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Stimulation and suppression of PCR-mediated recombination

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ABSTRACT

Recombination, or chimera formation, is known to occur between related template sequences present in a single PCR amplification. To characterize the conditions under which such recombinant amplification products form we monitored the exchange of sequence between two homologous templates carrying different restriction sites separated by 282 bp. Using a typical cycling program the rates of recombination between the two restriction sites were 1 and 7% using Taq and Vent polymerases respectively over 12 doublings. However, by using long elongation times and cycling only to the mid-point of the amplification recombination could be suppressed below visual detection with both polymerases. Conversely, cycling programs designed to promote incomplete primer elongation and subsequent template strand exchange stimulated recombination to >20%.

INTRODUCTION

The phenomenon of chimera formation, or recombination, between similar sequences during amplification by PCR has long bedeviled biologists. Saiki *et al.* (1) noted that PCR performed with the Klenow fragment of DNA polymerase I resulted in chimeras of the different alleles being amplified. Subsequently there have been a number of reports of chimera formation using both Taq and Vent polymerases (2–4). This phenomenon of chimera formation between related sequences has been attributed to incomplete primer extension in the elongation phase of the PCR cycle: if multiple related sequences are present in the reaction as template the incompletely extended primer can anneal to a different template in a subsequent cycle and be completely extended. This results in a chimeric product, equivalent to one in which recombination had occurred between the two parent templates at the point where elongation ceased on the first.

Given the propensity of related sequences to recombine in this manner, it is important for those attempting to clone an individual gene from cells containing multiple alleles or from a sample containing more than one related species to understand the conditions leading to chimera formation and how best to avoid them. For workers in the field of *in vitro* evolution, however, PCR-mediated recombination could potentially be exploited as a rich source of diversity from which to select novel molecules. *In vitro* evolution (or SELEX) has been immensely successful in finding novel nucleic acid aptamers and enzymes (5–9) and related techniques for *in vitro* evolution of proteins have been successful in directing modification of proteins (10,11).

In carrying out an *in vitro* evolution experiment a large pool of different DNA sequences (or a pool of RNA molecules or proteins derived from the DNA pool) is sifted for those molecules with a defined property. In the case of selection for RNA or protein the DNA molecules encoding the selected molecules are recovered by reverse transcription or by one of several mechanisms for linking protein and its encoding nucleic acid respectively (10,11). Those DNA molecules encoding the activity are then re-amplified by PCR and subjected to a new round of selection. After several rounds of selection has simplified the pool to a smaller number of more active molecules the pool is often subjected to mutagenesis to increase the number of different sequences related to active molecules in the hope of finding ever more active variants. Because recombination or 'shuffling' of related molecules has been shown both theoretically (12) and experimentally (13,14) to allow significantly more rapid evolution than mutagenesis alone, it would be greatly advantageous to be able to efficiently build recombination into the selection procedure itself.

At present random recombination between homologous DNA sequences can be carried out *in vitro* by limited digestion with DNase followed by gel purification of short fragments. These randomly overlapping fragments are then subjected to PCR in the absence of primers, allowing them to prime off one another to re-assemble full-length molecules, each individual now consisting of sequences derived from different parent molecules (13,14).

Here we report that by altering the PCR cycling conditions to favor incomplete extension and subsequent priming of those incompletely extended products we can force an increased recombination rate. Under these modified conditions we obtain >20% recombination between two sites separated by 282 bp after ~12 doublings. In addition, we detail conditions under which the recombination rate can be suppressed significantly below that found using typical conditions.

MATERIALS AND METHODS

PCR templates and primers

The template used for PCR was derived from pRSET-B (Invitrogen), a pUC-based plasmid. Using the 5′ primer 5′-TCCTGGTTACTACACGACG and the 3′ primer 5′-ATAAC-TACGATACGGGAGCG the 590 bp fragment from position 1270 to 1860 was amplified and then separately mutagenized by PCR to introduce a *Sma*I site at position 1333 and a *Pvu*II site at position 1614. The *Sma*I site was introduced by PCR using a

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'+SmaI' primer extending from the 5′-end of the fragment to position 1351, identical to the fragment sequence with the exception of a G→C change at position 1333 to introduce a *Sma*I site. The *Pvu*II site was introduced in three PCR steps. PCR was performed using a 5′ '+PvuII' primer, corresponding to the sequence from 1595 to 1633 with the exception of C1616G, A1618T and C1619G changes to introduce the *Pvu*II site, and the above 3′ primer, resulting in a 3′-fragment of the 590 bp sequence containing a *Pvu*II site at its 5′-end. The corresponding 5′-fragment was created by PCR using the complement of the above '+PvuII' primer and the 5′ primer, resulting in a 5′-fragment of the 590 bp fragment with the corresponding *Pvu*II site at its 3′-end. After gel purification both fragments were subjected to PCR with the 5′ and 3′ primers to regenerate a full-length 590 bp fragment, now containing a *Pvu*II site at position 1614. The two 590 bp fragments used as template therefore differ in only four positions, to encode a *Sma*I site at position 63 in the first and a *Pvu*II site at position 345 in the second.

PCR equipment and protocols

PCR reactions were carried out using a Hybaid Omnigene Thermal Cycler. PCR reactions were set up in 100 ml, using 2 ng total DNA, usually 1 ng each of the SmaI and PvuII fragments, 0.4 mM each dNTP, 1 µM each primer, the buffer supplied with the enzyme and, for Taq reactions, magnesium chloride added to a final concentration of 2.3 mM. Magnesium sulfate concentration in the Vent reactions was 2 mM. Two units of Vent DNA polymerase (New England Biolabs) and 5 units of Taq DNA polymerase (Promega) were added per 100 ml reaction according to the manufacturer's suggestion. Reactions were overlaid with 50 ml mineral oil. Cycling protocols used are described in the legend to Figure 2.

Recombination was monitored by the appearance of a restriction fragment derived from the PCR product containing both *Sma*I and *Pvu*II sites. Agarose gels were run in the presence of ethidium bromide and the relative intensities of DNA bands measured using an Alpha Innotech Corporation IS1000 Digital Imaging System. Absolute values were calculated by comparison with known standards.

Calculations

In order to calculate the degree of recombination the intensity of each band in a lane was measured using the spot denzo function of the Alpha Innotech Corporation IS1000 Digital Imaging System, providing raw data in relative units. A single background measurement was taken in the middle of each lane between the 345 and 282 bp bands. For experiments in which recombination was high only bands in the last (low loading) lane of each experiment were used; for those in which recombination was too low to be easily detected in the last lane the intensity of the 282 band and background were measured in a lane with more total loaded DNA and then normalized to the remaining values by the difference in loading.

Approximately 7% of the SmaI template and 3% of the PvuII template is refractory to cleavage in the absence of recombination (compare Fig. 2, lanes 3, 4, 9 and 10); this is thought to derive from wild-type template lacking either site contaminating each template preparation. The contribution of this contaminating wild-type template to uncleavable product after mixed template PCR was subtracted before any further data manipulation.

A sample calculation for the data in Figure 2, lane 13 (shown in Table 1) is provided. Background for this lane was 62. Because the ethidium bromide fluorescence of each fragment is proportional to its length, after subtraction of background for each band intensity the values were normalized to full-length by the ratio of the size of the full-length fragment to the fragment in question. The normalized values were added and percent of each of the total was calculated. The input template is contaminated with ~5% wild-type fragment lacking any restriction sites; this is subtracted from the value of the uncleaved 590 bp band.

Table 1.

Fragment	Raw data	Data minus background	Normalized to full-length	Percent of total
590	249	187	187	25 – 5 = 20
527	214	152	170	22
345	156	94	161	21
282	80	18	38	5
245	146	84	202	27

The existence of recombinant product lacking any restriction sites is masked by the formation of heteroduplex, as described in Results, so an initial estimation of the total amount of recombinant product was taken as twice the value for the 282 band, for a total of 10% recombinant product in the sample shown. This can be taken as a minimum estimate for the extent of recombinant product formation, but in cases where heteroduplex formation is significant heteroduplex formation reduces the amount of cleavable recombinant product significantly: because the recombinant product containing both *Sma*I and *Pvu*II sites is relatively rare nearly all heteroduplex formation between a recombinant strand containing both sites and some other strand will result in a fragment that does not contain both full restriction sites, so the amount of product in the 282 bp band is reduced by approximately the total rate of heteroduplex formation. The degree of recombinant product in the uncleaved 590 bp band should be equivalent to that corresponding to the 282 bp band, so it can be subtracted from the value for the 590 bp band for an estimate of the amount of heteroduplex product in the 590 band. As described in Results, the amount of uncleavable heteroduplex product represents half of the total heteroduplex product, so the total heteroduplex product is then twice the amount calculated to be in the 590 bp band. In the case shown below the amount of recombinant product in the 590 bp band represents 5% of total products. The amount of heteroduplex in the 590 bp band is then 15%, giving a total number of heteroduplex products of 30%. The intensity of the 282 bp band has therefore been reduced by ~30% from the actual value. Correcting for this gives a value of 7%, which when doubled to account for both reciprocal recombinant products gives a corrected approximate value of 14% total recombinant product formation, as compared with 10% without taking heteroduplex formation into account. Repeating the calculations using the new value for the recombinant products did not change the results significantly.

RESULTS

Recombination during PCR is thought to occur when elongating polymerase pauses or disengages from the template before elongation is complete. If this incompletely elongated strand primes synthesis on a non-identical template in a subsequent round a chimeric molecule is produced.

To explore ways in which we might enhance or suppress this recombination we engineered either a *Sma*I or *Pvu*II restriction

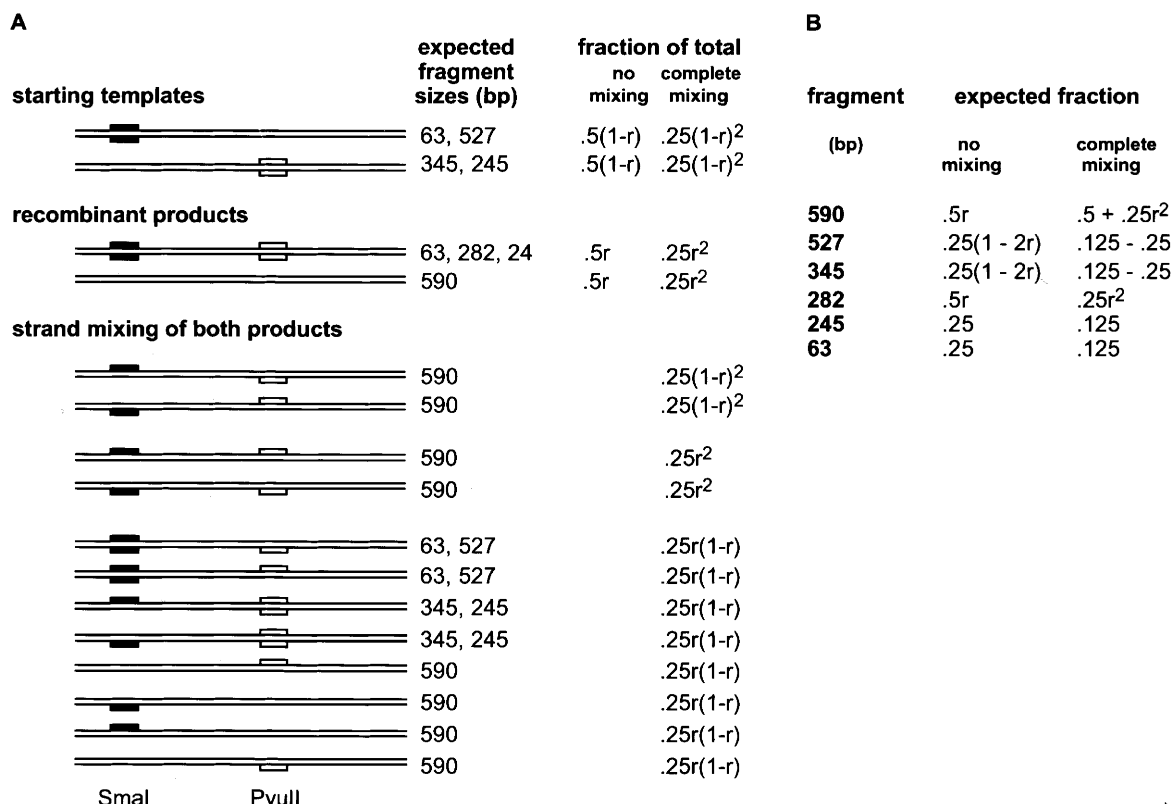

Figure 1. (**A**) Schematic representation of PCR templates used for amplification and the expected products for recombination and strand mixing. DNA templates for amplification were generated as described in Materials and Methods such that they carried either a *Sma*I or a *Pvu*II site. The fraction of each product was calculated for a given level of recombination (r = fraction of each template undergoing recombination) assuming either no strand mixing or complete random assortment of strands (see text for a more complete description). (**B**) Calculated molar proportion of each fragment expected.

enzyme site into a 590 bp DNA fragment at positions 63 and 345 respectively (Fig. 1A). When both fragments are included as template in a single PCR reaction recombination between the two sites produces products containing either both or neither site. We assessed the degree of recombination by measuring the relative intensity of a novel band derived from the recombinant DNA fragment containing both *Sma*I and *Pvu*II sites.

This assessment is complicated by the fact that late in a PCR reaction the high product concentration results in frequent strand re-annealing with another full-length complementary strand rather than with a primer for subsequent elongation. Where there are related but non-identical sequences in the reaction this re-annealing produces heteroduplex products, which in this case results in fragments that may contain a restriction site on only one strand, preventing cleavage (see Fig. 1 and below).

Factors that might affect the degree of incomplete elongation include the processivity of the enzyme and the length of time provided for elongation. In addition, slow cooling between the denaturation step and the annealing step could be expected to favor annealing of incomplete products over the more abundant primer. Because incompletely elongated product will have a much higher annealing temperature to the template than unextended primer, slow cooling between the denaturation and annealing steps should allow incompletely elongated product to bind template and begin further elongation before the annealing temperature of the primer is reached. To test these possibilities we assayed the degree of recombination produced with either Taq or Vent polymerases using: (i) a standard cycling program; (ii) a program designed to minimize partial elongation by providing a long elongation time; (iii) two different cycling programs designed to favor recombination by promoting incomplete elongation and by favoring annealing of those incompletely elongated products relative to the primer.

In Figures 2 and 3 we show the cleavage products of PCR DNA produced by either Taq or Vent polymerases using either a standard PCR cycle or one designed to facilitate recombination. The recombinogenic PCR cycle (shown schematically on the right in Figs 2 and 3) can be considered as a standard PCR cycle interrupted by an additional denaturation step: after a brief annealing step the temperature is immediately raised to the denaturation temperature to discourage complete elongation. After denaturation the reactions are cooled briefly to 72°C to allow incompletely elongated products to anneal to complementary full-length strands under conditions where primer is not able to compete for binding. The cycle is then continued from the annealing step.

The standard PCR cycle produces ~1% recombination over 25 cycles using Taq polymerase and 7% using Vent (Figs 2 and 3), while the cycle to promote recombination results in 14 and 19% recombination respectively (details of the calculations are found in Materials and Methods). The amount of product was quantitated by comparison with standard curves and the number of doublings that occurred in each reaction was calculated to be ~12.

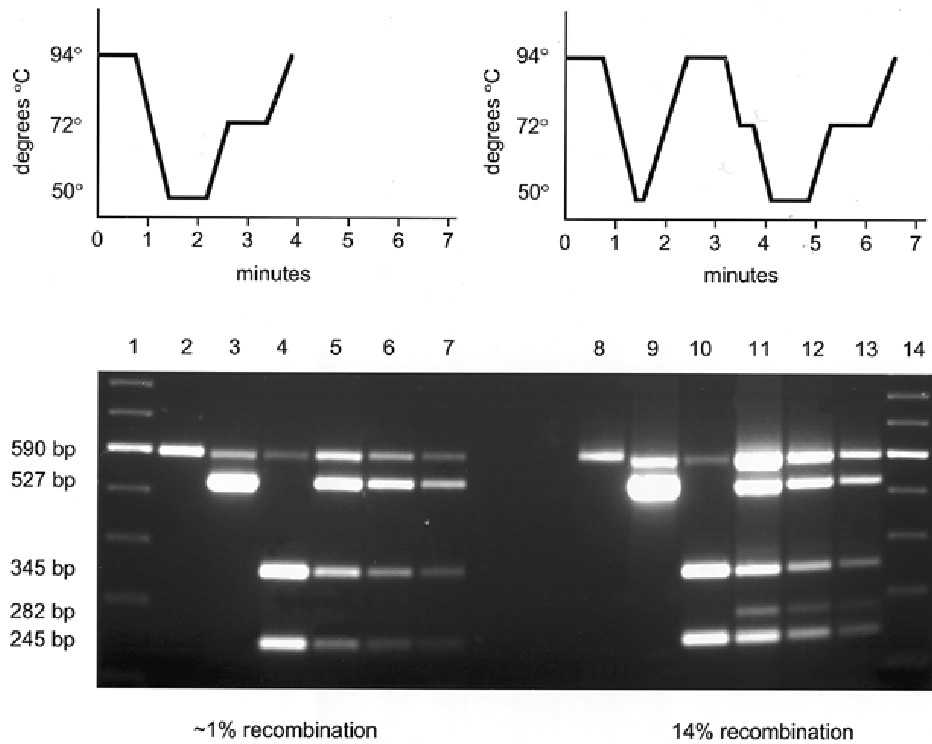

Figure 2. PCR-induced recombination as a function of cycling protocol. Two nanograms of template consisting of either SmaI or PvuII template alone or mixed in equal concentration were amplified with Taq polymerase using the PCR protocol depicted above each set of lanes. An equal portion of the PCR products was then subjected to complete digestion with both *Sma*I and *Pvu*II. Digestion products were analyzed by agarose gel electrophoresis in the presence of ethidium bromide. Fragment concentrations were estimated by measuring the fluorescence intensity of individual bands and comparing them with known standards (digital image capture and analysis performed with the Alpha Innotech IS1000 Imaging System). A 5-fold dilution of the uncleaved PCR product was loaded for comparison. Lanes 1 and 14, 100 bp marker; lanes 2 and 8, uncut PCR products at a 5-fold dilution relative to lanes 3–5 and 9–11; lanes 3 and 9, *Sma*I digest of amplified SmaI fragment; lanes 4 and 10, *Pvu*II digest of amplified PvuII fragment; lanes 5–7 and 11–13, 2-fold serial dilutions of *Sma*I/*Pvu*II-digested mixed fragment PCR reactions.

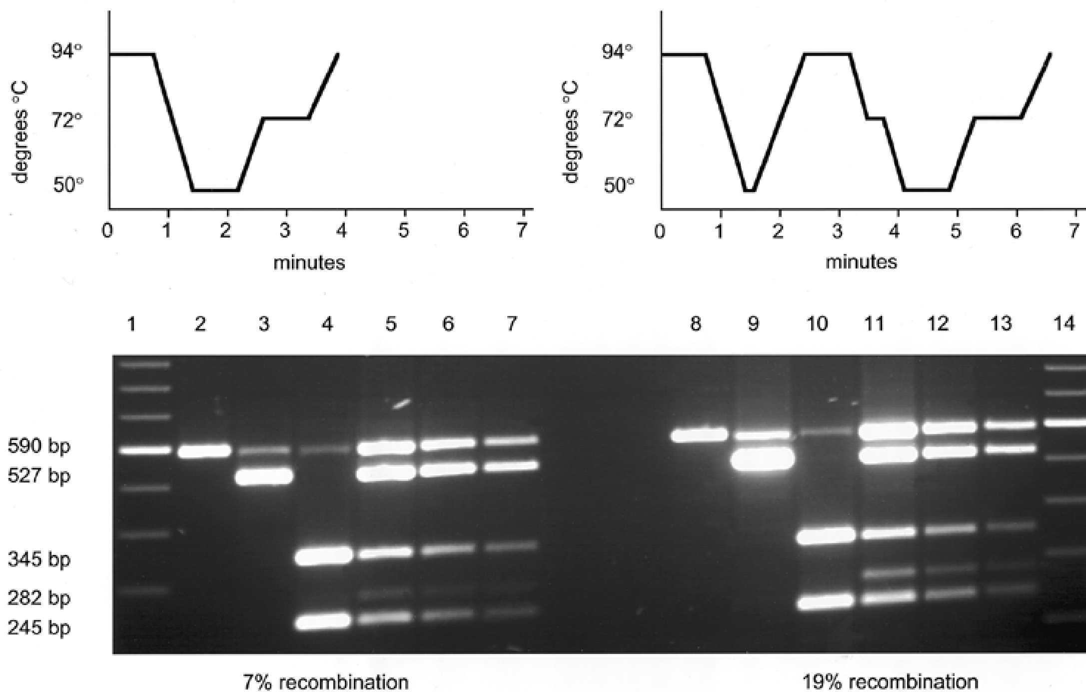

Figure 3. Identical to Figure 2 except that Vent polymerase was used rather than Taq polymerase for PCR reactions.

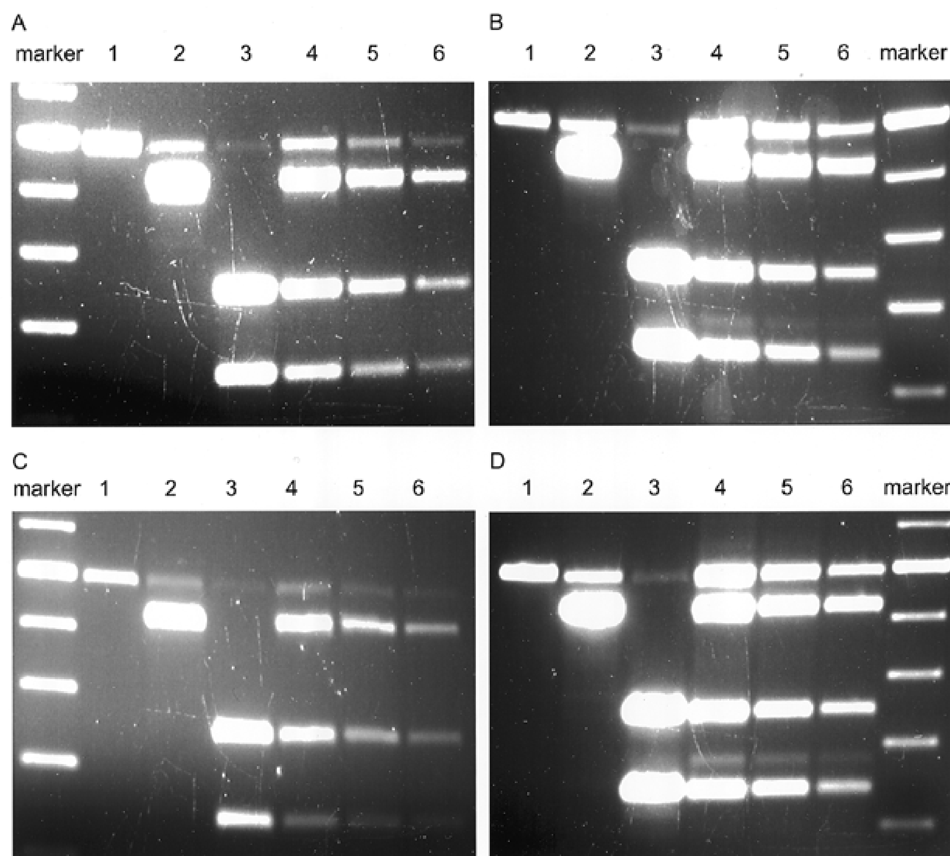

Figure 4. Effect of elongation time and cycle number on recombination. PCR reactions were performed using the standard cycle described in Figure 2 except that the elongation step was extended from 45 s to 3 min. Pictures were taken using the highest exposure and contrast practicable in an attempt to visualize recombination products. For comparison with the other figures the recombination products visible in (B) and (D) are not visible under normal contrast. Note that under these conditions of high contrast imperfections in the gel and UV light source are clearly visible. (**A**) PCR using Taq polymerase to the mid-point of amplification (the point at which half as much product is formed as at the end-point; 20 cycles). (**B**) PCR using Taq polymerase to 25 cycles. (**C**) PCR using Vent polymerase to the mid-point of amplification (13 cycles). (**D**) PCR using Vent polymerase to 25 cycles. Lanes 1, uncut PCR product; lanes 2, *Sma*I-digested amplified SmaI fragment; lanes 3, *Pvu*II-digested amplified PvuII fragment; lanes 4–6, 2-fold serial dilutions of *Sma*I/*Pvu*II-digested mixed amplification.

Note that when PCR product generated from the combined templates is cleaved with both enzymes a substantial fraction of DNA is refractory to cleavage, even when little or no recombination has occurred. This is because in the later stages of an amplification product accumulates to a degree that re-annealing competes significantly with primer binding and extension. In this case strand mixing results in formation of heteroduplex products that contain restriction enzyme recognition sequences on one strand but not the other (Fig. 1). In Figures 4 and 5 it can be seen that the degree of heteroduplex formation increases with increasing cycle number, as is expected due to higher product concentration. (There is a small amount of contaminating wild-type template lacking either restriction site in the SmaI and PvuII template preparations; see for example Figs 2 and 3, lanes 3, 4, 9 and 10. The contribution this contaminating wild-type template makes to uncleaved material in mixed template PCR reactions is accounted for in all calculations as detailed in Materials and Methods.)

Regardless of the extent of recombination, re-annealing results in a re-assorting of restriction site halves to 25% SmaI/SmaI, 25% PvuII/PvuII and 50% SmaI/PvuII duplexes. Therefore, the amount of heteroduplex product from a mixed amplification uncleaved by either enzyme represents only 50% of the total re-annealed product formed in the final cycle. Because the recombined product represents a minority of sequences, re-annealing will be nearly exclusively with a non-homologous strand, preventing cleavage by one enzyme. This leads to an underestimation of the total extent of recombination by approximately the degree to which the final products derive from re-annealing as opposed to primer extension (in the extreme case, dropping from a level of $0.5r$ to $0.25r^2$; Fig. 1). For example, in lane 5 of Figure 3 only 2.1% of the PCR products are cleaved by both enzymes and therefore 2.1% should also be cleaved by neither. However, after correction for contaminating wild-type template 23% of the products are not cleaved by either enzyme, indicating that ~40% of the products derive from re-annealing [(23 – 2.1%) × 2]. Therefore, the doubly cut band only represents ~60% of the actual recombinant product containing both sites, giving a corrected value for recombination to yield products containing both sites of 3.5% and for total recombination of ~7%.

Marton *et al.* (15) report that DNA nicking promotes recombination, presumably because priming on a nicked template produces an incompletely extended product that can serve as a primer on a new template in the next round. We noted that our recombinogenic cycle holds the template at 94°C for twice as long as the standard cycle and that the resulting extra nicking of the template might be responsible for the recombinogenic effect of the cycle. To test this we heated the template under PCR buffer conditions for 20 min at 94°C before amplifying it using the standard cycle. This produced no excess recombination (data not

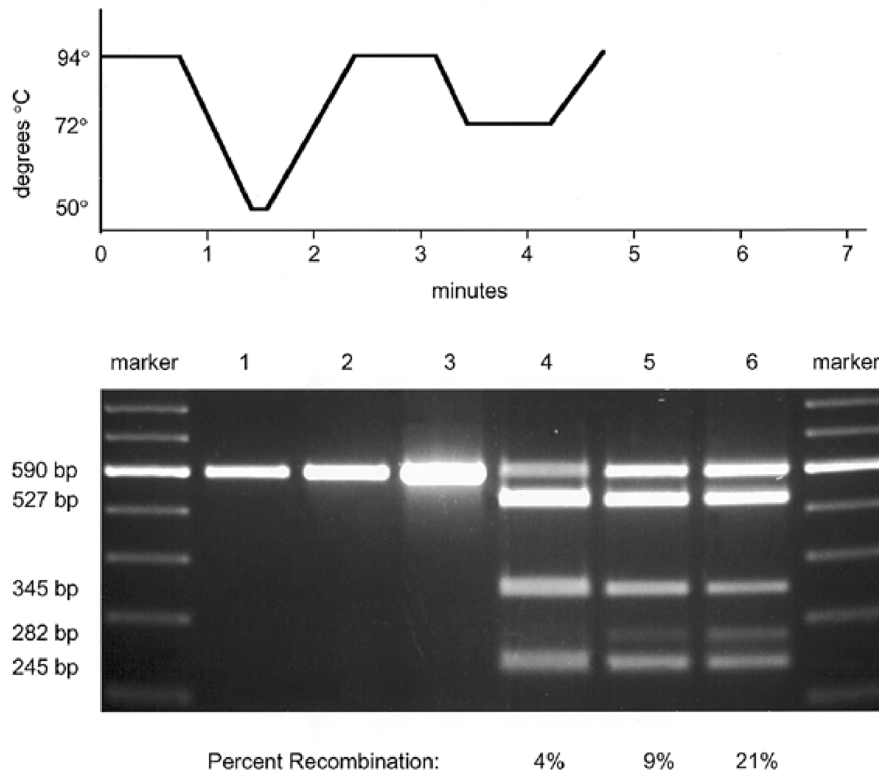

Figure 5. Recombination extent with increasing cycle number using a recombinogenic program. PCR was carried out with Taq polymerase using the following cycle: 94°C 45 s, 50°C 10 s, 94°C 45 s, 72°C 45 s for either 20, 25 or 30 cycles, followed by three cycles of 94°C 45 s, 50°C 1 min, 72°C 3 min. Lanes 1 and 8, 100 bp marker; lanes 2–4, product from 20, 25 and 30 cycles respectively; lanes 5–7, *Sma*I/*Pvu*II-cleaved products from 20, 25 and 30 cycles respectively. In lanes 5–7 equivalent amounts of cleaved product were loaded to allow direct comparison.

shown), suggesting that in our case excess recombination produced by the recombinogenic cycle is due to something other than prolonged heating.

Evidence suggests that both short extension times and over-PCR contribute to recombination (2,3). To test these parameters we carried out PCR amplification using a 3 min rather than 45 s extension time and sampled the reactions at the amplification mid-points (the point at which half as much product was formed as at the end-point) as well as at the end of 25 cycles. Control experiments showed that the mid-points of amplification under our conditions occurred at 20 cycles for Taq and 13 cycles for Vent (Fig. 4).

Under these conditions recombination was visible at 25 cycles for both the Taq and Vent reactions and was calculated to comprise ~0.5–1% of products for both enzymes, compared with 1 and 7% for Taq and Vent respectively using a 45 s elongation time, suggesting that longer elongation times may in fact reduce recombination. However, at the mid-points of both reactions recombinant product could not be identified visually even using high exposure times and the most optimal contrast (compare Fig. 4A with B and C with D). The products at the mid-points of the reactions were the result of 9.7 doublings for both the Taq and Vent reactions, while the end-point products after 25 cycles were the result of 11.5 and 11.7 doublings respectively, suggesting that what recombination does occur does so only late in the amplification program.

To test the possibility that recombination also occurs late in the programs designed to promote recombination we used a modified program in which the standard program was interrupted by a single denaturation step after the short annealing step. This was carried out using Taq polymerase for 20, 25 or 30 cycles, followed by three standard cycles, which were found to be necessary to obtain clean product (Fig. 5). Although the amount of total product only doubled with each five cycles between cycles 20 and 30, the amount of recombination rose strikingly from 4 to >20%.

This tendency for recombination to occur late in amplification probably explains the high degree of recombination with Vent polymerase we found using the standard PCR program (Fig. 3), as the mid-point for product formation occurs for Vent much earlier than that for Taq (cycle 13 versus 20). Supporting this explanation, when we carried out the standard program using Taq polymerase for 30 cycles rather than 25 the rate of recombination was greatly increased (data not shown).

DISCUSSION

In the course of evolving a novel molecule by the process of *in vitro* evolution a random or highly variable pool of molecules is subjected to selection for functional variants. Repeated cycles of selection followed by amplification of selected molecules by PCR results in a pool of much reduced complexity in which many molecules have the selected property. In order to more completely search the sequence space near active molecules pools are often subjected to mutagenesis in later cycles of selection, allowing yet more active variants to be selected (8). However, because the likelihood of a molecule acquiring a disadvantageous mutation is much greater than that of an advantageous one, any single molecule is unlikely to simultaneously acquire many ‘up’ mutations without concomitant ‘down’ mutations. Recombination

between individual related variants solves this problem by allowing the progressive accumulation of 'up' mutations in one molecule while simultaneously excluding 'down' mutations.

It has been suggested on theoretical grounds that the successful selection of highly complex functional molecules from sequence pools of presently available complexity must in fact be in part dependent on recombination during PCR (14). In the late stages of a selection, when the pool contains many related molecules, recombination would allow re-assortment of variation within a sequence family to allow a much more intensive examination of the sequence space around a particular consensus sequence than that afforded by simple mutation. The significant rate of recombination found using standard PCR cycling programs lends support to this hypothesis.

To take advantage of this phenomenon we have explored ways of specifically promoting PCR-mediated recombination to more efficiently search sequence space during an *in vitro* selection experiment. We find that cycling programs using a very short elongation time and a subsequent slow cooling to the annealing temperature can promote recombination between two sites separated by 282 bp to produce >20% recombinant product molecules, nearly half of the maximum achievable (50%). Half of the recombination events in this system will be between templates containing the same restriction site, producing a 'chimera' with a sequence identical to the parents. Therefore, if 20% of the products are found to be chimeras containing either both or no sites, a minimum of 40% of the total product molecules in the reaction are in fact derived from some recombination event, giving a recombination rate of 0.14% per base. This number is likely to be a minimum estimate, because the final number of chimeric molecules containing two restriction sites upon which the recombination rate is based is reduced by second recombination events that remove one of the sites.

Insofar as recombination occurs to at least some extent at most positions in the amplified sequence, the resulting PCR products will provide broad variation for continued selection during *in vitro* evolution. Results reported by Yang *et al.* (3) on recombination between two distinct HIV1 *tat* gene sequences indicate that although recombination occurs more frequently in some regions than others, recombination junctions may be relatively evenly spread throughout the sequence being amplified.

If this assumption holds, PCR-mediated recombination may serve as a convenient alternative to the more technically elaborate 'DNA shuffling' approach to recombinant mutagenesis (13,14), as the former procedure can be incorporated into the PCR amplifications required for *in vitro* selection with virtually no additional experimental effort.

Many *in vitro* selections are carried out using shorter molecules than were used in this study, however, a recombination rate similar to that reported here under 'recombinogenic' conditions would still result in ~10% chimera formation per PCR amplification for molecules with 70 nt between primer binding sites.

Interestingly, most recombination appears to occur very late in the amplification using either the standard or recombinogenic amplification programs. It has been suggested that the increasing template to primer ratio may allow partially elongated primer to better compete for template binding (2). However, the observation that most recombination also occurs late using a program specifically designed to favor annealing of partially elongated primer throughout the amplification suggests that this is not an important effect in our system. Alternatively, the increasing concentration of DNA in later cycles may reduce the effective polymerase concentration such that polymerase non-processivity is no longer effectively countered by rapid polymerase re-association.

Significantly for *in vitro* selection applications, most chimeric molecules are therefore derived directly from independent recombination events, rather than from amplification of chimeric molecules produced earlier in the amplification. To the extent that recombination sites are well distributed across the template, the complexity of the recombinant pool will therefore correspond closely to the number of recombinant molecules obtained.

CONCLUSIONS

Recombination during PCR reactions can be promoted or suppressed by choice of cycling program. By using cycling programs designed to promote incomplete elongation and subsequent priming by incompletely elongated products recombination can be stimulated to account for a significant proportion of the final products. The use of such recombinogenic PCR programs should facilitate optimization of complex activities during *in vitro* selection.

As has been previously suggested, we find that extended elongation times reduce recombination, as might be expected if polymerase pausing leads to slow elongation of some templates. More strikingly, we find that recombination does not seem to occur to a significant extent until the products have accumulated to relatively high concentrations, whether using a typical cycling program or the recombinogenic programs detailed here. These findings suggest that those who wish to avoid recombination between related sequences should employ PCR programs with increased elongation times and the absolute minimum number of cycles necessary to produce usable product.

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REFERENCES

1 Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) *Science*, **239**, 487–491.
2 Meyerhans,A., Vartainian,J.-P. and Wain-Hobson,S. (1990) *Nucleic Acids Res*., **18**, 1687–1691.
3 Yang,Y.L., Wang,G., Dorman,K. and Kaplan,A.H. (1996) *AIDS Res. Hum. Retroviruses*, **12**, 303–306.
4 Bradley,R.D. and Hillis,D.M. (1997) *Mol. Biol. Evol*., **14**, 592–593.
5 Tuerk,C. and Gold,L. (1990) *Science*, **249**, 505–510.
6 Joyce,G.F. (1992) *Scient. Am*., **267**, 90–97.
7 Szostak,J (1992) *Trends Biochem. Sci*., **17**, 89–93.
8 Breaker,R. and Joyce,G. (1994) *Trends Biotechnol*., **12**, 268–275.
9 Kumar,P.K.R. and Ellington,A.D. (1995) *FASEB J*., **9**, 1183–1195.
10 Clackson,T. and Wells,J.A. (1994) *Trends Biotechnol*., **12**, 173–184.
11 Hanes,J and Plückthun,A. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 4937–4942.
12 Holland,J.H. (1992) *Scient. Am*., **267**, 66–72.
13 Stemmer,W.P.C. (1994) *Nature*, **370**, 389–391.
14 Stemmer,W.P.C. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 10747–10751.
15 Marton,A., Delbecchi,L. and Bourgaux,P. (1991) *Nucleic Acids Res*., **19**, 2423–2426.