Constructing DNA by polymerase recombination

A.A.Yolov and Z.A.Shabarova
Department of Chemistry, M.V.Lomonosov State University, Moscow 117234, USSR

Received November 23, 1989; Revised and Accepted February 20, 1990

ABSTRACT

Polymerase-mediated recombination based on DNA polymerase chain reactions (PCRs) has been used to carry out directed joining at a preset point of two DNA fragments initially contained in a plasmid and a single-stranded synthetic DNA. The process includes copying of these fragments by PCR with generation of an overlapping homologous region. Such overlap of 12 base pairs in length was found to be sufficient to provide further DNA joining also by use of PCR.

INTRODUCTION

The gene-engineering methods currently used for obtaining recombinant DNA are chiefly based on cleaving the DNA with restriction enzymes and joining the fragments with DNA ligase. Because of their being sequence-dependent such methods have many limitations and do not yet allow one to obtain recombinant DNA of any preset structure. A breakthrough in this field is the development of DNA polymerase chain reactions (PCRs) (1) which make it possible not only to copy a specified fragment of any DNA (including total genomic DNA) but also to make chainges in or additions to the ends of this fragment (2). Two recent papers (3, 4) have described the directed joining of DNA fragments by PCR-copying of latters with generation of an overlapping region to provide their further joining. Such new variety of PCR — polymerase-mediated recombination — was used by us for joining of two DNA fragments initially harboured by plasmid and synthetic DNA at a strictly preset point. Also considered in this paper was the question of the length of overlap that allows the polymerase recombination to proceed.

MATERIALS AND METHODS

Initial DNAs. The synthetic 184-membered single-stranded DNA

3′ ATTGTGGCACGCACAACTGATAAAATGGAGACCGC-CACTATTACCTAGGTACATGATTCCTCCATGGTACCG-CCATGCCTATAGCTATTTGCCACAGGAGCTCGCGATA-CCAGAGATCTGGTATCTAGAATCTCCAGCTTTAAGTG-GACCTTAAGTTTCCGAGGAAAACCTCGGAAAAAAAA-AG 5′

syn-ssDNA

was made in our laboratory by chemical synthesis of oligonucleotide blocks followed by DNA-ligase assembly, and kindly provided by T.S.Oretskaya, T.G.Reintamm, and E.A.Kubareva. The plasmid pMZ41 (see Fig. 2 below) was constructed by inserting the plasmid pMM52 (5) fragment encoding the beginning of the phage λ *cro* gene mRNA into the polylinker region of plasmid pTZ18R directly after the phage T7 RNA polymerase promoter, and kindly provided by E.A.Skripkin (M.V.Lomonosov State University).

Oligodeoxyribonucleotide primers rU(5′-5′)d(pATGCTTCCG-GCTCGTATGTT) (P1), d(CCTTAGTACATCCTATAGTGA) (P2), d(TCCAGGTGAATTTCGACCTCTA) (P4), d(AGTCA-CGACGTTGTAAAACG) (P5), d(TAATACGACTCACTAT-AG) (P6), d(GATGTACTAAGGAGGTACCATGGCGG) (P7), d(CTCCCTATAGAGTCGTATTA) (P8) and d(ACACAGGA-AACAGCTATGAC) (RP) were synthesized in our laboratory in a Victoria-4M automatic synthesizer and isolated by HPLC as described earlier (6, 7) by T.S.Oretskaya, T.G.Reintamm, and E.M. Volkov. The primer d(TAATACGACTCACTATA-GGATGTACTAAGGAGGTACCATGGCGG) (P3) was obtained by linking primers P6 and P7 on the P2 template with the use of DNA ligase according to the established procedure, and isolated by gel electrophoresis.

Thermostable DNA polymerase from *Thermus thermophilus* (Tth-polymerase) was kindly provided by S.A.Zozulya (Branch of the Institute of Bioorganic Chemistry, USSR Academy of Sciences).

Polymerase chain reactions (PCR) were carried out in 100 μl of 67 mM Tris-HCl pH 8.8, 6.7 mM $MgCl_2$, 16.6 mM $(NH_2)_4SO_4$, 10 mM mercaptoethanol, 170 μg/ml bovine serum albumin, 0.05 mM each of dATP, dTTP, dCTP and dGTP. The reaction mixture contained 10−30 ng of plasmid or synthetic DNA, 100−150 pmoles each of two oligonucleotide primers, one of them 5′-endlabelled with ^{32}P-phosphate, and 5 units of Tth-polymerase. The mixture in a tube was overlaid with 30 μl of mineral oil and subjected to 15 or 20 cycles of PCR: denaturation for 1 min at 95°C, hybridization with primers for 2 min at 50°C, polymerase synthesis for 2 min (last cycle 10 min) at 70°C. For analysis and isolation of reaction products the electrophoresis in 8% polyacrylamide gel (50 mM Tris-borate pH 8.3, 1 mM EDTA) with subsequent autoradiography were used. The radioactivity of the isolated product was used to determine its yield with respect to the primer and its overall amount. The protocol was similar for joint copying of two DNA fragments used at 0.2−2 pmoles each.

RESULTS AND DISCUSSION

1. Principle and design

The process of PCR-mediated DNA joining was considered in recent reports (3, 4) as association of two DNA fragments with homologous sequences at one of ends during denaturation-

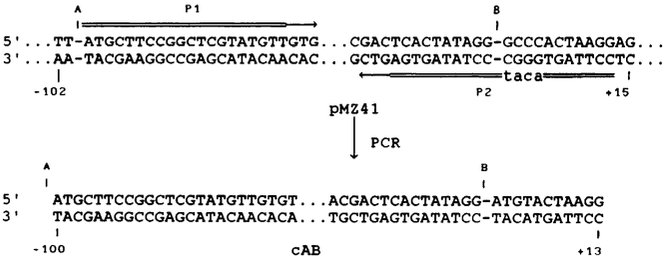
reannealing followed by extension by polymerase reaction. These reports appeared when our experiments described below were already under work. In our opinion, the mechanism of this process may be interpreted simpler (Fig. 1). If the initial DNA is present as two molecules and two respectively complementary primers, usual PCR cycles would yield single-stranded DNA copies (from the primer to the end of the respective chain) with a linear increase in the total amount of DNA. However, if the initial DNAs have a 'tail-to-head' identity as shown in Fig. 1, such a process will develop. In this case there will be copies with complementary 3'-ends, whereby they will be able to associate and be extended on each other's template in the 3' direction, to and including the 5'-terminal template sequences determined by the respective primers. Owing to the presence of free primers, such extended DNA will become capable of multiplying exponentially in the PCR and therefore will become a certainly prevailing component of the reaction mixture. This DNA will comprise the non-overlapping regions of the initial DNA molecules joined by their matching region and flanked by the respective primers.

We have used such an approach to perform joining of two DNA fragments initially contained in different molecules, i.e. polymerase recombination *in vitro*. This process is schematically depicted in Fig. 2 as applied to our experiment. The plasmid fragment AB (positions −100 to +2 from the transcription initiation point) containing the T7 RNA polymerase promoter preceded by a region including the sequence of the M13 reverse primer (RP) was joined at the point B (junction of positions +2 and +3) to the ds-copy of the central fragment BC in *syn-ssDNA*. At the first stage AB and BC fragments are copied by PCR using special primers to introduce additional regions adjacent to point B; each of these extensions corresponds to the B-terminal sequence of the other initial fragment. Thus, both copies (cAB and cBC, respectively) contain 30 bp-long homologous overlapping regions (−17 to +13) allowing them to be joined as described above (Fig. 1) to obtain the target product (AC in Fig. 2).

Since for a practical use of polymerase recombination it is very important to define the required length of overlap we have carried out also the joining of the same AB and BC fragments using their shorter copies cAB' and cBC'. As can be seen below, the overlap between cAB and cBC' is 12 bp-long (+2 to +13). cAB' and cBC have longer (22 bp, −17 to +5) overlap, but the latter includes two base pairs at positions +3 and +4 which are not the same in these fragments.

2. DNA copying and joining by PCR

To obtain cAB copy of pMZ41-harboured fragment AB the conventional PCR was carried out in the presence of primers P1 and P2. Since P2 is not wholly complementary to the corresponding pMZ41 region (positions −8 to +13), four nucleotides given in lower-case letters in P2 were changed in the copy (cAB):



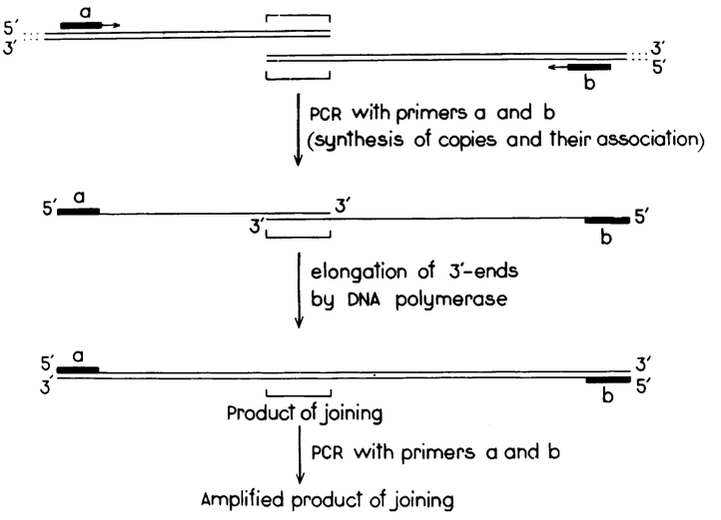


Figure 1. Scheme of the process taking place in joint PCR copying of two DNA fragments having an overlapping region (denoted as ı_____ı). Here and henceforth ⟶ are the oligonucleotide primers, the arrow indicating the direction of possible polymerase-mediated extension (5'→3').

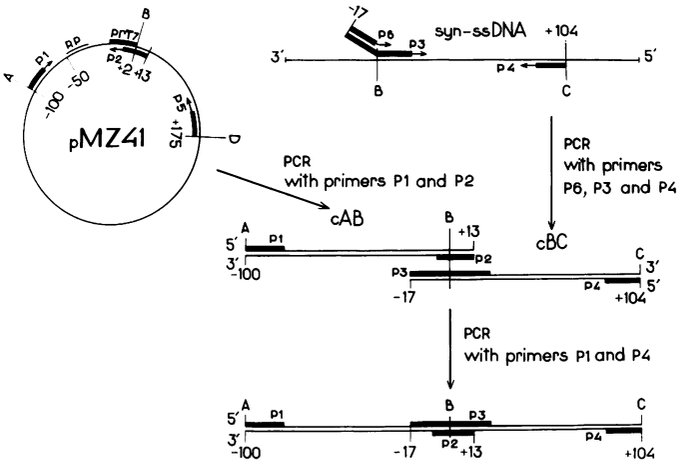
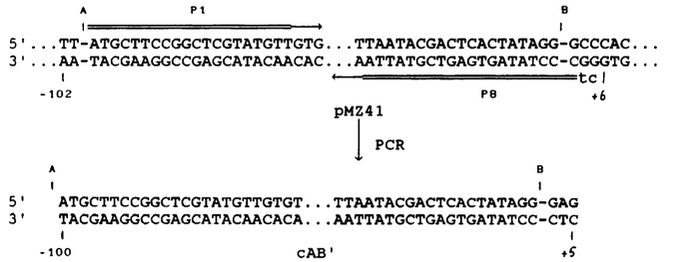


Figure 2. Scheme of joining two selected DNA fragments (AB in plasmid pMZ41, BC in the *syn-ssDNA*; B is the junction point) by polymerase recombination.

Such a way was also used to obtain cAB'. In this case the P2 primer was replaced by P8:



As a control, the pMZ41 fragment AD of 275 bp (−100 to +175) was copied without any alterations by PCR with primers P1 and P5. The AD fragment contains the T7 promoter preceded

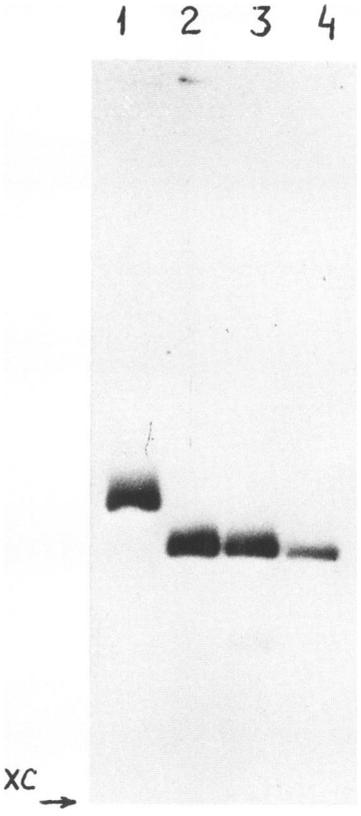
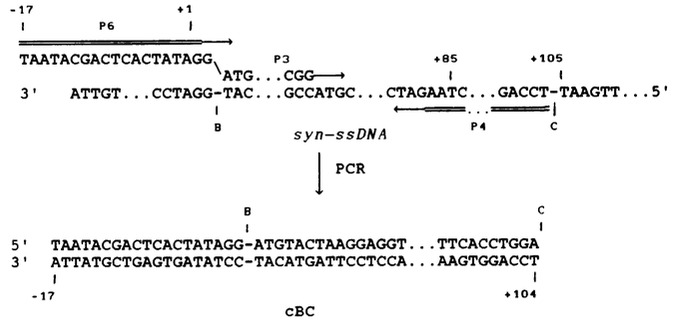
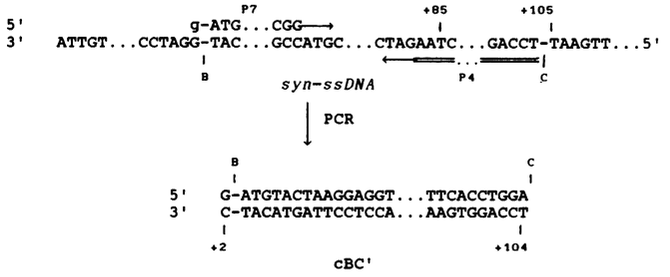


Figure 3. Autoradiography of gel electrophoresis of the products of PCR copying (20 cycles, for conditions see 'Materials and Methods') of fragment AD (0.2 pmoles) in the presence of primers P1 and 5′-^{32}P-endlabelled P5 (1), mixtures of cAB and cBC (2), cAB and cBC′ (3) or cAB′ and cBC (4), 0.2 pmoles each in all cases, in the presence of P1 and 5′-^{32}P-endlabelled P4 (2). XC is the position of xylene cyanol.

by a plasmid region and followed by a sequence coding for a 175-membered mRNA. The yields of PCR products after isolation from the gel reached 30% with respect to the primer (see 'Materials and Methods'). The BC fragment of *syn-ssDNA* was also copied by PCR, using as the 'left-hand' primer a 1:5 mixture of P3 and P6 (P6 is the 5′ region of the 45-membered P3), and P4 as the 'right-hand' primer:

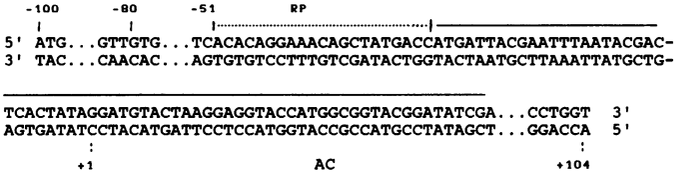


The result was a double-stranded copy (121 bp-long cBC) containing the T7 RNA polymerase promoter (−17 to +2) followed by a 102-nucleotide sequence of *syn-ssDNA*. Replacement of P3 and P6 mixture by P7 results, as expected, in the formation of shorter analogue of cBC − 103 bp-long cBC′:



The nucleotide sequence of the DNA fragments produced by PCR were confirmed by sequencing according to Sanger *et al.* (8) with the use of 5′-^{32}P-endlabelled primers RP, P2 or P6.

Joint PCR copying of cAB and cBC was carried out with primers P1 and P4. For control, the continuous AD fragment was amplified under the same conditions (20 cycles of PCR). As can be seen in Fig. 3, the PCR amplification of the mixture of cAB and cBC results in efficient formation of a product similar in electrophoretic mobility to the target product of polymerase recombination, the 204-bp-long fragment AC (see Fig. 2):



The product structure was confirmed by Sanger sequencing (8) with the use of 5′-^{32}P-endlabelled RP. This primer is complementary to the pMZ41 fragment AB (positions −51 to −32). The sequence read from the autoradiograph of the sequencing gel corresponds to the expected one (denoted by the line above) and comprises the plasmid fragment (T7 RNA polymerase promoter with an upstream region) followed by the ds-copy of *syn-ssDNA* joined to it at the preset point B. Thus, the data obtained confirm the feasibility and efficiency of polymerase recombination.

Figure 3 indicates that cAB and cBC′ as well as cAB′ and cBC are also efficiently joined in the reaction described above yielding the same fragment AC. The latter was proved also by Sanger sequencing. This fact allows us to conclude that generating a 12 bp-long overlap is sufficient for DNA construction by polymerase recombination. It is interesting that 22 bp-long overlapping regions in cAB′ and cBC (−17 to +5) are not completely complementary — there are differences at positions +3 and +4 (compare the sequences given above). Since PCR-mediated joining results in formation of the same product AC, it is to be concluded that the sequence of product in this case was determined by cBC and not by cAB′. Such a result can be expected because overlapping regions of single-strand copies formed on the first step of joint PCR (see Fig. 1) of cAB′ and cBC are completely complementary near position −17 (3′-end of cBC ss-copy) while complementarity near position +5 (3′-end of cAB′ ss-copy) is destroyed.

From our viewpoint, polymerase recombination can be used

for constructing DNAs of any preset structure as well as for directed DNA mutagenesis. A quite essential point is that PCR copying at the first stage allows the necessary fragments to be chosen from any single- or double-stranded DNA. By such a way more than 10^6-fold multiplication of selected fragment can be achieved (1). To perform polymerase recombination, however, in many cases much lower multiplication will be sufficient. In our opinion, the latter will possibly allow PCR-generated misincorporation of nucleotides to be minimized (1).

We have joint just two DNA fragments, but a process analogous to that presented in Fig. 1 can as well be envisaged for joining three or more fragments. Furthermore, polymerase recombination can also serve as a basis for designing a new means of constructing recombinant and mutant RNAs. Indeed, DNA can easily be transcribed into RNA if a promoter of an appropriate RNA polymerase (e.g. that of phage T7, see the cBC synthesis above and (2)) is introduced into this DNA with the use of the same PCR. In this case, the whole process will include conversion of the original RNAs into cDNAs by known techniques, joining the necessary fragments of these cDNAs and other DNAs by polymerase recombination to make a cDNA to the target RNA, and synthesis of the latter by transcription. Work along these lines is already under way in our laboratory.

ACKNOWLEDGEMENTS

The authors wish to thank Drs T.S.Oretskaya, T.G.Reintamm, E.A.Kubareva and E.M.Volkov for the synthetic single-stranded DNA and oligonucleotide primers, Dr E.A.Skripkin for the pMZ41 plasmid, and Dr S.A.Zozulya for the Tth-polymerase.

REFERENCES

1. Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.F., Higuchi,R., Horn,G.T., Mullis,K.B. and Ehrlich,H.A. (1988) *Science* **239**, 487–491.
2. Stoflet,E.S., Koeberl,D.D., Sarkar,G. and Sommer,S.S. (1988) *Science*. **239**, 491–494.
3. Higuchi,R., Krummel,B. and Saiki,R.K. (1988) *Nucl. Acids Res.* **16**, 7351–7367.
4. Horton,R.M., Hunt,H.D., Ho,S.N., Pullen,J.K. and Peace,L.R. (1989) *Gene* **77**, 61–68.
5. Skripkin,E.A., Bogdanova,S.L., Kopylov,A.M. and Bogdanov,A.A. (1986) *Dokl. Akad. Nauk SSSR* **287**, 237–241.
6. Gryaznov,S.M., Chernov,I.P., Potapov,V.K., Purmal,A.A., Metelev,V.G., Yolov,A.A. and Shabarova,Z.A. (1986) *Bioorgan. Khimiya* **12**, 1604–1611.
7. Volkov,E.M., Romanova,E.A., Krug,A., Oretskaya,T.S., Potapov,V.K. and Shabarova,Z.A. (1988) *Bioorgan. Khimiya* **14**, 1034–1039.
8. Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5464–5467.