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Detection of single DNA base differences by competitive oligonucleotide priming

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ABSTRACT

Synthetic DNA oligonucleotides can serve as efficient primers for DNA synthesis even when there is a single base mismatch between the primers and the corresponding DNA template. However, when the primer–template annealing is carried out with a mixture of primers and at low stringency the binding of a perfectly matched primer is strongly favored relative to a primer differing by a single base. This primer competition is observed over a range of oligonucleotide sizes from twelve to sixteen bases and with a variety of base mismatches. When coupled with the polymerase chain reaction, for the amplification of specific DNA sequences, competitive oligonucleotide priming provides a simple general strategy for the detection of single DNA base differences.

INTRODUCTION

Techniques enabling the rapid detection of single DNA base changes are important tools for genetic analysis (1). When the precise DNA base change in a mutation is known allele specific oligonucleotides (ASO's) can identify the unique sequences by differential hybridization under stringent conditions (2). Typically, 18–20 base oligonucleotides are constructed with perfect complimentarity to either a normal (wild-type) or mutant sequence. The DNA for analysis is tethered to a solid support and hybridized separately to the radioactive probes. The homology between each ASO and the test sequence is then revealed by sequential washings of the hybrids at high stringency so that the mismatched probe is washed free while the perfect match remains bound.

In order to simplify the detection of single DNA base changes we have used an alternative strategy employing mixtures of synthetic DNA oligonucleotides as primers for DNA synthesis. An example of the basic principle is outlined in Fig. 1. Two synthetic oligonucleotide primers are mixed in a single annealing reaction with a DNA template. Each of the primers is capable of priming DNA synthesis at the same site. However, when one primer is perfectly complementary to the DNA template it can bind in preference to a primer that differs by a single base. The use of a third oligonucleotide primer (common primer) allows the identification of the successfully competing primer by the polymerase chain reaction (PCR) (3–6). When the primer-annealing reactions are carried out at low stringency and with an excess primer to template ratio, the perfectly matched primer can be favored with a level of discrimination greater than 100:1. The perfectly matched primer will also compete successfully when it is at low abundance (*i.e.*, in the presence of up to a 100-fold excess of a mismatched primer) or when the correct match is only one of a four member mixed oligonucleotide family. We also show here that successful competitive oligonucleotide priming (COP) is dependent on the length of the primers and that the COP

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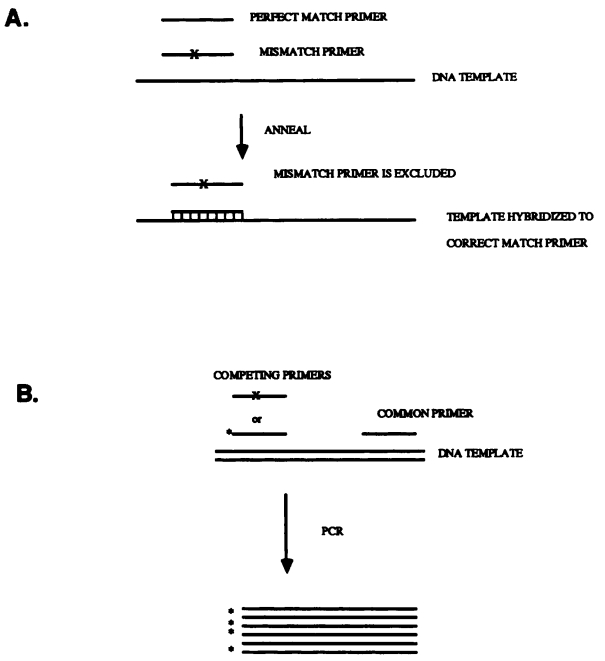

Figure 1. Overview of the general strategy for detection of single DNA base differences by competitive oligonucleotide priming (COP). **A.** DNA template is mixed with two oligonucleotides that differ by a single DNA base. If one oligonucleotide is a perfect match to the DNA template it will bind in preference to a mismatched oligomer. **B.** The correct match primer may be identified by differentially labeling the two oligonucleotides and including a third 'common' oligonucleotide primer. The common primer and the 'successful' COP primer are incorporated into a DNA fragment generated by PCR. Identification of the incorporated COP primer infers the template sequence.

system can be coupled to the PCR to detect single DNA base changes in mammalian genomic DNA.

MATERIALS AND METHODS

Oligonucleotide primers (Table 1) were synthesized on an Applied Biosystems 380B oligonucleotide synthesizer using β-cyanoethyl phosphoramadite chemistry. Mixed oligonucleotides were synthesized using the 380B mixed (competitive) coupling functions. The relative efficiency of addition of individual bases during mixed synthesis has been the subject of previous reports by the manufacturer (7). Following synthesis, oligonucleotides were deprotected in ammonium hydroxide for 6–12 hours at 55°C, dried, dissolved in formamide and purified by denaturing polyacrylamide gel electrophoresis. The oligonucleotides were electroeluted from gel slices and finally desalted over an NENsorb column (Dupont).

The PCR for the amplification of the competitive priming events was carried out either with the large fragment from *E. coli* DNA polymerase I (Klenow) (United States Biochemical Corporation; USB) or with the heat stable DNA polymerase from *Thermus aquaticus* (Taq) (Perkin Elmer/Cetus). Klenow reactions were in a final volume of 100

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TABLE 1. OLIGONUCLEOTIDE PRIMERS

Number	Sequence[a] (5′ to 3′)	Length	Template
1	CCCAGTCACGACGTT	15	M13 'Common'
85	AGCTCGGTACCC	12	M13 polylinker
86	AGCTCGG(TA)AC(CG)C	12	"
98	CGAGCTCGG(TA)AC(CG)C	14	"
99	TTCGAGCTCGG(TA)AC(CG)C	16	"
100	AATTCGAGCTCGG(TA)AC(CG)C	18	"
89	AATTCGAGCTCGGTACCCGG	20	"
90	AATTC(GC)AGCTCGG(TA)AC(CG)CGG	20	"
92	CAAGTGAATGTC	12	OTC mutation (+)
93	CAAGTTAATGTC	12	OTC mutation (spf)
94	CTGTCCACAGAAACAGGC	18	OTC 'Common'
246	GGCGATGTCAATAGGACTCCAGATG	25	HPRT genomic
352	CCACGAAGTGTTGGATATAAGC	22	HPRT genomic
383	TAATGACACAAACATG	16	HPRT Mutation (+)
384	TAATGACATAAACATG	16	HPRT Mutation (−)

[a]Parentheses denote mixtures of bases at a single position

μl containing 30 mM Tris-acetate, pH 7.9, 60 mM sodium-acetate, 10 mM magnesium-acetate, 10 mM dithiothreitol, 1.5 mM each of dATP, dCTP, dGTP, dTTP, 4 μM of each primer (or primer family) and 0.5 to 1.0 μg of DNA template (3). To initiate the Klenow catalyzed PCR reactions, DNA was denatured in 0.4 N NaOH for 5 minutes at 25°C, neutralized with 1/10 volume of 2M ammonium-acetate, pH 4.5, and precipitated with 2.5 volumes of ethanol. The pellet was resuspended in the PCR mix and annealed at 28°C for 3 min. before the addition of 5 units of enzyme. The PCR proceeded with 2 min. polymerization at 28°C, 2 min., denaturation at 105°C (in a heat block containing glycerol) and 30 sec. annealing at 28°C before fresh enzyme was added. The Taq PCR followed the procedure of Kogan *et al.* (5) except that the concentration of each primer was 1.0 μm. Temperature cycling of 37−55°C, 30 sec.; 65°C, 3 min; 92°C, 1 min. was controlled by an automated thermocycler (Perkin Elmer/Cetus).

Oligonucleotide primers were labeled at the 5′ terminus with T4 polynucleotide kinase (USB) to a final specific activity of 25 Ci/mMol. PCR products were analyzed on either a 4% NuSieve agarose (Marine Colloids) or 12% polyacrylamide gels and dried for autoradiography. Plasmid DNA was prepared by two rounds of cesium chloride/ethidium

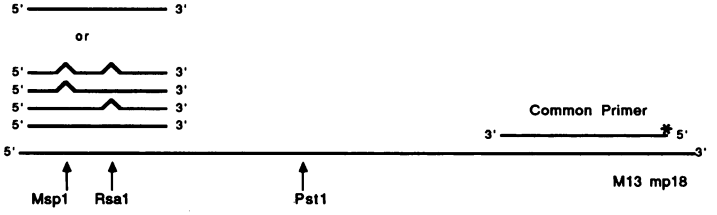

Figure 2. Strategy for analysis of competition between closely related oligonucleotide primers for an M13 (mp18) DNA template. The M13 multiple cloning site was PCR amplified with a radiolabelled (*) 'common' primer and either a perfectly matched opposing primer or a mixture of opposing primers that included single base mismatches to the DNA template. Incorporation of mismatched primers would lead to loss of restriction endonuclease recognition sites.

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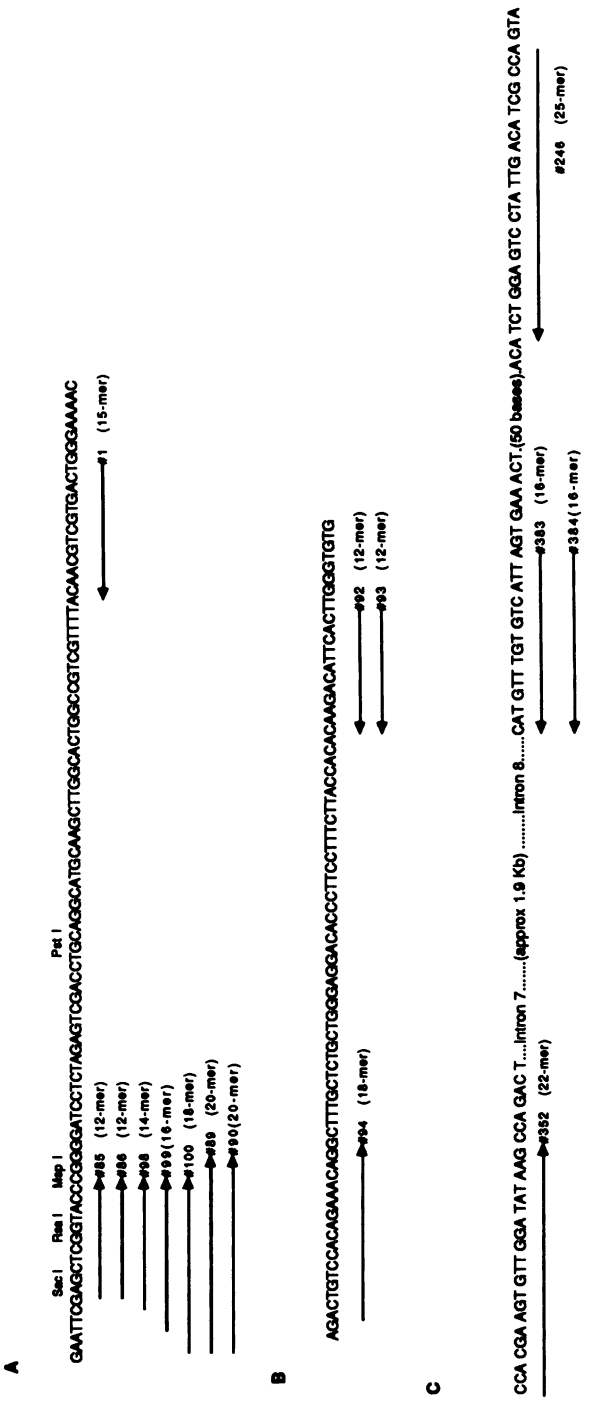

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bromide density gradient centrifugation and human genomic DNA was prepared from transformed human lymphoblasts using an Applied Biosystems 340A DNA extractor.

RESULTS

Competition Between Oligonucleotide Primers of Different Length

Single stranded DNA (ssDNA) from the filamentous phage M13 (mp18) (8) was employed to first demonstrate the efficiency of the COP method. The scheme is illustrated in Fig. 2. The general strategy was to amplify a region of the mp18 multiple cloning site (polylinker) with primers that would either faithfully copy the restriction endonuclease recognition sequences or destroy the sites because of incorporation of oligonucleotide primers that included mismatches to the mp18 template. The DNA sequence of the template in the region bound by the competitive and common primers is shown in Fig. 3a.

The 'common' primer (# 1, Table 1, Fig. 3) was a 15 base oligonucleotide that is often used as a universal DNA sequencing primer. Primers # 85, 86, 89, 90, 98, 99 and 100 each overlap the opposite end of the mp18 polylinker where the SacI, RsaI and MspI restriction endonuclease recognition sites are located. Each of the primers were constructed so that they would be complementary to the products of extension of primer # 1 through the mp18 polylinker. Primers # 85 (12-mer) and # 89 (20-mer) were completely homologous to mp18, and therefore could provide perfect copies of the ssDNA template. In contrast, primers # 86, 98, 99, 100 and 90 were constructed as mixtures. Each contained two or more positions at which two nucleotides were added during synthesis. For example, primer # 86, contained a mixture of A and T at position eight and C and G at position eleven. Thus, primer # 86 had a complexity of four members, one with complete homology to the corresponding region of mp18, two with base mismatches (A:A, G:G) that altered single restriction endonuclease recognition sequences (RsaI or Msp1) and one that altered both the enzyme sites.

Primer # 1 was radiolabeled at the 5′ terminus and employed in separate PCR reactions with each of the other primers and mp18 ssDNA template. The reaction products were either directly analyzed by gel electrophoresis and autoradiography, or first digested with a restriction endonuclease that identified a site within the amplified region. As expected the primer pair # 1/85 generated an 85 bp fragment that was able to be digested to completion by PstI yielding a 48 bp radiolabeled fragment and by RsaI or MspI generating 77 or 74 bp products, respectively (Fig. 4a). Thus, the perfectly matched primer # 85 was incorporated and faithfully reproduced the restriction endonuclease recognition sites from within its sequence. Somewhat surprisingly an identical result was obtained using the primer mixture # 1/86. As only 25% of the primer # 86 family is expected to be perfectly homologous to the mp18 template the presence of RsaI and MspI recognition sites within the amplified product indicated preferential incorporation of the perfectly matched primer relative to the family members with single or double DNA base mismatches. The apparent discrimination afforded by the competition for the correct match (*i.e.*, the relative incorporation of the perfect match *vs* a mismatch) is greater than 100:1 and even a prolonged

Figure 3. DNA sequence of the DNA templates used to analyze primer competition. Arrows showing the oligonucleotide primers point 5′ to 3′. The sequence of the individual oligonucleotides are shown in Table One. **A.** M13 mp18 polylinker region. **B.** Murine ornithine transcarbamylase cDNA (9) **C.** Human hypoxanthine phosphoribosyltransferase (HPRT) exon sequences. The primers for the amplification of the human genomic DNA were each complementary to exon sequences.

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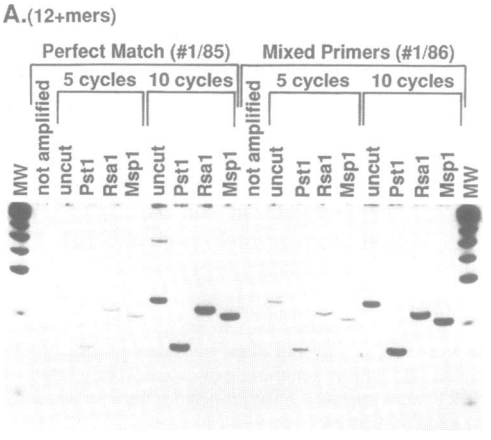
exposure of the gel illustrated in Fig. 4a failed to show any evidence of the primer pair # 1/86 product being refractory to RsaI or MspI digestion.

The successful competition of a correct primer varies to some extent with the overall length of the oligonucleotide. When the perfect match 20-mer # 89 was employed in the reaction the expected 91 bp product was generated and was digested to 81, 77 and 74 bp by SacI, RsaI or MspI, respectively. In contrast primers # 1/90 generated a 91 bp product that could not be completely digested with either SacI, RsaI or MspI (Fig. 4b). As the amplification product was completely digested at the PstI site that lies between primer # 1 and # 90 and would be expected to be faithfully copied, the failure of the sample to digest completely with SacI, RsaI or MspI is most likely due to the incorporation of the mismatched members of this primer family. Primer # 90 contained eight different species as compared to the 12-mer (# 86) that had a complexity of only four. However, we present evidence below that the incorrectly matched primer may be present at more than 100 fold excess without reducing the successful competition of the 'correct' primer. Therefore, the most likely explanation for the incorporation of mismatched primers in the # 1/90 reaction is that a single base mismatch within a 20 base oligonucleotide provides less potential for displacement or competition than a single mismatch within a 12-mer.

Oligonucleotides # 98, 99 and 100 were constructed to represent families of 14, 16 and 18-mers with a complexity of four members each. Digestion of the products of PCR amplification of each of these in conjunction with primer # 1 and the mp18 template revealed that the correctly matched oligonucleotides were predominantly incorporated although a small amount of material that was resistant to enzyme digestion was generated from the 18-mer family (Fig. 4c). We conclude that effective competition by a correct match primer may occur within a family of 12-mers, that the discrimination is greatly reduced within families of 20-mers and predict that the precise relationship between length and discrimination will vary depending on individual base mismatches and their sequence context.

Competitive Oligonucleotide Priming for Mutation Detection

The COP system was adapted for the identification of a known DNA single base change that leads to ornithine transcarbamylase (OTC) deficiency in the sparse fur (*spf*) mouse (9). Oligonucleotides were synthesized to overlap the C to A transversion (Table 1, Fig.



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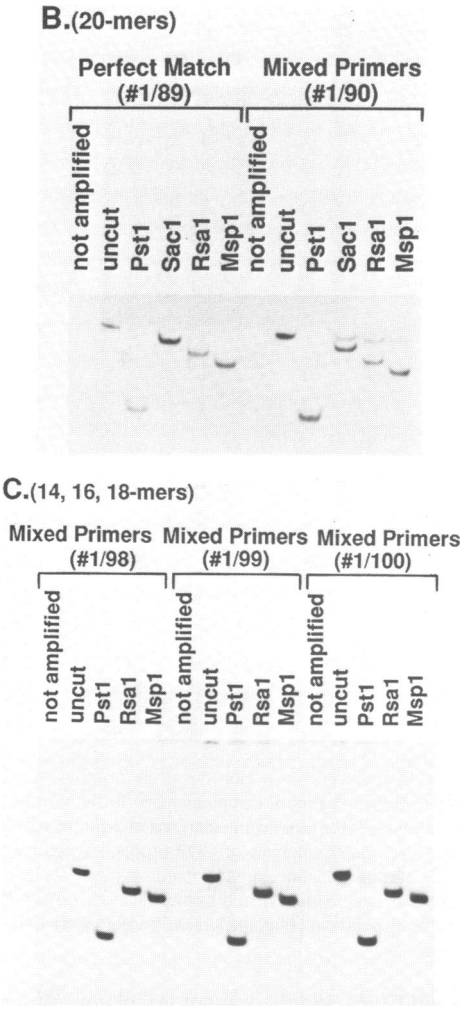

Figure 4. Competition between closely related primers for the M13mp18 single stranded DNA template. A complete explanation is given in the text. The results of restriction endonuclease digestion of amplified M13 DNA using **A.** 12 base, **B.** 20 base and **C.** 14, 16 and 18 base oligonucleotides as COP primers are shown. The incorporation of mismatched oligonucleotides leading to loss of a restriction endonuclease recognition sites is observed when the competing oligonucleotides are 20-mers (**B.**, last three lanes).

3b). Primers #92 and 93 are 12-mers that match the wild-type and *spf* sequences, respectively and #94 is a common 18-base primer complementary to the OTC cDNA in an opposite orientation to primers #92 and 93. Ten cycles of PCR were performed with cloned wild-type OTC cDNA or the *spf* OTC cDNA as template using primers #94 and an equimolar mixture of #92 and 93. A trace of radiolabeled primer #92 or 93 was used to monitor the competition between the two oligonucleotides.

Fig. 5a shows an ethidium bromide stained 4% NuSieve agarose gel used to analyze PCR products generated from primers #94/92/93. A fragment of the predicted size is

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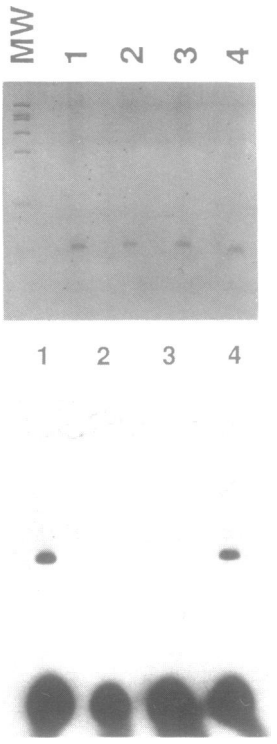

Figure 5. Identification of a C to A transversion in cloned murine ornithine transcarbamylase (OTC) cDNA. Four COP reactions containing competing primers complementary to the normal and mutant OTC sequences (#92/93) and a common primer (#94) (Table One) were analyzed by agarose gel electrophoresis. **Upper Panel**: Ethidium bromide-stained agarose gel electrophoresis of COP products (negative image). **1**, normal template, normal match primer labeled, **2**, normal template, mutant match primer labeled, **3**, mutant template, normal match primer labeled, **4**, mutant template, mutant match primer labeled, **Lower Panel**, Autoradiograph of the dried agarose gel, showing that the radiolabeled oligonucleotide primers were only incorporated in the presence of perfectly matched DNA templates.

generated from both the wild-type and the mutant templates. When this gel was dried and exposed to X-ray film only the fragments that were generated in reactions containing a radiolabeled primer that was a perfect match to the template had incorporated radioactivity (Fig. 5b). A prolonged exposure revealed faint bands from mismatch incorporation but indicated that the level of discrimination was greater than 100 to 1.

Preference for the 'correct' oligonucleotide primer

The strong preference of a DNA template for a 'correct' primer was further demonstrated by competing a perfectly-matched oligonucleotide primer with an excess of a mismatch oligonucleotide (Fig. 6). The preference of the cloned wild-type OTC template for the perfect match primer #92 above #93 when the two oligonucleotides were present in equimolar ratios (Lanes 1 and 2) was reduced only slightly when the radiolabeled mismatch (#93) was present in a 100-fold molar excess (Lane 3). At a 1000:1 ratio, where the molar concentration of the correct primer was approximately that of the DNA template (Lane 4) the mismatch was incorporated with a still lower efficiency than when the mismatch

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Figure 6. Successful primer competition in the presence of an excess of mismatched primer. The experiment is similar to that illustrated in Fig. 5 and a complete description is given in the text. Lanes 1 and 2 show incorporation and exclusion of radiolabeled perfect match and mismatched primers, respectively. Lane 3 indicates the level of incorporation of a radiolabeled mismatched primer present in a 100-fold excess above a perfectly matched, unlabeled primer (4.0 μM *vs* 40 nM); Lane 4, mismatch present at 1000-fold excess (4.0 μM *vs* 4.0 nM),; Lane 5, mismatch alone (4.0 μM). Lane 6 shows the exclusion of a radiolabeled mismatched primer (4.0 μM) that was annealed to the DNA template for 3 min before addition of an equimolar amount of the correct match primer and initiation of the reaction.

primer was present alone (Lane 5). In a further reconstruction experiment the template was annealed to the radiolabeled mismatched oligonucleotide for the usual 3 min. before an equimolar amount of the correct primer was added. DNA polymerase was added after 1 minute more annealing and the PCR was carried out as before. Surprisingly, the correct match primer was predominately incorporated (Lane 6) reflecting the ability of the perfectly matched primer to displace any mismatched primer that might have been bound.

Detection of Single DNA Base Differences in Genomic DNA Using Taq DNA Polymerase

To test whether the COP mutation detection system could be adapted to conditions that enable the use of the heat stable Taq DNA polymerase (5,6), oligonucleotides complementary to normal or mutant human hypoxanthine phosphoribosyltransferase (HPRT) sequences were constructed (Table 1, ref 9). The oligonucleotides (16-mers) differed by a single base (C *vs* T) at the eighth position and had previously been employed as ASO hybridization probes to identify the corresponding normal and mutant alleles in a family study of HPRT deficiency (10). To enable COP analysis of the G to A transition an approximately 1950 base region of the HPRT gene containing the known mutation site was first PCR-amplified from genomic human DNA samples using primers #246/352. This fragment appeared homogeneous when analyzed by agarose gel electrophoresis. Five percent of the initial reaction products were then taken to initiate a further 10 rounds of PCR, with each of the allele-specific COP oli-gonucleotides (#383/384) and a common (#352) primer present. Four COP reactions were performed with either the correct match or mismatched primers radiolabeled at the 5′ terminus and with either the normal or mutant 'preamplified' alleles as DNA templates. Analysis of each of the COP reactions by agarose gel electrophoresis and ethidium bromide staining revealed predominant products of the expected size. When the gel was dried and exposed to X-ray film it was found that the only fragments that were radiolabeled were the expected sized products from reactions where the radiolabeled primers perfectly matched the DNA templates. Thus the normal and mutant alleles were each correctly identified (Fig. 7).

DISCUSSION

We have demonstrated competition between closely related synthetic oligonucleotide primers

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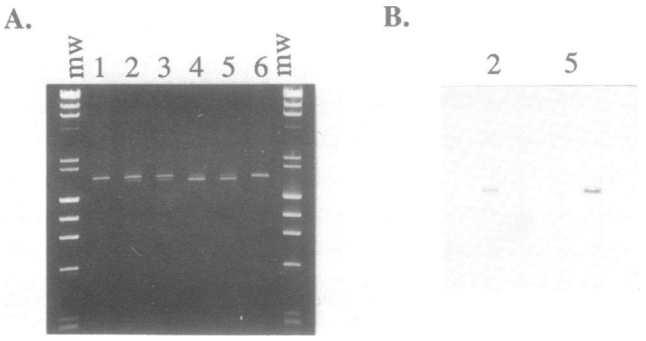

Figure 7. Identification of a single DNA base change in human DNA by COP, using Taq DNA polymerase. Four COP reactions containing competing primers complementary to normal and mutant HPRT gene sequences (#383/384) and a common primer (#352) (Table One) were performed using 'preamplified' human genomic DNAs as templates. **Left**: Ethidium bromide-stained agarose gel electrophoresis of PCR and COP products. Lanes 1 to 6 show the products of PCRs used to 'preamplify' the regions surrounding the normal and mutant alleles, respectively. Lanes 2–5 show COP products. **2**, normal template, normal match primer labeled, **3**, normal template, mutant match primer labeled, **4**, mutant template, normal match primer labeled, **5**, mutant template, mutant match primer labeled, **Right**: Autoradiograph of the dried agarose gel, showing the preferential incorporation of perfectly matched radiolabeled oligonucleotide primers.

for a single DNA template. When coupled to PCR, COP provides a simple method for the identification of single DNA base differences and thus represents an alternative to ASO probing for the analysis of mutations for which the precise DNA sequences can be predicted. In contrast to ASO probing the COP procedure does not require the use of solid filter supports and is technically more simple to perform. The COP strategy may therefore be favored for the routine analysis of known single DNA base substitutions or polymorphisms.

Successful COP has been shown to occur when competing oligonucleotides differ by single T-G, T-A, C-T or C-G base changes that generate A-A, G-G, G-A, T-C, C-A and T-G mismatches between the oligomers and their corresponding DNA templates. This represents six of the twelve possible base mismatches that may perturb normal Watson–Crick DNA base pairing. If reciprocal mismatches are equivalent (*e.g.*, A-T *vs* T-A) then only T-T and C-C mispairings are not described in this study. In other experiments C-C and G-G mismatches have been identified by COP (J. S. Chamberlain, *personal communication*) but it is likely that the general efficiency of the primer competition will be determined by both the mismatched base that is involved and its surrounding sequence context. Therefore many mutations in different sequences may need to be examined before a base mispairing that cannot be identified by COP could be found. The M13mp18 DNA template amplification/restriction strategy described here, coupled with mixed oligonucleotide synthesis represents a convenient method for further study of these relationships.

The competing oligonucleotide primers described above are short (12–16 nucleotides). Although this length exceeds that necessary for efficient priming of DNA synthesis it is less than the usual length employed for ASO probing. The efficiency of the competition can be reduced when the oligonucleotide primers are 20-mers but COP is still effective with 16-mers. As a general rule we are continuing to construct 16 base oligomers for

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mutation identification as they can function both as ASO probes and competing primers, to further test the method.

An important feature of this study was the adaptation of Taq DNA polymerase to the COP reactions. If COP is to be a favored method of mutation detection then the procedure should be more simple to perform than alternatives that offer the same specificity of DNA sequence discrimination. The substitution of Taq in place of Klenow and the accompanying development of automated thermocyclers has enabled the widespread acceptance of PCR technology. The demonstration that a single DNA base difference can be identified by COP using Taq allows a similar protocol for point mutation detection to that used for routine PCR DNA amplification. The region containing the mutant sequence is first amplified and a small aliquot of the PCR product taken to initiate the COP reaction. The radiolabeled COP products are then analyzed by gel electrophoresis and autoradiography to identify the individual alleles.

A possibly important determinant of the relative hybridization efficiency of competing oligimers may be the position of the individual base mismatches within the oligonucleotides. In addition, the occurrence of a mismatch at the 3′ terminus of an oligonucleotide may inhibit primer extension, provided the DNA polymerase used lacks a 3′ to 5′ exonuclease proofreading activity. Although this schema offers a strategy for mutation detection that is similar to COP in the manipulations and reagents that are required the underlying mechanism would be fundamentally different. In that case an oligonucleotide primer would not be required to bind specifically to the correctly matched allele, which contrasts to the central feature of the COP mechanism.

A mutation detection technique requiring a 3′ base mismatched oligonucleotide and DNA ligase has been recently described (11). The method allows mismatch detection by failure of head-to-tail ligation of two synthetic oligonucleotides at the site of a mutant DNA base. Excellent discimination between each of the 12 possible base mismatches and the corresponding perfect matches has been reported. The ligation method has a similar advantage to COP in that solid filter supports are not necessarily required. Unlike the primer competition reactions the ligation conditions are not so easily compatible to PCR buffers and therefore more extensive sample manipulation may be required for the analysis of PCR amplified DNA sequences.

There are many technical refinements that could be adapted to improve the COP method. More than two oligonucleotides can simultaneously compete for the same DNA priming site and multiple fluorescently labeled oligonucleotides (12) could be used to simultaneously test regions with a high degree of genetic heterogeneity. The maximum number of oligonucleotide species that could be employed in a single reaction has not yet been established but the observation that competition can occur when a mismatched primer is present at 100-fold higher abundance than the correct match suggests that even very highly polymorphic loci may be amenable to single analyses. Addition of a biotin residue to the 5′ terminus of a common primer (13) could further facilitate the method by allowing rescue of a PCR-amplified fragment that has incorporated the successfully competing, differentially labelled primer by an avidin bound support. In this case the final analysis of the COP products would not require gel electrophoresis and could be monitored by measurement of the radioactive or fluorescent incorporation of the support matrix. Such refinements may eventually lead to the complete automation of DNA base difference detection for genetic disease diagnosis and the analysis of other DNA sequence polymorphisms in complex genomes.

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