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Enhancement of PCRs by Partial Restriction Digestion of Genomic Templates

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ABSTRACT

Genomic DNA preparations derived from mammalian cells can often exhibit poor template activity in PCR, particularly when carried out on target sequences present at low copy number. Using genomic DNA bearing SV40 sequences integrated into host chromosomal DNA at low copy number as a target, we show that template efficiency can be dramatically enhanced after treatment of the genomic template with restriction enzymes for varying periods of time. Also, our results indicate that, while template activity was enhanced by all of the restriction enzymes tested, optimal digestion time varied for each enzyme.

INTRODUCTION

The polymerase chain reaction (PCR) is a widely utilized technique for rapid and specific amplification of target DNA sequences. Despite the fact

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that conditions for PCR optimization have been extensively studied, a number of DNA templates remain refractory to amplification or do not yield specific, reproducible amplification products (2). In particular, the genomic DNAs from higher eukaryotes often prove difficult to work with because of their size and complex secondary structure. The experiments presented here were initiated because of the difficulties encountered in utilizing simian virus 40 (SV40) sequences integrated into human genomic DNA at low copy number as a PCR template. Our results show that limited restriction endonuclease digestion of the genomic template can dramatically increase the yield of specific PCR product(s) and that such pretreatment might prove to be of general utility when working with complex templates.

MATERIALS AND METHODS

Isolation of Genomic DNA

Genomic DNAs were isolated as described previously (4,5). Briefly, approximately 10^8 cells in standard T75 tissue culture flasks were rinsed with phosphate-buffered saline (PBS), followed by incubation overnight at 37°C in 10 mL of extraction buffer (100 mM Tris-HCl, pH 7.9, 10 mM NaCl, 10 mM EDTA and 0.5% sodium dodecyl sulfate [SDS] with 100 µg/mL of proteinase K). The resulting suspensions were extracted twice using gentle agitation with an equal vol of phenol/chloroform-isoamyl alcohol (24:1). DNA was precipitated with 2 vol of ethanol, washed with 70% ethanol, air-dried and resuspended in 1 mL of Tris-EDTA (TE). RNase A was added to a final concentration of 10 µg/mL and incubated at 37°C for an additional 30 min. The phenol/chloroform-extraction and the ethanol-precipitation steps were repeated, and DNA was resuspended in 0.5 mL of TE (pH 7.5) and dialyzed against >3000 vol of TE.

Restriction Endonuclease Digestion

Restriction digestion was carried out with 2 U of the indicated restriction enzyme (New England Biolabs, Beverly,

MA, USA) and 5 µg of genomic DNA from cell lines 130 and 98 at the 131st and 101st serial passages, respectively, using buffers supplied with the enzymes. Restriction enzymes were inactivated by heating the digests at 68°C for 15 min before carrying out PCR.

PCR

PCR was carried out on a Genius Thermal Cycler (Technique, Princeton, NJ, USA) using the Expand™ High Fidelity PCR System (Boehringer Mannheim, Indianapolis, IN, USA) according to the directions recommended by the manufacturer at a final Mg^{2+} concentration of 1.5 mM. Amplification was carried out in thin-walled PCR tubes using 5 µg of template and SV40-specific oligonucleotides 19 (5'-GATTAAAATCATGCTCC-3') and 222 (5'-CTGAGGCGGAAAGAACCAGCTGTGGAATGTGT-3') as amplification primers (Figure 1). The reaction mixture was then completed with 10× reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.3, 15 mM $MgCl_2$), dNTPs (at a final concentration of 400 µM each) and sterile H_2O for a total of 50 µL. PCRs were covered in oil and heated to 95°C for 2 min followed by primer annealing at 50°C for 40 s and primer extension at 68°C for a period of 2 min. Cycling was carried out at 94°C

for 40 s followed by temperature steps of 50°C for 40 s and 68°C for 2 min for a total of 37 cycles.

Blot Hybridization

Products of the PCR and restriction fragments from genomic DNAs were separated by electrophoresis through 0.8% agarose gels and then transferred to nitrocellulose and hybridized to a ^{32}P -labeled, full-length SV40 probe as described previously (4,7).

RESULTS AND DISCUSSION

Our laboratory has utilized the PCR technique as a means of amplifying SV40 sequences present at low copy number in genomic DNAs, prepared from lines of viral-immortalized human keratinocytes (6). In contrast to free viral DNAs, amplification of viral sequences present in integrated form in genomic templates often proves difficult. Testing the hypothesis that DNA, nicked at sites adjacent to the amplicon-defined segment might enhance template efficiency, we carried out PCR on genomic DNA templates that had been subjected to partial-restriction digestion. For the experiments shown below, we used genomic DNA isolated

Figure 1. Location of PCR primers on SV40 genome. Amplification primers 19 (5'-GATTAAAATCATGCTCC-3') and 222 (5'-CTGAGGCGGAAAGAACCAGCTGTGGAATGTGT-3') were used in all the experiments. For reference, location of the origin of replication (ORI) and the *Bam*HI site are shown.

from line 130 of SV40-transformed human keratinocytes at the 131st serial passage and from cell line 98 at the 101st serial passage. As is generally true for papova virus-immortalized cell lines carried to high serial passage levels in vitro, the viral genomes exhibit defective replication origins and are present in an integrated form at low copy number (1,3). Figure 2 shows the time-dependent restriction digestion of 130 DNA by *Xba*I. Digestion was ex-

amined at various time points over a 2-h period. Southern blot hybridization indicated that digestion was near completion within this time frame and that the viral sequences are contained in a single supra-genomic band with an apparent size of approximately 10 kb. Figures 3 and 4 show representative experiments in which limited digests of the 130 genomic DNA were used as PCR templates, using amplification primers covering a 1492-nucleotide (nt)

Figure 2. (A) Time-dependent restriction digest of genomic DNA from SV40-transformed human keratinocyte cell line 130. The digest was performed using the enzyme *Xba*I; aliquots were removed at the different time intervals indicated. Eight microliters of each sample were then run on a 0.8% ethidium bromide-agarose gel using fragments from λ DNA digested with *Hind*III as size markers. Digestion times are indicated for lanes 1–6. (B) Southern blot hybridization was conducted using a 32 P-labeled, full-length SV40 genomic DNA as a probe. The autoradiograph shown on the right represents a 24-h exposure.

Figure 3. (A) PCR products obtained using *Xba*I-digested genomic DNA. All samples shown are PCR products using different digestion times up to 240 min. Samples were run on an 0.8% ethidium bromide-agarose gel. The marker used was a mixture of pBR322 DNA cleaved with *Bgl*II and *Hin*FI. The 1492-bp PCR product is indicated by the arrow. The results demonstrate that at time zero (uncut DNA), no PCR products are obtained, and that the best results are achieved at 90 min of digestion. (B) Southern blot hybridization showing bands hybridizing to the 32 P-labeled, full-length SV40 genomic DNA probe. The exposure time for the blot was 3 h.

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segment of the viral early region (Figure 1). Even a brief treatment of the genomic template with *XbaI* or *SacI* (enzymes with no recognition sites in SV40) dramatically enhanced amplification of the viral sequences, while, in contrast, little or no detectable amplification was seen with undigested DNA in these experiments (Figures 3; Figure 4, A–C). Surprisingly, template treated with either restriction enzyme for a 60-min period did not show any of the enhancement of the template activity observed at either longer or shorter treatment times, up to 240 min. Conversely, treatment with *BamHI*, an enzyme with an SV40 restriction site 2242 nt distal to the target region, lead to enhancement of template activity after 60 min of treatment, but did not at 15, 30, 90 or 120 min of treatment (Fig-

ure 4, D–F). To rule out the possibility that the observed enhancement might reflect idiosyncrasies associated with viral sequences in line 130, we tested another cell line using *XbaI*. Figure 5 shows that treatment of line-98 genomic DNA with *XbaI* produced two distinct bands at 90 min of digestion, while no product was observed at any of the other time points. The larger band was of the size expected for intact viral sequences (1492 kb), while the smaller band migrated at about 670 kb. Interestingly, this smaller band had never been seen in our previous PCRs using this same cell line without restriction enzyme treatment.

Techniques for preparing genomic DNAs from higher eukaryotes are tailored to minimize nicking caused by physical manipulation of the samples

during extraction. However, these procedures often result in viscous suspensions in which some of the DNA can be incompletely solubilized or partially aggregated. Conditions for PCR might be enhanced by small amounts of nicking by making the target template sequences more available for reaction or optimizing interaction with primers. The presence of aggregates might explain the unexpected finding that template activity can sometimes exhibit multiple peaks of enhancement, such as seen in Figure 4. Thus, the purity of the DNA sample could dictate how effective the restriction treatment will be and therefore define the optimal digestion time for PCR products to be obtained. We have tested 3–4 different genomic preparations from each of three different lines of SV40-transformed human

Figure 4. PCR of partial restriction digests of DNA from SV40-transformed human keratinocyte cell line 130, using the enzymes *SacI* and *BamHI*. DNAs were digested with the indicated restriction enzymes for periods ranging up to 240 min and then run on an 0.8% ethidium bromide-stained agarose gel. The marker used was a mixture of pBR322 DNA cleaved with *BglI* and *HinI*. (A) Digests using the restriction enzyme *SacI*, which does not cut SV40 DNA. Digestion times are indicated above the lanes. (B) Southern blot hybridization using ^{32}P -labeled, full-length SV40 genomic DNA as a probe, with an exposure time of 24 h. (C) The same results shown using a 48-h exposure time. (D) Digests using *BamHI*, a single cut enzyme for SV40 DNA. (E) Southern blot hybridization of the PCR of the *BamHI* digest after a 24-h exposure time. (F) The same results shown using a 48-h exposure time.

Figure 5. (A) PCR products obtained using *Xba*I-digested genomic DNA from cell line 98. All samples shown are PCR products using different digestion times up to 240 min. Samples were run on an 0.8% ethidium bromide-stained agarose gel. The marker used was pBR322 DNA cleaved with *Bgl*II and *Hin*fl. Two PCR products are observed at 90 min. (B) Southern blot hybridization using a 32 P-labeled, full-length SV40 genomic DNA probe. The exposure time for the blot was 24 h.

keratinocytes. In each case, enhancement of amplification of the viral templates was always observed within 2 h of restriction enzyme treatment and often after only 15 min. Because restriction sites for enzymes with hexameric recognition sequences are expected to occur on the average of only once in every 4⁶ bp, it is likely that any given restriction enzyme will not cut within a typical amplification primer-defined DNA segment. Therefore, identification of enzymes, which do not cut within the PCR target, can usually be accomplished by the testing of a small panel of candidate enzymes, even in cases where no sequence information for the target segment is available. Use of restriction enzymes with low cutting efficiencies, such as rare cutters, would also minimize this possibility. Selection of low-efficiency enzymes might also provide the additional advantage of preventing the digestion of the template too rapidly, so that an optimal digestion-time range can be selected.

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