Volume 283, number 2, 227–229 FEBS 09763 June 1991

ADONIS 001457939100472B

Simple approach for oligonucleotide-directed mutagenesis of any double-stranded circular DNA

V.L. Drutsa and V.R. Kaberdin

A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Leninskiye Gory, Moscow 119899, USSR

Received 28 March 1991

A new site-directed method for introducing mutations into any region of plasmid vector close to the unique restriction site is decribed. It is based on the use of 5'-phosphorylated mutagenic and nonphosphorylated auxiliary oligonucleotides and a specific combination of enzymatic procedures including 'nick-translation' as a key step. The method efficiency was demonstrated by constructing the deletion-insertion mutation which creates the consensus Pribnow box in a promoter-testing plasmid. The yield of the target mutation was up to 85–95%.

Oligonucleotide-directed mutagenesis; Pribnow box; Nick-translation

1. INTRODUCTION

We have shown earlier that insertion of synthetic fragments containing the Pribnow box (TATAATG) into DNA resulted in the creation of the transcription initiation signal in the region of insertion [1–3]. To continue these investigations we have developed a simple and efficient method of site-directed mutagenesis, applicable, in particular, for precise introduction of short synthetic fragments in close vicinity of any unique restriction site of a double-stranded circular DNA.

2. MATERIALS AND METHODS

Restriction endonucleases *Hin*dIII, *Sal*I, *Pst*I, *Bgl*II, *Eco*RI (20–50 units/μl), T_4 polynucleotide kinase (2.4 U/μl), T_4 DNA ligase (20 U/μl), *E. coli* DNA polymerase I (10 U/μl) and the Klenow fragment of *E. coli* DNA polymerase I (10 U/μl) were purchased from Fermentas (USSR); restriction endonuclease *Bst*EII (10 U/μl) from BRL (UK); S1 nuclease (300 U/μl) and exonuclease III (20 U/μl) from Boehringer (Germany); deoxyribonucleoside-5'-triphosphates and ATP from Sigma (USA); SeaKem-agarose from Marine Colloids (USA); MacConkey agar, bacto-triptone, yeast extract from Difco (USA); [γ-^{32}P]ATP, spec. act. >1000 Ci/mmol from Isotope (USSR).

Oligodeoxyribonucleotides were synthesized automatically by the phosphoramidite method and then purified by electrophoresis on a 20% denaturing polyacrylamide gel (PAAG). Sequencing of oligonucleotides was carried out according to the procedure of Maxam and Gilbert [4].

The plasmid pHD-001-11-14, a pBR322 derivative carrying the ampicillin resistance gene and *E. coli* gal operon with the promoter region replaced by polylinker from M13mp8 DNA, and the host *E. coli* F165' (gal$^-$) strain were kindly donated by Professor H.-J. Fritz (Goettingen, Germany). The 'dyt' media (16 g bacto-triptone, 10 g yeast extract and 5 g NaCl per liter of water) was used for bacteria growth. The large-scale preparation of plasmid was performed according to [5].

All enzymatic treatments and annealing of oligonucleotides to DNA were carried out according to standard protocols ([5] and elsewhere).

For DNA preparation covalently closed circular plasmid pHD-001-11-14 (40 μg), purified by CsCl gradient ultracentrifugation [5], was cleaved at the unique site with *Hin*dIII (200 units, 37°C, 2 h), extracted with phenol-chloroform (1:1) and precipitated with ethanol. The linearized plasmid (25 μg) was treated with exonuclease III (50 units, 37°C, 5 min) in 200 μl of buffer, containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, extracted with phenol, phenol-chloroform and ethanol precipitated. To estimate the extent of exonuclease III hydrolysis the aliquot (1 μg) of DNA obtained was treated with S1 nuclease, and the resulting double-stranded DNA was analyzed by electrophoresis on a 0.8% agarose gel using the original plasmid and *Bst*EII digest of λ phage DNA as size markers. Under the conditions described the length of protruding single-stranded 5'-ends generated by exonuclease III appeared to be about 500 nucleotides.

For mutation construction 2 μg of exonuclease III treated plasmid DNA were annealed with 2.8×10^{-3} A_{260} of 29-mer mutagenic oligonucleotide (10-fold molar excess) and 1.6×10^{-3} A_{260} of 17-mer auxiliary oligonucleotide (10-fold molar excess) in a total volume of 50 μl (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mM EDTA) by heating to 65°C for 15 min and cooling to 20°C for 3 h. Then DNA ligase (20 U) and ATP (1 mM) were added and ligation proceeded for 2 h. Upon the addition of 0.25 mM of each of the dNTPs and the Klenow fragment of DNA polymerase I (15 units) the mixture was incubated for 3 h. Then more DNA ligase was added (20 units) and the incubation continued for 2 h. All reactions were carried out at room temperature. The reaction mixture was extracted with phenol-chloroform (1:1) and DNA was precipitated with ethanol. The pellet was redissolved in 50 μl of the same buffer, containing 0.25 mM of each of the dNTPs, treated with DNA polymerase I (10 units, 16°C, 1 h) and used directly for transformation.

The cloning was performed using the $CaCl_2$ transformation technique [5]. The *E. coli* F165' (gal$^-$) cells with plasmids accepted were

Correspondence address: V.L. Drutsa, A.N. Belozersky Laboratory, Bldg 'A', Moscow State University, Leninskiye Gory, Moscow 119899, USSR

selected on MacConkey agar indicator plates, containing galactose (1%) and ampicillin (50 μg/ml), according to their colour (red, white). The plasmid DNA from various clones was purified by express alkaline-lysis method [5], digested with endonucleases *Sal*I, *Pst*I, *Bgl*II or *Hin*dIII and analyzed by electrophoresis on a 0.8% agarose gel. The mutagenized region of plasmids from several selected clones was sequenced using the Maxam and Gilbert method [4]. The protocol of operations was as follows: the plasmid DNA was digested with *Eco*RI, dephosphorylated, 5'-^{32}P-labelled, digested with *Bst*EII, electrophoresed in denaturing 8% PAAG and a small ^{32}P-labelled *Eco*RI/*Bst*EII fragment was isolated and sequenced.

3. RESULTS AND DISCUSSION

Several methods for constructing site-specific mutations in double-stranded DNA vectors have been described [6–9]. Nevertheless, in contrast to highly efficient and widely used methods of the site-specific mutagenesis on single-stranded DNA templates, the mutagenic strategies for plasmid DNA are still either nonefficient or may be realized only in specially constructed systems. As an alternative to highly specialized and complicated methods we propose here a simple and highly efficient scheme of oligonucleotide-directed site-specific mutagenesis, based completely on the well-known routine procedures of recombinant DNA technique and applicable for any double-stranded circular vehicle.

According to the scheme proposed the initial double-stranded covalently-closed circular DNA is cleaved at a chosen unique restriction site in the close vicinity of the region to be mutagenized and then treated with exonuclease III to generate protruding single-stranded 5'-termini, one of which includes the region to be mutagenized. Then the DNA obtained is annealed with the 5'-phosphorylated mutagenic oligonucleotide, complementary (except for the site to be changed) to one of these termini, and the nonphosphorylated auxiliary oligonucleotide, perfectly complementary to the 5'-terminus of the opposite DNA strand. Upon annealing with DNA, these oligonucleotides are able to restore the original restriction site. The hybrid obtained is recircularized with DNA ligase, single-stranded regions are filled in with the Klenow fragment of DNA polymerase I, and the nick adjacent to the phosphorylated 5'-end of the mutagenic oligonucleotide is repaired with DNA ligase. The DNA obtained is a heteroduplex molecule with a mismatched region in mutation construction site and a nick in the wild-type strand adjacent to the nonphosphorylated 5'-end of the auxiliary oligonucleotide. The 'shift' of this nick through the region to be mutagenized by DNA polymerase I treatment (the reaction of nick-translation) converts the wild-type strand into a mutant one.

To demonstrate the method efficiency we have carried out an experiment on the pHD-001-11-14 plasmid, convenient for detecting the promoter functioning by gal operon expression: insertion of 9 base pairs instead of 2 in the polylinker region. This change results in the creation of the consensus Pribnow box TATAATG, which, we have assumed, should create the transcription initiation signal upstream the gal operon, and the colonies harbouring plasmids with the target mutation

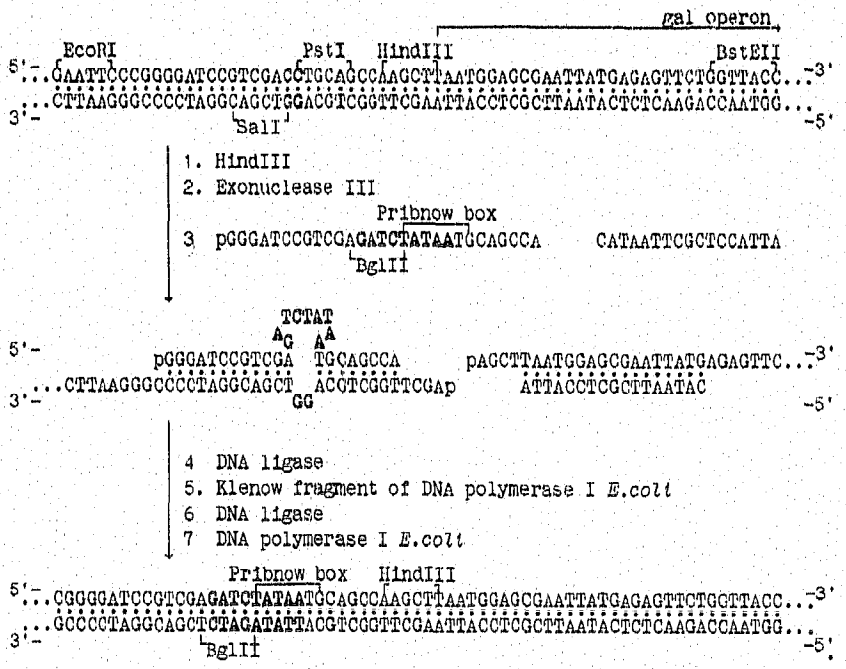

Fig. 1. The structures of the original plasmid, DNA-oligonucleotides complex and final mutant plasmid around the modified region. The restriction sites, synthetic oligonucleotides and the main steps of the mutagenesis are shown.

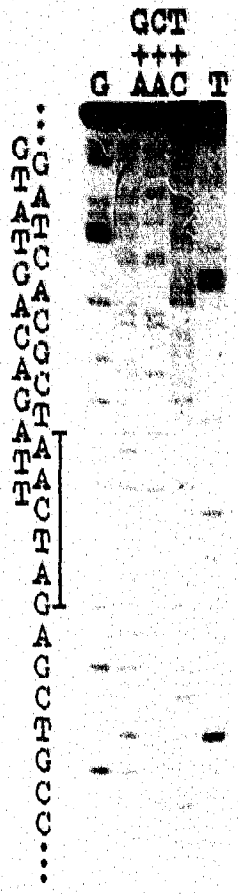

Fig. 2. The sequence analysis (electrophoresis on a 10% PAAG) of the [^{32}P]*Eco*RI/*Bst*EII fragment of the mutant plasmid. The letters above correspond to base-specific cleavage reactions: G, 40 mM dimethylsulphate, 20°C, 1 min; A+G, 50% formic acid, 60°C, 1 min; A+C, 1.2 M sodium hydroxide, 90°C, 10 min; C+T, 60% aqueous hydrazine, 37°C, 2 min; T, 0.3 mM potassium permanganate, 20°C, 2 min. The target insert is marked by bar.

could be identified by their red colour on MacConkey agar. As a result of mutagenesis the Pribnow box and *Bgl*II site should appear while *Sal*I and *Pst*I sites are removed. The structures of the original plasmid, DNA-oligonucleotides complex and final mutant plasmid around the modified region, restriction sites, synthetic oligonucleotides (5′-phosphorylated mutagenic 29-mer and nonphosphorylated auxiliary 17-mer) and main steps of mutagenesis are shown in Fig. 1.

Upon cloning, both red and white colonies were formed on MacConkey agar plates, the yield of red colonies being 85–95% in several lines of the experiments. The red colour was an indication of gal operon expression in the mutant plasmid. The subsequent analysis has confirmed the assumption that the Pribnow box was responsible for creation of the transcription initiation signal.

The restriction analysis of plasmid DNA, isolated from more than hundred clones, has shown that the *Hin*dIII site was present in plasmids of more than 98% of the clones; plasmids of more than 85–95% of the clones possessed the newly created *Bgl*II site and lacked the *Sal*I and *Pst*I sites. The mutagenized regions of plasmid DNAs of several selected clones were sequenced (see Fig. 1). The results of restriction and sequence analysis confirmed the construction of target mutants and strictly correlated with the phenotype of colonies. The yield of target mutants may be estimated as 85–95%.

In conclusion it should be noted that the elaborated method is of general applicability. It may be used to construct any other mutation types (base substitutions, deletions, insertions) in the vicinity of any unique restriction site in plasmid DNA. Moreover, this approach is also applicable in case the distance from the mismatch to the restriction site cannot be covered by a single piece of synthetic DNA. Then three primers are required: one mutagenic and two auxiliary oligonucleotides. Both synthetic oligonucleotide and single-stranded product of the asymmetric polymerase chain reaction may be used as mutagenic primer. At present this idea is being studied.

REFERENCES

[1] Koroleva, O.N., Drutsa, V.L. and Shabarova, Z.A. (1985) Biopolym. Kletka 1, 307–311.
[2] Koroleva, O.N., Drutsa, V.L. and Shabarova, Z.A. (1988) Biopolym. Kletka 4, 7–14.
[3] Koro[illegible], O.N. and Drutsa, V.L. (1991) FEBS Lett. 278, 207–210.
[4] Maxam, A.M. and Gilbert, W. (1980) in: Methods in Enzymology, vol. 65 (Grossman, I. and Moldave, K.N. eds) pp. 499–560, Academic Press, New York.
[5] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[6] Schold, M., Colombero, A., Reyes, A.A. and Wallace, R.B. (1984) DNA 3, 469–477.
[7] Efimov, V.A., Mirskikh, O.V., Chakhmakhcheva, O.G. and Ovchinnikov, Yu.A. (1985) FEBS Lett. 181, 407–411.
[8] Palermo, D.P. and Hess, G.F. (1987) DNA 6, 273–279.
[9] Bellini, A.V., De Ferra, F. and Grandi, G. (1988) Gene 69, pp. 325–330.