Vector-Swapping Strategy with an Exonuclease in Cloning and High Expression of Small PCR Products

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The need for designing new peptides of biological, pharmacological or clinical importance is increasing. To fulfill this need, a detailed knowledge of the structure-function relationship of known bioactive proteins is a prerequisite, and to obtain such information, we face challenges of inserting and deleting specific sequences into or from known proteins and expressing these modified proteins in cells. Polymerase chain reaction (PCR) (4) is essential for accomplishing the former procedure, and it is also a useful method for localizing the target function in short sequences, such as oligopeptides. Such studies require handling of small PCR products coding for the target areas of the proteins. These products are usually separated and isolated on polyacrylamide or agarose gels prior to expression in cells. However, this procedure is laborious and time-consuming and often results in a low recovery of the products.

We can solve this problem by devising a new method to clone and express small DNAs that does not require any separation and isolation steps. The cloning and expression strategy are schematically outlined in Figure 1. Two commonly used vectors were selected for cloning and expression, the pCR[®]II vector for cloning (donor) and the pMAL[™]-c2 vector for expression (acceptor). pCR[®]II and pMAL-c2 vectors were obtained from Invitrogen (Carlsbad, CA, USA) and New England Biolabs (Beverly, MA, USA), respectively. These vectors share an ampicillin-resistance (*amp^r*) gene. This shared gene in the cloning vector was selectively destroyed after pCR[®]II cloning. In this cloning step, the PCR products are amplified using PCR primers flanked with *Bam*HI recognition sequence. This flanking sequence was designed so as to adjust the reading frame of the PCR

products to that of the *malE* gene in the pMAL-c2 vector.

Another technique was devised for direct PCR cloning and expression of a concerned protein. In this procedure, PCR primers were designed to incorporate unique restriction endonuclease sites at their 5' ends without altering the translation reading frame of the protein (2). PCR products were then cleaved by the endonuclease to create sticky ends, followed by a direct ligation to an expression vector that had been cut with the same endonuclease. However, many restriction endonuclease sites fail to be cleaved when their recognition sequences are located within a few base

pairs of the end of a DNA fragment (2). One way to overcome this difficulty is to clone the PCR product as a blunt-ended fragment before its expression. However, in this case, an additional procedure is required in which a 3' overhang of the PCR products should be enzymatically removed using an enzyme such as Klenow or T4 DNA polymerase (6).

The usefulness of the innovated method was tested for the small PCR products (207 and 285 bp) derived from human fibronectin cDNA (1). In brief, the DNA fragments were amplified by PCR by using cDNA clones encoding human fibronectin, *Taq* DNA poly-

Figure 1. Schematic representation of cloning and expression of PCR products by selective destruction and reconstitution of *amp^r* in vectors. A PCR product that contains the *Bam*HI recognition sequence at both ends and single 3' A overhangs was subcloned into pCR[®]II with 3' T overhangs. The plasmid DNA from the pCR[®]II recombinant clone was sequentially subjected to enzymatic digestion with *Xmn*I and *BAL*-31 to destroy *amp^r*. For expression in bulk of MBP fusion proteins, the PCR product was then excised by *Bam*HI digestion and used for subcloning into the *Bam*HI site of pMAL-c2, which locates near the 3' end of the *malE* gene.

merase (Perkin-Elmer, Norwalk, CT, USA) and two *Bam*HI site (underlined)-flanked primers: 5' sense primer F17 (CCGGATCCGGCTACCGTGTGGATGTGAT, 28-mer) and 3' antisense primer F24 (CCGGATCCTATCTGGGCCCCGAGGTGGAG, 28-mer) for experiment 1; 5' sense primer F18 (CCGGATCCAGGAACACCTTTGCAAGAAGT, 28-mer) and 3' antisense primer F24 for experiment 2, respectively. The primers were synthesized on a Model 391 DNA Synthesizer (PE Applied Biosystems, Foster City, CA, USA). The PCR was carried out in 25 μ L of solution consisting of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1 mM each dNTP, 2.5 ng template fibronectin cDNA (pFH 100; Reference 1), 1 pmol of each primer as described above and 1 U of *Taq* DNA polymerase. Amplification was performed for 25 cycles at 94°C, 55°C and 72°C, each for 30 s, and terminated after the final incubation

at 72°C for 10 min. The product of experiments 1 and 2 encodes a part of the fibronectin type III₄ and type III₅ with the length of 285 and 207 bp as determined by agarose gel electrophoresis (5), respectively. The products were directly ligated to dT-tailed pCRII donor vector bearing both *amp*^r and the kanamycin-resistance (*kan*^r) gene and were transfected to One Shot™ INV α F' *E. coli* (Invitrogen). The recombinant clones were selected in medium containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and ampicillin. DNAs were prepared from 2 mL of white colonies by the standard miniprep method (5) and divided into 200-ng aliquots that were subjected to enzymatic digestion with *Xmn*I and *BAL*-31 (both from New England Biolabs) to destroy *amp*^r. As a control experiment, aliquots of pCRII recombinant DNAs were digested to completion with *Bam*HI without treat-

ment of *Xmn*I and *BAL*-31. If the destruction is not included at this step, undesirable self-ligation of vectors alone will occur at the *Bam*HI site, which will eventually give a high background of *amp*^r clones at the next step of subcloning. For this purpose, the donor vector containing the PCR insert is finally digested to completion by a restriction enzyme, *Xmn*I, to introduce an entry site of *BAL*-31 nuclease within the *amp*^r region and subsequently digested with the nuclease to destroy the *amp*^r but not the insert DNA. A reaction with *BAL*-31 was performed in a mixture consisting of 12.5 mM CaCl₂, 12.5 mM MgCl₂, 0.6 M NaCl, 1 mM EDTA and 20 mM Tris-HCl, pH 8.0 at 30°C. Samples were withdrawn at different times and placed into 25 mM ethylene glycol-bis (β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid to inactivate the enzyme and subjected to agarose gel electrophoresis to calculate

the time required to remove the desired number of nucleotides from the linearized DNA. Empirically, about a 700-bp deletion is sufficient. The resulting nuclease digests with the desired deletion were then treated with saturated phenol/chloroform and were precipitated with ethanol. They were digested again to completion by *Bam*HI, treated with saturated phenol/chloroform and subjected to a ligation reaction with the acceptor vector (phosphatase-treated, *Bam*HI-cut expression vector pMAL-c2), which enabled us to produce fibronectin fragment peptides as fusion proteins of the maltose-binding protein (MBP) (3) in *E. coli*. In the ligation reaction, an equimolar ratio of vector to insert is used at a final concentration of 10 µg/mL. Cells were transformed with the ligation products and plated on agar medium containing ampicillin for the first selection. The kanamycin resistivity of the randomly chosen amp^r colonies was examined on separate agar medium containing kanamycin (25 µg/mL). The colonies thus selected have an amp^r and kanamycin-sensitive (kan^s) genotype and contain the original PCR product cloned in the expression vector, which is verified by PCR insert screening (4) using the same PCR primers containing the *Bam*HI site as described above. Apparently, this method is not applicable to PCR products that contain *Xmn*I sites. In such a case, other restriction enzyme sites such as *Sca*I, which is located near the *Xmn*I site and within the amp^r gene of the donor vector, could be used.

When the recombinant pCRII DNA, which had been digested with *Xmn*I and *BAL*-31, was used for cloning of a PCR-generated, 207-bp fibronectin insert, the percentages of kan^r and kan^s colonies among a total of 17 amp^r clones was zero and 100%, respectively. In contrast, the percentages of kan^r and kan^s colonies among the total amp^r clones was 96% and 6%, respectively, when the recombinant DNA was used for expression without *Xmn*I and *BAL*-31 digestion. It is evident from the two experiments that positive colonies with expression of amp^r/kan^s were obtained from pCRII clones only when the clones were digested with *Xmn*I and *BAL*-31. Efficiency of cloning as amp^r/kan^s clones was quite low in the

experiments, in which the recombinant donor DNAs were not digested with the two enzymes, indicating that PCR DNA inserts preferentially self-ligated to the donor vectors under the conditions used in the present study. Therefore, it was concluded that the deletion of amp^r genes of the donor vector is indispensable for an efficient and selective subcloning of the insert into acceptor vector. A similar result was obtained for cloning a PCR-generated, 285-bp fibronectin insert (data not shown), suggesting that the cloning efficiency is not significantly changed when PCR products with different lengths were applied.

The subsequent PCR screening analysis (4) of the positive clones was performed using the 5' sense primer *malE* (GGTCGTCAGACTGTTCGATGAAGCC, 24-mer; New England Biolabs) and the F24 primer under the same PCR conditions as described above. After annealing the *malE* primer, the 3' end is 80 bp from the *Bam*HI sites within the *malE* gene of a pMAL-c2 vector. The results showed that more than 80% of the positive clones contain inserts.

Fusion protein analysis of positive clones showed successful expression of the insert, as expected (data not shown), confirming that the PCR products were cloned for expression in the predicted manner without isolating the insert DNA. The yield of the MBP fusion proteins was quite high and was usually in the range of 10–40 mg/L culture.

There have been variations in the method for inactivating the antibiotic-resistance genes at a swap of vectors. Commonly, two vectors have been utilized that do not share the resistance genes to maximize the chance of subcloning from one to another (5). However, a high expression of proteins in *E. coli* has not been accomplished with this method. In contrast, our method used two vectors that bear shared antibiotic-resistance genes and appears to be simple, convenient and most useful for efficient cloning and bulk expression of small PCR fragments (less than 300 bp). Mutants produced by in vitro mutagenesis of biologically important proteins can also be efficiently cloned and expressed, which contributes to ex-

amination of the structure-function relationship of the wild-type proteins. We can chemically synthesize peptides corresponding to the DNA fragments smaller than 60 bp, and this method should also be applicable for expressing such short nucleotide fragments.

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