Nucleic Acids Research, 1994, Vol. 22, No. 4 695–696

Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing

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Received December 21, 1993; Accepted January 19, 1994

Here we report on the modification of a genomic sequencing protocol, published by Frommer *el al.* (6), which allows the determination of the methylation status of cytosine residues on individual chromosomes. The method is based on PCR amplification from chemically modified genomic DNA, in which unmethylated cytosine residues have been converted into uracil by deamination. Sequencing of individual PCR products establishes the position of each 5-methylcytosine on individual chromosomes (6).

On single-stranded DNA, a high concentration of sodium bisulphite at pH 5.0 induces deamination of cytosine residues but not of 5-methylcytosine residues (1–4). In double-stranded DNA, the rate of cytosine deamination is less than 0.1% of the rate in single-stranded DNA (4,5).

We have applied bisulphite genomic sequencing to study DNA methylation in the mouse Insulin-like growth factor 2 (*Igf2*) gene (7). Initially, we chose the published reaction conditions (6), but used glass beads for the subsequent purification of the DNA. On several independent reactions, not all the unmethylated cytosine residues had become deaminated. In the sequenced PCR products clustered stretches of DNA remained unmodified, presumably because they had become double-stranded during treatment (Fig. 1A). We therefore introduced the following modifications in the protocol which resulted in full chemical conversion in most of the PCR products analysed (7, Fig. 1B): 1, DNA was alkaline-denatured directly prior to treatment; 2, the DNA concentration was decreased and the bisulphite concentration increased and 3, treatment was performed at a lower temperature to increase the extent of cytosine sulfonation at pH 5.0 (5) and to reduce annealing of single-stranded DNA sequences during treatment. These modifications did not affect 5-methylcytosine residues which remained unconverted (7).

The improved protocol is as follows: 1) Genomic DNA (2 μg) is digested with an endonuclease which gives a small fragment comprising the sequence of interest, phenol-extracted twice, dissolved in 100 μl deionised water, transferred to a siliconised 1.5 ml micro-centrifuge tube and denatured by adding 11 μl 3 N NaOH and incubating at 37°C for 20 min. 2) The tube is placed on ice and 1.1 ml of 3.5 M $NaHSO_3$/1 mM Hydroquinone, pH 5.0 is added. The solution is overlayed with 150 μl mineral oil and incubated in the dark for 24 h at 0°C. (the $NaHSO_3$/Hydroquinone solution is prepared immediately prior to use with all the components at 0°C when mixed: dissolve 8.1 g of $NaHSO_3$ (Sigma; assume $NaHSO_3/Na_2SO_3$ to be 1/1) in 18 ml deionised water, adjust to pH 5.0 with 5 N NaOH, add 1 ml 20 mM Hydroquinone solution and adjust the volume to 20 ml). 3) The sample is removed from underneath the oil and transferred to a siliconised 1.5 ml tube. DNA is extracted from the solution (for 30 min at 4°C, in the dark) with 20 μl of glass milk ('Geneclean II' kit; Stratech Scientific Ltd., London), glass beads are three times rinsed with 'Geneclean New Wash' and air dried; DNA is then dissolved in 100 μl deionised water. 4) Desulfonation is performed by adding 11 μl of 2 N NaOH followed by incubation at 20°C for 10 min. 5 M Ammonium-acetate (pH 7.0) is added to a final concentration of 3 M and the DNA is precipitated with 3 volumes of ethanol, dissolved in 100 μl of

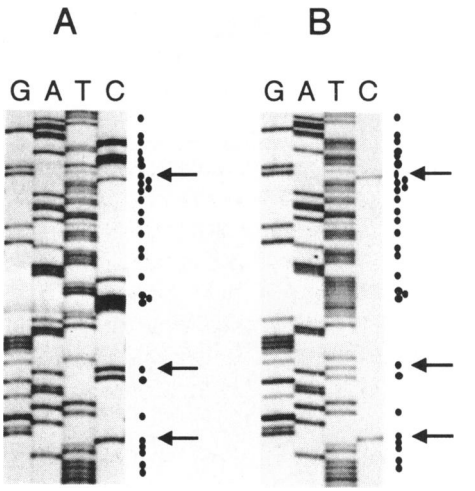

Figure 1. Methylation analysis by bisulphite genomic sequencing. **A)** DNA sequence of a PCR product amplified from mouse DNA which had been bisulphite-modified according to the Frommer *et al.* (6) protocol: 10 μg of DNA was neutralised and precipitated after alkaline denaturing, and incubated in 1.2 ml of 3.1 M $NaHSO_3$/0.5 mM Hydroquinone, pH 5.0 for 18 h at 50°C (6). After treatment, the DNA was purified using glass beads (see modified protocol). The (partially) modified sequence shown is amplified from the upstream region of the mouse *Igf2* gene (7). Dots indicate cytosine residues in the original sequence and arrows indicate the position of CpG dinucleotides. **B)** The same sequence was PCR amplified from mouse DNA which had been bisulphite-treated using the modified protocol. Here, small patches of untreated DNA were found only in some 10% of the PCR products analysed. The sequence of one cloned PCR product is shown: all unmethylated cytosines have been deaminated and two of the three CpGs were methylated in the original genomic sequence from which this PCR product was amplified.

deionised water and stored at −20°C. 5) PCR amplification is from 4 μl of modified DNA with a pair of strand-specific primers which contain cloning restriction sites (i.e. *Eco*RI, *Xba*I) at the 5′ end (6,7). PCR products are ethanol-precipitated, digested with the appropriate restriction enzymes, gel purified and cloned into M13mp19. 6) Single-stranded DNA isolated from individual recombinant phages is sequenced by the dideoxy nucleotide chain-termination method.

ACKNOWLEDGEMENTS

We thank Dr M.Frommer for communications to us about bisulphite genomic sequencing prior to publication. This work was supported by fellowships from the EMBO (to R.F.) and the European Communities (to J.W.).

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