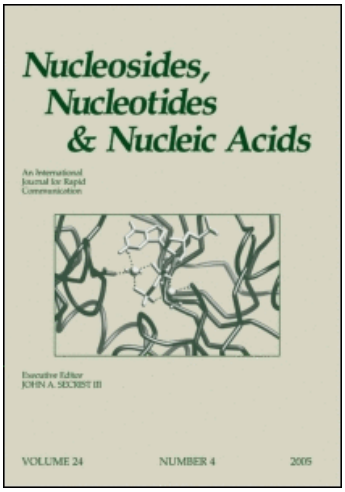

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A New Approach to Revealing Point Mutations in DNA Analyzed by Colorimetric Detection

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ABSTRACT

A new approach to detection of point mutations in an amplified DNA was developed. The approach is based on highly selective ligation (T4 DNA ligase) of a tandem of short oligonucleotides one of which contains the biotin group. The ligation product is formed only when the hybridization complex DNA/tandem is formed and the tandem is perfect. The hybridization complex DNA/(biotinylated ligation product) was separated from the biotinylated component of the tandem by UV-immobilization of the reaction mixture on a nylon membrane. The immobilized hybridization complex was detected colorimetrically by a streptavidin-alkaline phosphatase cojugate with a chromogenic substrate.

Key Words: T4 DNA ligase; Ligation; Hybridization; High-specific probe; Colorimetric detection; Point mutation; HIV; ΔF508.

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INTRODUCTION

The nucleic acids ability to form complementary complexes with oligonucleotide probes underlies many methods of DNA diagnostics.[1,2] Among them various hybridization methods play an important role. Hybridization is monophasic when carried out in solution and heterophasic when a DNA sample (direct hybridization) or a probe (reverse hybridization) is attached to a solid support. The advantage of heterophasic hybridization is the possibility to fix the hybridization complexes only on the phase and eliminate other components. The hybridization in solution is faster than on solid phase but for detection the hybridization product should be separated from the free probes or analyzed nucleic acids.

Hybridization methods usually apply relatively long probes which are able to strongly bind with DNA. The binding of these probes is selective only under certain conditions and moreover, efficient single mismatch discrimination can be achieved not for every nucleotide sequence (for example, in Ref. [3]).

Reliability of the results with the use of oligonucleotide probes may be improved via additional reactions with participation of specific enzymes sensitive to mismatches in the complexes of oligonucleotide and DNA template.[4–7] As shown in our previous work[8] formation of a «wrong» product may be excluded when three short oligonucleotides in tandem: octamer-tetramer-octamer are ligated on a DNA template when a mismatch is located in the complex of the central tetranucleotide.

In this work we used a the approach of colorimetric detection of such high specificity complexes of DNA with probe by UV-immobilization on nylon.

MATERIALS AND METHODS

Amplification of DNA

Amplification of DNA fragments was carried out with total DNA isolated either from cell cultures infected with the corresponding strains (HIV-1 strains bru and rf) or from blood of patients (CFTR). The reaction mixture (50 μl) contained primers (20 pmol each), 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 5 mM $MgCl_2$, 0.1% Tween-20, 0.2 mM each of the four dNTPs, and 1.5 units of Taq DNA polymerase. The 135-bp and 180-bp DNA fragments of HIV-1 strains bru and rf correspondingly were obtained as described previously.[9] DNA fragments of CFTR gene 230, 227 bp and 405, 402 bp were obtained using pairs of primers TGCCTGGCACCATTAAAG, GGGTAAGCTACTGTGAATGGAT and TGCCTGGCACCATTAAAG, CAATGGT-TATTTATATGGCAGC appropriately.

Oligonucleotide Ligation

The standard reaction mixture (15 μl) contained 10^{-5} M tetranucleotide, 10^{-7} (unless otherwise specified) biotinylated octanucleotide, template DNA ($\sim 10^{-7}$ M amplification PCR fragment denatured by heating for 5 min at 100°C with tandem),

25 units of T4 DNA ligase, 10 mM $MgCl_2$ 0.1 M NaCl, 10 mM DTT, 1 mM ATP, 20 mM Tris-HCl (pH 7.5). Ligation was carried out at 25°C for 30 min.

UV-Immobilization and Visualization

The ligation mixture was applied on a nylon membrane (Hiju Kalur, Estonia). Membranes were UV-irradiated with two low-pressure Hg lamps (EDB-30, distance 17 cm), washed twice with buffer APT-7.5 (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 0.5% Tween-20) for 10 min, and treated with Stv-AP [1 mg in 1 ml of buffer AP-7.5 (APT-7.5 without Tween-20)] at 60 ml/cm^2 for 30 min at 22–25°C. Excessive Stv-AP was removed. The membranes were washed thrice with buffer APT-7.5 for 5 min and once with buffer AP-9.5 (AP-7.5 with pH 9.5) for 5 min, and treated with chromogenic substrates [1 ml of buffer AP-9.5 + 4 ml of 75 mg/ml NBT in 70% DMFA (aqueous solution) + 8 ml of 50 mg/ml BCIP in 50% DMFA (aqueous solution)] at 60 ml/cm^2. The ''development'' reaction was carried out for 10–20 min and stopped by washing the membrane with distilled water. The staining intensity was registered with a scanner, the data obtained were processed using the Gel-Pro software to estimate the integral optical density (IOD).

RESULTS AND DISCUSSION

Our approach to reliable detection of analyzed DNA is based on colorimetric detection of its hybridization complexes with a high-specificity oligonucleotide probe

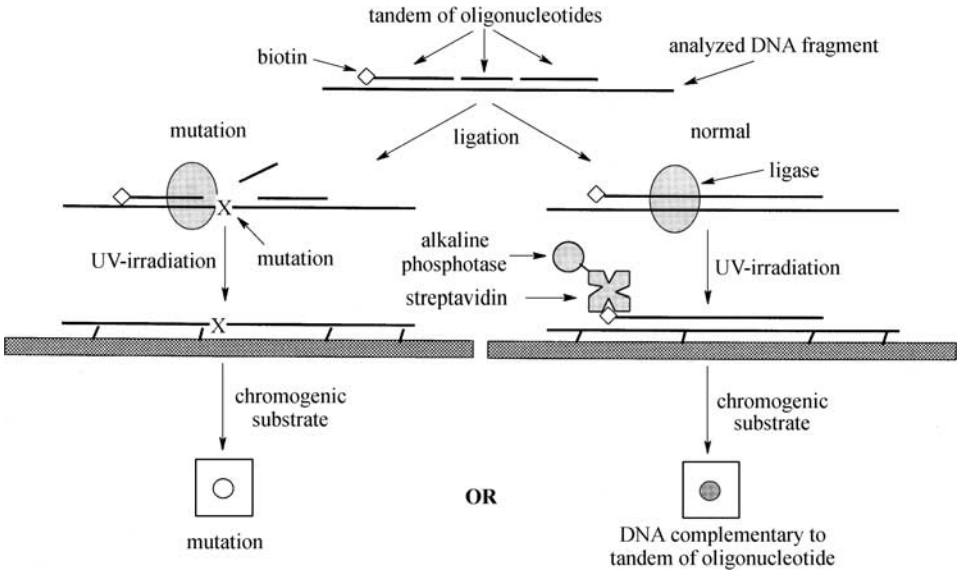

Figure 1. General scheme of the method for mutation revealing.

obtained by template ligation in solution of three short oligonucleotides one of which contains a biotin residue. To separate two biotinylated oligonucleotides: product of ligation and component of tandem we used the method of immobilization of long molecules of DNA on nylon under UV-irradiation.[10] We assumed that the long template DNA can be preferably immobilized due to its higher molecular weight and can retain the product of ligation on the membrane by strong complexation whereas the biotinylated component of the tandem forming a much less stable complex and can be easily removed by washing.

The treatment of membrane containing dots with biotinylated hybridization complexes by conjugate streptavidin-alkaline phosphatase and chromogenic substrate results in the coloration of the corresponding dots (Fig. 1).

PCR fragments of HIV-I genome (fr-135) and also normal (fr-230, fr-405) and ΔF508 mutant (fr-227, fr-402) human CFTR gene were used as a DNA template. For each analyzed sequence of the DNA-fragments corresponding tandems of three short oligonucleotides I, II, and III were designed (Fig. 2).

First we selected the optimal concentration of (Bio)pN_8 for prevention of non-specific signal, the intensity of which is dependent on the extent of immobilization of this component. For this purpose we compared the coloration intensity in dots containing mixtures of DNA and tandem after incubation with ligase and without it (Fig. 3) using different concentration of the biotinylated oligomer (Bio)pN_8. The optimal ratio between specific and nonspecific signal was observed with 10^{-7} M concentration of biotinylated component of the tandem.

The level of UV-immobilization of DNA depends on its length but for short oligonucleotides the nucleotide composition plays an increasingly important role.[11] Therefore the influence of nucleotide composition of biotinylated oligomer on the level of nonspecific signal was investigated using 3′- and 5′-biotinylated components of

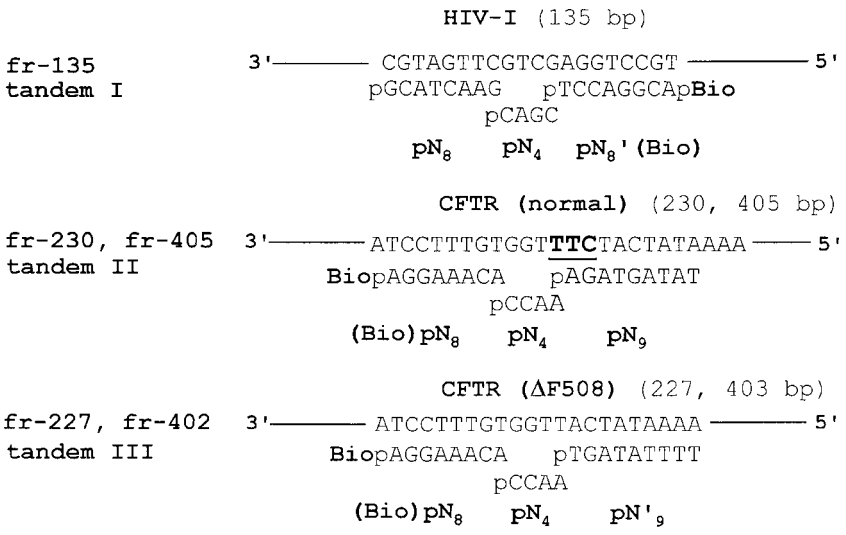

Figure 2. Tandems of oligonucleotides complementary to DNA fragments: HIV-I 135 (I), CFTR gene normal (II) and deletion (III).

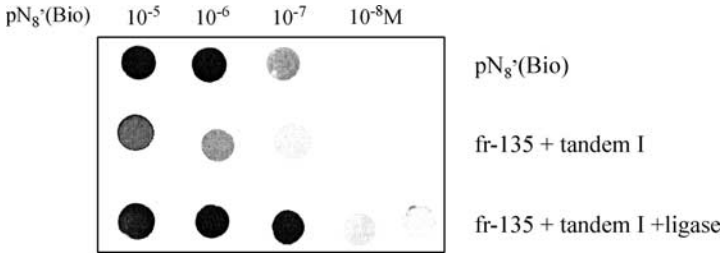

Figure 3. Optimization of pN_8'(Bio) concentration for specific detection of the tandem I ligated on the DNA template fr-135.

tandems II and III The aqueous solutions of these oligonucleotides with concentrations from 10^{-6} to 10^{-8} M were spotted on nylon. After UV-irradiation of membrane and color developing of dots it was found that efficiency of immobilization of the oligonucleotides is different (Fig. 4). As the oligonucleotides have similar length one can suppose that the presence of thymidines in the sequence increases the efficiency of UV-immobilization.

Thus designing the tandems it is necessary to consider the nucleotide composition of the biotinylated oligonucleotide because the level of its immobilization predetermines the intensity of nonspecific signal.

It was shown previously that the ligation of the tandem of three short oligonucleotides allows one to reveal point mutations in DNA.[8,9] The possibility to detect a point mutation using this approach was investigated on an example of the detection of three nucleotides deletion ΔF508. We used DNA fragments (fr-405 and fr-230) of the normal gene CRTF and the corresponding DNA fragments (fr-402 and fr-227) of the gene containing the deletion. The revealing of the deletion in DNA fr-227 and fr-402 was carried out by ligation of the tandem I complementary to the sequences M23 (deletion) and the tandem II complementary to the sequences M26 (normal). For comparison we analyzed fr-405 and fr-230 of the normal gene by ligation of these tandems. Figure 5 shows that the coloration occurred only in the dots where the ligated tandems were complementary to the DNA-fragments. The results obtained indicate that PCR fragments of various lengths can be used for analysis.

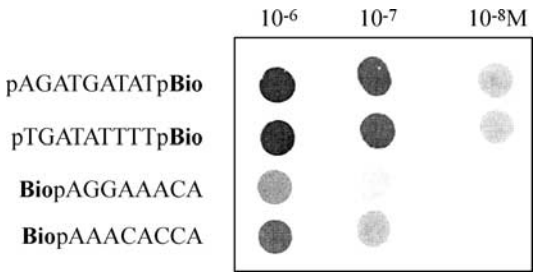

Figure 4. Influence of the nucleotide composition of biotinylated probes on the intensity of nonspecific colorimetric signal. In the dots of membrane: spotted biotinylated components of tandems II and III (aqueous solutions, 10^{-6}–10^{-8} M) of after UV-irradiation and revealing.

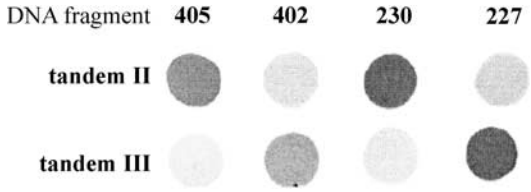

Figure 5. Discrimination of CFTR DNA-fragments (N, ΔF508). Nylon membrane after revealing spotted dots of the mixture after ligation of tandem II or III on PCR fragments fr-405, 230, 402 and 227.

Thus three nucleotide deletion and insertion can be reliably detected by the developed approach. The single nucleotide substitution in the DNA sequence is more difficult for detection among point mutations. The sensitivity of our method to single nucleotide substitution was assayed with the use of fr-135 *bru* fragment of HIV-1. The tandem 1 with tetranucleotide pCAGC was complementary to DNA template. The other 12 tandems contained tetranucleotides with any possible single-base substitutions (Fig. 6). Thus, we ligated 13 systems, including 12 with mismatches between the tetranucleotide and the template.

The coloration occurs only in the case of correct complementation of tetramer to DNA-fragment. In the other cases the colorimetric signal is much lower (Fig. 7).

The developed method was used for distinguishing the amplified DNA fragments of two HIV-I strains (*bru* and *rf*). Single-base substitution was located opposite to octamer in the site of ligation. Even in this case no coloration is observed when a non-complementary tandem was used.

Thus, our approach allows one to detect a single nucleotide substitution, deletion, or insertion in DNA fragments.

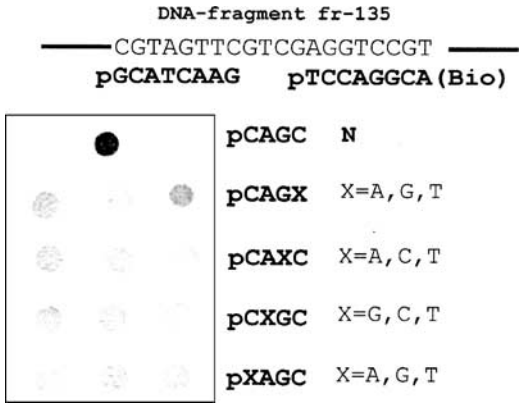

Figure 6. Discrimination of a single base substitution in the binding site of the tetranucleotide. Membrane after UV-irradiation and revealing of ligation mixture of DNA fr-135 and tandem I (13 tandems containing different tetramers).

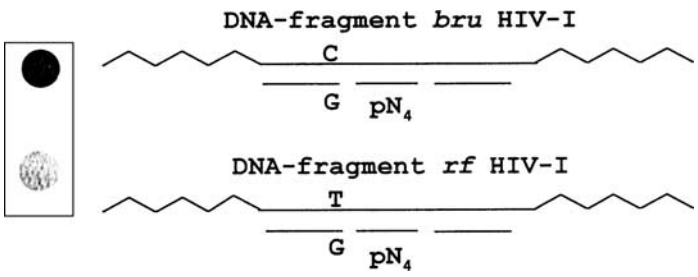

Figure 7. Discrimination of PCR-fragments of bru and rf HIV I strains. Membrane after UV-irradiation and revealing of ligation mixture.

This method does not require any expensive reagents or equipment. The approach can be used for developing test-systems for medical diagnostics of human genetic diseases, identifying strains of microorganisms, and for research in population genetics.

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