**Table 2.** List of chemicals reported to alter epigenetic statuses.

| Action | Chemical | Characteristics | Reference |
|---|---|---|---|
| DNA hypermethylation | Butyrate | Short-chain fatty acid | (Boffa, Mariani and Parker, 1994) |
| | 4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) | Tobacco-specific carcinogen | (Pulling *et al.*, 2004) |
| | Phenobarbital | Antiepileptic agent | (Bachman, Phillips and Goodman, 2006) |
| | Vinclozolin | Antiandrogenic compound | (Anway *et al.*, 2005) |
| | Diethylstilbestrol | Synthetic estrogen | (Bromer *et al.*, 2009) |
| DNA hypomethylation | 5-Azacytidine, 5-Aza-2′-deoxycytidine | Cytidine analog | (Egger *et al.*, 2004) |
| | 5-Fluoro-2′-deoxycytidine | Cytidine analog | (Jones and Taylor, 1980) |
| | 5,6-Dihydro-2′-azacytidine | Cytidine analog | (Curt *et al.*, 1985) |
| | Zebularine | Cytidine analog | (Cheng *et al.*, 2003; Holleran *et al.*, 2005) |
| | Ethionine | Methionine analog | (Shivapurkar, Wilson and Poirier, 1984) |
| | Arsenic compound | Metal compound | (Zhao *et al.*, 1997;Reichard, Schnekenburger and Puga, 2007) |
| | Valproic acid | Antiepileptic agent | (Detich, Bovenzi and Szyf, 2003) |
| | Procainamide | Antiarrhythmic agent | (Lee *et al.*, 2005; Segura-Pacheco *et al.*, 2003) |
| | Procaine | Anesthetic agent | (Villar-Garea *et al.*, 2003) |
| | Hydralazine | Antihypertensive agent | (Segura-Pacheco *et al.*, 2003) |
| | 6-Mercaptopurine | Anticancer agent | (Hogarth *et al.*, 2008) |
| | 6-Thioguanine | Anticancer agent | (Hogarth *et al.*, 2008) |
| | Psammaplins A | Antibiotic agent | (Pina *et al.*, 2003) |
| | (-)-Epigallocatechin-3-*O*-gallate (EGCG) | Major polyphenol from green tea | (Fang *et al.*, 2003) |
| | RG108 | DNMT inhibitor | (Brueckner *et al.*, 2005) |
| | SGI-1027 | DNMT1 inhibitor | (Datta *et al.*, 2009) |
| | Bisphenol-A | Synthetic estrogen | (Bromer *et al.*, 2010) |
| Alterations of histone modifications | Butyrate | short-chain fatty acid | (Stadtman and Barker, 1949) |
| | Trichostatin A | Microbially derived compound | (Yoshida *et al.*, 1990) |
| | Valproic acid | Antiepileptic agent | (Kramer *et al.*, 2003) |
| | Suberoylanilide hydroxamic acid (SAHA) | Hydroxamic acid | (Kelly *et al.*, 2003) |
| | Depsipeptide | Microbially derived compound | (Furumai *et al.*, 2002) |
| | Nickel compound | Metal compound | (Chen *et al.*, 2006) |
| | Chromium compound | Metal compound | (Zhou *et al.*, 2009) |
| | Arsenic compound | Metal compound | (Zhou *et al.*, 2009) |
| | Cobalt compound | Metal compound | (Li *et al.*, 2009) |
| | Cocaine | Crystalline tropane alkaloid | (Maze *et al.* 2010 |

traps DNMT1, which is subsequently degraded by proteasome (Ghoshal *et al.*, 2005). This leads to depletion of DNMT1 in a cell, and passive DNA demethylation is resultantly induced. There are many other chemicals reported to induce changes in epigenetic modifications (Table 2), but their direct action or indirect action through gene expression changes should be carefully evaluated.

## 5 EPIGENOMIC ANALYSIS IN TOXICOLOGY

Epigenomic alterations are deeply involved in carcinogenesis and possibly in other disorders. In addition, there are a large number of non-mutagenic carcinogens (Snyder and Green, 2001), some of which exert their carcinogenic action by inducing

cell proliferation. It seems reasonable to consider a possibility that some of the non-mutagenic carcinogens exert their action by epigenetic mechanisms. In this context, epigenomic analysis seems essential in toxicology, which has just started. Unfortunately, few reliable and sensitive methods specifically designed for toxicological analysis have been reported yet, and ordinary procedures for epigenetic and epigenomic analysis are used also for toxicological analysis. Their brief principles and efforts in development of convenient assay systems are described.

## 5.1 Principles of DNA Methylation Analysis

Methods can be divided into those for analysis of specific genomic regions and those for genome-wide analyses. DNA methylation at specific genomic regions is analyzed mainly based upon two principles of methylation detection; methylation-sensitive restriction enzymes, and bisulfite modification of DNA (Figure 8). Some restriction enzymes, such as *Hpa*II and *Sma*I, have recognition sequences with CpG sites, and cannot cleave if the CpG site is methylated. Bisulfite

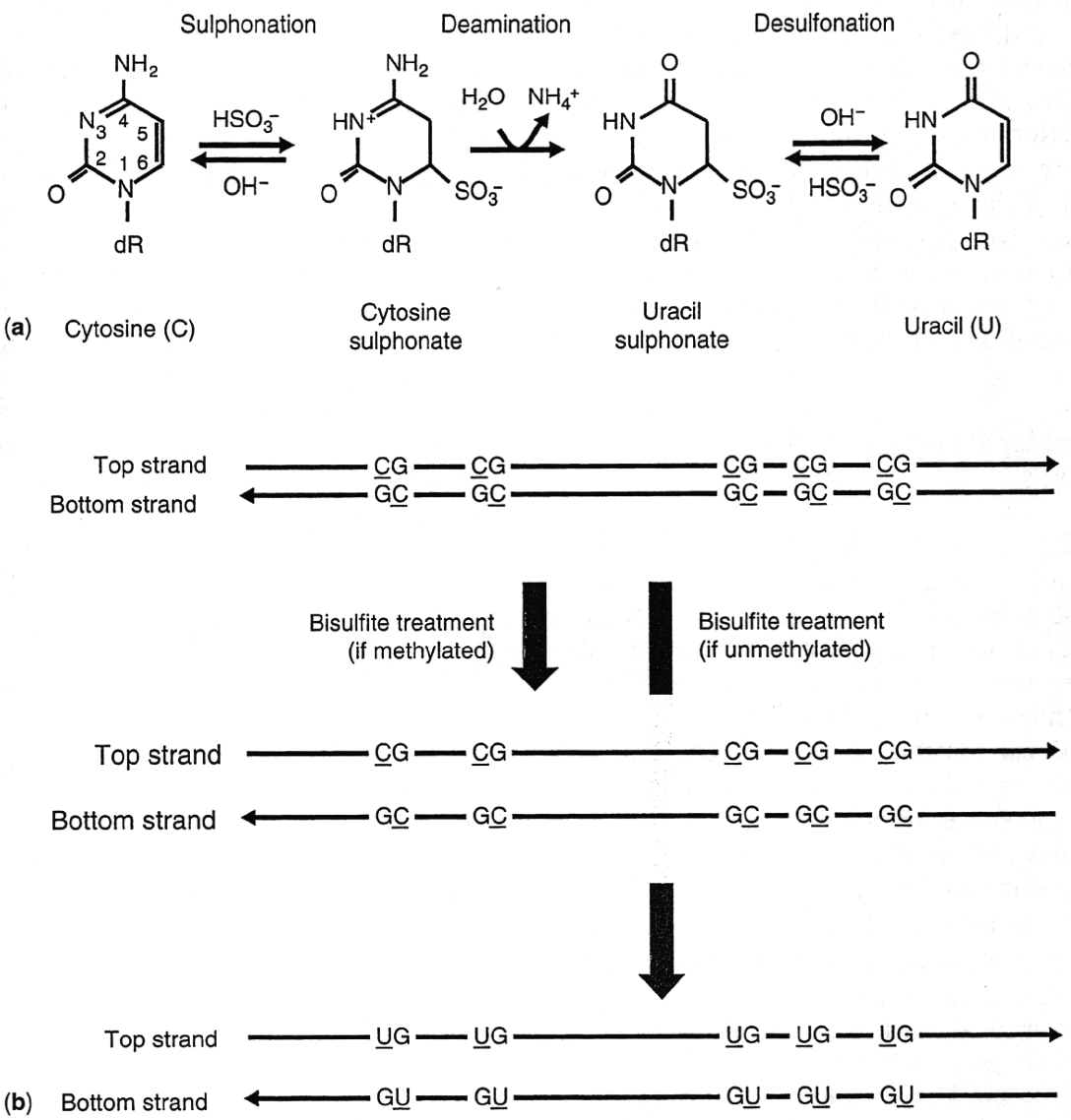


**Figure 8.** Principle of bisulfite modification: (a) chemical reactions for unmethylated cytosine; (b) sequence changes produced by bisulfite modification of methylated and unmethylated DNA. Different sequences are produced from methylated and unmethylated DNA, and the difference can be detected by various modalities.

modification takes advantage of different efficiency in converting cytosine to uracil, which is very efficient for unmethylated cytosines but very slow for methylated cytosines. After bisulfite conversion, the top and bottom strands are no longer complementary. Methylated and unmethylated DNA will produce different sequences after the conversion, and the difference can be detected by various techniques, such as sequencing, allele-specific PCR, restriction digestion, and pyrosequencing. Depending upon the purpose of experiments, appropriate techniques should be selected, considering the required amount of DNA, flexibility in selection of CpG sites to analyze, how quantitative the method is, technical complexity, and the cost.

Genome-wide analyses are generally composed of a step of detection of DNA methylation and another step of genome-wide analysis (Ushijima, 2005; Laird, 2010). The methylation detection can be performed using affinity-based methods, such as use of anti 5-methylcytidine antibody and affinity column with methylated DNA binding domains, but also using methylation-sensitive restriction enzymes and bisulfite conversion. The detection step can be performed using microarray or next-generation sequencers.

## 5.2 Principles of Histone Modification Analysis

Methods for histone modification analysis can be divided into: (i) those for analysis of global contents of histone modifications within a cell; (ii) those for analysis of histone modifications for a defined genomic region; (iii) those for histone modifications of defined genomic regions in a genome-wide manner. Global contents of histone modifications within a cell are mainly analyzed by immunohistochemistry and Western blotting. In contrast, histone modifications in defined genomic regions are analyzed by chromatin immunoprecipitation (ChIP). All of these methods are based upon the recognition of histone modifications by antibodies, and their specificity is critical for successful analysis.

The ChIP method can detect physical interactions between histones containing a specific modification and genomic DNA within a cell (Figure 9). The ChIP method is composed of four steps including: (i) preparation of fragmented chromatin from cells; (ii) immunoprecipitation by using a specific antibody; (iii) purification of immunoprecipitated (IP) DNA; (iv) analysis of IP DNA (Lee *et al.*, 2006). Fragmented chromatin is usually prepared by cross-linking DNA and histones by formaldehyde, followed by a fragmentation step by sonication or micrococcal nuclease. Immunoprecipitation is performed using a specific antibody, and then the immuno-complex of chromatin and antibody is collected and purified. IP DNA is analyzed by PCR of a specific genomic region, or by microarray or next-generation sequencers for a genome-wide analysis (Barski *et al.*, 2007; Lee *et al.*, 2006; Wang *et al.*, 2008).

## 5.3 Screening Methods for Epimutagens

A major reason why only a limited number of chemicals are reported to have epigenetic actions (see Section 4.6) is the lack of easy-to-use assay systems for chemicals' capacity to induce epigenetic alterations. For mutagens, there are various *in vitro* assays, using bacterial cultures or mammalian cells, and also *in vivo* assays using genetically-engineered animals (MacGregor, Casciano and Muller, 2000) (Table 3). In contrast, very limited assay systems are available for epimutagens. To construct an assay system for epimutagens, considerations should be given to what target genomic region is used as a marker for epigenetic effects, such as DNA demethylation and methylation, and what reporter

**Table 3.** Characteristics of assay systems for mutations and epigenetic alterations.

| | Mutation assays | Assays for epigenetic alterations |
|---|---|---|
| Bacterial system | *Reversion in S. typhimurium* (Ames test) | Essentially impossible |
| Mammalian cell | *HPRT* or *TK* mutations<br>Chromosome aberration test<br>Mouse lymphoma assay<br>Measurement of UDS | Under development (see text) |
| *In vivo* Assay | Micronucleus test<br>Mouse specific locus test<br>Tg mice for a marker gene (Big Blue, gpt-Δ, Muta-mouse etc.) | Not available yet |

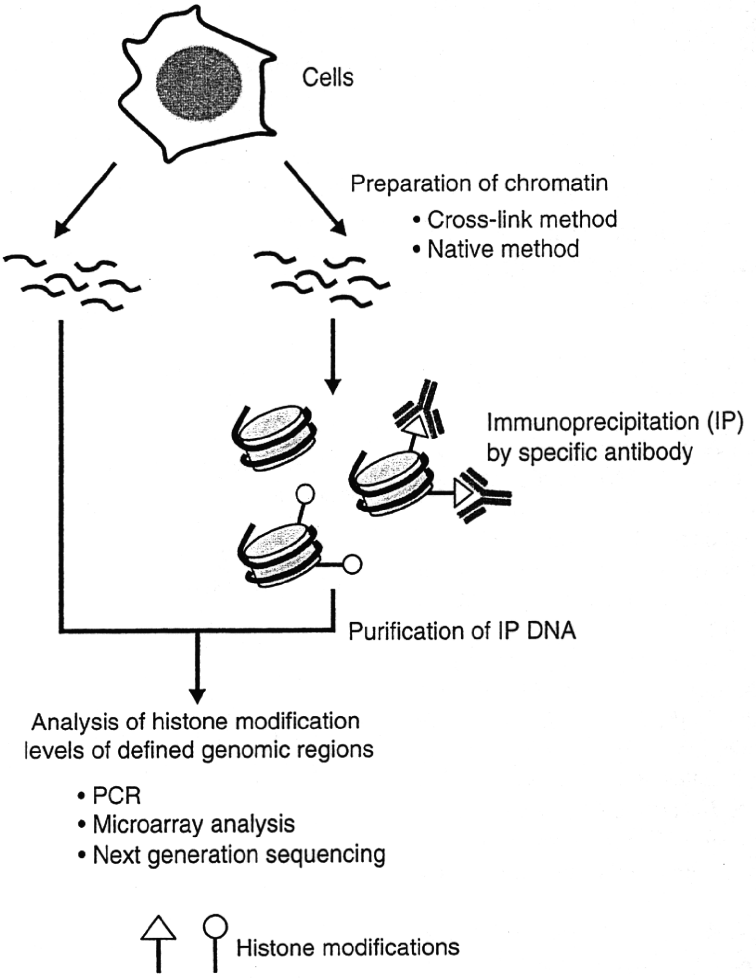


**Figure 9.** Principle of chromatin immunoprecipitation (ChIP). Fragmented chromatin is prepared, and then immunoprecipitated (IP) by using a specific antibody. DNA purified from the IP chromatin is used for analysis of histone modification levels for defined genomic regions by several technologies such as PCR, microarray, and next generation sequencing.

system is used. For screening purposes, a convenient and reliable assay system is essential.

So far, assay systems only for DNA demethylating agents have been reported. Three systems have been reported using a promoter of an exogenous gene and a reporter gene (Biard *et al.*, 1992; Cervoni and Szyf, 2001; Fan *et al.*, 2005). Among these, Fan *et al.*, 2005 successfully identified 5-bromo-2′-deoxyuridine (BrdU) as an anti-silencing agent without changing DNA methylation status. These exogenous promoters have a concern that they have epigenetic modifications different from endogenous genes. From this aspect, two assay systems are reported using a promoter of an endogenous gene (Okochi-Takada *et al.*, 2004; Oyer *et al.*, 2009). In addition to these efforts to use specific exogenous and endogenous promoters, hypomethylation of repeat sequences is also proposed as a precursor of toxicity (Carnell and Goodman, 2003).

## 6 EPILOGUE

Epigenomic alterations are important for cancer and possibly for other disorders. Nevertheless, epigenomic toxicology has just started, and scientists are not armed well yet. Application of findings in epigenetics and epigenomics to toxicology is now an exciting task.

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CHAPTER 8

# Analysis of Gene-specific DNA Methylation

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## INTRODUCTION

Gene- or region-specific DNA methylation analysis is necessary in various situations, and a variety of methods are available. It is important to become familiar with the characteristics of each technique, including the required amount of DNA, flexibility in selection of CpG sites to analyze, how quantitative the technique is, technical complexity, and the cost (Table 8.1). For example, if one wants to analyze DNA methylation as a cause of gene silencing, a specific region that controls gene expression should be analyzed [1], and a method with flexibility in selecting a region to analyze should be used. If one aims for diagnostic applications, a method that is highly accurate should be adopted.



In this chapter, we first introduce principles of DNA methylation analysis, and then summarize characteristics of individual methods. Finally, we will provide tips necessary to perform bisulfite sequencing, methylation-specific PCR (MSP), and quantitative MSP.

## PRINCIPLES OF DNA METHYLATION ANALYSIS

DNA methylation can be analyzed based on several principles that differentially recognize 5-methylcytosine ($C^m$) from cytosine (C). The first principle depends upon methylation-sensitive restriction enzymes whose activity is affected by the presence of a methyl group on a cytosine at a CpG site(s) within restriction sites (Fig. 8.1A). The vast majority of methylation-sensitive restriction enzymes, such as *Hpa*II and *Sma*I, are inactive on methylated CpG sites, but a unique methylation-sensitive restriction enzyme, *Mcr*BC, is inactive on unmethylated CpG sites. Differential cleavage can be detected by Southern-blot hybridization.

The second principle depends on bisulfite-mediated DNA conversion. This treatment converts unmethylated C into uracil (U) very rapidly, whereas it converts methylated C extremely slowly [2]. Under optimized conditions, a difference in methylation status of a CpG site can be converted into a difference of sequence, UpG or CpG. Once a difference of methylation status is converted into a difference of DNA sequence, it can be detected by various techniques, such as bisulfite sequencing, methylation-specific PCR (MSP), real-time MSP, combined bisulfite restriction analysis (COBRA), pyrosequencing, and MassARRAY® analysis (Table 8.1).

Third, methylated cytosines can be specifically recognized by an anti-methylcytidine antibody or a methylated DNA binding (MBD) protein. After appropriate shearing of DNA,

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**TABLE 8.1** Characteristics of Methods for Gene-specific Methylation Analysis

| | Amount of DNA Required | Flexibility in Selection of a Region Analyzed | Quantification | Ease of Use | Cost | Application |
|---|---|---|---|---|---|---|
| Southern-blot hybridization | Large | Low | No | Intermediate | Low | Detection of methylation/unmethylation at specific CpG sites |
| Bisulfite sequencing | Small | High | No | Intermediate | Low | Analysis of methylation pattern on individual DNA molecules |
| COBRA | Small | Low | Yes | Easy | Low | Detection of DNA molecules methylated/unmethylated at a specific CpG site |
| MSP | Small | High | No | Easy | Low | Detection of DNA molecules methylated/unmethylated at a specific region |
| Real-time MSP using SYBR Green I | Small | High | Yes | Easy | Low | Quantitative analysis of DNA molecules methylated/unmethylated at a specific region |
| MethyLight | Small | Very high | Yes | Easy | Intermediate | Quantitative analysis of DNA molecules methylated/unmethylated at a specific region |
| Pyrosequencing | Small | High | Yes | Intermediate | High | Quantitative methylation analysis of multiple CpG sites |
| MassARRAY® | Small | High | Yes | Easy | High | Quantitative methylation analysis of multiple CpG sites |

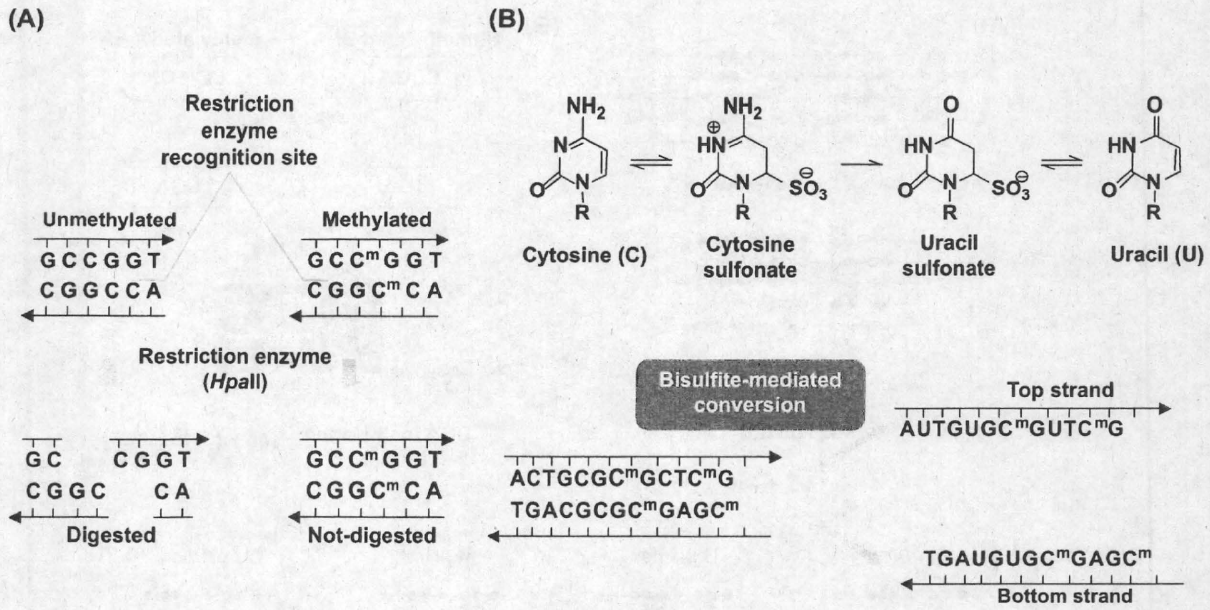


**FIGURE 8.1**

(A) Methods of DNA methylation detection. Detection by methylation-sensitive restriction enzymes. Genomic DNA is digested with a methylation-sensitive restriction enzyme (*Hpa*II in this figure) when its restriction site (CCGG) is unmethylated, but not digested when the site is methylated. Whether genomic DNA is digested or not represents the methylation status in the original DNA. $C^m$ stands for methylated cytosine. (B) Detection by bisulfite-mediated DNA conversion. Unmethylated cytosines are converted very rapidly into uracil by deamination whereas methylated cytosines are converted extremely slowly. Therefore, a difference in methylation status of a CpG site can be converted into a difference of sequence, UpG or CpG. After bisulfite-mediated DNA conversion, the upper and lower strands are no longer complementary.



methylated DNA can be collected using these affinity methods. This principle is mainly used for genome-wide screening techniques [3].

Fourth, the fraction of methylcytosine in the entire genomic DNA can be measured by HPLC or mass spectrometry [4]. Since this method does not contain sequence information, this can be used solely to measure global methylation levels.

## CHARACTERISTICS OF INDIVIDUAL TECHNIQUES

### Southern-blot Hybridization

Southern-blot hybridization for DNA methylation analysis is based on DNA digestion by a methylation-sensitive restriction enzyme and subsequent hybridization using a probe for a specific genomic region [5]. The methylation status of a restriction recognition site can be detected by monitoring the band positions of DNA fragments flanking the restriction sites. The advantage of this technique is its quantitative results reflecting the amounts of digested and undigested DNA molecules. Southern blot analysis is especially useful for analysis of repetitive sequences because multiple similar sequences in the genome can be analyzed by a single probe. On the other hand, this technique analyzes only a limited number of CpG sites located within restriction recognition sites, and requires a large amount of high-quality DNA. Although this technique was frequently used before bisulfite conversion-based techniques became popular, it has recently been used only occasionally.

### Bisulfite Sequencing

Bisulfite-converted DNA is amplified by PCR using primers located in genomic regions lacking CpG sites. The PCR product is then sequenced, usually after cloning of the PCR product, and CpG sites within the amplified region are interrogated (Fig. 8.2A) [6]. Cytosine

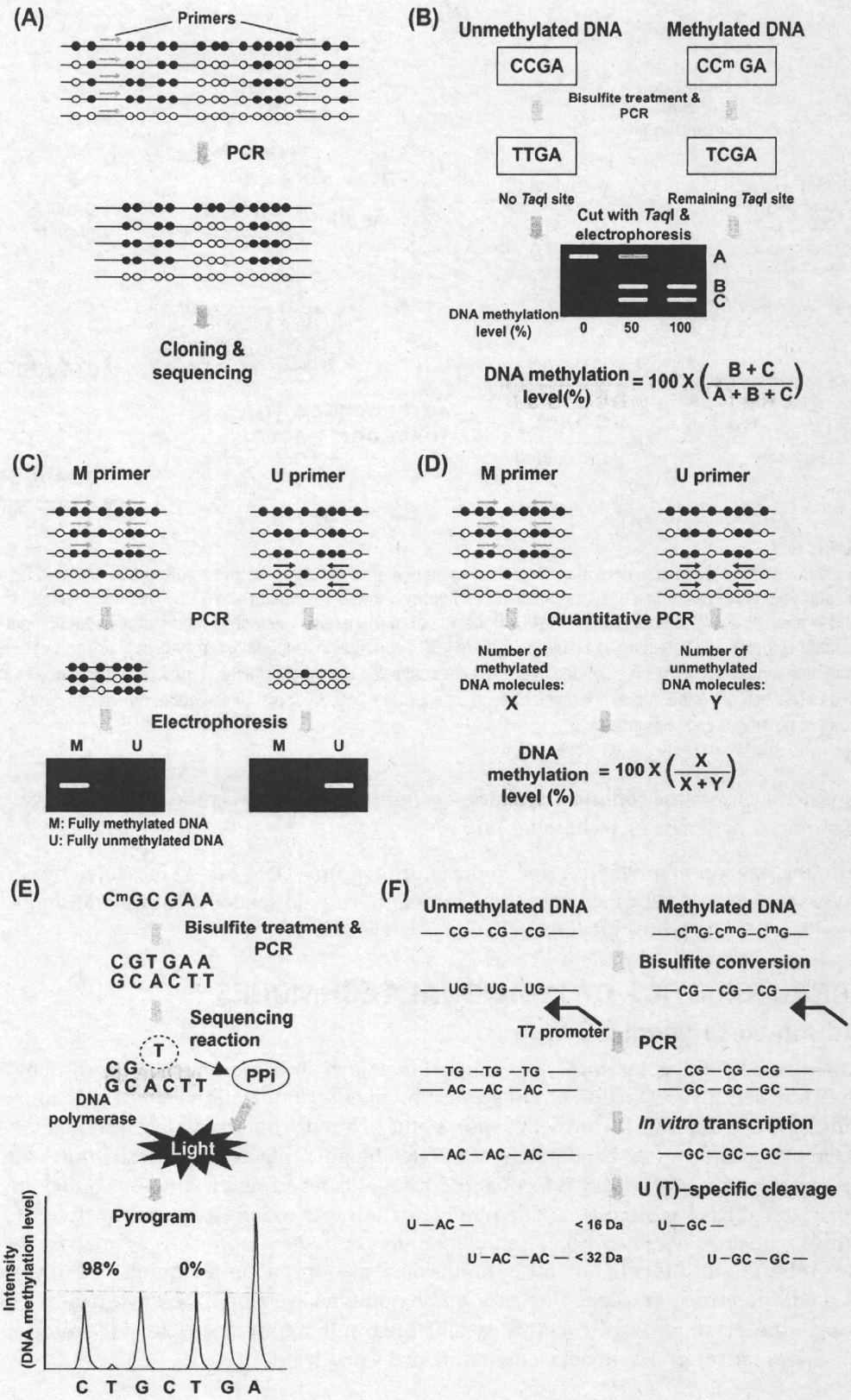


**FIGURE 8.2**

**Principles of individual techniques for DNA methylation analysis. Methylated and unmethylated CpG sites are shown by closed and open circles, respectively.** (A) Bisulfite sequencing. Bisulfite-converted DNA is amplified by PCR with primers covering no CpG sites. The PCR product is cloned, and individual clones are sequenced. This technique

(C) and thymine (T) at a CpG site in the converted DNA show methylated and unmethylated C, respectively, in the original DNA. This technique enables us to investigate the methylation status of every single CpG site between the primers, and how multiple CpG sites in a single DNA molecule are methylated. DNA methylation of almost any region can be analyzed using this method. A possible disadvantage is that this technique is labor-intensive, requiring that at least 10 clones per single sample be sequenced. There are also some technical pitfalls that will be described later.

### Combined Bisulfite Restriction Analysis (COBRA)

The COBRA technique is based on the appearance or disappearance of a restriction enzyme recognition site after bisulfite conversion (Fig. 8.2B) [7]. By quantifying the ratio of digested and undigested PCR products, the ratio of methylated and unmethylated DNA molecules can be quantified. This technique is suitable for detecting the methylation level of a CpG site quantitatively, and has the advantage of ease of procedure. Since multiple CpG sites within a small genomic region are coordinately methylated or unmethylated [4,8], analysis of a single CpG site can predict the methylation status of the surrounding region. A disadvantage is that CpG sites that can be analyzed by COBRA are limited.

Recently, a modified protocol for COBRA, Bio-COBRA, was developed [9]. Bio-COBRA incorporates an electrophoresis step of the digested PCR product in a microfluidics chip, such as Bioanalyzer (Agilent), and provides rapid and quantitative assessment of DNA methylation statuses in a large sample set.

## Methylation-specific PCR (MSP)

This technique interrogates methylation statuses of several CpGs at primer sites by performing PCR with primers specific to methylated or unmethylated sequences and observing the presence or absence of a PCR product (Fig. 8.2C) [10]. If both forward and reverse primer regions are methylated, intervening CpG sites are also likely to be methylated. DNA molecules with mosaic methylation patterns at primer sites are not amplified. This technique has high flexibility in selecting a genomic region to analyze because PCR primers can be designed at arbitrary positions, even if the region to be analyzed is CpG-rich, and it is technically simple. At the same time, MSP can easily produce false positive and false negative results. Therefore, it is critically important to use the optimal number of PCR cycles and annealing temperatures with appropriate negative controls, which will be described in the third section of this chapter.



### Real-time MSP and MethyLight

Real-time MSP is performed by real-time detection of MSP products. By comparing amplification of test samples with standard samples that contain known numbers of DNA

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provides a methylation pattern of individual DNA molecules at single CpG resolution. (B) COBRA. Bisulfite-converted DNA is amplified by PCR with primers covering no CpG sites, and the PCR product is digested with a restriction enzyme (*TaqI* in this figure). In the COBRA assay shown here, if the cytosine in the CpG site is methylated, the restriction site will remain. On the other hand, if the site is unmethylated, the restriction site will disappear. Quantitative analysis of methylation levels is achieved by subsequent gel electrophoresis and measurement of cleaved and uncleaved bands. (C) MSP. Methylation statuses at several CpGs within primer sequences are interrogated by performing PCR with primers specific to methylated or unmethylated templates and monitoring the presence or absence of a PCR product. PCR conditions are optimized using fully methylated DNA and fully unmethylated DNA. (D) Real-time MSP. The numbers of methylated and unmethylated DNA molecules are quantified by real-time MSP. (E) Pyrosequencing. C/T polymorphisms in the PCR product are investigated by measuring pyrophosphate released at individual sites. The amount of pyrophosphate is converted into a light signal, and then shown as a pyrogram. (F) MassARRAY®. The PCR product amplified from bisulfite-converted DNA is transcribed *in vitro*, and cleaved by RNase A. The difference in the mass of a product with C and that with T (16 Da) is detected by MALDI-TOF mass spectrometry.

molecules, numbers of methylated and unmethylated DNA molecules can be quantified (Fig. 8.2D). A methylation level can be calculated based on these numbers of DNA molecules. PCR products can be detected by an intercalating dye like SYBR® Green I (real-time MSP), or by a TaqMan probe (MethyLight) [11]. Since a TaqMan probe anneals only to a specific sequence (methylated or unmethylated sequence), MethyLight has higher specificity than quantitative MSP although a TaqMan probe is costly. Intercalating dye can detect even non-specifically amplified DNA and primer dimers, and confirmation of specific amplification by melting analysis of the PCR product is essential. It is reported that the use of a new fluorescent dye, such as SYTO-82, can produce more accurate melting results [12]. The real-time MSP and MethyLight techniques have a lot of flexibility in selecting a genomic region to analyze, as does MSP, and are accurate and sensitive in quantifying DNA methylation levels. The high accuracy and sensitivity of these techniques make them suitable for analysis of a large number of clinical samples.

### Pyrosequencing

Pyrosequencing detects methylation levels of individual CpG sites in a PCR product obtained by primers common to methylated and unmethylated sequences after bisulfite conversion. The amounts of C and T at individual sites are converted into the amounts of pyrophosphates released using the primer extension method, and their amounts are accurately quantified bioluminometrically using the Pyrosequencer system (QIAGEN) (Fig. 8.2E). The advantages of pyrosequencing are its accurate quantitative results and ease of daily procedure. However, design of suitable primers is difficult, depending upon the local sequence, and an instrument specifically designed for this analysis is unavoidably necessary.

### MassARRAY®



MassARRAY® also detects methylation levels of individual CpG sites in a PCR product using primers common to methylated and unmethylated sequences after bisulfite conversion. In this technique, the PCR is performed with a reverse primer coupled with a T7 promoter tag. The PCR product is transcribed *in vitro* using a single dNTP analog, which can be substituted for its rNTP. The *in vitro* transcript is then cleaved by RNase A, which digests at pyrimidine bases, in a base-specific manner (Fig. 8.2F). If dCTP was used during the *in vitro* transcription, the RNase A will cleave at every uracil. A difference in the mass of product with C and that with T (16 Da) is detected by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. MassARRAY® is a powerful technique to quantitatively investigate DNA methylation statuses of multiple CpG sites in a large number of samples, but has a disadvantage in the cost of the instrument.

## TIPS FOR BISULFITE SEQUENCING

Bisulfite sequencing is capable of analyzing detailed DNA methylation patterns of individual DNA molecules in given regions of the genome. It also provides quantitative information on the ratio of methylated and unmethylated DNA molecules. At the same time, although this technique is generally considered as technically simple, caution must be exercised to obtain unbiased results.

### PCR Conditions for Unbiased Amplification

It is well known that, depending upon PCR conditions, there can be a PCR bias that leads to preferential amplification of either unmethylated or methylated DNA [13,14]. In most cases, unmethylated DNA is preferentially amplified, but methylated DNA can be preferentially amplified with specific primers [13]. To avoid this PCR bias, a PCR condition that equally amplifies fully methylated and fully unmethylated DNA controls should be established by selecting an optimal primer set and an optimal annealing temperature (Fig. 8.3A) [14].

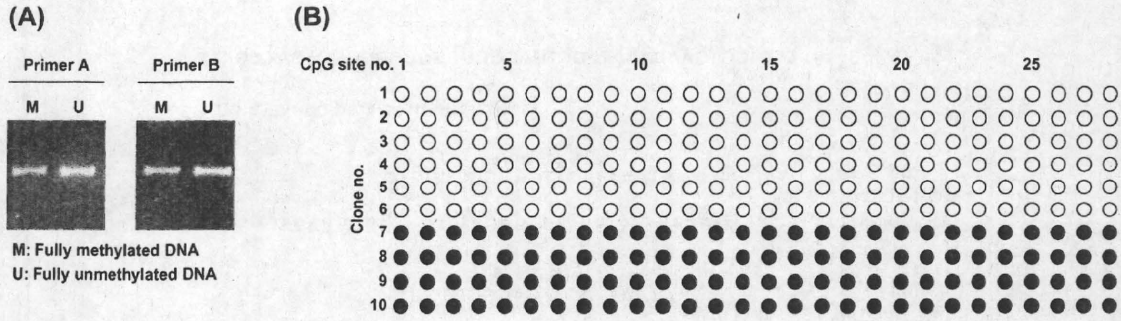


**FIGURE 8.3**

**Optimization of bisulfite sequencing.** (A) Comparison of two primer sets for bisulfite sequencing. The influence of primers on PCR efficiency was examined using fully methylated DNA (S) and fully unmethylated DNA (G). Primer A predominantly amplified unmethylated DNA, whereas primer B equally amplified both methylated and unmethylated DNA. (B) Confirmation of unbiased amplification. Methylated and unmethylated cytosines are shown by closed and open circles, respectively. The proportion of methylated clones was 40%, indicating appropriate PCR conditions and unbiased amplification were achieved.

Fully methylated DNA can be prepared by treatment of DNA with *Sss*I methylase (*Sss*I), and fully unmethylated DNA can be prepared by amplifying normal DNA with a GenomiPhi DNA amplification kit (GenomiPhi). When accurate estimation of the ratio of methylated and unmethylated DNA is necessary, control DNA containing an equal number of fully methylated and unmethylated DNA molecules should be prepared by mixing such DNA, and simultaneously analyzed to obtain a ratio of 40% to 60% (Fig. 8.3B).

### PCR Cycles to Avoid Artifacts

Even if optimal PCR conditions are used, PCR cycles should be minimized as long as a sufficient amount of a PCR product for cloning is obtained. Excessive PCR cycles cause denaturation of the PCR product in the absence of Taq polymerase activity, and produce the amplification of chimeric products and even PCR products that were not present in the template DNA. Excessive PCR cycles also exaggerate the difference in PCR efficiency between methylated and unmethylated DNA.



## TIPS FOR MSP AND QUANTITATIVE MSP

MSP is flexible in selecting regions for analysis and can be performed with ease and at a low cost. Real-time MSP provides accurate, sensitive, and quantitative assessment of DNA methylation levels. Under good conditions, DNA methylation levels obtained by real-time MSP have a variation ≤20% of the mean methylation level. To maximize these advantages, there are some tips for conducting MSP and real-time MSP.

### Primer Design

A genomic region should be carefully selected as in other analyses, and primers specific to methylated or unmethylated DNA should be designed in the same region. The 3′ end of a primer should be located at a polymorphic C/T site, and multiple CpG sites should be located near the 3′ end (Fig. 8.4A). Difficulty in designing primers specific to unmethylated DNA is frequently encountered, and use of the other DNA strand (bottom strand) is often helpful.

### PCR Conditions for Specific Amplification

The annealing temperature and magnesium concentration should be optimized using the fully methylated and fully unmethylated DNA controls. A good condition for primers specific to methylated DNA shows ample amplification of fully methylated DNA and no

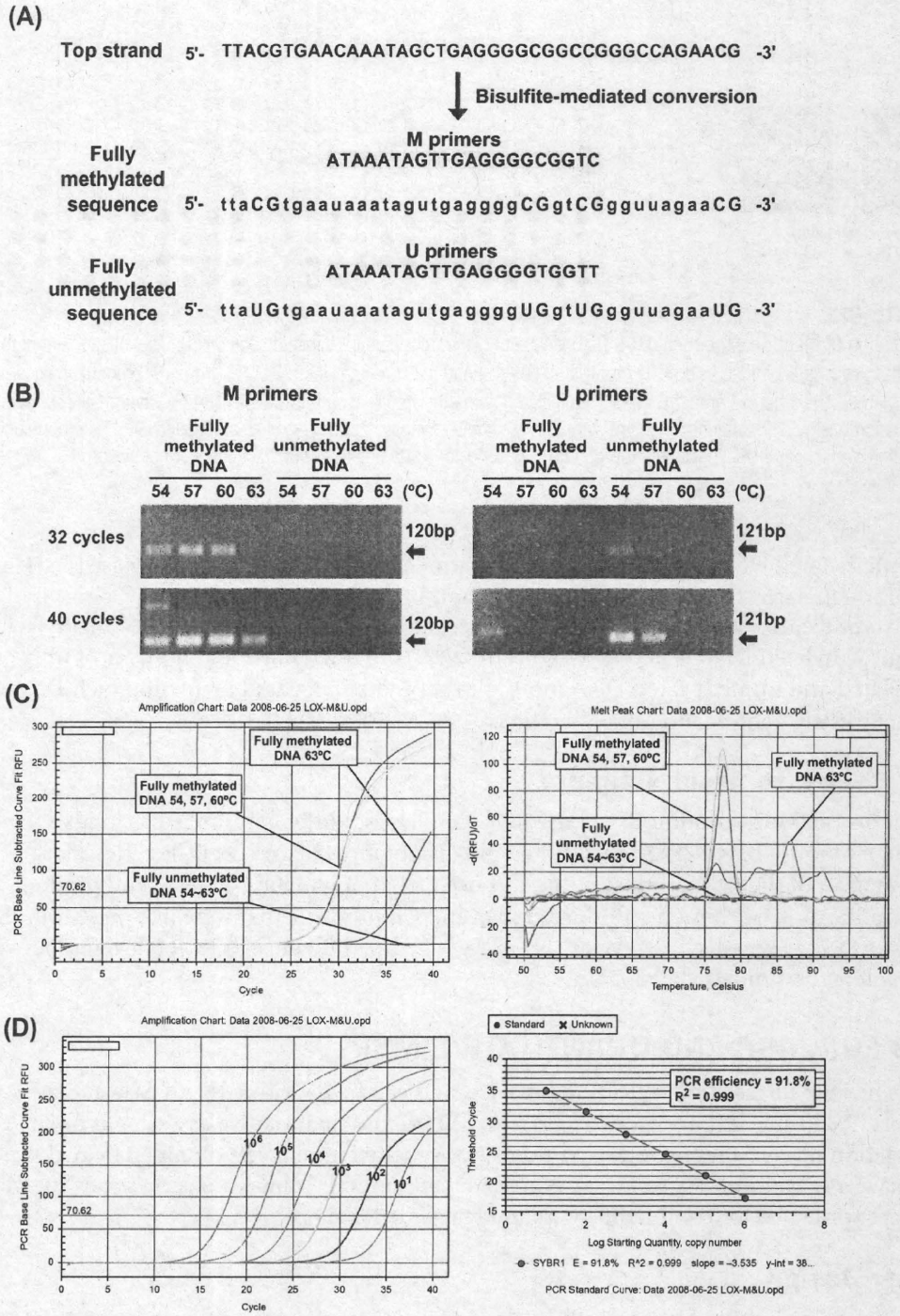


**FIGURE 8.4**

**Optimization of real-time PCR conditions.** (A) Primer design for MSP and real-time MSP. Primers specific to methylated and unmethylated DNA (M and U primers, respectively) should contain multiple CpG sites near and at their 3′ ends, and are desirably located in the same region. (B) Optimization of the annealing temperature for MSP. For the M primer, annealing temperatures of 57 and 60°C did not amplify fully unmethylated DNA, but amplified fully methylated DNA with good efficiency. For the U primer, only an annealing temperature of 57°C yielded specific and efficient amplification. (C) Optimization of the annealing temperature for real-time MSP. The real-time PCR amplification curve showed high PCR efficiency under annealing temperatures of 54, 57, and 60°C. The melting curve showed a single peak, thus specific amplification, under annealing temperatures of 54, 57, and 60°C. If multiple good annealing temperatures are available, a higher temperature is preferable for specificity. Optimal conditions in real-time MSP are occasionally different from those in MSP even if the same primer set is used. (D) Real-time MSP using standard DNA. Correlation using multiple standard DNA (R2) was >0.98, and PCR efficiency was >80%.

amplification of fully unmethylated DNA (Fig. 8.4B). A good condition for primers specific to unmethylated DNA amplifies fully unmethylated DNA, but not fully methylated DNA.

In the case of real-time MSP, the best conditions can be determined by the amplification curve and the melting curve (Fig. 8.4C). The amplification curve under good conditions shows a steep rise at an early PCR cycle, and a flat plateau. The melting curve under the best PCR conditions shows a single sharp peak.

### Preparation of Standard DNA

To quantify DNA methylation levels by real-time MSP, standard DNA with known numbers of DNA molecules is necessary. This can be prepared in two ways. First, the PCR product can be purified by a gel-filtration column to remove unused nucleotides and primers. Second, the PCR product of MSP is cloned into a plasmid, and the plasmid is linearized by a restriction enzyme. Since the molecular weight of the PCR product or the plasmid with the insert can be calculated, the number of DNA molecules in a measured weight of solution can be calculated. Preparation of standard DNA by cloning a PCR product has the advantage of accuracy and availability of a large amount of standard DNA, but has the disadvantage of being a complex procedure.

### Quantity of Template DNA

Both MSP and real-time MSP can achieve high sensitivity, such as detecting one methylated DNA molecule among 1000 molecules. However, substantial loss in the number of DNA molecules that can serve as a PCR template takes place during bisulfite-mediated conversion. Namely, although the weight of DNA decreases only slightly, the number of template DNA molecules measured by quantitative PCR decreases down to 5 to 10% of DNA before the treatment [15]. Therefore, caution must be exercised as to how many copies of template DNA are present in a PCR solution. Supposing that one human haploid genome weighs 3.6 pg and that 10% of DNA molecules are recovered as a template for PCR after bisulfite-mediated conversion, only 28 molecules are available for PCR of a single target sequence in a DNA sample that originated from 1 ng of genomic DNA before bisulfite treatment. If one wants to have a sensitivity of 1%, 1000 molecules (10 methylated molecules) in a PCR solution will be necessary, and this corresponds to 36 ng DNA in a reaction.



## EPILOGUE

Regional DNA methylation analysis is applied not only for basic research but also for diagnostic purposes. Selecting an appropriate technique and conducting experiments under good conditions are required to obtain reliable data. We hope that this chapter will help investigators to select appropriate techniques.

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