Convenient modification of the method for oligonucleotide-directed in vitro mutagenesis of cloned DNA

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A new modification of the oligonucleotide-mediated mutagenesis technique has been developed. The proposed methodology has been used to produce specific base changes in the double-stranded plasmid DNA. For this purpose, special cloning vectors have been constructed using the synthetic oligodeoxyribonucleotides. The developed method allows the production of mutant DNA from those of the wild-type with a yield of 10–20%.

Recombinant DNA *Oligonucleotide-directed mutagenesis* *Colony screening*

1. INTRODUCTION

Nowadays, site-directed mutagenesis of genes is successfully applied for studying structure-function relations in corresponding proteins [1]. Mutations in structural genes allow the production of proteins with specific amino acid changes that in some cases might result in obtaining modified molecules with increased or novel biological activities [2–4]. Moreover, the directed mutagenesis together with the data on primary and spatial protein structure provides information on the role of particular amino acids in the functioning of protein active sites, assembling of its subunits, etc., thus providing a method to study the mechanism of protein action.

Mutational changes in known DNA sequence can be effected in a number of ways [1,5], but the most precise way to introduce specific mutations in cloned DNA fragments is through the use of synthetic oligodeoxyribonucleotides as site-specific mutagens [6]. The general strategy of oligonucleotide-directed mutagenesis involves an application of synthetic oligonucleotide carrying the desired mutation (point substitution of the heterocyclic base, deletion or insertion) as a primer to direct the DNA synthesis on a single-stranded circular DNA template. So, the oligonucleotide is incorporated into the second DNA strand. After ligation, transformation of this closed circular heteroduplex DNA into *E. coli* cells followed by in vivo DNA replication resolves this heteroduplex into mutant and original, wild-type gene. The mutant can be easily identified against a background of nonmutant DNAs by hybridization with the same oligonucleotide [5–7].

To carry out oligonucleotide-directed mutagenesis, two systems are generally employed: single-stranded DNA phages (such as bacteriophage M13 and its derivatives, or ϕX174) or single-stranded circular plasmid DNAs. The latter are produced from the supercoiled double-stranded plasmid DNA by introducing nick(s) into one of the plasmid chains under the action of some restriction endonucleases in the presence of ethidium bromide (or by DNase I) with the following digestion of the nicked strand with exonuclease [2,8]. However, in this case the yields of mutants are low (on average, from 0.01 to 1%). The application of single-stranded DNA phages is more effective (the yields

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are 2–5%) but this approach has also a number of disadvantages, in particular, long duration of the experiment and necessity of special treatment for enrichment of the material with mutant DNA. Moreover, in both cases application of DNA polymerase for the primer elongation on the extensive, completely single-stranded circular template leads to appearance of false mutations. Decreasing the yield of the desired mutation also occurs, owing to the presence in the reaction mixture of many DNA molecules with an incompletely repaired second strand, since DNA polymerase has difficulties in passing over template regions with secondary structure, in particular origin of replication [9,10].

In order to increase the yield of mutation and simplify the general methodology, we have designed a modified procedure for changing specific nucleotides in a DNA sequence with high efficiency. The method is based on the application of a linear double-stranded plasmid DNA with a single-stranded sequence in the region for mutagenesis.

2. MATERIALS AND METHODS

Restriction endonucleases, *E. coli* exonuclease III, *E. coli* DNA polymerase I (Klenow fragment) and deoxynucleoside triphosphates were obtained from P-L Biochemicals. [γ-^{32}P]ATP (~5000 Ci/mmol) was from Amersham. T4 polynucleotide kinase, T4 DNA ligase and T4 DNA polymerase were isolated as described [11]. The oligodeoxyribonucleotides were synthesized by the rapid *N*-methylimidazolide phosphotriester method [12].

To prepare circular heteroduplex DNA, recombinant plasmid (2 μg) was treated with *Hpa*I in 20 μl of 20 mM Tris–HCl (pH 7.5)/1 mM dithiothreitol/50 mM NaCl/6 mM $MgCl_2$, or *Sma*I in the same volume of 20 mM KCl/10 mM Tris–HCl (pH 8.0)/6 mM $MgCl_2$/1 mM dithiothreitol, for 1 h. Then 6–8 units of exonuclease III (or 10 units of T4 DNA polymerase) were added. The reaction mixture was incubated at 25°C for 2–10 min. The reaction was stopped by heating to 60°C and the solution was extracted twice with phenol and then once with diethyl ether. The DNA was precipitated by adding 0.2 vol of 1 M sodium acetate and 2 vols ethanol. After centrifugation, the pellet was washed with ethanol, dried and dissolved in 50 μl of 50 mM Tris–HCl (pH 7.9)/10 mM $MgCl_2$/20 mM NaCl. The synthetic oligonucleotide (40–200 pmol) was added. Then the mixture was heated to 50–55°C for 5 min and allowed to cool slowly to room temperature. The four deoxynucleoside triphosphates were added to yield a final concentration of 300 μM, ATP to 100 μM and DTT to 1 mM. After the addition of *E. coli* DNA polymerase I (Klenow fragment) (30 units) and T4 DNA ligase (10 units), the reaction mixture was incubated for 5–6 h at 20°C.

4–5 μl of this mixture was used directly to transform competent *E. coli* HB101 cells. Competent cells were prepared and transformations were done according to [13]. A second cycle of transformation was usually done after a quick plasmid preparation [14]. Colony screening and hybridization with ^{32}P-labeled oligonucleotide were done as described earlier [15].

To determine the exact time of exonuclease digestion, a linear form of recombinant plasmid (10 μg) was treated with exonuclease III (40 units) at 25°C, or with T4 DNA polymerase (50 units) at 37°C, in 150 μl of 20 mM Tris–HCl (pH 7.5)/10 mM $MgCl_2$/50 mM NaCl/1 mM dithiothreitol. Aliquots (30 μl) were taken each 2–5 min, and 1 M sodium acetate (pH 4.5) (2 μl), 1 M NaCl (5 μl), 0.1 M $ZnCl_2$ (2 μl), H_2O (10 μl) and 1 μl (4 units) of S_1 nuclease (Sigma) were added to each sample. After incubation at 20°C for 15 min, the reaction was stopped by the addition of EDTA. To determine the size of the double-stranded DNA obtained after the removal of the single-stranded regions by S_1 nuclease, the samples were subjected to 1.5% agarose gel electrophoresis. The starting linear plasmid DNA and the *Hin*dIII digest of lambda DNA were used as DNA length markers.

3. RESULTS AND DISCUSSION

The proposed modified procedure involves using specially constructed cloning vectors pBRS1, pHS1 and pHS2. These plasmids are derivatives of pBR322 in which the *Eco*RI-*Hin*dIII region has been replaced by synthetic duplexes carrying *Sma*I, *Hpa*I and *Xho*I sites. Schemes for production of the plasmids are shown in fig.1. The substitution of the *Eco*RI-*Hin*dIII region in plasmid pBR322 for the synthetic duplex consisting of oligonucleotides d(AATTCCCCGGG) (I) and d(AGCTTCCCGGGG) (II) led to pBRS1 which had the *Sma*I

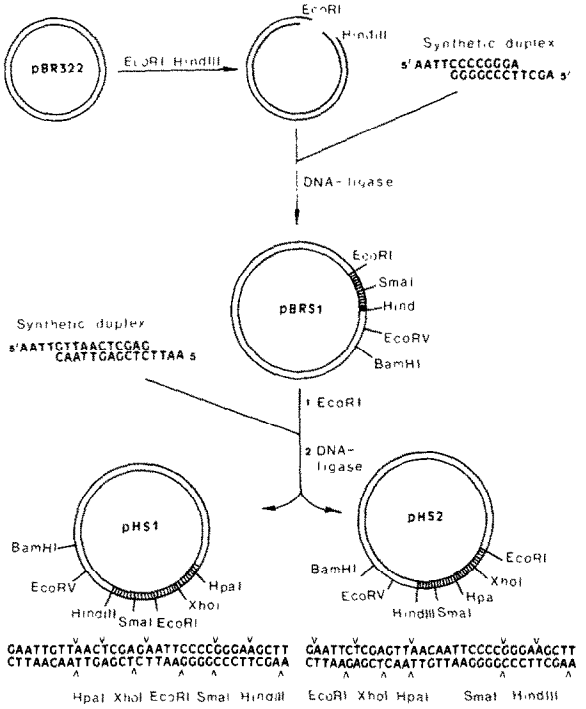

Fig.1. Scheme for construction of recombinant plasmids pBRS1, pHS1 and pHS2. Relevant restriction sites are indicated.

site. Then, insertion of the double-stranded fragment consisted of 15-mers d(AATTGTTAACTCGAG) (III) and d(AATTCTCGAGTTAAC) (IV) into the *Eco*RI site of pBRS1 resulted in the formation of the plasmids pHS1 and pHS2 containing the unique *Hpa*I and *Eco*RI sites. These vectors differ only in orientation of the 15-bp insertion, and they are obtained simultaneously by inserting this synthetic duplex into pBRS1. After the transformation of *E. coli* cells with this mixture, they were separated on the level of plasmid DNAs isolated from the colonies chosen by hybridization with ^{32}P-labeled oligonucleotide (III). Then they were identified by the restriction analysis and the direct determination of the primary structure of the *Eco*RI-*Hin*dIII fragment.

According to a procedure developed by us, the DNA fragment to be mutagenized is cloned in the vectors pBRS1, pHS1 and pHS2 using any suitable restriction site located in the *Eco*RI-*Bam*HI region situated close to *Sma*I, *Hpa*I and *Eco*RV sites of these plasmids. The recombinant plasmid obtained is digested with one of these enzymes to produce double-stranded DNA with blunt ends. This linear DNA is a substrate for *E. coli* exonuclease III (or

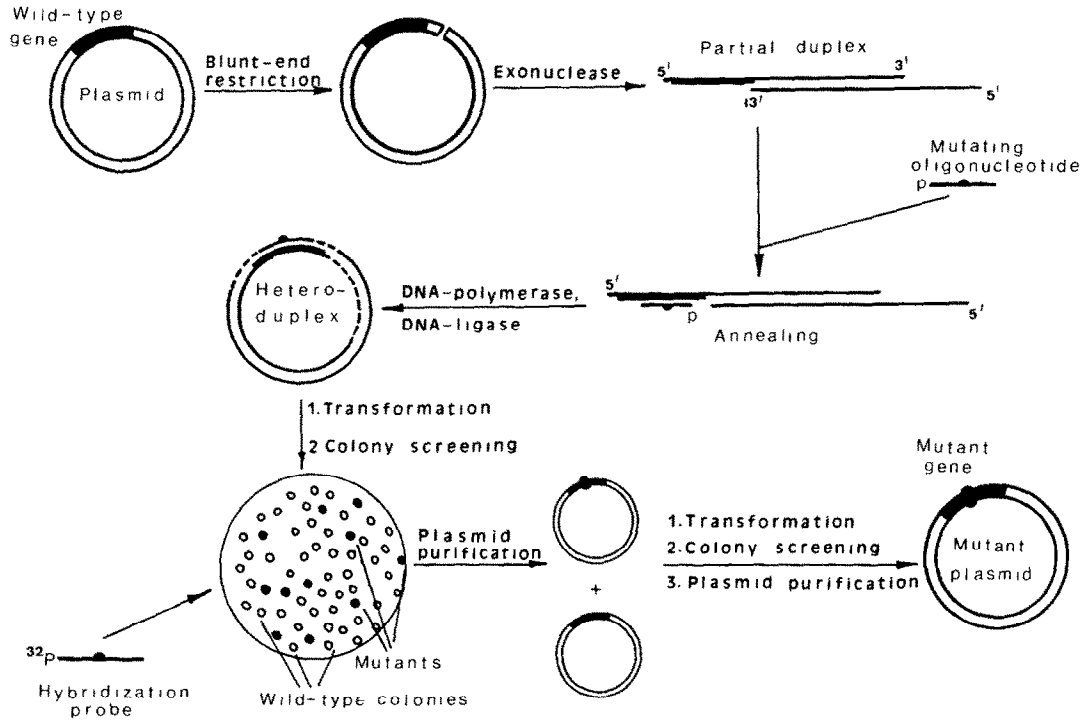

Fig.2. Overall scheme for oligonucleotide-directed mutagenesis of double-stranded linear plasmid DNA.

T4 DNA polymerase). The digestion in controlled conditions produces duplex with protruding single-stranded 5′-ends which include the site of the proposed mutation. Degradation of the DNA outside this region, where the sequence alters, occurs to a minimal degree (fig.2).

Limited degradation of DNA under the exonuclease action is achieved by using the controlled amounts of the exonuclease added to the reaction mixture and by the fixed time of its action. Usually, the incubation of DNA at 25°C for 5 min with 3–4 units of *E. coli* exonuclease III per 1 μg DNA leads to cleavage of about 300–400 residues from the 3′-ends of the duplex chains. T4 DNA polymerase works slower and cleaves off the same number of residues for 20 min. The exact time of exonuclease treatment should be determined empirically as described in section 2.

The next step includes annealing the synthetic oligonucleotide, which contains the desired mutation, with the plasmid DNA prepared as described above and the following completion of single-stranded regions of the molecule by the action of *E. coli* DNA polymerase I (Klenow fragment) in the presence of T4 DNA ligase. The latter provides the closing of the obtained heteroduplex into the cyclic molecule.

The obtained heteroduplex DNA is used to transform *E. coli* cells. Usually, about several thousand colonies grown for 16–18 h of incubation on LB-medium containing 1% agar and ampicillin (25 μg/ml). Several hundred (more often 200) clones are screened by hybridization with the same ^{32}P-labeled oligonucleotide-mutagen as a highly specific probe. Hybridization is carried out as described [15]. The temperature of the hybridization will vary with the sequence and length of the oligonucleotide and the type of mutation. As a rule, mutant colonies identified by colony hybridization after the first transformation with heteroduplex DNA contain a mixture of wild-type and mutant plasmids. For their separation a second transformation of *E. coli* with plasmid preparation isolated from these 'mixed' colonies is necessary. As shown in fig.3, a second screening allows identification of 'pure' mutant colonies which usually compose from 40 to 60% of all the secondary obtained colonies.

The experiments carried out showed that under the applied conditions the yields of mutant cloned after the first transformation of bacteria by heteroduplex DNA are up to 10–20% of the total amount of colonies. Thus, partial removal of one of the strands in plasmid DNA which affects mainly region of the desired mutation considerably decreases the possibility of formation of unplanned, 'false' mutations at other sites of the plasmid. Besides, it facilitates the mission of DNA polymerase and does not affect the origin of replication of the vector molecule.

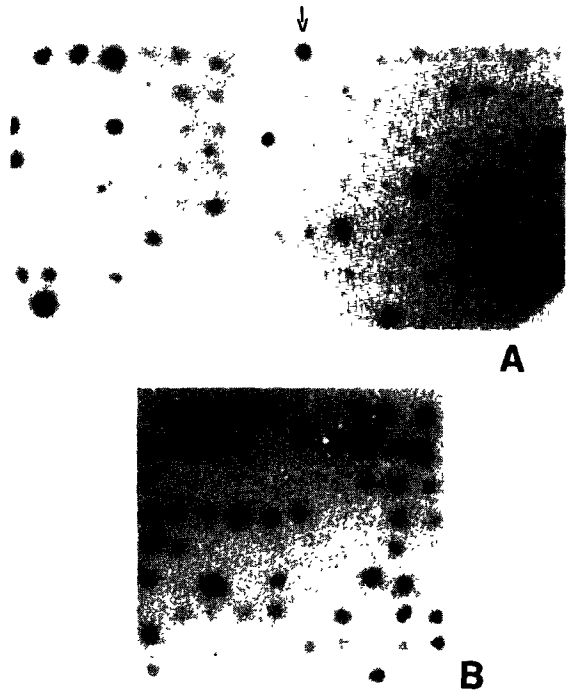

Fig.3. Autoradiograms showing the result of a colony screen by hybridization in situ with a ^{32}P-labeled 18-base oligonucleotide to select mutants of the gene for human proinsulin. (A) Screening after a first transformation of *E. coli*, plasmid DNA isolated from colony shown by an arrow was used for second transformation; (B) screening after a second transformation, the darkest colonies proved to be the desired mutants.

The procedure described above was used to introduce mutations into the gene for human proinsulin [16], promoter G2 of bacteriophage fd DNA [17] and other cloned synthetic DNAs. Autoradiograms obtained at determination of the sequence of human proinsulin gene region before and after insertion of mutation are presented in fig.4.

It must be noted that this technique can be ex-

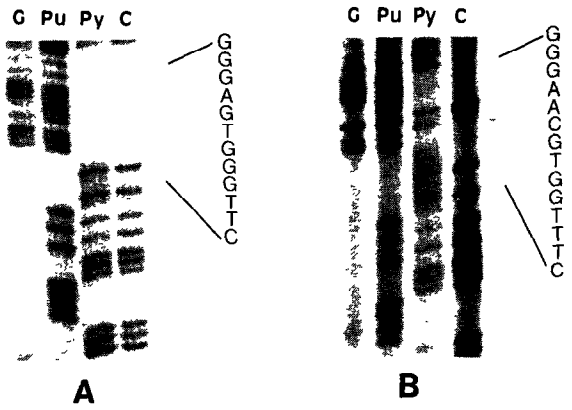

Fig.4. Maxam-Gilbert sequence analysis gel showing the part of the synthetic gene for human proinsulin containing a mutation (A) and the initial gene (B).

tended to any DNA sequence which has a unique site of restriction endonuclease generating blunt ends. It can be also applied to replicative form of phage DNAs (e.g., derivatives of M13 phage DNA: M13mp8, M13mp9, M13mp12, etc.) having polylinker sequences suitable for cloning and unique sites of blunt-ended restriction enzymes.

REFERENCES

[1] Harris, T. (1982) Nature 299, 298–299.
[2] Dalbadie-McFarland, G., Cohen, L.W., Riggs, A.D., Morin, C., Itakura, K. and Richards, J.K. (1982) Proc. Natl. Acad. Sci. USA 79, 6409–6413.
[3] Wilkinson, A.J., Fersht, A.R., Blow, D.M., Carter, P. and Winter, G. (1984) Nature 307, 187–188.
[4] Kin-Ming Lo, Jones, S.S., Hackett, N.R. and Khorana, H.G. (1984) Proc. Natl. Acad. Sci. USA 81, 2285–2289.
[5] Smith, M. (1982) TIBS 7, 440–442.
[6] Adelman, J.P., Hayflock, J.S., Vasser, M. and Seeburg, P.H. (1983) DNA 2, 183–193.
[7] Larson, G.P., Itakura, K., Ito, H. and Rossi, J.J. (1983) Gene 22, 31–39.
[8] Wallace, R.B., Johnson, P.F., Tanaka, S., Schold, M., Itakura, K. and Abelson, J. (1980) Science 209, 1396–1400.
[9] Nilsson, B., Uhlen, M., Josephson, S., Gatenbeck, S. and Philipson, L. (1983) Nucleic Acids Res. 11, 8019–8030.
[10] Osinga, K.A., Van der Blick, A.M., Van der Horst, G., Groot Koerkamp, M.J.A., Tabak, H.F., Veeneman, G.H. and Van Boom, J.H. (1983) Nucleic Acids Res. 11, 8595–8608.
[11] Dolganov, G.M., Chestukhin, A.V. and Shemyakin, M.F. (1981) Eur. J. Biochem. 114, 247–254.
[12] Efimov, V.A., Reverdatto, S.V. and Chakhmakhcheva, O.G. (1982) Nucleic Acids Res. 10, 6675–6694.
[13] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in: Mlecular Cloning, pp. 86–96, 249–255. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[14] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523.
[15] Efimov, V.A. and Chakhmakhcheva, O.G. (1982) Bioorgan. Khimia, 8, 2084–2093.
[16] Ovchinnikov, Yu.A., Efimov, V.A., Ivanova, I..N., Reverdatto, S.V., Skiba, N.P. and Chakhmakhcheva, O.G. (1984) Gene 31, 65–78.
[17] Ovchinnikov, Yu.A., Efimov, V.A. and Chakhmakhcheva, O.G. (1979) FEBS Lett. 100, 341–346.