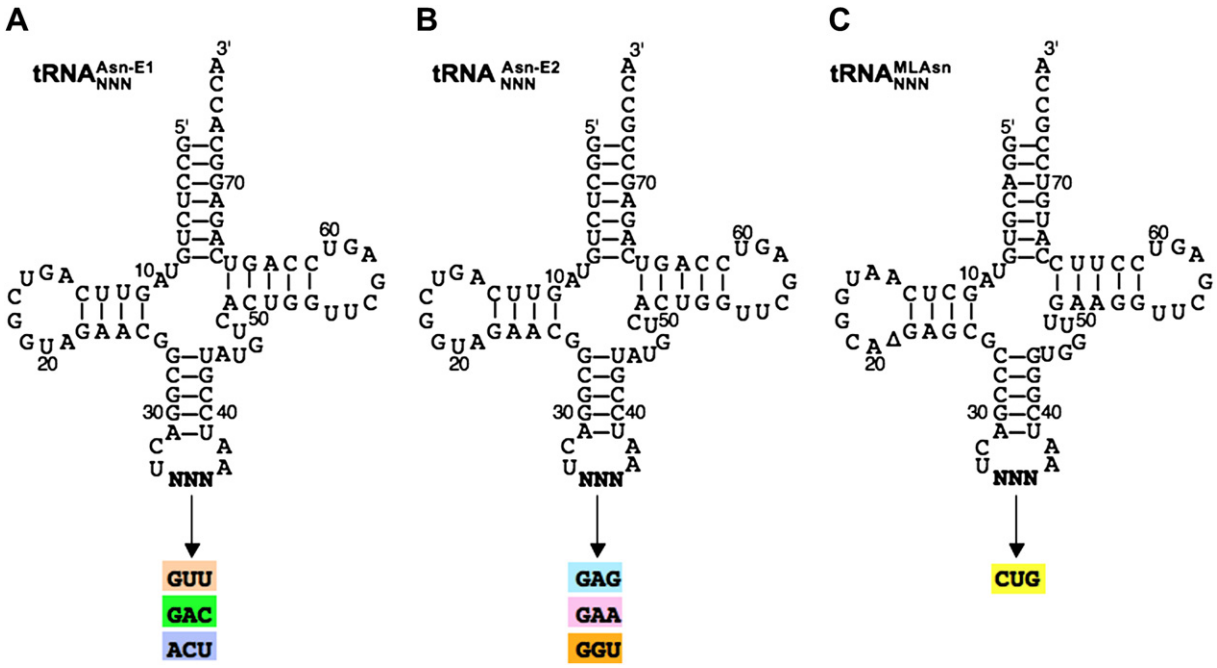

Figure 3. Three tRNA Body Sequences Used in This Study

(A) $tRNA^{Asn\text{-}E1}_{NNN}$.

(B) $tRNA^{Asn\text{-}E2}_{NNN}$.

(C) $tRNA^{MLAsn}_{NNN}$. NNN indicates anticodon and each anticodon is color-coded to pair with the corresponding codon shown in Figure 2B.

full-length peaks of E7–E9 were not detected (Figure S2). We wondered whether the lack of a full-length peak of E7–E9 was due to a significant reduction in polyester expression. Tricine-SDS-PAGE analysis showed that the expression level was slightly diminished as the polyester became longer, yet a detectable level of expression was observed for E6–E9 (Figure 4B, lanes 12–19). Due to the fact that [^{14}C]Asp was incorporated into the C-terminal FLAG sequence, we concluded that the polyester-FLAG could be expressed in all cases. We thus speculated that the full-length E6–E9 might be difficult to ionize under ordinary MALDI-TOF conditions. Unfortunately, attempts under more stringent ionization conditions, such as longer/stronger irradiation or the use of other matrices, resulted in fragmentation of the polyesters (data not shown).

Due to the difficulty in ionization of polyesters longer than a pentapolyester, we modified the strategy to obtain evidence for longer polyesters by MALDI-TOF analysis. The full-length polyester was hydrolyzed under basic conditions to generate shortened fragments. Because the FLAG peptide region could not be hydrolyzed under such conditions, we expected that $(ha)_n$-FLAG (n indicates the number of α-hydroxy acyl groups [ha] remaining after the base hydrolysis) could be detected by MALDI-TOF. Indeed, upon analysis of the hydrolyzed products, we were able to observe the $(ha)_2$-FLAG product as a major peak for E6–E9, occasionally with a minor peak of ha-FLAG (Figure 5B, E6–E9). Most importantly, the observed ms for each of the $(ha)_2$-FLAG products hydrolytically generated from the full-length E6–E9 was consistent with the expected ms of $(ha)_2$-FLAG encoded by the respective mRNA sequence (Figure 4A). Taken together, our findings show that polyesters can be continuously synthesized up to dodecapolyester in accordance with the mRNA template.

DISCUSSION

The flexizyme system is a highly flexible acylation tool that enables us to charge various acids onto any desired tRNAs. By means of this system, we were able to readily prepare tRNAs charged with a variety of α-hydroxy acids (ha-tRNAs). The various ha-tRNAs were then utilized in a special reconstituted cell-free translation system in which unneeded amino acids and their corresponding ARSs are withdrawn from the translation mixture (referred to as the *w*PURE system). Removal of such components creates vacant codons which can then be reassigned to specific α-hydroxy acids using the flexizyme system to generate ha-tRNAs that designate the respective codons. Thus, by combining these two technologies, we could use ribosomes for the synthesis of mRNA template-designed polyesters. Our data clearly show that polyesters of up to 12 α-hydroxy acids in length could be synthesized with the length and sequence defined by the mRNA template.

Currently, it is not clear what hindered the synthesis of polyesters longer than 12 α-hydroxy acids, yet based on this study and our other unpublished work on the incorporation of exotic amino acids into the nascent peptide

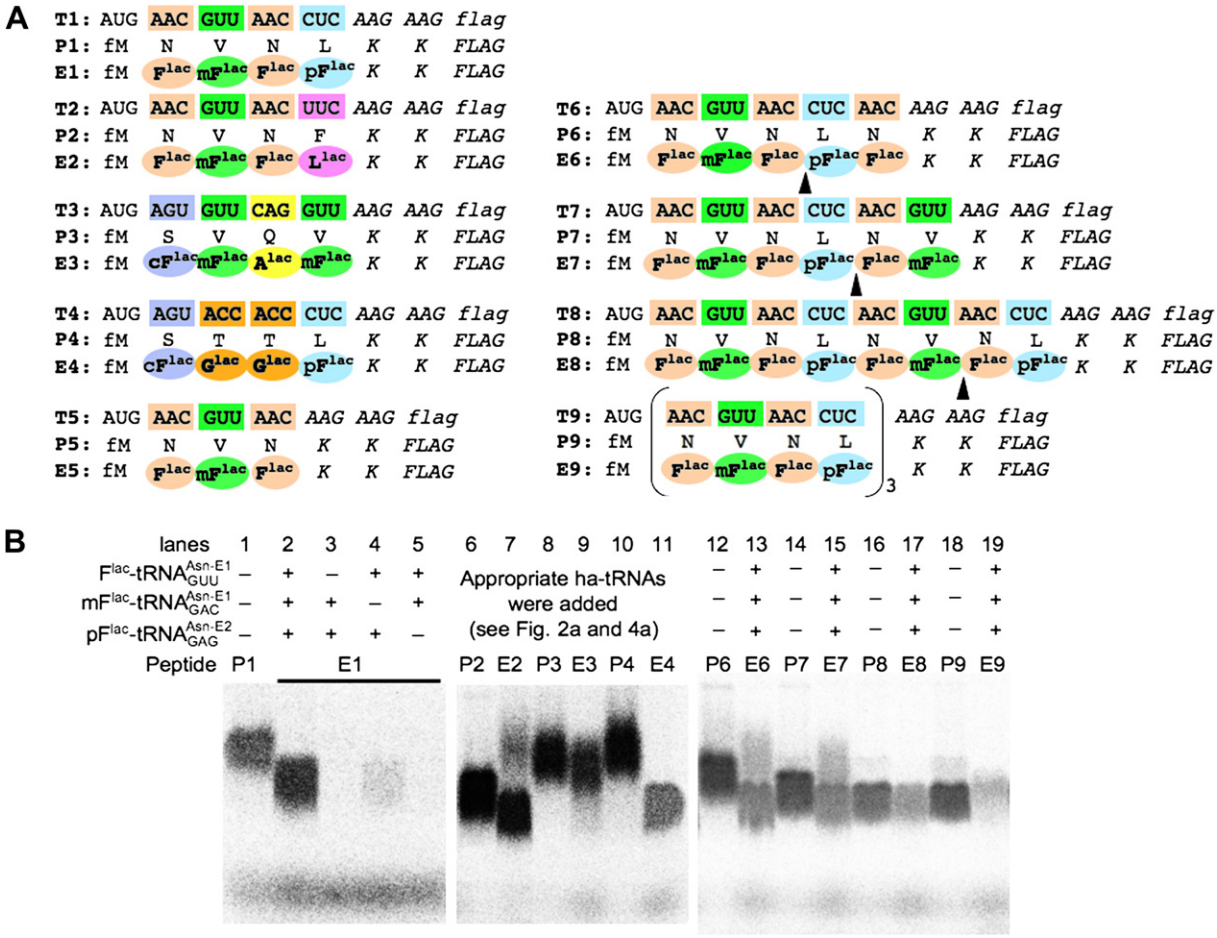

Figure 4. Polyester Synthesis

(A) Sequences of mRNA templates (T1–T9), polypeptides (P1–P9), and polyesters (E1–E9). Reprogrammed codons in mRNA (rectangles) and α-hydroxy acids (circles) are highlighted in colors matching those in Figure 2B. Black arrowheads indicate the positions of hydrolyzed ester bonds that give $(ha)_2$-FLAGs observed in Figure 5B.

(B) Tricine-SDS-PAGE analysis of the expressed products. The products, labeled with [^{14}C]Asp in the C-terminal FLAG peptide, were detected by autoradiography. Due to the basic conditions (pH 8.5) of the tricine-SDS-PAGE running buffer, each observed band derived from E1–E9 was likely the corresponding $(ha)_n$-FLAG peptide(s) generated by the hydrolysis of full-length polyester. Note that the mobility of the peptide or polyester-peptide hybrid was dependent upon its composition, that is, the net charge or hydrophobicity, and therefore the peptide mobility did not accurately reflect the peptide length. The combination of ha-tRNAs used in each lane for polyester synthesis was as follows: lane 7, F^{lac}-$tRNA^{Asn\text{-}E1}_{GUU}$, mF^{lac}-$tRNA^{Asn\text{-}E1}_{GAC}$, and L^{lac}-$tRNA^{Asn\text{-}E2}_{GAA}$; lane 9, cF^{lac}-$tRNA^{Asn\text{-}E1}_{ACU}$, mF^{lac}-$tRNA^{Asn\text{-}E1}_{GAC}$, and A^{lac}-$tRNA^{MLAsn}_{CUG}$; lane 11, cF^{lac}-$tRNA^{Asn\text{-}E1}_{ACU}$, G^{lac}-$tRNA^{Asn\text{-}E2}_{GGU}$, and pF^{lac}-$tRNA^{Asn\text{-}E2}_{GAG}$.

chain, we propose the following possibility. According to various experiments examining the kinetics of a single elongation of an α-hydroxy acid, the ester bond transfer rate is estimated to be at least one order of magnitude slower than its peptide counterpart [20, 21]. Moreover, the dissociation constant of F^{lac}-$tRNA^{Phe}$ (K_d = 30 μM) with EF-Tu-GTP is about two orders of magnitude higher than that of Phe-$tRNA^{Phe}$ (K_d = 100 nM) [22]. Even though we attempted to compensate for the poor affinity of ha-tRNA for EF-Tu by increasing the concentration of ha-tRNA, these efforts were insufficient. Therefore, this combination of a slow esteryl transfer rate for α-hydroxy acids with the poor affinity of ha-tRNA for EF-Tu likely causes the ribosome to stall during the translation of long polyesters, possibly resulting in the frequent dissociation of the translation complex and polyesteryl-tRNA molecule from the mRNA template. Our observation that polyester yield correlated with polyester length appears to support this hypothesis. Detailed studies in the future should provide not only more insights into the mechanism but also insights into how to engineer the translation system toward a more efficient synthesis of longer polyesters.

One obvious strategy toward generating a more efficient translation system for polyester synthesis would be to engineer EF-Tu or orthogonal tRNAs to have a tighter binding affinity [23, 24]. Another possible strategy is to generate a mutant ribosome capable of catalyzing the esteryl transfer reaction more efficiently [25–27]. Thus, if we were able to optimize the affinity of ha-tRNA for EF-Tu and ribosomes by selection or increase the

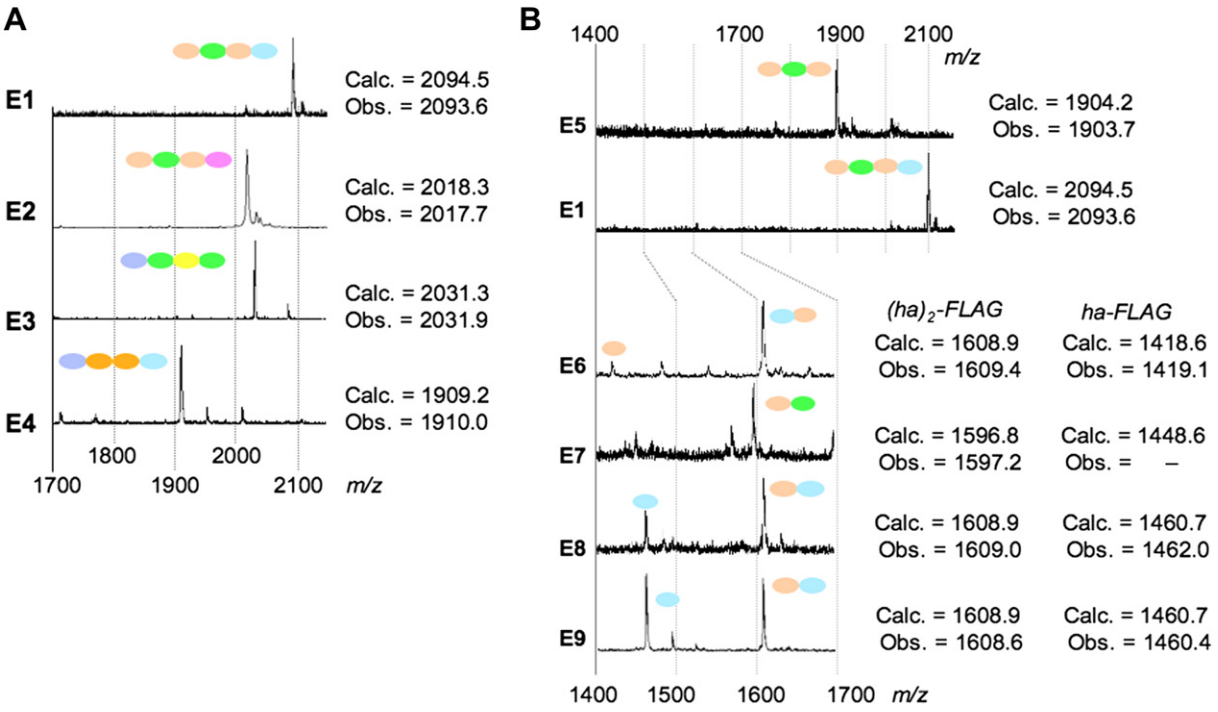

Figure 5. MALDI-TOF Analysis of Polyesters

(A) MALDI-TOF spectra of E1–E4. Calculated mass (M+H) and observed mass (M+H) are presented with the polyester moiety color-coded based on the reprogrammed genetic table shown in Figure 2A.

(B) MALDI-TOF spectra of E1 and E5–E9. Calculated mass (M+H) and observed mass (M+H) of full-length (E1 and E5) or $(ha)_2$-FLAG and ha-FLAG (E6–E9) are shown for each spectrum. The fragmented N-terminal products were removed during the FLAG-purification step, and therefore only the C-terminal fragments appear in the spectra.

efficiency of ribosomes for the esteryl transfer reaction, the translation system could be turned into a more efficient template-directed synthesizer for polyesters or even other nonpeptide biopolymers.

In light of the fact that polyesters have significantly different properties compared to peptides in terms of plasticity and rigidity, it is intriguing to investigate polyesters and polyester-polypeptide hybrid biopolymers engineered with a wide variety of side chains in strictly controlled sequences and lengths. However, because it has been difficult to synthesize such biopolymers with a variety of side chains, such biopolymers have not been well studied. The methodologies described in this report should open the door for the synthesis of a wide variety of polyesters and hybrid polymers, and thus will enable us to screen them for new possible functions and applications in the future.

SIGNIFICANCE

We have reported the mRNA-directed synthesis of polyesters, composed of several different α-hydroxy acids, whose sequence and length are fully controlled through reprogramming of the genetic code. This approach should be extendable to programmed synthesis of other types of backbone-modified biopolymers, such as polypeptide-ester hybrids and N-methyl-polypeptides.

EXPERIMENTAL PROCEDURES

α-Hydroxy Acid Substrate Synthesis

L-phenyllactic acid (F^{lac}) and lactic acid (A^{lac}) were purchased from Sigma-Aldrich and glycolic acid (G^{lac}) was purchase from Wako, Japan. *p*-isopropylphenyllactic acid (pF^{lac}), *p*-methoxyphenyllactic acid (mF^{lac}), and *p*-cyanophenyllactic (cF^{lac}) acid were synthesized from *p*-isopropylbenzaldehyde, *p*-methoxybenzaldehyde, and *p*-cyanobenzaldehyde, respectively, as previously reported [28]. *p*-isopropyllactic acid (L^{lac}) was synthesized as previously described [29]. All of the phenyllactic acid derivatives were converted to cyanomethyl ester (LG^1), and G^{lac}, A^{lac}, and L^{lac} were converted to dinitrobenzyl ester (LG^2) by the procedure reported elsewhere [14, 17].

Hydroxyacyl-tRNA Synthesis

For the hydroxyacylation of phenyllactic acid-LG^1 derivatives (F^{lac}, mF^{lac}, pF^{lac}, and cF^{lac}) eFlexiresin was used, whereas for other α-hydroxy acid-LG^2 derivatives (G^{lac}, L^{lac}, and A^{lac}) dFlexiresin was used. Due to the fact that tRNA hydroxyacylation was performed in the same manner regardless of either eFlexiresin or dFlexiresin, we refer to these resins commonly as Flexiresin in the protocol described below. Note that a general protocol for the preparation of Flexiresin is described in Supplemental Experimental Procedures. Hydroxyacylation was carried out as follows: a solution of 20 μl of 50 μM tRNA and 3 μl of 1 M Tris-HCl (pH 8.0) was heated at 95°C for 2 min and cooled to room temperature over 5 min. Six microliters of 3 M $MgCl_2$ was added to this mixture and the resulting solution was then loaded onto 10 μl (resin volume) of Flexiresin. After a suspension was made, the mixture was placed on ice and incubated for 5 min. The charging reaction was initiated by the addition of 1 μl of 300 mM α-hydroxy acid activated with an appropriate leaving group (LG^1 or LG^2) in

DMSO, and the mixture was incubated for 3 hr on ice. When *p*-isopropyllactic acid-LG[2] (L^{lac}) was used as a substrate, the reaction was incubated for 16 hr. Following the incubation, the supernatant was removed and Flexiresin-bound ha-tRNA was eluted with 30 μl of elution buffer (50 mM HEPES-K [pH 7.5], 10 mM EDTA) four times. To the pooled eluate 13.3 μl of 3 M NaOAc (pH 5.2) was added, and the resulting solution was precipitated with 280 μl of ethanol. After centrifugation, the resulting pellet was washed with 70% ethanol and dried under vacuum. Typically, the resulting ha-tRNA was dissolved in 0.5 μl of water to perform the expression of polyesters.

mRNA-Directed Polyester Synthesis

The *w*PURE system contains all the necessary components for translation except for AlaRS, ArgRS, AsnRS, CysRS, GlnRS, GluRS, GlyRS, HisRS, IleRS, LeuRS, PheRS, ProRS, SerRS, ThrRS, TrpRS, ValRS, and all 20 standard amino acids. To 12.6 μl of the above *w*PURE system was added 3.0 μl of 0.4 μM template DNA, 0.5 μl of 180 mM EDTA (pH 8.0), 3.0 μl of the mixture of Met, Tyr, and Lys (2 mM each), and 2.4 μl of 2 mM Asp (for MALDI-TOF analysis) or 500 μM [^{14}C]Asp (for tricine-SDS-PAGE assay). The mixture of ha-tRNAs (approximately 1000 pmol each) dissolved in 1.5 μl of 1 mM NaOAc (pH 5.2) was added to 3.5 μl of the above stock solution (total 5 μl translation volume) and the resulting mixture was incubated for 3 hr at 37°C. The synthesis of polyesters was then analyzed by the following procedures.

SDS-PAGE Analysis

The gel dimensions were as follows. The lengths of separation and stacking gels were approximately 55 and 10 mm, respectively, and the width and thickness were 83 and 0.75 mm, respectively. Separation gels contained 15% acrylamide (acrylamide:bisacrylamide = 19:1), 1 M Tris-HCl (pH 8.5), 0.1% SDS, and 13% glycerol, whereas the stacking gels contained 4% acrylamide (acrylamide:bisacrylamide = 29:1), 0.75 M Tris-HCl (pH 8.5), and 0.075% SDS. Electrophoresis was performed for 80 min under 20 mA constant mode. The anode running buffer was 200 mM Tris-HCl (pH 8.9), whereas the cathode running buffer contained 100 mM Tris, 100 mM tricine, and 0.1% SDS (the resulting buffer was approximately pH 8.3). The products labeled with [^{14}C]Asp in the C-terminal FLAG peptide were quantified by autoradiography using an image analyzer (FLA-5100, Fuji, Japan).

MALDI-TOF Analysis

The expressed product was incubated with FLAG-M2 agarose (Sigma) and the resin was washed with 50 μl of washing buffer (50 mM MOPS-K [pH 7.0], 150 mM NaCl). The immobilized product was eluted with 10 μl of 0.2% TFA. The purified product was then desalted with $ZipTip_{\mu\text{-}C18}$ (Millipore), and eluted with 1 μl of a 50% MeCN, 0.1% TFA solution saturated with the matrix *R*-cyano-4-hydroxycinnamic acid. MALDI-MS measurement of each product was performed using Autoflex II TOF/TOF (Bruker Daltonics) under the linear/positive mode and externally calibrated with Substance P (1347.5354 Da), Bombesin (1619.8223 Da), ACTH clip (1–17) (2093.0862 Da), and ACTH clip (18–39) (2465.1983 Da) as standards.

Supplemental Data

Supplemental Data include two figures and Supplemental Experimental Procedures and can be found with this article online at http://www.chembiol.com/cgi/content/full/14/12/1315/DC1/.

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