Exploiting structural differences among heteroduplex molecules to simplify genotyping the DQA1 and DQB1 alleles in human lymphocyte typing

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ABSTRACT

A novel approach to DNA probe hybridization and heteroduplex analysis, termed directed heteroduplex analysis (DHDA) is presented here to illustrate its utility in simplification of human lymphocyte antigen (HLA)-typing. By strategic labeling of single-stranded probe sequences, DHDA allows the identification of specific heteroduplex structures that contribute to the differentiation of DQA1 and DQB1 alleles. Because of the high degree of polymorphism among major histocompatibility complex class II second exon sequences, this analysis of 50 different heteroduplex molecules provides evidence of the importance of unpaired bases and mismatched base pairs and their effect on heteroduplex electrophoretic-mobility differences. This strategy is further used to genotype accurately a family for DQA1 which was previously analyzed by sequence specific oligonucleotide (SSO) probe hybridization. To differentiate by SSO-typing among the DQA1 and DQB1 alleles analyzed in this study requires the use of 23 different probes. Equivalent results are obtained by DHDA using only three probes. Therefore, this study suggests that accurate HLA-typing can be simplified by DHDA. Additionally, DHDA may be useful for differentiation of DNA sequence polymorphisms in other genetic systems.

INTRODUCTION

The cell surface glycoproteins encoded by the major histocompatibility complex class II (MHC class II) genes are responsible for activating CD4+ lymphocytes through antigen presentation (1, 2) and play important roles in transplantation immunology (3–5), in determining the nature of clinical manifestations in response to infectious agents (6–9), and in autoimmunity (10). The polymerase chain reaction (PCR) (11) facilitates rapid identification of the DNA sequence polymorphisms within the second exons of MHC class II genes. These genes encode the portion of mature MHC II proteins responsible for antigen presentation to T-cell receptors during initiation of an immune response. Identifying this molecular diversity within MHC class II molecules has been motivated in large part by the clinical significance of matching donor and host in solid organ (i.e. kidney, heart, lung, liver) and bone marrow transplants. Advantages of PCR based human lymphocyte antigen (HLA)-typing over serological or mixed lymphocyte reaction assays include increased specificity and sensitivity in detecting most allelic polymorphisms based upon DNA sequence differences. PCR based haplotyping has also been successfully applied to a wide variety of human populations because DNA sequence polymorphisms fail to occur in the conserved regions used as PCR primer annealing sites of genomic templates (12). In contrast, the utility of reagents for immunologically based HLA-typing are compromised by unidentified polymorphisms seen in non-European populations (13).

Detection of differences among PCR amplified MHC II alleles has employed the differential hybridization of sequence-specific oligonucleotide (SSO) probes to PCR products amplified by locus-specific primers (14). Because SSO detection involves the use of well over 100 probes (DRB1 = 66; DQA1 = 10; DQB1 = 13; DPA1 = 4; DPB1 = 26) (15), complete MHC class II haplotyping becomes complicated not only by the numbers of reagents required, but also because of the different hybridization and washing conditions required for probe specificity. These factors have influenced the development of alternative methods for allelic differentiation based upon amplified fragment length polymorphism (AFLP) analysis (16), single stranded conformational polymorphism (SSCP) (17), heteroduplex analysis (18–21), or denaturing gradient gel electrophoresis (22). Each of these alternative approaches reveals polymorphic differences among alleles following gel electrophoresis; the banding patterns, however, are frequently very complex even in homozygotes.

This study presents a novel PCR-based approach termed directed heteroduplex analysis (DHDA) employing the strategic PCR-based labeling of three DNA probes to achieve accurate and simple genotyping of all DQA1 and DQB1 alleles. Heteroduplex molecules (HDs), formed between a labeled allelic probe sequence and unlabeled allelic PCR products from
individual human samples, can be visualized by autoradiography because the resulting HD pattern is comprised of a single product in homozygous individuals or two products in heterozygous individuals. To verify the sensitivity and specificity of this new approach, we used DHDA to genotype a previously characterized three generation family at DQA1. Analysis of the relative gel-mobility differences between individual HDs suggests that the number, spacing and chemistry of unpaired or non-standard Watson-Crick base pairs influence the stability of HDs. Therefore, this study also suggests that strategic positioning of mutations may lead to new approaches for detecting DNA sequence polymorphisms through DNA probe hybridization.

**MATERIALS AND METHODS**

**DNA samples**

Human genomic DNA was prepared from Epstein–Barr virus-transformed B lymphoblastoid cell lines (BLCL) following proteinase K (100 µg/ml; sodium lauryl sulfate (SDS) (0.1%) lysis and phenol chloroform extraction (23). BLCL, homozygous for DQA1 and DQB1 were obtained from the 10th International Histocompatibility Workshop (24). Additional BLCL representing members of Family 104 were obtained from the CEPH (Centre pour l’Etude du Polymorphisme Humain) repository.

**PCR amplification**

PCR amplification of DQA1 and DQB1 second exon fragments was directed by previously defined primers that anneal to highly conserved regions overlapping the 5' and 3' intron-exon borders. These include: the DQA1 specific primers (GH26 [ + strand] 5'-CCTAAGCTTGTGCTGAGGTTGAACACTTGTACCCAG-3’ and GH27 [ - strand] 5’-CCAAGCTTACGGATCCTGCAG-3'); and DQB1 specific primers (DQB1 A [ + strand] 5'-CCTAAGCTTCTGTGCTGCTACTCTACCAACGG-3’ and DQB1 B [ - strand] 5’-CCAAGCTTCTGTAGGTAGGTTTGCAC-3'). All primers were modified by the addition of a synthetic 5' HindIII site. PCR amplifications were carried out in a solution (100 µl) containing 10 pmol of the appropriate + strand and - strand primers, 10 mM Tris–HCl, 1.5 mM MgCl₂, 0.01% gelatin, 100 µM dATP, dGTP, dCTP, TTP, 2.5 units of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), and 100 ng of purified human genomic DNA or 2 µl of an M13 phage stock (1 x 10^10 plaque-forming units/ml) containing a single DQA1 or DQB1 functional or pseudogene allele (probe template). The temperature-cycling conditions for DQA1 amplification was 94°C for 30 seconds (denaturation), 50°C for 30 seconds (annealing) and 72°C for 30 seconds (extension) for 40 cycles. For DQB1 the same conditions were used except that the annealing temperature was changed to 58°C. All amplification reactions were performed in the Perkin-Elmer 9600 Turbo PCR machine (Cetus, Emeryville, CA). Unlabeled PCR products from individual human samples were concentrated 4-fold by ethanol precipitation and resuspension in 25 µl of TE pH 7.6.

**Radioactive PCR amplification**

Two different strategies were utilized for the incorporation of radioactive nucleotides into PCR products derived from probe templates. Double-stranded labeling of the PCR products was performed by reducing the dATP concentration to 50 µM and adding 10 µCi of [α-32P]dATP (3000 Ci/mMole) as modifications to the reaction conditions (see above). Single-stranded labeling of the PCR products was achieved by utilizing + or - strand primers (10 pmoles) that had been labeled with 70 µCi [γ-32P]ATP (3000 Ci/mMole) (Amersham Co., Arlington Hts., IL) and treated with T4 polynucleotide kinase using conditions recommended by the manufacturer (U.S. Biochemicals, Cleveland, OH). Double-strand and single-strand labeled PCR products were diluted by the addition of 200 µl of TE pH 7.6 prior to DHDA.

**Cloning of PCR products**

Following amplification from human genomic DNA templates, non-radioactive PCR products were concentrated by ethanol precipitation and subjected to HindIII digestion in 20 µl following the suppliers’ recommended protocol (Boehringer-Mannheim, Indianapolis, IN). As the HindIII recognition site is not found within known MHC class II second exons, this treatment modifies only the PCR products at the synthetic HindIII sites introduced at the 5’ end of each primer sequence. HindIII digested MHC class II PCR products were separated from the primers and small HindIII digestion products by electrophoresis on a 2% agarose gel. The DQA1 and DQB1 PCR products were further purified from excised agarose and cloned into M13mp19 RF, that had been previously treated with HindIII and calf intestinal alkaline phosphatase (Boehringer-Mannheim, Indianapolis, Indiana). The DNA sequence of individually cloned MHC class II second exons was determined by standard dideoxy-nucleotide chain termination sequencing (25).

**DHDA**

Five µl of the unlabeled PCR products (derived from individual human genomic DNA templates concentrated 4-fold following ethanol precipitation) were mixed with 5 µl of the labeled PCR products (derived from specific DQA1 or DQB1 M13 phage stocks). One drop of mineral oil was added to overlay the mixture. Following a two minute incubation at 97°C to denature the double-stranded PCR products, HD formation was promoted by slowly cooling the reactions to room temperature (ramp time from 97°C to room temperature was 45 minutes). HD solutions (7 µl) were mixed with sample dye buffer (0.25% bromophenol blue; 0.25% xylene cyanol FF; 30% glycerol) (3 µl) and samples of 3.5 µl were loaded onto a 5% polyacrylamide (19:1(acrylamide: bis acrylamide)), 2.7 M urea gel (21), unless noted otherwise. Electrophoresis was performed on 50 cm sequencing gels at 35 mAmps for 5 hours in 1 x TBE. The temperature of the gel was maintained between 40 and 45°C. Gels were dried in vacuo prior to autoradiography.

**RESULTS**

DNA probe development applied to directed heteroduplex analysis (DHDA)

In contrast to previous studies (18-21) in which heteroduplex formation has been applied to HLA analysis, this study has employed strategic labeling of single stranded probe sequences to simplify the HD banding patterns and facilitate HLA-typing, referred to as directed heteroduplex analysis (DHDA). Functional DQA1 alleles, 0102 and 0501, and the pseudogene, DQB2, second exon fragments were cloned individually into M13mp19 and sequenced to verify identity with previously analyzed alleles (26) before serving as probe templates for DHDA HLA-typing. The DNA sequence of the probe M13-DQA1*0501 was compared to major functional DQA1 alleles to identify the
number, positions and nucleotides involved in base pair mismatching in resulting HD molecules formed between each functional allele and both the + and the − strands of the probe (Fig. 1A and 1B). The number of pairwise differences between DQA1 alleles and M13-DQA1*0501 ranges from 8– 29 (3.5 – 12.9%) over a total length of 225 nucleotides. Base pair mismatches have been subclassified by thermodynamic stability as determined by Aboul-ela et al. (27), as ‘stable’ (black letters/white background) or relatively ‘unstable’ mismatches (white letters/black background). Total numbers of base pair mismatches, and stable versus unstable mismatches, for each allelic HD product are provided in Fig. 2 below panels B and C. Additionally, a three base pair deletion (***) occurs in DQA1*0201, 0401, 0501 and 0601.

Correlating predicted DQA1 allelic HD polymorphisms with electrophoretic-mobility differences

As segments of single-stranded DNA within HDs causes gel retardation (during electrophoresis), relative to completely double-stranded DNA, and because the predicted DQA1 HDs (Fig. 1A and 1B) exhibit numerous differences in the positions of unpaired and mismatched base pairs, it was hypothesized that each HD would vary in the organization of its double and single stranded segments. If so, these unique DQA1 HD electrophoretic mobilities would permit genotypic analysis of this individual genetic locus (Fig. 2). As seen in the HD banding patterns for homozygous typing cell lines (HTCLs; representing the most frequently observed DQA1 alleles) when probed with
M13-DQA1*0501, every DQA1 allele, with the exception of DQA1*0601, can be distinguished by the unique mobility of one or both of its HD bands. In Fig. 2A, HDs are detected following double-strand probe labeling. The resulting pattern for each allele includes two HD bands (A+ :P- and A− :P+; A = HTCL allelic strand and P = probe strand). When the P+ strand (Fig. 2B) or the P− strand (Fig. 2C) are labeled independently the detected DQA1 HD polymorphism for each allele includes only one of the HD bands. Thus, it becomes possible to correlate the significance of unpaired and mismatched nucleotides in each allelic HD (identified in Fig. 1A and 1B) with relative mobility differences of the two products detected for each allele (intra-allelic HDs) and among products for other alleles (inter-allelic HDs). For example, when comparing relative effects of gel retardation among the intra-allelic HDs, the HD with the highest ratio of unstable:stable mismatches (Fig. 2, bottom) exhibits the greatest relative gel retardation (exception is DQA1*0301).

To verify the presence of DQA1*0501 through detection of an informative HD, samples can be probed with M13-DQA1*0102 for genotyping analysis, as seen in Fig. 3. Coincidently, M13-DQA1*0102 also detects a more distinctive HD polymorphism between DQA1*0101 and DQA1*0102 (see Fig. 3, Lanes 1 and 2 marked 0101 and 0102, respectively).

DHDA genotyping of DQA1 and DBQ1

Application of DHDA to genotyping of a previously characterized family was used to test the specificity of allelic identification by comparing HDs between HTCLs and previously SSO-genotyped Family 104, as detected by M13-DQA1*0102 (Fig. 3). Thus, grandparental alleles DQA1*0401 or 0601 (FAFA-10414 DQA1*0401 or 0601/0401 or 0601) and DQA1*0201 (MOFA-10414 = DQA1*0101/0201) were inherited by their son, FA-10401 (DQA1*0201/0401 or 0601), as expected. Further, all offspring from FA-10401 and MO-10402 (DQA1*0301/0501) also resulted in predictable combinations of their parental DQA1 alleles (either DQA1*0201 or 0401 or 0601 and DQA1*0301 or 0501). Therefore, DQA1 genotyping by DHDA was 100% consistent with the SSO-genotyping results.
Because differentiation is not linked to detection, DHDA can be accomplished using low stringency solution hybridization and relatively fewer numbers of probes. This feature of DHDA eliminates control of hybridization and washing conditions required for SSO probe hybridization specificity and eliminates the maintenance of the large collection of SSO probes. Allelic differentiation based on electrophoretic-mobility differences in DHDA also permits simultaneous positive identification of multiple alleles following a single probe hybridization in contrast to the necessity of using a complete SSO probe series, thus providing a means for more efficient utilization of precious DNA samples. Additionally, identifying novel alleles is based on positive detection of HD products with unique electrophoretic-mobilities.

An advantage of the SSO-typing system is based on non-radioactive detection. DHDA can be easily converted to non-radioactive reagents through strategic use of biotinylated primers and strepavidin-linked enzyme detection systems.

**Strategic differences between DHDA and other PCR-based approaches to HLA-typing**

Comparisons to other PCR-based HLA-typing techniques further illustrate the superiority of DHDA. DHDA does not involve additional enzymatic modification of locus-specific PCR products as required for allele-specific identification in AFLP methodologies. Advantages of DHDA over single-strand conformational polymorphism (SSCP) and conventional heteroduplex analysis (HDA) are based upon the complexity of heterozygous banding patterns. By SSCP unique allelic mobility differences are less obvious than by DHDA (especially with respect to DQBI) and therefore allelic differentiation is more accurate by DHDA.

When compared to HDA, DHDA represents a significantly improved alternative. First, identifying alleles in homozygous individuals by HDA is not possible because the only product formed is a homoduplex. Homoduplex molecules, regardless of sequence differences, exhibit identical electrophoretic mobilities when they are the same length. Clearly, DHDA differentiates homozygous individuals (Figs. 2, 3 and 4). Regarding the complexity of genotyping heterozygotes, in both DHDA and HDA, all possible homoduplex and HD molecular combinations are allowed to form in each individual reaction. The important difference lies in which HD molecules are detected. In HDA the difference lies in which HD molecules are detected. In HDA the detected bands will represent HDs formed between alleles A and B (A+:P- and A-:B+) in addition to the homoduplex on techniques that incorporate label into all double-stranded DNA molecules, the number of different HD banding patterns is equal to the number of possible heterozygous combinations (28 for DQA1 and 78 for DQBI). To pursue locus-specific genotyping of unknown samples would then require a prohibitively large number of positive allelic controls. In DHDA the detected products will represent the HDs formed between unlabelled alleles, A and B, and the labeled probe sequence, P (A+:P- and B+:P- or A-:P+ and B-:P+). Since all of the detected products form in reference to the labeled probe sequence, the number of different HD banding patterns is reduced to the number of alleles at any given locus (8 for DQA1 and 13 for DQBI). By using known heterozygous samples or by combining even larger groups of defined alleles the number of lanes per gel dedicated to positive controls can be further reduced to the desired number.

**DISCUSSION**

Strategic differences between DHDA and SSO-based HLA-typing. This study utilizes a novel strategy for simplifying DQA1 and DQB1 genotyping, termed DHDA. This method provides significant potential for overall application to HLA-typing. A comparison of DHDA to SSO-typing identifies numerous advantages of this strategy. First, complete differentiation of 7 of 8 DQA1 alleles and 12 of 13 DQB1 alleles was accomplished with 3 probes (DQA1 = 2 and DQB1 = 1). For comparable results at least 23 SSOs are required (DQA1 ≥ 10 and DQB1 ≥ 13) (21). The reduced number of probes needed for DHDA-typing is based on the fundamental differences in the application of DNA probes in DHDA and SSO analyses. SSO-typing relies on probe hybridization to detect and differentiate alleles. DHDA-typing relies on probe hybridization to detect alleles, but differentiation of alleles is based on the polymorphic electrophoretic mobilities of individual allelic HD molecules.

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Advantages of pseudogene-based probes in DHDA

The strategic use of the pseudogene-based probe provides two further advantages to genotyping individual loci. First, each functional allele is detected as a polymorphic HD product. Thus, homozygotes will be distinguished from heterozygotes by the presence of one instead of two HD products in their DHDA autoradiogram, unless products for two allelic HDs exhibit the same electrophoretic-mobility. As shown in the differentiation of DQB1 alleles (Fig. 4), this technical problem may be resolved by using the opposite strand probe. Second, each of the MHC class II loci have associated pseudogenes and conserved PCR primer annealing sites permit amplification of both the functional and pseudogene alleles. By using the pseudogene as the probe, unlabeled pseudogene PCR products amplified from individual human samples will be pulled into the homoduplex band and out of the heteroduplex field where they may obscure visualizing the functional allelic HDs.

Molecular analysis of HD structures

Conceptually, gel retardation of HDs (relative to homoduplexes) is due to regions of the molecule which are single stranded. DNA sequence comparisons between the probe M13-DQA1*0501 and the eight functional DQA1 alleles (Fig. 1A and 1B) suggest that the structure of these HD molecules is likely to be affected by: 1) deletions; 2) the number of base pair mismatches; 3) the intervals between mismatches; 4) the A-T versus G-C richness of the sequence flanking mismatches; and 5) the chemical stability between mismatched base pairs.

These predictions are supported by the data provided in Figs. 2 and 3. In Fig. 2, all HD molecules formed between the alleles without the deletion between nucleotides 133–135 (not deleted at nucleotides 133–135 = Δ) (DQA1*0101, 0102, 0103 and 0301) and the probe, M13-DQA1*0501, which is deleted at nucleotides 133–135 (Δ +), are affected by a 3 base bulge and demonstrate greater gel retardation than HDs formed between Δ + alleles (0201, 0401 and 0601). Consistent with this observation, when the probe is Δ − (M13-DQA1*0102), all Δ + alleles demonstrate greater gel retardation relative to the Δ − alleles (see Fig. 3). The presence of frameshift mutations, such as this, have been shown to affect HD structure in various ways (29–31).

Of relevance here, the juxtaposition of deleted and inserted nucleotides force kinks into the structures of resulting HDs because of ‘bulged-out’ (30), unpaired nucleotides. As kinked molecules do not move through the polyacrylamide matrix as easily as molecules without kinks, the kinked HD molecules exhibit relatively greater amounts of gel retardation.

The effect of individual mismatches and combinations of mismatches contribute to additional structural differences and promote unique HD mobilities that serve to differentiate individual alleles further. For example, it is generally observed that HDs with the greatest number of mismatches show the greatest gel retardation. Interestingly however, intra-allelic HDs are affected by the same number of base pair mismatches, yet some exhibit dramatically different electrophoretic mobilities (alleles 0101, 0102 and 0103). When compared to the position of the homoduplex band detected in lanes marked 0501, gel retardation of the A−:P+ HDs for 0101, 0102 and 0103 (Fig. 2B) is approximately two-fold greater than observed for the A+:P− HDs (Fig. 2C). Since the primary difference between intra-allelic HDs is the bases present at unpaired and mismatched positions, this suggests that the chemical stability between mismatched base pairs is an important factor in HD structure. Aboul-e1a et al. have measured the thermodynamic stabilities of all dinucleotide combinations when positioned in the center of a 9-mer (+ strand sequence dCAAXA4G; − strand sequence dCTYGT3G; in 1M NaCl, pH 7.0) (27). This study found that mismatches involving G-residues were relatively more stable than mismatches involving C-residues (stabilities of A-A and T-T mismatches were comparable to C-T and T-C mismatches) (27). While the sequences and buffer conditions involved in DQA1 heteroduplex formation differ, the observations synthesizing the number, spacing, sequence context and thermodynamic stabilities of base pair mismatches appear to be consistent with the hierarchy of base pair stability proposed above. Therefore it might be predicted that regions of HDs which are most likely to be single stranded are those which contain the most destabilizing combination of base pair mismatches.

The data compiled below panels B and C in Fig. 2 suggest that the majority of the inter-allelic and intra-allelic mobility differences may be explained by the number of unstable mismatches relative to stable mismatches; the greater this ratio the greater the observed gel retardation. Thus, the A−:P+ HDs exhibit greater gel retardation than the A+:P− HDs for alleles 0101, 0102 and 0103 while the converse is observed for alleles 0201, 0401 and 0601. For alleles 0101, 0102 and 0103 the A−:P+ HD molecules are affected by more unstable than stable mismatches (the majority of which [10] are observed within the 37 nucleotides flanking the deleted bases 133–135; see Fig. 1A). In contrast, the corresponding A+:P− HDs are affected by more stable than unstable mismatches (Fig. 1B). For alleles 0201, 0401 and 0601 the A−:P− HDs show greater numbers of unstable mismatches and therefore greater relative gel retardation than their A−:P+ intra-allelic counterparts.

The DQA1*0301 HDs are an exception to these observations. Here A−:P− is affected by relatively fewer unstable, as compared to, stable mismatches (unstable = 9; stable = 12) yet it displays greater gel retardation than A−:P+ (unstable = 12; stable = 9). To explain this inconsistency we note that the looped out bases are comprised of 3 purines (AGA) in A−:P− while the complementary pyrimidine bases (TCT) are unpaired in A−:P+. The relative effects of unpaired purines versus pyrimidines have recently been used to explain the HD electrophoretic mobility differences observed in detecting the cystic fibrosis mutation and other experimentally derived mutations (29, 30). In these systems unpaired purines always lead to greater gel retardation than the reciprocal pyrimidine bulges.

Finally, the effects of individual base pair mismatches can be observed since some alleles differ from each other at only one or two nucleotide positions. Specifically, alleles DQA1*0101 and 0102 differ by a G to C transversion at nucleotide 67; alleles DQA1*0102 and 0103 differ by an A to T transversion at nucleotide number 41 and by a G to A transition at nucleotide 89; alleles DQA1*0401 and 0601 differ by an A to T transversion at nucleotide 41. When M13-DQA1*0102 is used as the probe, 0101 is differentiated from 0102 (which is detected only as a homoduplex) in only the A−:P+ HD (Fig. 3 lanes 1 and 2; marked 0101 and 0102, respectively). Here the mismatch at nucleotide 67 is C-C (unstable) in contrast to a G-G (stable) in the A−:P− HD. The mismatched base pairs between M13-DQA1*0501 and DQA1*0102, 0103, 0401 and 0601 will be treated together. By comparing the mismatches between the probe (M13-DQA1*0501) and DQA1*0601 it appears that neither unstable mismatch A-A (in A−:P+) nor T-T (in A−:P−)
promotes mobility differences which allow differentiation between 
DQA1*0401 and 0601. This suggests that the same mismatch 
between M13-DQA1*0501 and DQA1*0103 will similarly result 
in no measurable differences between DQA1*0102 from 0103. 
Therefore it is suggested that the A+P− mobility difference 
between the DQA1*0102 and 0103 HDs in Fig. 2B must be due 
to the A-C mismatch (unstable) at nucleotide 89. The reciprocal 
G-T mismatch (stable) between the probe and 0103 in the 
A−P+ HD does not change the mobility of 0103 relative to 
0102 (Fig. 2C). These observations suggest that in the sequence 
contexts where they are found the C-C mismatch (nucleotide 67 
in DQA1*0101) and the A-C mismatch (nucleotide 89 in 
DQA1*0103) result in a single stranded ‘bubbles’ (30) and 
promote HD gel retardation, whereas the G-G mismatch 
(nucleotide 67 in DQA1*0101), the A-A and T-T mismatches 
(nucleotide 67 in DQA1*0103 and 0601) and the G-T mismatch 
(nucleotide 89 in DQA1*0103) may result in stable non-standard 
Watson–Crick base pairs and have no observed effect on HD 
mobility.

It appears that this type of analysis of the factors affecting HD 
structure may help to direct mutagenesis of specific probe 
sequence motifs to further differentiate alleles (DQA1*0401 
and 0601) which differ by only a single nucleotide. Based on 
the observations presented in this study if the + strand of the M13 
based probe could be changed to a C residue the mismatch for 
DQA1*0601 would be A-C while the mismatch for DQA1*0401 
would be T-C. Since the A-C mismatch appears to be 
characterized by the greatest instability, differentiation between 
DQA1*0401 and 0601 might be observed. A further application 
of these observations to a larger task is in regard to differentiating 
between the highly variable DRB1 and DPB1 alleles. Because 
of the large number of alleles observed at these genetic loci it 
may be possible to use the existing functional alleles or 
pseudogenes as informative probes for successful genotyping 
through the DHDA strategies described in this study.

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