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Rapid generation of incremental truncation libraries for protein engineering using α-phosphothioate nucleotides

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ABSTRACT

Incremental truncation for the creation of hybrid enzymes (ITCHY) is a novel tool for the generation of combinatorial libraries of hybrid proteins independent of DNA sequence homology. We herein report a fundamentally different methodology for creating incremental truncation libraries using nucleotide triphosphate analogs. Central to the method is the polymerase catalyzed, low frequency, random incorporation of α-phosphothioate dNTPs into the region of DNA targeted for truncation. The resulting phosphothioate internucleotide linkages are resistant to 3′→5′ exonuclease hydrolysis, rendering the target DNA resistant to degradation in a subsequent exonuclease III treatment. From an experimental perspective the protocol reported here to create incremental truncation libraries is simpler and less time consuming than previous approaches by combining the two gene fragments in a single vector and eliminating additional purification steps. As proof of principle, an incremental truncation library of fusions between the N-terminal fragment of *Escherichia coli* glycinamide ribonucleotide formyltransferase (PurN) and the C-terminal fragment of human glycinamide ribonucleotide formyltransferase (hGART) was prepared and successfully tested for functional hybrids in an auxotrophic *E.coli* host strain. Multiple active hybrid enzymes were identified, including ones fused in regions of low sequence homology.

INTRODUCTION

We recently introduced a combinatorial approach to generate fusion libraries between two gene fragments called incremental truncation for the creation of hybrid enzymes (ITCHY) (1). In contrast to DNA shuffling and related methods (2–4), ITCHY does not rely on the parental genes containing regions of DNA sequence homology to create crossovers. Instead, incremental truncation libraries are generated by digestion of the parental genes with exonuclease III under controlled conditions. Over the course of the truncation small aliquots are removed and quenched at a frequency such that, theoretically, every single base deletion of the two gene fragments will be collected. Fusion of the truncated gene fragments by blunt end ligation then generates the ITCHY library.

Although the ITCHY methodology has been successfully implemented in the search for functional hybrid enzymes (1), the method has a lengthy protocol and requires extensive time point sampling. We herein report a novel, alternative procedure to create ITCHY libraries using nucleotide triphosphate analogs such as α-phosphothioate dNTPs. Key to the procedure is the random, low frequency incorporation (spiking) of the target DNA segment with α-phosphothioate nucleotides. These nucleotide analogs have been shown to protect the DNA from exonuclease digestion (5), thus leading to the desired variation in truncation length upon nuclease treatment.

Spiking of the targeted DNA with α-phosphothioates can be achieved in two ways. The first approach involves an initial exonuclease treatment, limited to the segment of DNA to be truncated, generating a single-stranded overhang. This overhang subsequently serves as a template in a DNA polymerase catalyzed fill-in reaction in the presence of phosphothioates (Fig. 1a–c). Alternatively, α-phosphothioate dNTPs are incorporated during PCR amplification of the entire plasmid (Fig. 2a–c). By adjusting the concentration ratio between natural dNTPs and αS-dNTPs in the polymerase reaction mixture the frequency of incorporation of phosphothioate internucleotide linkages can be controlled. Next, the DNA is submitted to exhaustive exonuclease treatment, hydrolyzing natural dNMPs but stopping upon encountering a phosphothioate internucleotide linker. Due to the random distribution of phosphothioates in the DNA, a truncation library consisting of all single base pair deletions is generated at once.

The implications of our alternative method to create incremental truncation libraries, termed THIO-ITCHY, go beyond the elimination of time point sampling. Since the generation of diversity is no longer a function of timed exonuclease digestion but instead based on the random distribution of the α-phosphothioate nucleotides, the two targeted gene fragments can be combined into a single vector and processed simultaneously. THIO-ITCHY therefore enables the creation of comprehensive incremental truncation libraries of both gene fragments in a

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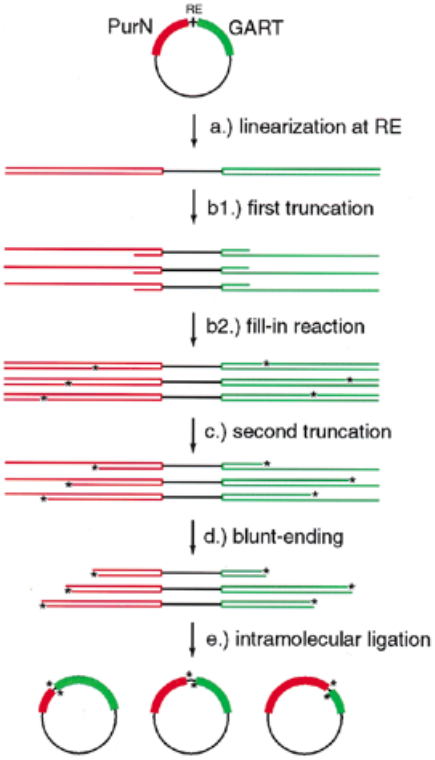

Figure 1. Schematic overview of THIO-ITCHY using α-phosphothioate nucleotide incorporation by primer extension. (a) Linearization of the starting plasmid by restriction digestion at the unique site between the two genes or gene fragments. (b1) Initial exonuclease treatment of the linearized plasmid with exonuclease III produces single-stranded overhangs, covering the entire length of the target region. (b2) The single-stranded target region serves as the template for polymerase catalyzed resynthesis of the complementary strand. The addition of small amounts of nucleotide analogs such as α-phosphothioate dNTPs to the reaction mixture results in random, low frequency incorporation of the analog into the newly synthesized strand as indicated by stars. (c) A second incubation of the plasmid with exonuclease III results in hydrolysis of standard dNMPs while the dNMP analogs block enzymatic degradation. (d) The single-stranded portions of the plasmids are removed enzymatically with mung bean nuclease. (e) The blunt-ended constructs are recircularized by intramolecular ligation.

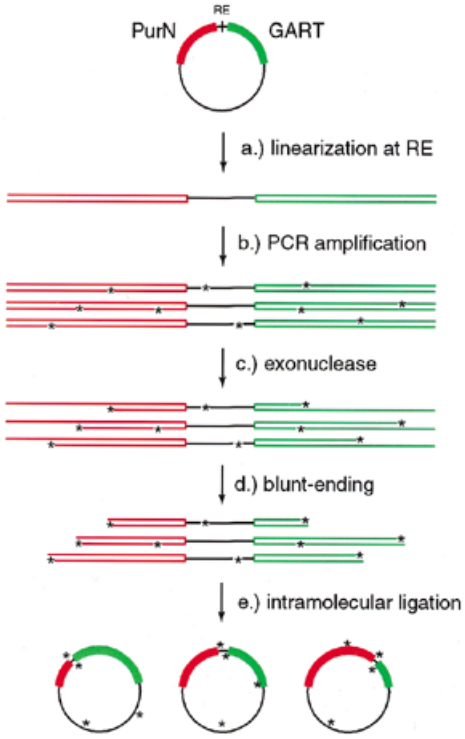

Figure 2. Schematic overview of THIO-ITCHY using α-phosphothioate nucleotide incorporation by PCR amplification. (a) Linearization of the starting plasmid by restriction digestion at the unique site between the two genes or gene fragments. (b) PCR amplification of the entire linearized vector in the presence of a mixture of dNTPs and αS-dNTPs as described in Materials and Methods. (c) Incubation of the plasmid with exonuclease III results in hydrolysis of standard dNMPs while the dNMP analogs will block enzymatic degradation. (d) The single-stranded overhangs of the plasmids are removed enzymatically with mung bean nuclease. (e) The blunt-ended constructs are recircularized by intramolecular ligation.

single reaction. Importantly, the advantages of THIO-ITCHY for creating incremental truncation libraries extend beyond experimental convenience. Additional diversification, desirable to widen and possibly accelerate the search for functional hybrids, can be accomplished by modification of DNA polymerase fidelity, resulting in random mutagenesis of the incremental truncation library.

MATERIALS AND METHODS

All enzymes used were purchased from New England Biolabs (Beverly, MA) unless otherwise indicated in the text. The α-phosphothioate nucleotides (racemic mixtures as well as *S*-stereoisomers) used in the experiments had been synthesized previously (6). Racemic mixtures of αS-dNTPs are also commercially available from Promega (Madison, WI) and Amersham Pharmacia (Piscataway, NJ). DNA samples were purified using the QIAquick gel and PCR purification kit (Qiagen, Valencia, CA). Where indicated, reactions were quenched by addition of PB buffer, supplied with the QIAquick PCR purification kit. The DNA was eluted from the spin columns using 50 µl of EB buffer (10 mM Tris pH 8.5).

Plasmid construction

pDIM-PGX (Fig. 3a) was constructed from pDIM-N2(PurN[1–144]) (7), containing an N-terminal fragment (*purN*[1–144]) consisting of the DNA coding for amino acid residues 1–144 of *Escherichia coli* GAR transformylase (PurN). Initially, the f1 region in pDIM-N2(PurN[1–144]) was removed by restriction digestion with *Kpn*I and *Nae*I. The overhangs were filled in by Klenow DNA polymerase treatment and the plasmid was cyclized, generating pDIM-N2(Δf1,PurN[1–144]). Next, the C-terminal human GAR transformylase (hGART) fragment [54–203], consisting of the DNA coding for amino acid residues 54–203 of hGART, was prepared by PCR, carrying a 28 nt linker region as a 5′-extension, flanked by *Bam*HI and *Bgl*II sites (Fig. 3b). Upon digestion with *Bam*HI and *Spe*I the hGART fragment was ligated into pDIM-N2(Δf1,PurN[1–144]) and transformed into *E.coli* DH5α-E (~10^{10} transformants/µg DNA; Gibco Life Technolo-

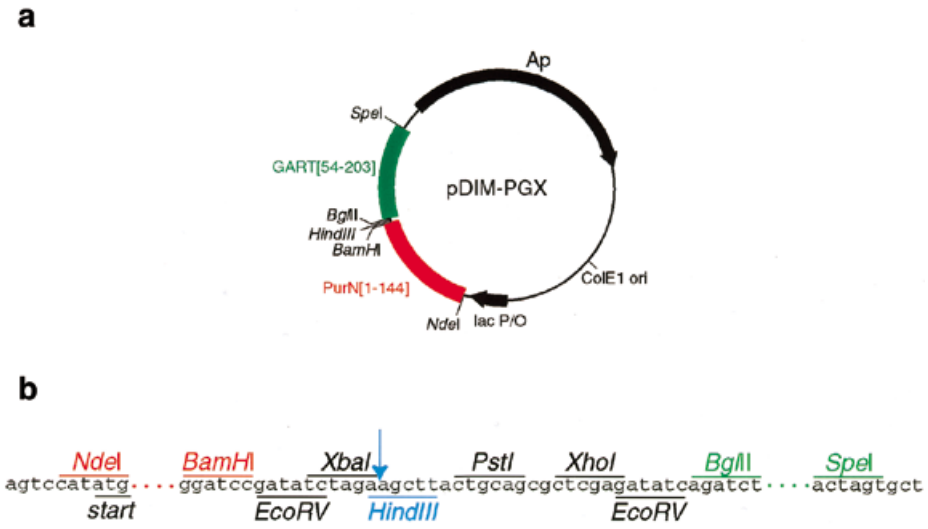

Figure 3. Description of pDIM-PGX: the two overlapping gene fragments are cloned in series within the same vector. The method requires only a single, unique restriction site between the two fragments (*Hin*dIII in pDIM-PGX). The *E.coli* PurN[1–144] fragment was inserted between *Nde*I and *Bam*HI sites (shown in red). A primer containing the multiple restriction sites cassette (*Eco*RV/*Xba*I/*Hin*dIII/*Pst*I/*Xho*I/*Eco*RV) as a 5′-overhang was used in amplification of the hGART[54–203] fragment (shown in green) which was cloned into the pDIM vector via *Bam*HI and *Spe*I sites. The plasmid carries an ampicillin resistance gene as selection marker (Ap) and has a ColE1 origin of replication. The fusion gene library is under control of the *lac* promoter (lac P/O).

gies, Rockville, MD). The resulting plasmid, pDIM-PGX, was isolated by large-scale plasmid preparation (Qiagen) and characterized by restriction analysis and DNA sequencing. Twenty micrograms of pDIM-PGX in 200 µl of NEB buffer 2 (1× = 10 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT) were linearized by restriction digestion with *Hin*dIII (60 U) and purified by agarose gel electrophoresis.

DNA spiking by exonuclease/Klenow treatment

Three micrograms of linearized DNA were mixed with 6 µl of 10× exonuclease III buffer (Promega) and the volume adjusted to 60 µl with water. The solution was pre-incubated for 15 min at 22°C, followed by addition of 260 U exonuclease III (Promega) and incubation at room temperature for 6 min. The average truncation rate under the described conditions was 50 bases/min (Fig. 1b). The reaction was quenched with 1 µl of 0.5 M EDTA pH 8, and the DNA QIAquick purified.

Resynthesis of the complementary DNA strands of the exonuclease-treated plasmid (50 µl) was performed by incubation with 3.75 U Klenow fragment (exo$^-$) in 10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, containing dNTPs (200 µM each) and αS-dNTPs (5 µM each, either the *S*-isomer or a racemic mixture) in a final volume of 150 µl for 10 min at 37°C (Fig. 1b). The reaction mixture was quenched by addition of PB buffer and the DNA QIAquick purified.

DNA spiking by PCR

α-Phosphothioate nucleotides were incorporated directly during PCR amplification of the linearized pDIM-PGX as shown in Figure 2. Ten nanograms of linearized plasmid were amplified with primers A (5′-TCCGGAGCTTCTAGATATC-GGATCCTTAGTCC-3′) and B (5′-AGGCCTCTGCAGCGC-TCGAGATATCAG-3′) (1 µM each) in 50 µl of reaction mixture [*Taq* DNA polymerase buffer (Promega) supplemented with 1 mM $MgCl_2$, 180 µM each dNTP, 20 µM each αS-dNTP and 2.5 U *Taq* DNA polymerase (Promega)]. The PCR program was 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, 270 s at 72°C, followed by 10 min at 72°C. After purification with the QIAquick kit, the amount of DNA was quantified by OD_{260} for adjustment of the amount of exonuclease in the following step.

Creation of an incremental truncation library

The solution of spiked DNA (50 µl) from either protocol was mixed with 120 U exonuclease III/µg 5′-end DNA (Promega) in the manufacturer's buffer (5.5 µl, 10×) (Promega) and incubated for 30 min at 37°C (Figs 1 and 2c). After quenching the reaction with PB buffer and QIAquick purification of the DNA, the single-stranded 5′-overhang was removed upon incubation with 2.3 U mung bean nuclease/µg DNA in the manufacturer's buffer (30 min at 30°C) (Figs 1 and 2d). The DNA was again QIAquick purified.

To improve the ligation efficiency, the plasmid library was blunt-ended with 4.5 U Klenow fragment in 6 µl of Klenow buffer (100 mM Tris–HCl pH 7.5, 50 mM $MgCl_2$) and dNTPs (final concentration 140 µM each nucleotide) for 10 min at 37°C. The DNA was QIAquick purified.

In the final step the plasmid library was cyclized by intramolecular ligation using 24 U T4 DNA ligase (Promega) in the manufacturer's buffer and 36 µl of 50% PEG (final volume 400 µl) overnight at 4°C (Figs 1 and 2e). Prior to transformation into *E.coli* DH5α-E the DNA was concentrated and desalted using QIAquick spin columns.

Selection of the THIO-ITCHY libraries

The incremental truncation library in DH5α-E was recovered and stored as described earlier (7). Following transformation into the auxotrophic *E.coli* strain TX680F′, selection of active hybrids was carried out as described previously (1).

RESULTS AND DISCUSSION

The feasibility of THIO-ITCHY as an alternative method to create incremental truncation libraries for protein engineering was demonstrated on a model system, using the N-terminal gene fragment of *E.coli* glycinamide ribonucleotide transformylase (PurN[1–144]) and the C-terminal portion of the human glycinamide ribonucleotide transformylase (hGART[54–203]). Both enzymes catalyze the transfer of a formyl group in the *de novo* purine biosynthesis pathway. Despite their high overall structural homology, the two sequences share only 50% identity at the DNA level (8). The PurN–hGART model system has previously been studied as proof of principle for other ITCHY protocols (1,9). Finally, the system offers a simple and straightforward procedure to identify functional hybrid enzymes through selection by functional complementation of the auxotrophic *E.coli* strain TX680F′.

Creation of THIO-ITCHY libraries

The method of creating incremental truncation libraries using α-phosphothioate nucleotides introduces several changes in the design of the vector containing the parental gene fragments. Most noticeably, the two genes are cloned in series within the same vector (Fig. 3), rather than on two separate plasmids as in the original two vector-based ITCHY (1). This allows simultaneous truncation of both gene fragments, since the fragment size distribution of the truncation library is no longer dependent on the duration of exonuclease digestion. Furthermore, the requirement for multiple, strategically placed restriction sites has been eliminated in THIO-ITCHY. Only a single unique cleavage site between the two gene fragments, for example the cloning site(s) of the target DNA, is required. Consequently, limitations to specific vector systems, suitable for incremental truncation, are eliminated, as demonstrated by the successful application of THIO-ITCHY in other expression systems such as the pET series (data not shown). Finally, the single vector design simplifies the library construction in the last step of the protocol by requiring only intramolecular ligation to recircularize the incremental truncation library.

The THIO-ITCHY protocol consists of five key steps (Figs 1 and 2). The method starts with linearization of the parental vector using the unique restriction site between the parental gene fragments. Gel purification of the digested product was required to remove trace amounts of incompletely digested vector which otherwise are carried through the remaining protocol and upon transformation bias the library towards untruncated material. The second step involves random incorporation of phosphothioates into the target DNA sequences. Two alternative approaches to spike the targeted DNA region were explored: primer extension (Fig. 1) and PCR amplification (Fig. 2).

Incorporation of phosphothioates by primer extension

Using exonuclease III, the two ends of the linearized plasmid, encoding the overlapping region between amino acid positions 54 and 144 (270 bp) of PurN and hGART, were converted into single-stranded DNA (Fig. 1b1). Exonuclease III under carefully chosen reaction conditions allows controlled 3′→5′ hydrolysis of double-stranded DNA (10,11). At 22°C in low salt buffer the enzyme hydrolyzes ~50 bases/min. The hydrolysis is quenched efficiently upon addition of EDTA. As reported for circular permutation-ITCHY (9), the application of QIAquick spin columns (see Materials and Methods) to purify the DNA intermediate from protein and EDTA proved simple and very efficient.

The single-stranded DNA portion then served as a template for the polymerase catalyzed resynthesis of the complementary DNA strand (Fig. 1b2). Use of a mixture of the four standard dNTPs spiked with small amounts of αS-dNTPs leads to random incorporation of the nucleotide analogs over the entire stretch of the resynthesized DNA. Several DNA polymerases, including Klenow fragment, T4 DNA polymerase, *Taq* DNA polymerase, Vent DNA polymerase and *Pfu* DNA polymerase, have been shown to successfully utilize phosphothioate analogs during template-directed DNA synthesis (12–14). However, none of the 3′→5′ exonuclease activities of Klenow, T4, Vent and *Pfu* DNA polymerases are capable of hydrolyzing the thiophosphate linkage. Upon completion of DNA synthesis this exonuclease activity engages in a process known as idling, the continued removal and resynthesis of the 3′-ends. This idling leads to the accumulation of phosphothioates at the 3′-ends of the resynthesized strands, biasing the resulting library towards full-length fragment sizes. Exonuclease-deficient variants of these polymerases must therefore be employed during synthesis of the complementary strand.

Another important consideration during the fill-in reaction is the ratio between dNTPs and dNTP analogs that are ultimately responsible for the diversity of the incremental truncation library. In theory, incorporation of a single dNMP analog over the length of the single-stranded DNA segment is desirable. In mathematical terms, the αS-dNTP to dNTP concentration ratio is inversely proportional to the length of the single-stranded DNA segment X scaled by a correction factor δ (see equation **1**). The correction factor δ represents the relative incorporation rates of dNTPs and αS-dNTPs. To a first approximation the incorporation efficiency of phosphothioates versus natural nucleotides by *E.coli* DNA polymerase I and *Taq* DNA polymerase indicates no apparent discrimination (δ = 1) (data not shown).

$$\frac{1}{X}\delta = \frac{[\alpha\text{S-dNTP}]}{[\text{dNTP}]} \qquad \mathbf{1}$$

However, earlier studies show that only the *S*-isomeric form of αS-dNTPs is utilized by DNA polymerases, while the *R*-isomer acts as a mediocre competitive inhibitor of the enzyme (13). The lower overall efficiency of incorporation of phosphothioate nucleotides by DNA polymerases in comparison to natural dNTPs must therefore be considered. This, as well as other non-specific effects, has led to an experimentally determined correction factor (δ = 7.5) (data not shown) for Klenow fragment (exo⁻).

Incorporation of phosphothioates by PCR amplification

Alternatively, introduction of α-phosphothioate dNTPs by PCR amplification of the entire vector sequence was successfully tested. While following the same rules and restrictions for dNTP:αS-dNTP ratios and polymerases as in the previously discussed primer extension experiments, the approach requires only nanogram quantities of the initial construct and requires even less hands-on time. On the other hand, one must consider the plasmid size and the error frequency of the utilized DNA polymerase. Although random mutagenesis in the target DNA

may be desirable, the approach inevitably introduces point mutations over the entire length of the plasmid that may disrupt or modulate other essential functions on the plasmid. Consequently, subcloning of the truncation library into a separate expression system may be necessary, especially for larger constructs or under deliberately chosen highly mutagenic conditions. In our model system *Taq* DNA polymerase, which has the lowest known error frequency of all commercially available exonuclease-deficient DNA polymerases, was utilized to amplify and spike linearized pDIM-PGX. Although not addressed specifically in this study, the reported experimental conditions result in an error frequency of 5×10^{-4} based on sequencing data from functional hybrid enzymes.

Creating the truncation libraries from phosphothioate-spiked DNA

The spiked DNA was then incubated a second time with exonuclease III under conditions of maximum activity (~450 bases/min) (Figs 1 and 2c). Only the randomly incorporated thiophosphate internucleotide linkages can halt degradation by the nuclease and protect the remaining plasmid from further hydrolysis. In control experiments plasmid DNA containing only standard nucleotides was removed with >99% efficiency, based on the number of colonies formed upon ligation and transformation of these samples.

The single-stranded 5′-overhang that remains after exonuclease treatment was removed upon incubation with mung bean nuclease (Figs 1 and 2d). Whereas initial experiments using S1 nuclease gave inconsistent and unreproducible data, the use of mung bean nuclease proved more efficient and reliable. Although direct ligation of the mung bean-treated DNA was successfully performed, the additional blunt-ending step by Klenow treatment increased the number of transformants 7-fold.

Following the described protocols we generated THIO-ITCHY libraries of PurN–hGART hybrid enzymes, consisting of $2–8 \times 10^5$ independent members. PCR analysis of the gene fusion product from randomly chosen library members indicated a linear size distribution over the expected range of truncation (data not shown). In addition, the distribution of crossovers between the parental gene fragments, as well as the variation in fragment size in the naïve (unselected) libraries, was investigated by DNA sequencing of several plasmids from randomly chosen colonies. The PurN–hGART fragment sizes and crossover points were established and plotted as shown in Figure 4 (red stars). Seven of the characterized sequences were found to be located in the desired sequence space between amino acid residues 54 and 144 while two library members were within the range of the standard deviation of the initial exonuclease digestion (indicated by the dashed line in Fig. 4). Two samples were found outside the expected sequence space. The random distribution over the sampled sequence space indicates no apparent bias towards particular regions within the gene fragments and, most importantly, towards constructs composed of equal sized fragments. Such would be indicative of carried over plasmid from the initial exonuclease treatment as a result of the synchronized hydrolysis of both 5′-ends by exonuclease. The experimental data in Figure 4 clearly show that the random incorporation of phosphothioates, followed by the exonuclease step, results in a random fragment size recombination between the two genes.

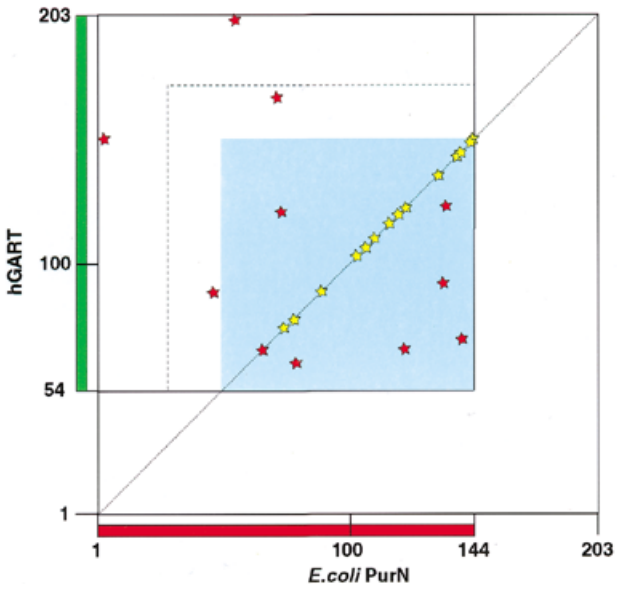

Figure 4. Distribution of naïve library members and functional hybrid enzymes in sequence space. The fragment size distribution of randomly chosen colonies from the unselected library (red stars), as well as after selection for functional complementation in the auxotrophic *E.coli* strain TX680F′ (yellow stars), is shown. The blue section outlines the targeted sequence space between amino acid residues 54 and 144 of the two fragments. The dashed square represents the region within the standard deviation of the stop point of the initial exonuclease treatment which must be added to the sampled sequence space. The diagonal marks the positions of exact alignment between the two parental genes. As previously observed for this model system (1,9), the majority of functional hybrid enzymes occur in positions of exact alignment and are therefore located on the diagonal.

Selection of functional hybrid enzymes

For the selection of catalytically active hybrid enzymes the plasmid library was recovered and transformed into the auxotroph *E.coli* strain TX680F′. Upon plating the transformants on minimal medium plates only bacteria which express hybrid enzymes capable of complementing the disrupted host GAR transformylase grow. Selection was performed by incubating the plates at 37°C, as well as under less stringent selection conditions at room temperature. The lower incubation temperature yielded approximately four times the number of colonies found at 37°C. Although the majority of the constructs selected at room temperature also grew at 37°C, additional temperature-sensitive hybrid enzymes were identified. As previously noted (9), the fusion points of the temperature-sensitive hydrids were exclusively located in the region between amino acids 80 and 103 (Fig. 5). Furthermore, sequence analysis of the naïve libraries identified an in-frame fusion construct (PurN[1–72]/GART[73–203] in Fig. 5) in the lower overlapping region (amino acids 55–80). Considering the absence of functional hybrid enzymes in that region, the result may indicate a structural inflexibility of that particular region.

Thirty-one colonies expressing functional hybrid enzymes were picked and analyzed by PCR and DNA sequencing. As in previous truncation experiments with the PurN–hGART model system, all except one construct were exactly aligned fusions. Crossovers between the parental gene fragments occurred

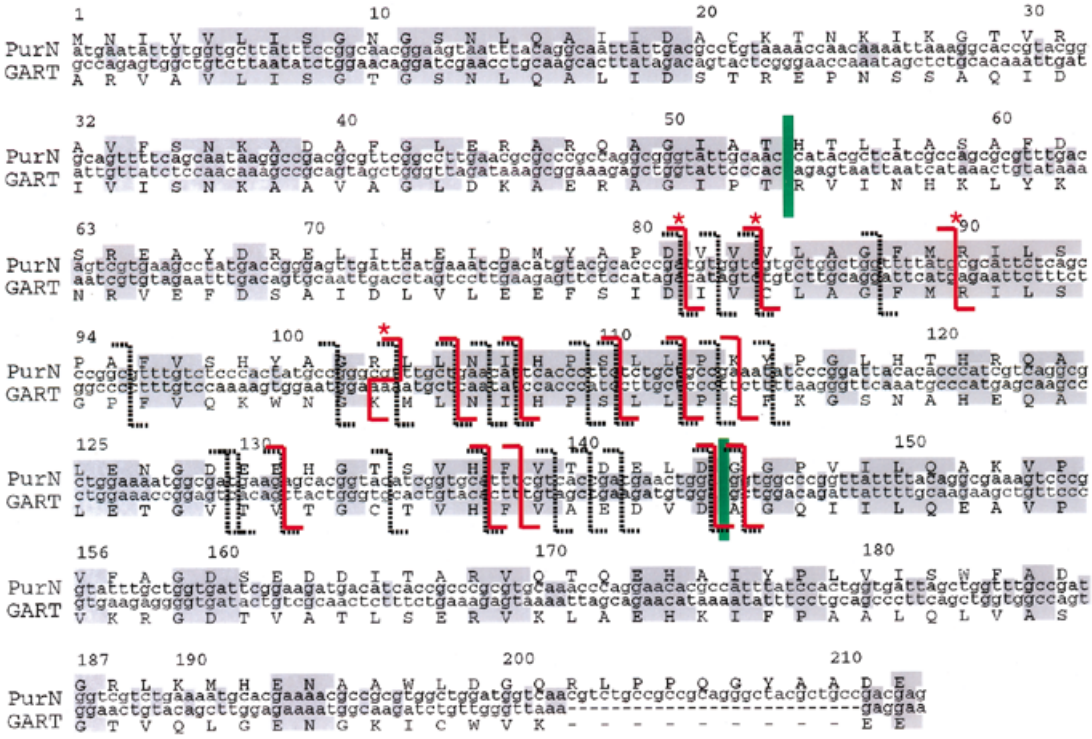

Figure 5. Fusion points of functional hybrid enzymes between *E.coli* PurN and human GART generated by THIO-ITCHY and identified by functional complementation in the auxotrophic *E.coli* strain TX680F′. Gray shaded areas indicate regions of DNA and protein sequence identity between the two aligned parental sequences. The green bars mark the end points of the two overlapping fragments. Fusion points of functional hybrid enzymes found by THIO-ITCHY are shown in red. Temperature-sensitive hybrid enzymes are marked with a star. For comparison, the summary of crossovers found upon selection of previous truncation libraries (1,9) are shown as black dashed lines.

within regions with different levels of homology (Fig. 5). Sequence analysis identified 14 distinct DNA fusion constructs, four of which were previously unknown. No mutations were identified in the gene fusions created using spiking by primer extension. In contrast, DNA sequence analysis of 10 functional hybrids created using spiking by PCR amplification showed a total of four point mutations in three of the sequences. Two of the mutations were silent (E44 and R168) and the other two occurred in the same construct (PurN[1–110]/GART[111–203], A145T/K157R). In the 31 sequences analyzed the entire range of functional crossovers from amino acid position 80 to 144 is represented and evenly distributed in the library. The frequency of functional hybrids per library size is similar to that observed in the original ITCHY approach (1).

Future developments

THIO-ITCHY offers the unique possibility of combining incremental truncation with random mutagenesis, resulting in increased diversity in the final library. As indicated by these initial studies, random mutagenesis during the polymerase catalyzed incorporation of the dNTP analogs is possible and results in further diversity of the sequence library. The fidelity of the polymerase during primer extension as well as amplification can be varied by partial substitution of magnesium with manganese, as well as variations in reaction buffer composition and temperature (15), increasing the mutation frequency to desirable levels.

Furthermore, it may be desirable to use thermostable polymerases such as *Taq* DNA polymerase for incorporation of phosphothioates using primer extension experiments for very long single-stranded templates. The higher reaction temperature allowed by thermostable polymerases should help to prevent premature termination of the fill-in reaction due to secondary structure formation of the single-stranded portions of the construct.

Conclusions

A new, fundamentally different approach to create incremental truncation libraries using α-phosphothioates has been successfully applied in the search for functional hybrid enzymes between the GAR transformylases from *E.coli* and human. As the newest addition to the incremental truncation technology, THIO-ITCHY offers quite a number of experimental advantages over ITCHY, including single vector design, lack of extensive time point sampling and a shortened protocol. However, the most important advantage of THIO-ITCHY arises from the possibility of combining incremental truncation with random mutagenesis, leading to further library diversity, which in turn can accelerate the identification of novel hybrid enzymes with improved function.

NOTE ADDED IN PROOF

The authors have recently become aware of a publication by King and Goodbourn (16) that uses α-phosphothioate

nucleotides to generate gene deletions, similar to the method reported by Eckstein and co-workers (12).

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