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Effect of 5-Bromo- and 5-Methyldeoxycytosine on Duplex Stability and Discrimination of the *Not*I Octadeoxynucleotide

QUANTITATIVE MEASUREMENTS USING THIN-LAYER CHROMATOGRAPHY*

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Octadeoxynucleotides based on the recognition sequence of the restriction endonuclease *Not*I were synthesized containing unmodified nucleotides and nucleotides with methyl and bromide additions at the C5 position of the pyrimidine ring of deoxycytosine. On annealing to single-stranded DNA bearing one *Not*I site, thin-layer chromatography (TLC) of the different oligonucleotides was used quantitatively to determine differences in dissociation temperature (T_d) and binding equilibrium. Buffers used in filter hybridization experiments could be used in this TLC system. In addition, actual hybridizations were carried out to filter-bound DNA with and without a *Not*I site. The incorporation of 5-methyldeoxycytosine and 5-bromodeoxycytosine led to a significant increase in stability of homoduplex formation during hybridization, due to a shift in the binding equilibrium and an increase of the T_d, thereby improving discrimination considerably. Some implications of the results for several techniques involving oligomer hybridization are discussed.

The use of oligonucleotides as probes and primers is now a major facet of molecular biology. The basis of this is the ability of oligonucleotides to differentiate between target and non-target sequences which differ by only one base. This has applications in the study of human genetic diseases (Rabin and Dattagupta, 1987), the screening of mutations synthesized by site-directed mutagenesis (Itakura *et al.*, 1984), and the direct recovery of sequences from genomic DNA using the polymerase chain reaction (Saiki *et al.*, 1985; Lee *et al.*, 1988). Techniques for producing the discrimination of perfect matches from single base mismatches have been proposed, based on hybridization conditions (Wood *et al.*, 1985) and a consideration of the relative stability of mismatches (Nozari *et al.*, 1986). However, there are cases where the probe sequence required precludes optimization of mismatch destabilizing forces, as is the case for the *Not*I recognition sequence (5′-GCGGCCGC-3′). This is of particular interest for screening large-insert genomic libraries for *Not*I sites (Estivill and Williamson, 1987) as these may be associated with coding sequences (Bird, 1986). Additionally, subsets of genomic libraries could be defined as *Not*I linking libraries (Poustka and Lehrach, 1986) for use in rapid chromosome "walking" strategies.

The *Not*I octadeoxynucleotide presents two major problems in terms of its use as a probe. First, due to the relatively small size of this oligonucleotide, homoduplexes are not particularly stable under standard conditions resulting in weak signals on autoradiography. Second, a subset of single base mismatches will form heteroduplexes that are difficult to distinguish from homoduplexes given the latter's relative instability, leading to a large proportion of false positives. The solution to these problems would be to increase the stability of the homoduplexes without increasing that of the heteroduplexes, such that hybrids could be washed at higher stringencies whilst maintaining a scorable signal. One way to do this is via the incorporation of modified nucleotides.

It has been demonstrated that the thermal stabilities of double-stranded polynucleotides are enhanced when halogenated pyrimidines are substituted for the naturally occurring bases (Massoulié *et al.*, 1966; Howard *et al.*, 1969) due to a stacking pattern which permits intimate contact between the halogen atom and the adjacent base (Bugg *et al.*, 1971). In addition, in aqueous solution, association of 5Br[1]-nucleosides occurs to a greater extent than that of an unmodified component (Ts'o, 1968).

In this paper we investigate the effect of the 5-methyl and 5-bromo substitutions of the cytosines in *Not*I oligomers on the stability of homo- and heteroduplexes, using thin-layer chromatography (TLC) for quantitative measurements. Continuous temperature gradients perpendicular to the direction of the TLC were applied by two aluminium plates, whose ends were thermostated. A similar device has proved to be useful in the determination of enzyme activities at different temperatures (Pohl *et al.*, 1982). Since a whole temperature range is examined in a single experiment, and since different probes can be used simultaneously, the method allows a very quick and easy accumulation of data. Complementary hybridization experiments to filter-bound DNA confirmed that the incorporation of 5Me-dC and 5Br-dC greatly improves the discrimination on hybridization of this probe to DNA targets with and without *Not*I sites.

EXPERIMENTAL PROCEDURES

Labeling Probes—5Br-dCTP and 5Me-dCTP were purchased from Pharmacia LKB Biotechnology Inc. All unmodified nucleotides were from Boehringer Mannheim. Probes (5′-GCGGCCGC-3′) were generated and labeled to high specific activity by a fill-in reaction in presence of modified or unmodified dCTP and [α-^{32}P]dGTP, using the template 5′-GCGGCCGCACCGGCCGGCC-3′ and primer 5′-GGCCGGCCGGUG-3′, followed by a release of the newly synthesized

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[1] The abbreviations used are: 5Br, 5-bromo; 5Me, 5-methyl; SDS, sodium dodecyl sulfate.

DNA by uracil-DNA glycosylase (Craig *et al.*, 1989), which was kindly provided by Tomas Lindahl (meanwhile the enzyme is also commercially available from Boehringer, Mannheim, FRG). The actual cleavage, after the removal of the uracil bases, is due to the presence of DNA endonuclease IV, which produces nicks on the 5′ side of the sugar-phosphate bond (Lindahl *et al.*, 1977). The labeled species were excised from a 20% acrylamide, 8 M urea gel and eluted in water overnight.

Thin-layer Chromatography—Plasmid pJDH118 (Hoheisel, 1989), containing a unique *Not*I site in its extended pUC18 polylinker, was cut with *Hin*dIII and subsequently digested with exonuclease III as described (Hoheisel and Pohl, 1986) to generate a single-stranded tail of about 400 nucleotides bearing one copy of the *Not*I sequence. 0.94 pmol (1.8 μg) of such DNA was mixed with 2.9 fmol of *Not*I oligomer (2×10^5 cpm Cerenkov-counting; final concentration 25 pM) in 116 μl of 180 μM NaCl, heated to 90 °C, slowly cooled down to 0 °C, and stored overnight at 2 °C. Dots of 0.5 μl were spotted 4 cm from the lower end of the TLC plate (20 × 20 cm; Polygram CEL300 DEAE/HR-2/15). The coated site was covered with Saran Wrap, and the plate was then sandwiched between two aluminium plates whose ends were kept at two different temperatures by circulating the liquid of two thermostated baths through them. TLC was carried out with 6 × SSC or 8 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate) for 20 min. Plates were exposed to Kodak XAR-5 film together with spotted dilutions of labeled oligomer of known concentration. The autoradiographs were scanned (LKB Ultroscan XL) to determine the amount of radioactivity remaining at the origins.

Filter Hybridization—DNA dot filters were prepared by spotting 100 ng of either pJDH118 (2.9 kilobases containing one *Not*I site) or pTZ18R (2.9 kilobases with no *Not*I site (Mead *et al.*, 1986)) onto nylon membranes (GeneScreen Plus, Du Pont-New England Nuclear). The DNA was fixed to the membranes using the manufacturers recommended conditions. Squares of 1 cm^2 were placed in 1.5-ml microcentrifuge tubes and pre-hybridized in 0.5 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA (Church and Gilbert, 1984) for 20 min at 27 °C. The pre-hybridization buffer was removed and replaced with 400 μl of the same buffer containing 1.5×10^6 cpm/ml (22 pM) of the appropriate oligonucleotide probe. Hybridization was carried out at 27 °C overnight, after which the filters were rinsed three times in 6 × SSC, 0.1% SDS at room temperature, followed by two washes of 5 min, each at 35 °C in the same solution. The filters were blotted dry and the relative number of counts quantitated using the AMBIS Radioanalytic Imaging System (AMBIS Systems Inc.) counting for 100 min. Then the filters were exposed to Kodak XAR-5 film, overnight at −70 °C using intensifying screens.

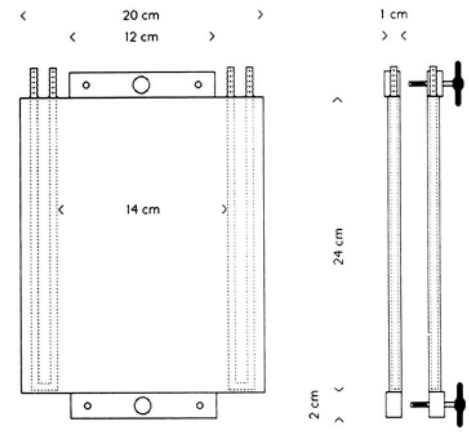

FIG. 1. **Schematic drawing of the aluminium plates used to establish a temperature gradient across TLC plates.** Distances are given. At both ends of each plate U-formed holes serve for circulating the liquid of two thermostated water baths through the plates, thereby keeping the ends at two different temperatures.

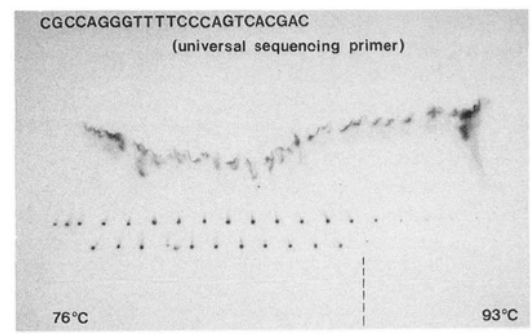

FIG. 2. **TLC of the universal sequencing primer.** A mixture of the 24-mer and the template DNA was spotted in two rows along the temperature gradient (76–93 °C). Development of the plate was in 8 × SSC. In the lower row of spots there is a sharp drop from 80% to no binding. Due to the presence of the free primer released from the lower row this effect is smoothed in the upper row. The line at 88.3 °C indicates the T_d as determined from six TLC experiments.

RESULTS

For the generation of quantitative data on duplex stability, we developed a TLC system which provided a simple and quick tool of measurement. A large number of samples can be run parallel in a single experiment thereby giving results which are directly comparable. The DEAE-cellulose plates allow the use of buffer systems actually used in filter hybridization. For octanucleotides a molarity of 0.65 M NaCl (*e.g.* 4 × SSC) was found sufficient to completely remove free oligomer from its origin. Therefore, if a mixture of single-stranded template DNA and oligomer was spotted, only such oligomer bound to the template remained, while any free probe was washed away by the chromatography buffer irrespective of the temperature. Since the plates were developed until all the free oligomer was removed from the origin (>5 min), the amount of probe remaining was not influenced by the uneven flow of the buffer.

A temperature gradient was applied by sandwiching the TLC plate between two aluminium plates (Fig. 1), which were kept at a different temperature at each end. The samples were spotted 4 cm from the lower end in order to give the chromatography buffer time to equilibriate to the temperature at each position of the gradient. Measurements could be carried out down to a temperature of −5 °C; below that the 8 × SSC buffer started freezing.

To test the technique, the 24-mer "universal sequencing primer" (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and 16-mer "reverse sequencing primer" (5′-AACAGCTATGACCATG-3′; both oligomers from New England Biolabs) were used, which have their complementary sequences on either side of the pJDH118 polylinker. The oligomers were kinase-treated, gel-purified, and subsequently annealed to single-stranded template which had been created with exonuclease III after cleavage of pJDH118 with *Eco*RI and *Hin*dIII, respectively (see "Experimental Procedures"). For the 24-mer universal primer the T_d in 8 × SSC was found to be 88.3 °C (±1.2 °C) (Fig. 2, *lower row*) with a sharp drop from 80% to no binding within about 1.7 °C. This effect could be smoothed by adding free oligonucleotide to the buffer to achieve a more accurate determination of the T_d. The easiest way to do so was by spotting a second row of oligomer in front of the row used for measurement (Fig. 2, *upper row*), so that only small amounts of oligonucleotide were needed. The reverse sequencing primer showed a much broader melting curve (Fig. 3) with the T_d being 63 °C (±0.6 °C).

To check the efficiency of incorporation by Klenow enzyme of the C5-modified nucleotides, the reverse squencing primer was annealed to single stranded pJDH118, its 3′ end 85 base pairs distant from the DNA terminus which had been created by the *Hin*dIII cut. In an extension reaction, similar to a standard sequencing reaction (Sanger *et al.*, 1977) but with no dideoxynucleotides present, the incorporation of dCTP, 5Me-dCTP, and 5Br-dCTP was compared at 20, 40, and 180

μM. No difference in the efficiency of the primer extension could be found (results not presented.)

The melting curves in 8 × SSC of *Not*I oligomers containing 5Me-dC or 5Br-dC, respectively, are shown in Fig. 4, in comparison to oligomer bearing unmodified dC. As an indication of the accuracy of the technique, when the melting curve of the unmodified oligonucleotide was repeated using [α-^{32}P]dCTP and unlabeled dGTP, the identical curve was obtained but the absolute number of counts at each spot increased by a factor of 4/3 (*e.g.* 338/256 cpm at 0 °C), since four labeled nucleotides were incorporated rather than three. Using labeled dGTP, as in the experiments shown here, there were only three labeled nucleotides per molecule, because the first dG nucleotide of the *Not*I oligomers always originated from the primer molecule used for their synthesis (see "Experimental Procedures").

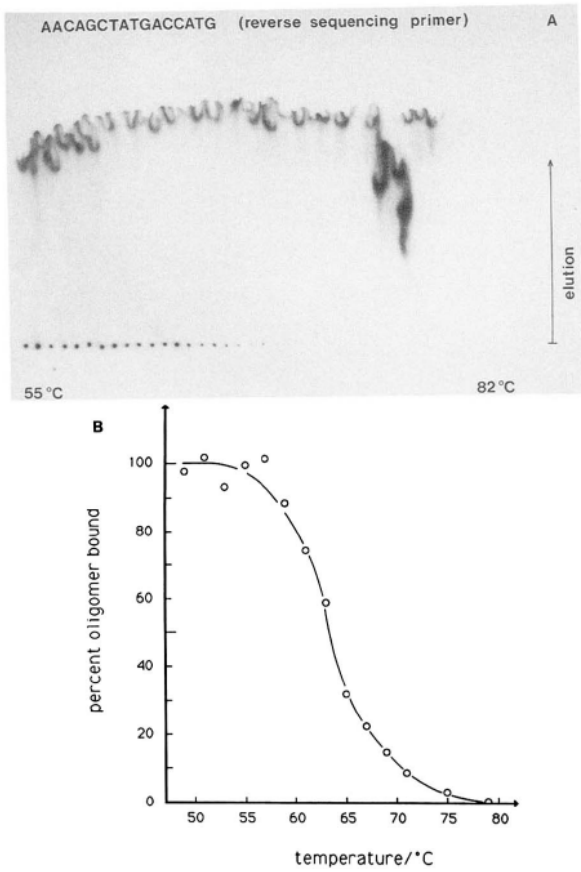

FIG. 3. *A*, TLC of the reverse primer. The 16-mer reverse sequencing primer kinased with ^{32}P was annealed to plasmid pJDH118 (see "Experimental Procedures") which had been labeled with ^{35}S to make it visible as well. TLC was in 8 × SSC with the temperature gradient being 55–82 °C. *B*, melting curve of the oligomer. To minimize the influence of the deviation in the amount of DNA spotted, the intensities of three spots were averaged to define the amount of oligomer bound at their average temperature. Since three TLCs as the one shown in *A* were run, each point represents an average from nine measurements.

Under the assay conditions of 1.32 M Na^+ (8 × SSC; Fig. 4) 31.8% of unmodified *Not*I oligomer were bound to the template at 0 °C. Bromo substitution or methylation at the C5 position increased these percentages over the entire temperature range, with, for instance, 43.9% (±1.5%) (factor 1.35) or 39.6% (±1.7%) (factor 1.21) of the molecules annealed to the template at 0 °C, the maximal difference occurring between 25 and 35 °C. Above a temperature of about 25 °C there is only a slight difference in the melting curves of the two modified oligomers.

Substitution of dC by its C5 modifications influenced not only the binding equilibrium but also the T_d of the oligonucleotides, rising by almost 10 °C from 27.4 to 36.5 °C in case of 5Me-dC and 37 °C for 5Br-dC. All measurements at probe concentrations between 10 and 25 pM resulted in the same melting curves for the three types of *Not*I oligonucleotides.

Experiments identical to those shown above were carried out at the lower salt concentration of 6 × SSC (0.99 M Na^+; Fig. 5). Due to the lower salt the amount of unmodified oligomer sticking to the template was reduced by a factor of 0.75 to an absolute value of 24% of the octamer still binding at 0 °C. The differences in the melting curves of 5Br- and 5Me-substituted oligonucleotides between 0 and 25 °C were not observed using 6 × SSC. Since the stability of the modified base homoduplexes are far less dramatically affected by the lower salt concentration, with the amount bound at 0 °C changed to 41.5% (±2.1%; Fig. 5), the ratios of signal intensities of modified to unmodified oligomer are considerably bigger than at higher salt concentration, *e.g.* 5-fold at 35 °C instead of 2.6-fold.

Complementary to the TLC experiments, actual filter hybridizations to DNA dot-blots were carried out. Fig. 6*A* shows a graph of the counts obtained after hybridization of *Not*I oligonucleotides to DNA targets with and without *Not*I sites. Again, the ratio of 4/3 in absolute counts between labeling with either radioactive dCTP (*dG*) or dGTP (*dC*) documents the accuracy of the measurements. Both 5Me-dC and 5Br-dC act to increase the amount of hybrid maintained after stringent washing at 35 °C, giving a 5-fold stronger signal with the

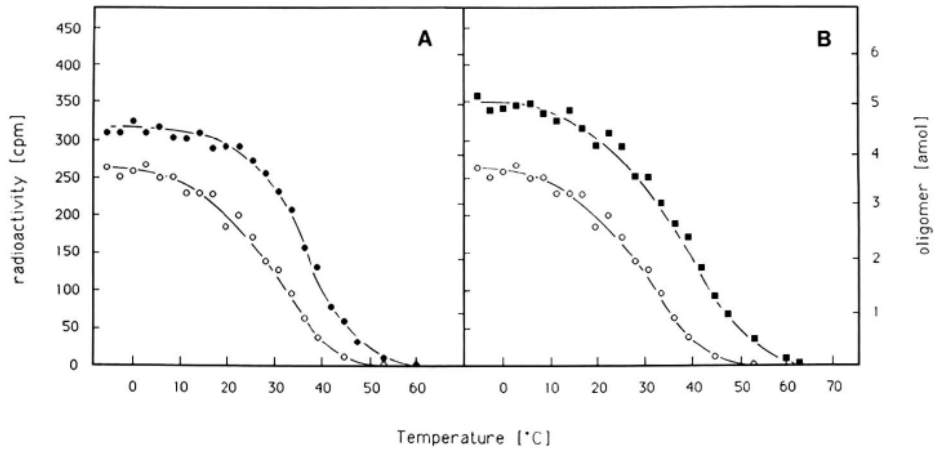

FIG. 4. **Melting curves of unmodified and modified *Not*I oligomers.** The melting curves in 8 × SSC are shown of *Not*I octamers containing 5Me-dC (●; *A*) or 5Br-dC (■; *B*) in comparison to that of the unmodified oligomer (○). Each point represents the average of six measurements.

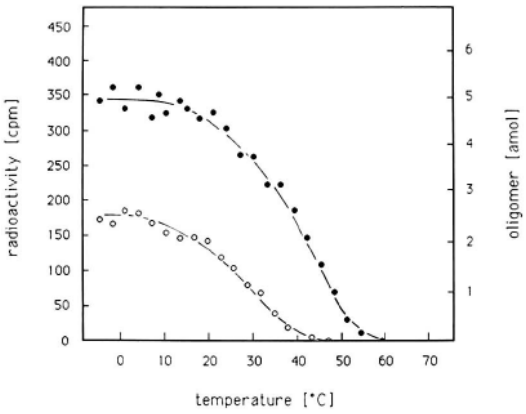

FIG. 5. **Melting curves of the *Not*I oligonucleotides in 6 × SSC.** TLC was carried out for 5Me- and 5Br-modified (●) and unmodified oligomer (○) as described under "Experimental Procedures." Each point represents the average of four measurements.

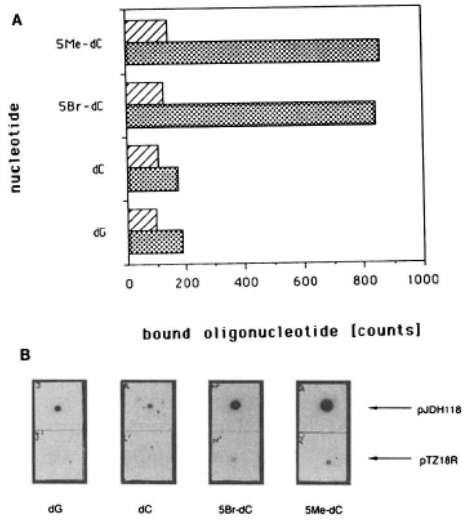

FIG. 6. *A*, the effect of incorporation of various nucleotides on the hybridization of the *Not*I oligonucleotide to positive and negative targets. The hybridizations were carried out on DNA dot-blots as described under "Experimental Procedures." The *columns* show the counts recorded by an AMBIS scanner within 100 min; *hatched columns* for hybridizations to pTZ18R (no *Not*I site); *dark columns* for those to pJDH118. Each column represents the average of three independent experiments. The background signal of machine noise and unspecific oligo-binding to the filter was approximately 300 counts (not shown). *dG* indicates a control labeled with [α-^{32}P]dCTP instead of [α-^{32}P]dGTP. *B*, autoradiograph of dot-blots after hybridization with oligonucleotide probes containing modified and unmodified nucleotides. The filters are some of those used for quantification on the AMBIS scanner. Some signal on the autoradiograph is due to dust which kept radioactivity even after the washing steps. This part of the signal was ignored in the quantification.

positive target, in accordance with the TLC data (Fig. 5) obtained at the same conditions (35 °C, 6 × SSC; the 0.1% SDS adds only 3.5 mM Na^+ ions). In contrast, there is only a 1.2–1.3-fold increase (±0.06-fold) in the amount of oligonucleotide binding to the negative target, indicating that heteroduplexes remain unstable despite the use of modified nucleotides. This means that for the *Not*I oligomer the substitution of dC by 5Br- or 5Me-dC nucleotides improved the discrimination between positive and negative targets by a factor of 3.9.

Fig. 6B shows the autoradiograph of some filters on overnight exposure. The increase in the signal strength obtained with probes containing 5Me-dC and 5Br-dC is obvious. A weak signal is also seen for these probes on the negative controls which might not be expected from the results shown in Fig. 6*A*. The small increase in counts between the hybridization of the unmodified oligonucleotide probe to the negative target and those obtained for the probes containing modified bases appears to result in a larger difference on autoradiography. This effect, however, is due to the threshold of activation of the x-ray film, with signals from the *dG* and *dC* pTZ18R filters falling below this level and thus giving a nonlinear response (data not shown).

DISCUSSION

The recognition of CpG-containing restriction endonuclease sites in genomic libraries *in situ* by oligonucleotide hybridization will aid the identification of coding sequences and produce linking libraries (Poustka and Lehrach, 1986) from existing genomic libraries. The major difficulty encountered is the low stability of the short oligonucleotides, used as probes, under conventional hybridization conditions.

By using the 5Me and 5Br substitution of the deoxycytosine nucleotide in the *Not*I octanucleotide sequence, a considerable improvement in duplex stability is achieved, particularly at higher temperatures. This is a result of an increase in both the T_d and the binding equilibrium, caused by a change in the stacking pattern (Bugg *et al.*, 1971). It has been demonstrated that stacking forces vary for different combinations of bases and are considerably dependent upon base sequence in oligonucleotides (Brahms *et al.*, 1967; Ts'o, 1968). Consequently, it can be expected that the effects the halogenated cytosines have on base stacking and thereby the duplex stability would be dependent on the local sequence and would vary from oligomer to oligomer. Since the substituted nucleotide triphosphates are incorporated by Klenow polymerase at rates similar to the unmodified dCTP, the latter can easily be replaced in extension reactions.

The measurements by TLC across a temperature gradient proved to be a very quick and reliable way to determine melting curves of even very short oligonucleotides. The method allows the use of probe concentrations identical to real filter hybridization experiments. It may well be possible to detect nonradioactively labeled oligomer instantly on the plate, measuring any influence of the label and simultaneously taking advantage of it for detection.

By using the modified nucleotides 5Me-dC and 5Br-dC it has been possible to increase the amount of oligonucleotide binding to a DNA target compared to unmodified bases. At the same time discrimination of heteroduplexes has also been improved with the background signal increasing only slightly. It should therefore be possible to use relatively short oligonucleotide probes for screening genomic libraries to detect sites of interest (for example, CpG restriction sites or promoter consensus sequences) which were hitherto unstable under standard hybridization conditions. Also, the improvement in discrimination combined with the increase of duplex stability may be employed for other techniques involving the hybridization of oligonucleotides, particularly the polymerase chain reaction. The incorporation of modified cytosine bases, as any other base modification with similar effects, should allow either more stringent conditions for the primer annealing, thereby improving the specificity of the duplex formation and suppressing background problems, or the use of shorter oligomers.

Another application is the mapping of genomic DNA using fingerprinting by hybridization (Michiels *et al.*, 1987; Craig *et al.*, 1990). It relies on the ability to selectively hybridize short oligonucleotides (nonanucleotides to dodecanucleotides) to *in*

situ filters of genomic cosmid libraries. The use of the C5-modified cytosines will allow the use of a wider variety of such oligonucleotides, of which several have been found not to be stable enough for discriminate hybridization on *in situ* filters.[2]

An improved sequencing strategy, based on standard procedures but using very short primer molecules (Studier, 1989), will certainly gain from an increased stability of the duplex formation. Base modifications may also be found crucial for proposed techniques of large-scale sequencing (Drmanac *et al.*, 1989; Khrapko *et al.*, 1989) by hybridization of genomic DNA fragments to a set of short oligonucleotides of known sequence (*e.g.* all 65,536 octanucleotides) bound to small surfaces. Due to the restrictions in both the length of the oligomers and the amount actually bound to the proposed "sequencing chip," in practice, the specificity and efficiency of binding will be essential.

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