JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

ARTICLE

pubs.acs.org/JACS

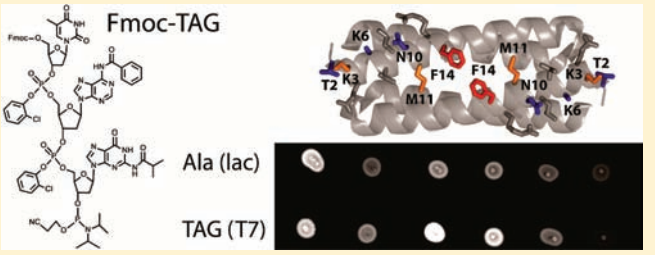
Synthetic Approach to Stop-Codon Scanning Mutagenesis

Lihua Nie, Jason J. Lavinder, Mohosin Sarkar, Kimberly Stephany, and Thomas J. Magliery*

Departments of Chemistry and Biochemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210, United States

Supporting Information

ABSTRACT: A general combinatorial mutagenesis strategy using common dimethoxytrityl-protected mononucleotide phosphoramidites and a single orthogonally protected trinucleotide phosphoramidite (Fmoc-TAG; Fmoc = 9-fluorenylmethoxycarbonyl) was developed to scan a gene with the TAG *amber* stop codon with complete synthetic control. In combination with stop-codon suppressors that insert natural (e.g., alanine) or unnatural (e.g., *p*-benzoylphenylalanine, Bpa) amino acids, a single DNA library can be used to incorporate different amino acids for diverse purposes. Here, we scanned TAG codons through part of the gene for a model four-helix bundle protein, Rop, which regulates the copy number of ColE1 plasmids. Alanine was incorporated into Rop for mapping its binding site using an in vivo activity screen, and subtle but important differences from in vitro gel-shift studies of Rop function are evident. As a test, Bpa was incorporated using a Phe14 *amber* mutant isolated from the scanning library. Surprisingly, Phe14Bpa-Rop is weakly active, despite the critical role of Phe14 in Rop activity. Bpa is a photoaffinity label unnatural amino acid that can form covalent bonds with adjacent molecules upon UV irradiation. Irradiation of Phe14Bpa-Rop, which is a dimer in solution like wild-type Rop, results in covalent dimers, trimers, and tetramers. This suggests that Phe14Bpa-Rop weakly associates as a tetramer in solution and highlights the use of Bpa cross-linking as a means of trapping weak and transient interactions.



INTRODUCTION

High-throughput sequencing, structural genomics, computational biology, and parallel approaches to protein interactions have transformed the pace at which we can understand the structure, function, and associations of a gene product. However, the detailed understanding of the function of any one protein, even if the structure is known, is still difficult and laborious. Alanine scanning mutagenesis remains the workhorse technology to estimate which protein side chains are critical for enzymatic activity, interactions, folding, and structure. Modern methods of site-directed mutagenesis and gene synthesis have greatly expanded the throughput of mutagenic scanning experiments, but for most laboratories this is still a laborious and time-consuming step.

A number of technologies have been introduced to speed up scanning mutagenesis, but they are often limited by the type, frequency, or specificity of mutation or by requiring a great deal more organic synthesis on an ongoing basis than is at the disposal of most molecular biologists. The ideal scanning library would offer production of variants with only the probe amino acid (e.g., Ala) or the wild-type amino acid at each position, a controllable number of mutations per gene (typically one), control of the possible locations for scanning and applicability to both large and small proteins, a minimal number of unique synthetic reagents, and compatibility with automated methods. Chatellier et al. have demonstrated a split-and-pool method which splits the resin for DNA synthesis into two columns where the wild-type codon and an alanine codon are synthesized separately, but mixing and splitting the resin requires manual intervention.[1] Gregoret et al. introduced a binomial mutagenesis method in which alanine was substituted for the wild-type amino acid by a single base change.[2] Therefore, it is straightforward to make alanine mutations with a probability of 0.5. This method produces a library that only contains the wild type and alanine mutants. However, most of the mutants have multiple alanine substitutions, and there are only seven amino acids that can be mutated with this method. Shotgun scanning was developed by Weiss and Sidhu[3] to provide a high-throughput alternative to traditional alanine scanning for mapping of the binding energy contributions of residues in protein−protein interfaces (hot spots[4]). The core of this method is the synthesis of the positions of interest using degenerate codons that include both Ala and the wild-type residue, using online mixing to produce stoichiometric mixtures of phosphoramidites at each nucleotide position. However, as there are not one-nucleotide differences between codons for all amino acids and Ala, some positions are varied to up to four amino acids (e.g., CG/AG/G is the closest match for Ala and Gln, but also codes for Pro and Glu). Without the use of a second set of diluted phosphoramidites, this method also produces clones with multiple mutations (more than $N/2$, depending on how many of the N sites code for two or four amino acids). Using dimethoxytrityl (DMT)-protected trinucleotide phosphoramidite synthons that correspond directly to the amino acid codons needed is the most direct route to control mutagenesis.[5−7] However, this is quite expensive, and no

Received: August 2, 2010
Published: March 31, 2011

ACS Publications © 2011 American Chemical Society

commercially available DNA synthesizer has 20 additional ports for the full complement of trinucleotides.

Alanine is not the only plausible residue for scanning; other types of systematic scans can augment traditional alanine scanning data. For example, Pal et al. have used their shotgun scanning method for "homologue scanning", mutating to a related amino acid instead of Ala, which reveals a hot spot different from that in pure Ala scanning (for example, it can reveal the function of Ala residues).[8] Cysteine scanning is a common method for understanding the topology of membrane proteins and provides a way to label proteins, albeit often not uniquely unless the protein naturally contains no Cys residues. One solution to this problem is instead to scan the protein with unnatural amino acids. Methodology developed in the Schultz group currently enables the insertion of approximately 70 different unnatural amino acids in bacteria and yeast, providing chemical analogues, biophysical probes such as fluorophores, unique reactive handles such as ketones, and photoactivatable amino acids such as photo-cross-linkers.[9] Indeed, incorporation of the photoaffinity label *p*-benzoylphenylalanine (Bpa)[10−12] has helped elucidate protein binding partners. Kauer et al. first synthesized Bpa and incorporated it into a 17-residue calmodulin binding peptide by solid-phase peptide synthesis.[13] Schultz and co-workers incorporated Bpa into *Escherichia coli* catabolite activator protein (CAP) by using stop-codon suppression. The mutant CAP (CAP-K26Bpa) formed a covalent complex with a DNA fragment containing the consensus operator sequence upon UV irradiation.[14] Recently, Mapp and colleagues have trapped the interaction of Gal4 and Gal80 with Bpa incorporation in yeast.[15]

Unnatural amino acids are inserted by engineered aminoacyl-tRNA synthetases (aaRSs) that uniquely acylate an engineered tRNA that decodes a four-base codon or, more commonly, the *amber* stop codon, TAG.[16] In addition, natural and engineered suppressor tRNAs can be used to insert 13 different amino acids in response to TAG (Ala, Cys, Ser, Leu, Glu, Gln, Tyr, Lys, Gly, His, Phe, Pro, Arg).[17] Thus, a library of *amber* stop-codon mutants is a very flexible one, in that it encodes a library of over 80 natural and unnatural amino acids by coexpression of the suppressing tRNA (and aaRS for unnatural amino acids). The TAG stop codon is the least used in *E. coli* and yeast, and good though variable suppression efficiencies around 25% are typical.

Here we demonstrate a DNA synthetic method of generating in-frame libraries of *amber* stop-codon variants of a gene. A single unique synthetic reagent is necessary for this method, a TAG trinucleotide phosphoramidite with base-labile 5′-hydroxyl protection (9-fluorenylmethoxycarbonyl, Fmoc), and it is fully incorporable into standard, automated DNA synthesis on a common commercially available apparatus with no manual intervention. The rate of mutagenesis can be controlled simply by dilution of the Fmoc-TAG, and because the method is synthetic, the possible sites of mutagenesis are set explicitly.

Our method is inspired by the work of Shortle and Soberon, who have exploited the orthogonality of standard DMT-protected mononucleotide phosphoramidites and Fmoc-protected mono-, di-, and trinucleotide phosphoramidites.[18−22] They have demonstrated that DMT removal from the 5′-hydroxyl group by trichloroacetic acid (TCA) is compatible with mild base (e.g., 1,8-diazabicyclo[5.4.0]undec-7-ene, DBU) and moreover that DBU does not deprotect any of the commonly used nucleobase protection of the exocyclic amines. This orthogonal protection enables the insertion of TAG after any number of DMT-protected couplings, which allows one to make an in-frame library of TAG mutations. However, in contrast to Shortle or Soberon, only a single Fmoc trinucleotide phosphoramidite (Fmoc-TAG) is required, and we report its synthesis and application for the first time.

To demonstrate the utility of this method, we generated a small library of in-frame TAG mutants of the gene for the four-helix bundle protein Rop, which modulates the copy number of ColE1 plasmids by binding to RNAs involved in plasmid replication.[23] Using an alanine suppressor tRNA and in vivo screen for Rop function, our study broadly confirms the conclusions of a previous in vitro site-directed alanine scanning study of Rop, although subtle but important differences are seen from the previous in vitro gel-shift results. One of the TAG mutants in the library was at the codon for Phe14, which is known to be critical for Rop function. Replacement of Phe14 with Bpa using unnatural amino acid suppression resulted in a surprising amount of activity for that variant. Moreover, when the purified protein was irradiated, covalent dimers, trimers, and tetramers were isolated, even though Rop is thought to be a dimer in solution. These results suggest that weak tetramers of Rop (or at least the Phe14Bpa mutant of Rop) are also present in solution and highlight the utility of Bpa cross-linking for elucidating weak and transient interactions.

EXPERIMENTAL SECTION

Synthesis of 5′-*O*-(9-Fluorenylmethoxycarbonyl)thymidine (1, Fmoc-T). This compound was synthesized by following the method of Lehmann.[24] Thymidine (50 mmol, 12.1 g) was dissolved in 100 mL of anhydrous pyridine. The solution was cooled to 0 °C on ice. Fmoc-Cl (51 mmol, 13.2 g) was added to the solution as a powder under magnetic stirring. The solvent was removed by rotary evaporation 30 min later. The mixture was dissolved in CH_2Cl_2 and purified by flash column chromatography with a gradient of CH_3OH (0−4%, v/v) in CH_2Cl_2. Fmoc-T (11.19 g, 24.1 mmol) was obtained as a white powder. Yield: 48%. (See the Supporting Information for full ^1H, ^{13}C, and ^{31}P NMR data, as appropriate, and mass spectrometric characterization of the compounds synthesized in this study.)

Synthesis of 5′-[5′-*O*-(9-Fluorenylmethoxycarbonyl)thymidin-3′-yl *o*-chlorophenyl phosphate]-6*N*-benzoyldeoxyadenosine (3, Fmoc-TA). Previously dried 1,2,4-triazole (32 mmol, 2.2 g) was dissolved in 80 mL of dry THF, and freshly distilled triethylamine (30.96 mmol, 4 mL) was added to the solution by syringe. 2-Chlorophenyl dichlorophosphate (15.48 mmol, 2.5 mL) was added to the mixture dropwise through a syringe under stirring. Ten minutes later, this solution was poured into a flask that contained previously dried Fmoc-T (5.6 mmol, 2.6 g), and the mixture was heated to 40 °C with an oil bath. The reaction was monitored by the disappearance of Fmoc-T from TLC within 2 h (a less polar spot than Fmoc-T can sometimes be observed). The solution was then cooled to room temperature and poured into a flask that contained previously dried 6*N*-benzoyldeoxyadenosine (7.9 mmol, 2.8 g), followed by addition of 1-methylimidazole (10 mmol, 0.8 mL) with a syringe. The reaction was completed 2 h later. After removal of the solvent THF, the mixture was dissolved in CH_2Cl_2 and washed with water. The organic phase was concentrated and subjected to flash column chromatography with a gradient of CH_3OH (0−7%) in CH_2Cl_2 for elution. A white gum (3 g) was obtained after removal of the solvent. Yield: 53.6%. (See the Supporting Information for NMR and MS data.)

Synthesis of 5′-[5′-[5′-*O*-(9-Fluorenylmethoxycarbonyl) thymidin-3′-yl *o*-chlorophenyl phosphate]-6*N*-benzoyldeoxyadenosin-3′-yl *o*-chlorophenyl phosphate]-2*N*-isobutyryldeoxyguanosine (4, Fmoc-TAG). This compound was

synthesized with the same procedure described for the Fmoc-TA. Fmoc-TA (8.9 mmol, 8.8 g) and 2*N*-isobutyryldeoxyguanosine (18.1 mmol, 6.11 g) were used as starting materials. A gradient of CH_3OH (0−9%) in CH_2Cl_2 was used as elution for flash column chromatography. A white gum (5.1 g) was obtained after removal of the solvent. Yield: 33.7%. (See the Supporting Information for NMR and MS data.)

Synthesis of 5′-[5′-[5′-*O*-(9-Fluorenylmethoxycarbonyl) thymidin-3′-yl *o*-chlorophenyl phosphate]-6*N*-benzoyldeoxy-adenosin-3′-yl *o*-chlorophenyl phosphate]-2*N*-isobutyryl-deoxyguanosin-3′-yl *O*-Cyanoethyl *N*,*N*-Diisopropylphosphor-amidite (5, Fmoc-TAG Phosphoramidite). The Fmoc-TAG (1 mmol, 1.5 g) was dried by coevaporation with anhydrous THF (5 mL) three times and dissolved in 10 mL of this solvent. *N*,*N*-Diisopropylethyl-amine (4.2 mmol, 0.73 mL) was added by syringe under argon protection and magnetic stirring, followed by syringe addition of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (3 mmol, 0.67 mL) dropwise. Half an hour later, the solvent was removed by rotary evaporation, and the mixture was then dissolved in CH_2Cl_2 which contained 8% (v/v) pyridine. The compound was purified with flash column chromatography; 8% pyridine in CH_2Cl_2 was used for elution. After removal of the solvent, the gum was dissolved in CH_2Cl_2 and precipitated with *n*-hexane three times. A white powder (0.7 g, 0.412 mmol) was obtained. Yield: 41.2%. (See the Supporting Information for NMR and MS data.)

Synthesis of *p*-Benzoylphenylalanine (Bpa). We adapted this from the method of DeGrado.[13] We synthesized the Bpa without purification of the intermediate product. Only the final product Bpa was purified by washing the solid with acetone and cold water sequentially. 4-Methylbenzophenone (9.65 g, 49 mmol), azobisisobutyronitrile (AIBN; 410 mg, 2.5 mmol), and *N*-bromosuccinimide (NBS; 8.9 g, 50 mmol) were dissolved in 300 mL of CH_3CN. The solution was heated to reflux for about 2 h. The acetonitrile solvent was removed by rotary evaporation. The solid mixture containing the product 4-(bromomethyl) benzophenone, was redissolved in 300 mL of ethyl acetate and washed with saturated $NaHCO_3$ solution and water sequentially. The roughly purified product was then dissolved in 300 mL of acetone, and diethyl acetamidomalonate (10.9 g, 50 mmol), K_2CO_3 (13.8 g, 100 mmol), and KI (0.83 g, 0.005 mmol) were added to the solution for the coupling reaction. The solution was heated to reflux overnight. The solid was filtered away. The acetone solution contained the coupling product. After the acetone was removed, the solid product was dissolved in 50 mL of 8 M HCl. The solution was heated to reflux for about 20 h for hydrolysis. The target product Bpa was precipitated out by neutralizing the solution with 8 M NaOH. Bpa precipitated when the solution was cooled. This was filtered and washed with acetone and cold water. (See the Supporting Information for NMR and MS data.)

DNA Synthesis and Purification. We synthesized the DNA oligonucletides using an ABI Applied Biosystems 392 DNA/RNA synthesizer with most reagents from Glen Research. The DNA sequence 5′-GAGATATACATATGGCTAGCC-3′ was synthesized from 3′ to 5′ with a 50 μmol C column (the first C was attached to the resin) using standard methods. DMT-A, -G, -C, and -T at 0.05 M were placed on ports 1−4, and Fmoc-TAG phosphoramidite at 0.05 M was on port 5. DBU at 0.1 M was on port 10 for deprotecting the Fmoc group. DBU was only directed into the column at the third cycle after Fmoc-TAG was mixed into the column (Figure 3). For the Rop library synthesis, 0.026, 0.038, and 0.05 M concentrations of Fmoc-TAG phosphoramidite were used in three different syntheses. The synthesized oligonucleotides were combined together for PCR amplification.

After the DNA was synthesized with the last DMT group on, the resin was resuspended in 1 mL of concentrated ammonium hydroxide in a tube with a screw cap. The solution was heated to 55 °C for 12 h. The ammonium hydroxide solution containing the DNA was then subject to an oligonucleotide purification column (OPC) by following the protocol from Glen Research Co.

Cloning of the Rop Library to the pAClac*Afl*III Vector. The synthetic DNA oligonucleotides were extended to a double-stranded cassette with a partially complementary oligonucleotide: gtaaagctca tcagcgtggt cgtgaagcga ttcacagata tctgcctgtt catccgcgtc cagctcgttg agtttctcta aaagcgttaa tgtctgactt ctg. The product was amplified by PCR with 5′ primer 5′-cacacaggaa acacgtatg-3′ and 3′ primer sequence 5′-ataatggcac ctcaatgatg atgatggtga tgtcctccga ggttttcacc gtcatctccg aaacgcgcga ggcaagaacg gtaaagctca tcagcgtgg-3′. After purification, these PCR products were digested and ligated into the pAClac*Afl*III vector at *Ban*I and *Afl*III sites (pAClac*Afl*III is a derivative of pAClac[25] with an *Afl*III site introduced for improved library cloning). The ligation product was transformed into DH10B *E. coli* and grown on LB agar containing kanamycin. After incubation overnight at 37 °C, the colonies were then spotted onto fresh LB kanamycin agar for colony sequencing (Genewiz Inc., South Plainfield, NJ).

Constructing the pMRH6sup3 Vector. A polycistronic suppression cassette was constructed by amplification of the *alaW amber* suppressor tRNA sequence from pAC-Ala(AS)[26] using three sets of oligonucleotides shown in the Supporting Information. The three sets of tRNA with a *proK* promoter and terminator sequence were amplified sequentially, digested, and ligated at internal *Bsm*AI and *Ear*I sites, and the final three-piece ligation product was gel purified, PCR amplified to append *Pst*I and *Bgl*II sites, and cloned into the pMRH6 vector at *Pst*I and *Bgl*II restriction sites (pMRH6 is a derivative of pMR101[27] designed for easy cloning of Rop variants with a TEV-cleavable N-terminal 6×His fusion).

Cloning the Rop-TAG Variants into the pMRH6sup3 Vector for *lac* or T7 Expression and Phenotypic Screening. Each of the six Rop-TAG mutants identified by sequencing from pACT7lac were PCR amplified with the *lac* promoter and terminator sequences and cloned into the pMRH6sup3 vector at the *Bst*EII and *Xba*I sites. For T7 expression of the six Rop-TAG mutants, each was PCR amplified to append *Afl*III and *Bam*HI sites and cloned into pMRH6sup3 at the *Afl*III and *Bam*HI sites. Due to the presence of an additional *Afl*III site in the sup3 cassette of the pMRH6sup3 vector, the cloning required first PCR amplifying and ligating the vector fragment between the two *Afl*III sites to the Rop-TAG gene(s). This two-piece ligation product was then cloned into pMRH6sup3 to produce the final constructs.

Activity Screening of the Rop-Ala Variants with GFP Fluorescence. The pMRH6sup3 plasmid containing the Rop-TAG gene and the pUCBADGFPuv plasmid[25] were cotransformed into DH10B cells previously lysogenized with the DE3 phage, which were grown on LB agar with kanamycin and ampicillin. After incubation at 37 °C, a single clone from each variant was restreaked onto LB agar containing 0.0005% arabinose, kanamycin, and ampicillin. Rop-AV[28] and a null linker were also cloned into the pMRH6sup3 vector as positive and negative controls, respectively. The plates were incubated at 42 °C for about 16 h. The fluorescence of the colonies was viewed under 365 nm UV light.

Cloning and Expression of F14Bpa. The F14Bpa gene with a C-terminal 6×His tag was cloned into a pET15b vector using restriction sites *Nco*I and *Bam*HI. A pSup-BpaRS-6TRN[29] vector harboring the stop codon suppressor tRNA was cotransformed into BL21(DE3) cells for expression of F14Bpa. Bpa was added to 2YT media to a final concentration of 2 mM. IPTG (0.1 mM) was used for the induction at $OD_{600} \approx 0.8$. The cells were harvested after incubation at 30 °C for 18 h. The protein was purified by Ni−NTA agarose (Qiagen) using standard methods.

Gel Filtration Chromatography Analysis of the Rop Variants. Four proteins were mixed as standards: albumin (65 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12 kDa), and aprotinin (6.5 kDa). The mobile-phase buffer was 40 mM phosphate buffer (pH 6.4) and 200 mM NaCl. All Rop variants and the standard mixtures were chromatographed under the same conditions (0.4 mL min^{-1}) with a Superdex 75 10/300 GL column (GE Healthcare).

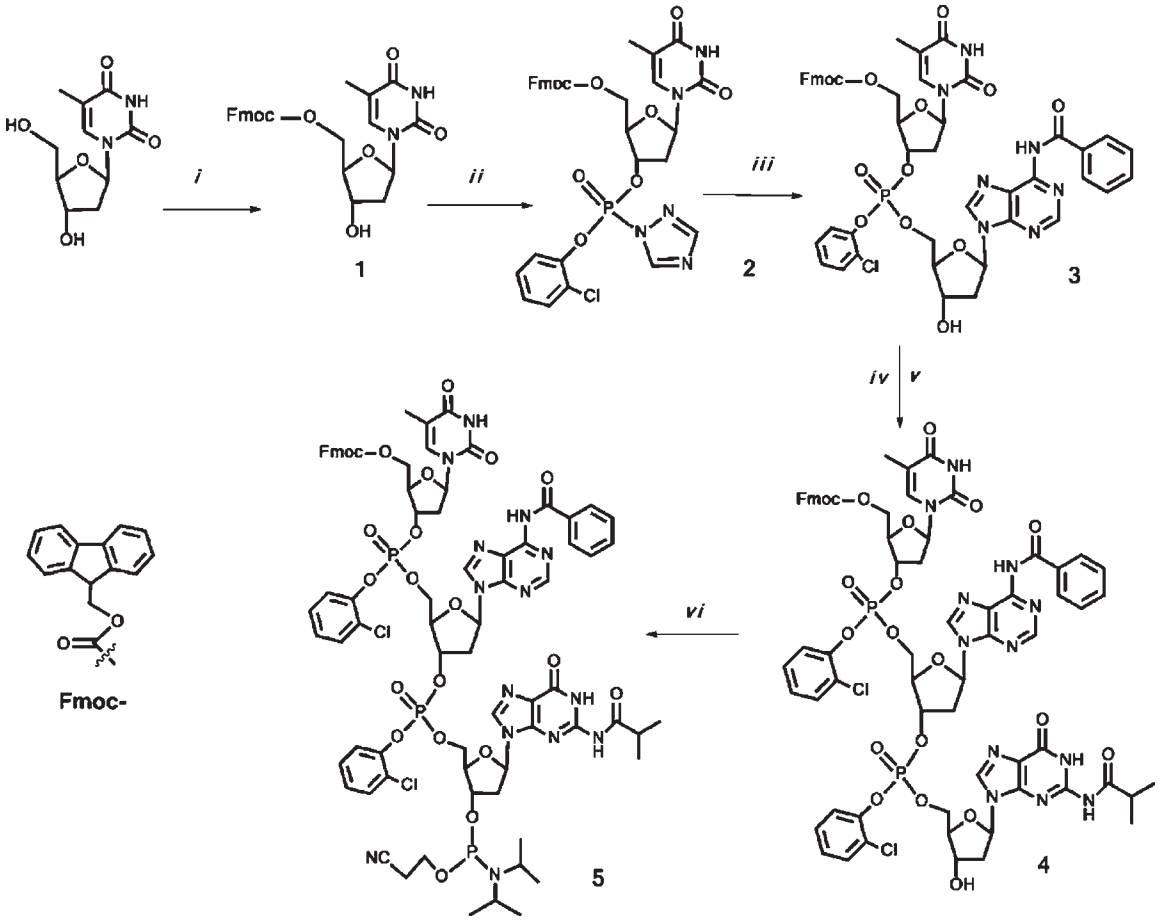


Figure 1. Synthesis of Fmoc-TAG phosphoramidite: (i) Fmoc-Cl, pyridine, room temperature; (ii) triazole, 2-chlorophenyl dichlorophosphate, triethylamine, THF, 40 °C; (iii) 6*N*-benzoyldeoxyadenosine, NMI, THF, room temperature; (iv) triazole, 2-chlorophenyl dichlorophosphate, triethylamine, THF, 40 °C; (v) 2*N*-isobutyryldeoxyguanosine, NMI, THF, room temperature; (vi) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, *N*,*N*-diisopropylamine, THF, room temperature.

Incorporation of Unnatural Amino Acid Bpa into Rop and Screening the Activity of Rop with GFPuv Fluorescence. To coexpress F14Bpa-Rop with GFPuv, we recloned the F14Bpa-Rop gene together with the T7 promoter into the pSup-BpaRS-6TRN vector at the *Pst*I restriction site. This pSup-RopF14Bpa plasmid and the pUC-BADGFPuv vector were cotransformed into DH10B(DE3) cells. The cells were grown on LB agar with ampicillin and chloramphenicol. Rop-AV and a null linker were cotransformed into DH10B(DE3) cells with pUCBADGFPuv vector as positive and negative controls, respectively. The cells were grown on LB agar with ampicillin and kanamycin. After incubation at 37 °C overnight, a single clone from each variant was streaked onto LB agar containing ampicillin, 2 mM Bpa, 10 μM IPTG, and 0.0005% arabinose. The plate was incubated at 42 °C for 16 h. The fluorescence of the clones was visualized with 365 nm UV excitation.

RESULTS

Synthesis of Fmoc-TAG Phosphoramidite. The synthesis of the Fmoc-TAG phosphoramidite has not been described, but other Fmoc-trinucleotide phosphoramidites (TNPs) and many DMT-TNPs have been reported.[5,19,22] Gaytan et al. have synthesized 20 Fmoc-trinucleotide phosphoramidites which code for the 20 natural amino acids.[22] The synthesis of Fmoc-trinucleotide phosphoramidites has suffered from low yields and low purity. The value of protecting the 3′-OH on the nucleotide with DMT during coupling reaction and removing the DMT with TCA afterward has been debated. Protecting the 3′-OH on the nucleotide with DMT avoids a 3′−3′ coupling reaction; however, the deprotecting reagent, TCA, is deleterious to purine bases and introduces other byproducts while decreasing the overall yield. We elected not to protect the 3′-OH group. The synthesis of Fmoc-TAG phosphoramidite is described in Figure 1. We found it was important to produce an intermediate phosphorylation reagent, (2-chlorophenyl)phosphoroditriazolide, in situ by titrating 2-chlorophenyl dichlorophosphate into triazole and triethylamine. The advantage of (2-chlorophenyl)phosphoroditriazolide is that, after the first triazolide is replaced by Fmoc-T, the reactivity of the second triazolide is decreased, and *N*-methylimidazole (NMI) is needed as a nucleophile catalyst for its substitution by 6*N*-benzoyldeoxyadenosine. Therefore, the chance of coupling two Fmoc-T moieties decreases. The lower reactivity of the second triazolide also favors nucleophilic attack from the primary 5′-OH over the secondary 3′-OH on 6*N*-benzoyldeoxyadenosine. Therefore, the main coupling product is 5′−3′ even without protecting the 3′-OH of 6*N*-benzoyldeoxyadenosine. The small amount of 3′−3′ coupling product can be easily removed by chromatography because it migrates faster than the 3′−5′ coupling product on the normal phase. We also found that increasing the reaction temperature to 40 °C at 3′-phosphorylation steps (ii and iv in Figure 1) decreases the reaction time from 4−5 to about 1.5 h.

dx.doi.org/10.1021/ja106894g |*J. Am. Chem. Soc.* 2011, 133, 6177–6186

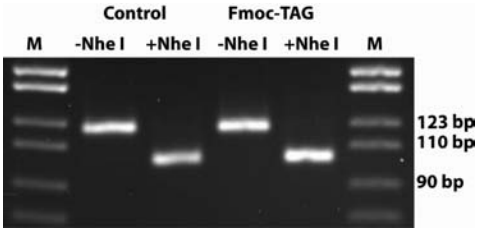


Figure 2. Digestion with *Nhe*I of PCR products synthesized with Fmoc-TAG phosphoramidite or commercially from standard methods.

Synthesis of a Single DNA Oligonucleotide with Fmoc-TAG Phosphoramidite. To adapt the Fmoc-TAG phosphoramidite to standard automated DNA synthesis, we made some small changes to the DNA synthesizer and its program. The standard ABI 392 DNA/RNA synthesizer has eight ports for nucleobase phosphoramidites. Therefore, it is easy for the Fmoc-TAG phosphoramidite to use one port and leave the other seven ports for DMT-mononucleotide phosphoramidites. The Fmoc deprotecting reagent, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), was used on port 10, which is usually used for concentrated ammonium hydroxide to cleave the oligonucleotide from the column after synthesis. A new function, "10 to column", was added to the DNA synthesis program when DBU was needed for deprotecting the Fmoc group. We combined the final cleavage and total deprotecting steps by suspending the resins into 55 °C concentrated ammonium hydroxide for 12 h after the DNA oligonucleotide was synthesized. Here, the DNA was synthesized with the last DMT group on for later purification.

To validate the DNA synthesized with the Fmoc-TAG phosphoramidite, we synthesized a 21-mer DNA oligonucleotide, 5′-GAGATATACATATGGCT**TAG**CC, containing an *Nhe*I restriction site (underlined sequence). The TAG in this DNA sequence (bold) was synthesized from the Fmoc-TAG phosphoramidite; the rest of the sequence was assembled from standard DMT-mononucleotide phosphoramidites. This DNA oligonucleotide was used as a forward primer for a PCR reaction, and a forward primer with the same sequence was purchased from Sigma Genosys as a positive control. Both of these forward primers were used separately to amplify a 119 bp fragment by PCR using the same reverse primer. It is important to realize that every molecule of PCR product contains the primer. As shown in Figure 2, lanes 2 and 4, a 119 bp fragment was amplified successfully by using both primers, indicating the synthesized primer annealed to DNA and was extended by polymerase as well as the standard primer. The PCR products were further digested with the *Nhe*I restriction enzyme to confirm the TAG codon existed in both DNA sequences. In lanes 3 and 5, it can be seen that the DNA was digested completely and produced a 104 bp fragment, demonstrating that the trinucleotide TAG was successfully incorporated into the DNA sequence and was recognized by the restriction enzyme.

Synthesis of the Rop Library with Fmoc-TAG Phosphoramidite. The purpose of the Fmoc-TAG phosphoramidite is to synthesize a DNA scanning library. The synthesis of a DNA library with Fmoc-TAG phosphoramidite and DMT-mononucleotide phosphoramidites is described in Figure 3. Three cycles were used to synthesize each codon. In the first cycle, the Fmoc-TAG phosphoramidite and the DMT-protected mononucleotide coding for the wild-type amino acid were mixed online and delivered to the column at the same time to introduce the TAG mutation. This was

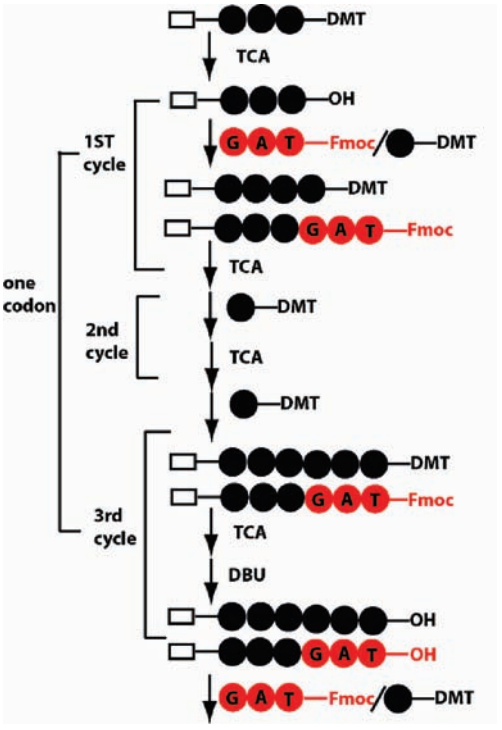


Figure 3. Scheme of DNA library synthesis with Fmoc-TAG trimer and DMT monomers.

followed by two cycles of standard DNA synthesis with only the DMT protecting group being removed by TCA to complete the synthesis of the wild-type codon. At the end of the third cycle, TCA and DBU were introduced into the column sequentially to remove both the DMT and Fmoc protecting groups. The resin then contained a mixture of DNAs with the wild-type and TAG codons in the same frame. The oligonucleotide could then be extended with another TAG codon insertion at any target position by repeating these three cycles. Notably, if the TAG were protected with DMT, further rounds of extension after TCA deprotection would yield genes in a mix of frames.

In principle, the ratio of products with TAG versus wild-type codons at each position and therefore the number of TAG codons in the whole gene can be controlled by adjusting the ratio of Fmoc-TAG to DMT-MNP (mononucleotide phosphoramidite). For example, if one wishes to scan the TAG through 10 positions, using 1/10 as much Fmoc-TAG would result in a 1:10 ratio of TAG to wild-type codon and an average of one TAG codon per clone. However, in reality, the Fmoc-TAG and the DMT-MNPs do not react equally. We tested the reactivity of Fmoc-TAG phosphoramidite against each DMT-MNP. The same concentrations of Fmoc-TAG phosphoramidite and DMT-MNP were mixed online and delivered to a column with an adenosine attached to the resin. The Fmoc-TAG and the DMT-MNP then competitively reacted with the adenosine and generated a mixture of dimer (XA) and tetramer (TAGA) products. The mixtures were analyzed by HPLC after being cleaved from the column and deprotected by concentrated ammonium hydroxide (Supporting Information Figures S18−S21). The reactivity of Fmoc-TAG against the DMT-MNPs was calculated by comparing the peak areas at 260 nm adjusted for the extinction coefficient (see the Supporting Information). From Figure 4, it can be seen that the reactivity of Fmoc-TAG

dx.doi.org/10.1021/ja106894g |*J. Am. Chem. Soc.* 2011, 133, 6177–6186

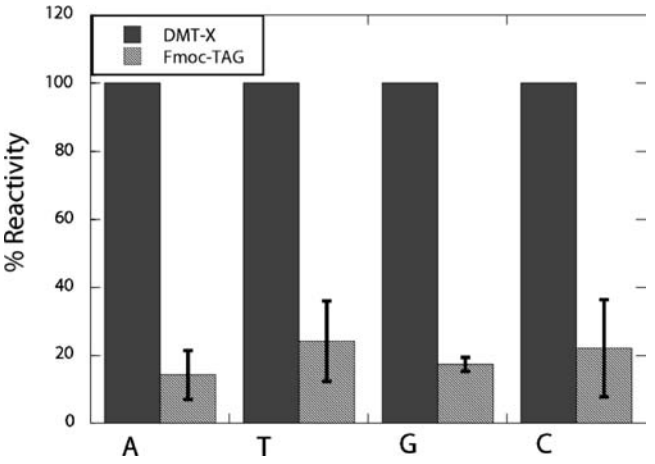


Figure 4. Reactivity of Fmoc-TAG phosphoramidite compared to DMT-mononucleotide phosphoramidite. The reactivity of each DMT-mononucleotide was normalized to 100% (dark gray bar) compared to that of Fmoc-TAG (light gray bar).

is ~20% of that of the DMT-mononucleotides, presumably due to the steric bulk. Consequently, about 5-fold more Fmoc-TAG is needed to achieve equal reactivity with the mononucleotides.

To validate the usage of Fmoc-TAG phosphoramidite in DNA library synthesis, we synthesized an 80 nt DNA library corresponding to the gene for Rop with TAG codon substitution at 10 different sites near the 5′ end of the gene: T2, K3, Q4, K6, T7, L9, N10, M11, R13, F14 (see Figure 5a). The oligonucleotide library was extended and amplified by PCR and cloned into a vector for screening. Of 30 sequenced clones, 27% had no TAG mutations, 60% had one TAG, 7% had two TAGs, and 7% had three TAGs. This is most consistent with a binomial distribution with an average 10% insertion rate for TAG at each site, which is the optimum for single insertions with 10 sites. We found that using fresh reagents and extending the DMT and Fmoc deprotection times from 3 to 6 s per cycle helped reduce insertions and deletions in the sequences. From this small selection of clones, six unique single TAG mutants were found.

Alanine Scanning Rop. To validate the biological relevance of the TAG-scanned Rop library, we constructed an alanine suppression plasmid that allows in vivo phenotypic alanine scanning of the TAG mutants. The alanine suppression construct from Miller et al.[17,26] was assembled by PCR into a polycistron containing three $tRNA^{Ala}$(CUA) genes in a manner similar to that of Schultz et al.[29] The final polycistronic cassette was then cloned into a pMR101-based vector (pMRH6). To test the TAG suppression efficiency of this pMRH6sup3 construct, the vector was transformed into a CSH108 strain[30] carrying a chromosomal *argE*(Am) marker and episomal *lacZ*8(Am) marker that allows genetic selection and blue/white screening for *amber* suppression. On minimal media plates containing kanamycin and X-gal, blue colonies appeared after two days of growth at 37 °C. However, CSH108 without the pMRH6sup3 plasmid (i.e., negative control) formed no colonies. The long incubation time is a result of slow suppression-dependent growth of this strain on minimal media. On LB plates containing kanamycin and X-gal, blue colonies appeared after overnight growth at 37 °C while the negative control, CSH108 without the pMRH6sup3 plasmid, had only white colonies.

The six unique TAG mutations corresponding to residues 2, 3, 6, 10, 11, and 14, and wild-type Rop, were cloned into pMRH6sup3 by PCR amplification of the genes along with a

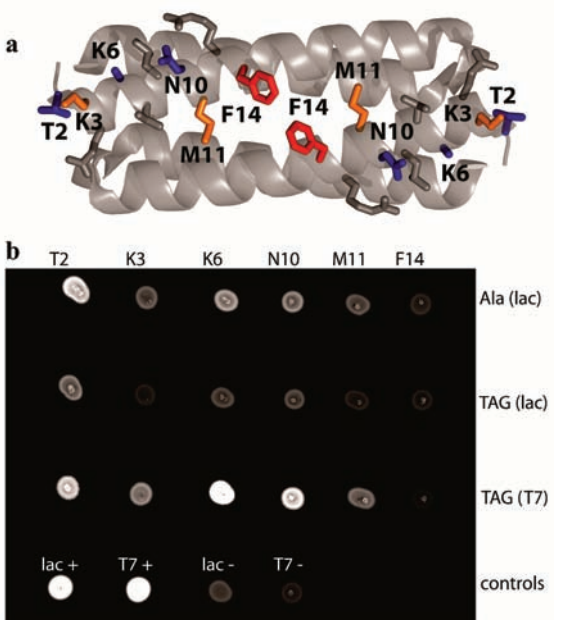


Figure 5. Alanine scanning the Rop binding site. (a) Ten residues were targeted for mutagenesis (see the text), which are color-coded red for no activity for the Ala mutant (see panel b), orange for weak activity, blue for significant activity, and gray for not observed in the small library. Rendered from PDB entry 1ROP using PyMOL. (b) The cellular fluorescence in the positive pUCBADGFPuv Rop screening strain is shown for each mutant. Ala (lac), for example, means an Ala codon appears in the gene and it is expressed from the *lac* promoter. The absolute amount of fluorescence depends on the suppression level and promoter strength, but the relative levels are consistent for each set. At the bottom, cells with wild-type Rop or a linker under each promoter are shown. The pMRH6sup3 plasmid containing different Rop variant genes and plasmid pUCBADGFPuv were cotransformed into DH10B(DE3) cells, and the cells were spotted on an LB agar plate supplemented with 0.0005% arabinose, kanamycin, and ampicillin and grown for 16 h at 42 °C. The fluorescence was visualized under 365 nm UV light. The colonies shown were all grown on the same agar plate.

flanking *lac* promoter and terminator from the pAClac clones. These cassettes were then cloned into the *Xba*I/*Bst*EII sites of pMRH6sup3 to allow *lac* expression of the Rop gene on the same p15A-based suppression plasmid. Magliery and Regan developed a cell-based screen for Rop function based on the expression of GFP from a ColE1 plasmid whose copy number is regulated by Rop.[25] Briefly, Rop significantly down-regulates that copy number of pUC-type ColE1 plasmids, particularly at 42 °C. Expression of GFP under the *araBAD* promoter along with the AraC regulator from a pUC plasmid results in strong cellular fluorescence with Rop activity and nonfluorescent cells without Rop activity at a particular concentration of arabinose. By transforming Rop variants into DH10B(DE3) containing the ColE1 reporter plasmid pUCBADGFPuv, we could screen for the in vivo activity of each alanine variant on the basis of GFP fluorescence. The fluorescence phenotypes suggested K3A, M11A, and F14A are inactive, while T2A, K6A, and N10A exhibited varying levels of fluorescence above the background. However, the levels of fluorescence of the three active variants were significantly below that of the positive control, wt-Rop, which we speculated might be due to suppression efficiency.

As a control, we mutated the six residues to alanine site specifically (using an Ala codon) and expressed them under

dx.doi.org/10.1021/ja106894g |*J. Am. Chem. Soc.* 2011, 133, 6177–6186

the *lac* promoter as a positive control. At the same time, we also expressed the TAG variants under a much stronger T7 promoter. These parallel screening contexts are shown in Figure 5b. The same qualitative pattern of cellular fluorescence is evident in each case, with the overall fluorescence being highest with the TAG-T7 constructs followed by the Ala codon−*lac* constructs and then the TAG−*lac* constructs. In all cases, the F14A mutant is completely inactive, indicating that the Phe14 is essential for binding as previously reported.[23] The K3A and M11A mutants show much lower levels of fluorescence in each context, indicating that although these residues play critical roles in binding, mutating them to alanine does not completely ablate Rop function. Interestingly, these in vivo phenotypes do not exactly match previous Ala scanning gel-shift results.[23] For example, N10 was identified as critical for binding, but here we see significant in vivo activity from N10A. Differences between the gel-shift and in vivo assays have been noted before.[25] For example, several variants in which large portions of the Rop core were replaced with Ala and Leu residues showed wild-type-like binding to small stem loops in vitro but no activity in vivo.

Incorporation of an Unnatural Amino Acid into Rop. One advantage of stop-codon scanning is that the same DNA library can be used to incorporate different natural or unnatural amino acids by using different stop-codon suppressors. For proof of principle, we incorporated a photoaffinity label unnatural amino acid, Bpa, into Rop at the F14 position. The benzophenonyl moiety can be excited to a diradical state by UV light, which can readily insert into C−H bonds, resulting in a covalent linkage. The Bpa used here was synthesized and used as a racemic mixture.

As the alanine scanning result indicated, F14 is very important for RNA binding. We wanted to determine the in vivo activity of F14Bpa-Rop using the same fluorescence screening as in alanine scanning. A cysteine-free Rop mutant including the T7 promoter was cloned into the Bpa suppression vector, pSup-BpaRS-6TRN, for compatibility with the Rop screening plasmid. The expression of F14Bpa-Rop from this construct was confirmed by small-scale culture. An active cysteine-free Rop variant, Rop-AV,[28] and a linker gene were used as positive and negative controls. This C38A C52V mutant of Rop was used as a background to remove any confusion in cross-linking experiments caused by oxidative dimerization.[28] Little cellular fluorescence is observed for the F14Bpa mutant (Figure 6a). It is also possible to examine the copy number of the ColE1 plasmid directly by preparation of the pUCBADGFPuv DNA. The pUCBADGFPuv plasmid level from F14Bpa is less than that of the negative control but much higher than that of the active Rop positive control (Figure 6b). This result confirmed that F14 is very important for Rop function, but it also suggests that low-level activity is possible with F14Bpa mutation.

As shown in Figure 7b, the two F14 residues from the two monomers lie near the dimer interface, so we speculated we might be able to capture the dimer covalently upon irradiation. Surprisingly, the F14Bpa protein formed covalent dimers, trimers, and tetramers upon irradiation with 365 nm UV light (Figure 7c). No significant amounts of higher oligomers were observed even upon extended irradiation (Figure S26, Supporting Information). We also analyzed the wild-type Rop, F14Bpa, and UV-irradiated F14Bpa-Rop with FPLC gel filtration chromatography (Figure 7d). The F14Bpa-Rop and wild-type Rop elute at the same volume, indicating they are the same size, approximately as expected for a dimer. This is consistent with gel filtration, analytical ultracentrifugation (AUC), and X-ray of

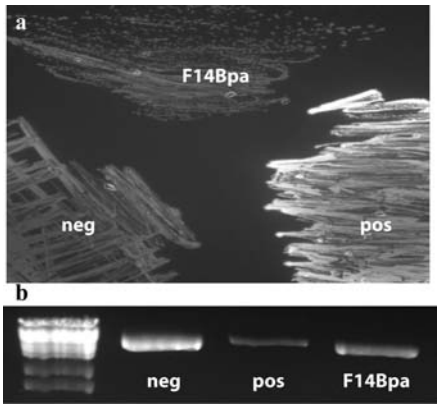


Figure 6. F14Bpa-Rop activity screening. (a) Fluorescence of clones containing the negative control, positive control, and F14Bpa. The plasmids were cotransformed into DH10B(DE3). The cells were grown for 16 h at 42 °C on LB agar supplemented with 2 mM Bpa, 0.0005% arabinose, 10 μM IPTG, and ampicillin. The fluorescence was visualized under 365 nm UV light. (b) Plasmid miniprep from the same number of cells of the negative control, positive control, and F14Bpa. The plasmid was digested with *Nco*I.

1ROP crystallographic evidence for wild-type Rop.[31−33] The UV-irradiated F14Bpa-Rop produced a new peak corresponding approximately to tetramers.

To rule out that the tetramerization of Rop is simply caused by nonspecific covalent cross-linking, we performed the UV irradiation both with an excess of BSA and in vivo by irradiating whole cells before purifying the F14Bpa-Rop via its C-terminal 6×His tag. In the presence of BSA, the formation of covalent dimers, trimers, and tetramers was evident in quantities comparable to those in the absence of BSA, and only a small amount of cross-linked BSA is visible. Likewise, the formation of dimers, trimers, and tetramers, but not higher oligomers, is evident from in vivo cross-linking, where the total protein concentration is very high, upward of 200 mg mL^{-1}.

These experiments do not necessarily imply that wild-type Rop also weakly tetramerizes; it is possible that the F14Bpa mutation causes or enhances tetramer formation. Although somewhat beyond the scope of the present study, we also performed formaldehyde cross-linking of wild-type Rop and F14Bpa-Rop at concentrations comparable to UV irradiation conditions, with and without BSA (see the Supporting Information). Under these conditions, oligomers as high as hexamers are visible by SDS−PAGE, and trimers and higher appear to be diminished somewhat in the presence of BSA for wild-type Rop but not F14Bpa-Rop. Significant cross-linking of BSA, even alone, is evident under these conditions. These results suggest that the F14Bpa mutation may be enhancing the formation of tetramers, but they do not definitively rule in or out the formation of wild-type Rop tetramers. Further studies based on this intriguing result are under way.

■ DISCUSSION

There are a number of methods to make mutational libraries to probe the structure and function of a protein, including error-prone PCR, cassette mutagenesis, binomial mutagenesis, and shotgun scanning with alanine or homologues. All of these methods have found important uses, but all also have some drawbacks that led us to develop this method. Purely random libraries require high-throughput screening of a large number of clones, and certain mutations (such as those that require two or

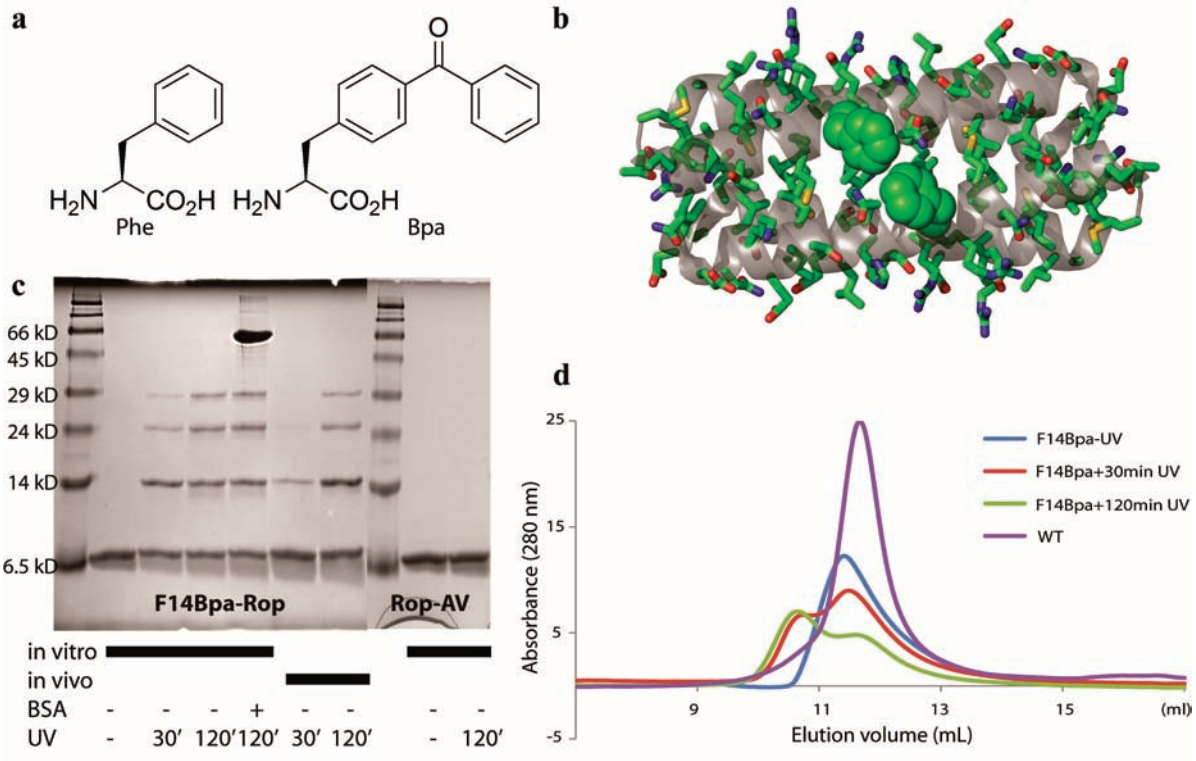


Figure 7. F14Bpa cross-linking of Rop. (a) Structures of Phe and Bpa. (b) The Rop dimer (1ROP) with the RNA-binding face toward the reader. Phe14 from each monomer (left and right) is shown in spheres. (c) SDS−PAGE of F14Bpa-Rop and wild-type Rop with increasing irradiation: lane 1, marker; lane 2, F14Bpa-Rop; lane 3, F14Bpa-Rop with 30 min of UV irradiation; lane 4, F14Bpa-Rop with 120 min of UV irradiation; lane 5, F14Bpa-Rop in the presence of an excess of BSA with 120 min of UV irradiation; lane 6, F14Bpa-Rop in intact *E. coli* with 30 min of UV irradiation; lane 7, F14Bpa-Rop in intact *E. coli* with 120 min of UV irradiation; lane 8, marker; lane 9, Cys-free Rop; lane 10, Cys-free Rop with 120 min of UV irradiation. (d) Gel filtration chromatography of wild-type Rop and F14Bpa-Rop with different UV irradiation times (0, 30, and 120 min).

three nucleotide changes) are very rare. Shotgun scanning has proven very useful, but most clones contain multiple mutations, and non-Ala mutations are common, complicating the analysis. Moreover, these libraries are static, meaning that if you wish to substitute another amino acid at each position, a new DNA library must be synthesized.

In contrast, our method gives complete control over which residues are mutated via the oligonucleotide synthesis, and the orthogonal protection strategy means that each position will be varied only between the wild-type codon and the stop codon and that all products will be in frame. The Fmoc-TAG phosphoramidite produces DNA that is replicated by DNA polymerase and quantitatively cleaved by a restriction endonuclease. A standard DNA synthesizer is easily modified to use the Fmoc-TAG phosphoramidite with a single reagent change that does not perturb the normal pattern of DMT-mononucleotide synthesis. By carefully determining the reactivity ratio of Fmoc-TAG phosphoramidite to the DMT-protected mononucleotide phosphoramidites, the mutagenesis rate of each clone can be controlled and set to approximately 1 (specifically, a binomial distribution with a maximum of 1). In this work we have assembled a small library from one oligonucleotide with scanned TAG mutations and three other homogeneous oligonucleotides, essentially using inside-out PCR. Inside-out PCR[34] and gene assembly akin to the reassembly step of DNA shuffling[35] can be used to efficiently synthesize genes at least up to 1 kb in a single step, enabling TAG scanning of larger proteins or sections of proteins in a single library using our methodology.

Shortle and Soberon have previously described the synthesis of Fmoc-protected mononucleotide and trinucleotide phosphoramidites, and DMT-protected trinucleotide phosphoramidites are now commercially available. We have made some improvements to the Fmoc-trinucleotide phosphoramidite synthesis here, and we have reported the synthesis of the Fmoc-TAG phosphoramidite for the first time, but the most important innovation over the previous synthetic approaches is that only a single synthesized reagent is necessary for this method. When multiple novel phosphoramidites are required, it both increases the cost and amount of labor and limits the compatibility with commercially available DNA synthesizers.

We have demonstrated the flexibility of these libraries in principle by showing both the scanning of alanine using an available Ala suppressor tRNA(CUA)[17] and the incorporation of Bpa into one library member using the BpaRS/tRNA(CUA) pair engineered by Schultz and colleagues.[29] With proper vector design, a single library could be scanned with over 80 natural and unnatural amino acids simply by cotransformation into different suppressor strains. Many amino acids can be scanned in *E. coli* or yeast, making a wide range of in vivo screens and display methods compatible for library sorting.

While we were developing this method, Cropp and colleagues also presented a molecular biology approach to TAG scanning using a transposon-based method and a selection step to ensure in-frame mutations.[36] We believe that this method and our method have complementary uses. For very large genes where little is known about which residues

to target, the transposon-based approach is appropriate. When one has sufficient knowledge to target a subset of sites for scanning (such as all surface positions), our synthetic method is advantageous, particularly for genes below 1 kb where gene assembly is facile.

Alanine scanning through the putative Rop RNA-binding site in combination with an in vivo screen for Rop function both broadly confirmed in vitro gel-shift data on Rop activity and at the same time revealed subtle differences. In particular, residue Phe14 was confirmed to be key to function, but other residues in a "stripe" along the helix bearing Phe14 have somewhat different results compared to in vitro findings.[23] This is likely due in part to the use of small (19 nt) model RNAs for the authentic (108 and 555 nt) inhibiting and priming RNAs of the ColE1 origin. This illustrates that the ease of combining our *amber* codon scanning method with in vivo screens in bacteria and yeast is an advantage. It is notable, however, that there is evidence of lower expression levels of the Ala mutants from TAG suppression than from decoding an Ala codon from the same promoter, as expected. This can be controlled by adjusting the strength of the promoter, as we demonstrated here. It is likely that there will be instances where poor suppression efficiency will result in a false-negative phenotype in any method using TAG suppression, and proper controls need to be constructed to interpret suppression screening results.

The Phe14Bpa mutation in Rop led to two surprises. The first was that it was at least weakly active in vivo. Previous studies have shown that F14Y is deficient in binding to a model of the substrate RNAs but able to bind similar RNAs with shortened or elongated loops, and F14W is completely deficient in RNA binding in vitro.[23] Even F14H is significantly reduced in RNA binding.[37] Recent structures of Rop and the F14Y and W mutants suggest that the larger indole ring promotes alternative conformations that might explain its total lack of RNA binding.[38] Apparently, some binding is still possible with the large benzophenonyl moiety. Even more striking, though, was that the irradiation of F14Bpa-Rop led to covalent dimers, trimers, and tetramers, even in the presence of competitor proteins in vitro and in vivo. The most straightforward interpretation of these data is that F14Bpa-Rop weakly associates into dimers of dimers. Such tetramers have never been observed in solution studies of wild-type Rop, although some Rop core mutants form higher oligomers.[32] In contrast, when Chin et al. made Bpa mutants of the GST dimer proximal and distal to the interface, dimer-sized adducts were observed for the proximal mutant and no adducts were observed for the distal one.[11] By gel filtration, we see only Rop-like dimers in solution for F14Bpa-Rop, and a second peak presumably corresponding to tetramers grows in upon irradiation. This study did not produce definitive evidence about whether wild-type Rop also weakly tetramerizes, although formaldehyde cross-linking suggests that the F14Bpa mutation may at least enhance the tetramerization. Further studies on wild-type Rop and the possible physiological significance of tetramer formation are therefore warranted. This discovery highlights the utility of Bpa cross-linking in the discovery of weak and transient interactions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Additional procedural details, spectroscopic characterization of synthetic products and intermediates, analysis of Fmoc-TAG reactivity, oligonucleotide primer sequences, plasmid maps, and gel filtration analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
magliery@chemistry.ohio-state.edu

■ ACKNOWLEDGMENT

This work was supported by NIH Grant R01 GM083114 (to T.J.M.) and by The Ohio State University. J.J.L. was an Ohio State NIH CBIP fellow and a fellow of the Great Lakes Chapter of the American Heart Association. K.S. was an Ohio State Chemistry NSF REU fellow. We are grateful to Peter G. Schultz for providing the BpaRS/tRNA(CUA) plasmids. The pAC-Ala-(AS) plasmid and CSH108 strain were provided by the *E. coli* Genetic Stock Center, Yale University. We thank Brandon Sullivan for helpful suggestions on the manuscript.

■ REFERENCES

(1) Chatellier, J.; Mazza, A.; Brousseau, R.; Vernet, T. *Anal. Biochem.* **1995**, *229*, 282.
(2) Gregoret, L. M.; Sauer, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4246.
(3) Weiss, G. A.; Watanabe, C. K.; Zhong, A.; Goddard, A.; Sidhu, S. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8950.
(4) Clackson, T.; Wells, J. A. *Science* **1995**, *267*, 383.
(5) Virnekas, B.; Ge, L.; Pluckthun, A.; Schneider, K. C.; Wellnhofer, G.; Moroney, S. E. *Nucleic Acids Res.* **1994**, *22*, 5600.
(6) Kayushin, A. L.; Korosteleva, M. D.; Miroshnikov, A. I.; Kosch, W.; Zubov, D.; Piel, N. *Nucleic Acids Res.* **1996**, *24*, 3748.
(7) Ono, A.; Matsuda, A.; Zhao, J.; Santi, D. V. *Nucleic Acids Res.* **1995**, *23*, 4677.
(8) Pal, G.; Fong, S. Y.; Kossiakoff, A. A.; Sidhu, S. S. *Protein Sci.* **2005**, *14*, 2405.
(9) Liu, C. C.; Schultz, P. G. *Annu. Rev. Biochem.* **2010**, *79*, 413.
(10) Chin, J. W.; Schultz, P. G. *ChemBioChem* **2002**, *3*, 1135.
(11) Chin, J. W.; Martin, A. B.; King, D. S.; Wang, L.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11020.
(12) Chin, J. W.; Cropp, T. A.; Anderson, J. C.; Mukherji, M.; Zhang, Z.; Schultz, P. G. *Science* **2003**, *301*, 964.
(13) Kauer, J. C.; Erickson-Viitanen, S.; Wolfe, H. R., Jr.; DeGrado, W. F. *J. Biol. Chem.* **1986**, *261*, 10695.
(14) Lee, H. S.; Dimla, R. D.; Schultz, P. G. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5222.
(15) Majmudar, C. Y.; Lee, L. W.; Lancia, J. K.; Nwokoye, A.; Wang, Q.; Wands, A. M.; Wang, L.; Mapp, A. K. *J. Am. Chem. Soc.* **2009**, *131*, 14240.
(16) Magliery, T. J. *Med. Chem. Rev.* **2005**, *2*, 21.
(17) Miller, J. H. *Methods Enzymol.* **1991**, *208*, 543.
(18) Sondek, J.; Shortle, D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3581.
(19) Gaytan, P.; Yanez, J.; Sanchez, F.; Mackie, H.; Soberon, X. *Chem. Biol.* **1998**, *5*, 519.
(20) Gaytan, P.; Yanez, J.; Sanchez, F.; Soberon, X. *Nucleic Acids Res.* **2001**, *29*, E9.
(21) Gaytan, P.; Osuna, J.; Soberon, X. *Nucleic Acids Res.* **2002**, *30*, e84.
(22) Yanez, J.; Arguello, M.; Osuna, J.; Soberon, X.; Gaytan, P. *Nucleic Acids Res.* **2004**, *32*, e158.
(23) Predki, P. F.; Nayak, L. M.; Gottlieb, M. B.; Regan, L. *Cell* **1995**, *80*, 41.
(24) Lehmann, C.; Xu, Y. Z.; Christodoulou, C.; Tan, Z. K.; Gait, M. J. *Nucleic Acids Res.* **1989**, *17*, 2379.

(25) Magliery, T. J.; Regan, L. *Protein Eng., Des. Sel.* **2004**, *17*, 77.
(26) Dorus, S.; Mimura, H.; Epstein, W. *J. Biol. Chem.* **2001**, *276*, 9590.
(27) Munson, M.; Predki, P. F.; Regan, L. *Gene* **1994**, *144*, 59.
(28) Hari, S. B.; Byeon, C.; Lavinder, J. J.; Magliery, T. J. *Protein Sci.* **2010**, *19*, 670.
(29) Ryu, Y.; Schultz, P. G. *Nat. Methods* **2006**, *3*, 263.
(30) Miller, J. H. *Short Course in Bacterical Genetics*; Cold Spring Harbor Laboratory Press: Plainview, NY, 1992.
(31) Banner, D. W.; Kokkinidis, M.; Tsernoglou, D. *J. Mol. Biol.* **1987**, *196*, 657.
(32) Munson, M.; Balasubramanian, S.; Fleming, K. G.; Nagi, A. D.; O'Brien, R.; Sturtevant, J. M.; Regan, L. *Protein Sci.* **1996**, *5*, 1584.
(33) Kresse, H. P.; Czubayko, M.; Nyakatura, G.; Vriend, G.; Sander, C.; Bloecker, H. *Protein Eng.* **2001**, *14*, 897.
(34) Gao, X.; Yo, P.; Keith, A.; Ragan, T. J.; Harris, T. K. *Nucleic Acids Res.* **2003**, *31*, e143.
(35) Stemmer, W. P. *Nature* **1994**, *370*, 389.
(36) Daggett, K. A.; Layer, M.; Cropp, T. A. *ACS Chem. Biol.* **2009**, *4*, 109.
(37) Christ, D.; Winter, G. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 13202.
(38) Struble, E. B.; Ladner, J. E.; Brabazon, D. M.; Marino, J. P. *Proteins* **2008**, *72*, 761.