Genotyping and haplotyping of polymorphisms directly from genomic DNA via coupled amplification and sequencing (CAS)

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ABSTRACT

Coupled amplification and sequencing (CAS) allows a segment of DNA to be sequenced directly from genomic DNA. An initial PCR amplification stage selects and amplifies the target. During a subsequent stage both strands of the target segment are sequenced simultaneously and amplified further. We show that CAS can readily identify variant base pairs. Genotyping of a population for known sequence variation can be achieved simply and directly from genomic DNA of each organism by performing CAS only for the variant bases. The procedure supercedes development and optimization of alternative typing assays based on oligonucleotide hybridization or ligation. In addition, we show that competitive oligonucleotide priming with allelic primers can be readily performed in concert with the second stage of CAS. The combination of techniques allows sequencing of a single chromosome from a heterozygous genomic sample and direct haplotyping of the polymorphism at the priming site with any others encompassed within the amplified segment.

INTRODUCTION

Coupled amplification and sequencing (CAS) is a process which allows simultaneous amplification and sequencing of the two complementary strands of a DNA segment (1). In the course of CAS, dideoxyribonucleotides are incorporated into DNA during each of fifteen thermally-cycled primer extension reactions proceeding concomitantly along both complementary strands of the segment.

We have now used CAS to characterize polymorphic sequences for genomic fragments and to type the polymorphism in each organism in the survey by performing *CAS genotyping* only for the variant bases and without need to develop alternative typing assays. In a further extension, we have performed CAS with allelic primers to haplotype polymorphisms within and downstream from the priming site. Typing of the polymorphism at the priming site was achieved by means of competitive oligonucleotide priming, an assay in which only one primer in a mixture of allelic primers is labeled and gives rise to a labeled product when a perfectly matched template is present but not

otherwise (2). With competitive priming during CAS it was possible to sequence either of the two chromosomes in a heterozygous individual directly from genomic DNA and to establish directly the linkage phase of multiple polymorphisms.

Studies in HOX2 cluster

We have analyzed a 300 bp sequence tagged site in the Homeo Box 2 cluster for sequence variation among the higher primates (3). The HOX2 fragment of the higher primates focuses into sharp bands on *denaturing gradient electrophoresis (DGE)*, a feature facilitating DGE screens for sequence variation. We have now analyzed the HOX2 fragment for intra-species polymorphism in each primate lineage. DGE screening in 8 chimpanzees (*Pan troglodytes*) detected a common bi-allelic polymorphism (4). In this study we use CAS to sequence the chimpanzee HOX2 segment and to characterize the variant bases giving rise to the DGE polymorphisms. Once characterized, we employed CAS genotyping to type several animals for the sequence polymorphism directly from genomic DNA.

Studies in β -globin cluster

The several known polymorphisms in a 3.1 kb intergenic region between human $\psi\beta$ and δ globin genes belong to one of only two common haplotypes, denoted 'R' and 'T' by Maeda et al (5). Our studies to date have focused on three polymorphic sites in the 770 bp inter-Alu segment within this region and showed that the postulated haplotypes apply in a broader sampling than that of Maeda et al (6). In the present study we characterize the fourth polymorphism known to be in this inter-Alu segment, a simple *T/G* transversion. Instead of developing oligonucleotide binding assays, we sought to genotype this polymorphism directly by sequencing the relevant bases using CAS of genomic DNA. By using the allelic oligonucleotide probes for the other positions as sequencing primers in a competitive priming assay we sought to haplotype the previously characterized allelic sites and the new polymorphism without reliance on family pedigrees.

MATERIALS AND METHODS

CAS genotyping of HOX2 polymorphism in chimpanzee

For CAS we used human primers *Pyg2* [5'-GCT CTA TAG G-AG GCC CTG AG-3'] and *Pyg3* [5'-GAG GCT GTT TAG ATG

AGA CA-3']; from their 5' ends, these primers delimit a 300 bp fragment 4 kb upstream from HOX2B (7). These primers define a 'sequence tagged site' (STS) in the HOX2 cluster (3) and have served to sequence via CAS the human fragment (1) as well as the homologous fragments in chimpanzee, gorilla and orangutan genomes (8).

From genomic DNA, CAS consisted of two stages:

Stage I—2 μ g genomic DNA of each chimpanzee was amplified enzymatically in a 250 μ l reaction mixture of the following reagents: 0.1 μ M (10 picomoles per 100 μ l) of each primer; 10 μ M of each dNTP; 1 nM (2U per 100 μ l) Taq polymerase (Cetus AmpliTaq); 50 mM KCl; 1.5 mM MgCl₂; 10 mM Tris pH 8.4; 0.01% gelatin. Stage I was performed in a programmable thermal cycler (Perkin Elmer, model 1050) for 15 cycles; each consisted of 1 min. denaturation at 94°C, 1 min. annealing at 55°C, and 1 min. polymerization at 72°C.

Stage II—The reaction mixture obtained from Stage I was divided into two 100 μ l aliquots. Each aliquot was supplemented separately with one or the other end-labeled primer. One volume received 10 picomoles of *Pyg2** (end-labeled *Pyg2*); the other, 10 picomoles of *Pyg3**. Each 100 μ l aliquot, thus 'boosted' with end-labeled primer, was subaliquoted into 4 volumes of 25 μ l each (one for each of the four ddNTPs). ddNTPs were added to their respective 25 μ l subaliquots to final concentrations of 80 μ M for ddG, 670 μ M for ddA, 670 μ M for ddT or 400 μ M for ddC. The reactions, overlaid with mineral oil, underwent 15 additional cycles with the same thermal profile as in Stage I.

CAS genotyping of a *T/C* polymorphism discovered in the HOX2 segment proceeded using the same reagent concentrations and thermal profile just detailed proceeding from 500 ng genomic DNA of each animal. Reagent amounts and reaction volumes for Stage I were one quarter of those described previously since only *T* and *C* reactions were performed with *Pyg2** during Stage II.

CAS haplotyping of β -globin polymorphisms in a human pedigree

We used two pairs of allelic oligonucleotides, namely, *GR1'* [5'-TTC ACT GTG TGT CAA AAT CT-3'] and *GR3'* [5'-TT-C ACT GTG TCA AAA TCT AG-3'] for one priming site and oligonucleotides *GR2* [5'-CAG GCT ATA TAT AAG AGT TTA-3'] and *GR4* [5'-CAG GCT GTA TTT AAG AGT TT-3'] for the other. From their 5' ends, primers *GR1'* and *GR3'* on one side and primers *GR2* and *GR4* on the other delimit a 270 bp fragment. *GR2/GR4* have been previously used as allele-specific oligonucleotide probes for two neighboring base pair substitutions treated as a single locus (base positions 928 and 932 according to the sequence of Maeda et al (9)). *GR1'/GR3'* are similar in sequence to oligonucleotides *GR1* and *GR3*, which have been previously used as allele specific probes (6) and allele specific PCR primers (10) for a TG polymorphic insertion (position 678). *GR1'* and *GR3'* contain the allelic sites in the middle of their sequences while *GR1* and *GR3* have the allelic sites near their 3' ends. CAS haplotyping was performed directly from genomic DNA for each of nine individuals in a four-generation Caucasian pedigree (6).

CAS was performed for persons A and I. The genotype of person A had been previously determined to be homozygous for the *GR1* and *GR2* sequences; person I had been typed as

homozygous for the *GR3* and *GR4* sequences (6). From genomic DNA, CAS consisted of two stages:

Stage I—2 μ g genomic DNA of each person was amplified enzymatically in a 250 μ l reaction mixture of the following reagents: 0.05 μ M (5 picomoles per 100 μ l) each of primers *GR1'*, *GR2*, *GR3'* and *GR4*; 10 μ M of each dNTP; 1 nM (2U per 100 μ l) Taq polymerase (Cetus AmpliTaq); 50 mM KCl; 1.5 mM MgCl₂; 10 mM Tris pH 8.4; 0.01% gelatin. Stage I was performed in a programmable thermal cycler (Perkin Elmer, model 1050) for 15 cycles; each consisted of 1 min. denaturation at 94°C, 1 min. annealing at 50°C, and 1 min. polymerization at 72°C.

Stage II—The reaction mixture obtained from Stage I was divided into two 100 μ l aliquots. When sequencing person A in the pedigree, one aliquot received 10 picomoles of *GR1'**; the other, 10 picomoles of *GR2**. When sequencing person I, one aliquot received 10 picomoles of *GR3'**; the other, 10 picomoles of *GR4**. Each 100 μ l aliquot supplemented with end-labeled primer was subaliquoted into 4 ampules containing 25 μ l each (one for each of the four ddNTPs). ddNTPs were added to their respective 25 μ l ampules to final concentrations of 80 μ M for ddG, 540 μ M for ddA, 540 μ M for ddT or 400 μ M for ddC. The concentrations of ddA and ddT were adjusted for the high A/T content (65%) of this segment and are 20% lower from previously reported conditions (1). The reactions underwent 15 further cycles with the same thermal profile as in Stage I but in a thermal cycler requiring no mineral oil (Perkin Elmer, model 9600).

CAS haplotyping of the globin segment proceeded using the same reagent concentrations and thermal profile just described starting from 500 ng genomic DNA of each person in the pedigree. Reagent amounts and reaction volumes for Stage I were one quarter of those described previously since only A and C bases were sequenced from the *GR2/GR4* site during Stage II. Competitive oligonucleotide priming (2) was performed in concert with Stage II of CAS by adding either *GR2** or *GR4** to the unlabeled *GR2/GR4* mixture present from the outset of Stage I. The presence (+) or absence (−) of A/C sequence tracks when reactions are boosted separately with either *GR2** or *GR4** typed the polymorphism at the *GR2/GR4* priming site as *GR2* +/− and *GR4* +/−. Sequences extended from successful priming are on the same chromosome as the allele at the annealing site.

Synthesis and labeling of oligonucleotide primers

Oligonucleotides were prepared by the solid-phase phosphoramidite method in an automated DNA synthesizer (Applied Biosystems) and purified by electrophoresis in a 7M urea, 20% polyacrylamide gel. Sequencing primers were labeled at their 5' ends with ³²P- γ ATP (Amersham) using T4 polynucleotide kinase (New England BioLabs) to a specific activity of 2–4 μ Ci/picomole.

Sample preparation for electrophoresis

At the end of Stage II, the reaction mixtures are lyophilized, and reconstituted in 5 μ l loading dye with 90% formamide. Stage II products in loading dye are boiled for 5 minutes. 2.5 μ l of each reaction is loaded in a 6% acrylamide, 8M urea gel and run at 45 Watts for 2.5 hrs. Gels are dried at 80°C for 2 hrs. and exposed to film for 12–72 hrs. at room temperature without intensifying screens.

RESULTS

CAS sequencing and genotyping of HOX2 polymorphisms in chimpanzee

We used CAS first to sequence the HOX2 fragment in chimpanzees homozygous for each DGE allele, namely, chimpanzees *Colin* and *109*. Two polymorphisms 47 bp apart were detected (Fig 1). Both polymorphisms are *T/C* transitions in the strand extended from *Pyg2*. Confirmatory *A/G* transitions are found in the complementary strand simultaneously extended from *Pyg3* during CAS. Referenced to the *Pyg2* strand, the linkage phase of the polymorphisms is *T-C* in one haplotype and *C-T* in the other.

The polymorphism at position 188 was typed in eight animals by performing CAS directly from genomic DNA with *Pyg2** (end-labeled *Pyg2*) but only for the two relevant bases, *T* and *C*. Close-ups of the track in question are shown in Fig. 2. In each panel, a different animal is typed. The upper *C* is invariant and provides a dosage control for the polymorphic lower *C*. Chimpanzees *105*, *109* and *Kasey* are homozygous for the lower *C* (*C/C*) while *Colin* and *Harv* are homozygous for *T* (*T/T*) at that position. Heterozygotes (*T/C*), namely, chimpanzees *107*, *Tank* and *Carl*, show both *T* and *C* bands at the same site. In

the *T/C* heterozygous animals, the intensity of the lower *C* is noticeably reduced in comparison with the invariant upper *C*.

Typing of the polymorphism at position 235 via the same process, but sequencing instead *A* and *G* with *Pyg3**, proved more difficult because the polymorphic base, the third *G* in a *GGG* track, is reduced in intensity even in the *G/G* homozygote (Fig. 1B). The *A* base at the polymorphic site was clear, however. *Colin* and *Harv* are *G/G* homozygotes since they lack this *A* while the other animals are either *A/G* or *A/A*. Chimpanzees *107*, *Tank* and *Carl* are *A/G* since their sequence had *C* at the relevant polymorphic position in the complementary *Pyg2* strand whereas *105*, *109* and *Kasey* are *A/A* since they lacked it (data not shown).

Concerted application of CAS and competitive priming for typing a β -globin polymorphic site

Competitive oligonucleotide priming allows typing of polymorphic bases by incorporation or exclusion of a labeled allelic primer in PCR product when amplification is carried out with a mixture of unlabeled allelic primers (2). This assay always generates a product, but the product is labeled only when the labeled allelic primer matches the template sequence. We reasoned that by simply having a mixture of unlabeled allelic primers during Stage I and by adding a single labeled allelic primer during Stage II, a competitive priming assay could be easily incorporated in CAS.

We performed competitive priming to type the polymorphic annealing site for primers *GR2* and *GR4*. This annealing site bears two neighboring polymorphisms at positions 928 and 932 which are treated as a single locus in the present analysis (Fig 3A). C-AS was performed with an equimolar mixture of unlabeled *GR2* and *GR4* oligonucleotides during Stage I followed by addition of either *GR2** or *GR4** during Stage II in a competitive priming assay. The presence or absence of a sequence ladder (rather than a labeled PCR product) typed the polymorphisms at the allelic priming site. We applied concerted competitive priming and C-AS to nine members of the same four-generation family used in previous studies (Fig 3B). Persons A, C, E, and F are homozygous *GR2* whereas person I is homozygous *GR4*. Persons

Figure 1. Polymorphic bases in HOX2 fragment in chimpanzees *Colin* and *109* determined via coupled amplification and sequencing (CAS) of genomic DNA. *Pyg2* and *Pyg3* are CAS primers for the two complementary strands of the segment. The animals had been originally typed as homozygotes for the alternative alleles of a common bi-allelic system detected via denaturing gradient electrophoresis (4). The sequence is numbered according to human sequence obtained by Ferguson-Smith (7) and deposited in the EMBL Data Library under accession number X57979. **Panel A.** *T-C* polymorphism in the strand extended from primer *Pyg2* (left) is confirmed by *A-G* polymorphism in the complementary *Pyg3* strand (right). **Panel B.** *A-G* polymorphism in the strand extended from primer *Pyg3* (left) is confirmed by *T-C* polymorphism in the complementary *Pyg2* strand (right). **Panel C.** Diagram of the polymorphisms discovered at positions 188 and 235 and of their relative location in the two complementary strands of the segment. Sequences for both strands are simultaneously generated during CAS.

Figure 2. CAS genotyping of the *T-C* polymorphism at position 188 in the *Pyg2* strand in eight chimpanzees. The upper *C* in each panel is invariant and provides a dosage control. (The full identification number of chimpanzees *105*, *107* and *109* is prefixed by *YB81*.)

B, D, G and H are GR2/GR4 heterozygotes. These results are identical to those found when GR2 and GR4 are used as allele-specific oligonucleotide probes in dot blot assays of PCR products amplified with primers flanking the 270 bp segment (6).

Figure 3. Schematic of β -globin segment characterized with CAS and competitive oligonucleotide priming in a small pedigree. Relative locations in the two complementary strands of the segment are highlighted for polymorphic sites. **Panel A.** A polymorphism was characterized at position 795. Basepair sites are numbered according to the sequence of Maeda et al for a 3.1 kb intergenic region between human $\psi\beta$ and δ globin genes (9). The two priming sites are polymorphic themselves: one at position 678; the other, at positions 928 and 932. Detail is provided only for positions 928 and 932 since these sites were typed by competitive priming with oligonucleotides GR2 and GR4 and haplotyped with the polymorphism at position 795. **Panel B.** Typing of polymorphism with concerted use of competitive oligonucleotide priming and CAS in a nine-member, four-generation family (6). From Stage I, CAS proceeded with an equimolar mixture of GR2/GR4 followed by boosting with either GR2* (end-labeled GR2) or GR4* during Stage II. The plus or minus refers to the presence or absence of sequence ladders.

Figure 4. Polymorphic bases at position 795 in β -globin fragment in persons A and I determined via CAS of genomic DNA. GR1'/GR3' and GR2/GR4 are mixtures of allelic primers used during Stage I. The choice of sequence primers for each individual in Stage II depended on the results of the competitive priming assay (previous figure). GR1' and GR3' or, alternatively, GR2 and GR4 are used as CAS primers for the two complementary strands of the fragment. **Panel A.** CAS with primers GR1'* and GR2* on person A. T at polymorphic position 795 in the GR1'* strand is confirmed by an A in the complementary strand extended from GR2*. **Panel B.** CAS with primers GR3'* and GR4* on person I. G at polymorphic position 795 in the GR3'* strand is confirmed by a C in the complementary strand extended from GR4*.

CAS sequencing of β -globin polymorphism in homozygous individuals

We used CAS to sequence the fourth polymorphism known to exist in the β -globin segment. As template, we utilized genomic DNA from persons A and I, known from our previous studies to be homozygous for alternative haplotypes defined by the other three polymorphisms in the β -globin segment (6). The polymorphic base, a T/G transversion, is located approximately midway in the segment, at base pair 795 (Fig 3A). We performed CAS with primers GR1' and GR2 on person A and found a T at the polymorphic locus in the GR1' strand confirmed by an A in the complementary strand extended from GR2 (Fig. 4A). We next sequenced person I with primers GR3' and GR4. At the polymorphic locus, this person bears a G in the GR3' strand, thus constituting the first G in a GGGG track (Fig 4B). The polymorphism is confirmed as a C in the complementary GR4 strand, constituting the last C in a CCCC track for this sequence. This result is in agreement with the expectation that person A is homozygous for the 'R' haplotype and person I is homozygous for the 'T' haplotype.

CAS haplotyping of two β -globin polymorphisms

We sought to genotype the polymorphism at position 795 and establish its linkage phase with the other polymorphisms at the GR2/GR4 annealing site for each member of the pedigree. We performed CAS only for bases A and C from genomic DNA with either GR2* or GR4* for homozygotes and separately with both GR2* and GR4* for heterozygotes. Persons A and I were also included in this series as controls.

Figure 5. CAS haplotyping in a small family of polymorphism at the GR2/GR4 priming site with the A/C polymorphism at position 795 of the β -globin fragment. GR1'/GR3' and GR2/GR4 are mixtures of allelic primers used during Stage I. Depending on the result of the competitive priming assay (Fig 3B) for each of nine individuals, either GR2* or GR4* was used as end-labeled sequencing primer during Stage II. Only A and C reactions were performed in each case. **Panel A.** Haplotyping of four GR2 homozygotes. **Panel B.** Haplotyping of GR4 homozygote. **Panel C.** Genotyping of a GR2/GR4 heterozygote with a mixture of GR2* and GR4*. **Panel D.** Haplotyping of four GR2/GR4 heterozygotes using both GR2* and GR4* as primer in separate Stage II reactions from a common Stage I pool. A/C tracks obtained with either GR2* or GR4* are shown for each person.

GR2 homozygotes, persons A, C, E, and F, showed a single A at the polymorphic site (Fig 5A). Person I, the sole GR4 homozygote, shows a single C (Fig 5B). These results establish the haplotypes as GR2-A and GR4-C, in agreement with the 'R', 'T' hypothesis. On the basis of Mendelian inheritance, the same haplotypes should apply to heterozygotes in the pedigree.

We next analyzed heterozygous persons B, D, G and H to genotype the polymorphism and to haplotype it with the priming site strictly by molecular means. In these experiments a common Stage I mixture for each person was then aliquoted into two ampules to be 'boosted' with either GR2* or GR4* during Stage II. Heterozygotes showed a sequence ladder with both GR2* and GR4*. When a mixture of GR2* and GR4* is provided during Stage II, the resulting sequence ladder shows both A and C at the allelic site at somewhat lesser intensity than the invariant bases (Fig 5C). However, in all four cases, sequencing only with GR2* revealed an A at the polymorphic base whereas sequencing with GR4* alone showed C (Fig 5D). The haplotypes are thus established molecularly by CAS in concert with competitive priming to be GR2-A and GR4-C, in agreement with the deduction from Mendelian inheritance.

DISCUSSION

CAS has many potential applications for evolutionary and medical genetics since it allows a given DNA segment to be readily sequenced for polymorphisms and mutations in any organism of a population. From genomic DNA sample to sequencing, CAS genotyping is executed with a single pair of primers and involves no cloning steps. In contrast, garnering this sequence information with classical cloning technology would have required libraries to be constructed and screened for each individual and enough clones to be sequenced to sample both chromosomes. Only sequencing guarantees 100% detection of the full extent of variation. For example, the two alleles of the chimpanzee HOX2 polymorphism were originally detected on denaturing gradient gels; only when sequenced with CAS were they found to differ from each other by two base pair changes. Once the nature of the polymorphism is defined, CAS can then be turned into an expedient genotyping device by performing the process only for the polymorphic bases in each individual studied.

CAS genotyping is especially valuable when the variant base pairs are not located within a common restriction site or within a domain surveyed by DGE. In those cases allele-specific oligonucleotide binding assays are often the favored alternative to type the polymorphisms (11). Reliable performance of such assays, however, requires synthesis of oligonucleotide probe for each allele as well as ascertainment of discriminating hybridization and washing conditions. Recently, oligonucleotide ligation assays have also been developed for large-scale automated typing (12), but these require synthesis of at least three oligonucleotides per bi-allelic system as well as empirical optimization of conditions. In contrast to all the above, CAS genotyping follows directly from sequencing conditions already elucidated to characterize the polymorphisms and may become the preferred method when a small number of organisms has to be genotyped for a distinct base pair change.

The only limitation of CAS genotyping lies in the nature of the autoradiographic banding pattern at the allelic site. As demonstrated by the multiple examples in the figures, the overall quality of the sequence ladders obtained from diverse genomic

DNA sources is quite high. Sample to sample variability is similar to that observed in any hybridization. However, in most sequences there may be some areas of compression or common termination. If an allelic site were to occur in such a region, the fidelity of a CAS assignment could be compromised by the sequencing artifacts. One such problem was illustrated by the HOX2 polymorphism at position 235. Such problems are usually obvious and can be overcome by sequencing the complementary strand.

We have previously developed molecular haplotyping strategies that do not rely on pedigrees. The single molecule dilution (SMD) method separates the maternal from the paternal chromosome by limiting dilutions of genomic DNA followed by biphasic booster PCR amplification (6). We expect SMD to be most valuable for haplotyping loci separated by as much as 100 kb (13). For haplotyping polymorphisms nested within PCR fragments, allele-specific amplification on the basis of 3' mismatches of one (10, 14, 15) or both (16, 17) PCR primers is simpler. Restriction digests or oligonucleotide probes could be used for typing of a nested polymorphism, but CAS with competitive priming would seem the method of choice. The method allows selective sequencing of one chromosome directly from genomic DNA of a heterozygous individual. The location of the allelic sites in the primer appears not to be critical since competitive priming relies on kinetic discrimination. The on-off rates for perfectly matched primers are much faster than for mismatched primers; a mismatched primer effectively fails to find a vacant template to anneal to. CAS in concert with competitive priming could have tremendous importance in the genotyping of highly polymorphic regions of the genome such as HLA and in the clinical study of genetic heterogeneity of inherited disease.

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