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PCR amplification of highly GC-rich DNA template after denaturation by NaOH

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DNA amplification via polymerase chain reaction has become an increasingly important tool of molecular biology (1). However, a high GC-content of template DNA may constitute a significant limitation of the technique. Previously, utilization of cosolvents, formamide (2) or glycerol (3) in the PCR was reported to significantly enhance specificity of the amplification. Most recently, a general method using native but not recombinant Pfu DNA polymerase was recommended to amplify regions of very high GC content (4). In the present study, we show that template denaturation with NaOH was required for PCR amplification of a highly GC-rich template.

We have been investigating the inherited polymorphisms and expression of a human endogenous retroviral sequence, HRES-1 (5, 6; EMBL accession number, X16514). PCR reaction was essentially carried out as described earlier (7). Our attempts to amplify a highly GC-rich region of HRES-1 (the GC content is 81% between nucleotide positions 1158 and 1266), using routine variations of PCR conditions such as $MgCl_2$, concentration (between 1.5–4 mM) and testing a wide range of temperatures for denaturation (95–97°C) and annealing (37–62°), failed to yield any amplification. PCR between nucleotide positions 400 to 1294 of HRES-1 utilizing primer pair HRES-1/FW (5′-ATGCCAGGACAGATGGAG-3′) and HRES-1/REV 5′-TGCGGGGGTCTTGAGGGT-3′) resulted in no amplification. As a template 25 pg to 250 ng of cloned HRES-1 containing plasmid, HRES-1/1 (5) was utilized while the concentration of primers was varied from 100 pM to 1 mM. The expected 895 bp fragment could not be detected by even Southern blot analysis using an end-labelled oligonucleotide, HRES-1/REVb (5′-ACTTGTACTTTGTATCAG-3′, corresponding to nucleotide positions 575–558). Addition to the reaction mixture of formamide (2.5–10%) or glycerol (5–10%), separately or in combinations, use of the Ampliwax/Hotstart System (Perkin Elmer Cetus, Norwalk, CT), boiling of the template for 5 min before PCR, or replacement of Taq with native Pfu DNA polymerase (Stratagene) using a two-step GC protocol (30 cycles with denaturing at 98°C for 1 min, annealing and extension at 70°C for 5 min) (4) did not result in a detectable amplification of HRES-1. In order to exclude errors in the sequence of the primers, they were used for double-stranded sequencing of HRES-1 denatured with 0.4 M NaOH using the Sequenase kit from USB (Cleveland, OH). Since clean sequencing reactions were obtained by all three HRES-1 primers, it was concluded that the primers effectively annealed to the NaOH-denatured HRES-1 templates. Thus, denaturation of the template may be the limiting factor of PCR of HRES-1. Accordingly, HRES-1/1 plasmid DNA was denatured in the presence of 0.4 M NaOH and 0.4 mM EDTA for 10 min at room temperature followed by addition of 1/10 volume of sodium acetate and precipitation in 2 volumes of alcohol. After washing the denatured DNA with 70% alcohol, it was resuspended in a PCR reaction mixture comprised of 1.5 mM $MgCl_2$, 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatin 200 uM of each dNTP, 1 uM of each primer, and 2.5 units of Taq DNA polymerase

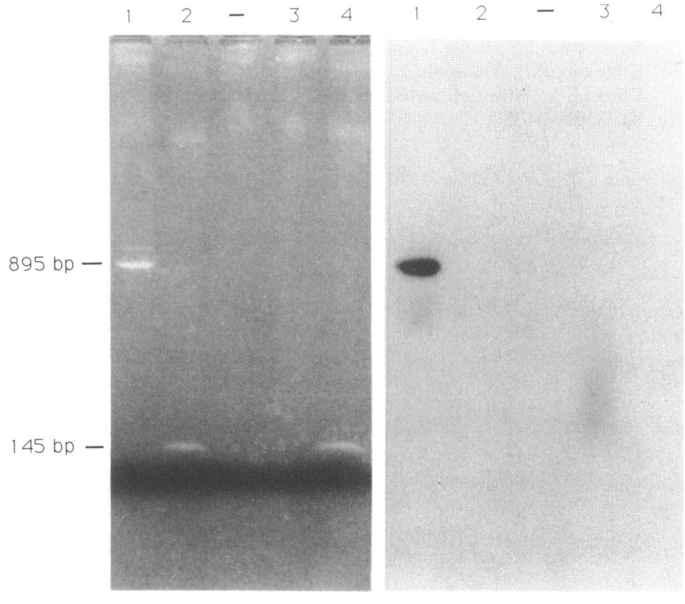

Figure 1. Effect of denaturing by NaOH on amplification of GC-rich DNA via PCR. PCR was carried out in a volume of 100 ul under standard conditions (6). Briefly, 30 cycles of PCR was performed with denaturation at 95°C for 1 min, annealing at 51°C for 1 min, and extension at 72°C for 1 min. 5 ul of each sample was run in a 2% agarose gel (left panel) which was blotted to a nylon membrane in 0.4 M NaOH, and hybridized to an end-labelled HRES-1/REVb oligonucleotide (right panel). Lanes are: 1 and 3, HRES-1/1 template amplified with HRES-1/FW and HRES-1/REV primers; 2 and 4, HRES-2 cDNA amplified with 4/20RFb and 4/2REVb primers. Templates for reactions 1 and 2 were denatured with 0.4 M NaOH prior to PCR.

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(Figure 1). As shown in Figure 1, following denaturation of the template by NaOH, HRES-1 was effectively amplified by PCR while no amplification of the HRES-1/1 plasmid template could be detected even by Southern blot analysis without pretreatment by NaOH. In order to demonstrate a general applicability of the method, efficient and specific amplification of a 70% GC-rich-region of human T-cell leukemia virus type-II (between nucleotide positions 1090 and 1206; 8) was accomplished following template pretreatment with, but not without, NaOH (data submitted but not shown). Denaturing by NaOH had no effect on amplification of a 145 bp fragment of another template, HRES-2, with a lower, 64%, GC content. Combination of NaOH pretreatment with addition of glycerol or formamide to the PCR reaction or replacement of Taq with Pfu DNA polymerase did not further improve amplification of HRES-1/1 (not shown). This underlines the importance of effective template denaturation prior to PCR. In summary, denaturing by NaOH was the only method that could make PCR amplification of highly GC-rich regions possible.

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