Regulation of average length of complex PCR product

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ABSTRACT

A method to achieve the preference towards longer products during PCR is described. The extent of this preference can be adjusted by slight variation of the PCR conditions. Being combined with the natural tendency of PCR to amplify shorter fragments more efficiently than longer ones, it allows one to regulate the average length of the complex PCR product over a very wide range to make it most suitable for further manipulations. The technique can be used for amplifying any complex DNA sample.

INTRODUCTION

In recent years the techniques for PCR amplification of long DNA fragments advanced significantly, especially with the invention of enzyme mixtures for 'long and accurate PCR' (1). Still, there is a well-known tendency of PCR to amplify shorter fragments more efficiently than longer ones. This is especially noticeable during the amplification of complex DNA mixtures containing more than one fragment. Such a situation arises during the preparation of amplified cDNA samples (2), during the amplification of cDNA ends (3–5) and during PCR-based genome walking (6). In all of these cases the bias towards shorter DNA fragments may pose significant problems during subsequent cloning and analysis of the PCR product. To avoid them, we developed a technique for introducing specific PCR conditions favouring the amplification of longer products from a complex DNA sample.

MATERIALS AND METHODS

For complex DNA samples obtained by PCR (for example, product of RACE or total cDNA amplification), the original PCR product was 'polished' by adding 4 U of T4 DNA polymerase to the completed PCR reaction (total volume 20–30 µl) followed by incubation at 37°C for 15 min. Then the DNA was purified using QIAquick Spin PCR purification kit (QIAGEN) and eluted with 10 mM Tris–HCl, pH 8.0 to obtain the DNA in a final concentration of ~10 ng/µl. Since the PCR primers lacked 5'-phosphate groups essential for ligation, the PCR product was phosphorylated. Purified DNA sample (8 µl) was mixed with 1 µl of 10× ligase buffer [containing ATP, New England Biolabs (NEB)] and 1 µl (5 U) of T4 polynucleotide kinase. The phosphorylation reaction was allowed to proceed for 30 min at 37°C, then stopped by heating at 70°C for 10 min. The ligation reaction was performed in the same tube by adding the following components to the reaction: 1 µl of 10× ligation buffer (NEB), 4 µl of 10 µM adapter (Fig. 2A), 4 µl of deionised water and 1 µl (2–4 U) of T4 DNA ligase (NEB). The ligation reaction was allowed to proceed for 16 h at 16°C or 3 h at room temperature, then stopped by heating at 70°C for 10 min. The ligation mixture was diluted 10-fold in water and 1 µl of the dilution was taken for subsequent PCR in a total volume of 20–30 µl. The PCR mixture contained 1× Advantage KlenTaq Polymerase Mix with provided buffer (Clontech), 200 µM dNTPs and an adapter-specific primer in appropriate concentration. The cycling profile (18–22 cycles; Hybaid OmniGene thermocycler, tube control mode) was: 95°C, 7 s; 65°C, 20 s; 72°C, 2 min for the distal primer and: 95°C, 7 s; 72°C, 2 min for the proximal primer.

In the model experiment, when the original cDNA sample was obtained by restriction endonuclease digestion, the protocol was similar but did not include stages of polishing and phosphorylation.

RESULTS AND DISCUSSION

Principle of the method

It was observed that, under certain conditions, amplification involving a single primer ('single-primer PCR') was less efficient for shorter molecules than for longer ones (7). This can be explained by the following (Fig. 1): a DNA fragment being amplified by a single primer contains inverted terminal repeats (ITRs) corresponding to the primer sequence. Thus, in a single-stranded form of this molecule its ends are represented by complementary sequences which tend to anneal to each other, the process competing with primer annealing. Evidently, the shorter the molecule, the higher the probability for its ends to meet and anneal and, therefore, the stronger the competition.

One specific case of a single-primer PCR should be mentioned which is the situation leading to PCR-suppression effect (PS-effect; 2,5,6). It occurs when the original ITR is about double the length of the PCR primer. In this case, the intramolecular annealing is so much more efficient than primer annealing that the amplification becomes completely (or, for very long fragments, almost completely) suppressed. When the ITRs equal the PCR primer in length, several factors can be identified (except the length of the fragment) which affect the final outcome of the competition. One of them is the GC-content of the ITR (and therefore of the primer). When the ITR and primer are represented by an oligo-dT stretch with a 5'-heel, only the shortest fragments (up to 300–400 bp) are suppressed (7). Meanwhile, as we demonstrate in the present work, with a very G/C-rich ITR and primer the suppression effect can be observed on longer fragments as well. But the most crucial parameter of the

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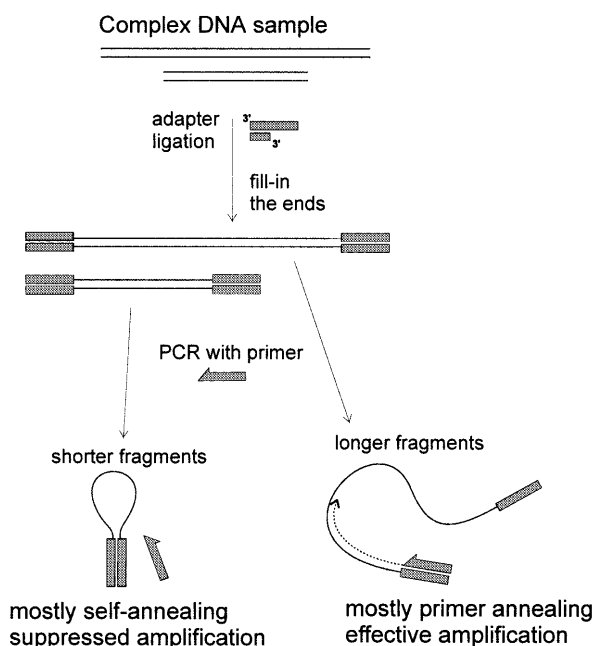
Figure 1. Schematic outline of the proposed method.

Figure 2. Application of the proposed technique to the model system—lambda DNA digested by *Hin*dII. (**A**) Oligonucleotides used in this work. (**B** and **C**) Amplification of the ligation mixture using distal and proximal adapter-specific primers respectively. On both panels: lane M contains 1 kb DNA ladder (Gibco BRL); lane λ/*h*II, the product of digestion (original DNA sample); other lanes, products of amplification using the corresponding primer in the concentration indicated above the lanes (μM).

single-primer PCR is primer concentration: its decrease shifts the equilibrium towards intramolecular annealing and therefore, the lower the primer concentration, the more pronounced is the suppression. This parameter can be easily varied to regulate the degree of suppression.

Thus, by means of attaching the ITRs to the complex DNA sample and amplifying it using a single ITR-specific primer in different concentrations, the two opposite PCR preferences—the 'natural' one towards shorter products and the introduced one towards longer products—can be combined in different proportions. As we show here, by using this approach it is possible to regulate the average length of the PCR product over a very wide range.

As for the way of attaching ITRs to a sample of DNA fragments, the ligation of pseudo-double stranded adapter (2,6; Fig. 2A) seemed to us the most universal, suitable for any DNA sample. The adapter is an equimolar mixture of two oligos of different length, the shorter being complementary to the 3' terminal part of the longer one. During the ligation reaction these oligos anneal to each other, forming a blunt end recognised by ligase, and the longer oligo becomes attached to the DNA fragment by its 3'-end (at both termini of the fragment). The shorter one is not ligated because it lacks a 5'-phosphate group (as well as the longer one). The DNA stretch complementary to the longer oligo is 'filled in' by Taq polymerase at the beginning of subsequent PCR.

In our work we used the 48-base-long adapter. Such a large adapter length allowed us to test two possible variants of single-primer PCR. The first one involved distal adapter-specific primer (Fig. 2A): in this case the ITRs were double the length
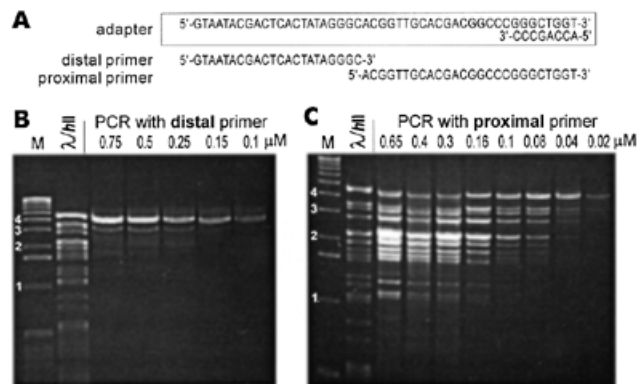
of the primer throughout the PCR and therefore true PCR-suppression effect was expected, meaning that only very long fragments could be amplified. The second variant involved proximal adapter-specific primer (Fig. 2A): in this case after the first successful primer annealing and elongation the ITRs became equal to the primer in length, and therefore less pressure on the shorter fragments was expected.

Model experiment

We tested the method on a model system using the DNA of phage lambda digested by *Hin*dII (a set of fragments from ~200 to 4500 bp long) as a complex DNA sample whose average length was to be regulated. After the ligation of the pseudo-double-stranded adapter, an aliquot of the ligation mixture was put into PCR with a distal (Fig. 2B) or proximal (Fig. 2C) adapter-specific primer in different concentrations. It was demonstrated that with distal primer only the longest bands of the pattern (longer than ~3.5 kb) can be amplified, while with proximal primer the shortest limiting length of a PCR product can be varied from ~0.6–0.8 to 2.5–3 kb. The correlation between the primer concentration and the degree of suppression is always the same in parallel experiments (data not shown), provided that the adapter and primers have the same sequence. Thus, the data presented in Figure 2 can be used as calibration charts for choosing a primer and its concentration for a particular task requiring length regulation.

Applications of the method

We found this simple technique especially useful in two particular cases. The first one is a difficult situation during RACE, when the cDNA end amplification results in a number of specific products, of which only the longest one represents the complete copy. The presence of several 5'-truncated cDNA variants is quite common during 5'-RACE of very long transcripts, and they are the first to suffer from incomplete reverse transcription
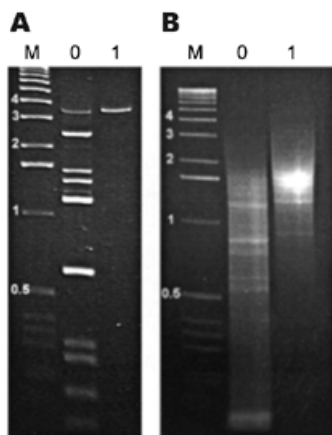
Figure 3. Applications of the described technique. (**A**) Extraction of the longest fragments from a complex RACE product. Lane M, 1 kb ladder (Gibco BRL); lane 0, original RACE product (a planarian cDNA for C-type lectin protein), obtained according to Matz *et al.* (5); lane 1, amplification of the longest fragments of the pattern according to the described technique, using proximal primer in 0.04 µM concentration. (**B**) Regulation of the average length of an amplified cDNA sample for library construction purposes. Lane M, 1 kb ladder (Gibco BRL); lane 0, the amplified cDNA sample prepared from total RNA of freshwater planarian using SMART cDNA Library Construction kit (Clontech); lane 1, biased cDNA sample amplified according to the described technique. In the PCR the proximal primer was used in 0.5 µM concentration.

and even slight mRNA degradation. In addition, a similar situation may arise during 3'-RACE of transcripts containing oligo-A tracks in the coding region: they serve as sites of oligo-dT primer annealing during the cDNA synthesis and thus lead to the generation of 3'-truncated cDNA variants. The example of a complex 5'-RACE product obtained according to Matz *et al.* (5) is shown in Figure 3A, lane 0. When the PCR product contains so many different fragments, the target band (the longest one)

is usually too weak for excision and purification from the agarose gel. The excision of the band followed by its re-amplification also may not work well because the contaminating fragments, which are much shorter, still would be preferentially amplified. Meanwhile, with the help of our technique, the longest fragments of this complex mixture can be easily obtained (Fig. 3A, lane 1).

Another convenient application of the technique is the preparation of amplified total cDNA for library construction purposes. Despite the fact that contemporary techniques for cDNA amplification produce samples with more or less natural length distribution (2), there still can be the over-representation of shorter cDNAs as a result of cloning of such samples, because shorter fragments are more effectively ligated into vector. Thus, the cDNA sample should be somewhat biased towards longer cDNAs to obtain natural length distribution upon cloning. This bias can be introduced by using our technique (the variant involving proximal adapter-specific primer), as is demonstrated in Figure 3B.

ACKNOWLEDGEMENTS

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