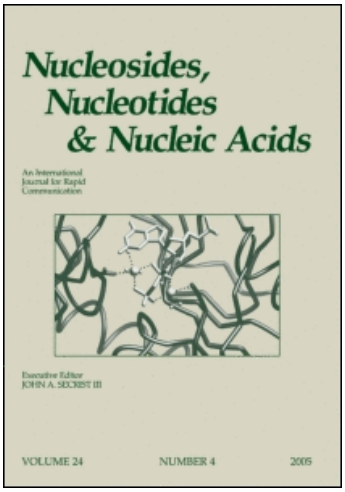

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Segregation of Partly Melted DNA Molecules

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SEGREGATION OF PARTLY MELTED DNA MOLECULES

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□ *Segregation of partly melted DNA molecules is a convenient and efficient method to isolate DNA fragments associated with CpG islands. The method stands on the observation that the electrophoretic mobility of partly melted DNA fragments in a denaturing gradient gel is low and that they persist in the gel so long as the remaining helical part is sufficiently resistant to strand dissociation and dissociates slowly. Such features are observed in DNA fragments derived from CpG islands. These DNA fragments are preferentially retained in a denaturing gradient gel after prolonged electric field exposure, permitting the enrichment of DNA fragments derived from CpG islands. The principle and practical application of this method are reviewed.*

Keywords dCpG islands; Denaturing gradient gel electrophoresis; Gene identification; Strand dissociation kinetics

INTRODUCTION

Polyacrylamide gel electrophoresis is a well-known means to separate DNA fragments. The most popular application of this system is the separation of double-strand or single-strand DNA molecules on the basis of their length. The physical basis of length-determined separation of linear helical molecules has been analyzed.[1–3] Double-strand DNA molecules are sensitive to length-determined separation under conventional experimental conditions if the molecule is completely helical. Denatured single-strand DNA is also amenable to length-determined separation. However, the mobility of DNA fragments in a polyacrylamide gel is also sensitive to the secondary structure. The relative electrophoretic mobility of nondenatured single-strand

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This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.

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DNA molecule is complex and hardly predictable. In certain systems, the deviation from linear structure affects the mobility of double-strand DNA fragments. For example, bending of DNA fragments is one such factors.[4] Later it was shown that the mobility of the fragment was low when the bend center was near the middle of the molecule, whose structure is more circular than linear.[5] Denaturing gradient gel electrophoresis (DGGE) is a modified version of polyacrylamide gel electrophoresis. The gel contains a linear gradient of chemical denaturant across the gel, either parallel or perpendicular to the applied electric field. DGGE is unique in that it permits sequence-determined separation of double-strand DNA fragments.[6]

The separation of DNA fragments by DGGE depends on the melting properties of DNA fragments.[7–9] Double-strand DNA is stabilized primarily by hydrogen bonding and base stacking. Distortion of hydrogen bonds between base pairs results in dissociation of the strands. Double-strand DNA fragments undergo strand dissociation when they are exposed to a strong dissociation condition, such as heat, non-aqueous solvents, or extremes of pH. When DNA fragments having domains with different T_ms are exposed to an increasing denaturing condition, the domain having the lowest T_m melts first, thereby a partly melted structure forms. The stability of this structure is our major concern in this article.

Conventionally, strand dissociation kinetics of partly melted DNA molecules was not deeply considered due to the lack of laboratory interest and an appropriate theory concerning the dissociation rate at temperatures lower than those sufficient to melt the entire molecule. Hence, the stability of the partly melted DNA molecules was not intensively investigated. However, experimental results and theoretical consideration of strand dissociation kinetics revealed an interesting feature of DGGE and led to the development of its novel application.[10] In this article, we discuss how reduced strand dissociation rate of partly melted DNA molecules has been applied to gene identification.

Denaturing Gradient Gel Electrophoresis

DGGE is different from other gel systems in that DNA fragments undergo helix-to-random coil transition in a gel. The transition results in change of forms from fully helical structure, then partly melted structure, and to fully dissociated structure. The intermediate partly melted structure is not always identified depending on the nucleotide sequence of the fragment. Since such behavior affects the electrophoretic mobility of the fragments, the analysis of DNA fragments by DGGE gives important information on melting behavior of DNA fragments.

In Figure 1A, a representative example of DGGE is shown (for details, see Myers et al., Abrams and Stanton, and Shiraishi[11–13]). The gel contains

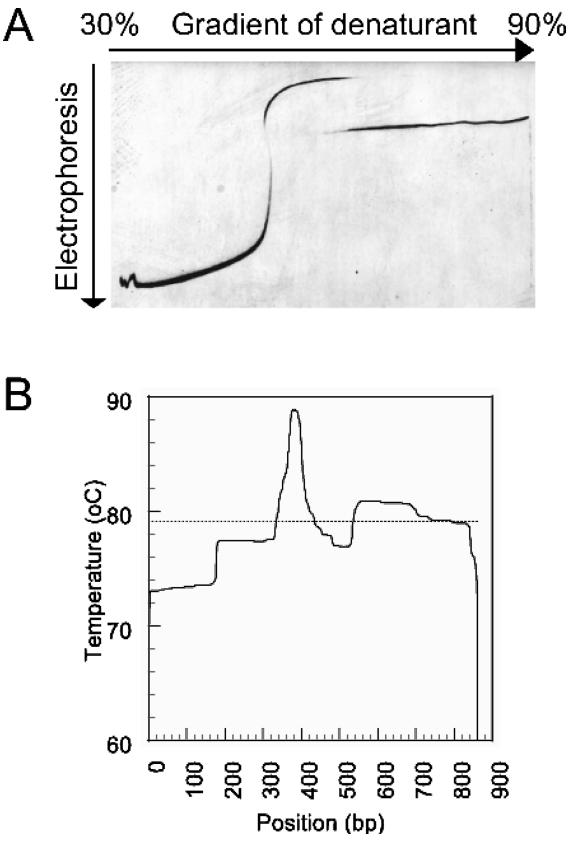

FIGURE 1 (A) A denaturing gradient gel analysis of the DNA fragment containing a part of the human *MYC* gene (a 863-bp *Pvu*II fragment covering positions 1976–2838, accession no. X00364). Approximately 5 μg of DNA fragments purified by agarose gel electrophoresis was loaded onto a denaturing gradient gel of 132 mm width, 150 mm length, and 0.75 mm thickness. The gel contains a 30–90% linear gradient of urea and formamide (100% denaturant is 7 M urea and 40% formamide). After loading, the gel was placed in a tank containing 11 l of buffer (40 mM Tris-HCl/20 mM sodium acetate/1 mM EDTA, pH 7.4). After 30 min run at 10 V/cm, the bath temperature was heated to 60°C, and run at the same voltage for 5.5 h. (B) Melting map of the same fragment. Calculation was performed using a program MELT94 (http://web.mit.edu/osp/www.melt.html). The dotted line indicates the temperature where dissociation constant is 10^{-6} M.

a linear gradient of chemical denaturant, urea and formamide; low at the left and high at the right. The gradient is equivalent to a linear thermal gradient of appropriate temperature range. DNA fragment was uniformly loaded onto the top of the gel. The gel was then placed in a bath maintained at high temperature and the voltage was applied. DNA fragments are double-stranded at low denaturant concentration and separated according to their length. The electrophoretic mobility of each fragment decreases markedly at a particular denaturant concentration, approximately at 50% denaturant (Figure 1A). This decrease in mobility is attributed to melting of strands of a part of the fragment. The electrophoretic mobility of this branched

molecule is much lower than that of fully helical molecule. Then there is a discontinuous transition and the new mobility increases to a level that is lower than the initial mobility but higher than the mobility of the partly melted structure. This increment in mobility is attributed to strand dissociation. The electrophoretic mobility of fully dissociated molecule is higher than that of partly melted molecule, although lower than that of fully helical molecule. Thus, change in mobility across the denaturing gradient closely correspond to the helical structure attributable to the denaturing equilibrium.[6]

The calculated melting map[14] predicts a melting behavior of DNA fragments having a defined sequence on the basis of Fixman-Freire modification[15] of Poland's algorithm.[16] A melting map of the corresponding DNA fragment is shown in Figure 1B. The contour shows the midpoint of the melting equilibrium at each base pair neglecting strand dissociation. The fragment consists of domains having different T_ms. The T_m of the domain covering positions 1–175 is the lowest, approximately 72.5°C. This region melts first and the mobility decreases abruptly upon melting of this domain. The calculated T_ms of substantial regions are higher than the equivalent temperature where the dissociation constant is 10^{-6} M (approximately 79.1°C). This calculation strongly suggests that such domains remain helical upon melting of the lowest melting domain, thus permitting retardation. The reduced mobility can be calculated by the following expression when the length of the melted region is much shorter than the total size.

$$\mu = \mu_0 \exp(-\mathrm{Lm}/\mathrm{Lr})$$

where μ is the mobility, μ_0 is the initial mobility at the fully helical state, Lm is the total number of non-helical base pairs, and Lr is a constant, taken as 75 here. This expression indicates that the residual mobility is determined by the length of the melted region and insensitive to the length of the remaining helical region. The calculation shows that the reduced mobility of this partly melted DNA molecule indicated in Figure 1 is 10% of the initial mobility.

Two DNA fragments that differ by as small as a single base in the lowest melting domain can differ in T_m. This difference is reflected to the retardation level of each fragment in parallel DGGE.[9] Hence, sequence alteration between fragments can be detected by DGGE as the difference in retardation level.[9,17] DGGE is frequently applied to the detection of sequence variation.[18]

Application to the Isolation of DNA Fragments Associated with CpG Islands

In spite of its utility in the detection of sequence variation, the analysis of intact genomic DNA by DGGE often eludes some sequence variations. This is because, in most cases, sequence variations occurring only in low melting domain(s) can be detected by DGGE. Those occurring in other domains

do not affect the retardation level unless domains having higher calculated T_ms remain helical. In addition, the detection of sequence variation is not achieved in regions where the double-strand does not melt uniformly. In order to overcome these problems, the attachment of an artificial G+C-rich sequence, termed a G+C-clamp, was devised.[19–21] The original genomic sequence having multiple melting domains often shows uniform melting when such a clamp was appropriately attached. A G+C-clamp is conveniently attached to the end of fragments by PCR and such treatment allows detection in sequence variation that cannot be otherwise detected.[22]

So far we took it for granted that the clamp was stable enough to maintain a partly melted form after prolonged exposure to an electric field. However, this is not always guaranteed. The interval between melting temperature and the temperature at an appropriate dissociation constant can be an indicator of such stability. Calculation showed that the decrease in the size of a G+C-clamp resulted in reduced interval between melting temperature and the temperature at which the calculated dissociation constant for the complete strand dissociation equals 10^{-6} M, although not experimentally validated.[19] This estimation suggests that the length of the clamp affects the stability. Another estimation shows that the stabilization of a 272-bp fragment conferred by the addition of a 30-bp clamp relative to that of the unclamped fragment is about 3°C when the dissociation constant is 10^{-4} M.[23] Experimental results show that this difference was adequate to enable the detection of point mutations occurred in the fragment. However, a rigorous theory concerning the dissociation rate at temperatures lower than those sufficient to melt the entire molecules is lacking and we can not discuss the stabilization effect quantitatively. The sequence and length of a clamp surely affects stability. Molecules with a relatively unstable clamp would not persist in a denaturing gradient gel for a long time because such fragments fade away through strand dissociation.

Consider a putative DNA fragment consisting of two domains that differ in base composition, one of which is G+C-rich. We may regard the higher melting domain as a potential G+C-clamp if the T_m of the other domain is substantially lower. When such fragments are subjected to DGGE, low residual mobility restricts migration into regions of more denaturing condition after partial melting. The same condition that affects retardation also increases the complete strand dissociation rate. On prolonged electric field exposure, partly melted DNA molecules undergo strand dissociation and fade away unless the stability of the remaining helical part is much higher than that of the already melted part. The retardation level depends on the least stable part, and the rate of strand dissociation is mainly controlled by the helical part.

The stable partly melted DNA molecule is especially expected for DNA fragments derived from the edge of CpG islands, which are very interesting vertebrate genomic regions. A CpG island is a chromosomal region

where nonmethylated CpG sites cluster significantly than elsewhere in the genome.[24] Most CpG islands are associated with 5′ region of genes and contain regulatory elements, such as a promoter. Since the identification of a CpG island from unsequenced DNA fragments permits the isolation of a novel gene,[25] the isolation of stable partly melted molecules in a denaturing gradient gel results in isolation of gene sequences. These considerations lead to the assumption that the analysis of the mixture of DNA fragments by DGGE would enrich G+C-rich fragments, many of which would be derived from CpG islands.

DNA fragments need to be digested to appropriate size that could be subjected to DGGE, approximately several hundred base pairs, because long DNA fragments tend to be resistant to strand dissociation regardless of their nucleotide sequence context. Preliminary experiments using DNA fragments having known CpG islands revealed that extensive fragmentation by digestion with restriction endonucleases, *Tsp*509I (AATT), *Mse*I (TTAA), *Nla*III (CATG), and *Bfa*I (CTAG), whose recognition sites appear relatively infrequently within CpG islands, yielded fragments that are appropriate for the DGGE analysis.[10] This treatment keeps the integrity of CpG island relatively intact, while majority of other chromosomal regions are severely fragmented. Such digests were subsequently subjected to DGGE to enrich CpG island fragments.

When DNA fragments containing the entire portion of human *ECGF*, *FOS*, *HRAS1*, *MYC*, and *MYCN* genes were analyzed, all predicted CpG islands were isolated in this way.[10] In Figures 2 and 3, a representative result is shown. A 6.5-kilobase *Bam*HI fragment containing the entire potion of the human *HRAS1* gene was analyzed. The gene consists of five exons (Figure 2A). Digestion with the four restriction endonucleases yields many fragments (Figure 2B). Computer analysis revealed that the predicted CpG island covered the 5′ region of the gene including the exon 0 (Figures 2B–E). The four enzyme-digest was analyzed by perpendicular DGGE, where the denaturant gradient is perpendicular to the electric field (Figure 3A). As inferred from Figure 1, different fragments undergo different melting profiles. In Figure 3B, the same digests were analyzed parallel DGGE, where the gradient of denaturant is parallel to the electric field. After a 6 h run, several fragments were detected as retained ones in the gel. However, many disappeared through strand dissociation during run, and only one fragment was persistently retained after a 15-h run. Further characterization revealed that this fragment was derived from the CpG island (Figure 2B). This procedure for CpG island enrichment was named segregation of partly melted molecules (SPM).

Conventional methods for CpG island isolation take advantage of the presence or clustering of recognition sites of some restriction endonucleases whose recognition sites frequently appear in CpG islands.[26] SPM is unique in that the identification of CpG island in independent of the presence of

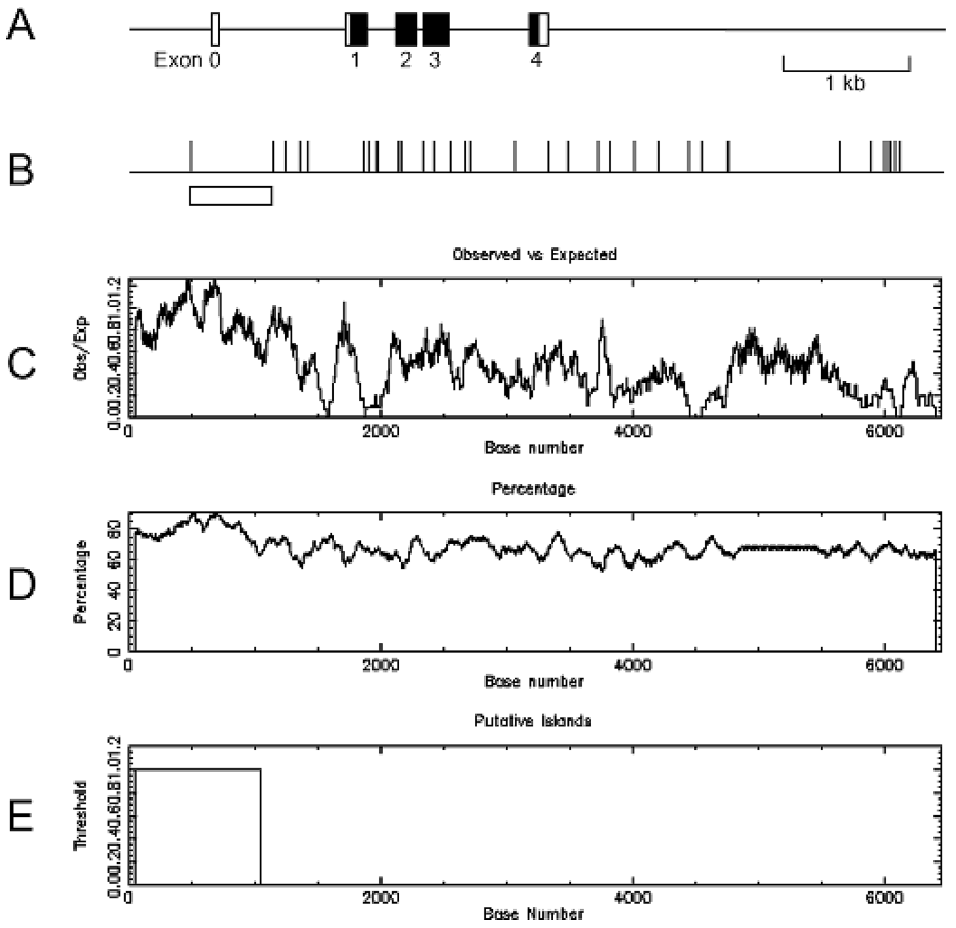

FIGURE 2 (A) The structure of the human *HRAS1* gene. (B) Recognition sites of restriction endonucleases *Tsp*509I, *Mse*I, *Nla*III, and *Bfa*I. The open box indicates the fragments that was recovered in the experiment shown in Figure 3B (positions 491–1146). (C) The observed versus expected ratio of the appearance of CpG dinucleotide. (D) G+C content of the fragment. (E) The position of the predicted CpG island (positions 48–1049). Calculations were done using programs at website http://www.ebi.ac.uk/emboss/cpgplot/.

recognition sites of specific restriction endonuclease(s) within the sequence. Furthermore, the entire procedure is very simple, just requiring digestion of DNA and gel electrophoresis.

By SPM analysis of contiguous bacteriophage P1 clones containing a 300-kb region of human chromosomal region 11q13, we have isolated all CpG islands associated with known genes residing in the analyzed region.[27] This result shows that SPM analysis is an efficient method for gene identification.

There are several experimental methods for gene identification from unsequenced DNA fragments. Representative ones are exon trapping,[28,29] cDNA selection,[30,31] and CpG island identification.[25] These methods

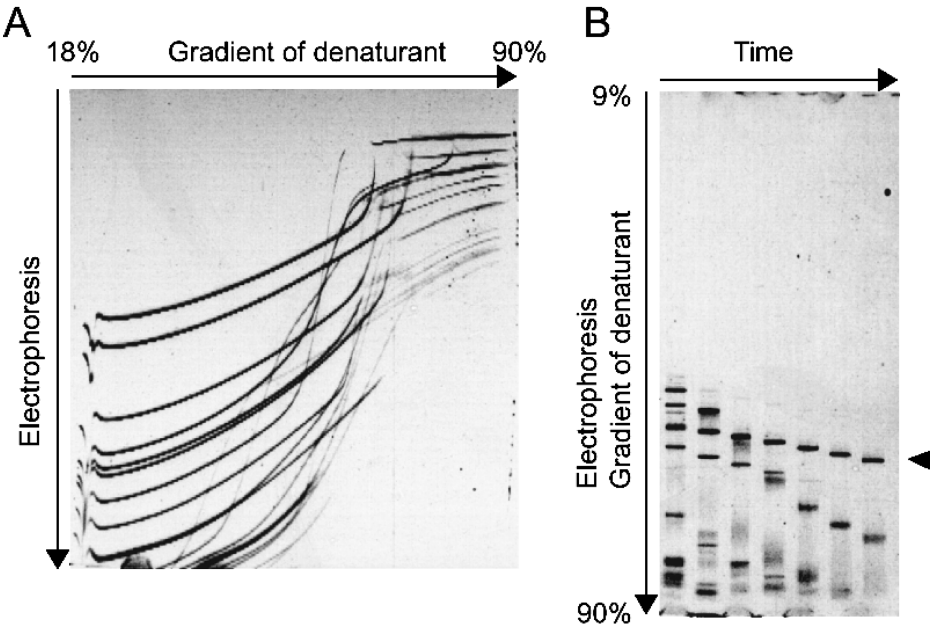

FIGURE 3 (A) A perpendicular DGGE of *Mse*I, *Nla*III, and *Bfa*I-digests of DNA fragments containing the entire portion of the human *HRAS1* gene (positions 1–6421, accession number V00574). Approximately 40 μg of digests was analyzed similarly as shown in Figure 1A. Jaggy curves were caused due to low resolution of the camera and are not the nature of the fragments. (B) A parallel DGGE of the same digest. Approximately 2 μg of digests was loaded onto a gel of a similar size as shown in Figure 1A containing 9%–90% gradient of denaturant and placed in a bath maintained at 60°C. Time of run is 6 h at the left and 15 h at the right. Samples were loaded every 90 min.

have played important roles for gene identification until long genomic sequences become available. At present, based on the availability of long genomic sequences, novel gene sequences can now be predicted by some algorithm.[32,33] On the other hand, experimental methods for gene identification are required. Accumulating evidence suggests that epigenetic regulation of gene expression is important. DNA methylation is an important epigenetic mechanism of gene regulation, and CpG islands are targets of that kind of study. No preexisting algorithm can predict methylation status. Various methods for CpG island detection is yet required. The combined use of SPM and a method to enrich methylated DNA for the epigenetic study will be described later.

Application to the Epigenetic Study

The determination of the entire human genomic DNA sequence revealed many important genetic information. However, DNA sequence itself is the starting point of the elucidation of genome function. Epigenetic information is an additional information that mediates genome function. Epigenetic

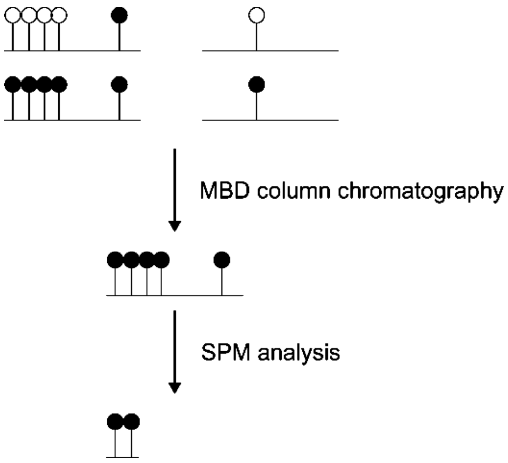

FIGURE 4 A scheme for the enrichment of methylated CpG islands by the combined use of MBD column chromatography and SPM. Open lollipops indicate nonmethylated CpG sites and closed lollipops indicate methylated CpG sites.

events are meiotically and mitotically heritable variation in gene activity that cannot be explained by the difference in DNA sequence. DNA methylation is a representative epigenetic event. DNA methylation in mammalian genome occurs at the position 5 of cytosine at CpG sequence. DNA methylation plays various biological roles, and its aberration is associated with disease. Aberrant methylation of CpG island is a representative feature observed in various human tumors.[34] CpG island methylation results in silencing of an associated gene and is considered to be an important mechanism in inactivation of tumor suppressor genes.

We intended a comprehensive isolation of methylated CpG islands in human cancer cells by the combined use of methyl-CpG binding domain (MBD) column chromatography and SPM method.[35,36] MBD column chromatography is a technique used to analyze the methylation status of DNA fragments primarily on the basis of the number of methyl-CpG sites within the fragments.[37] The combined use of MBD column chromatography and SPM is expected to provide important information to identify novel tumor suppressor genes. Highly methylated genomic DNA in cancer cells were enriched by MBD column chromatography and cloned (Figure 4). The cloned fragments were subjected to the SPM analysis (Figure 4) and DNA fragments associated with methylated CpG islands in cancer were successfully isolated.[36]

There are several merits in the combined use of MBD column chromatography and SPM method for the isolation of methylated CpG islands. First, these methods are insensitive to the presence or methylation status of recognition sites of restriction endonucleases. This feature is completely different from conventional methods with similar applications.[38] Secondly, methylated genomic fragments and cloned fragments do not undergo strand

dissociation, reannealing, and PCR. This feature is advantageous for comprehensive isolation since many DNA fragments, especially PCR-resistant G+C-rich fragments, can be lost during these steps.

Concluding Remarks

Physical properties of DNA have been extensively investigated and some of them have been applied to solve biological problems. We believe that there are many more interesting nature of DNA that can be applicable to the elucidation of unsolved problems in biology. The analysis of the structure of DNA is important by itself and of biological significance. Nucleic acid molecules are still an important research target.

SPM is a convenient and efficient method for gene identification. Further refinement of the rationale will give better insight of strand dissociation kinetics and provide novel application of the method.

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