RAPID COMMUNICATION

Asymmetrically Primed Selective Amplification/Temperature Shift Fluorescence Polymerase Chain Reaction to Detect the Hemoglobin Constant Spring Mutation

By Gerald L. Kropp, Suthat Fucharoen, and Stephen H. Embury

Hemoglobin (Hb) Constant Spring is an α-thalassemic hemoglobinopathy that is a major cause of severe α-thalassemia in Southeast Asians. The difficulty of diagnosing Hb Constant Spring using standard electrophoretic methods has led to interest in DNA-dependent diagnostic methods. The methods developed have had to contend with the high degree of homology of the α2-globin gene (the site of the Hb Constant Spring mutation) and the α1-globin gene. We have developed a single reaction polymerase chain reaction-based method that uses asymmetric priming and a temperature shift to accomplish dual ends, selective amplification of α2 but not α1 DNA and discrimination of normal and Hb Constant Spring α2 genes by allele-specific fluorescence polymerase chain reaction. Advantages of this method over previous approaches include avoiding radioisotopes, precluding the need for electrophoresis, and serving as its own control for successful amplification. It is readily applicable to routine diagnosis, population screening, and prenatal diagnosis.

Funding sources: Supported in part by Grant Nos. HL20985 and HL34408 from the National Institutes of Health, Bethesda, MD.

From the Divisions of Hematology of the Departments of Medicine, San Francisco General Hospital, The University of California San Francisco; the Northern California Sickle Cell Center, San Francisco, CA; and Siriraj Hospital and Mahidol University, Bangkok, Thailand.

Submitted March 4, 1991; accepted April 2, 1991.

Supported in part by Grant Nos. HL20985 and HL34408 from the National Institutes of Health, Bethesda, MD.


Address reprint requests to Stephen H. Embury, MD, Bldg 100, Room 263, San Francisco General Hospital, San Francisco, CA 94110.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1991 by The American Society of Hematology.

© 1991 by The American Society of Hematology.
Fig 1. Strategy for selective α2-globin DNA amplification and allele-specific fluorescence PCR diagnosis of Hb Constant Spring using asymmetric priming and a temperature shift. This is a diagram of the α2 (leftward, 5')-globin gene showing 5' untranslated sequences (5' UT), the three exons (EX 1, 2, and 3) separated by two introns (IVS-1, IVS-2), and 3' untranslated sequences (3' UT). The unlabeled primers that direct selective amplification of α2 sequences are an antisense primer shown as a thick arrow below 3' UT and a sense primer shown as a thin arrow above IVS-2. These locations are where the α2 and α1 loci lack homology, providing the basis for selective α2 PCR. The relative thickness of the arrows represents asymmetric priming with limiting amount of the sense primer. Below the EX 3 and the translation stop codon the two thick arrows that diverge at their 3' ends represent the fluorescently labeled primers complementary to normal α2 or αβ. Their thickness demonstrates that they are not present in limiting amount compared with the unlabeled antisense primer. The “F” indicates that the normal primer is 5' tagged with fluorescein and the “R” that the αβ primer is 5' tagged with rhodamine. Products of 165 bp result from PCR with these labeled sense primers and the unlabeled antisense primer, as demonstrated by the line below.

Primers: Sequences of the primers are shown in Table 1. Oligonucleotides were synthesized on a Cyclone model DNA synthesizer (Milligen Biosearch, Novato, CA) as described, except for the source of the 5' amine linker (β-LINK N-TFAc hexanolamine linkers; Milligen Biosearch). Fluorescent dyes were n-hydroxy succinamide esters of fluorescein and rhodamine (Applied Biosystems, Foster City, CA).

Reaction conditions: All four primers were added at the beginning of the reaction so that both α2 amplification and allele-specific fluorescence PCR were performed in a single reaction. Template DNA was obtained using standard phenol-chloroform extraction from peripheral blood leukocytes of controls known to have zero, one, or two αβ genes. Each reaction contained 0.5 μg template DNA, 50 pmol sense unlabeled primer, 2 pmol antisense unlabeled primer, 15 pmol of fluorescein-labeled normal α2 primer, 45 pmol of rhodamine-labeled αβ primer, and 1.5 U Taq polymerase (Amplitaq; Perkin Elmer Cetus, Norwalk, CT) in a 50-μL reaction. Amounts of the labeled primers were estimates. Their relative amounts were determined empirically as those that produced distinctly yellow product from heterozygous αβ template. They reflect the relative efficiencies of these primers in this reaction. The reaction mixture consisted of 80 mmol/L Tris pH 9.0, 20 mmol/L (NH₄)₂SO₄, 20 mmol/L NaCl, 3 mmol/L MgCl₂, 5 pmol T4 gene 32 protein (US Biochemical, Cleveland, OH), 50 mmol/L trimethylammonium chloride, and 50 μmol/L each 2' deoxyadenosine 5' triphosphate, 2' deoxycytosine 5' triphosphate, 2' deoxyguanosine 5' triphosphate, and 2' deoxythymidine 5' triphosphate (Perkin Elmer Cetus).

Table 1. Nucleotide Sequences of the Four PCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>5' to 3' Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabeled sense</td>
<td>TGCAGGGCTGAGCCGACACTG</td>
</tr>
<tr>
<td>Unlabeled antisense</td>
<td>ATTCGGGACAGAGAGAACC</td>
</tr>
<tr>
<td>Fluorescein-labeled normal α2 sense</td>
<td>&quot;F&quot;-CCTCAGAAATACGTG</td>
</tr>
<tr>
<td>Rhodamine-labeled αβ sense</td>
<td>&quot;R&quot;-CTTCAAAATACGTG</td>
</tr>
</tbody>
</table>

The sequence of each primer is shown in 5' to 3' order. “F” and “R” represent the 5'-linked fluorescein and rhodamine, respectively. The two bases underlined indicate the 3' divergence of the two labeled primers.

Fig 2. Photograph of electrophoretic gel containing fluorescent products of this reaction. The positions of the origin, 165-bp products, and unlabeled primers are labeled. The green product in lane 1 is from normal control DNA (genotype αα/αα), the red product in lane 2 is from heterozygous Hb Constant Spring DNA (genotype αβ/αβ), and the yellow product in lane 3 is from heterozygous Hb Constant Spring DNA (genotype αβ/αα). The DNA size markers are not shown.
The reaction was performed using a DNA Thermal Cycler (Perkin Elmer Cetus). DNA was denatured at 95°C for 5 minutes in the first cycle and for 1 minute thereafter. Annealing and extension were both at 72°C for a total of 1 minute for the first 10 cycles. For the next 40 cycles, annealing was at 50°C for 30 seconds, and extension was at 72°C for 30 seconds.

The fluorescent nature of the PCR product could have been documented by spectrofluorometry or color photography of the gel. We elected to separate our product and primers by electrophoresis on 10% polyacrylamide gels and perform color photography of the gel to demonstrate our results in detail.

RESULTS

Figure 2 demonstrates that normal control DNA (genotype αα/αα) directed amplification of green product, homozygous Hb Constant Spring DNA (genotype αα/αα) of red product, and heterozygous Hb Constant Spring DNA (genotype αα/αα) of yellow product, each seen as discrete 165-bp fragments. Unincorporated primers were well separated from the product by electrophoresis.

DISCUSSION

The method we have developed for detecting α<sup>3</sup> affords the advantages of avoiding radioisotopes and difficult technical procedures such as electrophoresis. Although we presented our data as a photograph of an electrophoretic gel to demonstrate the details of our procedure, results of this assay can be recorded using spectrofluorometry or color photography of PCR product in solution after separation from unincorporated primers, thereby avoiding electrophoresis. Allele-specific fluorescence PCR has been described as the preferred method for detecting α<sup>3</sup> in areas where its diagnosis would have the greatest impact on health care. We have refined this method in a manner that uses asymmetric priming and a temperature shift, whereby in a single reaction high annealing temperature favors selective α2 amplification and lower temperature favors allele-specific fluorescence PCR. This method is simpler than methods requiring restriction digestion, electrophoresis, and allele-specific hybridization, multiple exacting hybridization conditions for selective amplification and for allele-specific hybridization, or allele-specific amplification of internal control sequences for interpretation. Our method has potential for application to routine diagnosis, population screening, and prenatal diagnosis in populations having a significant prevalence of Hb Constant Spring.

ACKNOWLEDGMENT

The authors thank Patti Taber for assistance in preparing the manuscript, and Carolyn Deevy and Carolyn Breaux for photographic assistance.

REFERENCES

6. Weiss I, Liebhaber SA: Destabilization of α-globin Constant Spring mRNA is mediated by translation into the 3′ noncoding region. Blood 76:80a, 1990 (abstr; suppl)
22. Embury SH, Kropp GL, Stanton TS, Warren TC, Cornett...

23. Chehab FF, Kan YW: Detection of sickle cell anaemia mutation by colour DNA amplification. Lancet 335:15, 1990


Asymmetrically primed selective amplification/temperature shift fluorescence polymerase chain reaction to detect the hemoglobin Constant Spring mutation

GL Kropp, S Fucharoen and SH Embury