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Mutagenesis directed by phosphotriester analogues of oligonucleotides: a way to site-specific mutagenesis in vivo

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A new approach to induce directed mutations in genes of study through simple cotransfection of *E. coli* cells by the mixture of primer and template was developed. This method is based on the use of synthetic phosphotriester analogues of oligonucleotides as site-specific mutagenic primers. The achieved yield of mutant clones was 2–3%.

Oligonucleotide-directed mutagenesis; Phage M13; β-Galactosidase; DNA sequencing; Mutagenesis; single-stranded DNA

1. INTRODUCTION

It has been known for a long while that natural polynucleotides have a mutagenic influence on the cell genome [1]. For example, while treating cells with transforming DNA carrying a mutant gene, chromosomal DNA becomes mutant for the same gene [2]. The method of 'marker rescue' used in the mapping of viral genes is also based on the mutagenic effect of DNA fragments [3]. It has been shown previously that polynucleotides of several hundred nucleotides and more in length have a noticeable mutagenic potential [3,4]. Mutagenesis has a low efficiency (not greater than 0.1%) if short synthetic oligodeoxy-ribonucleotides in vivo are used [4,5]. To increase the yield of mutants and mutagenesis specificity it has been necessary to work out special methods of enzymatic synthesis of oligonucleotides up to a full-length mutant copy of the starting DNA in vitro [4,6]. One of the reasons for low efficiency of oligonucleotide-directed mutagenesis in vivo is the sensitivity of short synthetic oligodeoxyribonucleotides to cell nucleases [4]. For the first time the present paper shows the possibility of high-effective induction of in vivo directed mutations in the genes through cotransfection of bacterial cells by the mixture of DNA and phosphotriester analogues of oligonucleotides [7] which serve as mutagenic primers, but which are proofed against destruction by nucleases.

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2. MATERIALS AND METHODS

Single-stranded DNA of M13pmB phage [8], *E. coli* strain JM103 [9], were used. Oligonucleotides, their synthesis and properties have been described previously [7] in detail.

Oligonucleotides were phosphorylated for mutagenesis as described in [6]. Mutagenesis has been carried out in the following way: 20 pmol of oligonucleotides (5′-phosphorylated or without 5′-terminal phosphate group) were added to 1 pmol of the M13mpB phage DNA in 10 μl of the solution containing 0.02 M Tris-HCl (pH 7.5), 0.05 M NaCl, 0.01 M $MgCl_2$, 1 mM dithiothreitol. The mixture was kept at 20–23°C for 15–45 min. An aliquot of the mixture was used for transforming competent cells of *E. coli* JM103. Transformation conditions and preparation of single-stranded phage DNA for structural analysis have been described elsewhere [10]. Mutants DNAs were sequenced by the dideoxychain-termination method [11]. A 13-mer [12] was used as primer for DNA sequence analyses.

3. RESULTS AND DISCUSSION

We have previously shown that phosphotriester analogues were more effective mutagens when compared to phosphodiester oligonucleotides in oligonucleotide-directed in vitro mutagenesis experiments [7]. Phosphotriester links protect the mismatched area from the 5′-3′ and 3′-5′ ex-

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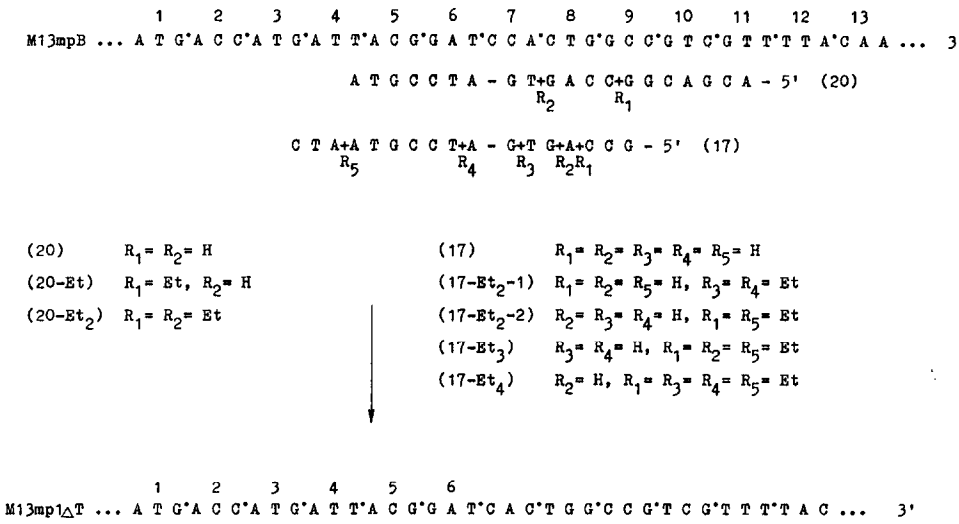

Fig.1. The structures of the mutagenic oligonucleotides and the sequence of the lacZ′ gene region coding for the amino-terminus of the α-peptide of β-galactosidase. Codons of the lacZ′ gene are marked with figures. R, substituents at phosphate groups; Et, ethyl residue.

onuclease activities of DNA polymerases [7] and probably from the repair enzymes of a cell. It was interesting for us to study the possibilities of the use of phosphotriester analogues of oligonucleotides for in vivo mutation induction.

The mutagenic activity of phosphotriester analogues has been tested in template DNA of M13mpB phage [8]. Fig.1 demonstrates the structure of the N-terminal site of the lacZ′ gene (coding for α-peptide of β-galactosidase) in M13mpB along with the structure of mutagenic oligonucleotides: 20-mer (20), its monoethyl (20-Et) and diethyl (20-Et_2) esters; 17-mer (17), its diethyl (17-Et_2-1 and 17-Et_2-2), triethyl (17-Et_3) and tetraethyl (17-Et_4) esters [7]. Oligonucleotides induce the deletion of the first or second nucleotide of the seventh codon of the Z' gene, resulting in the M13mp1 ΔT deletion mutant of a different phenotype (Lac^-) [8]. Complexes of oligo nucleotides with M13mpB phage DNA were used for transforming competent cells of *E. coli* JM103. A relative number of the mutant colonies served as a measure of efficiency of the mutagenesis induced with oligonucleotides [13]. The results of the analysis are summarized in table 1 which demonstrates that the best mutagenic properties in vivo are displayed by stereoisomers of the diethyl ester of 17-Et_2-2 and those of the triethyl ester of 17-Et_3 of the similar structure. As the mutagenic activities of these analogues are almost identical in vitro [7] and in vivo tests, we conclude that the main property defining the efficiency of mutagenesis is the presence of ethylated phosphate groups distant from the molecule ends by 3 nucleotides. The yield

Table 1

The efficiency of oligonucleotide-directed mutagenesis in vivo

Oligonucleotide[a]	The yield of mutants (%)[b] induced by	
	5′-phosphorylated oligonucleotides	Non-phosphorylated oligonucleotides
20	0.3 ± 0.1	0.1 ± 0.1
20-Et	0.5 ± 0.1	0.8 ± 0.1
20-Et_2	0.7 ± 0.1	1.0 ± 0.2
17	0.2 ± 0.1	–
17-Et_2-1	0.1 ± 0.1	–
17-Et_2-2(1)	1.4 ± 0.2	–
17-Et_2-2(2)	2.5 ± 0.5	0.7 ± 0.2
17-Et_3(1)	0.8 ± 0.1	–
17-Et_3(2)	1.9 ± 0.4	–
17-Et_3(3)	2.0 ± 0.3	–
17-Et_4	not found	–

[a] The structure is depicted in fig.1. The number of the fraction in isolating stereoisomers by reversed-phase chromatography is given in parentheses [7]

[b] Arithmetic mean and standard error according to the results of 3–4 individual experiments: 5–8 inoculations of *E. coli* JM103 cells separately transfected and characterized by different times of production were carried out in every test. Spontaneous background of Lac^- mutants is <0.1%

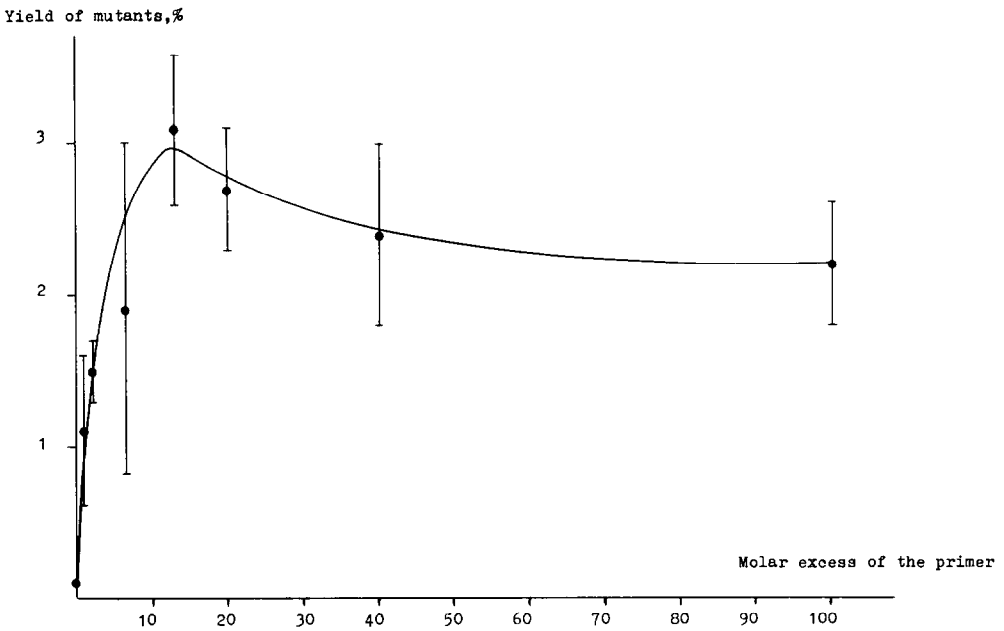

Fig.2. The dependency of the mutant yield upon the primer:template molar ratios in forming the 5′p-17-Et$_2$-2(2):M13mpB DNA complex. The diagram shows the results of 5-6 individual tests.

of mutants achieved 2-3% using these analogues which corresponds to an average value obtained in the majority of experiments on oligonucleotide-directed mutagenesis. Fig.2 demonstrates that a 10-13-fold excess of primer in reference to the template DNA is sufficient for effective mutagenesis in the conditions suggested.

With the 5′-phosphorylated analogue 17-Et$_2$-2(2) we have obtained mutants by adding the oligonucleotide and M13mpB phage DNA to *E. coli* JM103 competent cells without preforming the DNA: primer complex in vitro. The yield of mutants is 1.2 ± 0.3% (data of 5 individual tests).

To prove specificity of mutagenesis we have selected mutant phage clones in each test and determined the primary structure of their DNA in the linkage site of oligonucleotides by the method of Sanger [11]. The majority of tests have shown the mutation determined by the structure of oligonucleotides (deletion C). Only the 17-Et$_2$-1 analogue resulted in the mutants containing the unplanned deletion of 262 nucleotides in length including the lacI gene end, regulatory region and a part of the structural region of the lacZ′ gene. The accurate structure of the DNA of the unplanned mutants and the description of the possible mechanism of their formation will be published later.

Thus we have proposed a new solution to the problem of site-localized mutagenesis. It is based on one of the methods of synthetic organic chemistry and presupposes the incorporation of protecting groups into the oligonucleotide-mutagene in order to provide stability against destructive agents such as cell nucleases. The present paper shows that the use of synthetic analogues of oligonucleotides provides a new opportunity for carrying out mutagenesis with genetic material of a vital cell.

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