MEDICAL SCIENCES (2000), 2, 11–14

Effect of single mismatches at 3′–end of primers on polymerase chain reaction

*Simsek M, Adnan H

تأثير عدم التوافق الاحادي عند نقطة الأنتهاء الثلاثي للمواد التمهيدية في سلسلة تفاعل البوليمارز (حافز البلمرة)

م. سمسك ، ح. عدنان.

الملخص: الهدف: معرفة مدى تأثير ثلاثة أنواع من عدم التوافق بين قواعد الحمض النووي (بيتاجين الهيموجلوبين) وقواعد الانتهاء الثلاثي للمحفز. الطريقة والنتائج: ظهر أن الارتباط الذي حدث بين زوجي القواعد الثلاث (ج/ج،ت/ج،أ/ج) ساعد في عملية التكاثر السريع للمنطقة المنشودة في جين بيتا الهيموجلوبين (الهدف)، وهي منطقة تحتوي على 268 قاعدة حامض نووي مزدوج، وذلك باستخدام سلسلة من درجات الحرارة تراوحت بين 45 و 65 درجة مئوية، وهي ما تعرف بدرجة حرارة التلاحم المتماثل. تلاحم قاعدتي المحفز والهدف (ت/ج) كان قويا مثل التلاحم الطبيعي الذي يحدث بين قاعدتي (س/ج) حدث هذا التلاحم في كل درجات الحرارة المذكورة سلفا لم ينتج من التحام القواعد الأخرى (ج/ج أو أ/ج) أي تفاعل يذكر سوى التفاعل الضعيف للقاعدة ج/ج عند درجتي حرارة 45 و 50 درجة مئوية.
الخلاصة: ان المحفزين اللذين أمكنهما التفاعل هما زوجي القواعد (أ/ج و ج/ج) وقد تم التفاعل مع المنطقة المنشودة من جين بيتا الهيموجلوبين.

ABSTRACT: ***Objective and Method –*** To investigate the effect of three different mismatches (G/T, G/A or G/G) at the 3•–end of a primer to amplify a 268 bp (base pair) region of the human •–globin gene using different annealing temperatures (45 to 65•C). ***Results –*** The primer with the G/T mismatch was as efficient as the normal primer (G/C match) in the amplification of a 268 bp product at all temperatures tested. However, the primers having G/A or G/G mismatches at the 3'-end did not produce any specific polymerase chain reaction (PCR) fragment at all the annealing temperatures used, except a barely detectable 268 bp product for the G/G mismatch at 45 and 50•C. ***Conclusion –*** We conclude that our PCR system was refractory to amplification when one of the primers contained a G/A or G/G mismatch at the 3•–end with template DNA.

KEY WORDS: PCR, mismatched primers, β–globin gene

Polymerase chain reaction (PCR) is a sensitive and powerful method by which specific sequences of template DNA can be amplified more than a million fold using 20–30 cycles of DNA synthesis.[1] Each cycle requires three stages: first, denaturation of DNA at high temperature (90–94ºC), second, annealing of oligonucleotide primers at a lower temperature (usually at 40 to 60ºC), and finally, extension of primers at 72°C by *taq* DNA polymerase, a heat stable enzyme. Most of the DNA polymerases used in PCR lack an intrinsic 3' to 5' exonuclease activity.[2] Thus, oligonucleotides with a mismatch at the 3'–end may be extended and serve as primers in the polymerase chain reaction (PCR). The efficiency of such primers would depend on many factors, including the nature of the mismatch, the kinetics of association and dissociation of primer-template DNA duplex at the annealing and extension temperatures, and the effect of a mismatch on the stability of the duplex DNA formed.

Several investigators have begun to evaluate the possible effects of mismatches in their primers.[3-5] It has been shown that a single mismatch at or near the terminal 3' base of a primer affects PCR more dramatically than those single mismatches located internally or at 5' end.[6] Using primers with one or more mismatches near the 3' end, allele specific amplification (ASA) has been developed[7] for the detection of mutations in genetic diseases.

In this study, we have evaluated the effect on PCR, of three different mismatches (G/T, G/A and G/G) located at the 3'–terminal end of primers which were used to amplify a small region in the human β-globin gene.

METHOD

DNA ISOLATION

Miller's salting-out procedure[8] was used to isolate genomic DNA from whole blood with ethylenediamine tetra acetic acid (EDTA) as anticoagulant. The

Department of Biochemistry, Sultan Qaboos University, P.O.Box: 35, Postal Code: 123, Muscat, Sultanate of Oman

*To whom correspondence should be addressed.

nuclei of white blood cells were isolated after lysis of cells with a non-ionic detergent. The isolated cell nuclei were suspended in a buffer containing an ionic detergent (SDS) and proteinase K which digests the proteins associated with chromosomal DNA. All the proteins were precipitated using high salt concentration (salting out) and the DNA in the supernatant was recovered by precipitation with ethanol. The amount of DNA obtained was around 5–10 μg per 0.5 ml whole blood.

DNA amplification by Polymerase Chain Reaction.

A small region (286 bp) of human β–globin gene was amplified using a pair of primers which were either completely complementary to the globin gene, or had a single mismatch at the 3'–end. Genomic DNA was amplified in 50 μl reaction volumes with 25 pmoles of each primer, 1 unit of ampli*Taq* DNA polymerase (Perkin-Elmer Cetus Inc.), 200 μM each of the four deoxynucleoside triphosphates, and a buffer containing 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatin, and 1 mM $MgCl_2$ (unless otherwise mentioned). All amplifications were started by the addition of the Taq DNA polymerase at 94°C (hot start) and continued for 30 cycles in a DNA thermal cycler (Hybaid Inc.) Each cycle of amplification contained the following steps: DNA denaturation: 2 minutes at 94°C, primer annealing: 2 minutes at 50°C, and primer extension: 3 minutes at 72°C. The PCR products were separated by electrophoresis on 2.5% agarose gels and detected by staining with ethidium bromide (0.5 μg/ml) for 15 minutes in electrophoresis buffer (40 mM tris acetate pH 8.0 and 1mM EDTA). A polaroid land camera (MP4 Kodak) was used to take pictures of the gels over a UV trans-illuminator in dark room.

Synthesis of DNA primers

Two sets of oligonucleotide primers were synthesised using a DNA synthesizer (Pharmacia, Gene Assembler Special 4). One set completely matched the template DNA while the other had an upstream primer with a mismatch at its 3'–end. If the A residue in the ATG initiation codon is taken as position 1 in the human globin gene, the down–stream primer (HG–P2) was complementary to the sense strand (positions 54 through 73), and had the following sequence: 5'–CAACTTCATCCACGTTCACC–3'. The upstream primer (HG–P1) complemented the anti-sense strand of the β–globin gene (from position –195 to –176) with the following sequence: 5'–AAGAGCCAAGGACAGGTAC–3'. The three different upstream primers (HG–$P1_A$, HG–$P1_G$, HG–$P1_T$) with a single mismatch were identical to the sequence of the HG–P1, except that they had an A, G or T residues, respectively replacing the terminal C at their 3'–end.

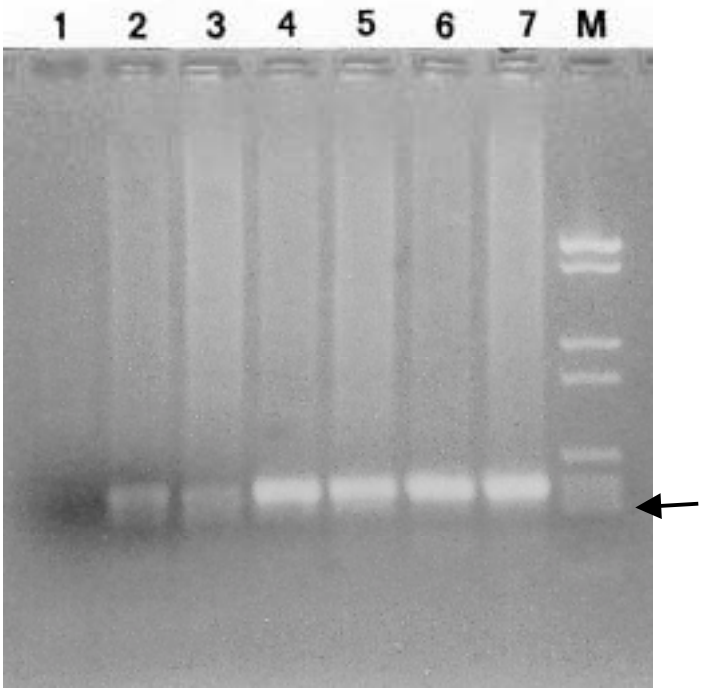

FIGURE 1. *The effect of Magnesium concentration on PCR.*

The reaction conditions were as described in the 'methods' section, using normal primers, except the variation in Mg^{2+} concentration. After 30 cycles of amplification, the PCR products were separated by electrophoresis on a 2.5% agarose gel (at 90 volts for 60 minutes) and detected by staining with ethidium bromide. The arrow shows the position of a specific PCR product (268 bp). Lane M contained DNA size markers. Lane 1 was negative control (without any template DNA added) in the PCR amplification. The reactions in lanes 2 to 6 contained different concentrations of Mg^{2+} ions: lanes 2 & 3 (0.5 mM),

RESULTS

The primers used in this work specified the amplification of a 268 bp region of the human β–globin gene. Before studying the effect of mismatches at the 3'–end of primers, an optimization for $MgCl_2$ was carried out since any PCR amplification is known to be very sensitive to magnesium ion concentration.[9] Using the normal primers, it was found that a detectable 268 bp PCR product was amplified at different Mg^{+2} ion concentrations ranging from 0.5 to 4.0 mM in 0.5 mM increments (data not shown). However, above 1.5 mM concentration, there were too many nonspecific products, and furthermore, the desired 268 bp product decreased in quantity with increased Mg^{+2} ion con-

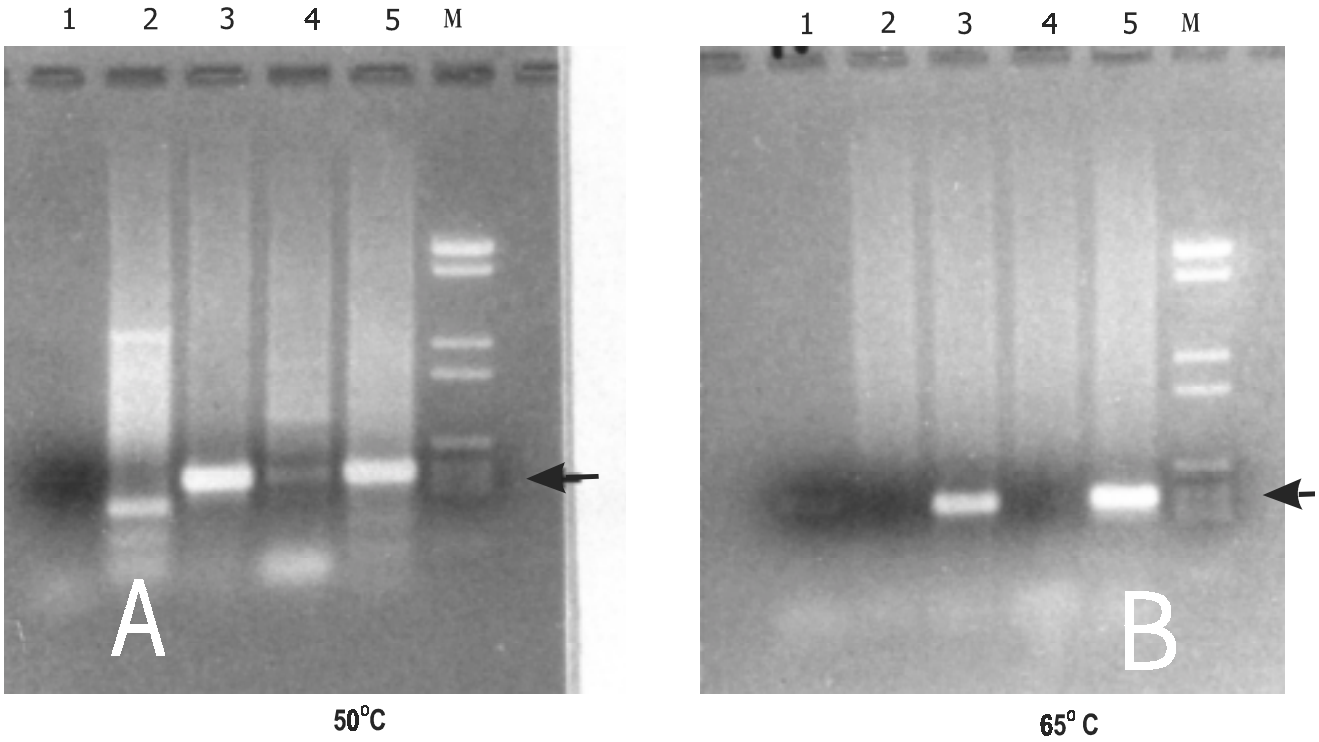

FIGURE 2. *The effect, on PCR, of single mismatches between the 3' base of a primer and template DNA at two temperatures*

The arrows indicate the position of the specific 268 bp PCR product. In both panels, lane M contained DNA sized markers. Lane 1 was negative control and lane 5 the positive control containing normal pair of primers. Lanes 2, 3 and 4 contained the mismatched primers, ending with G/A, G/T and G/G, respectively.

centration. Figure 1 shows a duplicate titration of magnesium ions at 0.5 to 1.0 mM range. There were no detectable non-specific products and the best amplificaton of the 268 bp product was achieved at 1.0 mM $MgCl_2$ concentration (figure 1, lanes 6 &7).

To study the effect of mismatches at the 3'–end of primers, we attempted to amplify the 268 bp DNA fragment at different annealing temperatures, from 45 to 65ºC using a normal downstream primer (HG–P2) together with a single mismatch containing upstream primer (HG–$P1_A$, HGP1T or HG–P1G). Having a G residue in the template DNA at the corresponding position, each of these primers would normally form 19 normal base pairs with the template, but in this case, resulted in a mismatch of G/A, G/T or G/G at the 3' terminal end, respectively. Figure 2 shows some representative results obtained with different primers at 50 and 65ºC. At both annealing temperatures, when normal primer pairs were used (figure 2, lane 5) the desired 268 bp DNA fragment was amplified satisfactorily without any detectable non-specific products. Interestingly, the primer with a G/T mismatch produced similar results (figure 2, lane 3). However, the results obtained with the primers containing either a G/A or G/G mismatch were entirely different. The primer with the G/A mismatch (figure 2B, lane 2) showed no detectable 268 bp product at 65ºC, a fairly distinct but a smaller PCR product than 268 bp at 50ºC, but was undetectable at 65ºC (Lanes 4 in figure 2B and 2A respectively).

DISCUSSION

We have studied here the priming efficiency of a 20 nucleotide long primer set which provided three different single mismatches (G/T, G/A and G/G) with the template DNA used. The G residue was located in the template (β-globin gene) while T, A or G were at the 3'–end of the primers.

Our results showed that the G/T mismatch primed the synthesis of a 268 pb specific PCR product, almost as efficiently as the normal primer (HG–P1) which contained a G/C match at the 3'–end. However, the priming efficiency of the primers with G/A or G/G mismatches was extremely poor, and resulted in the production of no specific PCR fragment that was detectable by staining with ethidium bromide.

The results obtained with all three mismatches were essentially the same as in figure 2 at different annealing temperatures tested (45 to 65ºC) except that the specific PCR product (268 bp) was barely detectable for the G/G mismatch at 45, 50 and 55ºC but for the G/A mismatch, no specific product was detectable

at all at any of the tested annealing temperatures. The G/A mismatch, however, produced a fairly strong and distinct product at 45ºC, sized <200 bp and was not produced at all at higher annealing temperatures (figure 2B, lane 2). We concluded that it must be a non-specific PCR product formed possibly due to dimerization of the primers.[2]

The priming efficiency of mismatched primers has already been studied by various groups. Our result with G/T mismatch agrees with that of Kwok[11] who showed that a single mismatch of T/T, T/C or T/G at 3'–end had no effect on the priming efficiency of their primers. The same group also studied a A/G mismatch, and our results agree with theirs. However, our results with the G/G mismatched primer (figure 2, lane 4) contradict some of the published work. For example, Newton[10] reported that a single G/G mismatch had very little effect on the priming efficiency of their primers, while we could get only a barely detectable specific PCR product at 45–55ºC, and none at 60 or 65ºC.

One potential application of mismatched primers has been towards identification of point mutations in genetic diseases. Using ASA primers which differed only by one base at 3'–end, like ours, Okayama[7] was able to detect three different point mutations in patients with an α1-antitrypsin deficiency. However, their results were contradictory to ours, as well as many other studies reported[10,11] in that 10 of the 12 possible mismatches, including a G/T mismatched primer, were all refractory to any detectable amplification.

However, it is difficult to make a direct comparison of the results reported with various 3' mismatched primers, due to differences in the PCR amplification conditions, especially the length and concentration of primers. In some studies, the primers were too short (12–16 bases)[12] and hence a 3'–mismatch may have more dramatic effects both on the stability of primer-template dublex and the extension of mismatched primer by DNA polymerase. On the other hand, if the primers are too long, like 30 bases used by Ehlen and Dubeau,[13] the effect of 3'–mismatch on PCR may be minimal. The primers used in this work were intermediate in size (20 bases long), a size commonly used by many research groups.

CONCLUSION

In summary, our upstream primers resulting in an A/G or G/G mismatch at the 3'–end with template DNA were refractory to amplification. But the same primer, when ended with a T/G mismatch, showed no refractoriness, and was as efficient as the normal primer for the amplification of a 268 bp DNA of the human β-globin gene.

REFERENCES

1. **Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA.** Primer-directed enzymatic amplification of DNA by a thermostable DNA polymerase. *Science* 1988, **239**, 487–91.
2. **Newton CR, Graham A., Enzyme Choice in PCR.** *Introduction to Biotechniques, Bios Scientific Publishers*, 1994, pp: 12–21.
3. **Gibbs RA, Nguyen PN, Caskey CT.** Detection of single base differences by competitive oligonucleotide priming. *Nucleic Acids Res* 1989, **17**, 2437–48.
4. **Eiken HG, Odland E, Boman H, Skjelkvale L, Engebretsen LF, Apold J.** Applications of natural and amplification created sites for the diagnosis of PKU mutations. *Nucleic Acids Res* 1991, **19**, 427–30.
5. **Tsai MY, Hanson NQ, Copeland KR, Beheshti I, Garg U.** Determination of a T/G polymorphism at nucleotide 3206 of the apolipoprotein cIII gene by amplification refractory mutation system. *Clin Chem* 1994, **40**, 2235–39.
6. **Lindeman R, Hu SP, Volpato F, Trent RJ.** PCR mutagenesis enabling rapid non-radioactive detection of common •thalassemia mutations in Mediterraneans. *Br J Haematol* 1991, **78**, 100–4.
7. **Okayama H, Curiel DT, Brantly ML, Holmes MD, Crystal RG.** Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. *J Clin Med* 1989, **144**, 105–13.
8. **Miller SA, Dykes DD, Polesky HF.** A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988, 1215–18.
9. **Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N.** Enzymatic amplification of β–globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science* 1985, **230**, 1350–54.
10. **Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalshekar N, Smith JC, Markham AF.** Analysis of any point mutation in DNA; the amplification refractory mutation system (ARMS). *Nucleic Acids Res* 1989, **17**, 2503–16.
11. **Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, Sninsky JJ**. Effects of primer-template mismatches on the polymerase chain reaction. *Nucleic Acids Res*, 1990, **18**, 999–1005.
12. **Wu DY, Ugozzoli L, Pal BK, Wallace RB.** Allele specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anaemia. *Proc Natl Acad Sci USA* 1989, **86**, 2757–60.
13. **Ehlen T, Dubeau L.** Detection of ras point mutations by PCR using mutations-specific, inosine containing oligonucleotide primers. *Biochem Biophys Res Commun* **160**, 441.