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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.**

The most widely used methods for protein mutagenesis are oligonucleotide-directed mutagenesis (1–5) and error-prone PCR (6, 7). Although recombination, with a low level of point mutation, was long ago demonstrated to be the preferred method for multiple cycles of mutagenesis (8–10), so far no methods have been developed for general, homologous recombination of DNA *in vitro*. A technically simple approach to recombination is reported, and its application to the design of linear sequences such as DNA, RNA, and proteins is explored (Fig. 1). The method involves digesting a large gene with DNase I to a pool of random DNA fragments (Fig. 2). These fragments can be reassembled into a full-length gene by repeated cycles of annealing in the presence of DNA polymerase. The fragments prime each other based on homology, and recombination occurs when fragments from one copy of a gene prime on another copy, causing a template switch.

The first experiment was an attempt to reassemble a 1-kb gene from short random fragments and then to determine the rate of mutations that may have occurred (Fig. 2). Next I tested whether recombination could be obtained between two genes, each carrying a stop codon marker at positions that differ by 75 bp (Fig. 3). Oligonucleotides were added to the pool of random fragments to determine whether they became incorporated into the gene (Fig. 4). Finally, I attempted to create a library of chimeras from a pair of homologous interleukin 1β (IL-1β) genes from different species (Fig. 5).

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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 AAAGCGTCGATTTTTGTGAT and ATGGGGTTCCGCGCACATTT (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 $MgCl_2$ 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 $MgCl_2$/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- to 200-bp fragments can yield a single PCR product of the correct size, 10- to 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 (*Bam*HI and *Eco*O109) and gel purification, the reassembled fragments were ligated into

Abbreviations: IL, interleukin; ds, double stranded; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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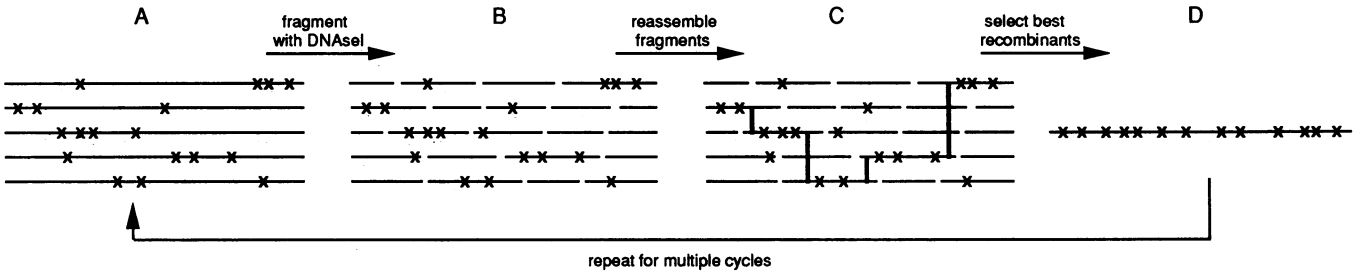


FIG. 1. (*A*) A pool of homologous genes with different point mutations is fragmented with DNase I. (*B*) For simplicity, all mutations shown are considered beneficial and additive. (*C*) Reassembly of the random fragments into full-length genes results in frequent template switching and recombination. A recombinant gene containing the four crossovers (thick lines) can be selected from the library of recombinants based on its improved function. (*D*) Selected pool of improved recombinants provides the starting point for another round of mutation and recombination. The recombination process alone causes a low level of point mutations but, if desired, additional mutations could be introduced by error-prone PCR or UV mutagenesis of the pool of genes.

pUC18 digested with *Bam*HI and *Eco*O109. After transformation and plating on plates with ampicillin, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), and isopropyl β-D-thiogalactopyranoside, the resulting colonies were analyzed for the presence of the *Hin*dIII/*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 *lacZα* (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 (*Eco*O109), 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 ATGTGCTGCAAGGCGATT, 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 Klenow 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 Klenow 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 (AACGCCGCATGCAAGCTTGGATCCTTATT and AAAGCCCTCTAGATGATTACGAATTCATAT), 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 *Sph* I, the fragments were ligated into *Xba* I/*Sph* I-digested pUC18. The sequences of the inserts from several colonies were determined by dideoxynucleotide DNA sequencing (United States Biochemical).

RESULTS AND DISCUSSION

Reassembly of the *lacZα* Gene. Fig. 2 shows a DNase I digest of a 1.0-kb sequence carrying the gene for the *lacZα* 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

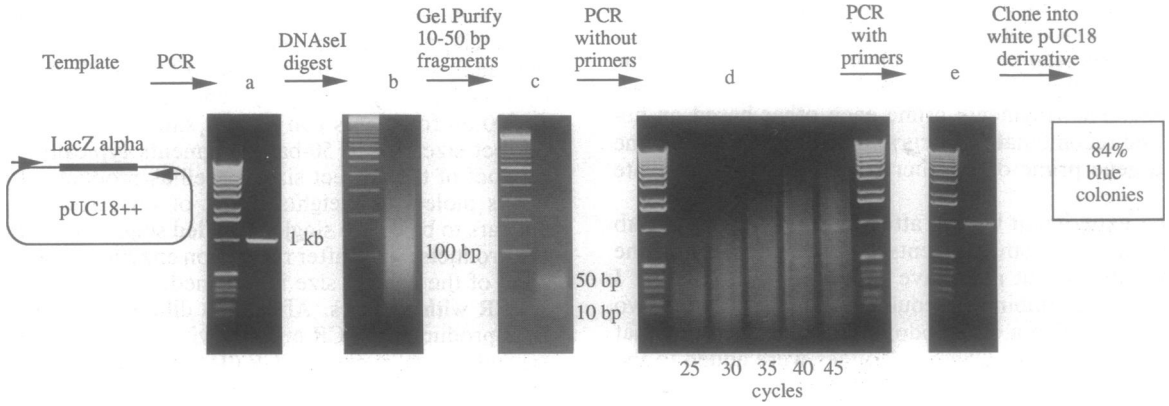


FIG. 2. Reassembly of a 1.0-kb gene from 10- to 50-bp random fragments. (*a*) A 1-kb DNA fragment encoding *lacZα* was amplified by PCR. After digestion of the gene with 0.15 unit of DNase I for 15 min at 20°C (*b*), fragments of 10–50 bases were purified from an agarose gel (*c*). (*d*) Purified fragments were reassembled into a full-length gene at a high fragment concentration (30 ng/μl) in the absence of primers. The average size of the PCR products increases gradually via the priming of one product on another. The heterogeneous appearance of the product is largely due to the partially single-stranded nature of the product. (*e*) After addition of primers and additional cycles of PCR, a single PCR product of the correct size is typically obtained. Cloning of this product into a plasmid yielded ≈84% light- to dark-blue colonies, reflecting mutations that occurred during the reassembly process.

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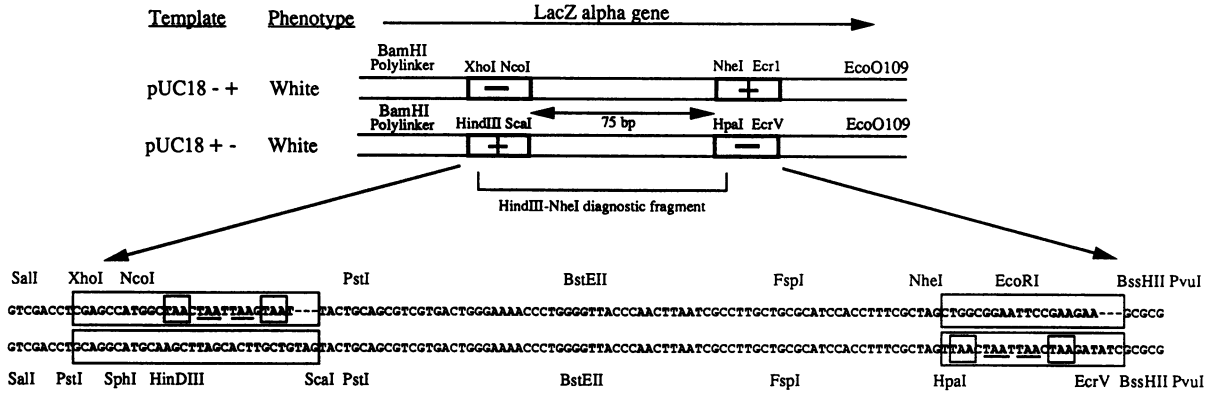
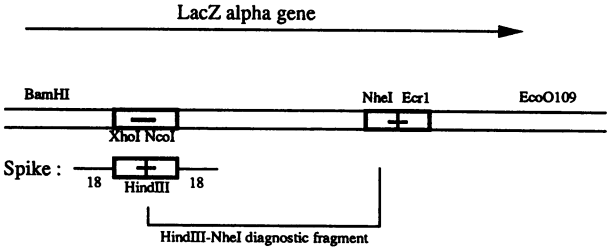


FIG. 3. Efficiency of recombination between two inactive *lacZα* genes, each carrying a single marker at a position that differs by 75 bases. Each marker is a 20- to 30-bp nonhomologous sequence with four stop codons, of which two are in the *lacZ* frame (small boxes), with one in each of the other two frames (underlined). The two 1-kb templates were mixed at a 1:1 ratio and shuffled. After cloning, 24% (n = 386) of the colonies had an active *lacZ* gene, resulting in blue colonies on X-Gal plates, which is near the theoretically expected value for complete recombination (25%). All 10 of the blue colonies contained the expected *Hin*dIII/*Nhe* I restriction fragment (data not shown).

$LacZ^+$, 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).



	% blue colonies
Control (no spiked oligo)	0.0 % (N=>1000)
Top strand spike	8.0 % (N=855)
Bottom strand spike	9.3 % (N=620)
Top and bottom strand spike	2.1 % (N=537)

FIG. 4. Mixtures of synthetic oligonucleotides can be added to the random fragments derived from a gene. In the reassembly reaction, these oligonucleotides can be incorporated instead of the original sequence. A 1-kb PCR fragment containing *lacZ*$^-$ from the pUC18−/+ plasmid (see Fig. 3) was digested with DNase I and small fragments were purified. A 66-mer oligonucleotide, containing 18 bases of homology to *lacZ* at both the 3′ and 5′ ends, was mixed in at a 4-fold molar excess. This oligonucleotide was designed to replace the 25-base heterologous *lacZ*-inactivating marker containing four stop codon mutations with wild-type *lacZ* sequence. The degree of incorporation of such oligonucleotides can be varied over a wide range by adjusting the molar excess, the annealing temperature, or the melting temperature of the homologous flanking sequence. Incorporation was less when both the top and bottom strand oligonucleotides were added, presumably because of competitive hybridization with the complementary oligonucleotide.

Crossover Between Two Markers in the *lacZ* Gene. Crossover between the two inactive *lacZα* 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 *Hin*dIII/*Nhe* I restriction fragment.

The whole 2.7-kb plasmids containing these same $LacZ^-$ markers were also efficiently reassembled from random 100- to 200-bp fragments. For reassembly of fragments derived from whole plasmids the theoretical end point is a single, large concatemeric molecule. As expected, concatemers of >20 kb were obtained, which, after digestion with a unique restriction enzyme, yielded the expected 2.7 kb; 11% (n = 328) of the resulting plasmids had recombined in the 75-bp area between the markers, resulting in a $LacZ^+$ phenotype.

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 $(13/14)^{14}$ = 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

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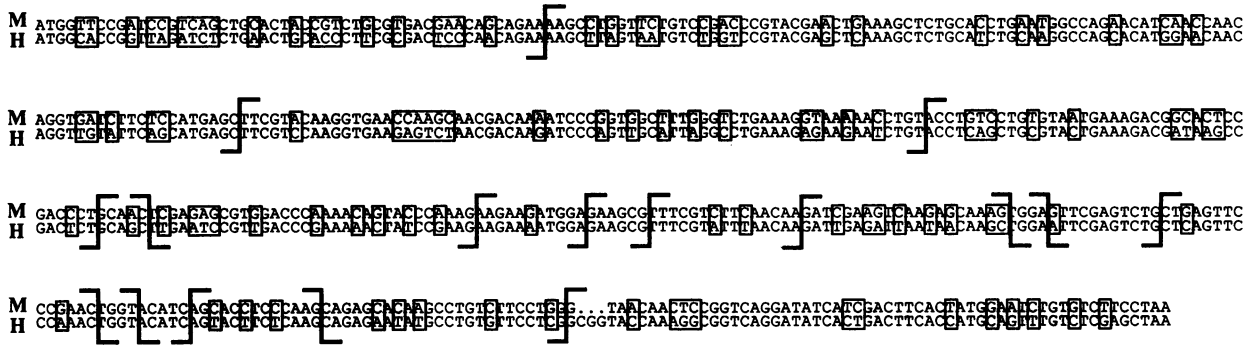


FIG. 5. Shuffling of a murine gene (M) with a human gene (H; with *E. coli* codon usage) for IL-1β represents an extreme example of shuffling, since the average length of sequence identity is only 4 bases. Crossovers could be obtained only by ensuring a low annealing temperature, which required the use of a manual PCR with a heat-labile, low-temperature DNA polymerase, such as the Klenow fragment of DNA polymerase I. Shown are the 17 crossovers that were obtained from DNA sequence analysis of nine colonies. Boxes show mismatched bases; thick bars represent crossovers. Surprisingly little sequence identity was sufficient to yield crossovers, presumably because of the tolerance for mismatched bases at the low annealing temperature.

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 differs for various 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).

Table 1. Mutations introduced by mutagenic shuffling

Transition	Frequency	Transversion	Frequency
G → A	6	A → T	1
A → G	4	A → C	2
C → T	7	C → A	1
T → C	3	C → G	0
		G → C	3
		G → T	2
		T → A	1
		T → G	2

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.

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 65°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 is it 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 reinserted 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.

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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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