Selective Enzymatic Amplification of \(\alpha_2\)-Globin DNA for Detection of the Hemoglobin Constant Spring Mutation

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

Hemoglobin Constant Spring is an elongation mutation of the \(\alpha_2\)-globin locus that results in a thalassemic phenotype. It has a high prevalence in Asian populations. When inherited with other \(\alpha\)-thalassemia determinants, the Constant Spring gene has the potential to cause severe forms of \(\alpha\)-thalassemia. Accurate diagnosis of the condition with standard hemoglobin electrophoresis is unreliable due to the small to undetectable amounts of the mutant hemoglobin present. Because of the extensive sequence homology of the \(\alpha_1\) and \(\alpha_2\) loci, allele-specific hybridization to total genomic DNA containing the Constant Spring gene would not distinguish between heterozygous and homozygous hemoglobin Constant Spring. Selective enzymatic amplification of \(\alpha_2\)-globin DNA sequences, however, allows unambiguous diagnoses to be made using allele-specific hybridization. This method is useful for providing accurate genetic counseling and prenatal diagnosis in populations and specific families in which precise diagnosis is important.

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MATERIALS AND METHODS

Experimental subjects. The subjects with Hb Constant Spring syndromes were patients in the Siriraj Hospital Hematology Clinic, Bangkok, Thailand. The hematologic data on which their diagnoses were based are shown in Table 1. These data were obtained using an electronic cell counter (Coulter model ZF-6, Coulter Electronics, Hialeah, FL), cellulose acetate Hb electrophoresis at alkaline pH,29 starch-gel electrophoresis,30 and alkali denaturation quantification of Hb F.31 Subjects 1 through 3 were homozygous for Hb Constant Spring (genotype \(\alpha_{CS}\alpha/\alpha_{CS}\alpha\), and subject 4 was heterozygous (genotype \(\alpha_{CS}\alpha/\alpha_{CS}\alpha\)). Homozygosity for \(\alpha_{CS}\alpha\), unlike for \(\alpha\), does not result in microcytosis.32 Globin gene mapping performed according to published protocols revealed a normal complement of four \(\alpha\)-globin structural genes32 for each of these subjects. Five DNA samples from individuals known to have a normal \(\alpha\)-globin genotype and no Hb Constant Spring were used as normal controls.

Three DNA samples from black individuals shown by gene mapping to have homozygous \(\alpha\)-thalassemia-2 (genotype \(-\alpha/\alpha\)) were used as negative controls for \(\alpha_2\)-DNA amplification. Mapping with the enzyme BglII demonstrated unambiguously that these single \(\alpha\) loci were of the rightward deletion or \(-\alpha\) variety.33 Based on a large population survey,34 these DNA samples from Black donors were assumed to contain the type 1 rightward deletion in which the \(\alpha_2/\alpha_1\) crossover responsible for the single \(\alpha\) locus occurred upstream of the Apal site in the \(\alpha_1\) second intervening sequence (IVS-2) and that the DNA downstream from this site was of the \(\alpha_1\) variety.

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Oligonucleotide design and synthesis. The experimental design of DNA amplification and allele-specific hybridization is shown in Fig 1. Selective amplification of α1 or α2 sequences encompassing their translation termination codons was directed by two 20 base primer pairs. These oligonucleotides were made complementary to opposite strands of a2 lack homology. The a2 is GAACC3'). The primer pairs. These oligonucleotides were made complementary to nucleotides 1 through 120 of the IVS-2 minus (complementary to mRNA) strand, and the a2 downstream primer (5'CCACGGGGG-GAGACCCGGGCGGCGGCTGCGCCCGC3') is complementary to nucleotides 126 through 145 of the 3'noncoding region plus strand. This pair directs amplification of a 318 nucleotide sequence. The a1 upstream primer (5'GGCCATGCTTCTTGCCCCT3'; a2 5'ATTCCGGGACAGAGA-TACGGGTGCAG3') is complementary to nucleotides 9 through 18 of the IVS-2 plus strand, and the a1 downstream primer (5'CCACGGGGG-GAGACCCGGGCGGCGGCTGCGCCCGC3') is complementary to nucleotides 67 through 86 of the 3'noncoding region plus strand. This pair directs amplification of a 275 nucleotide sequence. The upstream member of each primer pair is complementary to IVS-2 sequences where a1 and a2 differ by 8 nucleotides; a1-Stop (5'CTCCAGCTTAACGGTATTT3') was made complementary to the minus strand; its single base mutation made a C-T mismatch with normal α1 DNA. The normal α1-Stop (5'CTCCAGCTTAACGGTATTT3') was made complementary to the plus strand; its termination codon first base made an A-C mismatch with α2 DNA. A probe complementary to the minus strand would have relied on a T-G mismatch and, because of T-G pairing with α2 DNA, would have created diagnostic ambiguity.

Oligonucleotides were synthesized using cyanoethylphosphoramidite chemistry on a Cyclone model DNA Synthesizer (Milligen/Biosearch, San Rafael, CA) and purified either by anion-exchange high-performance liquid chromatography (HPLC) with a 3040L54 Vydac column (The Separations Group, Hesperia, CA) or by passage over an oligonucleotide purification cartridge (Applied Biosystems, Foster City, CA). Each method yielded a single 254-nm peak on HPLC.

Enzymatic DNA amplification. DNA was amplified enzymatically using the Gene Amp DNA amplification reagent kit with the thermostable enzyme Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) according to the method recommended by the manufacturer with the Mg2+ concentration adjusted to 4 mmol/L. The 100-μL reaction contained 50 pmol of each primer and 1 μg genomic DNA template. Amplification was performed using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) for 30 cycles with denaturation at 94°C for ten minutes in the initial cycle and two minutes in each subsequent cycle, primer annealing at 80°C for two minutes, and primer extension at 72°C for two minutes in all cycles except for ten minutes in the last cycle.

Amplification products were analyzed by electrophoresis at 70 V

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**Table 1. Hematologic Data and Hemoglobin Analysis**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Genotype</th>
<th>Age/Sex</th>
<th>Hb</th>
<th>Rbc</th>
<th>Hct</th>
<th>MCV</th>
<th>MCH</th>
<th>MCHC</th>
<th>Hb Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. S.S.</td>
<td>αα/αα</td>
<td>25/M</td>
<td>10.0</td>
<td>4.6</td>
<td>36</td>
<td>82</td>
<td>22</td>
<td>28</td>
<td>(CS + A2) + A-</td>
</tr>
<tr>
<td>2. J.A.</td>
<td>αα/αα</td>
<td>42/F</td>
<td>9.6</td>
<td>3.5</td>
<td>30</td>
<td>89</td>
<td>27</td>
<td>32</td>
<td>(CS + A2) + A-</td>
</tr>
<tr>
<td>3. S.P.</td>
<td>αα/αα</td>
<td>29/F</td>
<td>8.7</td>
<td>3.4</td>
<td>30</td>
<td>92</td>
<td>26</td>
<td>29</td>
<td>(CS + A2) + A-</td>
</tr>
<tr>
<td>4. S.K.</td>
<td>αα/αα</td>
<td>39/M</td>
<td>13.1</td>
<td>4.8</td>
<td>40</td>
<td>88</td>
<td>27</td>
<td>33</td>
<td>(CS + A2) + A-</td>
</tr>
</tbody>
</table>

Hb, hemoglobin; Hct, hematocrit; MCV, mean cellular volume; MCH, mean cellular Hb concentration; Rbc, red blood cell count.

*Hb Constant Spring was detected by starch-gel electrophoresis only.

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**Fig 1. Strategy for differential α2- and α1-globin DNA amplification and detection of α2 αα and normal αα αα Constant Spring-amplified DNA.** The IVS-2, third exon (exon 3), and 3' noncoding sequences of the α2 (below) and α1 (above) globin genes are diagrammed as parallel lines. The TAA in the α2 locus indicates its translation termination codon, and the1/2 AA in the α1 locus indicates the normal (TAA) and the Constant Spring (CAA) codon at position 142. The finer line between α1 and α2 indicates the degree of homology shared by the two loci horizontal segments indicating areas of perfect homology and irregular segments indicating nonhomologous sequences. Arrows (top) represent the α1-specific primer pair that directs amplification of a 275-bp fragment; arrows (bottom) α2-specific primer pair that directs amplification of a 318-bp fragment. Serpentine lines (top, α1 and bottom, α2) demonstrate the position of allele-specific probes used to distinguish α2- from α2-amplified DNA. The second serpentine line (bottom, CS-Stop or α3-Stop) shows the position of the allele-specific probes used to distinguish Constant Spring from normal α2-amplified DNA.
for 2.5 hours in a 2% NuSieve, 1% SeaKem agarose (both from FMC BioProducts, Rockland, ME) minigel containing 0.5 μg/mL ethidium bromide in a Tris (0.089 mol/L)-borate (0.089 mol/L)-EDTA (0.01 mol/L), pH 8.5 buffer with a Hoefer model HE 33 electrophoresis unit (Hoefer Scientific Instruments, San Francisco).

**Radiolabeling hybridization probes.** Oligonucleotide hybridization probes were end-labeled either at the 5' end with adenosine 5'[-32P] triphosphate (Amersham, Arlington Heights, IL) using T4 polynucleotide kinase (5' DNA Terminus Labeling System, Bethesda Research Laboratories, Gaithersburg, MD) or at the 3' end with adenosine 5'-α[-32P] triphosphate (Amersham, Arlington Heights, IL) using terminal deoxynucleotidyl transferase (TdT) in the 3' end-tailing method, each to a specific activity of ~1.5 x 10^6 cpm/ng. Radiolabeled products were separated from uninorporated triphosphates by passage over a G-25 Quick Spin Column (Boehringer Mannheim Biochemicals, Indianapolis).

**Slot-blot hybridization.** Aliquots of each 100-μL amplification reaction mixture were denatured in NaOH 0.3 mol/L at 94°C for five minutes, neutralized with an equal volume of 6 x SSC (SSC is NaCl 0.15 mol/L, Na citrate 0.015 mol/L, pH 7.0), and vacuum-blotted onto Nytran filters, pore size 0.45 mm (Schleicher and Schuell, Keene, NH) with a Manifold II Slot-Blot Apparatus (Schleicher and Schuell, Keene, NH). The filters were prehybridized for one hour at 43°C in 5 x NaCl 0.18 mol/L, NaHPO₄ 0.01 mol/L, EDTA 0.001 mol/L, pH 7.4 (SSPE), 5 x Denhardt's solution (0.02% Ficoll, molecular weight (mol wt) 360,000, all from Sigma Chemicals, St Louis), 0.5% sodium dodecyl sulfate (SDS), and 10 μg/mL yeast tRNA (Sigma).

The filters were hybridized overnight with 1 to 2 x 10^6 cpm radiolabeled probe prehybridization solution added. Hybridization temperatures were 55°C for the α₁, α₂, and CS-Stop probes or 49°C for the α₂-Stop probe. After hybridization, the filters were washed ten minutes at room temperature with 2 x SSPE, 0.1% SDS, and once for one minute with 5 x SSPE, 0.1% SDS at 55°C for those hybridized with the α₁, α₂, and CS-Stop probes or 49°C for those hybridized with the α₂-Stop probe. Hybridization was detected by autoradiography using Dupont Cronex film and Dupont Lightening Plus intensifying screens (E.I. Dupont de Nemours, Wilmington, DE) after exposure for one to 72 hours at ~70°C for 32P-labeled probes and room temperature for 35S-labeled probes.

**RESULTS**

**Differential amplification of α₁ and α₂ DNA.** The fidelity of α₁ and α₂ amplification was determined in two ways. First, DNA samples assumed to lack α₂ sequences in regions defined by our primer pairs were used as negative control templates for α₂ amplification (described in Experimental Subject sections above). These DNA samples contain only α₁ sequences in the region defined by our α₂ primers and should not support amplification of α₂ DNA. Figure 2 shows that the α₂ primers directed amplification of a 318-base pair (bp) DNA fragment when the genomic DNA templates contained the normal complement of four α-globin genes (genotypes αα/αα, αCSα/αα and αCSα/αCSα) but of no visible product when the DNA template was presumed to lack α₂ sequences in this region. The α₁ primers directed amplification of a 275-bp fragment with either type of DNA template. These data demonstrated that our two primer pairs direct amplification of discrete DNA fragments and that in the absence of complementary template sequences our α₂ primers do not direct promiscuous DNA amplification.

Second, we used α₁- and α₂-specific probes in slot-blot hybridization to assay for the presence of amplified α₁ and α₂ DNA. Figure 3 shows that αα/αα, αCSα/αα and αCSα/αCSα DNA amplified by the α₁ primers hybridized only with the α₁-specific probe and that the same DNA amplified by the α₂ primers hybridized only with the α₂-specific probe. These data confirmed that both primer pairs direct faithful DNA amplification.

**Allele-specific hybridization to amplified Constant Spring or normal α₂ DNA.** Figure 4 shows that amplified α₂ DNA from an individual with two normal α₂-globin genes (genotype αα/αα) hybridized only with the normal α₂-Stop probe and not the CS-Stop probe, from a subject with heterozygous Hb Constant Spring (genotype αCSα/αα) hybridized with both the normal α₂-Stop and the CS-Stop probes, from a subject with homozygous Hb Constant Spring (genotype αCSα/αCSα) hybridized with the CS-Stop probe.

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**Fig 2.** Products of α₂- and α₁-globin DNA amplification. Approximately one-tenth of the α₂-DNA-amplification products was subjected to electrophoresis in an ethidium bromide-permeated gel and photographed using ultraviolet illumination. Product sizes (in bp) are shown and sequences in the region defined by our primers (genotype type I. αα/αα). Lane 7 contains DNA size markers. (B) Genomic DNA templates and primer pairs for amplification products shown in each lane were (1) αα/αα × α₁ primers, (2) αCSα/αCSα × α₁ primers, (3) αCSα/αα × α₁ primers, (4) αCSα/αα × α₁ primers, (5) αCSα/αCSα × α₁ primers, (6) type I, αCSα/αCSα × α₁ primers, and (7) type I, αCSα/αCSα × α₁ primers. Lane 8 contains DNA size markers.
but not the normal $\alpha_2$-Stop probe, and from a subject with the rightward (presumed type I) $-\alpha\text{/}\alpha$ genotype with neither probe. Amplified $\alpha_2$ DNA from two other subjects with the $\alpha^{CS}\alpha/\alpha^{CS}\alpha$ genotype also hybridized only with the CS-Stop probe (data not shown).

**DISCUSSION**

We achieved faithful amplification of $\alpha_2$-globin DNA sequences separate from $\alpha_1$ sequences, which allowed us to use allele-specific hybridization to distinguish the presence of 0, 1, or 2 $\alpha^{CS}$ genes at the $\alpha_2$ locus. The high degree of sequence homology shared by the duplicated $\alpha_1$ and $\alpha_2$-globin genes\[^9\text{-}2\] confounds the diagnosis of $\alpha$-globin gene mutations using allele-specific hybridization of genomic DNA. A recent report described a method using combined restriction digestion and allele-specific hybridization,\[^40\] but the diagnostic approach used entails technical vagaries and requires far greater quantities of DNA than the PCR-based method we have used. We synthesized primer pairs complementary to IVS-2 and 3' noncoding sequences in which $\alpha_1$ and $\alpha_2$ are poorly homologous\[^8\text{-}20\] to direct selective enzymatic amplification of $\alpha_2$ and $\alpha_2$ DNA. Our $\alpha_2$ primers provided essentially pure $\alpha_2$ DNA lacking sufficient quantities of $\alpha_1$ sequences to confound the allele-specific hybridization to $\alpha^{CS}$. The importance of DNA diagnosis for the Constant Spring syndromes is underscored by the unreliability of detecting Hb Constant Spring with standard electrophoretic techniques\[^4\text{-}5\text{-}2\] and the clinical relevance of these syndromes in populations with high frequencies of $\alpha$-thalassemia and Constant Spring genes.\[^3\text{-}5\] Moreover, as a result of the recent emigration of many Southeast Asians to North America,\[^41\] births of individuals with these severe conditions have been observed outside of Southeast Asia.\[^42\]

The importance of reliably detecting Hb Constant Spring is further illustrated by the greater clinical severity of $\alpha$-thalassemia syndromes in which a single normal $\alpha$-globin gene is associated with a nondeletion $\alpha$-thalassemia gene ($\alpha^{ND}$) rather than a deleted gene.\[^3\text{-}8\text{-}11\] Subjects with the Constant Spring Hb-H disease syndrome (genotype $--/\alpha^{CS}\alpha$) are more anemic and have more Hb-H than those with deletional Hb-H disease (genotype $--/\alpha$).\[^8\text{-}9\] More important, nondeletional genotypes predicted to cause Hb-H disease ($--/\alpha^{ND}\alpha$) have actually resulted in hydrops fetalis.\[^10\text{-}11\] The cause of this greater severity is best understood in terms of the differential expression of the two $\alpha$ loci. Normally, the expression of the $\alpha_1$ gene exceeds that of the $\alpha_2$ gene by two to three times,\[^13\text{-}4\] perhaps due to transcriptional interference of $\alpha_1$ expression by $\alpha_2$ transcripts.\[^46\] With dele-

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*Fig 3. Hybridization of $\alpha_1$- and $\alpha_2$-specific probes to DNA amplified with $\alpha_1$- or $\alpha_2$-specific primers. Autoradiograph shows hybridization of filter-bound amplified $\alpha_2$ DNA with a radiolabeled $\alpha_2$-specific hybridization probe (A) and with a radiolabeled $\alpha_1$-specific hybridization probe (B) and of filter-bound amplified $\alpha_1$ DNA with a radiolabeled $\alpha_2$-specific hybridization probe (C) and a radiolabeled $\alpha_1$-specific hybridization probe (D). DNA templates for amplification contained both $\alpha_1$ and $\alpha_2$ loci. Numbered series indicate amplified DNA samples applied to the filters in decreasing aliquots of the 100-µL reaction volume (5.0, 2.5, 0.5, 0.25, and 0.05 µL).*

*Fig 4. Constant Spring genotypic assignments using hybridization of Constant Spring-specific and normal $\alpha_2$-specific hybridization probes to DNA amplified with $\alpha_2$-specific primers. Autoradiograph shows hybridization of radiolabeled Constant Spring-specific (CS-Stop) and normal $\alpha_2$-specific ($\alpha_2$-Stop) hybridization probes to filter-bound $\alpha_2$ DNA amplified from DNA of individuals with heterozygous Hb Constant Spring (genotype $\alpha^{CS}\alpha/\alpha\alpha$), homozygous Hb Constant Spring (genotype $\alpha^{CS}\alpha/\alpha^{CS}\alpha$), the normal $\alpha$-globin genotype ($\alpha\alpha/\alpha\alpha$), and homozygous rightward deletion $\alpha$-thalassemia-2 (genotype $-\alpha/-\alpha$, presumably type I). The slot-blotted DNA samples, labeled according to genotype, were 5-µL aliquots of the 100 µL $\alpha_2$-specific amplification mixtures.*
tion of the \(\alpha_x\) gene, there is a two-fold compensatory increase in \(\alpha_x\) expression.\(^{47,48}\) No such compensation occurs when a nondeletional gene occupies the \(\alpha_y\) locus,\(^{49}\) thus accounting for a lower overall \(\alpha\)-globin expression and greater clinical severity.

The diagnostic technique we have developed was made possible by recently developed molecular methods.\(^{24,38}\) It provides a reliable means of diagnosing Hb Constant Spring. This method can also be used with fetal DNA for prenatal diagnosis in couples at risk for severe \(\alpha\)-thalassemia syndromes involving the \(\alpha^{CS}\) gene. The recent development of methods of amplifying DNA from small volumes of blood dried on filters\(^{50}\) and of nonisotopic detection of hybridization probes\(^{26,28}\) will extend the availability of our method to populations in which \(\alpha^{CS}\) poses a substantial health problem.

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