ANALYTICAL BIOCHEMISTRY **257,** 203–209 (1998)
ARTICLE NO. AB972558

A Positive Selection Vector for Cloning of Long Polymerase Chain Reaction Fragments Based on a Lethal Mutant of the *crp* Gene of *Escherichia coli*

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Received September 23, 1997

We have constructed a cloning vector with a tight positive selection for recombinant clones in *Escherichia coli.* The positive selection pressure results from a lethal mutation within the *E. coli* gene coding for the catabolite gene activator protein CAP, which is disrupted whenever a fragment is successfully inserted. Here, we show that this "suicide" vector, pCAP^s, is suitable for cloning of PCR products as long as 9.3 kb into several unique restriction sites which are scattered throughout the lethal gene.

Cloning genes in plasmids of *Escherichia coli* is the key to recombinant DNA techniques. Many cloning systems have been developed and are used for this purpose, such as the well-known pUC vectors (1).

In some cases, the cloning of the gene is the bottleneck of the whole experiment, and here a higher efficiency in cloning leads to faster results. This efficiency is dependent upon the efficiency of ligation, the efficiency of transformation of *E. coli* cells, and the efficiency of the selection of those *E. coli* transformants that contain the desired insert. Improving the latter will improve the efficiency of the entire experiment.

For experiments in which cloning is not the most critical step, improving the efficiency of selection will allow the use of less efficient ligation reactions and transformation reactions, for example, by using unpurified DNA fragments from a PCR or from less competent host cells. This can be less expensive and, again, faster.

There are many ways to improve the efficiency of selection, with varying success and limitations: some methods need extra enzymatic treatment of the vector after linearization, like dephosphorylation (2), which makes religation of the vector impossible but allows ligation of the vector with insert DNA. Other methods destroy a reporter gene (or its promoter) and screen for clones without the phenotype of this reporter. An example of this is the use of α-complementation of β-galactosidase, which is known as "blue/white screening" [for a review see Ref. (3)].

The latter approach can lead to a very high background of nonrecombinant clones because all religated vectors can be transformed into host cells and yield clones showing the phenotype of the reporter. Therefore, several approaches that use genes which are toxic for the host cells have been developed (4–7). This results in a powerful selection for vectors with insert DNA. These so-called "positive selection" cloning systems have the advantage that they produce virtually no background clones, because religated vectors carrying the intact toxic gene are lethal to the host cell. The only clones observed with these positive selection cloning systems carry vectors ligated with DNA fragments. However, most of these positive selection cloning systems depend on special media or biochemicals, or are limited to a small number of host strains (4–7).

Here, we introduce a new cloning vector, pCAP^s ("s" stands for "suicide"), which expresses a toxic mutant of the catabolite gene activator protein CAP[3] [also known as the cyclic AMP receptor protein CRP, a pleiotropic

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[3] Abbreviation used: CAP, catabolite gene activator protein.

0003-2697/98 $25.00

TABLE 1

E. coli Strains Used for Cloning with pCAP^s

E. coli strain	Genotype	References
BMH8117	*Δ(lac–pro) thi supE nal*	(18)
DH5α	*supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* [*ϕ*80 *lacZΔM15*]	(19)
JM83	*ara Δ(lac–proAB) strA thi-1* [*ϕ*80 *lacZΔM15*]	(1, 20)
XA3DII	*Δ(lac–proAB)X111 argEam metB ara rpoB nal supD Δcrp-45 strA fnr-1 zci*::Tn*10* (tet^s) *cya*	(9)
XL1-Blue MRF′ Kan[a]	*Δ(mcrA)183 Δ(mcrCB–hsdSMR–mrr)173 supE44 endA1 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI^{q}ZΔM15* Tn5 (Kan^r)]	

[a] XL1-Blue MRF′ Kan was obtained as competent Epicurian Coli cells from Stratagene.

gene regulator; for review see (8)]. We show here that pCAP^s is a suitable tool for highly efficient cloning of DNA fragments in commonly used *E. coli* strains without the need for special media or screening systems.

MATERIALS AND METHODS

Strains and Plasmids

The bacterial strains used are listed in Table 1. Vector pBG2E181Q is described in Ref. (9). This vector is a high-copy-number plasmid carrying a mutant *crp* gene. A point mutation in codon 181 results in an E to Q substitution, which renders the protein toxic in the presence of cellular cAMP.

Reagents

Taq DNA polymerase, *Pwo* DNA polymerase, T4 DNA polymerase, Expand High Fidelity PCR System, High Pure PCR Product Purification Kit, High Pure Plasmid Isolation Kit, human placental genomic DNA, λ DNA, Rapid Ligation Kit, and restriction enzymes were from Boehringer Mannheim, Mannheim, Germany. T4 DNA ligase was from New England Biolabs, Schwalbach, Germany. QIAprep Spin Miniprep Kit and Qiagen-Tip 100 and 500 were from Qiagen, Hilden, Germany.

Cloning

Vectors. pBG2E181Q (9) and pCAP^s DNA (see Results) were prepared from *E. coli* strain XA3DII according to standard protocols (2) or with QIAprep Spin Miniprep Kit or Qiagen-Tip 100 or 500 according to the manufacturer's manual.

The vectors pBG2E181Q and pCAP^s were linearized using the blunt-cutting restriction enzyme *Mlu*NI or the 4-bp-overhang producing restriction enzyme *Not*I or *Pst*I. After digestion, *Mlu*NI was inactivated by incubation for 15 min at 65°C, *Not*I was inactivated by incubation for 20 min at 65°C, and the *Pst*I digestion was diluted 50-fold.

Cloning of DNA Fragments

21-bp Fragments. Oligonucleotides for cloning were produced with an Applied Biosystems 380 A oligonucleotide synthesis machine. They were annealed to yield double-stranded DNA fragments with either *Not*I-compatible cohesive ends

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5′-GGCCCTACTGCAGGTTCTACG-3′
    3′-GATGACGTCCAAGATGCCCGG-5′
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or *Xho*I-compatible cohesive ends

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5′-TCGAGAGCACACGTGCTGGAC-3′
    3′-CTCGTGTGCACGACCTGAGCT-5′.
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These 21-bp fragments were used for testing the engineered *Not*I or *Xho*I site, respectively. They were ligated with 5 ng *Not*I (*Xho*I)-digested pCAP^s DNA at an approximately 5- to 10-fold molar excess for 16 h and transformed into *E. coli* DH5α.

0.25-kb Fragments. Plasmid piWiT10WL1 (10) digested with *Pst*I or *Stu*I yields 13 identical 0.25-kb fragments with sticky ends or blunt ends, respectively. They were purified from agarose gels, ligated with 5 ng *Pst*I- or *Mlu*NI-digested pBG2E181Q DNA at an approximately 5- to 10-fold molar excess for 16 h, and transformed into *E. coli* BMH8117, DH5α, and JM83.

Mixture of 8.2- to 19.4-kb fragments. λ DNA was digested with *Sma*I and purified with the High Pure Plasmid Isolation Kit according to the supplier's manual. The resulting fragments have blunt ends and their sizes are 8.2, 8.6, 12.2, and 19.4 kb. Fifty nanograms of this mixture of DNA fragments and 1 ng of *Mlu*NI-linearized pCAP^s were ligated for 30 min using the Rapid Ligation Kit and transformed into *E. coli* XL1-Blue MRF′ Kan.

PCR Fragments

The PCR fragments used are shown in Table 2. These were amplified from λ DNA or human placental genomic DNA. Purification of PCR products was performed as described in the manual of the High Pure

TABLE 2

PCR Products Used to Test the Cloning Efficiency of the pCAP^s Cloning System

Fragment size (kb)	Template DNA	Primer	DNA Polymerase[a]
0.5	λ	5′-GATGAGTTCGTGTCCGTACAACT-3′, 5′-GGTTATCGAAATCAGCCACAGCG-3′	*Pwo* DNA pol
1.7	Human	5′-TGGTGCCACGTGCTGAAGAA-3′, 5′-GACTTCAAATTTCTGCTCCTC-3′	*Pwo* DNA pol, *Taq* DNA pol, Expand High Fidelity PCR System
4.8	Human	5′-GGAAGTACAGCTCAGAGTTCTGCAGCACCCCTGC-3′, 5′-GATGCGAAACTGAGGCTGGCTGTACTGTCTC-3′	Expand High Fidelity PCR System
5.0	λ	5′-GATGAGTTCGTGTCCGTACAACT-3′, 5′-ACTCCAGCGTCTCATCTTTATGC-3′	*Pwo* DNA pol
9.3	Human	5′-GGAAGTACAGCTCAGAGTTCTGCAGCACCCCTGC-3′, 5′-CAAAGTCATGCGGCCATCGTTCAGACACACC-3′	Expand High Fidelity PCR System

[a] The Expand High Fidelity PCR System uses an enzyme blend from *Taq* DNA polymerase and *Pwo* DNA polymerase.

PCR Product Purification Kit. Note that this does not include a gel-purification step.

The Expand High Fidelity PCR System uses an enzyme blend of *Pwo* DNA polymerase and *Taq* DNA polymerase. It generates a mixture of fragments having either blunt ends or deoxyadenylate extensions at the 3′ end. Therefore, the 4.8- and 9.3-kb PCR fragments were polished using T4 DNA polymerase as described (11) to increase the percentage of blunt-end DNA fragments, leading to an increased ligation efficiency. After polishing, the PCR product was further purified as described in the manual of the High Pure PCR Product Purification Kit.

Fifty to five hundred nanograms of purified and 1–4 μl of unpurified PCR products were ligated to 1 ng of *Mlu*NI linearized vector (pBG2E181Q or pCAP^s) with the Rapid Ligation Kit in a 5- to 30-min incubation step. Ligation products were transformed into *E. coli* XL1-Blue MRF′ Kan.

Transformation

Except for *E. coli* XL1-Blue MRF′ Kan cells, competent *E. coli* cells were produced with the Frozen Storage III method (12). Transformation was performed as described in the suppliers manual or in (12), respectively.

Transformed cells (40–100 μl) were plated on LB plates (13) with 100 μg/ml ampicillin and incubated at 37°C for 16–18 h. Clones were analyzed by restriction analysis of miniprep plasmid DNA, except for the clones with the 21-bp insert, which were analyzed by sequence analysis.

DNA Sequencing

DNA sequence analysis was performed using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with analysis on an ABI Prism 377 DNA Sequencer. pCAP^s plasmid DNA was prepared with QIAprep Spin Miniprep Kit or Qiagen-Tip 100, and the complete sequence of both strands was determined with the following primers: 5′-GAT CGG GGT GAT AAT CAG-3′, 5′-TGC CAC AGA GCC TTT AAC-3′, 5′-ATT TCT TTA CCC TCT TCG TC-3′, 5′-TGA AAT ACC GCA CAG ATG-3′, 5′-ATA AAG ATA CCA GGC GTT TC-3′, 5′-TGG TAA CAG GAT TAG CAG AG-3′, 5′-GCT CAG TGG AAC GAA AAC-3′, 5′-ATC AGC AAT AAA CCA GCC-3′, 5′-GCA GCA CTG CAT AAT TCT C-3′, 5′-AGA TCC AGT TCG ATG TAA CC-3′, 5′-TGC CAC CTG CTA AGA AAC-3′, 5′-GTT TTC ACC GTC ATC ACC-3′, 5′-GTT TAC GGC ACT CGT TAA TC-3′, 5′-CTA GTG AGA AAG TGG GCA AC-3′, 5′-TGC CAC ATT CAT AAG TAC CC-3′, 5′-ACC GTA TTA CCG CCT TTG-3′, 5′-CAG CGT GAG CTA TGA GAA AG-3′, 5′-CGG ATC AAG AGC TAC CAA C-3′, 5′-TTA TCT ACA CGA CGG GGA G-3′, 5′-AGC TGA ATG AAG CCA TAC C-3′, and 5′-ACA TCG AAC TGG ATC TCA AC-3′. The sequencing data were assembled and analyzed using the Wisconsin Package 9.0, Genetics Computer Group, Madison, Wisconsin.

RESULTS

Plasmid pBG2E181Q carries a mutant *crp* gene, which codes for a CAP protein with the substitution E181Q (9). *E. coli cya*$^+$ strains such as DH5α do not grow when transformed with plasmid pBG2E81Q, but *E. coli cya*$^-$ strains such as XA3DII grow normally and produce plasmid DNA and CAP E181Q protein (9). When cAMP is added to liquid media, *E. coli cya*$^-$ strains transformed with pBG2E181Q DNA stop growing. From this, we conclude that the CAP E181Q · cAMP complex is toxic to *E. coli* cells.

To test whether this vector can be used in a positive selection system, we analyzed the efficiency of cloning into it under various conditions. We inserted both purified and unpurified 0.5-kb PCR fragments produced

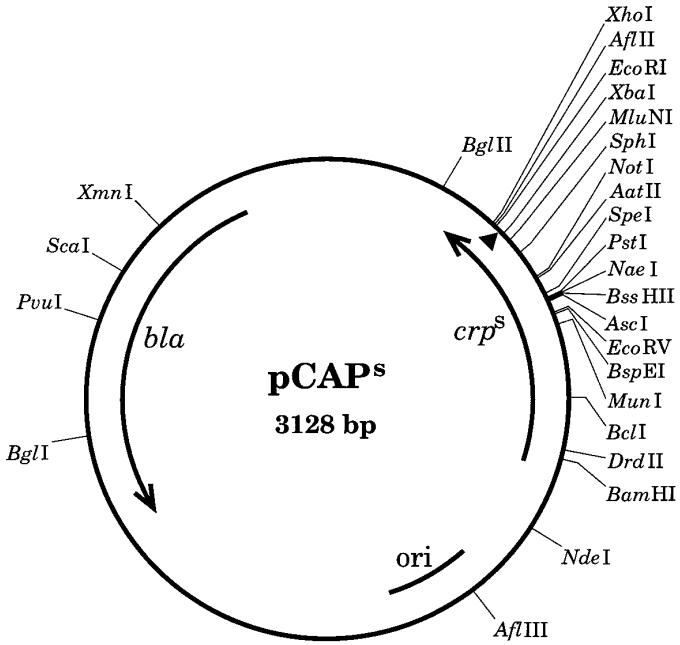

FIG. 1. Structure of plasmid pCAP^s. It has a size of 3128 bp. ori, origin of replication; *bla*, β-lactamase gene (ampicillin resistance); *crp*^{s}, gene coding for the cyclic AMP receptor protein suicide mutant; filled triangle, location of the E181Q mutation. All restriction endonuclease sites shown are unique. The sequence of pCAP^s is available at the EMBL nucleotide sequence database under Accession No. AJ001614.

with *Pwo* DNA polymerase into the unique *Mlu*NI site. *E. coli* strain XL1-Blue MRF′ Kan was used as host. With purified PCR fragments, 48 of 48 analyzed clones contained the insert. With nonpurified PCR fragments, 16 of 16 analyzed clones contained the expected insert.

We then tested whether this positive selection cloning system is suitable in host strains other than *E. coli* XL1-Blue MRF′ Kan and whether it is suitable for sticky-end cloning in other unique cloning sites in the *crp* gene. We analyzed the cloning efficiency of 0.25-kb *Pst*I or *Stu*I fragments cloned into pBG2E181Q which was linearized with *Pst*I or *Mlu*NI, respectively. The tested *E. coli* strains were BMH8117, DH5α, and JM83. A large majority of the analyzed clones contained the expected insert. A typical example is the result obtained with *E. coli* DH5α: here, with 0.25-kb *Pst*I fragments 100 of 108 analyzed clones were positive and with 0.25-kb *Stu*I fragments 96 of 108 clones were positive.

Construction of the Vector pCAP^s

To improve the cloning system, we introduced silent mutations into the vector pBG2E181Q by PCR mutagenesis or cloning of synthetic oligonucleotides. The map of the resulting vector pCAP^s with 19 unique restriction sites suitable for cloning is shown in Fig. 1.

As the 8-bp-long *Not*I recognition sequences are less frequent in any natural DNA than 6-bp recognition sequences, *Not*I is preferentially used for cloning of DNA fragments. Since it was impossible to introduce a *Not*I site into the *crp* gene without amino acid sequence alteration, we compared the primary sequence to the crystal structures (14, 15) in order to find regions in which amino acid replacements might be tolerated. We chose threonine at position 140 and replaced it by serine, thereby generating a *Not*I site. The lethality of the resulting *crp*^{s} gene was unaffected.

Nineteen unique restriction sites are distributed throughout the coding region of the *crp*^{s} gene (Fig. 1). The restriction sites *Mlu*NI, *Not*I, *Pst*I, *Xho*I, and others can be used for cloning of DNA fragments with the appropriate cohesive or blunt ends; for excision of the cloned inserts the adjacent restriction sites can be used.

Cloning Efficiency of pCAP^s

Short DNA Fragments

To test whether the positive selection is functional with very short in-frame insertions, we cloned 21 bp of synthetic DNA into the *Not*I site of plasmid pCAP^s, thereby inserting the amino acid sequence PTAGSSG between G141 and R142 of the CAP^s protein. Insertion of the DNA fragment in the opposite (anti-sense) direction results in a stop codon after the first inserted amino acid and thereby yields a truncated CAP protein. After cloning in *E. coli* DH5α, we sequenced the plasmid DNA of 15 individually transformed colonies. All of them carried the expected insert. In 7 clones, the DNA fragment was inserted in the sense orientation and in 8 clones in the anti-sense orientation. From this, we conclude that even a small DNA fragment with a sequence that allows a readthrough can be efficiently cloned with pCAP^s.

We also tested the *Xho*I site which is located nine codons C-terminal to the CAP^s mutation that leads to the lethal phenotype. We again inserted seven codons in frame: ESTRAGL or ESSTCAL, respectively. Of the 24 colonies that were analyzed, 18 contained the insert. From this we conclude that even a small insertion into the *Xho*I site is sufficient to disrupt the structure of the CAP^s protein and to interfere with its lethal properties.

Long DNA Fragments

We tested whether the pCAP^s cloning system is suitable for cloning blunt-end DNA fragments by using *Sma*I-digested λ DNA, which fragments are as large as 8.2–19.4 kb. From 12 analyzed clones, 5 clones contained a 8.6-kb insert and 1 clone contained a 12.2-kb insert. Therefore, it is possible to clone large blunt-end DNA fragments with pCAP^s.

TABLE 3

Cloning Efficiency with 1.7-kb Fragments

DNA polymerase	Purified	Colonies/Plate[a,b]	Positive/8 Analyzed Clones[b]
Pwo DNA pol	Yes	74, 481, 273, 64	8, 7, 7, 8
	No	37, 138, 95, 102	5, 7, 7, 5
Expand High Fidelity PCR System	Yes	19, 12, 11, 18	6, 0, 0, 6
	No	11, 11, 20, 24	4, 0, 0, 4
Taq DNA pol	Yes	7, 31, 16, 7	3, 4, 4, 1
	No	8, 11, 14, 9	1, 0, 0, 2

[a] For every plate, 12 amol linearized pCAP^s was used.
[b] The results of each of four independent experiments are shown.

Cloning Efficiency of PCR Fragments

We have demonstrated that the vector pBG2E181Q is a highly efficient tool for cloning short blunted PCR products, independent of their purity (see above). Therefore, we next tested the influence of the length of the PCR product, its purity, and the percentage of the blunt-ended fraction of PCR products on the cloning efficiency of the vector.

1.7-kb PCR Fragments. The 1.7-kb DNA fragments were produced in three different ways: with *Pwo* DNA polymerase, with *Taq* DNA polymerase, and with a blend of both, the High Fidelity Expand PCR System. These DNA fragments were used in both purified and unpurified form in cloning experiments. For each experiment, 1 ng linearized vector was ligated to the PCR product, and one-tenth of the ligation mix was transformed into *E. coli* XL1-Blue MRF′ Kan. One-quarter of the transformed culture was plated onto a single petri dish, such that each plate finally contained 25 pg vector DNA corresponding to 12 amol or 7 million molecules. Transformation efficiencies varied from 7 to 8.5×10^8 colonies/μg pUC18 DNA.

The results demonstrate that all tested DNA fragments can be cloned into this vector. The colonies/plate and the numbers of positive/eight analyzed clones are listed in Table 3. Purified PCR products were cloned with efficiencies of 88 to 100% when *Pwo* DNA polymerase was used. The High Fidelity Expand PCR System and *Taq* DNA polymerase were less efficient.

With unpurified PCR products, the corresponding efficiencies were also lower (Table 3). The data reflect that the purity and the percentage of blunt-end PCR products increase the efficiency of the cloning experiments. Note that here, and subsequently, we have not gel-purified PCR fragments prior to cloning. This is reflected by the less than 100% cloning efficiencies, in that negative clones contained shorter inserts probably derived from PCR by-products.

5.0-kb PCR Fragments. To figure out how efficient the cloning of PCR fragments larger than 1.7 kb is, we cloned 5.0-kb λ PCR fragments. These DNA fragments were produced with *Pwo* DNA polymerase and were purified. After ligation to pCAP^s and transformation of *E. coli* XL1-Blue MRF′ Kan, 9 of 15 analyzed clones contained the expected insert. With unpurified PCR products no positive clones were obtained.

4.8-kb and 9.3-kb PCR Fragments. Large PCR fragments can be amplified by using enzyme blends of different DNA polymerases (16). For the amplification of the 4.8- and 9.3-kb fragments we used a blend of *Taq* DNA polymerase (exhibiting a high processivity) and *Pwo* DNA polymerase (exhibiting 3′–5′ exonuclease activity). *Taq* DNA polymerase has been shown to add an extra nucleotide at the 3′ end of DNA, and DNA polymerases with 3′–5′ exonuclease activity generate blunt-end PCR fragments (17). DNA fragments generated by blends of these enzymes are mixtures of both species of PCR products (data not shown). An additional polishing step was applied to increase the percentage of blunt-ended PCR products.

Two of five analyzed clones contained the expected 4.8-kb PCR product, and 2 of 12 analyzed clones contained the expected 9.3-kb insert.

DISCUSSION

The pCAP^s positive selection cloning vector is based on the *crp*^{s} gene coding for a mutant CAP protein in which E181 has been replaced by Q (9). E181 is the second residue of the recognition helix (14, 15). In high amounts, this CAP^s protein is lethal for most *E. coli* strains (9). Since the lethality is not observed in *E. coli* strains which are defective in the adenylate cyclase gene (*cya*) and therefore are unable to produce the coactivator cyclic AMP, this lethality seems to be connected to the DNA binding activity of the CAP^s · cAMP complex (9). The CAP^s protein has a broadened DNA binding specificity and binds tightly to variants of the CAP site, which are barely recognized by the wild-type CAP protein (9). We suggest that CAP^s binds to one or very few sites on the *E. coli* chromosome at which it either activates a gene, resulting in a growth-inhibit-

ing or lethal overproduction of its product, or represses the expression of a protein essential for cell growth.

We have analyzed the efficiency of this positive screening system with different *E. coli* strains and different DNA fragments. Our results show that *E. coli* strains with or without an F factor and with or without the *lacI*^{q} gene can be used with the pCAP^s cloning system. The only requirement for the toxic effect of the CAP^s protein is the presence of cAMP. As this is produced endogeneously by most of the *E. coli* strains which are commonly used for cloning, we expect that most of these strains are suitable for the pCAP^s positive selection cloning system. Conversely, pCAP^s plasmid DNA can be propagated in all tested *cya*-defective *E. coli* strains, such as XA3DII, without negative selection pressure.

The use of the natural cofactor of the toxic gene product CAP^s, cAMP, has the advantage that no induction and no special selection conditions are necessary. Therefore, no special media, biochemicals, or temperature shifts are needed. The vector pCAP^s has no multiple cloning site, but is engineered so that it has several unique restriction sites scattered throughout the toxic *crp*^{s} gene, including the 8-bp recognition sequences for *Not*I and *Asc*I. These sites can be used for cloning of both blunt- and sticky-end DNA fragments effectively, as is shown by our tests with *Pst*I, *Not*I, *Mlu*NI, and *Xho*I.

One important characteristic for any positive selection system is the tightness of the selection or the frequency of escape. For example, in systems which have a multiple cloning site close to the promoter at the 5′ end of the lethal gene, cloning of DNA fragments in frame or with promoter activity could interfere with the selection. Cloning of fragments which are linked to the following lethal gene might be impossible if the resulting fusion protein retains its lethal properties. On the other hand, it is probably not possible to insert a multiple cloning linker into the middle of a lethal gene without destroying the lethality. We solved this problem by exploiting the degeneracy of the genetic code to introduce unique restriction sites into the gene at various positions at which any insertion is highly likely to disrupt the lethal gene product.

We tested this by introducing 21 bp, i.e., seven codons, into the *Xho*I site, which is the most C-terminal site within the *crp*^{s} gene, and found that the insertion abolished the toxicity of the CAP^s gene product, although it is located nine codons downstream of the E181Q substitution. Likewise, we inserted seven codons into the *Not*I site which was generated at the expense of one amino acid substitution in α helix D of the DNA binding domain (14, 15). The threonine to serine amino acid substitution did not affect the toxic properties of the *crp*^{s} gene. However, the seven-amino-acid insertion did interfere with the CAP^s activity. From this, we conclude that the pCAP^s cloning system is as tight as such a cloning system can be.

In addition to the unavoidable occurrence of spontaneous point mutations which can inactivate the CAP^s protein, there remains one possibility of obtaining viable clones without insert. This will occur whenever two vector molecules are ligated to each other in opposing orientations. At present there seems to be no way to exclude this type of reaction, but its frequency can be reduced efficiently by adding only small amounts of vector DNA to the ligation reaction. Thus, 1–5 ng (0.5–2.5 fmol) pCAP^s DNA per 10–20 μl and a 5- to 10-fold molar excess of fragments gave satisfactory results in our hands.

Our results show that the pCAP^s cloning system is suitable for efficient cloning of restriction fragments as long as 12.2 kb as well as of PCR fragments of up to 9.3 kb. Highest efficiencies are obtained with short blunt-end PCR products that are amplified by *Pwo* DNA polymerase. An additional purification of the PCR products enables the cloning of larger PCR products with acceptable efficiency.

Due to the terminal transferase activity of *Taq* DNA polymerase, a majority of PCR products amplified with this enzyme have a 3′-A overhang (17). These PCR products cannot be cloned into blunt-end cloning sites. As is shown here, it is possible to clone PCR products made with *Taq* DNA polymerase, however, with lower efficiency. In two of four experiments, positive clones were obtained (Table 3). The failure of two experiments probably reflects small deviations in the amounts of input template or primer DNA or *Taq* polymerase, which resulted in a more complete addition of the extra nucleotide to the PCR products. From this we conclude that the positive selection system is powerful enough to clone the low percentage of blunt-end PCR products obtained with *Taq* DNA polymerase (17) without an additional polishing step.

For long PCR, a blend of *Pwo* and *Taq* DNA polymerases is used to achieve efficient amplification of the target template (16). In experiments with long-range PCR products made with enzyme blends, we polished the 3′-A overhangs to increase the amount of blunt-end PCR fragments. In doing this, we cloned 4.8- and 9.3-kb PCR fragments, and 2 of 5 and 2 of 12 analyzed clones, respectively, carried the insert. One possible reason for a reduced efficiency with long PCR products could be that irregular short PCR by-products are produced; we have not included a gel-purification step in the experiments presented here. These by-products compete for ligation with the vector and result in negative clones. This could explain why 6 of 12 analyzed clones contained one of the 8.6- to 12.2-kb-long λ *Sma*I fragments, but only 2 of 12 analyzed clones contained the 9.3-kb PCR product.

The high ratio of positive clones, despite the absence of a gel-purification step, argues for the use of this positive selection system for routine cloning of DNA fragments. They can be either excised from the pCAP^s vector by restriction endonuclease digestion or reamplified by PCR. With appropriately designed primers containing SP6 or T7 RNA polymerase sites, it is possible to (re)amplify the cloned DNA fragments and to perform *in vitro* transcription and translation for further analysis (data not shown).

ACKNOWLEDGMENTS

We thank Jutta Kun, Karin Otto, Daniela Tils, and Gudrun Zimmer for excellent technical assistance and Andrew Barker for helpful comments on the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 274.

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