Nucleic Acids Research, 1997, Vol. 25, No. 17 3537–3542

Re-usable DNA template for the polymerase chain reaction (PCR)

Sabina N. Sheikh and Philip Lazarus*

Department of Pathology and Laboratory Medicine, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140, USA

Received April 7, 1997; Revised and Accepted July 7, 1997

ABSTRACT

DNA covalently bound to an uncharged nylon membrane was used for consecutive amplifications of several different genes by PCR. Successful PCR amplifications were obtained for membrane-bound genomic and plasmid DNA. Membrane-bound genomic DNA templates were re-used at least 15 times for PCR with specific amplification of the desired gene each time. PCR amplifications of specific sequences of *p53*, *p16*, CYP1A1, CYP2D6, GSTM1 and GSTM3 were performed independently on the same strips of uncharged nylon membrane containing genomic DNA. PCR products were subjected to restriction fragment length polymorphism analysis, single-strand conformational polymorphism analysis and/or dideoxy sequencing to confirm PCR-amplified gene sequences. We found that PCR fragments obtained by amplification from bound genomic DNA as template were identical in sequence to those of PCR products obtained from free genomic DNA in solution. PCR was performed using as little as 5 ng genomic or 4 fg plasmid DNA bound to membrane. These results suggest that DNA covalently bound to membrane can be re-used for sample-specific PCR amplifications, providing a potentially unlimited source of DNA for PCR.

INTRODUCTION

Since its first report by Mullis and colleagues in 1987 (1), PCR has become one of the most powerful and widely used techniques in molecular biology. Thousands of scientific papers are published each year using this technique as a tool for molecular research as well as for clinical applications, such as disease diagnostics (2,3), occult tumor cell detection (4–6) and prenatal diagnosis of a variety of human genetic disorders (7,8). Therefore, refinements in PCR protocols can improve DNA analysis and detection for a broad spectrum of medical and research purposes.

The amount of genomic DNA required for PCR ranges, in general, from 50 to 500 ng, with each amplification requiring fresh DNA as template. As the amplification of different genes often requires optimization and standardization, suitable quantities of DNA are required for analysis. However, circumstances may limit the availability of DNA, particularly for DNA isolated from individual tissue samples, including biopsies and archival tissue, where specimen quantities are usually finite and low in quantity. Therefore, re-use of a DNA sample for repetitive PCR amplifications and for amplification of DNA sequences from different genes would decrease the difficulties associated with limited DNA availability for all aspects of genetic testing by PCR.

In this report we describe a simple, novel PCR methodology where DNA, covalently bound to non-charged nylon membranes, serves as template for PCR amplification. We demonstrate that membrane-bound DNA can serve as template for repetitive PCR amplifications of multiple genes without cross-contamination between samples and can be utilized for: (i) genotyping polymorphic alleles, such as those present in specific xenobiotic metabolizing enzyme genes (XMEs); (ii) mutational analysis of oncogenes or tumor suppresser genes, such as *p53* and *p16*.

MATERIALS AND METHODS

Isolation and purification of DNA

Squamous cell carcinomas of the oral cavity were obtained by surgical resection from patients at Memorial Sloan-Kettering Cancer Center (New York, NY) as part of a large-scale study examining molecular markers of oral cancer susceptibility as previously described (9,10). These samples were selected based upon their unique genetic characteristics with respect to gene mutations and/or XME genotypes (see Table 1). All tumors were immediately frozen at –70°C after surgical resection. Slices (100–500 mg) of partially thawed tumor tissue were snap-frozen in liquid nitrogen and pulverized prior to homogenization in proteinase K (0.1 mg/ml)-containing TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). DNA was extracted as previously described prior to quantitation by spectrophotometery (11).

Plasmid pHp53E1CAT, which contains human *p53* exon 1 sequences immediately upstream of the chloramphenical acetyltransferase gene (*CAT*), was kindly provided by Stephen Strudwick (Temple University). Plasmid DNA was purified using a DNA purification kit (Qiagen) as per the manufacturer's instructions and quantitated by spectrophotometery.

Slot blot preparation and DNA immobilization

Duralon-UV membranes (Stratagene) were initially soaked in deionized, double-distilled water, followed by rinsing in 6× 0.9 M NaCl, 90 mM sodium citrate, pH 7.0, (SSC) buffer prior to slot blot assembly. Wells were washed with 500 µl 6× SSC containing

*To whom correspondence should be addressed. Tel: +1 215 707 5114; Fax: +1 215 707 2781; Email: plazarus@vm.temple.edu

0.05% bromophenol blue dye under low vacuum. DNA samples (either genomic or plasmid DNA, as indicated in the text) were diluted to 0.1 μg/μl in TE and denatured in 0.3 M NaOH at 60°C for 1 h. Denatured DNA was cooled on ice, diluted 1:1 (v/v) with 12× SSC and applied to slot blot wells under low vacuum in a total volume of 200 μl. An equal volume (200 μl) of 6× SSC buffer (–DNA) was applied to negative control wells. Membrane-bound DNA was immobilized by UV crosslinking in a UV Stratalinker 2400 (Stratagene).

Hybridization analysis

Slot blot membranes containing plasmid or genomic DNA were hybridized with ^{32}P-labeled antisense *CAT* (+50 to +70 nt relative to the translation initiation start site; GTACATTGAGCAACTG-ACTG) or human β-actin (+3 to +34 nt relative to the translation initiation start site; CGTTGTCGACGACGAGCGCGGCGATAT-CATCA) probes respectively, using standard procedures (11–13). Membranes were washed with 6× SSC for 2 min at 22°C, followed by 10 min at 45°C and then exposed for autoradiography.

PCR amplifications

All PCR amplifications were performed in a GenAmp 9600 thermocycler (Perkin Elmer) using *Taq* DNA polymerase (Boehringer Mannheim). The standard PCR was composed of a 100 μl reaction volume containing either 100 ng free DNA in solution or DNA blotted and covalently bound to nylon membranes as described above. For covalently bound DNA, membrane fragments corresponding to individual slots were cut using an autoclaved razor. The razor was flamed before each use, with the negative control slot without DNA cut last to assure that cross-contamination between samples was not a factor.

p53 exons 5 and 8 were individually PCR amplified as previously described (9,14). PCR amplification of *p53* exon 5–exon 7 sequences was performed using the Expand™ Long Template PCR System (Boehringer Mannheim) at an annealing temperature of 64°C with the following primers: sense, –43 to –24 nt relative to the 5′-end of exon 5, TTGTGCCCTGACTTT-CAACT; antisense, +8 to +27 nt relative to the 3′-end of exon 7, TGTGCAGGGTGGCAAGTGGC. PCR amplification of *p53* exon 1 was performed using pHp53E1CAT as template with reaction conditions similar to those described for *p53* exon 8 (9,14). Primers used for *p53* exon 1 were: sense, –220 to –200 nt relative to the translation start site, TGTCATGGCGACTGTCC-AGCT; antisense, –44 to –20 nt relative to the translation start site, CCAATCCAGGGAAGCGTGTCACCGT. *p16* exon 2 was PCR amplified using the following primers: sense, –26 to –7 nt relative to the 5′-end of *p16* exon 2, TCTGACCATTCTGTTC-TCTC; antisense, +32 to +53 nt relative to the 3′-end of *p16* exon 2, CTCTGAGCTTTGGAAGCTCTCA. *p16* PCR amplifications were performed as described above for *p53* exon 8 (9,14) in the presence of 5% formamide with an annealing temperature of 58°C for 20 s. CYP1A1 exon 7, GSTM1 exons 4–5 and GSTM3 exons 6–7 sequences were PCR amplified for 36 cycles as previously described (10,15). CYP2D6 exon 5 sequences were PCR amplified as performed for *p53* exon 5 using variable cycle numbers (as indicated in the text) with the following primers: sense, –25 to –7 nt relative to the 5′-end of exon 5, TGAGACCCCGTTCTGTCTG; antisense, +2 to +20 nt relative to the 3′-end of exon 5, ACCGTGGCAGCCACTCTCA. CYP2D6 intron 3–exon 5 sequences were PCR amplified using the same antisense primer in combination with the sense primer GCCTTCGCCAACCACTCCG (–18 to +1 nt relative to the 3′-end of exon 3), utilizing the Expand™ Long Template PCR System as described above. Between 10 and 25% of all amplifications were electrophoresed on 8% polyacrylamide gels to verify the integrity of the PCR bands. Gels were visualized over UV light after staining with ethidium bromide. Where indicated, PCR band intensities (PBI) were determined by computer scanning of gel images using the Photoshop/NIH Image 1.61 analysis system for McIntosh.

To prevent cross-contamination during PCR, all amplifications were performed using fresh, sterile, autoclaved tips, tubes and double-distilled water. Careful attention was given throughout to prevent cross-contamination between samples during DNA purification and isolation. All equipment utilized for tissue blending and homogenization were washed in a bath of concentrated chromic:sulfuric acid, rinsed three times in autoclaved double-distilled water and once in 70% ethanol, air dried and autoclaved. Forceps for membrane handling were autoclaved between experiments and flamed between the handling of each membrane sample. The membrane strips containing immobilized DNA were stripped twice with 150 μl double-distilled water at 65°C for 10 min after each PCR amplification. DNA-bound membrane strips were stored in water at 4°C. Extreme care was taken to avoid cross-contamination between membrane samples.

Genetic analysis

Mutations in *p53* exons 5 and 8 and *p16* exon 2, as well as genotyping analysis of the polymorphic CYP1A1^{Val} allele (codon 462 of exon 7) were screened by single-strand conformational polymorphism (SSCP) analysis as previously described (9,10,14). *p53* and *p16* mutations were verified by dideoxy sequencing as previously described (15). The GSTM3 YY1 polymorphism was assessed by restriction fragment length polymorphism analysis of GSTM3 PCR-amplified fragments digested with *Mnl*I (1 U/25 μl reaction) for 16 h at 37°C (16). The GSTM1 null (0/0) polymorphism was detected by co-amplification with the homologous GSTM4 gene [serving as a positive control for PCR amplification for GSTM1 (0/0) samples] in a three primer-based assay as previously described (10,17).

RESULTS

Slot blot/hybridization and PCR amplification using membrane-bound plasmid DNA

Serial dilutions (10–0.16 μg) of pHp53E1/CAT were blotted onto an uncharged nylon membrane, UV crosslinked and subjected to hybridization with ^{32}P-labeled *CAT* primer as described in the Materials and Methods. A correlation was observed between hybridization signal intensity and quantity of DNA, as shown in Figure 1A. Membrane pieces corresponding to each slot were carefully cut and used for PCR amplifications of *p53* exon 1 sequences. PCRs using these plasmid DNA-bound membrane strips as template resulted in amplification of the desired product (219 bp; Fig. 1B). Upon further titration, a decreased intensity of PCR banding after gel electrophoresis and ethidium bromide staining was observed, beginning at 40 pg pHp53E1/CAT DNA (Fig. 1C), although a detectable 219 bp band was observed for membranes containing as low as 4 fg plasmid DNA. *p53* exon 1

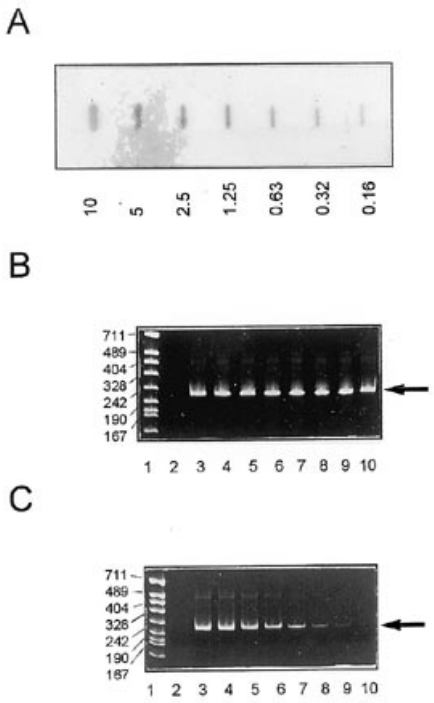

Figure 1. Slot blot/PCR amplification of plasmid DNA. (**A**) Serial dilutions of pHp53E1/CAT plasmid DNA (10–0.16 µg, as indicated) were blotted on an uncharged nylon membrane and hybridized with ^{32}P-labeled CAT probe as described in Materials and Methods. (**B**) PCR amplification of *p53* exon 1 sequences (219 bp, indicated by arrow) were performed for corresponding amounts of membrane-bound pHp53E1/CAT DNA as shown in (A). Ten percent of total PCR product was separated by electrophoresis and stained as described in Materials and Methods. Lane 1, SK/*Hpa*II DNA marker; lane 2, negative control (membrane with no DNA); lane 3, 10 µg pHp53E1/CAT; lane 4, 5 µg pHp53E1/CAT; lane 5, 2.5 µg pHp53E1/CAT; lane 6, 1.25 µg pHp53E1/CAT; lane 7, 0.63 µg pHp53E1/CAT; lane 8, 0.32 µg pHp53E1/CAT; lane 9, 0.16 µg pHp53E1/CAT; lane 10, 100 ng unbound (free) pHp53E1/CAT DNA. (**C**) Ten percent of PCR amplifications of *p53* exon 1 sequences (219 bp, indicated by arrow) was electrophoresed and stained as described in (A), using membrane strips blotted with the following amounts of pHp53E1/CAT DNA: 8 ng (lane 3), 4 ng (lane 4), 400 pg (lane 5), 40 pg (lane 6), 4 pg (lane 7), 400 fg (lane 8), 40 fg (lane 9) and 4 fg (lane 10). Lane 1, SK/*Hpa*II DNA marker; lane 2, negative control (membrane with no DNA).

sequences were verified by dideoxy sequencing (results not shown). These data suggested that the same membrane-bound DNA samples can be utilized for both hybridization studies as well as PCR.

Re-usability of membrane-bound genomic DNA as template for PCR

DNA samples prepared from three oral cavity tumors (MSK73, MSK75 and MSK80) were chosen for this study and were analyzed for mutations in *p53* and *p16* gene sequences and known polymorphisms present in the GSTM1, CYP1A1 and GSTM3 genes. These samples were chosen based upon the fact that each DNA sample exhibited unique genetic characteristics with respect to gene mutations and/or XME genotypes. The mutation/genotype status of each DNA sample, elucidated in PCRs using free, unbound DNA as template, is shown in Table 1. Ten micrograms of each genomic DNA sample were blotted and crosslinked to nylon membrane as described in the Materials and Methods. Membrane strips corresponding to each slot were carefully cut and used for successive PCR amplification of several different gene sequences. The membrane pieces with bound DNA were stripped between each PCR amplification and used for 15 consecutive PCRs. The results of alternate amplifications are shown in Figure 2A. An initial PCR was performed for simultaneous amplification of GSTM1 (275 bp) and GSTM4 (202 bp) (lanes 3–5). Subsequent amplifications using the same DNA-bound membrane strips were also performed for *p53* exon 8 (223, lanes 6–8), *p16* exon 2 (384 bp, lanes 9–11), *p53* exon 5 (261 bp, lanes 12–14), GSTM3 (lanes 15–17) and CYP1A1 exon 7 (see SSCP analysis, Fig. 3A; the electrophoresis of CYP1A1 exon 7 PCR product on 8% non-denaturing polyacrylamide is not shown). For GSTM3, electrophoresis was performed after restriction enzyme digestion with *Mnl*I. To determine the re-usability of membrane-bound DNA template, some gene sequences were amplified more than once (data not shown). PCR fragments of the appropriate size were amplified in all rounds of PCR for all membrane-bound DNA samples. Negative control membrane pieces were also re-used in PCRs to control for cross-contamination between samples. No amplification was observed for negative control membrane strips (i.e. without DNA) for any round of PCR (round 1 amplification shown in Fig. 2A, lane 2; results not shown for negative control amplifications for other PCR rounds).

Table 1. Mutational and genotyping data of DNA samples using free DNA as template for PCR

DNA sample	Mutations	Genotype of oral tumor samples		
		CYP1A1	GSTM1	GSTM3
MSK73	Wild-type *p53* and *p16*	Ile/Ile[a]	(0/0)[b]	A/A
MSK75	*p53* exon 8, codon 278, C→T	Ile/Ile	[+]	A/B
MSK80	*p16* exon 16, codon 102, G→A	Val/Ile	[+]	A/B

[a]Ile, isoleucine; Val, valine.
[b](0/0), null genotype; [+], heterozygous (+/0) and/or homozygous (+/+) genotype.

PCR was also attempted on membranes with titrated amounts of bound genomic DNA as template. As shown in Figure 2B, accurate genotyping for GSTM1 was performed using 3.0, 1.0 and 0.1 µg membrane-bound genomic DNA. Both GSTM1-null (0/0) (sample MSK73, lanes 4–6) and GSTM1-positive (275 bp; sample MSK75, lanes 8–10) genotypes were clearly discernible using as little as 0.1 µg membrane-bound DNA as template for PCR, with the positive control GSTM4 gene amplified in all cases. These data suggest that successful PCR amplification can be obtained when using quantities of membrane-bound DNA similar to the levels used in standard PCRs with free, unbound genomic DNA as template.

To better evaluate the sensitivity of membrane-bound PCR, we examined PCR amplification of CYP2D6 exon 5 sequences using 5 and 20 ng of both membrane-bound and free genomic DNA. As shown in Figure 2C, a CYP2D6 exon 5-specific amplimer (222 bp) was observed for PCRs with both 5 and 20 ng membrane-bound and free DNA used as template, with bands observed as early as 32 cycles for the two DNA samples examined. Increased levels of background amplification were observed in PCRs using these lower DNA amounts (i.e. as compared with PCRs utilizing ≥100 ng DNA; results not shown), but this background was observed for both membrane-bound and free DNA. Background banding

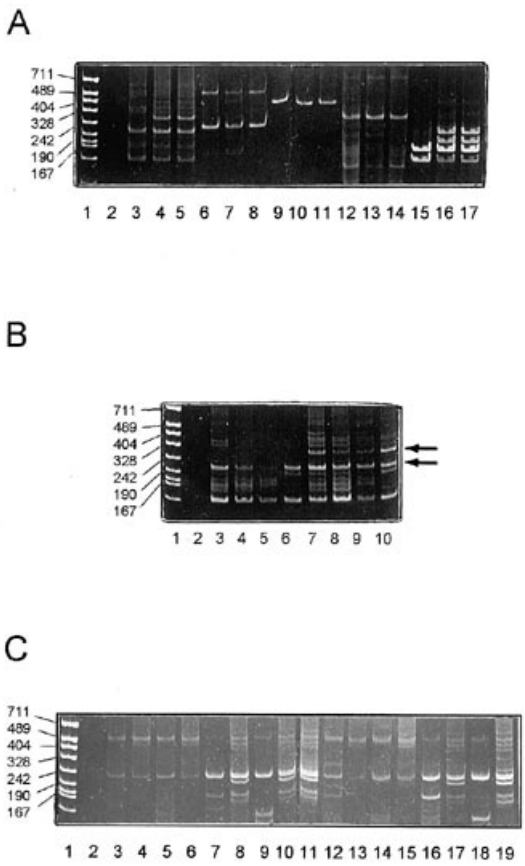

Figure 2. Sequential PCR amplification of several different gene products using membrane-bound genomic DNA as template. (**A**) Oral tumor genomic DNA (10 µg)-bound membrane strips were utilized in up to 15 sequential PCR amplifications of various gene products. Shown are 10–25% of PCR products from five of these different amplifications. Membranes were stripped between each PCR amplification as described in the text. Lane 1, SK/*Hpa*II DNA marker; lane 2, negative control (membrane with no DNA); lanes 3–5, GSTM1 and GSTM4, co-amplified (see text, PCR amplification 1); lanes 6–8, *p53* exon 8 (PCR amplification 3); lanes 9–11, *p16* exon 2 (PCR amplification 5); lanes 12–14, *p53* exon 5 (PCR amplification 7); lanes 15–17, GSTM3 (PCR amplification 9). Lanes 3, 6, 9, 12 and 15, genomic DNA from tumor sample MSK73; lanes 4, 7, 10, 13 and 16, genomic DNA from tumor sample MSK75; lanes 5, 8, 11, 14 and 17, genomic DNA from tumor sample MSK80. (**B**) Oral tumor DNA samples MSK73 and MSK75 were used in GSTM1/GSTM4 PCR co-amplifications using 3 (lanes 3 and 7), 1 (lanes 4 and 8) and 0.1 µg (lanes 5 and 9) membrane-bound DNA. Shown is 30% of total PCR after electrophoresis and staining as described in Materials and Methods. PCR using 0.1 µg free DNA is shown in lanes 6 (MSK73) and 10 (MSK75). Lane 1, SK/*Hpa*II DNA marker; lane 2, negative control (membrane with no DNA). Upper arrow, GSTM1; lower arrow, GSTM4. (**C**) Oral tumor DNA samples MSK73 (lanes 3, 4, 7, 8, 12, 13, 16 and 17) and MSK75 (lanes 5, 6, 9, 10, 14, 15, 18 and 19) were used for PCR amplifications of CYP2D6 exon 5 sequences (222 bp, indicated by arrow) using 20 (lanes 3–10) or 5 ng (lanes 12–19) membrane-bound (lanes 3, 5, 7, 9, 12, 14, 16 and 18) or free (lanes 4, 6, 8, 10, 13, 15, 17 and 19) DNA. Shown is 10% of PCR products after 36 (lanes 3–6 and 12–15) or 50 cycles (lanes 7–10 and 16–19) of PCR. Lane 1, SK/*Hpa*II DNA marker; lane 2, negative control (membrane with no DNA); lane 11, positive control for PCR using 100 ng genomic DNA sample MSK75.

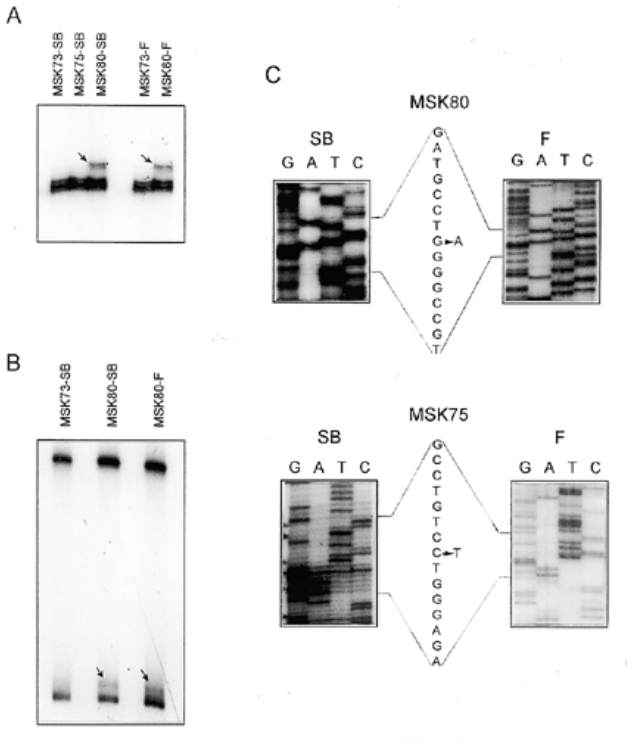

Figure 3. PCR amplification of membrane-bound genomic DNA for genetic screening. (**A**) SSCP analysis of exon 7 CYP1A1 sequences PCR amplified using slot-blot membrane-bound (SB) or free (F) oral tumor genomic DNA as template as described in the text. DNA-bound membranes were: (i) used as templates in nine previous PCR amplifications for all samples; (ii) were utilized for the PCR amplifications shown in Figure 2. Arrows indicate the polymorphic CYP1A1^{Val} allele. (**B**) SSCP analysis of *p16* exon 2 sequences PCR amplified using membrane-bound (SB) or free (F) oral tumor genomic DNA as template as described in the text. DNA-bound membranes were used for four previous PCR amplifications for all samples as shown in Figure 2. Arrows indicate shifted bands. (**C**) Comparison of sequences of PCR products obtained using bound (SB) or free (F) DNA as template for PCR. SSCP shifted bands were dideoxy sequenced after low melting point agarose purification of PCR samples as previously described (15). The wild-type gene sequence is described between the corresponding sequencing panels. Mutated nucleotides are indicated by an arrow. (Top) *p16* exon 2; (bottom) *p53* exon 8.

appeared to be less in membrane-bound PCRs as compared with PCRs with free DNA as template after 50 PCR cycles (compare lanes 7, 9, 16 and 18 with lanes 8, 10, 17 and 19). Successful amplifications were also performed for each of six attempts at PCR utilizing these same DNA-bound membrane strips (results not shown). In addition, the CYP2D6 exon 5 band intensity after 36 cycles of PCRs using 100 ng membrane-bound DNA (result not shown) was similar to that observed after 50 cycles of PCR using 5 ng membrane-bound DNA (lanes 16 and 18), suggesting that efficient amplification can be obtained for membrane-bound PCR using as little as 5 ng template by optimizing PCR conditions such as cycle number.

The efficiency of membrane-bound DNA PCRs was determined by examining the relative ethidium bromide stained PBI of PCRs utilizing the same amounts of membrane-bound or free DNA (data summarized in Table 2). The relative PBI ratios for 100 ng membrane-bound DNA (Fig. 2B, lanes 5 and 9) versus 100 ng free DNA (Fig. 2B, lanes 6 and 10) were 0.19 for DNA sample MSK73 and 0.66 for MSK75. This ratio increased to 1.1 and 2.9 for 20 ng DNA [compare lanes 3 and 5 (membrane-bound) with lanes 4 and 6 (free), Fig. 2C] and 3.6 and 1.4 for 5 ng DNA [compare lanes 12 and 14 (membrane-bound) with lanes 13 and 15 (free), Fig. 2C] for the same two DNA samples, respectively. These data suggest that the efficiency of PCR appears to increase

in reactions utilizing lower amounts of membrane-bound DNA as template. This is consistent with the fact that amplifications using 10 µg membrane-bound DNA resulted in PCR bands which were often the same or only marginally greater than that observed for PCRs using 100 ng free DNA.

Table 2. Efficiencies of membrane-bound:free DNA PCRs

DNA (ng)	DNA sample	
	MSK73	MSK75
100	0.19[a]	0.66
20	1.1	2.9
5	3.6	1.4

[a]Indicated are the ratios of membrane-bound:free DNA PBI as determined by computer scanning of gel images (see Material and Methods). Ratios were determined for PCRs of GSTM4 exon 4–5 sequences (100 ng DNA; Fig. 2B; indicated by lower arrow) or CYP2D6 exon 5 (20 and 5 ng DNA; Fig. 2C).

We extended our analysis of membrane-bound PCR to determine the utility of this technique for the amplification of larger DNA fragments. We attempted PCR amplification of a 898 bp fragment comprising CYP2D6 intron 3–exon 5 sequences and a 1126 bp fragment comprising *p53* exon 5–exon 7 sequences, using either membrane-bound (10 µg) or free (100 ng) DNA. Although bands corresponding to CYP2D6 or *p53* sequences were observed in most cases for both membrane-bound and free DNA PCRs, the efficiency of PCR was greatly reduced with all membrane-bound DNA samples tested (results not shown). These results suggest that membrane-bound PCR may be less efficient than free DNA PCR when amplifying longer fragments.

Genetic screening of PCR products obtained using membrane-bound genomic DNA as template

Each of the tumors used for this analysis were from individuals exhibiting specific genotypes for polymorphic XME genes (see Table 1). PCR fragments generated by amplification of DNA-bound membrane strips were screened by RFLP or SSCP followed by dideoxy sequencing to confirm gene sequences. As shown in Figure 2A (lanes 3–5), electrophoresis of PCR-amplified products for the GSTM1 polymorphic gene shows that sample MSK73 is homozygous null for GSTM1 (0/0), while both samples MSK75 and MSK80 exhibit GSTM1-positive genotypes, as indicated by the presence of the GSTM1-specific 275 bp band. The non-polymorphic GSTM4 gene was amplified in all samples as an internal control for PCR amplification (17). Some background amplification was observed in GST PCR amplifications (see Fig. 2A and B, lanes 3–5) due, in all probability, to the use of three primers in this multiplex PCR assay. However, this background was observed for PCRs using both membrane-bound and free DNA as template. In addition, RFLP analysis of GSTM3 gene sequences was performed after *Mnl*I digestion of PCR-amplified product (Fig. 2A, lanes 15–17). The A/A genotype (digested fragments at 11, 51, 86 and 125 bp) was exhibited for MSK73 and the A/B genotype was observed for samples MSK75 and MSK80 (digested fragments at 11, 51, 86, 125 and 134 bp). The upper 200 bp fragment observed after RFLP of *Mnl*I-digested PCR amplifications of both membrane-bound and free DNA templates exhibiting the AB genotype is similar to that reported previously and has been suggested to be due to amplification of GSTM3-like sequences (16). Also, SSCP analysis of CYP1A1 exon 7 PCR-amplified sequences showed that DNA samples MSK73 and MSK75 exhibit a homozygous (Ile/Ile) genotype, whereas MSK80 is heterozygous (Ile/Val), as shown in Figure 3A. Together, these results are in agreement with PCR results obtained for all polymorphic XME genes using free, unbound DNA for all samples tested (see Table 1).

We also studied these membrane-bound tumor DNA samples for mutations in the *p53* and *p16* tumor suppresser genes, since each tumor was shown to possess a specific mutational spectrum, as shown in Table 1. SSCP analysis of *p53* and *p16* showed identical shifts with membrane-bound versus free DNA in PCR amplifications of both *p16* (Fig. 3B) and *p53* (data not shown). The SSCP shifted bands were sequenced for both *p53* and *p16*. Identical sequences were observed for PCR amplifications performed with either membrane-bound or free DNA as template, with MSK75 exhibiting a C→T transition in exon 8 of the *p53* gene and MSK80 exhibiting a G→A transition in exon 2 of the *p16* gene (Fig. 3C). MSK73 exhibited both wild-type *p53* and *p16* (results not shown). Similar to the situation observed when using free DNA samples as template for PCR, wild-type sequences were also observed for PCR amplifications of *p53* exon 5 for all three membrane-bound tumor DNA samples (results not shown). No significant artifactual band shifts or sequencing results were observed for any of the PCR samples amplified using membrane-bound PCR. Together, these results demonstrate that the sequences obtained for PCR-amplified bands using membrane-bound DNA as template were identical to those obtained using free, unbound DNA as template.

DISCUSSION

PCR is a method of choice for molecular biologists and is widely used for several types of research and clinical applications. In this report we describe a novel methodology, where membrane-bound DNA template can be re-used for PCR amplification of several different gene sequences. To our knowledge this is the first study reporting the use of a re-usable source of DNA as a template for repetitive PCR amplification. We demonstrate that DNA cross-linked by UV light to a solid support such as a nylon membrane could be used for multiple PCR amplifications without any effect from previous reactions. By SSCP screening and DNA sequencing we showed that PCR of membrane-bound DNA results in identical sequences to free DNA PCR amplifications without artifactual polymorphic amplifications caused by UV crosslinking of the DNA to the membrane. In addition, successful amplification was obtained for PCRs with as low as 5 ng membrane-bound DNA and band intensities from PCRs using lower amounts of membrane-bound DNA approached that of PCRs using higher amounts of membrane-bound DNA simply by increasing the cycle number. Furthermore, the efficiency of PCR at lower DNA amounts (i.e. 5–20 ng) approached that of free DNA. The relatively inefficient amplification observed when using higher amounts of membrane-bound DNA for PCR may be due to the fact that although more DNA is loaded into the well, the well surface area remains unchanged. Therefore, less surface area would be available for DNA binding, potentially resulting in increased secondary and tertiary formations within the membrane-bound DNA, which could significantly decrease the relative amounts of DNA available for PCR amplification. Inefficient PCR

from membrane-bound DNA was reported previously (18), but this was for membrane-bound PCR using a relatively high amount (2 μg) of a cDNA preparation (and not genomic DNA) as template. Together, the results obtained in this study suggest that membrane-bound PCR can be a highly sensitive and efficient technique.

A drawback to this methodology may be that, due to the nature of DNA binding to a solid support such as a nylon membrane, PCR amplification of longer fragments (i.e. >850 bp) appears to be much less efficient than for shorter sequences (<300 bp). We have not fully tested the parameters of membrane-bound PCR for amplification of longer fragments, but PCR amplification of longer fragments is likely to be dependent upon a number of factors, including length of sequence, sequence specificity, DNA quality, PCR conditions, etc. Our data suggest that although membrane-bound PCR can be used for amplification of longer DNA fragments, it appears to be more problematical than when amplifying shorter fragments. More extensive analysis is required to more fully assess the utility of this technique for amplification of larger PCR fragments.

We demonstrate that PCR using membrane-bound DNA as template is highly specific and can be utilized for both genotyping studies as well as mutational analysis. In addition, we demonstrate that one can combine this PCR procedure with membrane hybridization techniques utilized in dot blot or Southern analysis. Therefore, this method may be useful for a variety of clinical and research purposes, particularly for studies involving PCR amplification of DNA isolated from archival tissue specimens or tissue biopsies, where the quantities of isolated DNA are often limited.

ACKNOWLEDGEMENTS

We thank Stimson P.Schantz for providing tumor tissue samples, Stephen Strudwick for kindly supplying plasmid pHp53E1/CAT and helpful discussions and Taji Kaur and Jong Park for supplying primers. We also thank Roy White for use of his McIntosh computer and Photoshop/NIH Image 1.61 gel analysis system. This work was supported by NIH grants CA59917 (P.L.) and DE/CA12206 (P.L.).

REFERENCES

1 Mullis,K.B and Faloona,F.A. (1987) *Methods Enzymol.*, **155**, 335–350.
2 Ghossein,RA. and Rosai,J. (1996) *Cancer*, **78**, 10–16.
3 Roberts,W.M., Estrov,Z., Kitchingman,G.R. and Zipf,T.F. (1996) *Leukemia Lymphoma*, **20**, 181–197.
4 Nollau,P., Jung,R., Neumaier,M. and Wagener,C. (1995*) Scand. J. Clin. Lab. Invest.*, **221** (suppl.), 116–121.
5 Pan,L.X., Diss,T.C. and Isaacson,P.G. (1995) *Histopathology*, **26**, 201–217.
6 Sleater,J.P., Segal,G.H., Scott,M.D. and Masih,A.S. (1995) *Modern Pathol.*, **7**, 593–598.
7 Bruce,I.J. (1993) *Sci. Prog.*, **77**, 183–206.
8 Lyons,J. (1992) *Cancer*, **15**, 1527–1531.
9 Lazarus,P., Garewal,H.S., Sciubba,J., Zwiebel,N., Calcagnotto,A., Fair,A., Schaefer,S. and Richie,J.P.,Jr (1995) *Int. J. Cancer*, **60**, 458–463.
10 Park,J.Y., Muscat,J.E., Ren,Q., Schantz,S., Harwick,R.D., Stern,J.C., Yoo,H.J., Richie,J.P.,Jr and Lazarus,P. (1997) *Cancer Epidemiol. Biomarkers Prevent.*, in press.
11 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Clonning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
12 Davis,L.G., Dibner,M.D. and Battey,J.F. (1986) *Basic Methods in Molecular Biology.* Elsevier, New York, NY.
13 Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Smith,J.A., Seidman,J.G. and Struhl,K. (1987) *Current Protocols in Molecular Biology*, Wiley, New York, NY.
14 Lazarus,P., Stern,J., Zwiebel,N., Fair,A., Richie,J.P.,Jr and Schantz,S. (1996) *Carcinogenesis*, **17**, 733–739.
15 Anderson,R.D., Bao,C.-Y., Minnick,D.T., Baxter,J., Veigl,M.L. and Sedwick,W.D. (1993) *Mutat. Res.*, **288**, 181–185.
16 Inskip,A., Elesperu-Camiruaga,J., Buston,N., Dias,P.S., Macintosh,J., Campbell,D., Jones,P.W., Yengi,L., Talbot,J.A., Strange,R.C. and Fryer,A.A. (1995) *Biochem. J.*, **312**, 713–716.
17 Cantlay,A.M., Smith,C.A.D., Wallace,W.A., Yap,P.-L., Lamb,D. and Harrison,D.J. (1994) *Thorax*, **49**, 1010–1014.
18 Kadokami,K. and Lewis, R.V. (1990) *Nucleic Acids Res.*, **18**, 3082.