RESEARCH

A Novel In Vivo Method to Detect DNA Sequence Variation

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Mismatch repair detection (MRD) is an in vivo method that uses a change in bacterial colony color to detect DNA sequence variation. DNA fragments to be screened for variation are cloned into two MRD plasmids, and bacteria are transformed with heteroduplexes of these constructs. The resulting colonies are blue in the absence of a mismatch and white in the presence of a mismatch. MRD is capable of detecting a single mismatch in a DNA fragment as large as 10 kb in size. In addition, MRD has the potential for analyzing many fragments simultaneously, offering a powerful method for high-throughput genotyping and mutation detection in a large genomic region.

The detection of mutations in genomic DNA plays a critical role in efforts to elucidate the genetic basis of human disease. Although many approaches are currently applied to the problem of mutation detection, no single technique provides a rapid method for screening large stretches of genomic DNA with high sensitivity and specificity (Grompe 1993). We have developed an in vivo bacterial assay, mismatch repair detection (MRD), that utilizes the *Escherichia coli* methyl-directed mismatch repair system to detect single-base mismatches in DNA. Unlike other DNA variation detection techniques, MRD can detect a single-base mismatch in up to 10 kb of DNA. In addition, MRD has the potential to examine many different DNA fragments simultaneously, providing a rapid method for screening large stretches of DNA for nucleotide sequence variation.

The normal function of the *E. coli* methyl-directed mismatch repair system is to correct errors in newly synthesized DNA resulting from imperfect DNA replication (Wagner and Meselson 1976). The system distinguishes unreplicated from newly replicated DNA by taking advantage of the fact that methylation of adenine in the sequence GATC occurs in unreplicated DNA but not in newly replicated DNA. Mismatch repair is initiated by the action of three proteins, MutS, MutL, and MutH, which lead to nicking of the

unmethylated, newly replicated DNA strand at a hemimethylated GATC site. The unmethylated DNA strand is then digested and resynthesized in a replication reaction in which the methylated strand is used as a template (Modrich 1991). The methyl-directed mismatch repair system can repair single-base mismatches and loops up to 3 nucleotides in length. Loops of 5 nucleotides and larger are not repaired (Parker and Marinus 1992). We have taken advantage of the inability of the mismatch repair system to repair loops of 5 nucleotides or greater to design two vectors that allow in vivo mismatch repair to be detected visually as a change in bacterial colony color.

Two pUC-derived plasmids, the blue (pMF200) and the white (pMF100) plasmid, are used in the MRD procedure. These plasmids are identical except for a 5-bp insertion into the *lacZα* gene of pMF100 (Fig. 1). This insertion results in white colonies when bacteria transformed with the plasmid are grown on LB plates supplemented with indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-thiogalactoside (IPTG). In contrast, bacteria transformed with the blue plasmid result in blue colonies when grown under these conditions. The initial step of the MRD procedure (Fig. 2) consists of cloning one of two DNA fragments to be screened for differences into the blue plasmid and cloning of the second DNA fragment into the white plasmid. The blue plasmid construct is then transformed into a *dam*⁻ bacterial strain, resulting in a completely unmethylated plasmid, whereas the white plas-

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Figure 1 The MRD vectors. pMF100 and pMF200 are derived from pUC19 with the multiple cloning site displaced from the *lacZα* region (Yanisch-Perron et al. 1985). In addition, the MRD vectors contain the *Bgl*I fragment (2166–472) and most of the multiple cloning site of pBluescript (Short et al. 1988). The multiple cloning sites of the MRD vectors do not have sites for the restriction enzymes *Xba*I, *Spe*I, *Bam*HI, *Sma*I, and *Apa*I; the *Eco*RI site is not unique. pUC19 multiple cloning sites (nucleotides 400–454) were replaced using 70-nucleotide-long oligonucleotides with a sequence containing 4 GATC sites. In addition, the sequence replacing the pUC19 multiple cloning sites in pMF200 has a 5-bp insertion as compared to pMF100 creating a nonfunctional *lacZα* in pMF200. The label loop is to indicate this difference between pMF100 and pMF200.

mid construct is transformed into a *dam*⁺ bacterial strain, resulting in a fully methylated plasmid. The two plasmids are then linearized, denatured, and reannealed, resulting in two heteroduplex and two homoduplex plasmids. Following digestion with *Mbo*I, which digests only unmethylated homoduplexes, and *Dpn*I, which digests only fully methylated homoduplexes, the remaining hemimethylated heteroduplexes are circularized, transformed into *E. coli*, and plated onto agar supplemented with X-gal and IPTG. In the absence of a mismatch between the two test DNA fragments, the 5-nucleotide loop in the *lacZα* gene that results from heteroduplex formation between the white and the blue plasmids is not repaired by the mismatch repair system. Subsequent plasmid replication produces both white and blue plasmids in a single colony, leading to a blue color. In contrast, if a mismatch is present in the heteroduplex DNA, a corepair event takes place that involves both the mismatch in the DNA as well as the 5-nucleotide loop in the *lacZα* gene. In this case, the

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unmethylated *lacZα* gene on the blue plasmid is degraded and replaced by the *lacZα* gene from the methylated strand of the white plasmid, resulting in a white colony. Previous in vivo studies have suggested that the corepaired segment of DNA is at least 1.5 kb (Carraway and Marinus 1993). We have found that corepair of a mismatch and the *lacZα* gene in the MRD system occurs even when the distance between them is 5 kb (see below).

RESULTS

Testing Known Mutations

As an initial test of the sensitivity and specificity of the MRD system, we tested the detection of a single-nucleotide mismatch in a 550-bp DNA fragment derived from the promoter of the mouse β -globin gene (Myers et al. 1985a). We used MRD to compare this DNA fragment, which contains a T at position –49 relative to the functional transcription start site of the gene, with a second DNA fragment identical in sequence except for a C at position –49. In this experiment, the mismatch was located ~700 bp from the 5-nucleotide *lacZα* loop in the vector. Comparison of the two DNA molecules by using MRD resulted in 90% white colonies. In contrast, comparison of the same two DNA molecules with no mismatch (–49T/–49T) resulted in only 7% white colonies (Table 1; Figs. 2 and 3). Comparison of all of the possible different single-nucleotide mismatches at position –49 using MRD revealed proportions of white colonies ranging from 80% to 90% (Table 1; Figs. 2 and 3). These results demonstrate that MRD can detect all of the different DNA variations possible at this position with high efficiency.

In an effort to establish the generality of the above results, we used the MRD system to detect a total of five additional single-nucleotide mismatches in two different DNA fragments (Table 1). Four of these mismatches are at different nucleotide positions in the human cystathionine β -synthase gene (Kruger and Cox 1995). The remaining one mismatch represents a single-nucleotide change in the human agouti gene (Wilson et al. 1995). In each case, we were able to detect the single-nucleotide mismatch (Table 1).

The detection of a single mismatch in 10 kb of DNA

In the experiments described above, we were surprised to observe that we were able to detect the

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mismatch even when it was as far from the loop as 2.3 kb. In addition, because the proportion of white colonies was >50%, corepair of the mismatch and the loop occurred irrespective of which side the mismatch was located relative to the loop on the unmethylated strand. In an effort to determine whether the efficiency of mismatch detection would remain high if the distance between a mismatch and the vector loop was even larger, we performed the following experiment. A 9-kb test DNA fragment derived from bacteriophage λ was cloned into the MRD plasmid system and compared with the same test DNA containing a 2-bp insertion located 5 kb from one end of the fragment. Because DNA molecules used for transformation are circular, a mismatch in a 10-kb fragment is always within 5 kb of the loop. The mismatch in this experiment was at least 5 kb away from the loop in either direction. In the presence of the 2-bp loop, 70% white colonies were produced, as compared with 10% white colonies in the absence of the mismatch. These results indicate that MRD can detect a mismatch in 10 kb of DNA.

Detecting variation in PCR products

Next, we investigated the utility of MRD for detecting unknown mutations in genomic DNA fragments generated by the polymerase chain reaction (PCR). PCR is a practical method for obtaining a particular genomic DNA fragment of interest from many different individuals. Recent advances in PCR technology make it possible to isolate DNA products >10 kb in length (Barnes 1994; Cheng et al. 1994). However, the introduction of errors during the PCR reaction severely limits the use of individual cloned PCR products for mutation detection, particularly in the case of long PCR products. In an effort to overcome this limitation, we have developed a protocol that uses MRD to enrich for molecules that are free of PCR-

Figure 2 (See facing page for legend.)

induced errors. Following this “cleaning” protocol, the cloned PCR products can be compared for DNA sequence differences by using the MRD procedure described above.

The basic principle underlying the MRD cleaning protocol is the fact that any single PCR-induced mutation makes up a very small fraction

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Table 1. Detection of Known Point Mutations Using MRD

Variation ^a	Fragment size ^b	Distance from loop ^b	Percent white colonies ^c
None ^d	0.55	N.A.	7
G/C ^d	0.55	0.7	89
A/T ^d	0.55	0.7	84
G/T ^d	0.55	0.7	82
A/C ^d	0.55	0.7	82
C/T ^d	0.55	0.7	90
None ^e	2.0	N.A.	8
A/C ^e	2.0	0.4	35
None ^f	2.2	N.A.	10
C/T ^f	2.2	2.3	83
G/A ^f	2.2	2.1	86
C/T ^f	2.2	1.6	81
T/C ^f	2.2	1.8	80

The mutations are in the order listed: C341T, G502A, C992T, and T833C.

^a(A/T) At the only position of variation between the two fragments compared, the *dam*⁻-grown variant has an A and the *dam*⁺-grown variant has a T at the same position on the same strand. Therefore, mismatches produced in such an experiment are A/A and T/T.

^bIn kilobases. (N.A.) Not applicable.

^cAt least 250 colonies were counted to determine the percentage.

^dExperiment using fragment of the mouse β -globin gene.

^eExperiment using fragment of the human agouti gene.

^fExperiment using fragment of the human cystathionine β -synthase gene.

Figure 3 Transformation plates of the different mismatches at position -49 of mouse β -globin promoter. The plate labeled T/T, containing a majority of blue colonies, represents the transformation with the nonmismatched control, the remaining plates, containing a majority of white colonies, represent transformation with mismatched molecules. Nomenclature of the different comparisons is as described in Table 1.

of all the molecules generated by PCR. As a result, when the products of a PCR reaction are cloned into the blue and the white MRD vectors and assayed as described above, the majority of products containing PCR-induced errors are present as heteroduplex molecules containing a mismatch and produce white colonies. In contrast, those PCR products with no PCR-induced errors con-

tain no mismatches and result in blue colonies. Given that not all mismatches are repaired with 100% efficiency, some blue colonies can be expected to contain PCR-induced errors following the first round of enrichment. However, if blue colonies are isolated and used in a second round of MRD cleaning, those molecules containing PCR-induced errors can be reduced even further.

Figure 2 The MRD procedure. (a) Formation of the heteroduplex. DNA from the unmethylated blue plasmid and the methylated white plasmid containing the fragments to be screened are linearized, denatured, and reannealed. The resulting molecules are fully unmethylated blue plasmid homoduplex, fully methylated white plasmid homoduplex, and hemimethylated heteroduplexes (two populations of heteroduplexes are formed). Only the heteroduplex molecules are left intact after treatment with *Mbo*I, which digests fully unmethylated DNA, and *Dpn*I, which digests fully methylated DNA. (b) Introduction of the heteroduplex into *E. coli* and detection of the variation. The heteroduplex molecules prepared in a are circularized with T4 DNA ligase and transformed into *E. coli*. In the absence of a mismatch, DNA replication in the bacteria generates both the blue and the white plasmid, producing a blue colony. In the presence of a mismatch, repair of the unmethylated blue strand of the heteroduplex using the white strand as a template generates the white plasmid only, producing a white colony.

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Because each blue colony contains both a blue and a white MRD plasmid (see above), the second round of MRD cleaning is carried out as follows.

Plasmid DNA isolated from blue colonies following the first round of cleaning is used to transform both *dam*⁻ and a *dam*⁺ bacterial strains. Although both blue and white colonies result from each transformation, only the blue colonies are isolated from the *dam*⁻ transformation and only the white colonies are isolated from the *dam*⁺ transformation. Plasmid DNA is prepared from such colonies, and heteroduplexes are isolated as described above. Blue colonies arising from transformation with these heteroduplexes are enriched further for the products free of PCR-induced error. For example, in an experiment in which 75% of molecules contain one or more PCR-induced errors following PCR, assuming 95% efficiency of mismatch repair and 10% frequency of white colonies in the absence of a mismatch, the expectation would be 10% blue colonies following one round of MRD enrichment, with 66% of the molecules in such colonies free of PCR-induced errors. If the plasmid DNA from the blue colonies were used for a second round of MRD enrichment, the expectation would be 41% blue colonies, with 96% of the molecules in such colonies free of PCR-induced errors.

As a test of the practicality and the efficiency of the MRD cleaning protocol, we isolated a 2-kb human chromosome 21-specific PCR product from each of the two chromosome 21 homologs of a single individual. The two chromosome 21 homologs were separated from each other in independent hamster-human somatic cell hybrid clones. Genomic DNA isolated from these somatic cell hybrid clones was the template of the PCR reactions. When the PCR products derived from each homolog were compared by using MRD as described above, ~10% blue colonies were observed in each case. Following two rounds of MRD cleaning, the proportion of blue colonies was 60%–80% (Table 2). In contrast, when these “cleaned” PCR products derived from the two homologs were compared with each other by using MRD, ~90% of the resulting colonies were white, indicating the presence of at least one single-base difference in the 2-kb PCR products derived from the two different chromosome 21 homologs. We have demonstrated independently the presence of at least one DNA sequence variation in these 2-kb PCR products by finding a *Hinf*I restriction fragment length polymorphism (RFLP) (data not shown). These results

Table 2. Detection of DNA Variation Following PCR Cleaning

Variants compared ^a	Experimental stage	Percent white colonies ^b
1/1	no cleaning	>90
2/2	no cleaning	>90
B1/B1	one cleaning round	70
B2/B2	one cleaning round	64
BlueB1/BlueB1	two cleaning rounds	38
BlueB2/BlueB2	two cleaning rounds	21
BlueB1/BlueB2	testing clean products	>90
BlueB2/BlueB1	testing clean products	>90

^a1 and 2 represent PCR products from two different homologs of chromosome 21 isolated in hamster-human somatic cell hybrids. (1/1) Comparison of a blue plasmid grown in a *dam*⁻ strain containing the PCR product from hybrid 1 with a white plasmid grown in a *dam*⁺ strain containing the PCR product from hybrid 1. (B1/B1) Comparison of blue *dam*⁻ grown plasmids obtained from the blue colonies of comparison 1/1 to white *dam*⁺-grown plasmids obtained from the same source. (Blue/B1/BlueB1) Comparison of blue *dam*⁻ grown plasmids obtained from the blue colonies of comparison B1/B1 to white *dam*⁺-grown plasmids from the same source. (BlueB1/BlueB2) Comparison of blue *dam*⁻-grown plasmids from the blue colonies of comparison B1/B1 to white *dam*⁺-grown plasmids obtained from the blue colonies of comparison B2/B2.

^bAt least 200 colonies were counted to determine the percentage.

demonstrate that MRD can be used to enrich for PCR products that are largely free of PCR-induced errors and that such products can be used in conjunction with MRD to detect human DNA sequence variation. In addition, we have used MRD in conjugation with the high-fidelity polymerase, *Pfu*, to analyze 2-kb PCR products for DNA variations without the need to perform PCR cleaning (M. Faham and D.R. Cox, unpubl.).

DISCUSSION

Current techniques for detecting unknown mutations in genomic DNA fall into three general classes. The first class of techniques, which in-

cludes single-strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), and heteroduplex analysis in gel matrices (Myers et al. 1985b,c; Orita et al. 1989a,b; Sheffield et al. 1989; Perry and Carrell 1992; White et al. 1992), detects conformational changes created by DNA sequence variation as alterations in electrophoretic mobility. These techniques are limited by the need to determine optimum reaction conditions for each DNA fragment and by a marked decrease in sensitivity with increasing DNA fragment size. The second class of techniques, which includes RNase A cleavage, chemical mismatch cleavage (CMC), and enzyme mismatch cleavage (EMC), uses chemicals or proteins to detect sites of sequence mismatch in heteroduplex DNA (Myers et al. 1985d; Cotton et al. 1988; Maschal et al. 1995; Youil et al. 1995). These techniques can be used to assay many different DNA fragments with a single set of assay conditions. In addition, they can be used to detect mutations in larger DNA fragments. However, even with this second class of techniques, the upper limit for the size of the screened DNA fragment is ~1 kb.

Unlike all of the techniques described above, which involve in vitro analyses of DNA to detect sequence variation, MRD utilizes an in vivo assay for detecting unknown mutations in genomic DNA. We have used this system to analyze a variety of heteroduplex molecules with inserts ranging in size from 550 to 9 kb, representing each of the four possible classes of single-nucleotide substitutions between the strands. All of the mutations tested, nine of nine point mutations (Table 1) and three of three deletions of 2–3 bp (M. Faham and D.R. Cox, unpubl.), could be detected unambiguously. Our data indicate that MRD can detect mismatches in DNA fragments of up to 10 kb in size. Thus, MRD overcomes one of the major limitations of techniques currently available for detecting unknown mutations.

In some cases of mutation detection (e.g., comparison of a patient's DNA with the patient's tumor DNA) and polymorphism detection (e.g., identification of a polymorphic marker for mapping a recombination breakpoint), the goal of the experiment is to identify a variant DNA fragment. In such cases, MRD's ability to detect DNA variation in long DNA fragments with high sensitivity is particularly useful. In other cases of mutation detection in human genomic DNA or high throughput genotyping, the experimental goal is

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to identify which one of many variations in a large genomic region is the disease-causing variation. To achieve this goal, one needs to test for the presence of the different identified variations in many people from the normal population. In such cases, an efficient analysis of many small fragments is more beneficial than the analysis of a long DNA fragment. MRD is well suited for this experimental problem, as the technique can be used to analyze many fragments simultaneously in a single experiment. In such an experiment, heteroduplexes are made between a pool of restriction fragments from the genomic region of interest of a "standard" and a "test". This is followed by ligation of these heteroduplexes into the hemimethylated MRD heteroduplex vector and transformation into *E. coli*. The resulting blue colonies contain DNA fragments that have no sequence variation between the tester and the standard, whereas the white colonies contain fragments with sequence differences. DNA prepared from the pool of blue colonies contains fragments of identity, whereas DNA prepared from the pool of white colonies contains fragments containing differences. Determination of whether a specific DNA fragment is present in the white pool or the blue pool indicates whether the fragment contains a variation. One can use agarose gel electrophoresis of a restriction digest that releases the insert fragments in the blue and the white pools to determine the pool in which each fragment is present. We performed this procedure to analyze up to 10 DNA fragments simultaneously for variation (M. Faham and D.R. Cox, unpubl.). To analyze more fragments, the resolution of the different fragments on agarose gels would be impractical. One can use the blue and white pool DNA as independent hybridization probes on a blot containing DNA from each of the different fragments. For each dot, the comparison of the hybridization signal produced by the blue probe with that produced by the white probe determines whether that fragment contains a variation. Such a procedure has the potential for detecting the presence of mutations in a region representing hundreds of kilobases of DNA or for genotyping many loci simultaneously. This approach is similar to that used in the genomic mismatch scanning (GMS) procedure for identifying regions of the genome identical by descent (Nelson et al. 1993). However, an important difference between GMS and MRD is that GMS yields a probe only for regions of identity, whereas MRD yields probes for both regions

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of identity and regions of difference. The ability to isolate both types of probes results in improved signal to noise as compared to the use of a single probe. Although the manual isolation of blue from white colonies becomes impractical as the number of colonies becomes large, the use of an automatic cell sorter or a selectable system to isolate blue from white colonies should allow for the analysis of very large numbers of colonies.

The detection of DNA variation by MRD is limited by the ability to obtain the specific DNA fragments to be analyzed from the patients of interest. However, several approaches are presently available to isolate the necessary DNA fragments, including long-range PCR with high-fidelity enzymes (e.g., *Pfu*) (Neilson et al. 1995), *recA*-assisted cleavage (Ferrin and Camerini-Otero 1991), and the use of a single set of oligonucleotide primers to amplify multiple specific fragments simultaneously by PCR (Brookes et al. 1994). In conjunction with these methods for isolating specific genomic DNA fragments, MRD provides a powerful technique for the detection of unknown mutations, the detection of DNA variation in large genomic regions, and high-throughput genotyping.

METHODS

Construction of MRD Vectors

pNEB193 (New England Biolabs) was digested with *AseI*, and the larger two fragments were ligated to each other leading to a construct, pNEB133, with a deletion of ~60 bp. pBCKS (Stratagene) was digested with *SpeI*, filled in with Klenow, digested with *SmaI*, and recircularized with T4 DNA ligase. The resultant clone ΔB was resistant to cleavage by *XbaI* in addition to the other expected enzymes (*SpeI*, *BamHI*, and *SmaI*). *BssHII* digest of ΔB was performed, and the smaller band (~150 bp) was gel eluted and ligated to *TfiI*-digested and Klenow-filled pNEB133. The resultant clone, pNEB133B, had the orientation of T3 being the far end from the *lacZα* gene. The *BspHI* fragment containing the ampicillin gene of pNEB133B was replaced with a PCR-generated fragment carrying the chlormphenicol-resistant gene producing pMF0. pNEB133B was digested with *ApaI*, the 3' overhang chewed with T4 DNA polymerase, a *BglII* linker added, and recircularization performed with T4 DNA ligase producing the clone pNEB133BB. The *BglII* fragment containing the M13 origin of replication from pBluescript was inserted instead of the *BglII* fragment of pNEB133BB, producing the clone pBBM. Two complementary oligonucleotides were cloned into an *EcoRI*-*HindIII*-digested pNEB133. One oligonucleotide had the following sequence: 5'-AATTCTGCACGGATCCACGC-GATCGCTCTGATCAGCAGATCTCACTG GTGACCTCT-TAATTAACAGCATGC-3'. The other oligonucleotide had the complementary sequence except it had the sequence AGCT as an overhang at its 5' end and it lacked a complementary to the last 4 nucleotides of the 5' end of the first

oligonucleotide. The resultant clone, pOI was digested with *BclI* and *EcoRI* and ligated to 2 complementary oligonucleotides that are identical to the sequence deleted in pOI except that they lack the 5 nucleotides GCACG, destroying the *BamHI* site and producing a clone, pOII, that has a deletion just upstream of the *EcoRI* site that makes *lacZα* in-frame. The *AflIII*-*BglII* fragment of pMF0 was replaced with the *AflIII*-*BglII* fragments of pOI and pOII producing two chlormphenicol-resistant clones that are identical except for a 5-bp insertion. The two clones were named pMF2 and pMF2-5; pMF2 produces white colonies in the proper medium and is the product of pOI, and pMF2-5 produces blue colonies in the proper medium and is the product of pOII. The smaller *EcoRI* fragment of pBBM was replaced with the small *EcoRI* fragments of pMF2 and pMF2-5 producing two ampicillin-resistant clones that are exactly identical except for a 5-bp insertion.

Testing Known Mutations

A 550-bp *Clal*-*SacI* fragment of four variants having A, G, T, or C at position -49 of mouse β-globin promoter, was cloned into *Clal*-*SacI* pMF100 and pMF200. The T and C variants were cloned into pMF100; the T, G, and A were cloned into pMF200. pMF200 clones were grown in a *dam*⁻ strain (SCS110) (Stratagene), and pMF100 clones were grown in a *dam*⁺ strain (DH5α) (GIBCO-BRL). The plasmid DNA was linearized with *AflIII* in 30-μl reactions. About equal amounts (estimated by gel electrophoresis of the linearized plasmids) were mixed, and the volume increased to 100 μl with TE buffer (10 mM Tris, 0.1 mM EDTA). The sample was then extracted with 100 μl of a 1:1 phenol/chloroform mixture, followed by extraction with 100 μl of chloroform. Five microliters of 0.5 M EDTA and 12.5 μl of 1 M NaOH were added, and the reaction left at room temperature for 15 min. The reaction was neutralized by the addition of 12.5 μl of 2 M Tris (pH 7.2, 125 μl of formamide was added, and the reaction incubated at 30°C for 1 hr. Chloroform extraction was performed twice, followed by ethanol precipitation. DNA was digested in a 20-μl reaction with *MboI* (5 units) for 1 hr, and *DpnI* (10–20 units) for 10 min at 37°C. The reaction was stopped by the addition of 1 μl of 0.5 mM EDTA. The intact heteroduplex plasmid was separated from the *MboI*- or *DpnI*-digested plasmid by agarose gel electrophoresis. The DNA was isolated from the gel slice and resuspended in 20 μl of water. Three microliters was used for a 20-μl recircularization reaction using T4 DNA ligase overnight at 16°C. Transformation of DH5α was performed with only 30 min recovery at 37°C after the heat shock. Transformation reactions were plated on LB agar plates with 50 μg/ml of carbinicillin, 64 μg/ml of X-gal, and 64 μg/ml of IPTG and incubated at 37°C overnight. *EcoRI*-*PvuII* fragments of the cystathionine β-synthase alleles were obtained from constructs of Kruger and Cox (1995). Adapters converting the *EcoRI* overhang to *NotI* overhangs were ligated on the fragments. These fragments were subsequently cloned in a *NotI*-*EcoRV*-digested pMF100. In addition, the wild-type control fragment was cloned in the pMF100. The MRD analysis was performed as in above. The control experiment was comparing the blue vector (pMF200) carrying the wild-type allele to the white vector (pMF100) carrying the same allele. The test experiment compared the blue vector carrying the wild-type allele to the white vector carrying another allele.

KpnI–*EcoRV* fragments of human agouti alleles were obtained from constructs of Wilson et al. (1995). These fragments were cloned in *KpnI*–*EcoRV*-digested MRD vectors. The wild-type allele was cloned in both pMF200 and pMF100. The mutant allele was cloned in pMF100 only. The MRD procedure was performed as described above. The control experiment compared the blue vector carrying the wild-type allele to the white vector carrying the wild-type allele. The test experiment compared the blue vector carrying the wild-type allele to the white vector carrying the mutant allele.

A 9-kb fragment of λ DNA was cloned in pMF1 (a relative vector to pMF2-5) producing the clone pB10. Partial digest with *BstBI* was performed. (Two *BstBI* sites were present: one in the chloramphenicol resistant gene, and the other ~5 kb in the insert.) Fill-in reaction was performed with Klenow, followed by recircularization with T4 DNA ligase and transformation into bacteria. Resultant clones were analyzed for the generation of a *NruI* site at the correct position; the correct clone was named pB10 + 2. A *NotI*–*KasI* fragment containing the insert of pB10 was cloned in a *NotI*–*KasI*-digested pMF2. The resulting clone pB210 was compared using MRD to pB10 and pB10 + 2. The MRD procedure was performed as described above.

Detecting Variation in a PCR Product

An *EcoRI* fragment of a cosmid was subcloned in pNEB193. Using sequence information of the clone, primers were designed to produce a PCR product of ~3 kb in size. The PCR reaction was performed with the enzyme mixture *rTth* + *Vent* (Perkin Elmer). The PCR product was extracted with an equal volume of a 1:1 mixture of phenol/chloroform and ethanol precipitated. Restriction digest with *HindIII* and *KpnI* was performed producing a fragment of ~2 kb in size. These fragments were cloned in *HindIII*–*KpnI*-digested vectors that are relatives to pMF100 and pMF200. Only 5% of the transformation mixture was plated, and the rest was grown directly in 5 ml of Luria broth (LB) containing 50 μ g/ml of carbinicillin. DNA was isolated from the transformation cultures and DNA of the pMF200 clones was transformed into SCS110 (*dam*[−] strain). Five percent of the transformation was plated and the rest grown directly in 5 ml of LB + 50 μ g/ml of carbinicillin. DNA isolated from these cultures was compared with DNA isolated from the pMF100 clones carrying fragments generated from the same source (i.e., the same somatic cell hybrid). The MRD procedure was performed as described above. About 50 blue colonies from this comparison were picked and grown in 5 ml of LB + 50 μ g/ml of carbinicillin. One microliter of a 1:1000 dilution of DNA isolated from these cultures was used to transform DH5 α (*dam*⁺ strain), and 2 μ l of the same dilution was used to transform SCS110 (*dam*[−] strain). White colonies from the first transformation and blue colonies from the second transformation were picked and grown in 5 ml of LB + 50 μ g/ml of carbinicillin. DNA isolated from these cultures was used to perform the MRD procedure. Subsequent to this second round, blue colonies were picked and grown, and their DNA was used to transform the two bacterial strains as described above. These DNA samples were used for comparison fragments generated from the same source and fragments generated from a different source (i.e., the other somatic cell hybrid).

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REFERENCES

- Barnes, W.M. 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proc. Natl. Acad. Sci.* **91**: 2216–2220.
- Brookes, A.J. et al. 1994. Cloning the shared components of complex DNA resources. *Hum. Mol. Genet.* **3**: 2011–2017.
- Carraway, M. and M.G. Marinus. 1993. Repair of heteroduplex DNA molecules with loops in *Escherichia coli*. *J. Bacteriol.* **175**: 3972–3980.
- Cheng, S., C. Fockler, W.M. Barnes, and R. Higuchi. 1994. Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci.* **91**: 5695–5699.
- Cotton, R.G.H., N.R. Roguies, and R.D. Campbell. 1988. Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its applications to the study of mutations. *Proc. Natl. Acad. Sci.* **85**: 4397–4401.
- Ferrin, L.J. and R.D. Camerini-Otero. 1991. Selective cleavage of human DNA: recA-assisted restriction endonuclease (RARE) cleavage. *Science* **254**: 1494–1497.
- Grompe, M. 1993. The rapid detection of unknown mutations in nucleic acids. *Nature Genet.* **5**: 111–117.
- Kruger, W.D. and D.R. Cox. 1995. A yeast assay for functional detection of mutations in the human cystathionine β -synthase gene. *Hum. Mol. Genet.* **4**: 1155–1161.
- Maschal, R.D., J. Koontz, and J. Sklar. 1995. Detection of mutations by cleavage of DNA heteroduplexes with a bacteriophage resolvase. *Nature Genet.* **9**: 177–183.
- Modrich, P. 1991. Mismatch repair. *Annu. Rev. Genet.* **25**: 229–248.
- Myers, R.M., L.S. Lerman, and T. Maniatis. 1985a. A general method for saturation mutagenesis of cloned DNA fragments. *Science* **229**: 242–247.
- Myers, R.M., S.G. Fischer, L.S. Lerman, and T. Maniatis.

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1985b. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acid Res.* **13**: 3131–3145.

Myers, R.M., N. Lumelsky, L.S. Lerman, and T. Maniatis. 1985c. Detection of single base substitutions in total genomic DNA. *Nature* **313**: 495–498.

Myers, R.M., Z. Larin, and T. Maniatis. 1985d. Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. *Science* **230**: 1242–1246.

Nelson, S.F., J.H. McCusker, M.A. Sander, Y. Kee, P. Modrich, and P.O. Brown. 1993. Genomic mismatch scanning: A new approach to genetic linkage mapping. *Nature Genet.* **4**: 11–18.

Nielson, K., J. Braman, and K. Kretz. 1995. High fidelity long PCR amplifications with pfu DNA polymerase. *Strategies* **8**: 26–33.

Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya. 1989a. Detection of polymorphisms in human DNA by gel electrophoresis as single-strand conformational polymorphism. *Proc. Natl. Acad. Sci.* **86**: 2766–2770.

Orita, M., Y. Suzuki, T. Sekiya, and K. Hayashi. 1989b. Rapid and sensitive detection of point mutation and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5**: 874–879.

Parker, B.O. and M.G. Marinus. 1992. Repair of DNA heteroduplexes containing small heterologous sequences in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **89**: 1730–1734.

Perry, D.J. and R.W. Carrell. 1992. A rapid and simple approach to the detection of DNA mutations in thromboembolic diseases. *J. Clin. Pathol.* **45**: 158–160.

Sheffield, V.C., D.R. Cox, L.S. Lerman, and R.M. Myers. 1989. Attachment of a 40-base-pair G + C rich sequence (G + C clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl. Acad. Sci.* **86**: 232–236.

Short, J.M., J.M. Fernandez, J.A. Sorge, and W. Huse. 1988. λ ZAP: A bacteriophage λ expression vector with *in vivo* excision properties. *Nucleic Acids Res.* **16**: 7583–7600.

Wagner, R. and M. Meselson. 1976. Repair tracts in mismatched DNA heteroduplexes. *Proc. Natl. Acad. Sci.* **73**: 4135–4139.

White, M.B., M. Carvalho, D. Derse, S.J. O'Brien, and M. Dean. 1992. Detecting single base pair substitutions as heteroduplex polymorphisms. *Genomics* **5**: 301–306.

Wilson, B.D., M.M. Ollmann, L. Kang, M. Stoffel, G.I. Bell, and G.S. Barsh. 1995. Structure and function of

ASP, the human homologue of the mouse agouti gene. *Hum. Mol. Genet.* **4**: 223–230.

Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequence of the M13mp18 and puc19 vectors. *Gene* **33**: 103–119.

Youil, R., B.W. Kemper, and R.G.H. Cotton. 1995. Screening for mutations by enzyme mismatch cleavage with T4 endonuclease VII. *Proc. Natl. Acad. Sci.* **92**: 87–91.

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