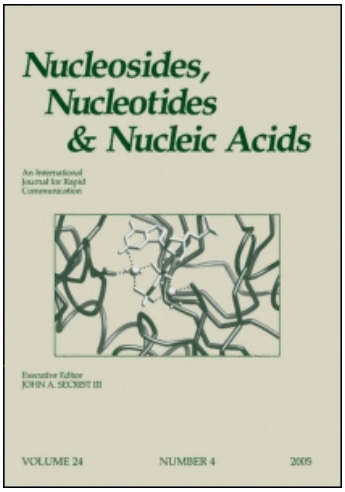

Nucleosides, Nucleotides and Nucleic Acids

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Nucleic Acids Probes in Diagnostic

Robert Teoule[a]; Hervé Bazins[b]; Brigitte Fouqué[b]; André Roget[b]; Sylvie Sauvaigo[b]
[a] Département de Recherche Fondamentale, Laboratoires de Chimie, Grenoble, FRANCE [b] CIS bio international, Laboratoire des Sondes Moléculaires Centre d'Etudes Nucléaires, Grenoble, FRANCE

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NUCLEIC ACIDS PROBES IN DIAGNOSTIC

Robert Teoule*
*Département de Recherche Fondamentale, Laboratoires de Chimie
Hervé Bazin+, Brigitte Fouqué+, André Roget+, Sylvie Sauvaigo+
+CIS bio international, Laboratoire des Sondes Moléculaires
Centre d'Etudes Nucléaires, BP 85X, 38041, Grenoble, FRANCE

ABSTRACT Some aspects concerning the development of nucleic acids probes in diagnostic are reviewed. The influence of base sequence, probe concentration and length on the melting temperature of DNA duplexes is discussed. Multiple probe labeling can be performed on a DNA synthesizer using modified nucleoside phosphoramidites bearing the reporter molecule. These probes have been successfully applied to the histological detection of a variety of neuropeptide mRNAs and neuropeptide receptors mRNAs. An hybridization format using PCR and solid support fixation of the amplified probe has been used for the typing of human papilloma viruses.

INTRODUCTION

The understanding of many aspects of human pathology has been revolutionized by advances in molecular biology. The genome of the living organisms contains a wealth of information and the identification of specific sequences contributes to the fundamental understanding of the etiology of diseases (for review see 1-10). The use of DNA probe techniques has expanded to many areas of medecine such as genetic diseases and predisposition, virology, parasitology, cancerology, tissue typing, epidemiology and forensic science.

The technique is based on nucleic acids hybridization which provides a convenient method for the detection and measurement of a defined oligonucleotide sequence in a mixture of heterologous sequences. Currently one of the most popular methods in laboratory research is dot blot hybridization. The main steps of the process are: a) denaturation of the DNA provided by the biological sample to be analysed; b) fixation of the single stranded DNA on a support to avoid renaturation; c) hybridization with the labeled probe d) washing of excess non hybridized probe and counting of the

radioactivity attached to the support. There are other non radioactive detection systems and hybridization procedures such as *in situ* hybridization with messenger RNAs on cells or tissue sections to determine gene expression, hybridization with chromosomal DNA for cytogenetic studies, sandwich hybridization, hybridization in solution with subsequent separation on hydroxyapatite, Northern and Southern blots.

However, technology suitable for research is not well adapted for large scale routine clinical applications and in commercial competition DNA probes suffer with monoclonal antibodies. There is a need to improve the ease of use, speed, reproducibility and sensitivity.

HYBRIDIZATION

To predict the melting temperature of oligonucleotides two empirical formula are often used. The very simple approximation Tm(°C)= 2(A/T)+4(A/G) 1, where G/C and A/T indicate the number of base pairs, is frequently used as a guide for short oligodeoxyribonucleotides of length between 10 and 20 nucleotides in 1.0 M Na^+ buffer.

However, the stability of the nucleic acid duplexes depends on several factors including the length of the double helix, base composition, ionic strength, temperature, presence of mismatched bases and denaturing agents. Consequently, the following empirical formula gives a better approximation:

Tm (°C)= 0.41(% G+C) + 16.6logM + 81.5 - 500/nt - 0.61(%F) - 1.2D 2

where Tm is the melting temperature, % G+C the percentage of G and C in the probe, M the monovalent ion concentration, nt the number of nucleotides in the probe, F the percentage of formamide in the buffer and D the mismatch percentage.

The equation holds between the limits $0.05M < Na^+ < 0.5M$ when the formamide is below 40% . Formamide facilitates DNA denaturation and it is commonly employed for *in situ* hybridization.

The empirical formula does not take into account the influence of base sequence and probe concentration. In water, nucleic acids duplex stability is mainly due to base stacking interactions and thus is related to base sequence. Interaction energies can be estimated as the sum of the energies of contributing complementary dinucleotide fragments (11). Breslauer et al (12) have predicted DNA duplex stability and melting behaviour from the base sequence and reported the complete thermodynamic library of the ten Watson-Crick DNA nearest-neighbor interactions.

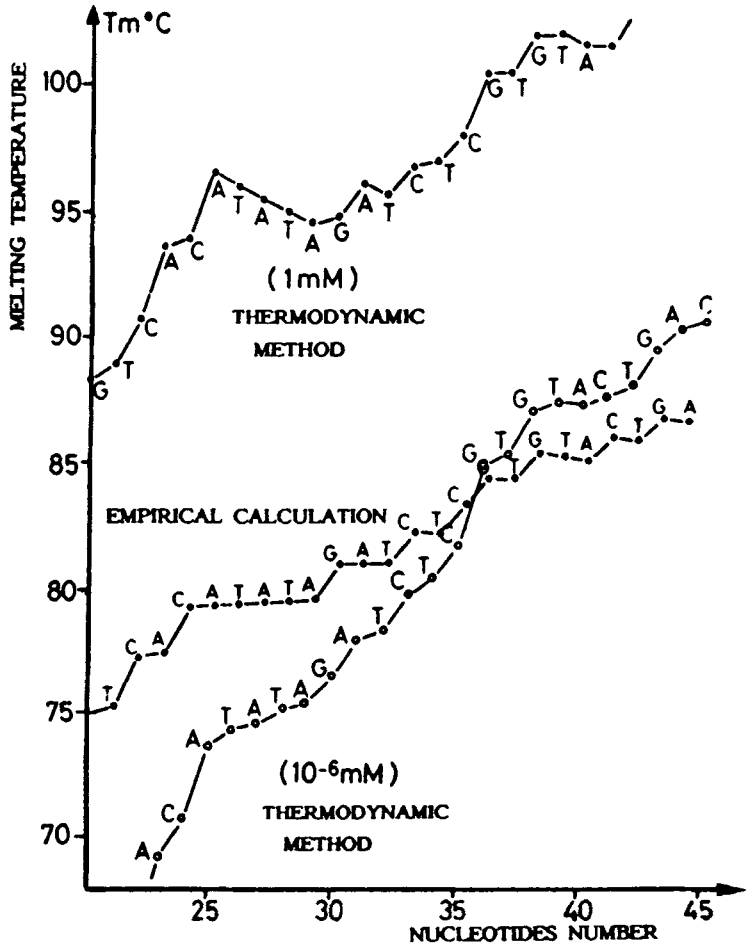

FIG. Melting Temperatures (pH 7, 1M Na^+) calculated for DNA probes of increasing lengths (5'------> 3') corresponding to the structures: d(AGTCATGATGTGACACTCTGTCACATATAGATCTCGTGTACTGACTGA CTGACTGACTGACATATGTCGCATCA). Calculations were performed by the empirical method 2 and the nearest-neighbor method (concentrations 1mM and 10^{-6}mM) for this series of oligonucleotides beginning at the same 5-'end and extending towards the 3-'side. Each oligonucleotide differs from the previous one by the addition of one nucleotide. In this figure only the Tm values for the medium sized duplexes are given.

A computer program to determine the melting temperature of oligodeoxynucleotides which differ from each other only by the addition of one base has been devised. Tm calculations obtained by the empirical method 2 and the nearest-neighbor method are given in figure for a series of oligonucleotides beginning at the 5'-end and extending towards the 3'-end. The curves calculated for concentrations of 10^{-3}M and 10^{-9}M by the thermodynamic method can be compared with that obtained with the empirical method 2. Melting Temperatures increase with oligonucleotide concentration and length. At the higher concentration (10^{-3}M) the addition of TATAG to the oligonucleotide GTCATGATGTGACACTCTGTCACA does not have a stabilizing effect and TATAG is not hybridized to the target. For the higher dilutions (10^{-9}M), melting temperatures are lower but every nucleotide of the sequence TATAGAT is hybridized.

Hybridization is usually carried out 20°C below the Tm. A difficult step involves a high stringency washing step, often for a short time under salt, formamide and temperatures close to the Tm of the desired duplex (13). Several washes of varying stringency are used to eliminate excess probe, non specifically bound probe and probe bound to partially homologous sequences in the target genome. The theory predicts that the best discrimination between correct hybridization at a fully complementary sequence in the target genome relative to hybridization at sequences not completely homologous occurs at the melting temperature. In figure inspection of the melting temperature curves at 1mM and 10^{-6}mM shows that discrimination is enhanced at low concentration.

To increase the hybridization rate it is possible to use a higher probe concentration, but, this is at the expense of discrimination. At room temperature the mismatches containing guanine (G.T,G.G,G.A) are more stable than those containing cytosine (C.A,C.C). At higher temperature pyrimidine-pyrimidine mismatches become the least stable (14).

Other computer programms based on different folding algorithms, search sequences in data bases, determine the self-complementarity of the oligonucleotide, palindromes in the sequence, and alternative (non-target) sites for the oligonucleotide. They are used as a guide to oligonucleotide sequence selection for PCR primers or hybridization probes (15).

Stability of RNA duplexes is now predicted with better accuracy using free energy parameters (16, 17). Terminal unpaired nucleotides at the end of the helixes contribute to their stability. As much as possible, in the

hybridization of DNA probes with viral, ribosomal, or messenger RNA, regions of RNA secondary structure have to be avoided (18).

LABELING

Radioisotopic labels have problems of safety, short shelf-life and waste disposal and in the recent years it has become clear that further development of nucleic acids as diagnostics tools is dependent upon their replacement with easily detected, stable, non radioactive tags. Various substances have been proposed as alternatives and most of them are derived from immunoassays technology. The ideal label should be easily attachable to the DNA probe, very sensitive, stable at the temperature required for the hybridization and able to produce a signal modulated by the hybridization, allowing the development of non separation assays. None of the direct labels fulfill all the criteria discussed above. The direct labeling of the probe with enzymes used in immunoassays such as alkaline phosphatase or horseradish peroxidase has proved difficult to realise due to low sensitivity or enzyme inactivation. The most widely used non radioactive labeling method involves biotinylated nucleotides. Biotin is a vitamin which has the property to bind with a high affinity constant to avidin ($k=10^{-15}$). Avidin (or streptavidin) is frequently linked to alkaline phosphatase or horseradish peroxidase. Haptenes detected with specific antibodies are used although they have a lower affinity constant ($k=10^{-10}$).

To improve the detection limit various methods have been proposed such as multiple labeling or labels providing direct or indirect signal amplification (enzymes). For example, multiple biotins can be attached along the chain when the probe is obtained by copying a nucleic matrix using biotinylated dUTP or dinitrophenyl dATP derivatives with DNA polymerases or reverse transcriptase (19, 20, 21). About one biotin per ten nucleotides can be inserted. Large DNA fragments can also be tagged by a photoactivable derivative of biotin (22) or by the bisulfite catalysed transamination of cytosine to insert fluorescent (23) or biotinylated (24) probes. However these methods do not work with short oligonucleotides. Non radioactive labeling of the 5'-end of an oligonucleotide can be performed in different ways (25-30). The use of nucleoside phosphoramidite or phosphonate derivatives bearing a N-protected aminoalkyl group on the base has been investigated (31-35). After deprotection and purification of the oligonucleotide, the amino group is reacted with a functionalized label. Note

that all these methods need an additional step to introduce the reporter group.

Labeled deoxynucleoside phosphoramidites bearing haptenes, biotin, fluorescent or others tags which can be efficiently and routinely used to prepare mono or polylabeled oligonucleotides directly on a DNA synthesizer have been developed (36). These modified phosphoramidites are assembled by the same procedure as the usual commercialized phosphoramidites. Insertion of a non radioactive label in the oligonucleotide chain can decrease the stability of the duplex and first labeling was intoduced only in non Watson-Crick positions, at the C-8 of adenine and the C-5 of thymine or uracil (19, 20). However, 6-Me adenine and 4-Me cytosine are natural bases and hybridize with thymine and guanine, respectively. The melting curves of oligonucleotides bearing 6-methyladenine or 4-methycytosine determined by ^{1}H-NMR experiments show that the duplex formation with a complementary chain depends on the position of the label in the oligonucleotide (37-39). Consequently, the best method is to insert the label(s) at the 5' or 3'-end of the oligonucleotide. It may be shown that the efficiency of the detection is proportional to the number of tags introduced provided that the spacing between the label and the oligonucleotide chain is sufficient (36). The avidin-biotin system requires a large arm between the biotin and the oligonucleotide.

AMPLIFICATION

Sensitivity is one of the major problems for routine use of non radioactive nucleic acids probes but, the situation has changed radically with polymerase chain reaction (PCR). The specificity of PCR amplification is based on two PCR primers which hybridize to opposite strands flanking the DNA fragment to be amplified. Repeated cycles of thermal denaturation, primer hybridization, and enzymatic extension result in an exponential augmentation of the selected sequence. The genetic information contained in the target sequence can be quickly amplified one million fold into large amounts of accessible and analyzable material (40). A single cell contaminated with a virus contains enough genetic material for identification. Generally purification of the DNA is not necessary. It is also possible to insert at the ends of the oligonucleotide new sequences and tags by the intermediacy of primers. These tags can be used for detection or separation of the amplified fragment.

A methodology named Amplicis simplifies the use of DNA probes in clinical environment. It exhibits a very high sensitivity, has a low background level and could be fully automatized (41). The method comprises the following steps: 1) Crude biological DNA to be assayed is submitted to a standard PCR procedure; 2) The mixture obtained is diluted at least 200 folds; 3) A small aliquote is subjected to a second amplification with two modified oligonucleotides as primers. One of the primers bears a biotin, the other a detection tag, and therefore, the duplex obtained after amplification has a biotin at one end and a detection label at the other end. To avoid the production of unfaithful copies the number of cycles in the second amplification cycles is limited ; 4) The duplex is captured on an avidin coated tube and excess primers washed off; 5) The labeled duplex remaining on the support is detected by ^{32}P or ^{125}I counting. Iodine-125 can be easily introduced to the oligonucleotide by iodination of a specific tag .

An hapten at the end of the double helix can be used as a reporter group by conjugation with an antibody coupled to an enzyme such as alkaline phosphatase or horseradish peroxidase. The enzyme action is detected as chromophore deposition or the release of colored, fluorescent, or luminescent species in solution. Generally, the direct detection of simple organic reporter groups on the oligonucleotides by fluorescence is not sufficiently sensitive.

Reports concerning the detection of viral diseases support the idea that PCR is more specific than other established detection methods. Dimerization of primers or false priming can produce unfaithful copies and, due to its extreme sensitivity, PCR is prone to contamination. To avoid false positive results strict rules must be followed and the data confirmed by independent experiments. However, the confirmation of PCR results is not always feasible. The advantage of the Amplicis technique is the possibility of automatisation which would avoid most of the contaminations due to incorrect handling of samples and reagents. The system is more sensitive than the classical dot blot hybridization assay and scarce sequences in crude DNA samples can be easily detected. The amplitude of the signal is very large (100-80000 cpm) and can be modulated by the quantity of radioactivity present in the second amplification. Other advantages of the procedure are a high percentage of fixation of the labeled duplex on the support which results in a small consumption of labeled primers and the elimination of most of the cellular contaminants which allows an easy detection with non radioactive labels.

APPLICATIONS

The use of DNA probes as a routine diagnostic reagent is now a reality and sometimes advances made in the research laboratory are rapidly transferred to the clinical environment. For example, in 1980, it was shown that a single-base-pair transversion (T-to-G) in the TATA box drastically decreased the efficiency of specific transcription of an eukaryotic gene (43). Only two years after the nucleotide sequence of a cloned human ß-globine gene from a man with $ß^+$-thalassemia major revealed a single base mutation located 32 nucleotides 5' to the mRNA cap site in the TATA box sequence (44). ß-Thalassemia which is among the most common inherited diseases may occur from either single base substitutions, deletions or insertions within or 5' to the ß-globine gene and presently over 30 mutations are detected (42).

The measurement of the myc oncogene mRNA amplification by using DNA probes bearing several biotins and synthesized with biotinylated nucleosides phosphoramidites (36) is an aid for making therapy decisions for neuroblastoma, one of the most common cancer of children (45). The Amplicis methodology has been applied to the detection and typing of human papilloma viruses which are associated with cervical cancer (41).

DNA probes bearing several biotins (36) have been used to investigate gene expression in tissue sections. The various inconveniences associated with radioactive probes, bio-hazards, autoradiography, limited histological resolution and delay in obtaining results were avoided. A great variety of polylabelled oligonucleotides complementary to peptides mRNAs, including oxytocine, vasopressine, vasopressine intron I, RD2 receptor, propiocortin, calcitonin, calcitonin gene related peptide, prodynorphin, and corticotropin releasing factor, were used for *in situ* hybridization in normal and tumoral tissues (46-50). These biotinylated oligonucleotides make it possible to anatomically investigate subcellular compartiments and the metabolism of mRNAs. For example, hybridization with D2 dopamine receptor mRNA and proenkephalin probes showed that most cells containing D2 receptor are enkephalin producing neurons (50).

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