Inhibition of PCR Amplification by a Point Mutation Downstream of a Primer

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ABSTRACT

A T→C point mutation is shown to specifically inhibit PCR amplification when compared to wild-type controls in exon H of the factor IX gene. Multiple primers of different lengths and locations were designed to examine this phenomenon. The experiments suggest that poor annealing and/or extension from the downstream primer are responsible for the observed inhibition and that the mutation can exert an inhibitory effect upon PCR amplification at a distance of at least 84 bp. The inhibition was not alleviated when amplification conditions such as annealing temperature, time of extension, type of DNA polymerase or concentration of DNA template, primer or DNA polymerase were varied. The inhibitory factor(s) are likely to be contained within the amplified segment itself because neither the use of a previously amplified PCR product as template for nested PCRs nor the restriction enzyme digestion of that previously amplified product relieved the inhibition of PCR amplification in the mutant sample. Computer analyses with the FOLDRNA and FOLDDNA programs did not reveal the mechanism of inhibition. Although dramatic inhibition, as shown here, may be uncommon, more subtle inhibition may be frequent. Documentation of differential amplification caused by a single-base substitution in template sequence has implications for certain commonly used PCR-based methods such as quantitative PCR, differential display and DNA fingerprinting. In addition, heterozygous single-base pair mutations downstream of a primer may be missed if the PCR is inhibited; alternatively, the mutation may appear to be homozygous if amplification of the mutated allele is selectively enhanced.

INTRODUCTION

While the polymerase chain reaction (PCR) method is an exceptional tool, the efficiency of amplification of PCR can be affected in many ways. For example, denaturation can be inhibited by regions of high G+C content, and primer annealing and elongation can be inhibited by template secondary structure. Heterozygosity of a sample can lead to the preferential amplification of one allele over another due to (i) differential denaturation caused by differences in melting temperature of two alleles (e.g., human leukocyte antigen [HLA] locus), (ii) length differences between alleles when Taq DNA polymerase is limiting (e.g., variable number tandem repeat [VNTR] analysis) and (iii) stochastic fluctuation in the number of copies of each allele when the initial number of templates is very small (2).

In an effort to develop a new PCR-based screening method for the detection of mutations, we discovered that PCR amplification was specifically inhibited in a hemophilia B patient with a T31340C mutation within the segment to be amplified. However, this inhibition was not observed in wild-type controls or 44 other hemophilia B patients with mutations in exon H of the factor IX gene. The data suggest that this mutation specifically inhibits primer annealing or extension during PCR. To our knowledge, this is the first report describing inhibition of PCR amplification by a single-base sequence change within the segment to be amplified.

MATERIALS AND METHODS

Oligonucleotides

The sequence and position of oligonucleotides D1–D7 are shown in Figure 1. The other oligonucleotide sequences and informative names are listed as follows: P1: F9(30646)-34D GGCTTTTTGGTCTGAAAAATATGCATTGGCTCTC; U1: F9(31454)-17U AACAAAAGATGGGAAAG; U2: F9(31584)-17U GACTGTAATTTCCTAAC; U3: F9(31645)-31U CTGATGGAAGAGTGGAGAATTTAACTTCAC. The precise sizes and locations of the PCR-amplified segments can be obtained from the informative names (1). As an example, for oligonucleotide P1, F9 represents the human factor IX gene, (30646) is the base number to which the 5' end of the primer anneals (numbering as described in Reference
PCR Amplification from Genomic DNA Templates

PCR amplification for the PCR inhibition experiments in Figures 2 and 4 were performed directly on genomic DNA templates. *Tth* and *Taq* DNA Polymerases (Boehringer Mannheim, Indianapolis, IN, USA) were used for comparison. The reaction conditions for *Tth* were 100 mM KCl, 10 mM Tris-HCl (pH 8.9 at 25°C), 1.5 mM MgCl$_2$, 50 µg/mL Rinder serum albumin (RSA), 0.05% (vol/vol) Tween® 20, 200 µM of each dNTP, 0.1 µM of each primer, 0.25 U of *Tth* DNA polymerase and 250 ng of genomic DNA.

The reaction conditions for *Taq* were 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, 200 µM of each dNTP, 0.1 µM of each primer, 0.5 U of *Taq* DNA polymerase and 250 ng of genomic DNA. Both mixtures contained a total volume of 25 µL. Amplified segments were electrophoresed through a 3% agarose gel in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 6 V/cm for 40 min. The gels were stained in 0.5 µg/mL ethidium bromide for UV photography.

DNA templates for the experiments shown in Figure 3 were prepared from PCR-amplified DNA products of wild-type and mutant human genomic DNA. Oligonucleotide primers P1 and U3 were used to amplify a 1-kb region of exon H in the human factor IX gene with *Taq* DNA polymerase. The PCR product was purified by a Centricon® 100 microconcentrator (Amicon, Beverly, MA, USA) to remove the unincorporated primers and dNTPs. The amount of recovered DNA was determined by spectrophotometry at 260 nm and diluted to 2 ng/µL.

PCR inhibition experiments were performed with the previously amplified PCR segments as template. The cycling conditions were the same as outlined above except only 12 cycles were performed. The PCR conditions were also the same as outlined above for *Tth* DNA polymerase, except the mixture contained 200 µM of each dNTP, 0.1 µM of each primer, 0.2 U of *Tth* DNA polymerase, 0.4 ng of PCR-amplified DNA and 1 µCi [$\alpha$-32P]dCTP (3000 cpm/mmol; Amersham, Arlington Heights, IL, USA) in a total volume of 20 µL. The labeled DNA samples were electrophoresed through a 3% agarose gel and stained with ethidium bromide for UV photography. The agarose gel was then dried and subjected to autoradiography and quantitative analysis by a PhosphorImager™ (Model 425B; Molecular Dynamics, Sunnyvale, CA, USA). The dried gel was exposed for 8 min, and the PCR yield was quantitated with ImageQuant™ software (Molecular Dynamics). The PCR yield was...
defined in arbitrary units relative to the background. Percent inhibition is defined as the ratio of the mutant PCR yield to the wild-type PCR yield using identical primers and conditions.

RESULTS AND DISCUSSION

Inhibition of PCR Amplification by a Downstream Mutation

The phenomenon of PCR amplification inhibited by a mutation downstream from a primer was first observed by us in an experiment involving multiplex PCR. When samples from multiple hemophilia B patients with known mutations in this region were amplified, certain segments were consistently absent from one patient with a T31340C transition. These segments should have been amplified with a downstream primer (Figure 1) whose 3′ end was 3 bp upstream from the mutation. To study this phenomenon in more detail, we synthesized oligonucleotides of different lengths and positions (Figure 1). Oligonucleotides D1–D4 were designed to examine the effect of primer length on the inhibition. These primers varied in size from 12–18 nucleotides, but the 3′ ends were all 3 bases away from the mutation site. Oligonucleotides D2 and D5–D7 were designed to examine the inhibitory effect of primer position. These primers were all 14 nucleotides in size and were located 3, 1, 12 and 84 bp from the mutation, respectively.

Figure 2 demonstrates the inhibitory effect that the mutation exhibits on PCR amplification. In this experiment, each downstream primer (D1–D7 and P1) was paired with the U2 upstream primer for both wild-type and mutant genomic samples. Efficient amplification was observed for all combinations using wild-type DNA. However, amplification of the mutant genomic DNA was inhibited completely by using primers D1, D2, D5 and D6. Partial inhibition was also observed when primers D3 and D4 were used. However, no inhibition was observed for primers D7 and P1. These data indicate that the inhibition of primer annealing and/or extension for the mutant sample was correlated with primer size and distance from the mutation. Figure 2 was duplicated using U1 and U3 upstream primers with similar results (data not shown). Similar results also were obtained when the above experiment was performed under the following conditions: (i) The annealing temperature was changed to 45°C and 55°C, (ii) the 72°C extension period was increased to 3 min and (iii) the concentration of the

![Figure 3](image-url) Quantitative analysis of PCR inhibition. DNA, previously amplified with P1 and U3 and *Taq* DNA polymerase, was diluted and amplified for 12 cycles using *Tth* DNA polymerase (see Materials and Methods). After agarose gel electrophoresis, PCR yields were quantitated by a PhosphorImager. A) The U2 upstream primer was used in all amplifications with downstream primers D1–D7 and P1. Quantitative analysis reveals all mutant reactions were inhibited except P1 when the previously amplified PCR DNA was used as template. B) The D2, D4, D6 and D7 downstream primers were paired with the U3 and U1 upstream primers, respectively. Quantitative analysis reveals that the mutant samples are inhibited, independent of which upstream primer is used. The combination of P1 and U3 serves as a control. W = wild type. M = mutant.

![Figure 4](image-url) Inhibition of mutant genomic sample using *Taq* DNA polymerase. The U2 upstream primer was used for all reactions. Inhibition of PCR amplification was also observed using *Taq* DNA polymerase. The combination of P1 and U2 serves as a control. Lane M = 120 ng of φX174/HaeIII DNA.
DNA polymerase was varied from 0.1 to 0.8 U/reaction (data not shown).

Quantitation of the Inhibition

Experiments were designed to quantitate the relative amounts of PCR inhibition (Figure 3) (see Materials and Methods for details on experimental design). Inhibitory effects were observed for primers D1–D6 when compared to wild-type controls. Moderate inhibition (50.8%) was also observed for D7, which was 84 bases away from the mutation. However, no inhibition was observed for P1, which was 663 bp from the mutation. These results were repeated three times with similar results.

To examine the possibility that the inhibition occurs in the first few cycles of PCR due to interaction with flanking sequence, the 1-kb segments amplified using P1 and U3 were also digested with the HphI restriction endonuclease. This digestion generated a 356-bp fragment for both the mutant and wild-type samples. This enzyme cleaves specifically at a site 50 bp 5′ to the mutation site (see Figure 1). When used as the template for the subsequent amplifications, inhibition of amplification was observed again for the mutant sample using primers D1–D6 (data not shown). These experiments suggest that inhibition is not dependent on the characteristics of sequences 5′ of the HphI site in the 1-kb amplified segment that was derived.

Effect of Upstream Primer Position

To assess the effect, if any, that the upstream primer may have on the observed inhibition, we used two different upstream primers (U1 and U3, 100 bp and 275 bp from the mutation, respectively) (Figure 3B). The template and protocol were the same as those used in Figure 3A. A pronounced inhibition of PCR amplification occurred in all of the reactions with the mutant sample involving primers D2, D4, D6 and D7 as compared to wild-type controls. Figure 3B demonstrates that the inhibition was independent of the upstream primer used.

Effect of DNA Polymerases

The efficiency of amplification may vary with the DNA polymerase. The experiments in Figures 2 and 3, which demonstrate PCR inhibition, utilized Tth DNA polymerase. Taq DNA polymerase was used to evaluate the effect of another DNA polymerase. Multiple amplifications directly from wild-type and mutant genomic DNA with Taq DNA polymerase were performed under a variety of conditions using different upstream primers. Amplification did not occur in wild-type samples using D1, D6 and D7. However, when amplification was possible for wild-type samples, inhibition was observed in the corresponding mutant samples (Figure 4). Pfu DNA polymerase was also analyzed with similar results (data not shown). These experiments suggest that, while some variation occurs due to differences in DNA polymerases, the inhibitory effect observed here is independent of the polymerase used.

Effect of Other Mutations

Two additional patients with the identical mutation at 31340 were examined to determine if the observed inhibition was specific to the sample analyzed above. The inhibition was also observed in these patients.

Seven other hemophilia B patients with mutations near 31340 were tested for inhibition of amplification. Four were point mutations 5′ to 31340 (G31276A, T31304C, C31317A and C31328T), one was a point mutation (C31356A), one was a three-base deletion (Δ31355-7) and one was a four-base insertion (31345) 3′ to 31340. No inhibition was observed in any PCR amplification utilizing these samples when compared to wild-type controls, suggesting that the inhibition is relatively specific to the mutation at 31340 and not to a larger region of the factor IX gene.

Analysis of DNA Secondary Structure

The FOLDRNA program (University of Wisconsin Genetics Computer Group, Madison, WI, USA) with standard RNA and also FOLDDNA folding energies (Marquis Gacy, personal communication) were used to examine the secondary structure of the region surrounding the mutation at 31340.
ondary structure specific to the mutant sequence was modeled. However, it was not drastically different from the wild-type sequence and likely does not affect the efficiency of PCR amplification (data not shown).

In an attempt to disrupt any secondary structure specific to the mutant sample, we designed four downstream primers identical to D3 to contain unique mismatches. In theory, the mismatches could destabilize a hairpin or other structure in the template, enabling the mutant sample to be amplified. These mismatched primers were paired with U1 primer for PCR amplification. Inhibition was observed for the mutant samples, while efficient amplification occurred for the wild-type samples (data not shown). These experiments suggest that the inhibition may be due to factors unrelated to template secondary structure.

The experiments above have demonstrated the inhibition of PCR amplification by a point mutation in the factor IX gene. Although the prevalence of such a dramatic inhibition may not be high, the incidence of more subtle differences in the efficiency of amplification of two samples with seemingly insignificant differences in DNA sequence may be significantly greater. This phenomenon should be considered when performing PCR-based methods that critically depend on the efficiency of PCR amplification.

REFERENCES


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Simple Method for Selective Amplification of cDNA from a Defined Promoter

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ABSTRACT

A simplified technique for the detection of transcripts from a defined promoter is described. After reverse transcription, a PCR target sequence is selectively added to the 3’ end of cDNA strands by DNA polymerase extension directed by an oligonucleotide template. Those cDNA molecules that do not have ends within a few nucleotides of the promoter start site are not extended and thus are excluded from subsequent amplification. Even when amplified products are visualized by ethidium bromide staining of agarose gels, this method requires only 1% of the RNA usually needed for detection of mRNA by standard RNase protection utilizing radiolabeled probes. In contrast to direct detection of cDNA by PCR, this procedure restricts amplification to a narrow subset of transcripts even when other overlapping colinear transcripts are present. We call this detection procedure specific amplification of cDNA ends (SPACE).

INTRODUCTION

While studying gene regulation in papilloma viruses, we sought a sensitive method for detecting transcripts initiating from a specific promoter whose start site may overlap with transcripts from adjacent promoters. To detect such transcripts, primer extension or RNase protection assays have been used with limited sensitivity. We describe here a polymerase chain reaction (PCR)-based amplification strategy that detects only cDNA molecules with defined ends. In contrast to the cDNA end amplification methods previously described [e.g., rapid amplification of cDNA ends (RACE) reviewed in Reference 3], this method exhibits specificity and is therefore referred to as specific amplification of cDNA ends (SPACE).

MATERIALS AND METHODS

Figure 1 diagramatically depicts