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PCR Mutagenesis-Based Method for Generation of Positive Controls for SSCP Analysis

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ABSTRACT

We have developed a primer-mediated PCR mutagenesis-based method for the generation of positive controls to test the sensitivity of single-strand conformation polymorphism (SSCP) or any other PCR-based mutation screening method. This technique is based on the incorporation of a third longer primer, containing a mismatched base, into the PCR along with the two wild-type primers normally used to amplify DNA fragments for SSCP analysis. The longer mismatch primer (LMP) shares the sequence of one of the wild-type primers and also contains 5 to 10 additional bases, which include the mismatched base. The resulting PCR product is identical in length and sequence to the wild-type template with the exception that the LMP base mismatch is incorporated into nearly 100% of the product. We have observed an altered SSCP mobility pattern in all cases where positive controls have been generated using this technique. We believe that the use of such *in vitro*-generated controls can contribute to the interpretation of band patterns and to the optimization of experimental conditions for SSCP to facilitate maximum detection of sequence variants.

INTRODUCTION

The definitive method for the detection of mutant alleles is the direct sequencing of polymerase chain reaction (PCR) products (10), but this is tedious, time-consuming and costly when working with a large number of samples. For this reason, several methods of screening for mutations in DNA sequences have appeared (3) with the aim of analyzing the greatest number of patients and loci in the shortest possible time and at reasonable cost. One of the techniques most commonly used is single-strand conformation polymorphism (SSCP) analysis (9). When faced with searching for allelic variants in a gene

that has not been analyzed previously, it is very beneficial to use positive control material, such as DNA from individuals with previously characterized mutations to be confident that the conditions used are sensitive enough to detect all variants. Otherwise, negative SSCP results could be due to inappropriate SSCP conditions rather than the absence of variants.

The incorporation of point mutations is a recurrent topic in PCR applications and is the simplest method for modifying DNA sequences during amplification (4). This technique is based on the principle of hybridizing primers with mismatched bases under determined conditions (7,8,13).

Here we present a simple PCR-mediated mutagenesis method for generating mutated controls to be used to evaluate the sensitivity of SSCP conditions. The addition of a third longer primer with a mismatched base in the area not shared by the wild-type primer, longer mismatch primer (LMP), to the PCR tube of an individual (negative control) permits the *in vitro* generation of a positive control during PCR. To our knowledge, this is the first report of PCR mutagenesis being used for the generation of positive controls to evaluate SSCP sensitivity.

MATERIALS AND METHODS

In Vitro Mutagenesis of the RDS/Human Peripherin Gene (Exon 1, bp 508-849)

We PCR-amplified part of exon 1 (bp 508-849) of the RDS/human peripherin gene (11) from 100 ng (1 μ L) of genomic DNA in a PCR with a final volume of 10 μ L, including 75 μ M of each dNTP (Boehringer Mannheim, Mannheim, Germany), 1 U *Taq* DNA Polymerase (Boehringer Mannheim), 1 μ L of 10 $\times$ PCR standard buffer (Boehringer Mannheim), 0.1 μ L of [α^{32}]-dCTP (Amersham International plc, Little Chalfont, Bucks, England, UK) and 5 pM of each wild-type primer, whose sequences are: sense: 5' AAGT-ATGCCAGATGGAAGCC 3'; anti-sense: 5' TCTGACCCCAGGACTGG-AAG 3'.

In addition to the two wild-type primers, 5 pM of a LMP containing a

nucleotide (nt) substitution (A for a G) at position 829 of exon 1 of the RDS/human peripherin gene are added to three different control reactions selected to be mutated. The sequence of the LMP is 5' TCTGACCCCAGGAC-TGGAAGTCACT 3'.

The underlined sequence is shared by both the wild-type antisense primer and the LMP. Extension of the LMP during the PCR generates a PCR product that contains an A to G transition at position 829 of exon 1 of RDS/human peripherin gene.

The PCR conditions are: an initial denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 65°C for 1.5 min and a final extension at 74°C for 5 min.

The 342-bp PCR product is larger than the optimum size needed for analysis by SSCP (3). Therefore, before SSCP analysis, the product is digested with an endonuclease (5). In this case, *MspI* (Boehringer Mannheim) was used according to manufacturer's recommendations. The digestion

Figure 1. SSCP analysis of exon 1 of the RDS/peripherin gene. Lanes 1–5 represent individuals with a normal band pattern for exon 1 of the RDS gene. Lane 1 shows a homozygote for the nt 558 C to T polymorphism; lanes 2–5 are heterozygotes for this polymorphism. Lanes 6, 6*, 7, 7*, 8 and 8* represent the band pattern for three unrelated control individuals. The asterisk indicates where the LMP was added to the PCR. A band, labeled M in lanes 6*, 7* and 8*, corresponds to the change (nt 829 G to A) introduced during the PCR. The bands labeled W form the usual SSCP band pattern for each of the fragments analyzed. The band labeled P appears in the 156-bp fragment and corresponds to the nt 558 C to T polymorphism. The individual in lanes 7 and 7* is homozygous for this polymorphism. The individuals shown in lanes 6, 6*, 8 and 8* are heterozygous for this polymorphism. The band AW has been observed occasionally in the 156-bp fragment and corresponds to an alternative configuration of the wild-type pattern.

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Table 1. Summary of Experimental Conditions for In Vitro Mutagenesis Experiments 2, 3 and 4

	Experiment 2	Experiment 3	Experiment 4
Target Sequence Analyzed	RDS/Peripherin (Exon 2)	RDS/Peripherin (Exon 2)	Rhodopsin Gene (intron C)
GenBank Accession No.	U07148	U07148	K02281
Wild-Type Primers Used ^a	Sense: 4–23 Antisense: 315–297	Sense: 4–23 Antisense: 315–297	Sense: 4054–4073 Antisense: 4387–4369
LMP Name (Sense/Antisense)	fLMP (sense)	rLMP (antisense)	4ms (sense)
LMP Sequence ^b	5' <u>AAGCCC</u> ATCTCCAG- CTGTCTGTTCCCTTT 3'	5' <u>TTACCCTCTACCC</u> CC- AGCTGCACAGGG 3'	5' <u>TGGAGGAGCC</u> ATGGT- CTGGACCTGGGTCCC 3'
PCR Conditions	[(94°C-30'')→(61°C-20'')→ (72°C-40'')] ×30	[(94°C-30'')→(61°C-20'')→ (72°C-40'')] ×30	[(94°C-30'')→(64°C-30'')→ (74°C-1')] ×30
Primer Concentration ^c	125 nM	125 nM	200 nM
Nucleotide Mutation ^d	nt 25 del T	nt 294 G to T	nt 4076 C to T
Mutation Type	Single-base deletion	Transversion	Transition
PCR Product Size	312	312	334
Endonuclease ^e	SacI	SacI	BglI
Digested Fragment Sizes ^f	180 /136	180 / 136	156 /178
SSCP Running Conditions	5W/17 hours	5W/17 hours	7W/15H 30'
Figure	2B, lane 6	2B, lane 7	2A, lane 5

^aAll primers used (including LMPs) have been generated in an Oligo 1000 DNA Synthesizer™ (Beckman Instruments, Fullerton, CA, USA) at 200-nM scale. Numeration of wild-type primers are according to GenBank accession numbers.

^bUnderlined sequence of the LMPs is shared with the corresponding wild-type primer (sense or antisense).

^cEach LMP assay needs three primers (sense, antisense and LMP) in equal concentration.

^dAccording to GenBank accession numbers.

^eEndonuclease digestions according to manufacturer's recommendations. All endonucleases provided by Boehringer Mannheim.

^fBold number indicates the digested fragments that contain the in vitro-generated positive controls.

generates two fragments of 156 and 186 bp; the 186-bp fragment harbors the base mutated in vitro. In the 156-bp fragment generated by this method, it is possible to detect the polymorphism (nt 558 C to T) described by Farrar et al. (1).

Digested samples are diluted to a final volume of 75 µL, then 3 µL of each sample are mixed with 3 µL of SSCP stop solution/loading buffer (95% formamide, 20 mM EDTA, pH = 8.0, 0.05% bromophenol blue, 0.05% xylene cyanole), denatured for 5 min at 97°C and chilled on ice. Samples are loaded on a 4-mm-thick, nondenaturing 0.5× MDE™ Gel (AT Biochem, Malvern, PA, USA) containing 10% glycerol and 0.6× TBE buffer (Boehringer Mannheim). Electrophoresis is carried out using 0.6× TBE buffer overnight at room temperature with the power fixed at 6 W. The gel is dried and subjected to

autoradiography at -80°C for 24 h. Figure 1 shows the results of this experiment.

In Vitro Mutagenesis of Additional DNA Target Sequences

To demonstrate that our experimental approach can be used to introduce point mutations into any DNA sequence, we include experimental details for three additional in vitro mutagenesis experiments in Table 1. A single-base deletion (Experiment 2) and a G to T transversion (Experiment 3) are created in exon 2 of the RDS/human peripherin gene. A C to T transition is introduced in intron C of the rhodopsin gene (6) (Experiment 4). Experimental conditions not shown in Table 1 are identical to those described above for exon 1 of RDS/human peripherin (bp 508–849). Figure 2 shows results of

SSCP analyses performed using the mutated segments. Each experiment has been repeated several times, obtaining identical results (data not shown).

RESULTS AND DISCUSSION

Figure 1 shows the results of SSCP analysis of exon 1 of the RDS/human peripherin gene. The lanes corresponding to the PCRs where the LMP was added (*) have an identical altered migration pattern for one of the bands of the 186-bp fragment, whereas the PCRs without the LMP share the normal pattern shown in lanes 1–5. It is interesting to note that in the reactions containing LMP, the wild-type band has completely disappeared. This suggests that close to 100% of the product has the mutation incorporated. In the lower section of Figure 1, the bands for the 156-bp

fragment are shown (for details see legend for Figure 1).

Figure 2 shows the results of three additional SSCP analyses using positive controls generated in mutagenesis Experiments 2, 3 and 4. Lane 5 of Figure 2A shows the SSCP pattern of an in vitro transition mutation (nt 4076 C to T) in intron C of the rhodopsin gene generated in Experiment 4 (for details see Table 1 and legend for Figure 2). Figure 2B shows SSCP analysis of exon 2 of the RDS/human peripherin gene and its flanking sequences. In this experiment, two different LMPs [one forward LMP (fLMP) and one reverse LMP (rLMP)] were used in two separate PCRs to test SSCP sensitivity in the two fragments (180 and 136 bp) generated after *SacI* (Boehringer Mannheim) digestion of the PCR product. The fLMP (Experiment 2) creates a base pair deletion at position 25 of exon 2 of the RDS/peripherin gene. The abnormal SSCP pattern generated can be seen in lane 6 of Figure 2B. In this case, the migration pattern is not altered; one of the 180-bp fragment bands that does appear in the wild-type SSCP pattern (lane 8), does not appear. This same pattern has been observed repeatedly (data not shown), and further experiments must be performed to evaluate the behavior of this single-base deletion under our SSCP conditions. The rLMP (Experiment 3) creates a single-base transversion (G to T) at position 294 (for details see Figure 2 and Table 1). This mutation generates an aberrant migration of both strands of the 136-bp fragment (Figure 2B, lane 7). In this case, the wild-type product is absent.

The sensitivity of the SSCP technique has not been studied sufficiently (10), and evaluation of SSCP sensitivity has been complicated by the absence of positive controls. In addition, "the use of positive controls is a valuable aid to confident interpretation" (12). Here we show four different experiments using primer-mediated in vitro mutagenesis with the same result: the generation of an aberrant SSCP pattern. Mutagenesis through the addition of an LMP in the PCR of a control individual represents a rapid, easy and economical method for producing positive controls for SSCP or any other PCR-based mutation screening methodology. This

mutagenesis approach should prove useful regardless of the pathology being studied.

Remarkably, our experimental approach permitted us to generate mutated controls in one step during the PCR. The mismatched base incorporated lies outside the wild-type primer sequence used to amplify the DNA segment. Both the wild-type primer and the LMP

that introduces the single base change are added to the PCR without the risk of reversion to the wild-type product during the PCR. A preceeding step to generate the mutation is not necessary. Since the LMP is always extended at the 3' end, the final product is identical in size to the control segment being amplified (with exception of deletions). More importantly, the mutation is

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introduced into the exact sequence context as the control template, and the DNA region to be screened.

The reason why the alteration is produced in 100% (or close to 100%) of the PCR product is not clear. Initially, we expected that adding two primers that recognize the same target in the DNA sample would result in competitive annealing (2) and the amplification of both products. However, our results suggest that the greater size of the LMP favors its annealing. This may be due to "co-annealing" of both primers, which obligates extension of the longer primer. On the other hand, an exponential loss of wild-type product could take place during PCR amplification.

With the use of our experimental approach, only positions that are close to the ends of PCR products can be mutated. Nevertheless, we feel that the gen-

eration of positive SSCP controls through the use of LMPs may be useful for interpreting band patterns and in setting up of individually optimized experimental conditions for a specific SSCP target fragment. This should help to reduce the number of false negatives when screening for mutations using SSCP or any other PCR-based mutation screening procedure.

Obviously this method does not solve the problem of SSCP controls completely. The detection of a single change does not mean that the conditions used can detect 100% of mutation possibilities. However, the observation of an aberrant SSCP pattern provides confirmation that the method has been performed correctly. In addition, a negative with an in vitro-mutated positive control serves as a signal that SSCP conditions should be revised. However, control materials may not always be available for the DNA region to be analyzed, particularly when screening for mutations not previously reported. In vitro-generated mutations can be used as a complement to a set of previously characterized mutated samples. In addition, in vitro-generated controls could be useful in the development of novel mutation detection methods.

Figure 2. Results of in vitro mutagenesis Experiments 2, 3 and 4. (A) SSCP analysis of exon 4 of the rhodopsin gene. Brackets and numbers at left indicates the position and size in base pairs of each DNA fragment generated after *Bgl*II (Boehringer Mannheim) digestion of the PCR product in Experiment 4. For details see Table 1. Lane 1: nondenatured sample; lanes 2-4: individuals with a normal SSCP pattern; lane 5: DNA sample mutated in vitro using LMP 4ms (generates nt 4076 C to T transition in the 156-bp fragment; arrow indicates the position of an altered band; lane 6: the same DNA sample shown in lane 5 without addition of LMP 4ms to the PCR. (B) SSCP analysis of exon 2 of the RDS/peripherin gene. Brackets and numbers at right indicate the position and size in base pairs of each DNA fragment after *Sac*I (Boehringer Mannheim) digestion in Experiments 2 and 3. Lane 1: nondenatured sample; lanes 2-4: individuals with a normal SSCP pattern; lane 5: nonamplified sample; lane 6: DNA sample mutated in vitro using rLMP, which generates nt 25 del T deletion in the 180-bp fragment; lane 7: the same DNA sample used in lane 6 but mutated in vitro using rLMP, which generates nt 294 G to T transversion in the 136-bp fragment; lane 8: the same DNA sample used in lanes 6 and 7 without addition of the LMP to the PCRs. Arrows indicates the positions of an absent band in lane 6 and the band shifts in lane 7.

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