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Combinatorics of peptide sextets encoded by a single microgene

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Abstract

Genetic information stored in DNA sequences is translated into protein by linking a triplet nucleotide sequence and an amino acid. Because the frames of the triplets can be configured in three ways, a total of six polypeptides, each with a different sequence, can be produced from a single double-stranded DNA molecule. We recently developed the **MolCraft** system [reviewed in K. Shiba, J. Mol. Catal. B 18 (2004) xxx], which enables us to make combinatorial polymers of three peptides translated from one strand of a double-stranded DNA molecule. To explore all the information that a single double-stranded DNA molecule encodes, we have now developed a new system, **La-MolCraft**, in which all six reading frames encoded by both strands are combinatorially polymerized using loop-mediated isothermal amplification of DNA (LAMP) [Nucl. Acids Res. 28 (2000) E63].
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1. Introduction

Analytical studies of the structures of existing proteins have shown that they are constructed from assemblages of smaller sub-structural units often referred to as “modules” [1] or “domains” [2,3]. Most likely, these sub-structural units correspond to smaller genetic units that have, through combinatorial polymerization, come to make up larger modern genes [4]. It is thus plausible that the genes encoding extant proteins developed from smaller primordial genes (microgenes) that emerged from random nucleotide sequences [5,6] or from repeats of short oligonucleotides [7,8] whose translational products (polypeptides) had only weak biological activities. Eventually, combinatorial assemblages of these primordial microgenes evolved into larger modern genes that have more convoluted and unequivocal activities. The advantage of such “hierarchical evolution” in the creation of new proteins has already been demonstrated in an in silico evolution experiment [9], and now much effort is being made to apply the concept of hierarchical evolution or “block shuffling” as an underlying principle of in vitro protein evolution [10–19].

MolCraft is a simplified protein evolution system based on a hierarchical approach that we recently developed [20,21]. In **MolCraft**, a short DNA sequence (microgene) is initially evolved in silico. This microgene is then tandemly polymerized using the MPR method [20] to prepare a library of larger artificial proteins. Because the MPR method randomly inserts or deletes nucleotides at end-joining junctions, translational products of the microgene polymers are combinatorial polymers of three reading frames of a single microgene. The fact that the artificial proteins produced with **MolCraft** have properties also observed in natural proteins suggests that “repetitiousness” contributes the emergence of structured proteins [22,23].

In **MolCraft**, a microgene unit is polymerized in a “head-to-tail” manner [20], which means that linkage between one reading frame coded by a “+” DNA strand and another coded by a “−” DNA strand (where “+” and “−” are arbitrarily designated) can never occur. Consequently, molecular diversity obtained from a single microgene is limited to the combinatorics of three frames but not the entire six frames that a single microgene possesses in its “+” and “−” strands.

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Our aim in the present study was to explore all of the information that a single microgene can encode by establishing a new microgene polymerization system in which all six reading frames of a microgene are combinatorially polymerized. For this purpose, we applied the recently established loop-mediated isothermal amplification of DNA (LAMP) method, which can produce inverted repeats of a target DNA through the use of a DNA polymerase and a set of four specially designed primers [24].

2. Experimental

2.1. Design of microgenes

We designed a 105 bp microgene, MG-51, within which we encrypted three biological functions, two into the +DNA strand and one into the −DNA strand. The encrypted motifs were: (i) a GluGlu epitope tag (EEEEYMPME) derived from a polyoma virus medium T antigen [25] in the +1 coding frame (shown in dark blue in Figs. 1 and 2); (ii) an S-peptide (KETAAAKFERQHMDS) corresponding to the N-terminal 1–15 residues of RNase S, which binds to S-protein (RNase S residues 21–124) to reconstitute RNase activity [26–28], in the −1 coding frame (shown in dark red in Figs. 1 and 2); and (iii) a LacZ mini-α motif (LQRRDWENPGT) derived from residues 11–21 of the LacZ α fragment in the +1 coding frame (shown in dark green in Figs. 1 and 2). The full-length LacZ α fragment is known to reconstitute β-galactosidase activity by combining with the ω-fragment of LacZ [29,30]; however, the functionality of the mini-α motif had not been investigated until the present study. Using the CyberGene program (unpublished), we chose codons for the three reading frames such that the microgene contained no termination codons within any of its six reading frames, or at junctions, and the peptides coded by the different coding frames had a propensity to form α-helix.

2.2. Preparation of LAMP template

Two single-stranded oligonucleotides, KY-1299 (5′-GGA AGA TCT GAG GAG GAG GAG TAT ATG CCG ATG GAG GCT GTC CAT ATG TTG GCG CTC GAA CTT CGC CGC-3′) and KY-1300 (5′-GCC GGT ACC TGT TCC AGG ATT TTC CCA GTC TCT TCG CTG CAA AAG GAG ACG GCG GCG GCG AAG TTC GAG CGC C-3′), which have a complement region at their 3′ ends, were synthesized. A 200 pmol aliquot of each oligonucleotide was heated to 95 °C for 3 min in 50 μl of 10 mM Tris–HCl (pH 8.0) and annealed by slow (2 °C/min) cooling to 37 °C. Double-stranded MG-51 was then synthesized by adding 10 units of *E. coli* Klenow fragment (New England

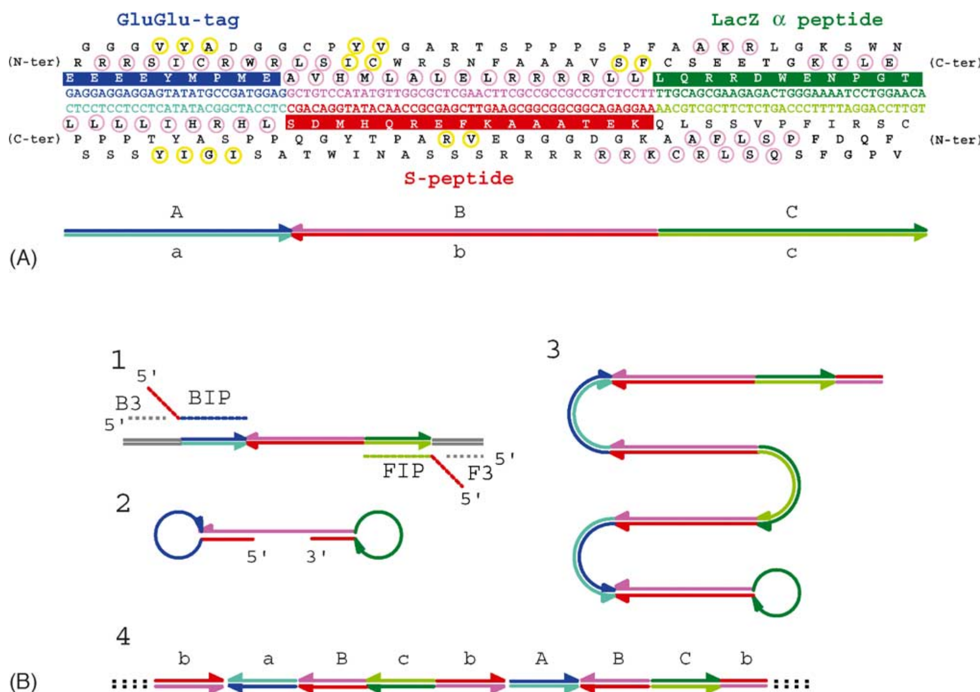

Fig. 1. A microgene and its polymerization using the LAMP method. (A) Sequences of MG-51 and its translation products. The GluGlu-tag peptide sequence is shaded in blue, and the corresponding sense and antisense DNA sequences are shown in blue and cyan, respectively. The S-peptide sequence is shaded in red, and the corresponding sense and antisense DNA sequences are shown in red and magenta, respectively. The mini-α motif is shaded in green, and the corresponding sense and antisense DNA sequences are shown in green and lime green, respectively. The predicted secondary structures of the peptide sequences (excluding embedded motifs) are showed in pink circles (alpha helix) and yellow circles (beta-strand). (B) Scheme for the LAMP reaction; details are provided in Notomi et al. [24]. (1) Four primers (BIP, FIP, B3 and F3) anneal to the target DNA and synthesize new DNA through strand displacement DNA synthesis. (2) Dumb-bell-like intermediate in the LAMP cycle. (3) Late stage intermediate in the LAMP cycle. (4) Schematic representation of the combinatorial assemblage of three encrypted motifs.

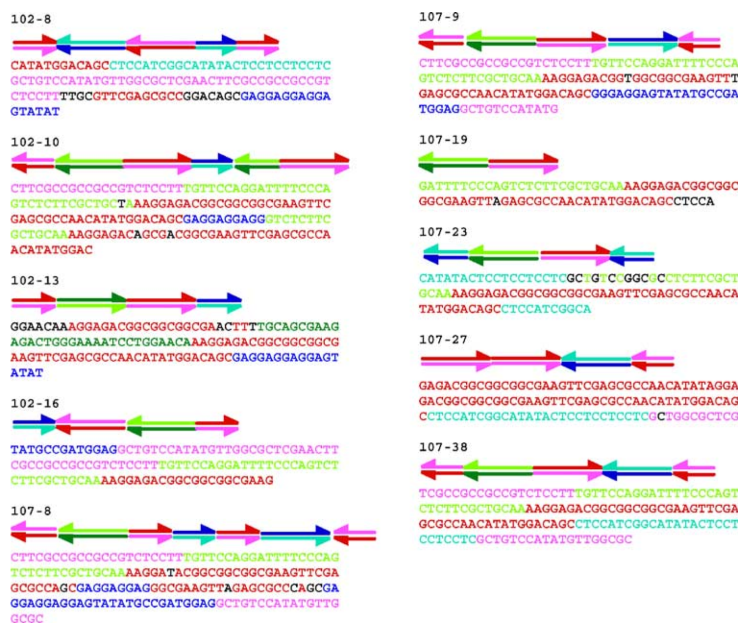

Fig. 2. Sequences of 10 cloned LAMP products. Nucleic acid sequences are colored as described in the legend to Fig. 1. The organization of the motifs is shown as in Fig. 1; the letters in black indicate nucleotide substitutions or sequences of unknown origin.

Biolabs, Beverly), 10 units of T4 polynucleotide kinase (TOYOBO, Osaka), final 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 μg/ml BSA, 200 μM ATP and 200 μM dNTPs to the annealed primers. The synthesized double-stranded DNA was phosphorylated at its 5′ end [31] and cloned into the *Sma*I site of pTZ19R [32], yielding plasmid pKK080. The integrity of the cloned MG-51 was confirmed by DNA sequencing using a DTCS cycle sequencing kit (Beckman Coulter, Fullerton) with a CEQ2000 XL genetic analyzer (Beckman Coulter).

2.3. LAMP reaction

The LAMP method requires a set of four specially designed primers for target amplification [24]. The primers used in the present study were: BIP, KY-1301 (5′-GAG CGC CAA CAT ATG GAC AGC GAG GAG GAG GAG TAT ATG CCG ATG GAG-3′); FIP, KY-1302 (5′-CTT CGC CGC CGC CGT CTC CTT TGT TCC AGG ATT TTC CCA GTC TCT TCG CTG CAA-3′); B3, KUNO-22 (5′-AGC GGA TAA CAA TTT CAC ACA GGA-3′); and F3, KUNO-21 (5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′). The LAMP reaction was initiated by mixing BIP (20 pmol), FIP (20 pmol), B3 (5 pmol) F3 (5 pmol) and pKK080 plasmid DNA (1 ng) in 25 μl of 40 mM Tris–HCl (pH 8.8), 20 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.2% Tween20, 0.8 M betaine, 1.4 mM dNTPs, 2 units of *Bst* (exo^-) DNA polymerase (New England Biolabs) and 2 units of Vent (exo^+) DNA polymerase (New England Biolabs). The amplification reaction was continued under isothermal conditions, 63 °C for 2 h (*Bst* DNA polymerase) or 12 h (*Bst* DNA polymerase + Vent DNA polymerase) and terminated by heating the reaction mixture at 80 °C for 10 min.

2.4. Cloning LAMP products

The end product of the LAMP reaction was a large stem-loop DNA that could not be cloned into a vector without modification. To obtain a double-stranded, blunt-ended DNA, the single-stranded loop regions were digested with Mung bean nuclease (MBN) [31]. Approximately 5 μg of the LAMP product were treated for 15 min at 25 °C with 5 units of MBN (TaKaRa-Bio, Shiga) in 50 μl of 30 mM sodium acetate (pH 5.0), 100 mM NaCl, 1 mM zinc acetate and 5% glycerol. After the reaction was terminated by addition of 1 μl of 0.5 M EDTA, the mixture was neutralized with 10 μl of 1 M Tris–HCl (pH 9.5), extracted using phenol–chloroform followed by chloroform, and separated using 2.0% TAE-agarose (NuSieve 3:1, BioProducts, Rockland) gel electrophoresis. DNA fragments 300–500 bp in size were recovered from the gel and purified using a GeneClean II kit (Qbiogene, Carlsbad). The purified blunt-ended, phosphorylated DNAs were then ligated into the *Sma*I site of pTZ19R [32].

2.5. Protein expression

The 158 bp *Hind*II–*Eco*RI fragment from pKK102-13, which contained the cloned LAMP product, was subcloned into the *Sma*I and *Eco*RI sites of pGEX-2T [33], yielding the GST-fusion plasmid pKK118. Similarly, the 134 bp *Asp*718–*Bam*HI fragment of pKK102-8 was purified and blunt-ended with Klenow fragment [31], cloned into the *Ava*I site of pGEX-2T, which had been blunt-ended with Klenow fragment prior to the ligation, to obtain pKK119. The integrity of pKK118 and pKK119 was confirmed by sequencing.

To express GST-fusion proteins, *E. coli* XL1-blue harboring pGEX-2T, pKK118, and pKK119 were inoculated into 1 ml of LB medium [31] containing 50 μg/ml carbenicillin (Sigma Chemicals, St. Louis) and incubated overnight at 37 °C with shaking. To induce the proteins, 1 ml of pre-warmed LB medium containing 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 50 μg/ml carbenicillin were added to the cultures, which were then incubated at 37 °C for an additional 2 h.

2.6. Detection of artificial proteins

Cells were harvested from 100 μl of IPTG-induced cultures by centrifugation, resuspended and boiled in 100 μl of SDS-PAGE sample buffer (0.125 M Tris–HCl, pH 6.8, 10% 2-mercaptoethanol, 10% sucrose, 0.02% bromophenol blue), after which 10 μl samples were subjected to SDS-PAGE using PAG-mini 15/25 pre-cast minigels (Daiichi Pure Chemicals, Tokyo). The gels were then stained with Phast-Gel Blue R stain Coomassie R 350 dye (Amersham Biosciences, Uppsala). For Western blotting, 1 μl samples were electrophoresed and then transferred to immobilon-P PVDF membranes (Millipore, Billerica) using with Panther semidry electroblotter (Owl Separation Systems, Portsmouth) and a semi-dry electroblot buffer kit (Owl Separation Systems). The primary antibodies used to detect recombinant proteins were anti-GST (Amersham Biosciences), anti-S-probe(K-14) (Santa Cruz Biotechnology, Santa Cruz), and goat anti-GluGlu (Bethyl Laboratories, Montgomery) antibodies; the secondary antibodies were HRP-conjugated anti-goat-Ig (Organon Teknica, Durham) and anti-rabbit-IgG (Amersham Biosciences) polyclonal antibodies. Immunoreactants were visualized using ECL-plus (Amersham Biosciences) and LumiVision Pro chemiluminescence detection CCD (Aisin Seiki, Aichi).

S-peptide complementation assays were carried out using an S-tag rapid detection kit (Novagen, Madison). Cells were harvested from 1 ml of IPTG-induced culture, resuspended in 200 μl of 1% SDS, and heated at 70 °C for 10 min to obtain a crude extract. Two microliters of the extract were then diluted 10 times with distilled water and mixed with 400 μl of 20 mM Tris–HCl (pH 7.5), 100 mM NaCl and 0.1 mg/ml poly(C). After prewarming the mixture to 37 °C, the RNase reaction was started by adding 500 ng of S-protein. The reaction was continued at 37 °C for 5 min and then terminated by adding 100 μl of ice-cold 25% trichloroacetic acid. After centrifugation, OD_{280} was measured as an index of ribonuclease activity.

3. Results

3.1. Combinatorial assemblages of frames coded from "+" and "−" strands

The LAMP method was developed by Notomi et al. with the aim of achieving rapid amplification of a target DNA under isothermal conditions [24]. One of its intriguing properties is that the resultant polymers contain inverted repeats of the target sequences, which enables physical linkage between frames encoded by "+" and "−" strands of the DNA (Fig. 1B). We have been interested in the possibility that this unique property of LAMP could be used to develop a novel protein evolution system in which all six reading frames from a single microgene could be combinatorially polymerized. What follows is a description of our pilot experiment.

We designed a 105 bp microgene (MG-51) in which the "+" strand coded for the GluGlu epitope motif [25] and part of the LacZ α-peptide [29,30], while the "−" strand coded for the S-peptide of RNase S [28] (Fig. 1A). After cloning MG-51 into a vector, its sequence was amplified using LAMP with four specifically designed primers, BIP, FIP, B3 and F3 (see Section 2). BIP and FIP contain sequences from both the "+" and "−" strands of the target and initiate the LAMP reaction; B3 and F3 were used for strand displacement synthesis [24] and were complementary to vector sequences in this experiment. The amplified DNAs produced with LAMP had a large stem-loop structure (Fig. 1B) that had to be degraded before the resultant blunt-ended products were cloned into a vector.

Although the LAMP reaction targeted to MG-51 produced apparently large DNAs that migrated very slowly when subjected to agarose gel electrophoresis (data not shown), we obtained no clones with inserts larger than 220 bp (data not shown). Our ability to obtain larger inserts was not improved by using *E. coli* host strains SURE or STBL2, which stabilize DNA having inverted or tandem repeats [34,35]. At the moment, we do not know what hindered the cloning of larger LAMP products, but it is plausible that recombination or deletion of palindromic and repetitive sequences might have reduced the size of stably maintainable sequences [36]. Despite this limitation, inserts with sizes ranging from 46 to 214 bp were successfully cloned, and sequence analyses revealed that polymerization of the "+" and "−" strands of MG-51 had occurred as expected (Fig. 2). For example, clone 102-8 starts with a "+" strand, shifts a "−" strand, and ends with a "+" strand (Fig. 2).

We did encounter two unexpected results, however. First, there were a large number of nucleotide substitutions in the clone products, in particular when *Bst* (exo^-) DNA polymerase was used as the sole polymerase in the LAMP reaction (data not shown). Most of nucleotide substitutions were C → A transversions at position 49 of MG-51, which is located at the very end of the BIP primer in the LAMP intermediate (Fig. 1B(2)), suggesting the terminal dA addition by *Bst* (exo^-) DNA polymerase [37] caused the substitutions. We then used a mixture of *Bst* (exo^-) and Vent (exo^+) DNA polymerase with the idea that the terminal dA could be edited by Vent (exo^+) DNA polymerase [38]. We found that this reduced, but did not completely suppress, the appearance of nucleotide substitutions (data not shown).

The second unexpected observation was that the polymers obtained contained many deletions, resulting in them having

pKK118

(GST)...GSPRLEDPRNKGDGGGELLQRRDWENPGTKETAAAKFERQHMDSEEEEYMVPSSNSS*

pKK119

(GST)...GSPGTIYSSSSLSGARTQKETAAAKFERQHMDSEEEEYMPMEAVHMGIPGIHRD*

(A)

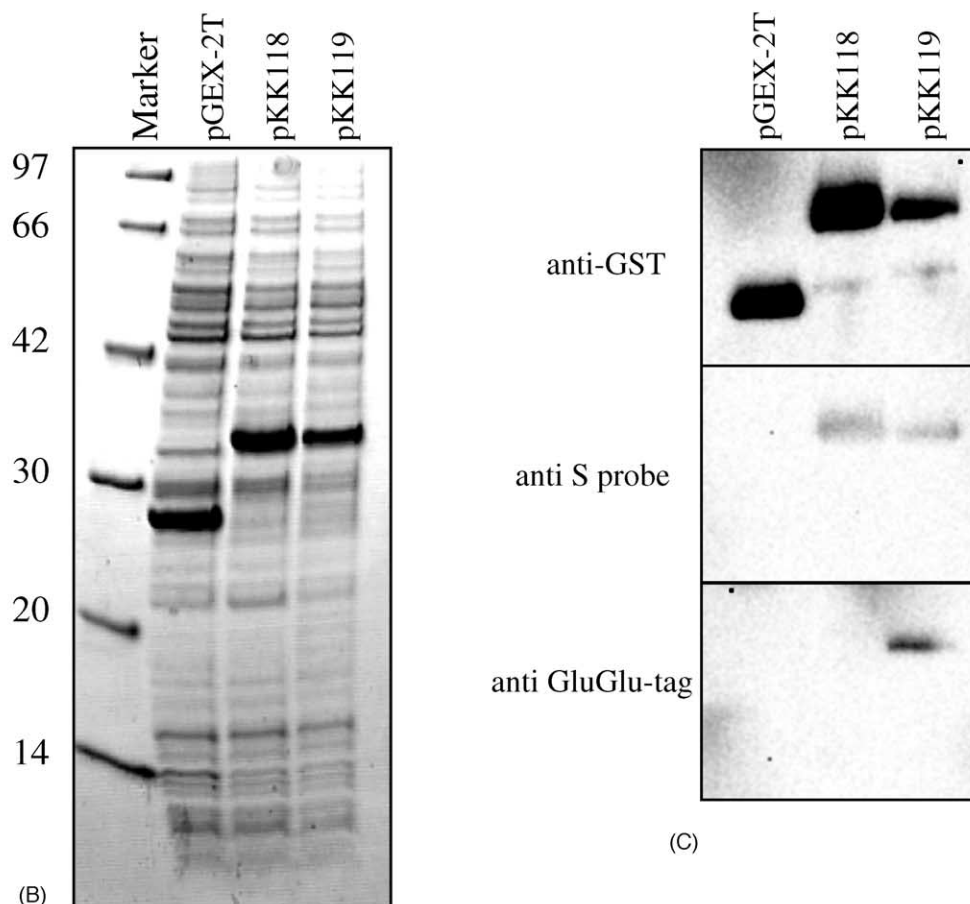

Fig. 3. Artificial proteins created by combinatorial assemblage of frames encoded by different strands of MG-51. (A) Primary structures of MG-51 polymers. (B) Total proteins from *E. coli* harboring the indicated plasmid were separated by SDS-PAGE and detected by CBB staining. Molecular weights of marker proteins are shown on the left. (C) Western blot of total proteins: GST (upper), S-peptide (middle) and GluGlu-tag (lower) were probed with the appropriate antibodies.

convoluted structures that were not seen in polymers created with **MolCraft**. Although the cause is not yet known, it might be that deletions were due to instability of inverted DNA repeats within the inserts.

3.2. Expression of encrypted activities on artificial proteins

To evaluate the functionality of the artificial proteins, we chose two clones, 102-8 and 102-13, and translated one reading frame from each as a fusion protein with GST (Fig. 3A). Both plasmids produced stable fusion proteins (Fig. 3B and C, top) whose apparent molecular weights agreed with those predicted from the primary sequences. The artificial protein from pKK119 contained both the S-peptide and the GluGlu epitope, which were encrypted into different strands of MG-51 (Fig. 1A), whereas the protein translated from pKK118 contained only the S-peptide. Western blot analyses with anti-S-peptide and anti-GluGlu epitope antibodies confirmed the presence of these motifs within the artificial proteins (Fig. 3C). Moreover, the S-peptides within both artificial proteins reconstituted RNase activity when combined with S-protein (RNase S residues 21–124) (Table 1), indicating that the expressed S-peptides were able to function in a complementary fashion. To our knowledge, these are the first examples of an active S-peptide located in the middle of a peptide, instead of at the N-terminal end [28].

The artificial protein produced from pKK118 contained the mini-α motif upstream of the S-peptide (Fig. 3A). How-

Table 1
S-peptide complementation assay

Crude extracts	OD_{280} for 5 min	Concentration of S-peptide (pmol/μl)
pGEX-2T	0.001	0
pGEX-2T + S-peptide[a]	0.740	0.10[a]
pKK118	0.820	0.11[b]
pKK119	0.645	0.09[b]

[a] 0.1 pmol of authentic S-peptide (KETAAAKFERQHMDSSTSAA) was added to pGEX-2T crude extract.

[b] Concentration of S-peptide in reaction mixtures (500 μl) as converted using the absorbance of pGEX-2T with S-peptide reacted mixture.

ever, the protein did not exhibit α-complementary activity, as *E. coli* expressing the LacZ ω-fragment and the artificial protein formed white colonies on a plate containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), a chromomeric substrate for LacZ. Apparently, the mini-α motif we used, which is a shorter version of LacZ α-fragment, was not active enough for α-complementation.

4. Discussion

We have successfully used the LAMP method, which forms concatenated inverted repeats of target sequences [24], to assemble reading frames coded from both strands of a single microgene. With this new methodology, we will be able to more fully explore the information encoded in both strands of a single microgene, something that was not possible with **MolCraft** [20,21]. We have named this new method **La-MolCraft**.

Several problems will need to be solved before the full potential of **La-MolCraft** is realized, however. First, we found that many nucleotide substitutions, presumably due to dA addition by *Bst* (exo^-) DNA polymerase [37], were introduced into the resultant polymers. Although we were able to diminish their number by using a mixture of *Bst* (exo^-) and Vent (exo^+) DNA polymerase, additional optimization using other types of DNA polymerase (e.g., KOD polymerase, which has both 3′–5′ exonuclease and chain replacement activity [39]) is needed.

Second, we encountered difficulty in cloning the LAMP products—i.e., we were only able clone products that were <220 bp in size. We believe this was because of the ability of the LAMP product to form stable stem-loop structures in regions of inverted repeats. An intensive search for the host strains and culture conditions needed to solve this problem is ongoing.

With this pilot experiment, we have shown that two epitopes can be actively expressed in artificial proteins (Fig. 3C). Moreover, the complementary activity of S-peptide with RNase was reconstituted on the proteins (Table 1). We also encrypted other potentially complementary activity in MG-51 by using a partial sequence of the LacZ α-fragment (mini-α-motif). It is well known that the full-length LacZ α-fragment can reconstitute β-galactosidase activity in vivo and in vitro by combining with the LacZ ω-fragment [29,30]. In the present study, however, artificial proteins containing the 11-amino acid mini-α motif were found to be inactive for α-complementation in vivo. This lack of complementation may be explained by the low affinity of mini-α fragment to ω-fragment, or, by the interference of segments from artificial proteins other than the mini-α sequence. Our next goal will be to select artificial proteins with activity complementary for the LacZ ω-fragment from a large pool of MG-51 LAMP products.

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