Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 298 (2002) 37–40
BBRC
www.academicpress.com

Limitations on the recombinant plasmid selection by Lac$^+$/Lac$^-$ colony phenotype detection

Nadezhda I. Gutkina, Natalia V. Varlakhanova, Marina V. Lysova, and Sergei P. Kovalenko*

Institute of Molecular Biology and Biophysics, Academy of Medical Sciences, Siberian Branch, Timakov str. 2, Novosibirsk 630117, Russia

Received 5 September 2002

Abstract

Lac$^+$/Lac$^-$ selection of recombinant plasmids based on the insertional inactivation of LacZα gene cannot differentiate recombinant clones in some cases. Several fragments of exon 11 of human *brca1* gene were cloned in LacZα-containing plasmids so that frameshift appeared at the 5′-end of the fragments tested but these fragments were in frame with the part of LacZα situated downstream of the polylinker. All plasmids except one caused blue colonies formation after being transformed in *Escherichia coli* LacZΔM15 cells in spite of the frameshift. The fact may be explained by reinitiation of translation within the mRNA transcribed from the inserted DNA fragments at in-frame AUG, GUG, and UUG. The data demonstrated limitations on the Lac$^+$/Lac$^-$ selection of LacZα-based recombinant plasmids.

Keywords: Lac$^+$/Lac$^-$ selection; Recombinant plasmid; brca1; LacZα; Translation initiation; Shine–Dalgarno sequence; *Escherichia coli*

LacZα-containing plasmids are widely used for the cloning of various DNA fragments [1–5]. The selection of *Escherichia coli* clones carrying recombinant plasmids is based on the change of colony phenotype (Lac$^+$ → Lac$^-$) which occurs due to the insertional inactivation of LacZα gene in the recombinant plasmid. Thus, after the transformation of ligation mixture, blue colonies on the indicator plates contain plasmids without insert, white colonies contain recombinant plasmids.

In spite of the simplicity and routing usage in various applications, the blue/white selection cannot differentiate recombinant clones in some cases [6–9]. So, the cloning of amplicons in pGEM-T Easy Vector (Promega) was reported to cause blue or pale-blue colonies in several experiments [8].

Our experiments on the cloning of human *brca1* gene fragments in pGEM7Zf plasmid also resulted in the formation of Lac$^+$ (not Lac$^-$ as expected) *E. coli* colonies carrying recombinant plasmids.

The present study is an attempt to find the reason for Lac$^+$/Lac$^-$ phenotype selection limitations in the LacZα-containing vectors' cloning.

Materials and methods

Enzymes and chemicals. Taq DNA polymerase, T4 DNA ligase, dNTPs, and the kit for DNA isolation were from "MedigenLab" (Novosibirsk, Russia). Restriction endonucleases, T4 polynucleotide kinase, and plasmid pUC19 were from "SibEnzyme" (Novosibirsk, Russia). Plasmids pGEM7Zf and pBluescript IISK were from "Promega" (USA) and Stratagene (USA), respectively. Guanidine thiocyanate, calcium chloride, peptone, yeast extract, and BactoAgar were from "Fluka" (Switzerland). Silica (Silicon dioxide) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were from "Sigma" (USA). Agarose, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), isopropyl-β-D-thiogalactopyranoside (IPTG), and the kit for PCR products purification with HiBind DNA columns (E.Z.N.A. Cycle Pure Kit) were from "Peqlab Biotechnology GmbH" (Germany).

Bacterial strain. The LacZΔM15 *E. coli* strain XL1-Blue (Stratagene, USA) was used as the host for all plasmids.

Genomic DNA isolation and amplification. Human genomic DNA was isolated from blood by using the kit for DNA isolation. The following primers were used to amplify exon 11 of *brca1* gene: (1) 5′GTG TCC CAA GCT TGG CTG CTT GTG AAT TTT CTG AGA CG;

* Corresponding author. Fax: +7-3832-334301.
E-mail address: skoval@online.sinor.ru (S.P. Kovalenko).

0006-291X/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0006-291X(02)02392-6

(2) 5′GCG TGA CTC TAG AGC GGG CAA AAA CCT GGT TCC. The primers were synthesized by "ARC Scientific GmbH" (Germany). PCR was performed in a 50-μl volume containing 16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8), 0.01% Tween 20, 4 mM $MgCl_2$, 300 μM of each dNTP, 0.5 μM of each primer, 20–50 ng genomic DNA, and 2 U Taq DNA polymerase. Reactions were performed in thermocycler AMP-4 ("Bis," Novosibirsk, Russia). PCR conditions were 94 °C/4 min, 65 °C/1 min, and 72 °C/2 min and then 34 cycles: 94 °C/1 min, 65 °C/1 min, and 72 °C/2 min. PCR product was purified by E.Z.N.A. Cycle Pure Kit according to manufacturer's instruction.

Plasmid constructions. Amplified exon 11 of *brca1* gene digested with *Eco*RI and *Asu*NHI was ligated into the vector pGEM7Zf double digested with *Eco*RI and *Xba*I. The resulting recombinant plasmid (pGEM7Zf+ex11) contained 2.97 kb-fragment of exon 11 in a single translation frame with LacZα (Fig. 1).

Competent cell preparation and transformation were conducted by $CaCl_2$ method [10]. The indicator plates contained 1.5% agar supplemented with ampicillin, tetracycline, X-Gal, and IPTG. Plasmid DNA was isolated by alkaline lysis method [10]. All other DNA manipulations were carried out in the standard manner [10].

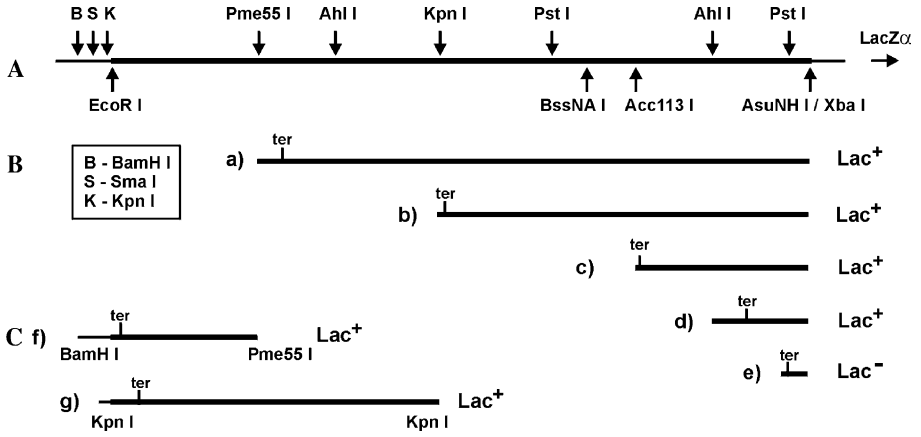

Fig. 1. DNA fragments of exon 11 of *brca1* gene fused with LacZα and tested on the ability to cause Lac^+ or Lac^- phenotype of LacZΔM15 *E. coli* cells. (A) The major part of exon 11 (*Eco*RI/*Asu*NHI) cloned in pGEM7Zf plasmid. Restriction endonuclease sites used for deletions introduction are designated by arrows. (B) Exon 11 DNA fragments situated downstream of the deletions introduced into the pGEM7Zf+ex11 plasmid: (a) *Sma*I–*Pme*55I deletion; (b) *Kpn*I–*Kpn*I deletion; (c) *Bss*NAI–*Acc*113I deletion; (d) *Ahl*I–*Ahl*I deletion; (e) *Pst*I–*Pst*I deletion. (C) Small fragments of exon 11 cloned in pBluescript IISK (f) and pUC19 (g) plasmids. Termination codons (indicated as ter) appeared due to the frameshift.

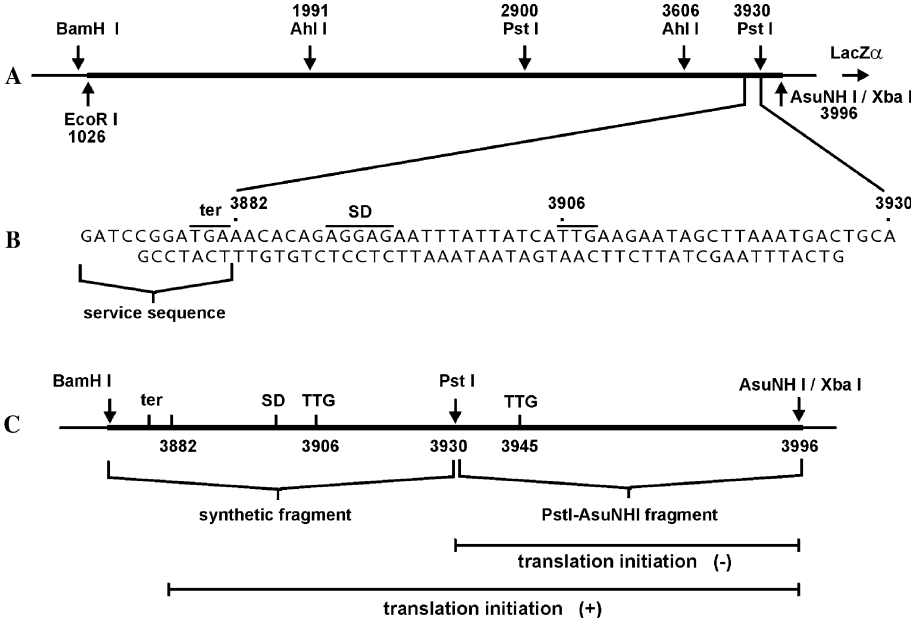

Fig. 2. Synthetic DNA fragment cloning in pGEM7Zf+ex11 plasmid digested by *Bam*HI and *Pst*I. (A) A major part of exon 11 (*Eco*RI/*Asu*NHI) cloned in the polylinker of pGEM7Zf plasmid. Numeration of nucleotides of exon 11 corresponds to *brca1* mRNA numeration (Accession No. U14680, GenBank). (B) Synthetic DNA fragment. The fragment contains a nucleotide sequence of exon 11 (from 3882 till 3930 positions) and a service sequence with TGA termination codon. (C) DNA insertion in the polylinker of pGEM7Zf comprising synthetic DNA fragment and *Pst*I–*Asu*NHI fragment of exon 11 of *brca1* gene. The resultant plasmid causes Lac^+ phenotype after being transformed in LacZΔM15 *E. coli* cells in spite of the termination codon occurrence just after *Bam*HI site.

Deletions were introduced into the cloned exon 11 by digestion of plasmid DNA pGEM7Zf+ex11 with the appropriate restriction endonucleases followed by ligation. Two restriction fragments of pGEM7Zf+ex11 (*Kpn*I–*Kpn*I and *Bam*HI–*Pme*55I) were subcloned into pUC19 and pBluescript IISK vectors, respectively.

Two complementary oligodeoxynucleotides were synthesized (BiosSet, Novosibirsk, Russia) to prepare a synthetic DNA fragment containing a part of exon 11 (Fig. 2). Oligonucleotides were phosphorylated with T4 polynucleotide kinase, then annealed, and ligated into pGEM7Zf+ex11 plasmid double digested with *Bam*HI and *Pst*I.

In all cases of plasmid constructing the restriction fragments were separated in 1% agarose and eluted from the gel using the kit for DNA isolation (MedigenLab). The appropriate restriction analysis was performed to confirm the structures of all recombinant plasmids.

β-Galactosidase activity assay. *Escherichia coli* XL1-Blue cells containing pGEM7Zf or pGEM7Zf+ex11 plasmids were grown up at 37 °C to optical density of 0.1–0.6 at 600 nm in LB medium, supplemented with ampicillin, tetracycline, and IPTG. The assays were performed as described by Miller [11] with ONPG as substrate. At least nine activity measurements for each strain were performed, the mean values and deviations were calculated by standard technique.

Results and discussion

A large part of exon 11 of human *brca1* gene (2.97 kb, *Eco*RI/*Asu*NHI) was cloned in pGEM7Zf plasmid and the resultant plasmid was designated as pGEM7f+ex11. The cloning was designed in-frame with LacZα gene. All colonies of XL-1 Blue *E. coli* cells with the recombinant plasmid were blue on the indicator plates in spite of the proved 2.97 kb DNA insert. The activity of β-galactosidase in *E. coli* cells with the recombinant plasmid (954 ± 67 Miller units) did not differ from β-galactosidase activity of the same cells with the original pGEM7Zf plasmid (827 ± 42 Miller units). So, in-frame insertion of 2.97 kb DNA fragment into pGEM7f plasmid did not influence the ability of the plasmid to cause β-galactosidase activity in LacZΔM15 *E. coli* cells. The fact may be explained by the formation of the fusion protein capable of effecting α-complementation in LacZΔM15 *E. coli* cells. The putative fusion protein contains on the N-terminal part either a complete aminoacid sequence coded by exon11 *brca1* gene fragment or a part of this sequence. In the last case, one should presume the translation initiation within mRNA coded by the fragment of exon 11 of *brca1* gene.

Several plasmids were constructed to detect the possibility of translation initiation within the fragment of exon11 of *brca1* gene. Five plasmids were constructed by the introduction of the following deletions into the pGEM7f+ex11 plasmid (Fig. 1):

(1) *Sma*I/*Pme*55 I; (2) *Kpn*I/*Kpn*I; (3) *Bss*NAI/*Acc*113I; (4) *Ahl*I/*Ahl*I 5) *Pst*I/*Pst*I.

All deletions resulted in the shift of the original LacZα open reading frame. In all cases stop codons appeared downstream of the deletion introduced (Figs. 1a–e).

Quite unexpectedly, Lac^- colony phenotype was observed in a single case only, namely in the case of the plasmid with *Pst*I/*Pst*I deletion. All other plasmids caused Lac^+ colonies phenotypes in spite of the LacZα open reading frameshifts.

The most probable explanation for the Lac^+ phenotype formation is the translation initiation within the fragments cloned, downstream of the frameshift. The hypothesis was supported by other experiments with the cloning of two DNA fragments of exon 11 in LacZα-containing vectors. In both cases the cloning was designed so that frameshift appeared at the 5′-end of the fragment cloned, but the fragment was in the single frame with the part of LacZα situated downstream of the multiple cloning site. Namely, *Bam*HI–*Pme*55I DNA fragment was cloned in pBluescript IISK plasmid digested by *Bam*HI and *Eco*RV simultaneously (Fig. 1f) and *Kpn*I–*Kpn*I fragment was cloned in pUC19 plasmid digested by *Kpn*I (Fig. 1g). In both cases the colonies harboring the plasmids demonstrated Lac^+ phenotype.

*Pst*I/*Pst*I deletion in pGEM7Zf+ex11 plasmid caused Lac^- phenotype of the colonies. In our experiments there was the only case where $Lac^+ \rightarrow Lac^-$ selection of recombinant clones was effective. This result indicates the absence of translation initiation signals within the region 3930–3996 of exon 11 (Fig. 1e). Meanwhile, the translation initiation presumably occurs in the region downstream of position 3606 (Fig. 1d), since *Ahl*I/*Ahl*I deletion introduced into the pGEM7Zf+ex11 plasmid resulted in the plasmid causing Lac^+ phenotype when transformed into the LacZΔM15 *E. coli* cells. According to these observations the translation initiation site is situated in the region between *Ahl*I and *Pst*I sites (in the region 3606–3930). There are no ATG or GTG codons in this region in-frame with the terminal part of LacZ, but several in-frame TTG codons may be found.

TTG (UUG) codon was described as the initiator codon in some bacterial genes [12–14]. The nearest to *Pst*I TTG codon (position 3906) was tested on the ability to initiate the translation. Two oligonucleotides were synthesized so that they could form a double-stranded DNA fragment containing the sequence of exon 11 from 3882 to 3930 (Fig. 2B). This DNA fragment was designed to contain terminator TGA codon at the upstream position and sticky ends for convenient cloning in pGEM7f+ex11 digested by *Bam*HI and *Pst*I. The cloning of the chemically synthesized fragment resulted in the formation of pGEM7Zf plasmid containing exon 11 fragment 3882–3996 (Fig. 2C). The phenotype of the colonies with the plasmid constructed was Lac^+ and that strongly indicates the possibility of translation initiation at TTG codon at position 3906.

It is generally accepted that the efficiency of initiation is affected by the sequences surrounding start-codon [14–18]. Shine–Dalgarno (SD) sequence located few bases upstream of initiation codon was reported to play

a crucial role in translation initiation efficiency [14,16,18]. The analysis of exon 11 nucleotide sequence revealed several SD-like sequences situated in the vicinity of the potential initiation codons (ATG/GTG/TTG). Quite remarkable to note that one SD-like sequence found in exon 11 has a very high homology to the sequence preceding ini-codon in mRNA of β-subunit of *E. coli* RNA-polymerase. As for TTG codon in 3906 position of exon 11, the obvious SD-like sequence (aggag) may be found 12 bp upstream of the codon (Fig. 2B). The fact favors the hypothesis of the translation initiation at TTG at 3906 position. In general, exon 11 contains several regions similar to the translation initiation sequences of *E. coli* (SD-like sequence with the adjacent initiation codon), some of them (or all of them) may be used by *E. coli* translation machinery for the effective translation initiation.

*Pst*I–*Asu*NHI fragment (positions 3930–3996) is the only DNA fragment in our experiments that does not effect the translation initiation (Figs. 1e and 2C). Meanwhile, this fragment contains the only TTG codon in 3945 position, the codon cannot serve as ini-codon in this case. The most probable explanation for that is the absence of SD-like sequences in the vicinity of this TTG codon.

Our results strongly indicate that Lac^+ phenotype of *E. coli* clones with the recombinant plasmids reflects the translation initiation within the fragments cloned.

So, the cloning of DNA fragments containing open reading frames in LacZα-based plasmids in the variety of cases cannot rely upon the phenotypic (Lac^+/Lac^-) selection of clones harboring recombinant plasmids. The problems with Lac^+/Lac^- recombinant clone selection may be explained by the following features of the LacZα system:

- the variety of polypeptides that may be added to N-terminus of α-peptide without the influence on its ability to participate in α-complementation;
- eukaryotic DNA fragments contain a lot of sequences recognized by *E. coli* translation machinery as translation initiation sites. These sequences consist of ATG/GTG/TTG codon with the adjacent SD-like sequences.

The practical outcome of our work is the precaution for the Lac^+/Lac^- selection in the case of the open reading frame-containing fragment cloning. These fragments should contain the proved termination codon(s) at the junction with the major part of LacZα gene. Alternatively, other selection cloning systems (without Lac^+/Lac^-) may be used.

References

[1] J.E. Cronan Jr., M.L. Narasimhan, M. Rawlings, Insertional restoration of β-galactosidase α-complementation (white-to-blue colony screening) facilitates assembly of synthetic genes, Gene 70 (1988) 161–170.
[2] A. Lönn, M. Gardonyi, W. van Zyl, B. Hahn-Hägerdal, R.C. Otero, Cold adaptation of xylose isomerase from *Thermus thermophilus* through random PCR mutagenesis, Eur. J. Biochem. 269 (2002) 157–163.
[3] J. Matsuda, M. Togo, S. Kagawa, J.E. Moore, PCR cloning of the resuscitation-promoting factor (Rpf) gene from *Micrococcus luteus*, sequencing and expression in *Escherichia coli*, Microbios 104 (2001) 55–61.
[4] L.R. Washburn, K.E. Weaver, E.J. Weaver, W. Donelan, S. Al-Sheboul, Molecular characterization of *Micoplasma arthritidis* variable surface protein MAA2, Infect. Immun. 66 (1998) 2576–2586.
[5] B. Yao, P. Sollitti, J. Marmur, Primary structure of the maltose-permease-encoding gene of *Saccharomyces carlsbergensis*, Gene 79 (1989) 189–197.
[6] J. Brosius, Superpolylinkers in cloning and expression vectors, DNA 8 (1989) 759–777.
[7] T.J. Close, J.L. Christmann, R.L. Rodriguez, M13 bacteriophage and pUC plasmids containing DNA inserts but still capable of β-galactosidase α-complementation, Gene 23 (1983) 131–136.
[8] S. Frackman, D. Kephart, Rapid ligation for the pGEM-T and pGEM-T Easy Vector systems, Promega Corporation, http://www.promega.com/pnotes/71/7807_08/7807_08.html.
[9] S.N. Slilaty, S. Lebel, Accurate insertional inactivation of lacZα: construction of pTrueBlue and M13TrueBlue cloning vectors, Gene 213 (1998) 83–91.
[10] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[11] J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1972.
[12] M. Kozak, Initiation of translation in prokaryotes and eukaryotes, Gene 234 (1999) 187–208.
[13] T. Meinnel, C. Sacerdot, M. Graffe, S. Blanquet, M. Springer, Discrimination by *Escherichia coli* initiation factor IF3 against initiation on non-canonical codons relies on complementarity rules, J. Mol. Biol. 290 (1999) 825–837.
[14] C. Stenström, E. Holmgren, L.A. Isaksson, Cooperative effects by the initiation codon and its flanking regions on translational initiation, Gene 273 (2001) 259–265.
[15] C. Stenström, H. Jin, L.L. Major, W.P. Tate, L.A. Isaksson, Codon bias at the 3′-side of the initiation efficiency in *Escherichia coli*, Gene 263 (2001) 273–284.
[16] J. Shine, L. Dalgarno, The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites, Proc. Natl. Acad. Sci. USA 71 (1974) 1342–1346.
[17] M. Dreyfus, What constitutes the signal for the initiation of protein synthesis on *Escherichia coli* mRNAs?, J. Mol. Biol. 204 (1988) 79–94.
[18] W.F. Jacob, M. Sauter, A.E. Dahlberg, A single base change in the Shine–Dalgarno region of 16S rRNA of *Escherichia coli* affects translational of many proteins, Proc. Natl. Acad. Sci. USA 84 (1987) 4757–4761.