Unexpected behavior of H2K^b mutant DNAs in denaturing gradient gel electrophoresis

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ABSTRACT

Denaturing gradient gel electrophoresis (DGGE) is based upon the different melting behaviors of DNA molecules in a chemical denaturant gradient according to their sequences. This technique has recently become a widespread tool to detect mutations. The introduction of a GC-clamp enables the detection of most single base differences between two DNA molecules. As a test system we have applied the polymerase chain reaction (PCR) in combination with DGGE to detect a number of mutations in the mouse H2K^b DNA sequence. A wide variety of spontaneous *in vivo* mutations of this haplotype have been reported in the C57BL/6J mouse strain and are clustered in the alpha 1 and alpha 2 domains. The combination of PCR and DGGE revealed almost all base changes present in the H2K^b mutants used. However, most of the PCR products of these mutants showed melting behavior which is not easily predicted. We suggest that in addition to current simple theory, which considers that the migration of a DNA molecule in a denaturing gradient depends primarily on its initial melting behavior, additional factors such as secondary structure in partially melted molecules may play a role and can be used to detect mutations.

INTRODUCTION

DGGE is an efficient and discriminating method to separate DNA double helices according to their melting properties, which are a function of base sequence and composition (1). When the double helix reaches the point in a denaturing gradient where the least stable domain starts to melt, the mobility of the molecule decreases dramatically. This 'bubbling' is highly dependent on the sequence; thus, single base changes affect the onset of this phenomenon and can be detected by the different mobilities of DNA molecules in a gradient (2). Since the original description (3) of this technique two extensions have been described which lead to an increase in its analytic power. First, a package of computer programs (MELT, TRAVEL, SQTHX)(4) can predict parts of DNA molecules with low or high melting temperatures (melting domains) based on sequence information, and is able to estimate mobility in a given gel to help in setting up

experimental conditions. Second, attaching a GC-rich segment (GC-clamp) to the original sequence makes visible single base changes in higher-temperature melting domains which otherwise might be disguised (5–7). At present, the combination of a GC-clamp with DGGE seems to be the most reliable of available rapid screening methods to search for mutations (8).

Major Histocompatibility Complex (MHC) class 1 genes encode intrinsic membrane proteins consisting of a leader sequence, three extracellular domains, a transmembrane segment and three cytoplasmic domains (9,10). In the mouse, this gene family comprises three loci (H2K, H2D and H2L), all of which are highly polymorphic. Many spontaneous *in vivo* mutations in the H2K^b gene of the C57BL/6J mouse strain have been detected (11,12) and sequenced. These mutations are clustered predominantly in the first two extracellular domains, alpha 1 and alpha 2, presumably because of the immunological consequences of such changes in terms of T cell recognition (13). To test the ability of DGGE to detect known mutations, we examined one previously unsequenced H2K^b mutant (bm2)(11) and nine already characterized H2 mutants. Six of the tested mutants were known to contain alpha 2 mutations (14–16) (see Table 1). Three alpha 1 mutations (bm3, bm8 and bm11)(12,14) in H2K^b and the H2D^{bm14} mutation (17) served as controls.

An additional consideration prompting this study is the issue of utilizing PCR in conjunction with DGGE. Several authors have questioned whether DNA polymerase errors during PCR amplification would be mistaken for mutations present in the starting DNA sample, and which DNA polymerase should be used to minimize this problem (18,19). We therefore performed DGGE analysis on known H2K^b mutant DNAs that had been amplified with PCR. The results obtained show that standard PCR in conjunction with DGGE can indeed be used to efficiently detect mutations in specific DNA sequences from even complex genomes.

MATERIALS AND METHODS

DNA

Genomic DNAs of the C57BL/6J strain and homozygous H2K mutants bm4, bm2 and bm14 (from The Jackson Laboratory), bm1, bm3, bm5, bm6, bm9, bm8, bm10 and bm11 (kindly

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provided by Dr. R. Melvold, Chicago) were extracted from frozen livers by standard procedures (20), quantitated by UV absorbance, aliquoted and stored at -20°C .

PCR

Primer oligonucleotides were synthesized by Oligos Etc. (Ridgefield, CT) and used without further purification. The upstream primer was provided with a 30-base GC-clamp at its 5'-end. Base sequences were as follows: upstream primer (50-mer) 5'-GCCCCCGCCCCGCCCCGCCCCGCCCCGCCCCGAGGCTCTCACACTATTCAGG-3'; downstream primer (20-mer) 5'-TATGGACTTCTTGCCCTTGC-3'. The PCR (21) conditions were those described in the AmpliKit[™] (Perkin Elmer). Briefly, each reaction (total volume 100 μl) contained 100 ng of genomic DNA, 0.4 μmol of each primer and 1.25 mM each dNTP (Perkin Elmer) in RX buffer (Perkin Elmer) leading to a final Mg^{++} concentration of 1.5 mM. Samples were

overlaid with 100 μl mineral oil (Sigma) and boiled for 5 minutes. Four units of Taq Polymerase (Perkin Elmer) were added to each sample. Forty cycles of amplification (95°C for 2'; 60°C for 1' and 72°C for 2') were carried out in a thermal cycler (Perkin Elmer). The amplification of genomic DNA was designed to yield a fragment of 289 base pairs (30 bases of GC-clamp plus genomic DNA from positions 885–1144, according to the numbering of ref. 14; Genbank accession numbers K00015 and V00746) which covers most of the alpha 2 domain. After PCR, 8 μl of the reaction volume were mixed with gel loading buffer (15% Ficoll 400, 0.2% bromophenol blue, 0.2% xylene cyanol and 25 mM EDTA)(22) and electrophoresed on a 4% agarose gel (1% agarose, 3% NuSieve agarose) to visualize and check product size. The gel was stained with ethidium bromide, UV-illuminated and photographed.

DGGE

We adopted DGGE conditions from ref. 1, using 7% acrylamide (acrylamide:bisacrylamide ratio 37.5:1) in TAE and a standard gradient of 40% to 80% denaturant (100% = 7M urea and 40% formamide). Eight μl of PCR products were mixed with 1 μl of loading buffer. Electrophoresis was performed at a constant voltage of 150 V (10 V/cm) and a constant temperature of 60°C . Gels were stained with ethidium bromide, UV illuminated and photographed. Experiments were repeated eight times with identical results. Essentially the entire DGGE gel is shown in all figures.

Computations

Computational calculations of melting behavior were carried out on an IBM PS/2 microcomputer using the program package MELT, TRAVEL and SQHTX (kindly provided by Dr. Leonard Lerman, MIT, Cambridge) according to the instructions given in ref. 4 and provided with the programs.

Table 1: Mutations in the H-2K^b alpha 2 domain

Mutants	Base Changes	Position in gene ²	Position in PCR product ³
bm5	A to T	962	108
bm6 and bm9	A to T	962	108
	T to C	976	122
bm4 ⁴	G to A	1099	245
	A to C	1102	248
	G to C	1108	254
bm10 ⁴	A to G	1102	248
	G to A	1108	254
	G to C	1115	261
bm1	A to C	1069	215
	A to T	1070	216
	A to T	1078	224
	G to A	1079	225
	A to T	1080	226

² The base positions in this column follow that given in ref. 14.
³ The base positions in this column correspond to those in Figure 1.
⁴ A to G (1132) and A to T (1136) base changes within the primer region are essentially not represented in the PCR product.

Figure 1A (upper). Melting map of the H-2K^b alpha 2 domain PCR products of the C57BL/6J compared to those of the bm1, bm4, bm5, bm6 and bm10 mutants as generated by the MELT program. The graph displays the temperature for each base of the fragments above which more than 50% of the DNA is predicted to be melted. All fragments express 3 melting domains (pieces of DNA with similar melting temperature): the first 30 bases constituting the GC-clamp, a distinct melting domain at 79°C to position 180 and a second domain with a melting temperature of 77°C . **Figure 1B** (lower). Calculated mobility plots of these fragments as assessed by the MTRV program. This program estimates the relative mobility of a given fragment at a particular temperature (equivalent to a denaturing percentage) based on the calculations of the melting maps. Once a fragment starts melting its mobility decreases sharply. These differences in mobility can be made visible in DGGE.

RESULTS

In order to derive initial experimental conditions for these tests, we calculated melting maps for the wildtype C57BL/6J and mutant bm1, bm4, bm5, bm6 and bm10 H2K^b alpha 2 domains, with the addition of a 30-base GC-clamp to all sequences (Figure 1A). Generally similar patterns were predicted for all mutant DNAs and the wildtype, namely, two melting domains near 78°C and the GC-clamp region which melts around 90°C. The base changes of the mutants with regard to the wildtype (Table 1) caused only subtle differences between the predicted melting profiles. Mutant bm6 showed a slightly higher stability in the first melting domain (positions 30 to 180) and mutants bm10 and bm4 were predicted to have higher and lower melting profiles, respectively, compared to C57BL/6J in the second melting domain (positions 180 to 270). In contrast, mutants bm5 and bm1 did not differ from the wildtype. Figure 1B shows that the differences in the melting maps were reflected in the computational simulation of mobility as a function of melting temperature. At a lower temperature level, bm4 was calculated to have a lower mobility in the gel (i.e. to slow down sooner in relation to the others). At higher temperatures, bm6 and bm10 were estimated to have a higher mobility than C57BL/6J. As in their melting maps, bm1 and bm5 showed the same curve as the wildtype. Since program SQHTX calculates the migration difference between a homoduplex and a heteroduplex different for previously chosen base positions in a sequence, no additional information could be obtained using this program since only

Figure 2. Electrophoresis of identical aliquots from the same PCR amplifications of the H2K^b alpha 2 domain on standard and denaturing gradient gel electrophoresis. Numbers of the lanes refer to the 'bm number' (eg. lane 2 is bm2), lane B refers to C57BL/6J and the unlabeled lane is the BRL 1 Kb DNA molecular weight ladder. **Figure 2A** (upper) Agarose gel. All PCR products exhibit the same size of approximately 290 base pairs. Electrophoresis was performed for 1 hour at 90 volts. **Figure 2B** (lower) DGGE gel (40% to 80% denaturant, run 4 hours at 150 volts). All known mutations in the H2K^b alpha 2 domain were revealed by different positions of the PCR products in the gel, with the exception of bm5 (lane 5) which stayed at the same position as the wildtype (B). Surprisingly, all mutant PCR products showed an upward shift.

homoduplex DNA fragments (PCR amplification products from homogeneous DNA samples that were not melted and reannealed with mutant nucleic acids to produce heteroduplexes) were utilized in these experiments.

We then performed standard electrophoresis and DGGE with the products resulting from PCR amplification of the H2K^b alpha 2 domain from genomic DNA of the wildtype and various mutants. The agarose gel in Figure 2A indicates that PCR successfully produced 12 DNA fragments of equivalent size (approximately 290 base pairs). Figure 2B shows the migration and melting behavior of the PCR products in a 40% to 80% denaturing gradient gel. As expected, the H2K^b alpha 2 PCR products of alpha 1 mutants bm3, bm8, bm11 and the H2D mutant bm14 migrated to the same position as the wildtype. The single bands of these products in both standard electrophoresis and DGGE confirm the uniformity of PCR amplification and DGGE analysis of the H2K^b alpha 2 target sequences from mouse genomic DNA. The H2K^{bm2} mutant that had previously been characterized immunologically but not molecularly showed different migration behavior than the wildtype in the DGGE gel, allowing the conclusion that this mutation is located in the alpha 2 domain of the H2K^b gene. Mutants bm6 and bm9, which possess an identical mutation (23), both had a sharp and clearly distinct additional band migrating several millimeters above the wildtype level in the gel. The PCR product from mutant bm5, which has a melting temperature-conserving A to T transition (causing an Tyr to Phe change at amino acid position 116) (24) showed the same mobility as that from C57BL/6J. The bm4 and bm10 mutations shared similar behavior, with neither forming a discrete band in the DGGE gel. Mutant bm1 migrated with its major portion at a position very high in the gel, and a minor part forming a discrete doublet near the wildtype level.

These actual DGGE migration behaviors of most of the mutant PCR products did not correspond to the theoretical calculations. To test whether the experimental results were dependent on non-equilibrium transitional phenomena, we performed DGGE on bm1, bm5, bm6 and the wildtype PCR products using different electrophoresis times (Figure 3). This experiment revealed that some of the unpredicted upward shifts (eg. mutant bm6) could best be seen at an optimal run time of 4 hours. With shorter or

Figure 3. Electrophoresis of the wildtype C57BL/6J and the H2K^b mutants bm1, bm5 and bm6 PCR amplification products in 40% to 80% DGGE at various run times. The numbers above the bars indicate run times from 3 to 5 hours, numbers 5, 6, 1 below the bars refer to specific mutants (B is the wildtype).

Figure 4. Comparison of the experimental migration speed of PCR amplified DNA fragments. In a 40% to 80% denaturant gradient, the wildtype (open squares) and the major upper bm1 PCR products (closed squares) followed similar patterns of initially rapid migration, then slowing down and finally speeding up again. The wildtype band was found to be an average of two centimeters further down in the gel than the major bm1 band at all times. With a 60% to 100% denaturant gradient, both bands migrated at the same linear pace at all run times (crosses).

longer run times, the unique upper bm6 mutant band seemed either to be not yet formed or to loose resolution and disappear; in either of these cases, no obvious shift with respect to the wildtype product was visible. In the case of the bm1 mutation, the unique major band was formed very rapidly at a high position in the gel. With longer run times this band continued to migrate slowly and then later increased its migration speed. (Figure 4 displays the pace of migration at different denaturing gradient levels.) As predicted by the computational simulation, the bm5 mutant PCR product moved at exactly the same position as that from C57BL/6J at all run times. In a similar experiment performed with a higher denaturing gradient (60% to 100%), these fragments continued to migrate but did not form bands clearly distinguishable from wildtype after run times from 2 to 6 hours. In this higher gradient, the migration speeds of all 4 tested fragments, including the bm1 mutant, were linear with time (Figure 4). Finally, to test whether the migration patterns shown in Figures 2 and 3 were concentration-dependent, ten-fold serially diluted samples of wildtype and mutants bm1, bm5 and bm6 (from the amounts shown in Figures 2 and 3 down to .001 of these amounts) were electrophoresed on similar DGGE gels, electroblotted onto nylon membrane, hybridized with a radiolabelled oligonucleotide probe internal to the amplified portion of the H2K^b alpha 2 domain and autoradiographically exposed. The migration patterns seen for these mutants in this experiment were identical to those shown in Figures 2 and 3 and were not affected by the amount of sample loaded (data not shown).

DISCUSSION

The actual migration behavior of mutants bm6/9, bm4, bm1 and bm10 in DGGE gels differed significantly from the computed predictions. As compared to mutant bm5, which has an A to T change at position 962 and which behaves identically to wildtype, mutants bm6 and bm9 might be expected to have a more stable double helix due to an additional T to C change at position 972 (see Table 1). This expectation was reflected in the theoretical calculation of Figure 1, which predicted a greater stability for

Figure 5. Examples of possible secondary structures formed by the bm6 (left) and the bm1 (right) mutations. The additional T to C mutation of bm6 with regard to mutant bm5 and the wildtype sequence may allow formation of a three-base stem loop stabilized by two base pairings not possible in the latter two molecules. In the bm1 mutation, five single base changes (A to C, G to A and three times A to T) create a structure capable of forming an eight-base stem loop held together by four classical base pairs and two weaker G-T interactions.

the bm6/9 mutant products and lower positions in the gel. However, the actual experiment showed a clearly detectable upward shift of the mutant bm6 and bm9 H2K^b alpha 2 PCR amplification products (Figures 2 and 3). A second class of discrepancy between expected and actual DGGE migration behavior was represented by the bm4 and bm10 mutations, which did not form any sharp major bands in practice, although both were predicted to migrate close to the wildtype in computer simulations. Finally, the mutant bm1 PCR product displayed its major DGGE band at a position much above that predicted by the computer programs.

The computer models used are based on the assumption that a given region of a nucleic acid molecule can exist in only two states, double helical or fully dissociated (25). Segments of a molecule in which a GC base pair is replaced by an AT pair or vice versa are thought to melt at different temperatures (positions in a denaturing gradient) than those of the reference molecule. The initial melting considerably slows down a DNA fragment's mobility until a final velocity in the gel is reached (1). A GC to AT substitution should lead to earlier melting and thus be made visible by an upward shift in the gel relative to the unchanged DNA fragment, while an AT to GC change should lead to greater stability and thus a downward shifted band in a DGGE gel. Such predicted alterations have been demonstrated experimentally using a beta globin promotor fragment (5). Since the results presented here for many of the H2K^b mutants are not consistent with the theoretical predictions, we conclude that the initial sequence dependent melting behavior of a DNA double helix is not the only determinant of the migration of a DNA molecule in DGGE. An apparent difference between experimental results and theoretical calculations has also been previously reported, and was correlated with an unusually slow approach of these fragments to equilibrium (26,27).

The PCR products from the H2K^b mutants studied here apparently existed in several distinct states different from that of the wildtype at different electrophoresis times. Mutant bm6 showed clearly different migration behavior from that of mutant bm5 and the wildtype only at a particular time, while the bulk of the bm1 mutant PCR product migrated initially more slowly,

and later more rapidly, than the wildtype. In addition to these temporal effects, the simultaneous existence of two distinct bands in mutants bm6 and bm9 at certain times (one indistinguishable from the wildtype band and another clearly several millimeters higher in the gel) and the lack of any distinct DGGE band with mutants bm4 and bm10 emphasize that PCR amplification products from homozygous DNA samples need not form single bands in DGGE. The most plausible explanation for how these unexpected observations could be produced in a sequence-specific fashion from mutations as subtle as a single base change out of several hundred is the temporary formation of stem loops during DGGE. Specifically, we suggest that by providing alternative structures that are intermediate in stability between normal and fully melted forms, the formation of temporary stem loops would allow an earlier transition (and upward DGGE shift) than the full melting of a domain. Current theory of DGGE acknowledges the possibility but does not integrate the influence of intermediate melting and secondary structures into predictions of DGGE migration of DNA molecules (4).

Possible examples of the types of secondary structures that may be formed by the PCR products studied here are presented in Figure 5. In the bm6 (and bm9) mutant, the single additional base change relative to mutant bm5 may allow a temporary stem loop which cannot be formed by the bm5 or wildtype PCR products. The DNA fragment amplified from the bm1 mutant could form a more stable secondary structure when partially melted than that which would be possible for the C57BL/6J PCR product. A variety of potential intermediate stem loops not possible for the wildtype are predicted by computer searches of the sequences of mutants bm4 and bm10 (not shown), and which might form a variety of differently migrating forms and lead to the observed lack of a clear band for these mutants in DGGE gels. (It is also worth pointing out that bm4 and bm10 also had mutations in the regions of PCR primer binding-see footnote to Table 1. These mutations did not prevent PCR amplification, and are unlikely to have caused the lack of defined DGGE bands via heteroduplex formation since heteroduplexes would accumulate linearly and represent only a minority of PCR products in comparison to homoduplex PCR products incorporating the primers, which would accumulate exponentially.) The possibility that these fragments formed a smear due to complete melting and strand separation is also highly unlikely because of the presence of the GC clamp. The lack of effect of sample concentration on these migration behaviors provides evidence that these patterns depend on intramolecular properties rather than on intermolecular interactions.

In summary, PCR followed by DGGE enabled the detection of 4 out of 5 classes of H2K^b mutants studied and allowed the rapid assignment of the bm2 mutation to the alpha 2 domain. Although we cannot fully explain the structural basis of all DGGE migration behavior of PCR amplification product at the present time, the reliable detection of known mutations by PCR-DGGE demonstrated here proves that this technique can be used experimentally to detect many or even most mutations in a homogeneous starting DNA fragment. The PCR-DGGE procedure is simple, efficient and rapid, and is thus amenable to mass screening for mutations in defined genomic regions.

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