Research Report

Pyrosequence-Based Typing of Alleles of the HLA-DQB1 Gene

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ABSTRACT

DNA typing of alleles of the highly polymorphic HLA-DQB1 gene was performed by Pyrosequencing™ using purified DNA from the 11th International Histocompatibility Workshop human cell lines and samples from the Children's Hospital of Pittsburgh registry of diabetics and their first-degree relatives. Pyrosequencing was optimized for genotyping exon 2 of the HLA-DQB1 gene, but the procedure should be applicable to other HLA loci. The 47 HLA-DQB1 alleles were readily identifiable, as were the 1128 potential allelic heterozygous combinations. The method required PCR conditions that specifically amplified DQB1 but not the pseudogene, DQB2. The new method of pyrosequence-based typing can be performed in 96- or 384-well format. The 61 polymorphic residues of DQB1 exon 2 were identified within four pyrosequencing reactions, obtained by a 70-nucleotide read length in each reaction, in about an hour's time. Allelic combinations of HLA-DQB1 most frequently found in the population of diabetics and their immediate family members were analyzed and successfully compared to typing of the DQB1 alleles by sequence-specific oligonucleotide probe protocols. Pyrosequence-based typing is compatible with genotyping of allelic combinations expected from heterozygous individuals, resulting in nucleotide resolution of the highly polymorphic HLA system. Using pyrosequencing, more than 750 sample wells can be processed in a working day, resulting in the identification of more than 50 000 bases.

INTRODUCTION

Nucleotide polymorphisms at certain HLA loci correlate with susceptibility to a variety of autoimmune diseases, such as type 1 (insulin-dependent) diabetes (recently reviewed in References 9 and 19). Genetic studies of diabetics and their immediate family members have provided critical information, identifying genetic markers for diabetes (3,4,6), with specific polymorphisms of the HLA-DQB1 locus being the strongest indicators of type 1 diabetes mellitus susceptibility (2,18,29).

Methods for screening the highly polymorphic HLA loci for alleles associated with disease are important for risk evaluation of genetically susceptible individuals. HLA-DQB1 alleles containing a polymorphism encoding the presence of a neutral amino acid residue (e.g., alanine, valine, or serine) at amino acid 57 represent one type 1 diabetes susceptibility marker, while an aspartate at the same position (Asp-57) confers resistance (2,15,16,27,29). However, sequence data, including that of the DQ and DRB alleles, result in haplotype information that can be used to better interpret the degrees of association and susceptibility of various genotypes for type 1 diabetes. Moreover, high-resolution typing of the HLA region is required for successful kidney (11,28) and bone marrow (7,12, 26) transplantation.

Advances in DNA sequencing technology via automated acquisition and analysis are important additions to the screening and characterization of genetic markers. Pyrosequencing™ (Pyrosequencing AB, Uppsala, Sweden) methodology represents an improvement to data acquisition, analysis, and allelic identification, including the advantages of real-time data output, resistance to sequencing artifacts associated with analysis by gel electrophoresis, no need for DNA fluorescent labeling, and compatibility with 96- and 384-well microplate formats (22,25).

Pyrosequencing was originally designed for expressed sequence tag sequencing in which short (roughly 10-nucleotide) stretches of DNA are analyzed. The pyrosequencing method (22) is a four-enzyme process combining the activities of DNA polymerase, ATP sulfurylase, luciferase, and apyrase. Briefly, the method can be described in four steps: (*i*) hybridization of sequencing primer to an ssDNA template; (*ii*) incorporation of a complementary nucleotide and release of pyrophosphate; (*iii*) use of ATP sulfurylase to convert pyrophosphate and exogenous adenosine 5′ phosphosulfate into ATP, followed by conversion into light via the activity of luciferase; and (*iv*) degradation of unincorporated dNTP by means of apyrase. Sequencing continues by reiteration of steps 2–4 using the next dNTP to be tested for incorporation into the nascent nucleotide chain. The data

Table 1. Oligonucleotide Primers Used for PCR and DNA Sequencing

Name	Sequence (5′→3′)	Comment
PCR Primers		
DQBamp-A	CCTGTGCTACTTCACCAACGG	DQB1 Forward Primer
DQBamp-B	Biotin-TEG-CTCGTAGTTGTGTCTGCACAC	DQB1 Reverse Primer
SR25	TTTGACCCCGCAGAGGATTTCGTG	DQB1 Forward Primer
SR22	Biotin-TEG-CTCTCCTCTGCARGATCCC	DQB1 Reverse Primer, Alleles 05/06
SR24	Biotin-TEG-CTCGCCGCTGCAAGGTCGT	DQB1 Reverse Primer, Alleles 02/03/04
Model DQB1 Oligonucleotides		
SR11	AGATGTGTCTGGTCACACCCCGCACGCGCTAGCGCGTGCG	Modeled after DQB1*05011
SR14	AGATGCTTCTGCTCACAAGACGCACGCGCTAGCGCGTGCG	Modeled after DQB1*0201
Pyrosequencing Primers for HLA-DQB1 Exon 2		
SR26	GACCCCGCAGAGGATTTCG	Nucleotide 10 to 9
SR27	CAACGGGACGGAGCGCGT	Nucleotide 41 to 58
PSQ1	ACGGAGCGCGTGCG	Nucleotide 48 to 61
PSQ3	TCGACAGCGACGTG	Nucleotide 106 to 119
SR30	GCCGAGTACTGGAACAGCC	Nucleotide 159 to 177

Primers DQBamp-A and DQBamp-B were designed by the 11th International Histocompatibility Workshop (8). Primers SR25, SR22, and SR24 were designed following the recommendations of Pera et al. (17) but with the additional constraint of avoiding formation of self-priming 3′ secondary structure during pyrosequencing. Primers SR26, SR27, PSQ3, and SR30 are the minimal primer set required for complete sequencing of DQB1 exon 2.

output is represented graphically by a "pyrogram" consisting of a plot of time versus intensity of light produced (23). Generation of light represents incorporation of a particular nucleotide. In our hands, pyrosequencing has been readily able to read DNA sequences of up to 70 nucleotides. An important advantage of pyrosequencing over other sequencing technologies is that nucleotides are tested individually for incorporation into nascent DNA. The result is that mixed populations of DNA expected from heterozygous individuals are sequenced independently, allowing the resolution of allelic combinations that are impossible to distinguish by conventional sequencing approaches.

Our study was meant to explore the use of pyrosequencing to identify alleles of the various HLA genes in which polymorphic DNA stretches of greater than 250 nucleotides are generally considered. Samples of genomic DNA prepared from human HLA homozygous cell lines developed by the 11th International Histocompatibility Workshop (8) and samples from the Children's Hospital of Pittsburgh (CHP) Histocompatibility Center study of diabetics and their first-degree relatives listed in the Allegheny County Diabetes Registry (6,13) were selected for genotype analysis. Pyrosequencing was initially used to identify alleles frequently detected with the CHP Histocompatibility Center study population, resulting in high-resolution typing of the highly polymorphic exon 2 region of the HLA-DQB1 gene. Pyrosequencing easily completed a 70-nucleotide read length of each sample in a 96-well sample tray within an hour's time, potentially yielding 768 sequence reactions in an 8-h shift or as many as 3072 when performed in a 384-well format, which is sufficient to fully sequence the polymorphic exon 2 region of HLA-DQB1 from roughly 768 individuals. Its accuracy and throughput make pyrosequence-based typing an important addition to the methods available for the identification of known markers in genetic studies and genotyping donors for transplant recipient matching.

MATERIALS AND METHODS

Materials

Genomic DNA samples were obtained from donors studied at the CHP Histocompatiblity Center among the diabetics and their first-degree relatives listed in the Allegheny County Diabetes Registry (6,13) or from lymphoblastoid cell lines made available on the occasion of the 11th International Histocompatibility Workshop that are each homozygous for one of the 12 different, most common, DQB1 alleles (8). Oligonucleotides were purchased from Operon Technologies (Alameda, CA, USA) or synthesized on-site using a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA) (Table 1). Biotinylated oligonucleotides were purified using HPLC. The pyrosequencer was purchased from Pyrosequencing AB. Reagents for pyrosequencing were obtained from Pyrosequencing AB and used as recommended. All other chemicals and reagents were of the highest quality obtainable.

Methods

PCR amplification was performed in 50-µL volumes containing *Taq* buffer (Applied Biosystems), 2 mM $MgCl_2$, 0.2 µM each dNTP, 0.2 µM forward and biotinylated reverse primers, 1 U *Taq* DNA polymerase, and 5 µL purified genomic DNA (200–500 ng DNA). Amplification included 96°C incubation for

3 min, followed by 32 cycles at 96°C, 55°C, and 72°C incubation for 30 s at each step. PCR cycling was followed by a final 5-min incubation at 72°C. Samples were then stored at -20°C.

Pyrosequence reactions were performed using 20–40 µL amplified DNA from the PCR mixture. Samples were mixed with 4 µL streptavidin-coated beads purchased from Amersham Biosciences (Piscataway, NJ, USA) in Binding Buffer (Pyrosequencing AB) for 10 min with vigorous mixing. Beads were transferred to a filtration apparatus, and the Binding Buffer was removed by vacuum. DNA attached to the streptavidin beads was denatured by incubation for 1 min in 50 µL Denaturation Buffer (Pyrosequencing AB) and washed twice with 150 µL Wash Buffer (Pyrosequencing AB). Beads were suspended in 50 µL Annealing Buffer (Pyrosequencing AB), and 40 µL were transferred to the 96-well PSQ™ plate (Pyrosequencing AB). The appropriate sequencing primer was added in a volume of 5 µL using a 3 µM stock solution. Primer annealing was performed by incubating the samples at 80°C for 2 min. Samples were allowed to cool for 5 min at room temperature (usually 24°C) and sequenced by pyrosequencing. A detailed description of the pyrosequencing reaction conditions has been recently published by Gharizadeh et al. (10). Pyrosequence data were quantified and background-corrected using Peak Height Determination Software v1.1 (Pyrosequencing AB).

Pyrosequence-based typing simulation software (unpublished data) was developed in Perl v5.6.1 provided by ActiveState Tool (Vancouver, BC, Canada) under the General Public License. Simulation software was used to generate the expected pyrosequence-associated peak heights by comparing nucleotide dispensation orders used during pyrosequencing to allelic sequence data. Pyrosequence-based typing simulation software also compared simulated peak height data for each possible heterozygous allelic combination to distinguish unique and ambiguous allelic combinations. Documentation for Perl can be found at http://www.perl. com. The pyrosequence-based typing simulation software is available upon request.

RESULTS

Oligonucleotide-Based Studies

Pyrosequencing has been applied here to the problem of human leukocyte antigen (HLA) typing. Experiments were designed to evaluate the method's suitability for reading nucleotide sequences modeled after HLA-DQB1 alleles *05011 and *0201 (oligonucleotides SR11 and SR14, respectively) (Table 1). Oligonucleotide samples were designed so that a hairpin structure, formed at the 3′-end, would allow self-priming for DNA sequencing (24). Pyrosequencing was performed on the individual oligonucleotides (Figure 1, A and B). For each sample, the complete 20-nucleotide read length was obtained, consisting of the expected signal intensities for incorporation of up to three identical nucleotides in a row (Figure 1A, dispensation G4). Sequencing of a solution containing an equal mixture of both oligonucleotides (Figure 1C) was performed to simulate the data expected from a heterozygous individual. The resulting pyrogram appeared to reflect correctly the expected signal intensities, as judged from an examination of the single sequence data (Figure 1, A and B). For example, in Figure 1C, oligonucleotide SR11 exhibited sequence-specific signal at dispensations G4, C19, and A21, while oligonucleotide SR14 resulted in specific signals at dispensations T1, C3, G12, and G22. The signal associated with the incorporation of the last nucleotide in the nascent chain was lower than expected, probably the result of poor processivity at this position.

Peak signal intensities from the pyro-

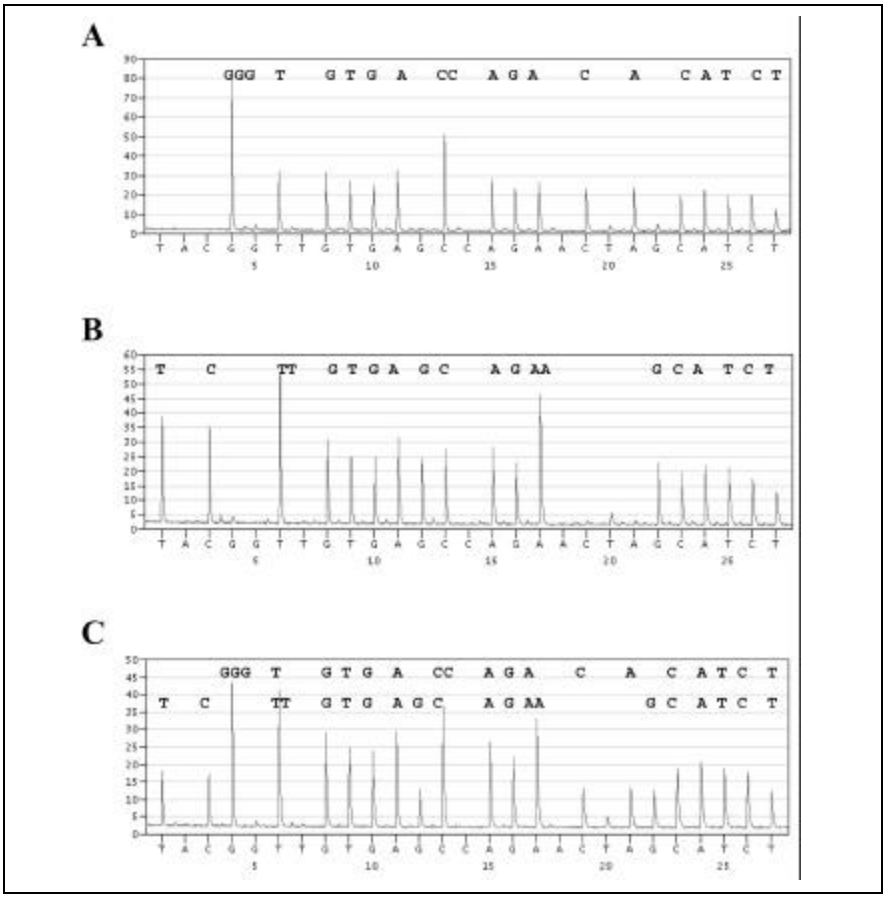

Figure 1. Pyrogram of model DQB1 oligonucleotides SR11 (A), SR14 (B), and the mixture of oligonucleotides SR11 and SR14 (C). Oligonucleotides were present at 0.2 µM when used alone and at 0.1 µM each when mixed together. Dispensations G4, C19, and A21 are specific for SR11, while T1, C3, G12, and G22 are specific for SR14. The oligonucleotide sequences are indicated in each panel. In panel C, the sequence of SR11 and SR14 are indicated in the top and bottom lines, respectively. Axes are nucleotide dispensation event (x-axis) versus observed signal intensity (y-axis).

grams in Figure 1 were quantified and background-corrected. Additional analysis was performed by comparison of the observed signal intensities from the mixture of oligonucleotides SR11 and SR14 (Figure 1C) with the calculated signal intensities obtained from sequencing these oligonucleotides individually (Figure 1, A and B). As illustrated in Figure 2, comparison of the observed and calculated signal intensities resulted in a linear relationship with a slope of about 1.04 and a correlation coefficient of approximately 0.99, indicating an additive, predictable signal. Successful analysis of mixed oligonucleotide sequences, modeled after DQB1 alleles frequently observed during genotyping of individuals from the CHP study of diabetics and their first-degree relatives, suggests that genotyping of heterozygous individuals is suitable to pyrosequencing methodology.

Pyrosequence-Based Typing of HLA-DQB1 Allele *0301

Genomic DNA containing the HLA-DQB1 gene was prepared by PCR amplification using exon 2-specific primers DQBamp-A and DQBamp-B used in the 11th International Histocompatibility Workshop (8). DNA obtained from the HLA homozygous cell line BM16 (DQB1*0301) resulted in a single PCR product of the expected molecular weight when analyzed by gel electrophoresis (data not shown). However, the resulting pyrogram indicated the presence of at least two sequences (Figure 3A), as would be expected from a heterozygous source. For example, the anticipated sequence obtained from the HLA-DQB1*0301 allele using oligonucleotide primer PSQ1 is initially TTATGTGA, while the observed sequence exhibited a strong G signal at dispensation G4. Moreover, the signal intensities at nucleotide dispensations T1, A2, A11, A33, G34, and A35 are of one range, while the other dispensations exhibited much higher intensity, as though a second sequence was present and at a higher molar concentration.

Quantification of peak heights, followed by subtraction of the signal expected from the DQB1*0301 allele, resulted in the unmasking of a DNA sequence associated with a slope of roughly 0.99 and a correlation coefficient of approximately 0.92, when the observed signal intensities were compared to the calculated intensities for the DQB1*0301 sequence and the second sequence (Figure 3A). Identification of the DNA sequence was per-

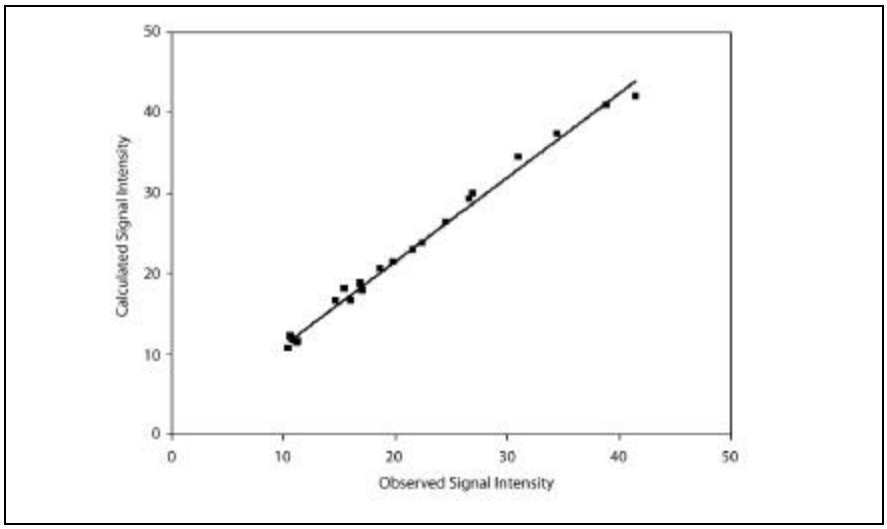

Figure 2. Plot of the observed signal intensity versus the calculated signal intensity for the oligonucleotide mixture of SR11 and SR14. Data were quantified from the pyrograms in Figure 1 using Peak Height Determination Software v1.1 and were background-corrected. Observed signal intensity was obtained using the peak height values from Figure 1C, while calculated signal intensity was determined using peak height values from Figure 1, A and B, assuming an equal molar ratio of the two oligonucleotides. Calculated signal intensity for each peak is defined as 0.5×[(observed signal intensity for SR11) + (observed signal intensity for SR14)]. Linear regression analysis indicated a correlation coefficient of 0.99 and a slope and intercept of 1.04 and 0.71, respectively.

formed by BLAST analysis (1) against the publicly available human database, resulting in a complete match to a 27-nucleotide region of the HLA pseudogene DQB2 (Figure 3A). Moreover, BLAST analysis using the PCR primer sequences resulted in a complete match to regions of the pseudogene such that the expected PCR product would have a base-pair length identical to that obtained from amplification of DQB1 (data not shown). Identification of the combination of sequences DQB1*0301 and DQB2 indicated the usefulness of PSBT in HLA genotyping. However, the 11th International Histocompatibility Workshop primers, while previously considered sufficient for other genotyping technologies, are inappropriate for sequence-based typing methods requiring specific amplification of the DQB1 locus (17), possibly having contributed to ambiguous genotype analysis of HLA-DQB1.

Reengineered PCR primers SR22, SR24, and SR25 (Table 1) were synthesized following the recommendations of Pera et al. (17), with the intention of maximizing specific amplification of the DQB1 locus, but modified to avoid formation of self-priming 3′ secondary structure that during pyrosequencing can lead to primer extension (24). PCR amplification of genomic DNA yielded a product of the expected molecular weight when analyzed by gel electrophoresis (data not shown). Pyrosequencing of the amplified DNA obtained from cell line BM16 (Figure 3B) resulted in a pyrogram for the expected DQB1*0301 allelic sequence, the result of a roughly 80% reduction in signal associated with the contaminating pseudogene (compare nucleotide dispensation G4 in Figure 3, A and B). Low-intensity signals at dispensations G4, C19, and G22 may indicate the presence of an additional contaminating PCR product, suggesting that further optimization of the PCR amplification step may result in improved data for pyrosequence-based typing.

Pyrosequence-Based Typing of Genomic DNA from the CHP Study Samples

Pyrosequence-based typing was performed, using DNA extracted from peripheral blood mononuclear cells of diabetic patients and their immediate family members, on allelic combinations frequently observed in the CHP study population (16). Figure 4 shows data representing a 70-nucleotide length read of exon 2 of the HLA-DQB1 gene for a HLA-DQB1*0302 homozygous individual (Figure 4A) and for a HLA-DQB1*0201-02+*0302 heterozygous individual (Figure 4B). Pyrograms exhibited the expected peak intensities for the homozygous and heterozygous combination (Figure 4). Amplified genomic DNA from the homozygous individual (Figure 4A) exhibited peak intensities corresponding to the expected sequence for the *0302 allele; variations in signal intensity correlated to the number of nucleotide residues incorporated.

Pyrosequencing exhibited a drop in overall signal during the complete 69-nucleotide dispensation cycle, which is probably the result of loss of apyrase activity (10). For the heterozygous individual (Figure 4B), signal intensities also correlated to the number of nucleotide residues incorporated, although for the combined *0201-02+*0302 sequence. Moderately intense signal at nucleotide dispensations A29 and A39 (Figure 4B) did not correspond to the expected allelic combination, suggesting the presence of a contaminating sequence, indicating that additional optimization of PCR conditions may become necessary to improve the accuracy of pyrosequence-based typing. Complete pyrosequencing of the 270-bp DQB1 gene exon 2 was performed using four sequencing primers (Table 1) and resulted in overlapping pyrosequence information (data not shown).

Pyrosequencing of genomic DNA obtained from individuals containing seven of the most common DQB1 allelic combinations (including the diabetogenic HLA-DQB1*0302 allele in combination with alleles *0201-02, *0301, *0302, or *0501) are summarized in Table 2. Genotyping was performed on samples from 49 individuals containing alleles accounting for greater than 80% of the total alleles observed in type 1 diabetics and greater than 70% of control

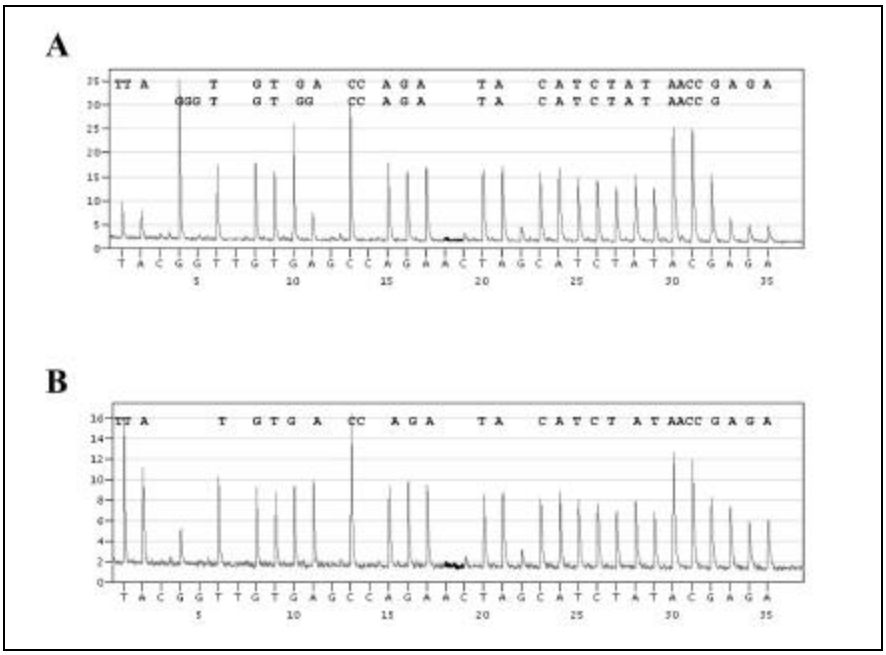

Figure 3. Pyrograms of HLA-DQB1 allele *0301 using 11th International Histocompatibility Workshop-recommended PCR primers DQBamp-A and DQBamp-B (A) or DQB1-specific PCR primers SR24 and SR25 (B). Pyrosequencing was performed on PCR-amplified DNA extracted from the HLA-DQB1*0301 homozygous cell line BM16 using DQB1-specific primers prepared at the 11th International Histocompatibility Workshop (DQBamp-A and DQBamp-B) (A) or primers engineered for this study (SR24 and SR25) based on the recommendations of Pera et al. (17) (B). Sequencing primer was PSQ1. Nucleotide sequences are indicated in panel A for DQB1*0302 (top sequence) and the pseudogene DQB2 (bottom sequence). The nucleotide sequence of allele DQB1*0302 is indicated in panel B. Axes are nucleotide dispensation event (x-axis) versus observed signal intensity (y-axis).

Table 2. Common DQB1 Alleles and Their Identity in Diabetic Families Sequenced in This Study

Number of Samples	SSOP-Identified Alleles[a]	PSBT-Identified Alleles[b]
Heterozygous		
6	*0201-02 + ***0201-03**	*0201-02 + ***0203**
14	*0201-02 + ***0302/07**	*0201-02 + ***0302**
6	*0201-02 + ***0602/111**	*0201-02 + ***0602**
6	***03011/012** + *0302	***03011** + *0302
7	***0302/07** + ***05011/012**	***0302** + ***05011**
4	***05011/012** + ***0602/111**	***05011** + ***0602**
Homozygous		
6	***0302/07**	***0302**

[a]Source of sequence-specific oligonucleotide probe (SSOP) genotyping: CHP Histocompatibility Center study of diabetic patients and their immediate family members. Ambiguously SSOP-typed alleles are indicted in bold.

[b]Each SSOP-identified subpopulation examined in this study resulted in an identical pyrosequence-based typing (PSBT)-identified genotype. Alleles resolved by PSBT are indicated in bold.

subjects from the CHP study population (5). Pyrograms readily distinguished these allelic combinations, leading to their immediate identification as well as being able to resolve ambiguous results obtained from sequence-specific oligonucleotide probe-based typing (Figure 4 and Table 2). An apparent advantage of pyrosequence-based typing, in contrast to hybridization-based methods, is that an oligonucleotide primer initiates the sequencing of a 70-nucleotide region of the allele, while hybridization by oligonucleotide probe identifies only the complementary annealing site. Predictions of the number of oligonucleotide probes required to identify the DQB1 allelic combinations included a high number necessary to distinguish 18 individual codon positions (14), which is a number of primers similarly required for analysis by mass spectrophotometry-based methods. Pyrosequence-based typing required fewer primers for genotyping of the highly polymorphic HLA locus than hybridization-based methodologies.

Simulation of pyrosequencing data was performed for the complete set of DQB1 alleles, using a computer algorithm to generate the predicted pyrograms for each allelic combination. Identification of unique pyrograms was performed by comparing the theoretical results of each possible allelic combination (data not shown). The 44 unique DQB1 exon 2 sequences resulted in 990 potential allelic pairs, of which 982

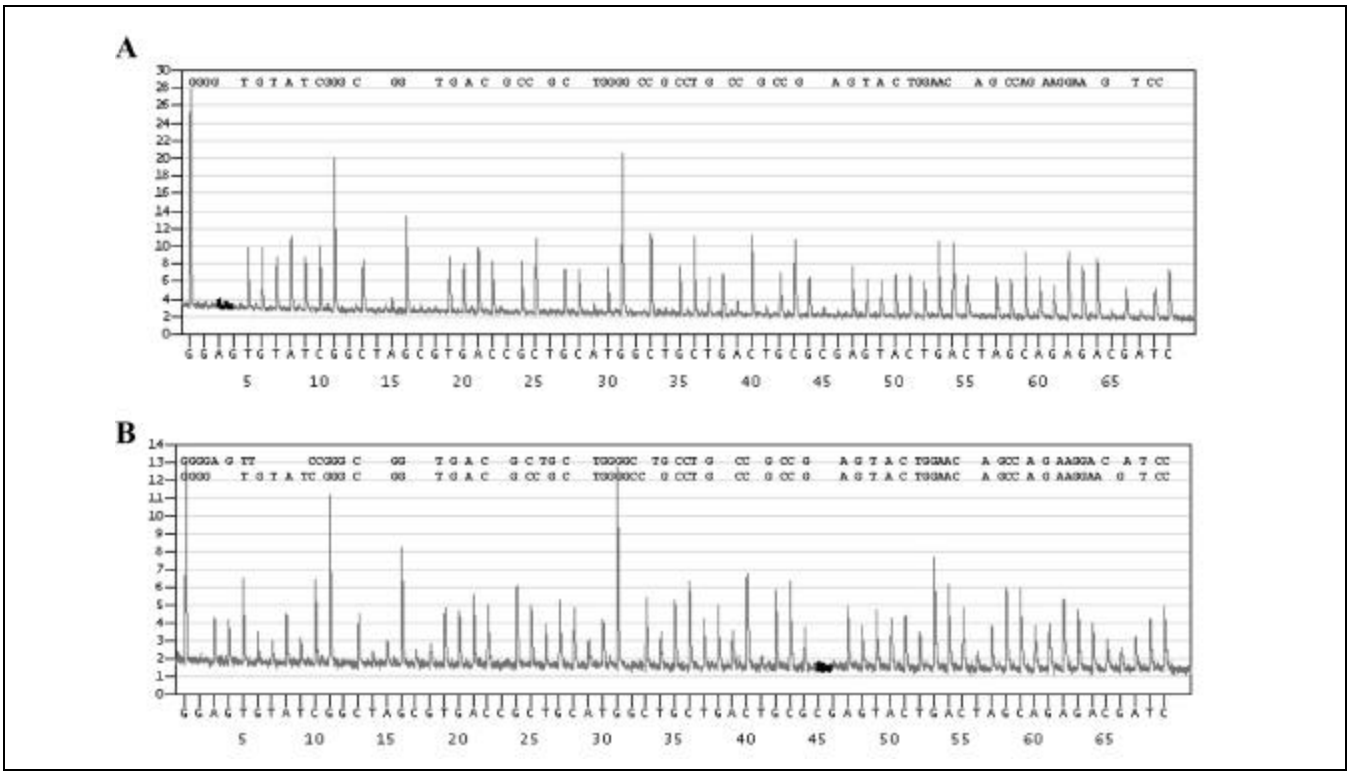

Figure 4. Pyrograms of HLA-DQB1 alleles prepared from peripheral blood mononuclear cells extracted genomic DNA samples from the CHP study of diabetics and their immediate family members. Pyrograms illustrate sequence data from a homozygous *0302 individual (A) and a heterozygous *201-02+*0302 individual (B). Nucleotide sequences are indicated for *0302 in panel A and for *0201-02 (top sequence) and *0302 (bottom sequence) in panel B. Read lengths were at least 70 nucleotides. Sequencing primer was PSQ3. Axes are nucleotide dispensation event (x-axis) versus observed signal intensity (y-axis).

gave unique pyrograms, a predicted success rate of roughly 99%. Eight allelic pairs yielded identical pyrograms and occurred in four sets: (*i*) *0201-02+*03011/09 or *0203+*0304; (*ii*) *0201-02+*03032 or *0203+*0302; (*iii*) *03011/09+*0302 or *03032+*0304; and (*iv*) *05012+*0610 or *0502+*0613. Changes to the pyrosequencing protocol (e.g., the order of nucleotide dispensation) may further optimize the ability to distinguish between the remaining allelic combinations (unpublished data). Amplification of DQB1 exons other than exon 2 will be required to distinguish among alleles *0201/*0202, *03011/*0309, and *06011/*06013. A similar analysis, based on methods used in sequence-based typing, has been posted at the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/) (20,21). In contrast to pyrosequence-based typing, sequence-based typing was predicted to result in 26 ambiguous allelic pairs when the DQB1 gene was examined.

DISCUSSION

Pyrosequencing methodology represents an improvement in automation, including the advantages of real-time data output, absence of sequencing artifacts associated with gel electrophoresis, no need for DNA fluorescent labeling, and compatibility with 96- and 384-well microplate formats. Originally formulated for sequencing short stretches of DNA, pyrosequencing has been evaluated for incorporation into genomic typing protocols with the goal of improving throughput and accuracy of HLA genotyping.

Pyrosequence-based typing of HLA alleles was performed on prevalent allelic combinations observed in diabetics and their first-degree relatives. Pyrograms confirmed the assignment of alleles obtained by sequence-specific oligonucleotide probe-based typing and provided unambiguous additional resolution. Complete sequencing of the highly polymorphic exon 2 of HLA-DQB1 was performed in four reactions, resulting in overlapping sequence information. Different reaction conditions may allow the sequencing of a longer stretch of DNA (10), further reducing the number of overlapping segments needed. Although the polymorphisms at some other HLA loci will result in a more complex analysis, particularly HLA class I loci, the results obtained by sequencing DQB1 alleles can be considered the first step to expand pyrosequencing applications to a complete HLA typing.

Optimization of the pyrosequence-based typing protocol allowed the rapid identification of alleles for the analysis of genetic predisposition to disease and

for histocompatibility typing. Considering that a single pyrosequencing instrument is capable of performing sequencing of a 96-well tray every hour, up to 15 000 sequence reactions can be analyzed within a month. On this basis, pyrosequencing methodology seems to possess all the attributes to enhance the throughput and specificity of HLA typing via the rapid acquisition of DNA sequence information.

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REFERENCES

1. **Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*:3389-3402.
2. **Boehm, B.O., B. Manfras, S. Seidl, G. Holzberger, P. Kuhnl, C. Rosak, K. Schoffling, and M. Trucco.** 1991. The HLA-DQ beta non-Asp-57 allele: a predictor of future insulin-dependent diabetes mellitus in patients with autoimmune Addison's disease. Tissue Antigens *37*:130-132.
3. **Cavender, D.E., D.K. Wagener, B.S. Rabin, D.J. Becker, T.J. Orchard, M.S. Eberhardt, R.E. LaPorte, A.L. Drash, and L.H. Kuller.** 1984. The Pittsburgh Insulin-Dependent Diabetes Mellitus (IDDM) study. HLA antigens and haplotypes as risk factors for the development of IDDM in IDDM patients and their siblings. J. Chronic Dis. *37*:555-568.
4. **Davies, J.L., Y. Kawaguchi, S.T. Bennett, J.B. Copeman, H.J. Cordell, L.E. Pritchard, P.W. Reed, S.C. Gough, et al.** 1994. A genome-wide search for human type 1 diabetes susceptibility genes. Nature *371*:130-136.
5. **Dorman, J.S., R.E. LaPorte, R.A. Stone, and M. Trucco.** 1990. Worldwide differences in the incidence of type I diabetes are associated with amino acid variation at position 57 of the HLA-DQ beta chain. Proc. Natl. Acad. Sci. USA *87*:7370-7374.
6. **Drash, A.L., R.B. Lipton, J.S. Dorman, D.J. Becker, R.E. LaPorte, T.J. Orchard, W.J. Riley, M. Trucco, and L.H. Kuller.** 1991. The interface between epidemiology and molecular biology in the search for the causes of insulin-dependent diabetes mellitus. Ann. Med. *23*:463-471.
7. **Erlich, H.A., G. Opelz, and J. Hansen.** 2001. HLA DNA typing and transplantation. Immunity *14*:347-356.
8. **Faas, S.J., R. Menon, E.R. Braun, W.A. Rudert, and M. Trucco.** 1996. Sequence-specific priming and exonuclease-released fluorescence detection of HLA-DQB1 alleles. Tissue Antigens *48*:97-112.
9. **Friday, R.P., M. Trucco, and M. Pietropaolo.** 1999. Genetics of Type 1 diabetes mellitus. Diabetes Nutr. Metab. *12*:3-26.
10. **Gharizadeh, B., T. Nordstrom, A. Ahmadian, M. Ronaghi, and P. Nyren.** 2002. Long-read pyrosequencing using pure 2′-deoxyadenosine-5′-*O*′-(1-thiotriphosphate) Sp-isomer. Anal. Biochem. *301*:82-90.
11. **Hsia, S., J.Y. Tong, G.L. Parris, D.D. Nghiem, E.M. Cottington, W.A. Rudert, and M. Trucco.** 1993. Molecular compatibility and renal graft survival—the HLA DRB1 genotyping. Transplantation *55*:395-399.
12. **Hurley, C.K., M. Maiers, J. Ng, D. Wagage, J. Hegland, J. Baisch, R. Endres, M. Fernandez-Vina, et al.** 2000. Large-scale DNA-based typing of HLA-A and HLA-B at low resolution is highly accurate specific and reliable. Tissue Antigens *55*:352-358.
13. **LaPorte, R.E., A.L. Drash, D. Wagener, T.J. Orchard, and L.H. Kuller.** 1982. The Pittsburgh insulin-dependent diabetes mellitus (IDDM) registries: the descriptive epidemiology of racial differences, p. 66-77. *In* G. Mimura, S. Baba, Y. Goto, and J. Kobberling (Eds.), Clinico-Genetic Genesis of Diabetes Mellitus. International Congress Series 597. Excerpta Medica, Amsterdam, The Netherlands.
14. **Luo, M., J. Blanchard, Y. Pan, K. Brunham, and R.C. Brunham.** 1999. High-resolution sequence typing of HLA-DQA1 and -DQB1 exon 2 DNA with taxonomy-based sequence analysis (TBSA) allele assignment. Tissue Antigens *54*:69-82.
15. **McDevitt, H.** 2001. Closing in on type 1 diabetes. N. Engl. J. Med. *345*:1060-1061.
16. **Morel, P.A., J.S. Dorman, J.A. Todd, H.O. McDevitt, and M. Trucco.** 1988. Aspartic acid at position 57 of the HLA-DQ β chain protects against type I diabetes: a family study. Proc. Natl. Acad. Sci. USA *85*:8111-8115.
17. **Pera, C., L. Delfino, A. Longo, M.P. Pistillo, and G.B. Ferrara.** 2000. Novel associations among HLA-DQA1 and -DQB1 alleles, revealed by high-resolution sequence-based typing (SBT). Tissue Antigens *55*:275-279.
18. **Pietropaolo, M., D.J. Becker, R.E. LaPorte, J.S. Dorman, S. Riboni, W.A. Rudert, S. Mazumdar, and M. Trocco.** 2002. Progression to insulin-requiring diabetes in seronegative prediabetic subjects: the role of two HLA-DQ high-risk haplotypes. Diabetologia *45*:66-76.
19. **Pietropaolo, M. and M. Trucco.** 2000. Major histocompatibility locus and other genes that determine the risk of development of type 1 diabetes mellitus, p. 399-410. *In* D. LeRoith, S.I. Taylor, and J.M. Olefsky (Eds.), Diabetes Mellitus: A Fundamental and Clinical Text, 2nd ed. Lippincott Williams and Wilkins, Philadelphia, PA.
20. **Robinson, J., A. Malik, P. Parham, J.G. Bodmer, and S.G.E. Marsh.** 2000. IMGT/HLA database—a sequence database for the human major histocompatibility complex. Tissue Antigens *55*:280-287.
21. **Robinson, J., M.J. Waller, P. Parham, J.G. Bodmer, and S.G.E. Marsh.** 2001. IMGT/HLA Database—a sequence database for the human major histocompatibility complex. Nucleic Acids Res. *29*:210-213.
22. **Ronaghi, M.** 2001. Pyrosequencing sheds light on DNA sequencing. Genome Res. *11*:3-11.
23. **Ronaghi, M., S. Karamohamed, B. Pettersson, M. Uhlen, and P. Nyren.** 1996. Real-time DNA sequencing using detection of pyrophosphate release. Anal. Biochem. *242*:84-89.
24. **Ronaghi, M., B. Pettersson, M. Uhlën, and P. Nyrën.** 1998. PCR-introduced loop structure as primer in DNA sequencing. BioTechniques *25*:876-878.
25. **Ronaghi, M., M. Uhlen, and P. Nyren.** 1998. A sequencing method based on real-time pyrophosphate. Science *281*:363-365.
26. **Rosner, G., B.S. Martell, and M. Trucco.** 2000. Histocompatibility, p. 233-251. *In* E.D. Ball, J. Lister, and P. Law (Eds.), Hematopoietic Stem Cell Therapy. Churchill Linvingstone, Philadelphia, PA.
27. **Todd, J.A., J.I. Bell, and H.O. McDevitt.** 1987. HLA-DQ β gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature *329*:599-604.
28. **Tong, J.Y., S. Hsia, G.L. Parris, D.D. Nghiem, E.M. Cottington, W.A. Rudert, and M. Trucco.** 1993. Molecular compatibility and renal graft survival—the HLA DQB1 genotyping. Transplantation *55*:390-395.
29. **Trucco, M.** 1992. To be or not to be Asp 57, that is the question. Diabetes Care *15*:705-715.

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