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Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: Evidence for solvent-induced bends in DNA heteroduplexes

(detection of mutations/DNA conformation/DNA bends)

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ABSTRACT **Several techniques have recently been developed to detect single-base mismatches in DNA heteroduplexes that contain one strand of wild-type and one strand of mutated DNA. Here we tested the hypothesis that an appropriate system of mildly denaturing solvents can amplify the tendency of single-base mismatches to produce conformational changes, such as bends in the double helix, and thereby increase the differential migration of DNA heteroduplexes and homoduplexes during gel electrophoresis. The best separations of heteroduplexes and homoduplexes were obtained with a standard 6% polyacrylamide gel polymerized in 10% ethylene glycol/15% formamide/Tris-taurine buffer. As predicted by the hypothesis of solvent-induced bends, when the concentration of either ethylene glycol or formamide was increased, the differential migration decreased. Also, single-base mismatches within 50 bp of one end of a heteroduplex did not produce differential migration. Sixty of 68 single-base mismatches in a series of PCR products were detected in some 59 different sequence contexts. The eight mismatches not detected were either within 50 bp of the nearest end of the PCR product or in isolated high-melting-temperature domains. Therefore, it was possible to predict in advance the end regions and sequence contexts in which mismatches may be difficult to detect. The procedure can be applied to any PCR products of 200–800 bp and requires no special equipment or preparation of samples.**

A number of attempts have been made to develop rapid techniques for the detection of single-base changes in DNA sequences (see ref. 1). Several of the techniques were based on the conformational changes produced by single-base mismatches in heteroduplexes that contain one strand of wild-type and one strand of mutated DNA (1–9). Although single-base mismatches produce measurable decreases in the thermal melting temperature of double-stranded DNA (10–12), the conformational changes that are produced below the melting temperature are relatively subtle (13, 14). The reactivity of some mismatched bases in heteroduplexes is altered so that several nucleases and chemical reagents can be used to distinguish DNA homoduplexes from some heteroduplexes (1–6, 15). In addition, there have been attempts to explore the possibility that single-base mismatches in double-stranded DNA can be detected by differential migration of heteroduplexes and homoduplexes in electrophoretic gels. For example, Bhattacharyya and Lilley (16) were unable to detect any differential migration by PAGE of 12 different single-base mismatches. Recently, however, White *et al.* (17) reported detection of eight of nine single-base mismatches in DNA heteroduplexes in which the mismatches were located in a 29-bp hairpin loop. Also, detection of some single-base mismatches in DNA heteroduplexes was recently observed by electrophoresis in a commercial proprietary gel (7–9).

During electrophoresis under the conditions commonly used to separate the double-stranded DNA by size, the external electrical field drags the chains through the fixed obstacles presented by the gel network (18–21). The rate of migration depends on both the contour length of the DNA and the end-to-end vector of the polymer in the direction of the electric field. The available analyses of the process, however, appear ". . . to be only at the beginning" (19) and are ". . . based on simple qualitative ideas that are evident simplifications . . ." (21). Also, relatively little is known about the effects of single-base mismatches on the conformation of double-stranded DNA below its melting temperature and under different solvent conditions. X-ray diffraction analysis of oligonucleotides indicated that mismatched bases remain within the helix with little distortion of the helix itself (13, 14), but the x-ray data may be biased in favor of undistorted structures because molecules with distorted helices probably do not crystallize readily. Also, only a limited number of solvent conditions can be used for crystallization. In the special case of oligonucleotides in which one strand contains an extra base not found in the complementary strand, NMR (22, 23) and x-ray (24) analyses showed that under some conditions the extra base remained within the helix and under others it was rotated out of the helix. When the extra base remained within the double helix, a bend of $\approx 20°$ was detected by NMR (22). Moreover, the bends created by an extra base in one strand were shown to retard electrophoretic mobility when the conformational effects of the bends were amplified by preparing concatamers of the appropriate length (25, 26).

Here we reasoned that either a "bubble" (Fig. 1) with both mismatched bases retained within the double helix or a "bulge" with both bases rotated out of the helix is unlikely to change the conformation of the molecule sufficiently to alter its electrophoretic mobility. In contrast, asymmetrical displacement of the two bases, so that one base is retained in the double helix and the other base is rotated out, is likely to produce a bend in the helix (Fig. 1) and thereby a large change both in conformation and electrophoretic mobility. Previous studies with solvents such as ethylene glycol and formamide demonstrated that these solvents produce subtle conformational changes in DNA (28–30) in a continuous process that begins well below the concentrations required for complete denaturation at moderate temperatures (30). Therefore, we tested the hypothesis that an appropriate system of mildly

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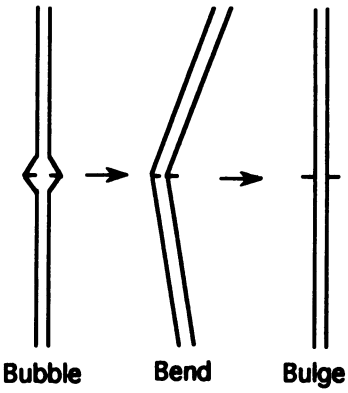

FIG. 1. Hypothetical scheme for how mildly denaturing conditions may alter DNA conformation. "Bubble" and "bulge" are defined as suggested by Bhattacharyya and Lilley (27). As discussed in text, the hypothesis explored here was that mildly denaturing solvent conditions promote rotation of one mismatched base out of the double helix to create a bend and that increased concentrations of the denaturing solvents promote rotation of both mismatched bases out of the helix to produce a bulge.

denaturing solvents can amplify the tendency of single-base mismatches to produce conformational changes such as bends in the double helix (Fig. 1) and thereby increase the differential migration of DNA heteroduplexes and homoduplexes during gel electrophoresis.

METHODS

PCR products of 485 bp each were generated from a set of four M13 phage DNA templates that were engineered to contain a different base at a single site (5). To prepare samples containing an excess of a single heteroduplex species, the same four M13 templates were used in an asymmetric PCR with a 50:1 molar ratio of the primers to generate single-stranded DNAs. The individual PCR products were then mixed in pairs of opposite sense and incubated at 68°C for 30 min to give samples that largely consisted of a single species of heteroduplex.

PCR products containing previously defined single-base differences were provided by several investigators in our own research group: Loretta Spotila and Jiapiao Zhuang, the type I procollagen COL1A1 (31); Charlene Williams, COL2A1 (32); and Helena Kuivaniemi and Gerard Tromp, COL3A1 (33) and elastin (34). Angela Christiano and Jouni Uitto of the Department of Dermatology, Jefferson Medical College, provided PCR products from the *COL7A1* gene (35). Steve Sommer (Mayo Clinic, Rochester, MN) provided PCR products from the factor IX-encoding gene (36).

Before electrophoresis, EDTA was added to each PCR product in a final concentration of 10 mM. Twenty microliters of each sample was heated to 98°C for 5 min followed by incubation at 68°C for 1 hr to generate heteroduplexes. Four microliters of the sample was mixed with 4 μl of 20% (vol/vol) ethylene glycol/30% (wt/vol) formamide containing 0.025% each of xylene cyanol FF and bromphenol blue. A standard DNA-sequencing gel apparatus was used with 37.5 × 45 cm glass plates and a 36-sample comb. A 1-mm-thick gel was prepared with 6% polyacrylamide and a 29:1 ratio of acrylamide to bisacrylamide, 10% (vol/vol) ethylene glycol (Sigma), and 15% (wt/vol) formamide (GIBCO/BRL) in 0.5× TTE buffer (1× TTE is 89 mM Tris/15 mM taurine buffer/0.5 mM EDTA, pH 9.0; United States Biochemical). The electrode buffer was 0.25× TTE in the upper chamber and 1× TTE in the lower chamber. The gel was preelectrophoresed at 45 W for 15 min, and the samples were electrophoresed at 45 W for 4 hr at room temperature. Monitoring with an embedded temperature-sensitive liquid-crystal strip (AT Biochem, Malvern, PA) indicated that the temperature was 35°C at the top of the gel and 38°C at the bottom. After electrophoresis, one glass plate was removed, and the gel on the second glass plate was stained with ethidium bromide at 1 μg/ml for 6 min followed by destaining for 12 min. The bands were visualized with a hand-held UV torch. The relevant section of the gel was cut, transferred with a piece of blotting paper to a transilluminator, and released from the paper by wetting it with water. The gel was then photographed under standard conditions.

RESULTS

Detection of All Mismatches in a Single Sequence Context. Initially, we tested several solvents known to alter the conformation of DNA (28–30) and then elected to use ethylene glycol and formamide primarily because they are compatible with PAGE. We also elected to use a Tris-taurine buffer instead of the more commonly used Tris-borate buffer because a commercial concern (United States Biochemical) recently directed attention to the adverse reactions of borate with alcohols such as glycerol. Preliminary experiments suggested that the best separations of heteroduplexes and homoduplexes were obtained with a 6% polyacrylamide gel polymerized in 10% ethylene glycol, 15% formamide, and 0.5× TTE buffer.

To test the system, four genetically engineered M13 phage (5) were used as DNA templates to prepare four PCR products with a different base at the same site in a sequence of -TCCNGGG- (nt 6328). The PCR products were mixed in pairs to generate six samples containing homoduplexes and heteroduplexes with all 12 possible single-base mismatches—i.e., the four possible bases in one strand mismatched with each of three noncomplementary bases in the opposite strand. The homoduplexes all comigrated, but at least one more slowly migrating band was observed in each of the paired samples containing heteroduplexes (Fig. 2). Therefore, all 12 possible single-base mismatches were detected. Control experiments demonstrated that the more slowly migrating bands were heteroduplexes and not single-stranded DNA because denatured single-stranded DNA from the same PCR products migrated at one-fourth the rate of the bands in Fig. 2 (data not shown).

To identify the specific heteroduplex bands, asymmetric PCR reactions were carried out with the same templates to generate an excess of one strand. Each strand was then mixed with a sample containing an excess of the opposite strand from a second M13 template. As a result, samples with single heteroduplexes with defined mismatches were generated. The heteroduplexes were retarded to different degrees relative to the homoduplexes (Fig. 3). There was, however, no obvious relationship between the size or other features of the mismatched bases and the relative retardation of the hetero-

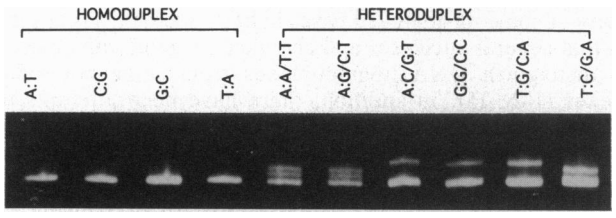

FIG. 2. Differential migration of DNA heteroduplexes and homoduplexes. Samples were PCR products prepared from four M13 templates that contained a different base at a single site (nt 6238) in the sequence context -TCCNGGG- (5). Pairs of PCR products were mixed and used to generate samples that contained equimolar amounts of two species of homoduplexes and two species of heteroduplexes (5).

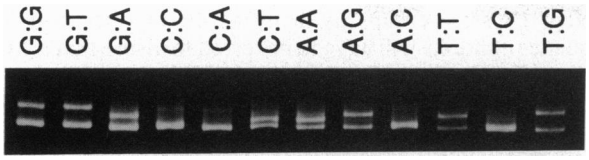

FIG. 3. Differential migration of defined DNA heteroduplexes. The samples were paired mixtures of asymmetric PCR products of opposite sense prepared from M13 templates, and therefore each contained an excess of a single heteroduplex.

duplexes. For example, the relative retardation of heteroduplexes containing identical pairs of purine and pyrimidine bases was G·G > T·T > A·A > C·C. Four heteroduplexes comigrated with the homoduplexes: C·C, C·A, A·C, and T·C. However, the comigration of these four heteroduplexes with homoduplexes did not limit detection of single-base differences between two DNA templates because the complementary heteroduplexes were detected in symmetrical PCR products (last four lanes in Fig. 2). Of special note was the observation that the heteroduplex containing a C·T mismatch with the cytosine in the sense strand and the thymine in the antisense strand migrated more slowly than the homoduplex, whereas the heteroduplex containing the T·C mismatch with the thymine in the sense strand comigrated with the homoduplex.

Two Tests of the Hypothesis of Solvent-Induced Bends. If the hypothesis of solvent-induced bends is correct (Fig. 1), an increase in concentration of the denaturing solvents should rotate both bases out of the double helix to create a "bubble" and abolish the differential migration of heteroduplexes and homoduplexes. Fig. 4 shows that the slower migration of a heteroduplex relative to a homoduplex decreased when the ethylene glycol content of the gel was increased from 10% to 20%, and it disappeared when the ethylene glycol concentration was increased to 30%. The loss of resolution was not explained by denaturation of the heteroduplexes because none of the DNA comigrated with single-stranded DNA (data not shown). The resolution of heteroduplexes from homoduplexes also decreased without denaturation of the heteroduplexes when the formamide concentration was increased from 15% to 25% and largely disappeared when it was increased to 30% (data not shown).

If the hypothesis of solvent-induced bends is correct (Fig. 1), a mismatch near one end of a DNA fragment should have much less effect on the differential migration than a mismatch in the interior of the same fragment. A single-base mismatch in a PCR product from a COL1A1 cDNA was not detected when the mismatch was 51 bp from an end of the 503-bp PCR product (Table 1). The same mismatch was detected under the same conditions when the same cDNA template was used to generate a 630-bp PCR product in which the mismatch was 81 bp from the nearest end.

Detection of Previously Defined Single-Base Differences in PCR Products. To test the electrophoretic conditions further, analyses were carried out on 58 additional PCR products that contained previously identified single-batch differences (Table 1). The PCR products ranged in size from 200 to 800 bp. The single-base mismatches were detected in all but seven of the PCR products. Analysis of the locations of the mismatches tested in Table 1 indicated that all 59 mismatches that were correctly detected were >50 bp from the nearest end of the PCR product. In contrast, the four mismatches in the factor IX-encoding gene that were not detected were all located within 50 bp of one end of the PCR products.

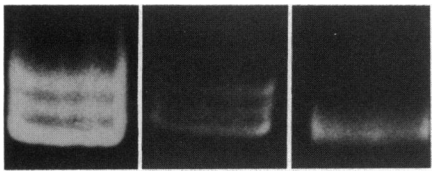

FIG. 4. Effect of increased concentrations of ethylene glycol. Left lane, 10% ethylene glycol; middle lane, 20% ethylene glycol; right lane, 30% ethylene glycol.

Table 1. Detection of previously defined single-base differences in PCR products

Gene	Single-base changes	Result: Correct detection	Result: False negative: End sequences*	Result: False negative: High-melting domain†
M13 phage	10	10	0	0
COL2A1	7	7	0	0
COL7A1	8	8	0	0
Elastin	1	1	0	0
Factor IX	35	31	4	0
COL1A1	2	1‡	1‡	0
COL3A1	5	2	0	3
Total	68	60	5	3

*Mismatches within 50 bp of the end of a PCR product.
†Mismatches in an isolated high-melting-temperature domain, as defined in Fig. 5 and Table 2.
‡Same mismatch was detected when present 81 bp from the end of PCR product but was not detected in a second PCR that placed the same mismatch within 51 bp of one end.

Predictability of Detection Based on Low- and High-Melting-Temperature Domains. As indicated in Table 1, three mismatches in PCR products of the *COL3A1* gene were not detected, even though the mismatches were located >50 bp from the nearest end of the fragment. Collagen genes are characterized by repetitive G+C-rich coding sequences (37). Therefore, we explored the possibility that the three mismatches in PCR products from *COL3A1* were located in unusually G+C-rich and stable sequences. As indicated in Fig. 5 and Table 2, the three mismatches in *COL3A1* that were not detected were all in isolated high-melting-temperature domains, as defined by a recent computer program for assessing the helical stability of DNA sequences (11). In contrast, the single-base mismatches that were easily detected were in domains with temperatures for 95% helicity that were no more than 4°C higher than the average value for the whole fragment (Fig. 5 and Table 2).

DISCUSSION

Further experiments will obviously be required to substantiate fully the hypothesis that DNA homoduplexes and heteroduplexes can be separated electrophoretically on the basis of solvent-induced bends in the double helix (Fig. 1). The hypothesis was strongly supported, however, by the observation that the differential migration was markedly reduced by increasing the concentration of either of the two denaturing solvents above an optimal level. Also, as expected on the basis of the hypothesis, a single-base mismatch that was 51 bp from one end of a DNA fragment did not produce differential migration, whereas the same mismatch was readily detected in the second PCR product from the same template that placed the mismatch 81 bp from the nearest end.

A large number of competing techniques are now available to screen or scan DNA fragments for single-base mismatches (for review, see ref. 1). One critical question about each of the techniques is whether it will detect all mismatches in all sequence contexts. The sequence context of a base mismatch clearly has an important effect on ease of detection by any physical, chemical, or enzymic method. As illustrated here,

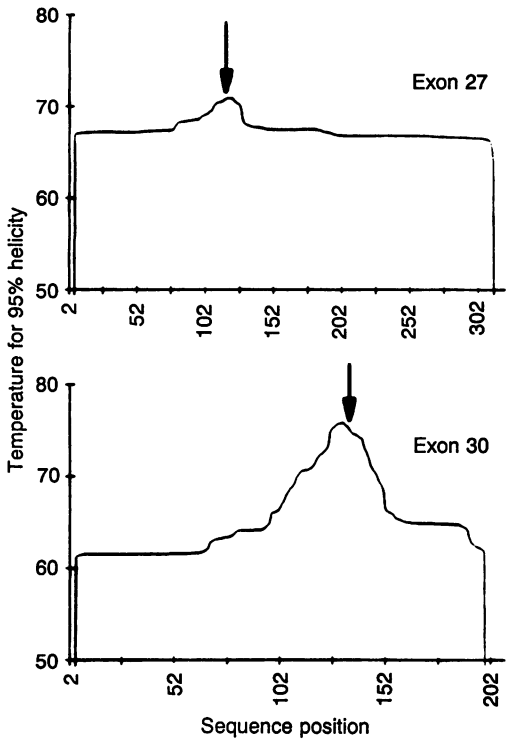

FIG. 5. Predicted melting profiles at 95% helicity for two PCR products from the *COL3A1* gene. Arrows indicate sites of single-base mismatches. The mismatch in exon 27 was detected. The mismatch in exon 30 was not detected.

a heteroduplex containing a C·T mismatch was detected by differential migration of the heteroduplex when the cytosine was in the sense strand but was not detected when the cytosine was in the antisense strand. These and other observations suggest as many as 5 nt flanking a base mismatch may influence the conformational change induced by the mismatch. Hence, perhaps as many as 4^{10} or over a million sequence contexts will have to be tested to insure that a given technique can detect all possible mismatches. Under the conditions developed here, 60 of 68 single-base mismatches were detected in some 59 different sequence contexts. Moreover, the results made it possible to predict in advance sequences in which mismatches were difficult to detect either because they were within 50 bp of the end of the DNA fragment or because they were in a high-melting-temperature domain readily defined by analysis of the sequence with a computer program (11). In our experience, isolated high-melting-temperature domains are relatively rare in PCR products. For example, we found only one or two such domains of <150 bp each in analysis of 15 PCR products spanning over 10,000 bp of cDNAs for the human *COL1A1* and *COL2A1* genes (ref. 31 and L. Spotila, A. Colige, L. Sereda, C. D. Constantinou, and D.J.P., unpublished work), and only one or two such domains of <150 bp each in 26 PCR products spanning >25,000 bp of genomic DNA for the human *COL2A1* gene (32). Also, it is very likely that mismatches in isolated high-melting-temperature domains will show differential migration of heteroduplexes when the PCR products are prepared with G·C clamps at one end, as now recommended for detection of single-base mismatches by denaturing-gradient gel electrophoresis (12) or by using different solvent conditions for the electrophoretic gel.

Of special note is that the procedure developed here can be applied to any PCR products of 200–800 bp and requires no special equipment or preparation of samples except for pouring a standard polyacrylamide gel in a modified solvent-buffer system.

Table 2. Detection of previously defined single-base mismatches in low- and high-melting temperature domains

Gene	Location of mismatch*	Temperature for 95% helicity, °C	
		Average	Mismatch site†
COL3A1	Exon 27	67.4	+3.4
	Exon 28	64.5	+7.7‡
	Exon 30	65.4	+10.0‡
	Exon 31	68.5	+6.7‡
COL2A1	Exon 5B	71.7	+6.5§
	Intron 9 (2)	70.4	+3.3
	Exon 11	73.0	+0.99
	Exon 32 (2)	74.5	+1.28
	Exon 48 (2)	75.3	+1.47
COL7A1	Exon 14	74.5	+0.10
	Exon 18	76.2	+0.51
	Exon 21	75.7	+2.92
	Exon 113	73.6	+2.08
	Exon 118	76.0	+1.16
M13	nt 6328 (6)	74.2	+1.14
Factor IX	Exon 8 (22)	68.5	+1.25¶

*Location defined by exon or intron. Values in parentheses indicate number of different mismatches in same exon when more than one was assayed.
†Temperature at site of mismatch less average temperature for entire PCR product.
‡Mismatches were not detected.
§Mismatch was poorly detected.
¶Value ± 0.60 SD.

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