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Modified oligonucleotides as a tool for DNA sequencing, fingerprinting and mapping

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Abstract

The synthesis of modified oligonucleotides bearing 5-methylcytosine and 2-aminoadenine instead of natural counterparts, which are characterised by enhanced strength of complementary DNA binding (strong binding oligonucleotides, SBO) has been developed. Advantages at SBO applications are described as hybridisation probes, probes for physical mapping, primers for DNA sequence and PCR amplification.

The progress, achieved over the last 15 years in the field of chemistry of oligonucleotide synthesis, resulted in appearance of great variety of techniques using the synthetic oligonucleotides in different areas of molecular biology. All the techniques depend on formation of duplexes "oligonucleotide-complementary DNA", the stability of which has a considerable effect on efficiency of such methods. Stability of the complementary complexes could be increased as a result of modification of the heterocyclic bases by means of changing cytosine and adenine by 5-methylcytosine and 2-aminoadenine (2,6-diaminopurine) respectively (1). It is known that stability of diaminopurine-thymidine pair becomes approximately equal that of the G-C pair due to appearance of new hydrogen bond (2). The increase of stability in case of 5-substituted pyrimidines is explained by hydrophobic interaction of methyl or others hydrophobic groups (bromine, propinyl) in the large groove of DNA helix (3).

Fig. 1 represents the schemes of synthesis of modified amidite components for oligonucleotide synthesis:

a) initial 5-DMTrT is converted to 5-methyl-5'-DMTr-cytidine by standard procedure via 4-substituted triazolide intermediate. The further blocking of aminogroup by benzoyl chloride and phosphitilation of 3'-hydroxy group by β-cyanoethyl diisopropylamino phosphochloridite result to 5-MeC amidite.

b) synthesis of 2,6-diaminopurine monomer appeared to be more complicated
and time consuming. The yield on the stage of dG conversion to diaminopurine does not exceed 50%, and there are some problems with blocking both aminogroups due to high acid lability of this nucleoside. The best result was achieved with dimethylacetamidine protection group. The structure of both monomers was confirmed by NMR spectroscopy. Solid phase oligonucleotide synthesis was carried out by standard amidite technique using the ASM-102 automatic synthesizer

Yields of condensation were more that 97%. After deprotection by 50% alcoholic solution of aminoethanol at 70°C for 1 hour the oligonucleotides were purified by denaturating polyacrylamide gel electrophoresis or HPLC.

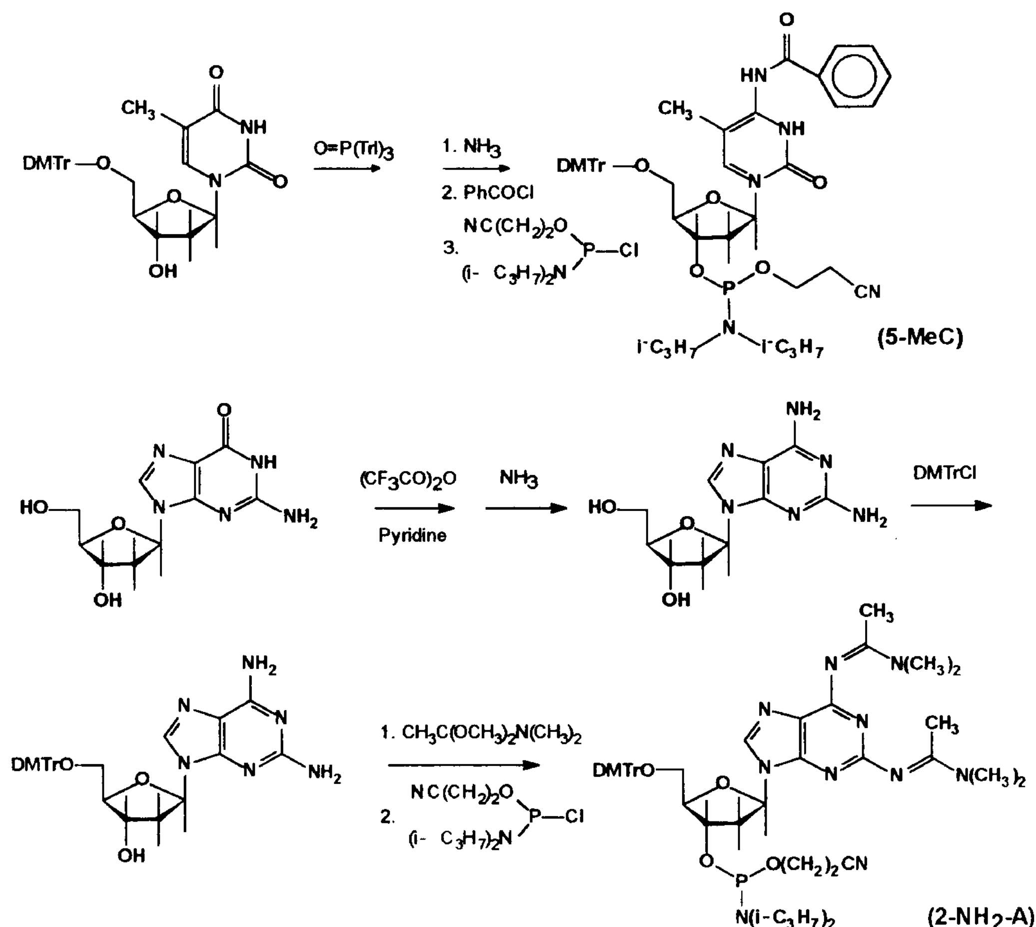

Fig. 1. Scheme of the synthesis of modified components for oligonucleotide synthesis

Measurements of melting temperatures of 17-mers with natural, C-modified and fully modified structures showed that the stability of duplexes increased from 68°C to 78°C in accordance with the approximate formulae:

Tm(modified oligo) = Tm(nonmodified oligo) + n,
where n = number of modified C and/or A

Encouraging results were obtained by with SBOs as hybridization probes in DNA fingerprinting (4). DNA fingerprinting is being used now in many different fields, such as population genetics, evolutionary biology and forensic and legal medicine, but application of the method is restricted by the amount of DNA samples available. Therefore the sensitivity of hybridization analysis in DNA fingerprinting is very important. Fig. 2 presents the Southern blot hybridization analysis of human DNA, isolated from 8 unrelated individuals, with modified and natural 15-mer $(CAC)_5$ probes. Hybridization analysis in the case of SBO is much more sensitive (more than 10 fold); the exposure time can be considerable shortened. The quantity of the DNA detectable by the modified probe can be as low as 0.5 microgram per lane. It is very important that the stronger binding of modified oligonucleotide does not affect the specificity of binding. The SBO can be especially useful when stability of duplex formed by oligos made of standard building blocks is not sufficient for the analysis.

Nevertheless the question remained whether the modified oligos could prime DNA synthesis and if so: a) what is the minimal length of oligos necessary for the efficient priming; b) what is their priming capacity in comparison to natural counterparts; c) whether they retain the specificity of priming.

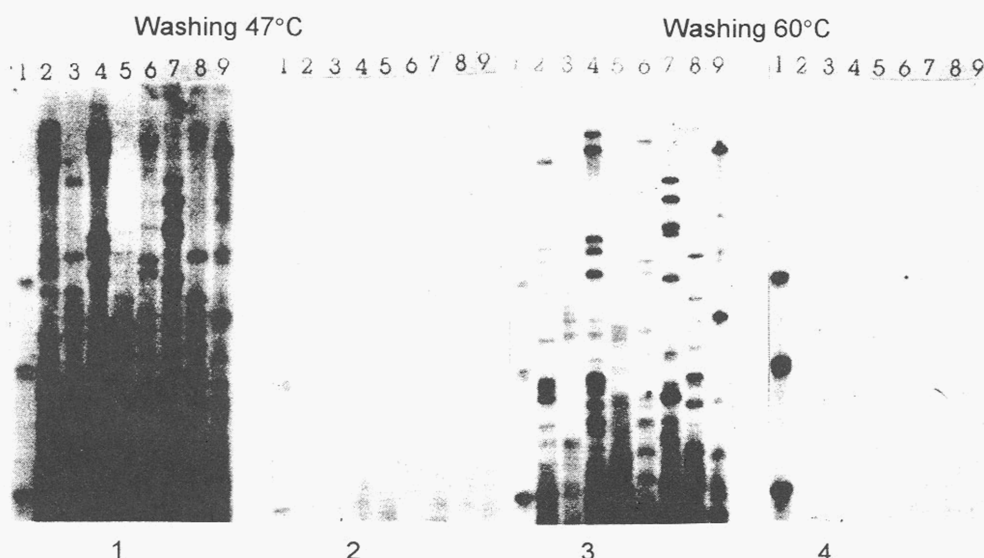

Fig. 2 Southern blot hybridisation analysis of human genomic DNA isolated from 8 unrelated individuals with modified (1,3 patterns) and nonmodified (2,4 patterns) $(CAC)_5$

To answer these questions we measured the efficiency of DNA synthesis primed by modified and natural penta- and hexanucleotides (5). Analysis of $\alpha[^{32}P]$-dATP incorporation into the newly synthesised chain (Fig. 3) allows us to conclude:

1. Pentanucleotides, either modified or natural, are rather inefficient as primers.
2. Hexanucleotides show rather good priming efficiency, but modified ones are considerably more active as primers.

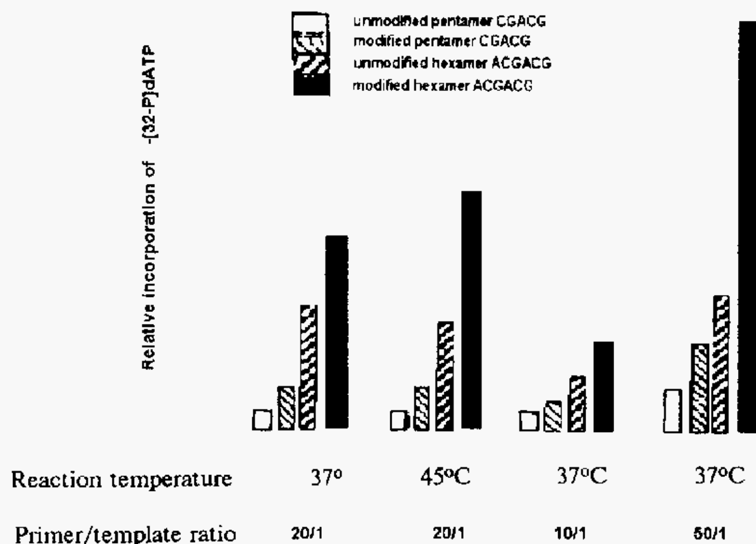

Fig. 3 Comparison of DNA synthesis primed by penta- and hexamers.

These results allowed us to apply the modified oligos to DNA sequencing with the use of short contiguous primers (Fig. 4a). This method was developed by W.Studier (6) and W.Szybalski (7) groups and based on primer walking sequencing with primers, composed of several short oligos. Oligos for such composite primers could be simply

chosen from a premade library and, consequently, the procedure could be robotized. However the method works only in 60-70% cases and the lowest length of such primers was demonstrated to be six bases (4096 components in a library) (6).

We also demonstrated that in several cases when hexamers of natural structure failed to prime DNA synthesis, SBO hexamers appeared to work. We tested this approach both with the model templates (DNA of M13 phage in single and double stranded forms) and by sequencing of unknown DNAs.

Earlier we had shown that sequences primed by the universal M13 sequencing modified 17mer were specific and even more intensive (8). Using SBOs as components of the composite primers we succeeded in reducing the lengths of the short constituents to five-mers which lead to reduction of a premade library to 1024 components (1). We compared different combinations of SBO and unmodified blocks as the composite primers and demonstrated that strenght of binding of the oligonucleotide in the 3'-outermost position is the most crucial for the quality of sequencing patterns.

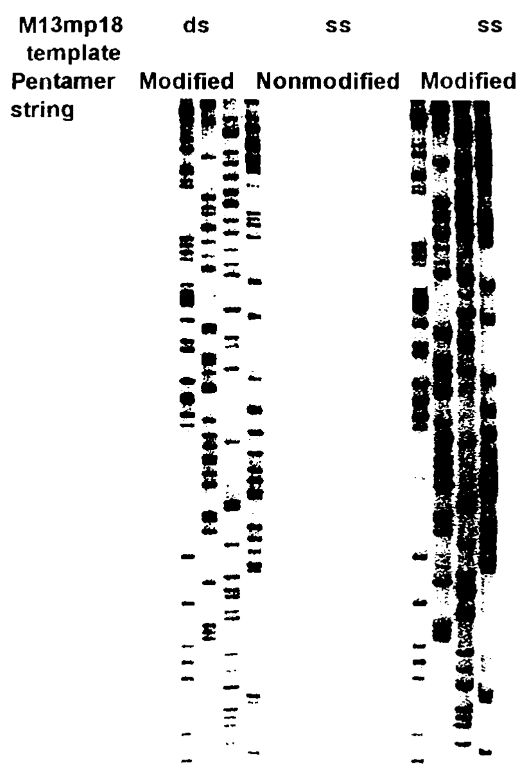

Fig. 4a
Priming by contiguous pentamer string sequencing
AAAAC/GACGG/CCAGT

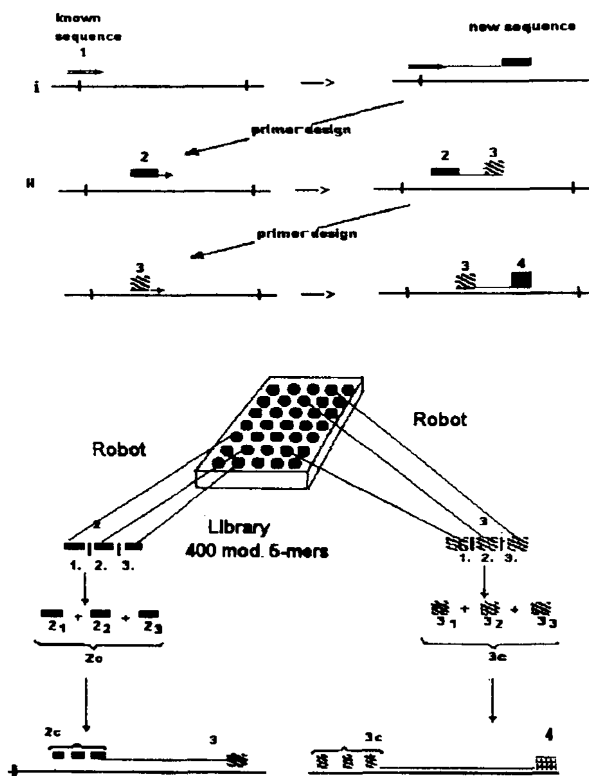

Fig. 4b
Strategy of primer walking

Another area demonstrating the advantages of SBO primers is PCR amplification. Primers with higher affinity to a complementary template allow to increase the yield of amplification products with the use of the same number of cycles as in the case of standard primers (Fig. 5a), or to reduce amount of the template; such primers should allow PCR amplification at higher annealing temperatures (Fig. 5b) or help to overcome the effect of primer binding suppression due to hairpin structures formation (Fig. 5c).

SBO application for physical mapping of DNA also seems very promising. The development of physical techniques for direct visualisation of complexes of oligonucleotides with the complementary DNA, such as

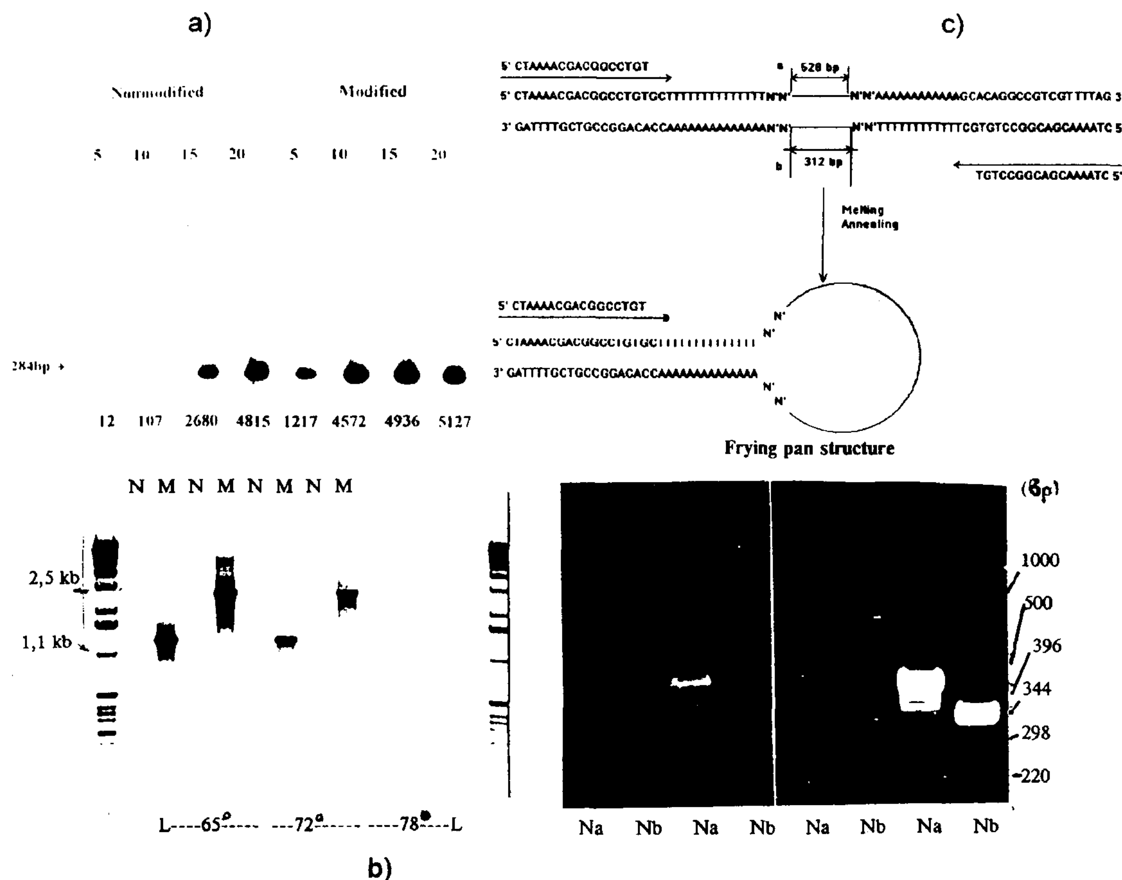

Fig 5 The application of SBO for PCR amplification
a) Influence of primer modification on the rate of PCR product accumulation
b)Temperature dependence of PCR amplification
c) PCR amplification of DNA fragments with secondary structure

electron microscopy or scanning tunneling or atomic force microscopes seems to open new opportunities for DNA physical mapping. To be valuable for comparative purposes, oligonucleotides used for DNA marking should be short enough to occur frequently and form a multicomponent pattern of the distribution in the DNAs under comparison. The oligonucleotides 4-6 residues long could meet all these requirements, however the standard procedure is completely unsuitable for sample preparation. To overcome this problem we propose a new method of localisation of hexanucleotide sequences on DNA template, which allows one to obtain the pattern of their distribution by means of routine procedures of electron microscopy (6).

The technique makes use of the specific initiation of the DNA polymerase-synthesis by short oligonucleotide primers and the visualisation of the ligands included in the newly synthesised DNA in the course of the synthesis (Fig. 6a). The examples of distributions of label positions on DNA samples and their computer development are given on Fig. 6b.

Patterns obtained in such a way with different oligomers and templates can be used for comparison of DNAs structures. The resolution can be improved by means of the synthesised DNA length and size of the electron label reduction. However resolution better than 50-100 residues with the classic electron microscopy can not be obtained. But there is a possibility to change dramatically the situation by development of new techniques, such as scanning tunneling or atomic force microscopy having atomic resolution.

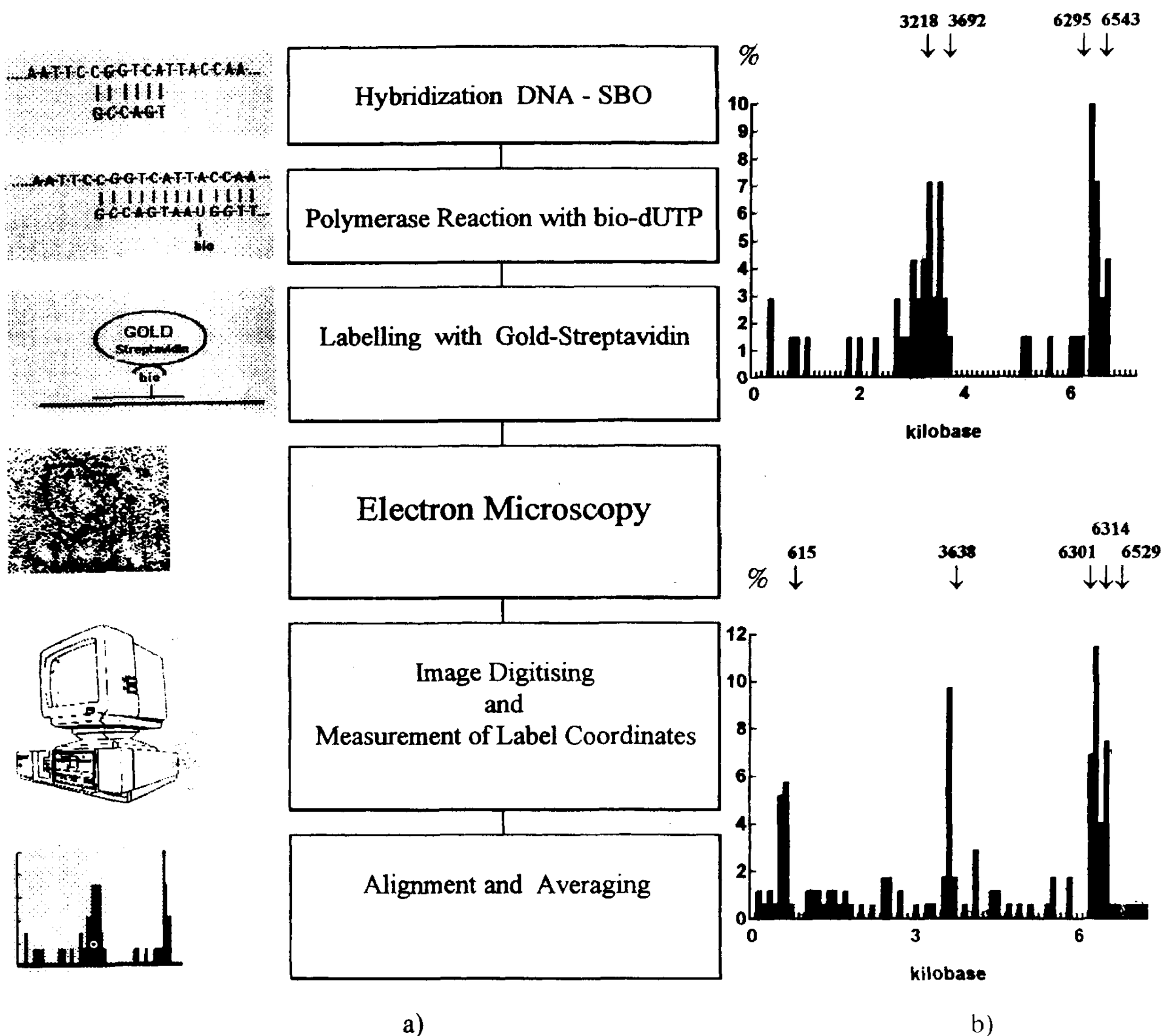

Fig. 6. Application of SBO for DNA physical mapping
a) The scheme demonstrating the principle of the technique
b) Histograms of the gold particles distribution along M13mp1,8 ssDNA hybridised with GCCAGT (top) and ACGACG (bottom) SBO

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