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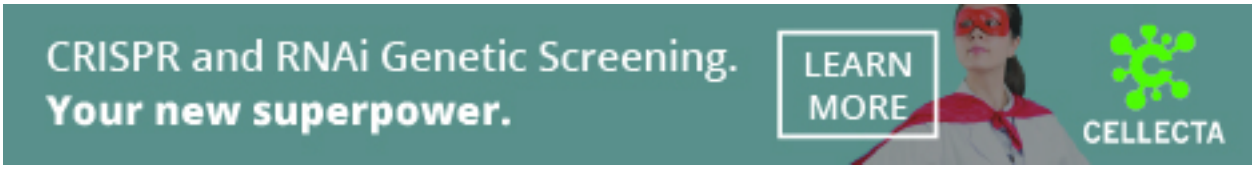

A Method for Specific Amplification and PCR Sequencing of Individual Members of Multigene Families: Application to the Study of Steroid 21-Hydroxylase Deficiency

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Mutations at the human HLA-linked *CYP21B* locus are responsible for 21-hydroxylase deficiency, a recessively inherited disorder of steroidogenesis. The scope for PCR-based analysis of the *CYP21B* gene has been restricted by the very high sequence homology between *CYP21B* and a closely related pseudogene, *CYP21A*. Here we describe a novel PCR sequencing strategy that allows the independent amplification of the entire *CYP21B* coding sequence and the subsequent enzyme-mediated conversion of the PCR product to a single-stranded form for dideoxy sequencing. We have used this approach to characterize the 21-hydroxylase deficiency allele associated with HLA-B55, the most frequent HLA marker associated with a *CYP21B* point mutation in the British population, and also an HLA-B35 associated allele of Asian origin. Allele-specific oligonucleotide (ASO) hybridization analyses have confirmed the selective amplification of *CYP21B* genes and the identity of the pathological mutations. The method can be adapted to permit selective amplification and PCR sequencing of individual closely related members of other multigene families and small-copy-number repetitive DNA families.

Selective in vitro amplification of individual members of a repetitive DNA family is often desirable for identifying locus-specific characteristics of interest such as map position, DNA structure, and gene expression in multigene families. In the case of high sequence homology between nonallelic members of a repetitive DNA family, primers that have been selected to amplify a specific locus may also bind to other members of the family. Ensuing amplification products will therefore constitute a mixture of nonallelic sequences and also artificial heteroduplexes formed between complementary strands of nonallelic sequences.

The above problem is particularly relevant to the human steroid 21-hydroxylase and complement C4 gene families. Each of these families is defined by highly related genes that are clustered in the HLA complex on the short arm of chromosome 6, and are flanked by highly polymorphic HLA genes both centromerically (e.g., HLA-DR genes) and telomerically (HLA-B, HLA-C, and HLA-A). Normally, the 21-hydroxylase/complement C4 gene cluster on chromosome 6 consists of two tandemly repeated units, each about 30 kb long and containing a 21-hydroxylase gene and a C4 gene[1,2] (see Fig. 1A). One unit contains a normally functional 21-hydroxylase gene, *CYP21B*, and a complement C4 gene, *C4B*. The neighboring unit contains a 21-hydroxylase pseudogene, *CYP21A*, whose nucleotide sequence shows about 97% identity to that of the *CYP21B* gene,[3–5] and the complement *C4A* gene which shows a similarly high degree of sequence identity to the *C4B* gene.

The number of tandem 21-hydroxylase/C4 gene units in a single cluster can vary from 1 to 4[6,7] and sequence exchange between loci is known to occur by unequal crossover[8] and may also occur by gene conversion-like events (for recent reviews, see refs. 9 and 10). Indeed, all recorded pathological mutations at the *CYP21B* locus are thought to have occurred by these mechanisms. In approximately 25% of cases, pathological mutation results from loss of all or part of the *CYP21B* gene sequence following deletion due to unequal crossover. Such pathological deletion haplotypes are often associated with specific HLA antigens such as HLA-Bw47 and HLA-B40(60). The remaining 75% of cases show pathological point mutations that are associated with a diverse set of HLA antigens. In each case that has been investigated so far, the mutation seems to have been copied from DNA sequence normally present in the *CYP21A* pseudogene, presumably by a gene conversion-like mechanism. Additional evidence for the role of gene conversion at this locus has been supplied by restriction fragment length

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variant (RFLV) (see ref. 6) analysis, allele-specific oligonucleotide (ASO) hybridization, and PCR-based studies.[9,10]

The variation in copy number and the sequence exchange between the 21-hydroxylase genes does not facilitate their analysis. In this paper we describe a PCR-based strategy that permits selective amplification of the entire *CYP21B* gene and its enzymatic conversion to a single-stranded form suitable for DNA sequencing.

MATERIALS AND METHODS

PCR Amplification

Genomic DNA was initially digested to completion with a fivefold excess of *Taq*I (Boehringer-Mannheim). In the initial PCR reaction 0.5 μg of *Taq*I-restricted DNA was amplified in a 100 μl reaction containing 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.4), 0.017% bovine serum albumin, 5% dimethylsulfoxide, 2 units of *Taq* polymerase (Boehringer-Mannheim), and 50 pmoles of each of the oligonucleotide primers: 5′-CGACAGCTAGATTTCCGGCTGGAATC-3′, 5′-CGAGTTCTCGGTGCCCTTCACGGAAAT-3′. Amplification involved 30 cycles of DNA denaturation (45 sec at 93°C), primer annealing (1 min at 65°C), and DNA synthesis (3 min at 72°C). The product of the initial PCR reaction was diluted 100-fold with distilled water and 1–5 μl (1/10000–1/2000 of the total product) used as a template for secondary PCR amplification with the internal primers 5′-CCCGTGATGCATATAGAGCATGGCTGTG-3′ and 5′-CACGGAAATGAAGCTGAGACC-3′. The reaction conditions were as above except that only 25 cycles were performed.

Preparation and Dideoxy Sequencing of Single-stranded DNA

The product of the secondary PCR reaction was purified by selective binding to and elution from a solid matrix (Biorad, Prep-a-gene) , and was digested to completion with a fivefold excess of *Nsi*I in a buffer specified by the manufacturers (New England Biolabs). The reaction mixture was diluted by the addition of an equal volume of 10 mM $MgCl_2$, 10 mM Tris·HCl (pH 8.0) and incubated for 1 hr at 37°C with exonuclease III (100 units/μg). The resulting single-stranded DNA was purified by phenolic extraction and ethanol precipitation and used as a template for dideoxy DNA sequencing (USB, Sequenase-2.0) using 16- and 17-nucleotide-long oligonucleotide primers complementary to conserved regions of the *CYP21B* gene antisense strand.

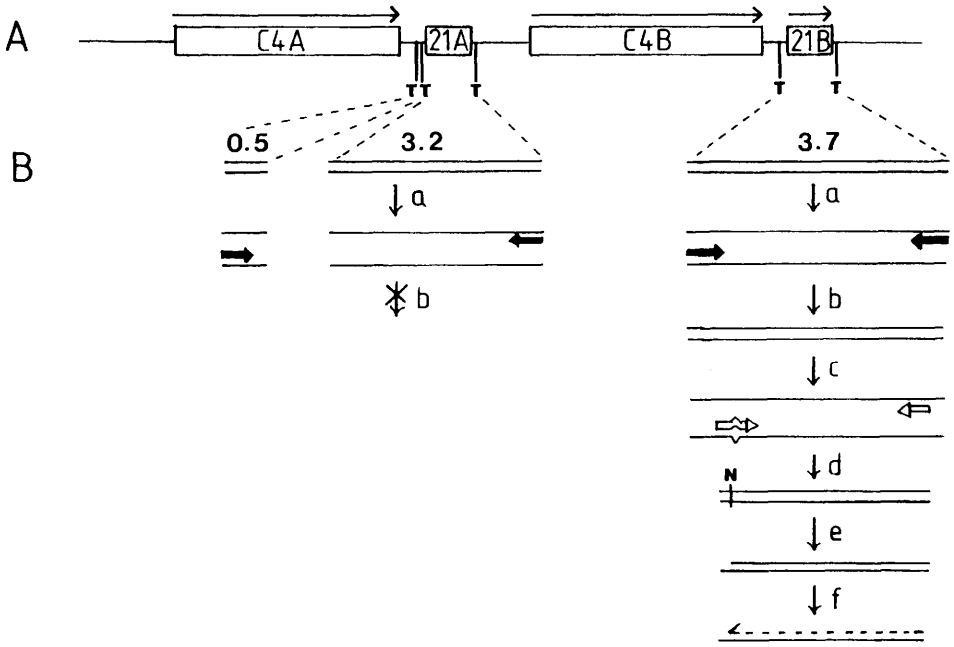

FIGURE 1 PCR sequencing strategy. (*A*) *CYP21* and *C4* gene organization. Arrows mark the direction of transcription of expressed genes. The direction from left to right is from telomere to centromere. (T) Relevant *Taq*I sites flanking the *CYP21* genes. (*B*) Selective amplification of *CYP21B* genes and enzymatic production of single-stranded DNA templates for DNA sequencing. Numbers refer to the size in kilobases of *Taq*I restriction fragments deriving from the *CYP21A* and *CYP21B* genes. Steps are: *a* and *b*) Selective amplification of CYP21B genes using primers derived from the region immediately adjacent to the flanking *Taq*I sites; (*c* and *d*) secondary PCR amplification using primers, one of which is deliberately mismatched to introduce a *Nsi*I site (N); (*e*) digestion with *Nsi*I; (*f*) digestion with exonuclease III.

Allele-specific Oligonucleotide Hybridization to PCR Products

Approximately 100 ng of product from the initial PCR reaction was denatured by the addition of NaOH to a final concentration of 0.5 M and slot-blotted in duplicate onto a nylon membrane (Amersham, Hybond N+). The membrane was incubated in a 5x SSPE, 5x Denhart's prehybridization mix (0.9 M NaCl, 50 mM NaH_2PO_4, 20 mM EDTA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, pH 7.4) for at least 2 hr at 50°C. Hybridization was subsequently carried out at 50°C overnight in the same solution containing 10 ng of oligonucleotide probe that had been end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. After hybridization, filters were washed with 5x SSPE (0.9 M NaCl, 50 mM NaH_2PO_4, 20 mM EDTA, pH 7.4) at room temperature and then with a tetramethylammonium chloride (TMA) wash solution (3 M TMA, 50 mM Tris-HCl pH 8, 2 mM EDTA) initially at room temperature and subsequently at 55–58°C.[11] Allele-specific oligonucleotides were as follows: 173N 5′-AGGTAACAGATGATGCTGC-3′; 173M 5′-GCAGCATCAACTGTTACCT-3′; 319M 5′-CAGCGACTGCAGGAGGAGC-3′; 319M 5′-GCTCCTCCTACAG-TCGCTG-3.′

RESULTS

The basis of the PCR sequencing method is outlined in Figure 1. Selective amplification of the *CYP21B* gene was achieved using as a template for PCR amplification, genomic DNA that had been digested to completion with *Taq*I. The primers used derive from sequences located in the immediate vicinity of well-conserved *Taq*I restriction sites that flank both the individual *CYP21A* and *CYP21B* genes at positions approximately 3.7 kb apart. *CYP21A* genes can be distinguished

TABLE 1. Nucleotide Alterations in Mutant *CYP21B* alleles

Position	Mutant	*CYP21B*	*CYP21A*
A. Mutant *CYP21B* allele on an HLA-DR4 B55 A11 haplotype			
Fourth nucleotide upstream of initiation codon	T	C	T
Codon 173 (172[a]) in exon 4	AAC Asn	ATC Ile	AAC Asn
Codon 454 (453[a]) in exon 10	TCC Ser	CCC Pro	CCC Pro
Codon 494 (493[a]) in exon 10	AGC Ser	AAC Asn	AAC Asn
B. Mutant *CYP21B* allele on an HLA-DR2 B35 A3 haplotype			
Thirteenth nucleotide upstream from intron 2—exon 3 junction	A	C	G
Codon 318 in exon 8	TAG Stop	CAG Gln	TAG Stop
Codon 493 in exon 10	AGC Ser	AAC Asn	AAC Asn

[a]Corresponding codon position in the majority of CYP21B genes; the mutant allele coincidentally has an additional codon because of a neutral length polymorphism in exon 1.

from *CYP21B* genes because they have an additional *Taq*I site located between the conserved *Taq*I sites. Consequently, digestion of genomic DNA with *Taq*I results in differential cutting: *CYP21B* genes are included in 3.7-kb *Taq*I fragments, and *CYP21A* genes are included in 3.2-kb *Taq*I fragments. Subsequent PCR amplification with the indicated primers will result in amplification of the *CYP21B*-specific 3.7-kb fragment. However, amplification of *CYP21A* genes does not occur because the PCR primers hybridize to sequence elements on different molecules; one primer hybridizes to a 0.5-kb *Taq*I fragment while the other hybridizes to the 3.2-kb *Taq*I fragment (see Fig. 1B, steps a and b).

Following selective amplification of *CYP21B* genes, the initial PCR product is used as a pool for secondary PCR amplification using nested primers, one of which is deliberately mismatched in order to introduce a *Nsi*I site at one end of the PCR product. *Nsi*I digestion of this product therefore results in a product with one blunt end and one overhanging 3′ end (Fig. 1B, step e). As sufficiently long 3′ single-stranded overhangs are not cleaved by exonuclease III,[12] the antisense strand, which has the overhanging 3′ end, will be resistant to exonuclease III digestion, whereas the sense strand will be susceptible to exonuclease III digestion. Following exonuclease III digestion, the resulting single-stranded antisense strand product can be used with sense strand-specific primers to permit DNA sequencing.

We have applied the above method to amplify and sequence the *CYP21B* genes in two 21-hydroxylase deficiency patients. One of the patients has the simple virilizing form of the disease in which homozygous deficiency in 21-hydroxylase activity leads to the accumulation of steroid precursors proximal to the enzyme block and excessive steroid androgen biosynthesis (for review, see ref. 13). This patient has an HLA-Bw47-bearing haplotype that carries a *CYP21B* gene deletion, and a DR4 C4B6 C4A4 B55 A11 haplotype. The latter haplotype bears a mutant *CYP21B* gene and is the most common extended haplotype of this kind in the U.K. population.[6] DNA sequencing of the 3.7-kb PCR product from this patient shows a *CYP21B* sequence with a few nucleotide changes (see Table 1). They include a T→A missense mutation at codon 173 that results in an isoleucine to asparagine amino acid change (Fig. 2, left panel). This change, which is alternatively found at codon position 172 of some *CYP21B* alleles due to length polymorphism in exon 1, has been previously shown to be pathological.[14–16] The mutation results in sequence identity in the region of the pathological mutation between the mutant gene and the corresponding *CYP21A* sequence, suggesting a gene conversion-like origin.

A second possible gene conversion event has introduced a T residue at a position four nucleotides upstream from the initiation codon. However, because we previously have observed this nucleotide change in an unaffected *CYP21B* deletion heterozygote (S. Collier, unpubl.), it seems likely to represent a neutral polymorphism at the *CYP21B* locus. The other two changes are missense mutations that

do not appear to have originated by gene conversion. Of these, the change observed in the second last codon (494) has previously been reported in one functional *CYP21B* gene as well as two mutant alleles,[5,15] suggesting that it does not contribute to pathogenesis. Additionally, although not previously reported, the change in codon 454 is unlikely to be pathogenic as we have previously observed this change in the single *CYP21B* gene of a deletion heterozygote who is clinically asymptomatic (S. Collier, unpubl.).

The second 21-hydroxylase deficiency patient has the "salt-wasting" form of the disease; in addition to excessive androgen biosynthesis, there is an inability to conserve dietary sodium as a result of a deficiency in aldosterone biosynthesis.[13] This patient is HLA-homozygous as a consequence of known parental consanguinity and has a single type of *CYP21B* gene located on an HLA-DR2 B35 A3 haplotype. DNA sequencing analysis of the PCR product from this patient has also revealed a *CYP21B* gene sequence that diverges from the sequence of normal *CYP21B* genes at a very small number of nucleotide positions (see Table 1). One of the changes is a C→T transition which results in a nonsense mutation at codon 318 (Fig. 2, middle panel). This mutation has previously been defined as pathological[17] and appears to be the result of gene conversion from the pseudogene. Other mutational differences include an A→G transition in the second last codon which, as described above, is not thought to be pathogenic. In addition, there is a C→A transversion in the second intron at a nucleotide position which is the location of a splice site mutation in some mutant *CYP21B* alleles.[4,5] The same nucleotide change, however, has been reported not to alter normal splicing of the *CYP21B* gene,[4] suggesting that it is does not contribute to disease.

As a check on the ability of the method to amplify *CYP21B* genes selectively, and as confirmation of the sequence data, we have probed the primary PCR product from both patients using a panel of allele-specific oligonucleotides. These comprised pairs of *CYP21B*-specific and *CYP21B*-mutant specific (*CYP21A*-specific) oligonucleotides for the two candidate sites of pathological mutation. As expected, the primary PCR product from the deletion heterozygote patient showed no evidence of hybridization with a *CYP21B*-specific ASO encompassing codon 173, but strong hybridization with the mutant-specific ASO for this region (Fig. 2, right panel). However, the same DNA showed positive hybridization with a *CYP21B*-specific ASO encompassing codon 318, but no hybridization with the equivalent *CYP21B* mutant-specific (= *CYP21A*-specific) ASO. Reciprocal results were obtained with the primary PCR product from the second patient. The lack of ambiguity in these results confirms the ability of the method to amplify *CYP21B* genes selectively.

DISCUSSION

Steroid 21-hydroxylase deficiency is the most outstanding example of disease due to intragenomic sequence exchanges; all pathological mutations

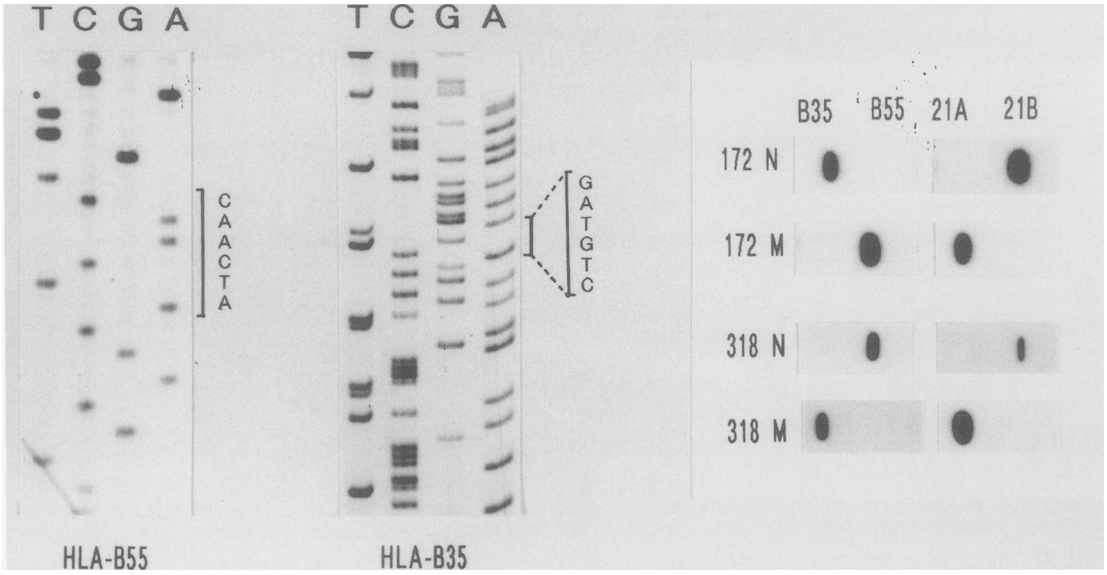

FIGURE 2 PCR sequencing and ASO hybridization analysis at sites of pathological mutation in mutant *CYP21B* genes. (*Left*) DNA sequence spanning a missense mutation in the mutant *CYP21B* gene of an HLA-B55 deletion heterozygote patient. The interpreted hexanucleotide sequence corresponds to codons 172 and 173 in this gene. (*Middle*) DNA sequence spanning a nonsense mutation in the mutant *CYP21B* gene of an HLA-B35 homozygous patient. The interpreted hexanucleotide sequence corresponds to codons 317 and 318. (*Right*) ASO hybridization. DNA sources as follows: B35, amplified *CYP21B* genes from an HLA-B35 homozygous patient; B55, amplified *CYP21B* gene from an HLA-B55 deletion heterozygote patient; 21A, control *CYP21A* gene DNA; 21B, control *CYP21B* gene DNA. ASOs were as follows: 172N and 172M, ASOs specific for a sequence encompassing codon 172 (173) in the normal *CYP21B* gene (N), or in the corresponding mutant (M) sequence; 318N and 318M, ASos specific for a sequence encompassing codon 318 (319) in the normal CYP21B gene (N), or in the corresponding mutant (M) sequence.

that have been recorded at the *CYP21B* locus appear to be due to gene conversion-like mechanisms (this paper and previous reports; for recent reviews, see refs. 9 and 10), or to gene deletion arising as a result of unequal crossover and probably also unequal sister chromatid exchanges.[8–10] Because all pathological point mutations at the *CYP21B* gene appear to arise by copying sequence from the *CYP21A* pseudogene, PCR-based assays of pathological mutation at the *CYP21B* locus have been restricted in scope because of co-amplification of *CYP21A* sequences.

To overcome this, PCR assays have recently been devised by taking advantage of intragenic locations where the CYP21A and CYP21B gene sequences are suitably divergent.[19,20] However, the use of intragenic primers to amplify *CYP21B*-specific sequence selectively is disadvantaged by the following: (1) the interpretation of the results is not facilitated by ignorance of the sequence corresponding to the primer binding sites of the *CYP21A* and *CYP21B* genes under study; (2) the PCR products are gene fragments so that assay pf mutations (by ASO probing or DNA sequencing) at different ends of the *CYP21B* gene may require interpretation of different PCR products which cannot be shown to have derived from the same gene. For example, chance occurrence by gene conversion of a *CYP21B*-specific primer binding site in a *CYP21A* gene may result in co-amplification of unwanted *CYP21A* gene sequence.

The method of PCR sequencing described in the present report overcomes these limitations. First, the PCR product encompasses the entire *CYP21B* gene. Additionally, the choice of primers from sequence adjacent to the flanking *Taq*I sites ensures immediate correlation with genomic *Taq*I Southern hybridization results. This is advantageous because genomic *Taq*I Southerns are almost universally used as an initial step in investigating the possibility of *CYP21B* gene deletion. Finally, the method described herein has the advantage that it permits rapid PCR sequencing of *CYP21B* genes; a full-length genic PCR product is converted enzymically to a single-stranded form suitable for DNA sequencing using *CYP21B*-specific primers. Although much attention has recently been paid to the extreme sensitivity of the PCR reaction, the potential for contamination of the primary 3.7-kb amplification product by *CYP21A* sequence does not appear to be a problem, as shown by the unambiguous demonstration of *CYP21B*-specific sequences by sequencing and ASO hybridization of the PCR products (see Fig. 2).

The above method can be modified to permit selective amplification of *CYP21A* genes. *CYP21B* genes, but not *CYP21A* genes, normally possess an internal *Kpn*I site. A preamplification step with *Kpn*I, instead of *Taq*I, leads to selective cleavage of *CYP21B* genes and subsequent selective amplification of *CYP21A* genes (Collier *et al.*, in prep.). The method can also be generally adapted to permit selective amplification and PCR sequencing in multigene families and other DNA sequence families in which there are a few individual members that are closely related in sequence. Unlike the ARMS amplification method which can discriminate between closely related sequences,[21] the method described in this paper does not require DNA sequence information to identify locus-specific differences; instead, knowledge of specific restriction site differences is sufficient. Additionally, because the method does not require the use of locus-specific primers, the primers can be designed from highly conserved regions to amplify full-length genes as in the present paper.

To adapt the method of locus-specific amplification to any small family of highly homologous DNA sequences, two conditions are ideally required. First, some DNA sequence should be known concerning the locus of interest to permit the synthesis of primers spanning the desired region. Second, the region to be amplified in the locus of interest should consistently lack one or more restriction sites that are known to occur in the equivalent region in other members of the family. Digestion with the relevant restriction enzyme or enzymes should then cleave within the region of interest in all members of the family except the desired locus.

The method for enzyme-mediated production of single-stranded PCR products can also be adapted to other loci. In the specific case of the *CYP21B* gene, we took advantage of a naturally occurring sequence that showed 5/6 matches with the *Nsi*I recognition sequence; the choice of a primer with an appropriate single mismatch then permitted the introduction of a *Nsi*I site during secondary amplification. To generalize the method, a single amplification step can be used. One primer can be designed to contain an additional sequence at its 5′ end that encompasses a recognition site for an enzyme that produces a suitably overhanging 3′ end (e.g., *Apa*I, *Ban*II, *Kpn*I, *Nsi*I, *Pst*I, *Sac*I, or *Sph*I). The choice of which restriction site to include in the primer is determined by restriction mapping; only recognition sites for enzymes that do not cut in the region between the primer binding sites can be used. Following amplification, digestion with the appropriate enzyme can then generate a PCR product with a single overhanging 3′ terminus which will be resistant to exonuclease III digestion.

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