DNA shuffling by random fragmentation and reassembly: 
*In vitro* recombination for molecular evolution

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**ABSTRACT**

Computer simulations of the evolution of linear sequences have demonstrated the importance of recombination of blocks of sequence rather than point mutagenesis alone. Repeated cycles of point mutagenesis, recombination, and selection should allow *in vitro* molecular evolution of complex sequences, such as proteins. A method for the reassembly of genes from their random DNA fragments, resulting in *in vitro* recombination is reported. A 1-kb gene, after DNase I digestion and purification of 10- to 50-bp random fragments, was reassembled to its original size and function. Similarly, a 2.7-kb plasmid could be efficiently reassembled. Complete recombination was obtained between two markers separated by 75 bp; each marker was located on a separate gene. Oligonucleotides with 3' and 5' ends that are homologous to the gene can be added to the fragment mixture and incorporated into the reassembled gene. Thus, mixtures of synthetic oligonucleotides and PCR fragments can be mixed into a gene at defined positions based on homology. As an example, a library of chimeras of the human and murine genes for interleukin 1β has been prepared. Shuffling can also be used for the *in vitro* equivalent of some standard genetic manipulations, such as a backcross with parental DNA. The advantages of recombination over existing mutagenesis methods are likely to increase with the numbers of cycles of molecular evolution.

My interest is in applying repeated cycles of DNA shuffling and selection to the molecular evolution (11–13) of genes, operons, and biosynthetic pathways. I recently demonstrated the utility of this approach with a β-lactamase system (14). While many different library formats for molecular evolution have been reported for polynucleotides (15–17) and peptides and proteins [phage (18–20), lacZ (21), and polysomes (22)], in none of these formats has recombination by random crossovers been used to deliberately create a combinatorial library.

**MATERIALS AND METHODS**

**Substrate Preparation.** The substrates for the shuffling reaction were 1.0-kb double-stranded (ds)DNA PCR products derived from pUC18 with the primer sequences AAAGCGTCCATTTTGTGAT and ATGGGGTTCGCGACACATT (Fig. 2). The removal of free primers from the PCR product by Wizard PCR (Promega) was found to be very important.

**DNase I Digestion.** About 2–4 μg of the DNA substrate(s) was digested with 0.0015 unit of DNase I (Sigma) per μl in 100 μl of 50 mM Tris-HCl, pH 7.4/1 mM MgCl₂ for 10–20 min at room temperature. Fragments of 10–50 bp were purified from 2% low melting point agarose gels by electrophoresis onto DE81 ion-exchange paper (Whatman), elution with 1 M NaCl, and ethanol precipitation.

**PCR Without Primers.** The purified fragments were resuspended in PCR mixture (0.2 mM each dNTP/2.2 mM MgCl₂/50 mM KCl/10 mM Tris-HCl, pH 9.0/0.1% Triton X-100) at a concentration of 10–30 ng/μl. No primers were added at this point. Taq DNA polymerase (Promega) was used at 2.5 units per 100 μl of reaction mixture. A PCR program of 94°C for 60 s; 94°C for 30 s, 50–55°C for 30 s, and 72°C for 30 s (30–45 times); and 72°C for 5 min was used in an MJ Research (Cambridge, MA) PTC-150 thermocycler. The PCR reassembly of small fragments into larger sequences was analyzed by taking samples after 25, 30, 35, 40, and 45 cycles of reassembly (Fig. 2). Whereas the reassembly of 100–200-bp fragments can yield a single PCR product of the correct size, 10–50-base fragments typically yield some product of the correct size as well as products of heterogeneous molecular weights. Most of this size heterogeneity appears to be due to single-stranded sequences at the ends of the products, since after restriction enzyme digestion a single band of the correct size is obtained.

**PCR with Primers.** After 1:40 dilution of this primerless PCR product into PCR mixture with 0.8 μM each primer and 15 additional cycles of PCR (94°C for 30 s, 50°C for 30 s, and 72°C for 30 s), a single product of the correct size is typically obtained (Fig. 2).

**Cloning and Analysis.** After digestion of the PCR product with terminal restriction enzymes (BamHI and EcoO109) and gel purification, the reassembled fragments were ligated into

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**Abbreviations:** IL, interleukin; ds, double stranded; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.
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resulting colonies were analyzed for the presence of the HindIII/Nhe I fragment, which is diagnostic for the +/− recombinant.

Whole Plasmid Reassembly. A 1:1 mixture of two 2.7-kb whole plasmids, pUC18+/− and pUC18−/+ , containing stop codons at different locations of lacZa (Fig. 3) was digested with DNase I and 100- to 200-bp fragments were purified as described above. The reassembly program was 60 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s plus 3 s per cycle. Gel analysis showed that most of the PCR product was >>20 kb. After digestion with a unique enzyme (EcoO109), most of the product consisted of a single band of the expected size, which was gel purified, ligated, transformed, and plated on LB/ampicillin/X-Gal plates as described above.

IL-1β Experiments. A murine IL-1β gene (BBG49) and a human IL-1β gene with Escherichia coli codon usage (BBG2; R & D Systems) were PCR amplified from plasmid templates using the primers TTAGGCACCCCAGGCTTT and ATGG-GCTGCAAGGCGATT, resulting in two 0.8-kb PCR fragments. The areas of sequence identity between the human and the murine IL-1β sequences are on average only 4.1 bases long (Fig. 5). To force recombination based on such short sequence identity, a very low effective annealing temperature was used. After the denaturation step, the tube was rapidly cooled in dry ice/ethanol and reheated to the annealing temperature. Then 1 unit of the Klengow fragment of DNA polymerase I was added. Because the enzyme is heat-labile, it needs to be added at every cycle. Preparation of dsDNA PCR products, DNase I digestion, and purification of fragments was as described for lacZ. Only the first 15 PCR cycles of the reassembly were performed with the heat-labile polymerase (manual PCR program: 94°C for 1 min, 10 s on dry ice/ethanol (until frozen), incubation for ~20 s at 25°C, addition of 1 unit of Klengow fragment, reaction for 2 min at 25°C (15 times)). After these 15 manual cycles, Taq polymerase was added and an additional 22 cycles of PCR without primers were performed (94°C for 30 s, 35°C for 30 s, no 72°C). The reaction mixture was then diluted 1:20, primers were added at 0.8 μM (AAGCCCGCATGAAGCTTG-GATCTTATT and AAAGCCCTCTAGATGATTAC-GAATTCATAT), and plus primer PCR was performed as described for lacZ. The second primer pair differed from the first pair only because a change in restriction sites was preferred. After digestion of the PCR product with Xba I and Sp h, the fragments were ligated into Xba I/Sp h-digested pUC18. The sequences of the inserts from several colonies were determined by dideoxy nucleotide DNA sequencing (United States Biochemical).

RESULTS AND DISCUSSION

Reassembly of the lacZa Gene. Fig. 2 shows a DNase I digest of a 1.0-kb sequence carrying the gene for the lacZa fragment. Gel-purified 10- to 50-bp fragments were reassembled (initially without primers) to a single PCR product of the correct size. Of the resulting colonies, 84% (n = 377) are
LacZ\(^+\), versus 94% without shuffling. When a high concentration of fragments (10–30 ng/μl) was used, the reassembly reaction was surprisingly reliable. By sequencing of random clones, it has been shown that the reassembly process introduces point mutations at a rate of 0.7% (n = 4437 bases; Table 1), which is similar to error-prone PCR (6, 7); 11/12 types of substitutions were found with no frameshifts (Table 1). Since the only difference compared to normal PCR was the use of small fragments, the rate of point mutagenesis may depend on the size of the fragments that are used in the reassembly. In contrast to PCR, DNA reassembly is an inverse chain reaction. In PCR the number of polymerase start sites and the number of molecules grows exponentially, whereas in DNA reassembly the number of start sites and the number (but not the size) of the molecules decreases over time. This reaction may perhaps be used for reassembly of genes from the highly fragmented DNA of fossils (23).

Crossover Between Two Markers in the lacZ Gene. Crossover between the two inactive lacZ\(^-\) genes from plasmids pUC18\(+/+\) and pUC18\(+/-\), each of which contains a single stop codon marker at positions that differ by 75 bases, was measured after shuffling (Fig. 3). The ratio of active, recombinant colonies was 24% (n = 386), close to the theoretically expected value of 25% for complete recombination. All 10 of the blue colonies assayed contained the diagnostic HindIII/NheI restriction fragment (data not shown).

Recombination of a Pool of Point Mutants. When 14 different point-mutated LacZ\(^-\) colonies, obtained from the experiment described in Fig. 2, were recombined as a pool, 34% (n = 291) of the resulting colonies were LacZ\(^+\). These colonies presumably arose by recombination of the DNA from different colonies. Assuming that the point mutations are spread out throughout the gene and that each of the 14 mutant plasmids contains only one knockout mutation, then for each marker there is a 13/14 chance of getting the wild-type version and the probability of getting all 14 wild-type versions in a single clone is (13/14\(^4\)) = 35%, close to the observed value of 34%. The close match between the predicted and the observed values of both LacZ shuffling experiments suggests that the fragments reassort freely. The 34% value contrasts with the expected rate of reversal of a single point mutation by error-prone PCR, which should be close to the published mutagenesis rate of 0.7% (7). However, a control experiment using error-prone PCR instead of shuffling was not feasible, because the 1-kb fragment proved too large to be generated by error-prone PCR.

Incorporation of Synthetic Oligonucleotides. Synthetic oligonucleotides can be added to the reassembly mixture and incorporated into the gene based solely on 15–20 bases of homology at the 5' and 3' flanking sequences (Fig. 4). Single-stranded DNA was incorporated more efficiently than dsDNA (8–9.3% versus 2.1%), presumably because of competitive hybridization. Mixtures of synthetic oligonucleotides, PCR fragments, or even whole genes can be mixed into another sequence library, and the insertion of one sequence cassette is independent from the insertion of a cassette in another part of the template (unpublished data). Thus, the
degree of recombination, the homology required, and the composition of the mixture can be independently and simultaneously varied along the length of the reassembled gene.

Creation of a Library of Chimeras of Two Related Genes. We attempted to create a library of chimeras between a human (with E. coli codon usage) and a murine IL-1β gene. Since the areas of sequence identity are on average only 4.1 bases long (Fig. 5), this represents an extreme case, exploring the minimum requirements for crossover. Crossovers could not be obtained by several approaches using Taq polymerase, even when a 10-fold excess of one of the IL-1β genes was used (data not shown). The explanation is that with any heat-stable polymerase, the cooling time of the PCR machine (94°C to 25°C at 1–2°C/s) causes the effective annealing temperature to be higher than the set annealing temperature. To force efficient crossovers based on such a low degree of homology, a very low effective annealing temperature is needed. By using the Klenow fragment of DNA polymerase I, a heat-labile polymerase, a low annealing temperature was obtained. Under these conditions, a total of 17 crossovers by DNA sequencing of nine colonies was obtained (Fig. 5). One of the crossovers was based on only 1 or 2 bases of uninterrupted identity, suggesting that some mismatches are tolerated if sufficient flanking homology is present.

Technical Issues. The removal of free primers from the PCR product before fragmentation proved to be very important. Without adequate primer removal, contamination with full-length template can lead to a low frequency of recombination. However, even restriction fragments or whole plasmids can be efficiently reassembled from their fragments, avoiding the potential contamination of PCR products with primers.

The preferred fragment size depends on the intended number of crossovers, which varies for different applications. For the in vitro evolution of a gene, for example, larger fragments, which result in one or two crossovers per gene, may be preferred (14).

The stringency of annealing during the reassembly PCR depends on the minimum degree of homology that should still yield crossovers. If the degree of homology is high, Taq polymerase can be used with an annealing temperature of between 45°C and 63°C. Crossovers occur when a partially reassembled PCR product primes on the homologous position of a related but not identical template (template switch). Crossovers based on identity of <15 bases appear more difficult to obtain. Control over the stringency of recombination is expected to be much greater with in vitro than with in vivo recombination.

Comparison to Other Mutagenesis Techniques. The two most widely used methods for protein mutagenesis are error-prone PCR and oligonucleotide-directed mutagenesis.

Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence (6, 7, 24, 25). Error-prone PCR can be used to mutagenize a mixture of fragments of unknown sequence. However, computer simulations (8–10) have suggested that point mutagenesis alone may often be too gradual to allow the block changes that are required for continued sequence evolution. The published error-prone PCR protocols (6, 7) do not allow amplification of DNA fragments >0.5–1.0 kb, limiting their practical application. In contrast, DNA shuffling can be applied to sequences >1 kb, has a mutagenesis rate similar to error-prone PCR, and also works with pools of unknown sequence. Repeated cycles of any mutagenesis strategy lead to an accumulation of neutral mutations, which, for example, may make a protein immunogenic. Only with DNA shuffling it is possible to remove such neutral mutations by backcrossing with excess parental or wild-type DNA (14).

In oligonucleotide-directed mutagenesis (1–5), a short sequence is replaced with a synthetically mutagenized oligonucleotide. This approach does not generate combinations of distant mutations and is combinatorial only within the limits of the oligonucleotide. The limited library size relative to the vast sequence space (13) means that many rounds of selection are unavoidable for protein optimization. Mutagenesis with synthetic oligonucleotides requires sequencing of individual clones after each selection round followed by grouping into families, arbitrarily choosing a single family, and reducing it to a consensus motif, which is resynthesized and reintroduced into a single gene followed by additional selection (3). This process constitutes a statistical bottleneck; it is labor intensive and not practical for many rounds of mutagenesis.

Table 1. Mutations introduced by mutagenic shuffling

<table>
<thead>
<tr>
<th>Transition</th>
<th>Frequency</th>
<th>Transversion</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>G → A</td>
<td>6</td>
<td>A → T</td>
<td>1</td>
</tr>
<tr>
<td>A → G</td>
<td>4</td>
<td>A → C</td>
<td>2</td>
</tr>
<tr>
<td>C → T</td>
<td>7</td>
<td>C → A</td>
<td>1</td>
</tr>
<tr>
<td>T → C</td>
<td>3</td>
<td>C → G</td>
<td>0</td>
</tr>
<tr>
<td></td>
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<td>G → C</td>
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A total of 4437 bases of shuffled lacZ DNA were sequenced. The mutation rate was 0.7%; 11/12 types of base substitutions were found, but there were no frameshifts.
Error-prone PCR and oligonucleotide-directed mutagenesis are thus useful for single cycles of fine tuning but rapidly become limiting when applied for multiple cycles. One apparent exception is selection of an RNA ligase ribozyme from a random RNA library using many rounds of amplification by error-prone PCR and selection (15). Based on the work of Holland (9), it is surprising that such a complex structure could be evolved without recombination. However, the occurrence of recombination in these experiments has not been ruled out. Using the lacZ assay (Fig. 3), recombination has been found to occur even during normal PCR at a frequency of ~0.03% per 25 cycles. Others have reported a rate of 5.4% recombinants under standard PCR conditions (26). Considering that >1000 cycles of PCR were required for selection of the RNA ligase, even such a low rate of recombination may have contributed significantly to evolution of the ribozyme.

A method that was developed for assembly of synthetic oligonucleotides into a full-length gene (27) could be used for recombination but differs fundamentally from DNA shuffling in that it uses known sequences and fixed junction sites. The chain shuffling of the heavy- and light-chain genes of antibodies is also conceptually different since it uses a single, fixed crossover site (28).

**Implications for Molecular Evolution.** The goal of applied molecular evolution (11–13) is to mimic the natural design process and speed it up by directed selection in vitro toward a simple, specific goal. Computer simulations of evolution, called genetic algorithms (8–10), have shown that recombination (with a low level of point mutagenesis) between individuals is sufficient for the evolution of complex linear sequences. Similarly, by mimicking the mechanisms by which bacteria have evolved a variety of resistance genes, the combination of episome transfer, recombination, point mutation, and selection should allow one to efficiently redesign genes, operons, pathways, and perhaps whole bacterial genomes for specific applications (14).

Limitations in genetic algorithms become more pronounced when more cycles are performed. The advantages of recombination, as well as the differences between different recombination formats, may become more pronounced with increasing numbers of cycles.

The ability to perform the molecular equivalent of some standard genetic matings by recombination in vitro, such as a molecular backcross, is likely to be useful. A molecular backcross is performed by repeated "mating" with the desired background while selecting for the mutations of interest (14). As in traditional breeding, this approach can be used to combine phenotypes from different sources into the background of choice.

Shuffling requires the presence of homologous regions separating regions of diversity. Scaffold-like protein structures may be particularly suitable for shuffling. The conserved scaffold determines the overall folding by self-association while displaying relatively unrestricted loops that mediate specific binding. Examples of such scaffolds are the immunoglobulin β-barrel, and the four-helix bundle (29).

It is of interest to note that the crossover, the essence of sexual recombination and a presumably complex behavior, actually occurs in naked DNA. Since sexuality appears to be a behavior that is inherent in the building blocks (13), recombination and sexuality are probably as old as DNA itself.

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