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High-Speed Conversion of Cytosine to Uracil in Bisulfite Genomic Sequencing Analysis of DNA Methylation

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Abstract

Bisulfite genomic sequencing is a widely used technique for analyzing cytosine-methylation of DNA. By treating DNA with bisulfite, cytosine residues are deaminated to uracil, while leaving 5-methylcytosine largely intact. Subsequent PCR and nucleotide sequence analysis permit unequivocal determination of the methylation status at cytosine residues. A major caveat associated with the currently practiced procedure is that it takes 16–20 hr for completion of the conversion of cytosine to uracil. Here we report that a complete deamination of cytosine to uracil can be achieved in shorter periods by using a highly concentrated bisulfite solution at an elevated temperature. Time course experiments demonstrated that treating DNA with 9 M bisulfite for 20 min at 90°C or 40 min at 70°C all cytosine residues in the DNA were converted to uracil. Under these conditions, the majority of 5-methylcytosines remained intact. When a high molecular weight DNA derived from a cell line (containing a number of genes whose methylation status was known) was treated with bisulfite under the above conditions and amplified and sequenced, the results obtained were consistent with those reported in the literature. Although some degradation of DNA occurred during this process, the amount of treated DNA required for the amplification was nearly equal to that required for the conventional bisulfite genomic sequencing procedure. The increased speed of DNA methylation analysis with this novel procedure is expected to advance various aspects of DNA sciences.

Key words: ammonium bisulfite; CpG island; epigenetics; 5-methylcytosine; sodium bisulfite

1. Introduction

The epigenetic regulation of gene activity is currently of great interest in biological research. Many modifications in chromatin structure are related to epigenetic regulation of gene activity. Of these, DNA methylation is considered to be important. Methylation of vertebrate DNA occurs at position 5 of cytosine moieties, predominantly in CpG dinucleotide sequences. Various methods have been developed to investigate the methylation status of specific genomic sequences.[1–3] Based on the underlying principles, these methods may be divided into three groups: (I) use of restriction endonuclease(s) whose activity is influenced by methylation of the recognition site, (II) use of chemical reactions that modify either cytosine or 5-methylcytosine, and (III) use of a protein that has different affinity to methylated and nonmethylated DNA.[1–3]

Of these, bisulfite genomic sequencing (group II) is a method most frequently used.[4] When nonmethylated cytosine is treated with sodium bisulfite, it is deaminated to form uracil.[5–7] 5-Methylcytosine reacts only very weakly to this reagent and remains largely intact.[7] Subsequent PCR amplification and nucleotide sequence analysis permit unequivocal discrimination of cytosine and 5-methylcytosine in genomic DNA.[4] However, there are a number of disadvantages to the currently used bisulfite genomic sequencing procedure. One of these is that the procedure is time consuming, often taking 16–20 hr to achieve a complete conversion of cytosine to uracil, although conversion of cytosine to uracil in 4 hr has been described.[8] As DNA methylation analysis is of potential clinical significance,[9] an accelerated conversion would be desirable for clinical applications as well as for basic science research.

We have previously reported that treatment of DNA with higher concentrations of bisulfite at elevated temperatures greatly enhances the conversion of cytosine to uracil, keeping 5-methylcytosine largely intact.[10] The underlying principle of this enhanced conversion is that

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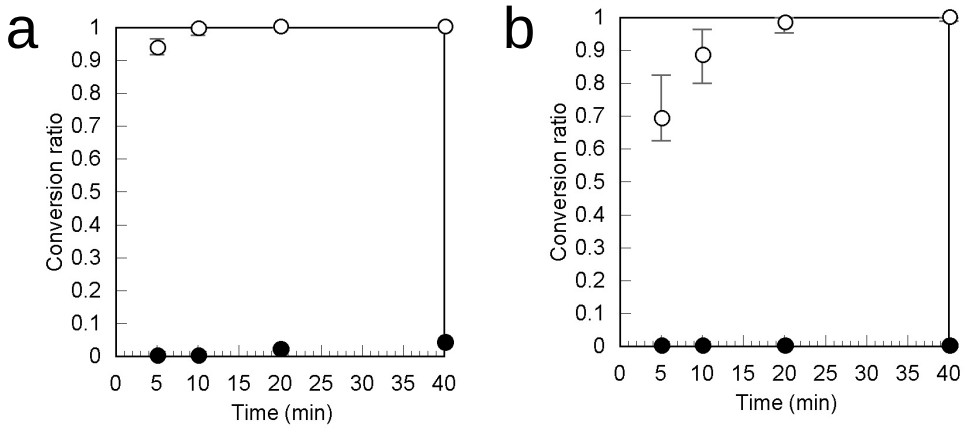

Figure 1. The analysis of the efficiency of the conversion. (a) Treatment with 9 M bisulfite at 90°C and (b) treatment at 70°C. Open circles indicate the ratio of conversion of nonmethylated cytosine to uracil per plasmid clone (e.g., the value 0.9 indicates 72 cytosine residues out of 80). Closed circles indicate the ratio of deaminated 5-methylcytosine in the entire population of plasmid clones that were sequenced (e.g., the value 0.02 indicates a total of 1 out of 50 5-methycytosines). Similar experiments were performed again using independently treated samples and a similar efficiency of conversion was confirmed (data not shown).

the rate of hydrolytic deamination of sulfonated cytosine, which is a rate-determining step of the overall reaction, is approximately proportional to the bisulfite concentration.[11] The uppermost concentration of sodium bisulfite attainable is approximately 5 M. However, we have found that a combined use of sodium bisulfite and other sulfite compounds permits the formation of solutions containing higher concentrations of bisulfite.[10] We prepared 10 M bisulfite solution by mixing sodium bisulfite, ammonium sulfite, and ammonium bisulfite.

In the current study, we have investigated the use of this 10 M bisulfite solution in the genomic sequencing analysis of various DNAs. Plasmid and human genomic DNAs were analyzed in terms of the efficiency of conversion (cytosine to uracil) and DNA degradation during this treatment.

2. Materials and Methods

2.1. Treatment of DNA with bisulfite

High molecular weight DNA of the MCF-7 cell line was prepared as described previously.[12] Plasmid pUCMyc65 DNA (accession no. X00364, positions 1–8078, cloned in *Eco*RI/*Hin*dIII sites of the plasmid vector pUC19) was digested with *Eco*RI/*Hin*dIII (New England BioLabs) and MCF-7 DNA was digested with *Tsp*509I (New England BioLabs). DNA was recovered by ethanol precipitation after phenol-chloroform extraction. A conventional bisulfite modification reaction was performed as described previously[13] with slight modifications. Briefly, 4 μg of the digested DNA was denatured in 50 μl of freshly prepared 0.3 N NaOH for 30 min at 37°C. The solution was mixed with 500 μl of 4 M sodium bisulfite/1 mM hydroquinone. Mineral oil was overlayed and the sample was incubated for 20 hr at 55°C in the dark. DNA was recovered using Wizard DNA Clean-Up System (Promega) and dissolved in 90 μl of water. The solution was mixed with 11 μl of freshly prepared 0.2 N NaOH and the mixture was incubated for 10 min at 37°C. DNA was recovered by adding 150 μl of 4 M ammonium acetate (pH 7.0), 10 μg of yeast tRNA (Sigma), and 750 μl of ethanol. The precipitate containing the DNA was dissolved in 50 μl (for plasmid DNA) or 16 μl (for MCF-7 DNA) of 10 mM Tris•HCl/1 mM EDTA (TE, pH 7.5). The preparation of 10 M bisulfite solution was as described previously.[10] Briefly, 2.08 g $NaHSO_3$ (Wako), 0.67 g $(NH_4)_2SO_3 \bullet H_2O$ (Wako), and 5.0 ml 50% $(NH_4)HSO_3$ (Wako) were mixed and heated at 90°C to obtain a solution of pH 5.2–5.3 (measured at ambient temperature). Treatment of DNA using 10 M bisulfite solution was performed as follows: 545 μl of 10 M bisulfite solution (maintained at 70°C) or 565 μl (maintained at 90°C) was added to the alkali-denatured DNA solution (50 μl) and the mixture was divided evenly into four tubes. Each mixture was incubated for a designated period in a water bath maintained at either 70°C or 90°C (mineral oil-overlaying was not performed, nor was hydroquinone addition). DNA was recovered and dissolved in an appropriate volume of TE so that the DNA concentration was equal in all samples.

2.2. PCR analysis and cloning of bisulfite-treated plasmid DNA

Bisulfite-treated pUCMyc65 was diluted serially (20 ng/ml to 20 pg/ml, 10-fold at each dilution) by TE containing 0.2 mg/ml of yeast tRNA. A region corresponding to positions 6209–6522 (accession no. X00364) was amplified by PCR. The PCR primers used in the experiments (shown in Figs. 1 and 2) were 5′-cggaattcTGGTGAGAGGAGTAAGGGTGG-3′ and 5′-cgggatccACAAAATTATCTCCCCAAA-3′ (lower case letters show adaptor sequences for restriction endonu-

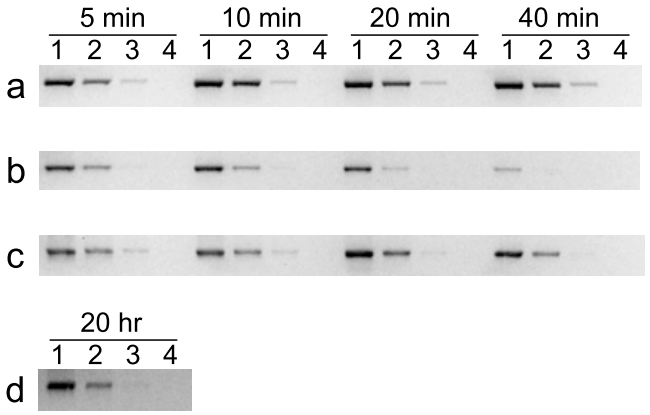

Figure 2. The analysis of the degradation of DNA. Twenty nanograms (lane 1), 2 ng (lane 2), 200 pg (lane 3), and 20 pg (lane 4) of bisulfite-treated and non-treated plasmid DNAs were subjected to PCR. (a) *Eco*RI/*Hin*dIII digests of pUCMyc65 treated with alkaline were recovered by ethanol precipitation. The recovered DNA (in neutral buffer) was incubated at 90°C for the indicated periods and recovered, treated with alkali, and precipitated with ethanol. (b) DNA fragments treated with bisulfite solution at 90°C. (c) DNA fragments treated with bisulfite solution at 70°C. (d) DNA fragments treated conventionally (3.6 M bisulfite, 20 hr at 55°C) and recovered, treated with alkali and precipitated with ethanol. (a)–(d) represent negative images of ethidium-stained gels manipulated by computer imaging software Adobe Photoshop® 7.0. Similar experiments were performed again using independently treated samples and a similar mode of degradation of DNA was confirmed (data not shown).

cleases). After incubation for 3 min at 95°C, 35 cycles of amplification (30 sec at 95°C and 1 min at 60°C) using AmpliTaq DNA polymerase Stoffel fragment (Applied Biosystems) was performed (the reaction conditions were those recommended by the manufacturer). Twenty nanograms, 2 ng, 200 pg, or 20 pg of treated DNA was used as a template. PCR products obtained using 20 ng of treated DNA was submitted to further analysis. The PCR product was then digested with *Eco*RI/*Bam*HI and the fragments were purified by agarose gel electrophoresis. The fragments obtained were cloned into *Eco*RI/*Bam*HI sites of the plasmid vector pUC119 (TaKaRa). Ten independent plasmid clones were selected and their nucleotide sequences were determined.

2.3. PCR analysis and cloning of bisulfite-treated high molecular weight DNA

Bisulfite-treated MCF-7 DNA was serially diluted by TE containing 1.25 mg/ml of yeast tRNA. After incubation for 3 min at 95°C, 20 cycles of the initial amplification (30 sec at 95°C; 30 sec at 55°C; and 30 sec at 72°C) using AmpliTaq DNA polymerase Stoffel fragment was performed. The reaction was "hot-started" as described.[13] Five hundred nanograms, 50 ng, 5 ng, or 500 pg of treated DNA was used as a template. PCR primers used for the initial amplification were CDH1-L1 and CDH1-R1 (*CDH1*) and RASSF1A-L1 and RASSF1A-R1 (*RASSF1A*) (Table 1). A semi-nested PCR was performed using 2 μl (*CDH1*) or 6 μl (*RASSF1A*) of the initial reaction mixture with conditions similar to those described above, except that a 30-cycle amplification was applied. PCR primers used in the semi-nested PCR were CDH1-L2 and CDH1-R2 (*CDH1*) and RASSF1A-L1 and RASSF1A-R2 (*RASSF1A*) (Table 1). PCR products obtained using 500 ng of treated DNA were subjected to nucleotide sequence analysis. Twelve independent plasmid clones were selected and their nucleotide sequences were determined.

3. Results and Discussion

3.1. Efficiency of conversion of cytosine to uracil

Ten volumes of 10 M bisulfite was mixed with one volume of DNA solution so that the final concentration of bisulfite was approximately 9 M. Representative results are shown in Fig. 1. One microgram of *Eco*RI/*Hin*dIII digest of plasmid pUCMyc65 was treated with bisulfite at different temperatures for different periods of time (Fig. 1). An appropriate region (positions 6209–6522) was amplified by PCR, and the nucleotide sequences of 10 clones were determined after cloning of the PCR product. The region contains 85 cytosine residues, 5 of which are known to be methylated (*Bst*NI sites). When the digest was treated at 90°C for a 5-min incubation, more than 90% of the cytosine residues in the analyzed region were converted to uracil (Fig. 1a). After a 10-min incubation, 99% of cytosine residues were converted to uracil (Fig. 1a) while a 20-min incubation resulted in 100% conversion (Fig. 1a). 5-Methylcytosine remained unmodified after the 10-min incubation (Fig. 1a). Of the 50 residues of 5-methylcytosine (five loci, 10 plasmid clones), one was converted to thymine at 20 min and two at 40 min (Fig. 1a). These results demonstrate that a complete conversion of cytosine to uracil can be achieved within 20 min without significantly influencing 5-methylcytosine.

When the DNA was treated at 70°C, a reduced rate of conversion was observed (Fig. 1b). After incubation for 5, 10 and 20 min, 70%, 88% and 98% cytosine residues were deaminated to uracil, respectively (Fig. 1b). All cytosine residues were converted to uracil after 40 min (Fig. 1b) and 5-methylcytosine remained completely unmodified even after 40 min (Fig. 1b).

3.2. Degradation of DNA

DNA degradation under acidic conditions is an additional potentially serious problem in the current practice of the bisulfite genomic sequencing technique.[14] Therefore, we examined how much DNA was degraded after our newly devised treatment. DNA samples were serially

Table 1. PCP primers used in the study shown in Figures 3 and 4.

Gene	Name of primers	Sequence (5' to 3')[a]	Positions (accession no.)
CDH1	CDH1-L1	ATTTAGTGGAATTAGAATAGTGTAGGTTTT	(791–820, L34545)
	CDH1-R1	CTACAACTCCAAAAACCCATAACTAAC	(1139–1165, L34545)
	CDH1-L2	cggaattcTTAGTAATTTTAGGTTAGAGGG	(837–858, L34545)
	CDH1-R2	cgggatcCTACAACTCCAAAAACCCATAACTAAC	(1139–1165, L34545)
RASSF1A	RASSF1A-L1	cggaattcGTTTTGGTAGTTTAATGAGTTTAGGTTTTTT	(18092–18122, AC002481)
	RASSF1A-R1	ACCCTCTTCCTCTAACACAATAAAACTAACC	(17741–17771, AC002481)
	RASSF1A-R2	cgggatCCCCACAATCCCTACACCCAAAT	(17918–17940, AC002481)

[a]Lower cases indicate adapter sequences for restriction endonucleases.

diluted and used for PCR, and minimum DNA concentrations that give PCR products were compared. Firstly, DNA was treated only with TE. Using a DNA sample incubated for 5 min at 90°C as a template, PCR product was detected when 200 pg of template DNA was applied (Fig. 2a, lane 3). PCR product was consistently detected using 200 pg of template DNA when samples treated with the buffer for longer periods were used (Fig. 2a). These results suggest that incubation for 40 min at 90°C at neutral pH does not cause degradation of DNA.

Using a DNA sample treated with 9 M bisulfite for 5 min at 90°C as a template, PCR product was detected only when 2 ng or a greater amount of DNA was applied (Fig. 2b, lanes 1 and 2). This result shows that a significant portion of DNA was degraded after a 5 min treatment with 9 M bisulfite at 90°C. A similar pattern was observed when DNA samples treated with 9 M bisulfite at 90°C for 10 and 20 min were used as template. Using a DNA sample treated with 9 M bisulfite for 40 min at 90°C, PCR product was detected only when 20 ng of template DNA was applied (Fig. 2b, lane 1). These results show that DNAs were gradually degraded and severely damaged after 40 min treatment. Using DNA samples treated with 9 M bisulfite for 5 min or 10 min at 70°C, PCR products were detected when 200 pg of template DNA was applied (Fig. 2c, lane 3). Using DNA samples treated with 9 M bisulfite for 20 min or 40 min at 70°C, PCR products were clearly detected when 20 ng and 2 ng of template DNA were applied (Fig. 2c, lanes 1 and 2). These results show that DNAs were less severely damaged when treated with 9 M bisulfite at 70°C compared to 90°C. Using a DNA sample obtained after conventional bisulfite modification (3.6 M bisulfite, 20 hr, 55°C), PCR product was detected when 20 ng and 2 ng of template DNA was applied (Fig. 2d, lanes 1 and 2). These results suggest the DNA damage after incubation with 9 M bisulfite for 40 min at 70°C is similar to the damage occurring in the conventional treatment.

Real-time PCR experiments were performed to provide a quantitative measure. However, consistent results were not obtained, presumably due to the presence of long consecutive A or T sequences that potentially hamper accurate PCR (data not shown).

We predicted that shorter periods of incubation would keep DNA more intact than conventional longer treatment. However, we found that after shorter periods of incubation with a higher concentration of bisulfite and at a higher temperature, DNA had undergone degradation as extensively as in the conventional treatment. We detected no pH change of the reaction solutions during the incubations. These results suggest that the degradation of DNA proceeds by a mechanism which differs from acidic depurination caused by oxidative decay of bisulfite. This problem must be overcome by further studies to enable DNA methylation analysis using smaller amounts of DNA obtainable from samples such as microdissected tissues, forensic materials, and even of DNA from a single cell.

3.3. Treatment of high molecular weight DNA with high concentrations of bisulfite

High molecular weight DNA obtained from MCF-7, a human breast cancer cell line, was digested with restriction endonuclease *Tsp*509I. After alkaline treatment, the denatured DNAs were treated with 9 M bisulfite for 20 min at 90°C or for 40 min at 70°C. It is reported that the CpG island of the *CDH1* gene and that of the *RASSF1A* gene are unmethylated and methylated in MCF-7 cells, respectively.[13,15] We investigated whether the methylation status of these CpG islands can be reproduced after 9 M bisulfite treatment.

After treatment with 9 M bisulfite, a 280-base pair (bp) fragment containing a part of exon 1 of the *CDH1* gene

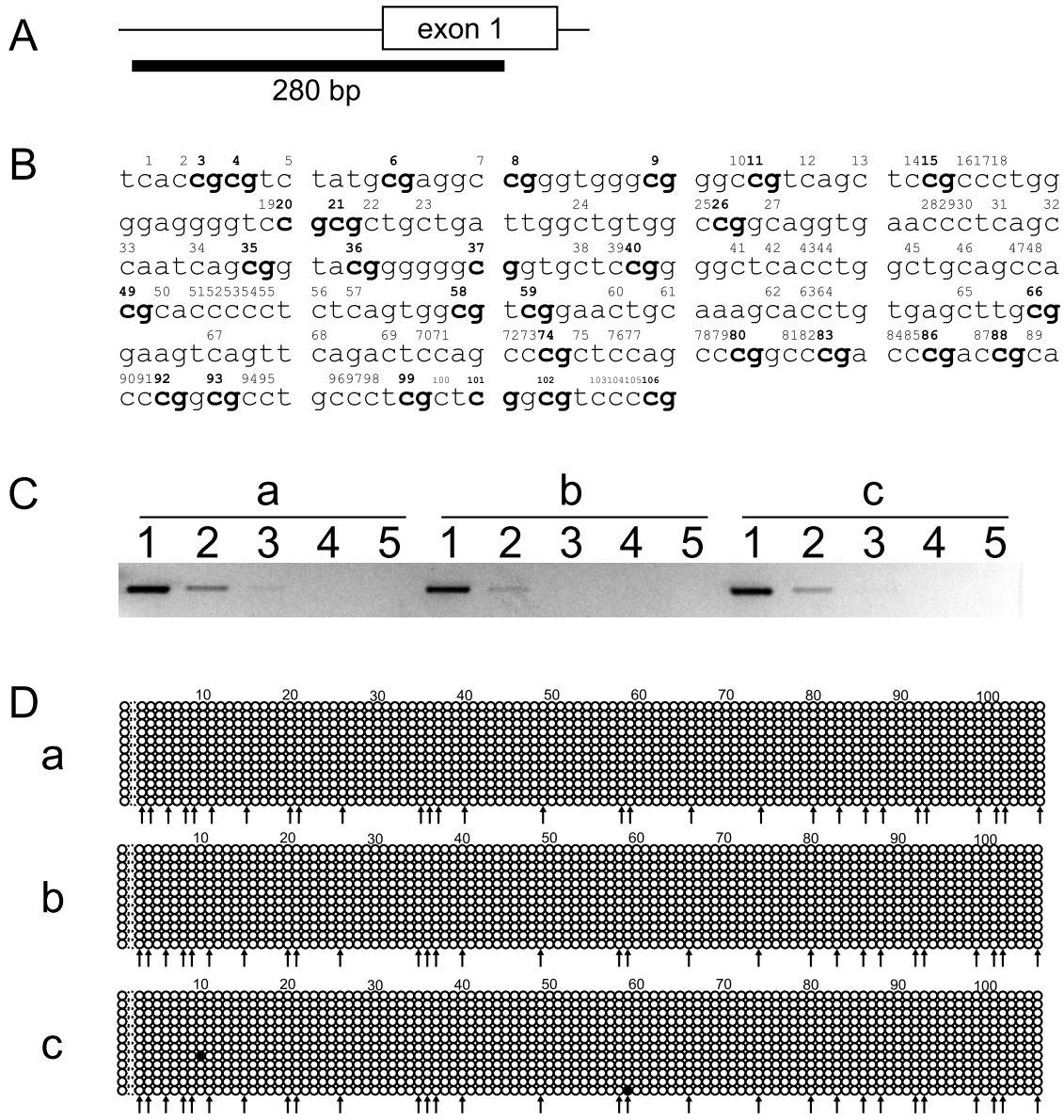

Figure 3. Bisulfite genomic sequencing analysis of the *CDH1* gene in MCF-7 cells. (A) The genomic region that was amplified. (B) Nucleotide sequence of the region. Bold characters indicate CpG dinucleotides. (C) Serial dilution of the template DNA. a, conventional treatment (3.6 M bisulfite, 20 hr, 55°C); b, 20 min at 90°C; c, 40 min at 70°C. Five hundred nanograms (lane 1), 50 ng (lane 2), 5 ng (lane 3), 500 pg (lane 4), and 50 pg (lane 5) of DNA was used as a template. (D) Nucleotide sequence analysis of plasmid clones. Each row indicates an independent plasmid clone. Open circles and closed circles indicate thymine and cytosine, respectively. The dotted circle at position 2 was not counted because this position was heterozygous (C/A) in the MCF-7 cells. Arrows indicate positions of cytosine at CpG dinucleotide.

was amplified (Fig. 3A). The same strand, as shown in Fig. 3B, was used as a template. We first analyzed how much DNA could be used as a template. Using MCF-7 DNA treated conventionally as a template, PCR product was clearly detected when 50 ng of DNA was applied (Fig. 3C, a, lane 2). Using MCF-7 DNA treated with 9 M bisulfite solution either for 20 min at 90°C (Fig. 3C, b, lane 2) or for 40 min at 70°C (Fig. 3C, c, lane 2) as a template, PCR products were detected when 50 ng of DNA was applied.

The PCR product obtained using 500 ng of DNA as a template in each experiment was cloned. Twelve independent plasmid clones were picked up and subjected to nucleotide sequence analysis. The strand contained 106 cytosine residues in the amplified region, 29 of which were located at CpG sites. When MCF-7 DNA was treated by the conventional method, all cytosine residues were converted to uracil in all 12 plasmid clones that were analyzed (Fig. 3D, a). Almost the same results were obtained when MCF-7 DNA treated with 9 M bisulfite solution either for 20 min at 90°C (Fig. 3D, b) or for 40 min at 70°C (Fig. 3D, c) was used as a template.

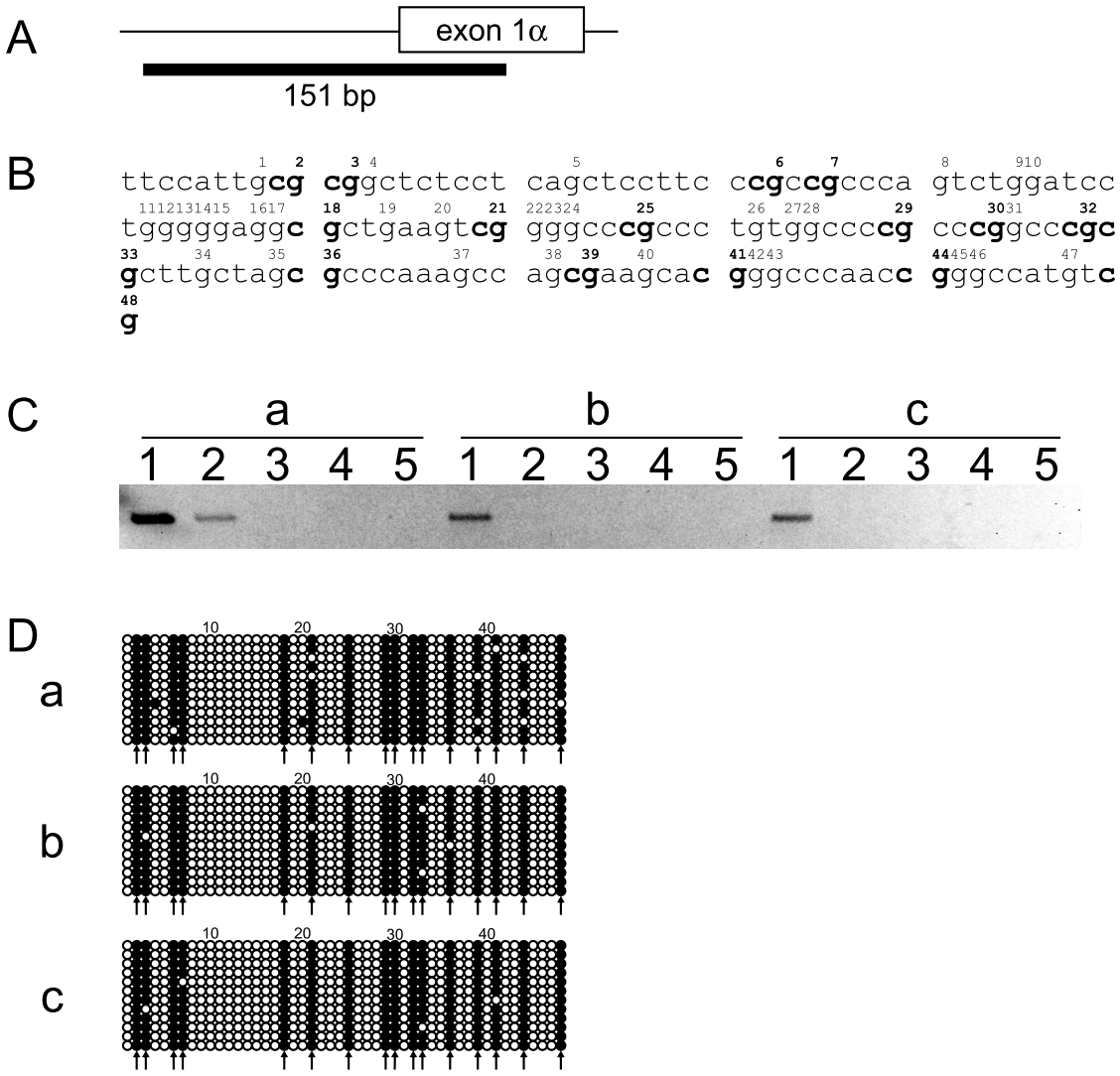

Figure 4. Bisulfite genomic sequencing analysis of the *RASSF1A* gene in MCF-7 cells. (A) The genomic region that was amplified. (B) Nucleotide sequence of the region. Bold characters indicate CpG dinucleotides. The complementary strand was used as a template. As a consequence, cytosine methylation status of the complementary strand is reflected as guanine residues. (C) Serial dilution of the template DNA. a, conventional treatment (3.6 M bisulfite, 20 hr, 55°C); b, 20 min at 90°C; c, 40 min at 70°C. Five hundred nanograms (lane 1), 50 ng (lane 2), 5 ng (lane 3), 500 pg (lane 4), and 50 pg (lane 5) of DNA was used as a template. (D) Nucleotide sequence analysis of plasmid clones. Each row indicates an independent plasmid clone. Open and closed circles indicate thymine and cytosine, respectively. Arrows indicate positions of cytosine at CpG dinucleotides.

These results suggest that treatment of human genomic DNA with 9 M bisulfite solution at an elevated temperature permits rapid conversion of cytosine to uracil.

We then analyzed the methylation status of the CpG island of the *RASSF1A* gene in MCF-7 cell. A 151-bp fragment containing a region of exon 1α was amplified (Fig. 4A). The complementary strand, as shown in Fig. 4B, was used as a template. Using MCF-7 DNA treated conventionally as a template, PCR product was detected when 50 ng of DNA was applied (Fig. 4C, a, lane 2). Using MCF-7 DNA treated with 9 M bisulfite solution either for 20 min at 90°C (Fig. 4C, b) or for 40 min at 70°C (Fig. 4, c) as a template, PCR products were detected only when 500 ng of DNA was applied (Fig. 4, b and c, lane 1). These results suggest that the mode of DNA degradation caused by treatment with 9 M bisulfite at an elevated temperature varies depending on nucleotide sequences.

The analyzed strand contains 48 cytosine residues in the amplified region, 16 of which are at CpG sites. When MCF-7 DNA was treated by the conventional method, almost all cytosine residues at non-CpG sites were converted to uracil in all 12 plasmid clones that were analyzed (Fig. 4D, a). In contrast, most cytosine residues at CpG sites remained unmodified (Fig. 4D, a). Almost the same results were obtained when MCF-7 DNA treated with 9 M bisulfite solution either for 20 min at 90°C (Fig. 4D, b) or for 40 min at 70°C (Fig. 4D, c) was

analyzed.

3.4. Conclusion

We have shown that the conversion of cytosine to uracil can be achieved within 20 min by treating genomic DNA with a highly concentrated bisulfite solution at an elevated temperature, while keeping 5-methylcytosine largely intact. These results show that bisulfite-modified DNA can be prepared in just a couple of hours commencing from the denaturation step. The increased speed of bisulfite genomic sequencing analysis is expected to promote many aspects of DNA research. For example, since aberrant DNA methylation is associated with various diseases, rapid analysis of methylation status of a patient's DNA is of clinical significance. Obviously, a shorter period for treatment is advantageous for automating the overall workup. This novel procedure will play an important role in DNA methylation studies.

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