Direct molecular haplotyping by melting curve analysis of hybridization probes: beta 2-adrenergic receptor haplotypes as an example

Genevieve Pont-Kingdon[1,*] and Elaine Lyon[1,2]

[1]Institute for Clinical and Experimental Pathology, ARUP Laboratories, 500 Chipeta Way, Salt Lake City, UT 84108, USA and [2]Department of Pathology, University of Utah, Salt Lake City, UT 84132, USA

ABSTRACT

Direct determination of the association of multiple genetic polymorphisms, or haplotyping, in individual samples is challenging because of chromosome diploidy. Here, we describe the ability of hybridization probes, commonly used as genotyping tools, to establish single nucleotide polymorphism (SNP) haplotypes in a single step. Three haplotypes found in the beta 2-adrenergic receptor (β2AR) gene and characterized by three different SNPs combinations are presented as examples. Each combination of SNPs has a unique stability, recorded by its melting temperature, even when intervening sequences from the template must loop out during probe hybridization. In the course of this study, two haplotypes in β2AR not described previously were discovered. This approach provides a tool for molecular haplotyping that should prove useful in clinical molecular genetics diagnostics and pharmacogenetic research where methods for direct haplotyping are needed.

INTRODUCTION

Genetic variations may cause disease, increase risk of disease or affect individual response to therapeutic treatment of disease. Recent data suggest that in some cases, a combination of multiple polymorphisms on the same DNA molecule (haplotype) is a stronger predictor of phenotypes than a single polymorphism (1–5). Establishing haplotypes is also a powerful tool in searching for determinants of complex diseases. Furthermore, phenotypes or disease risk can be due to the synergic effect of several mutations in the same gene (6–9). Single nucleotide polymorphisms (SNPs) are the most abundant variants and genotyping tests are available for many disease-associated SNPs, but establishing haplotypes in individuals is significantly more difficult.

Indirect haplotyping methods, such as linkage studies, require samples from multiple family members who are often unavailable for testing, and the accuracy of algorithms that infer haplotypes from multiple polymorphisms in the population is insufficient for an individual clinical evaluation (10–12). Direct molecular methods that unambiguously determine haplotypes present in an individual are more suitable for a clinical setting. Because of human diploidy, both copies of the chromosomes (or genes or chromosomic regions) need to be separated before or during analysis. The diploid copies can be physically separated by transferring single chromosomes in hybridoma cell lines (13), cloning in microorganisms (14), or diluting and analyzing single DNA molecules (15–19). Alternatively, the different alleles can be analyzed individually using PCR-based technologies, such as the widely used allele-specific PCR and its variations (20–25), the recently described 'long-range PCR–intramolecular ligation' technique (26,27) and allele-specific pyrosequencing or hybridization (28,29). However, non-PCR methods are very time consuming and the above mentioned PCR techniques can be difficult to optimize.

Here, we utilize hybridization probes and the principle of fluorescence resonance energy transfer (FRET) (30) to determine the phase of SNPs amplified on a single PCR product. In this method, a fluorescein-labeled probe (donor) and acceptor probes (labeled with either LCred640 or LCred705) are annealed adjacently on a template to generate a fluorescent signal. When heated, probes melt from the template, resulting in loss of fluorescent signal. Melting temperatures (T_ms) that reflect the stability of the probes are specific to their length, their nucleotide composition and their homology with the template. Melting curve analysis of hybridization probes is used extensively as a genotyping tool, and we demonstrate SNP haplotyping using the probes. For demonstration, we used three SNPs from the well-documented haplotypes of the beta 2-adrenergic receptor (β2AR) (1,25,31).

*To whom correspondence should be addressed. Tel: +1 801 583 2787, ext. 2546; Fax: +1 801 584 5109; Email: pontkig@aruplab.com

MATERIALS AND METHODS

Samples

Samples used for this study were from DNA de-identified following the Institutional Review Board protocol. They were extracted from whole blood with the MagNa Pure LC DNA Isolation Kit I (Roche Applied Science). DNA panels HD04, HD32 and HD100 from different ethic groups were purchased from Coriell (Coriell cell repository, Camden, NJ).

PCR, melting conditions and analysis for the haplotyping assays

All PCRs were performed in capillary tubes with 10 µl reactions on a LightCycler Instrument (Roche Applied Science) using the 5.32 run version with automated gain adjustment.

PCR and haplotyping of the β2AR gene was performed using ∼50 ng of genomic DNA template in 1× LightCycler-DNA Hybridization Master Hybridization Probes (Roche Applied Science) adjusted to a final $MgCL_2$ concentration of 3 mM with the forward and the reverse primers [β2AR-F: 5′-GCAGAGCCCCGCC-3′ and β2AR-R: 5′-AAA-CACGATGGCCAGGAC-3′ (1)] at 0.5 µM each and the probes at 0.2 µM each. The following conditions were used for the reaction: denaturation at 95°C for 0 s, annealing at 60°C for 10 s and extension at 72°C for 15 s for 40 cycles. Programmed transition rates were 20°C/s from denaturation to annealing and from extension to denaturation and 2°C/s from annealing to extension. The amplification cycles were followed by a melting cycle, in which DNA was denaturated at 95°C for 30 s, cooled to 30°C using a rate of 1°C/s and held for 120 s. Temperature was then raised to 75°C with a transition rate of 0.1°C/s. Fluorescence was continuously monitored during the melt.

Melting curves were converted into negative derivative curves of fluorescence with respect to temperature (−dF/dT) by the LightCycler Data Analysis software. All analyses were performed with background correction and color compensation.

Melting analysis of oligonucleotide series

Oligonucleotides complementary to haplotype 2 and haplotypes 4 at SNPs 46 and +79 were synthesized and dHPLC purified at the DNA-peptide core facility at the University of Utah. Ten microliter reactions containing 20 pmol of oligonucleotides and 2 pmol of the anchor and the reference probes for SNP 46/+79 in 1× PCR buffer were placed in capillaries and melted as described above.

Allele-specific amplification and analysis

To confirm the haplotypes determined by the haplotyping probes, allele-specific amplification (20) was performed. As described previously (23), three reactions were performed per sample. The non-allele-specific primers are forward: CTTCCAGGCGTCCGCTC and reverse: CATTGCCAA-ACACGATGGCC. The two allele-specific reactions rely on the polymorphism at position −47. The two allele-specific forward primers are CCGTGGGTCCGCCC (haplotype 2) and CCGTGGGTCCGCCT (haplotypes 4 and 6). Reactions were performed with the following condition using pure *Taq* Ready-To-Go PCR beads (Amersham Biosciences):

denaturation at 95°C for 10 s, annealing at 70°C for 30 s and extension at 72°C for 30 s for 40 cycles. Product of the allele-specific reactions were analyzed by dideoxynucleotide sequencing and/or by melting of the SNP 46/+79 probe pair. For this, 8 µl of PCR was mixed with 1 µl of each probe in capillaries and melted in a LightCycler as described above.

RESULTS

Determination of β2AR haplotypes using two pairs of SNPs and haplotyping probes

The different associations of 12 SNPs in the promoter and 5′ region of the β2AR gene define 13 different haplotypes (1). Polymorphisms and haplotypes in this region have been linked to individuals' response to β-agonists used in asthma treatment (1,32). SNPs at positions −20 (T/C), +46 (A/G) and +79 (C/G) allow differentiation of the three main haplotypes in several ethnic groups (1) (Figure 1A). It should be noted that these alone are not able to distinguish the main haplotypes (2, 4 and 6) from less common ones (1). These SNPs are therefore used here only as a proof of principle. SNP −47 is also listed on Figure 1; it is not tested by the probe assay but latter for haplotyping confirmation by allele-specific amplification. The three SNPs (−20, +46 and +79) are amplified on a 218 nt long PCR fragment and pairs are analyzed independently using hybridization probes that analyze either the polymorphisms at position −20 and +46 or probes that interrogate position +46 and +79 (Figure 1B). Because the distance between the SNPs is greater than can be tested under one continuous probe, sequences present in the template between the SNPs were omitted within the haplotyping probes (Figure 1B). These deletions are 55 nt long in the SNPs −20/46 probe and 22 nt long in the SNP 46/+79 probe.

Analysis of 30 random samples with each probe set revealed six derivative melting curves profiles. For each sample, using either of the probe sets, one or two curves are observed (Figure 1C and D). Genotypes corresponding to each melting curve were determined by sequencing the PCR products obtained from samples revealing single melting curves (samples 152, 144 and 137 on Figure 1C and D). Sequencing data (data not shown) indicate that these samples are homozygous at each SNP locus and sequences correspond to haplotype 2, 4 or 6. The stability of the probes with the different haplotypes is determined by the number of matches between the probes and the haplotypes. The probes are the most stable when hybridized with haplotype 2 (no mismatches except the 55 or 22 nt intervening sequences). Haplotype 6 that mismatches with the probes at position −20 or +79 has an intermediate stability and haplotype 4 that mismatches with the probes at both SNP positions is the least stable.

In 20 samples, two derivative curves are observed. Melting profiles correspond to the presence of every possible association of the three haplotypes (i.e. hap2/hap4, hap2/hap6 and hap4/hap6). Confirmation of haplotypes in these samples will be described latter.

Fluorescence due to FRET is lost as the two interacting fluorophores are separated. The observation that the polymorphism located downstream of the deletion relative to the FRET activity affects the stability of the probe (compare
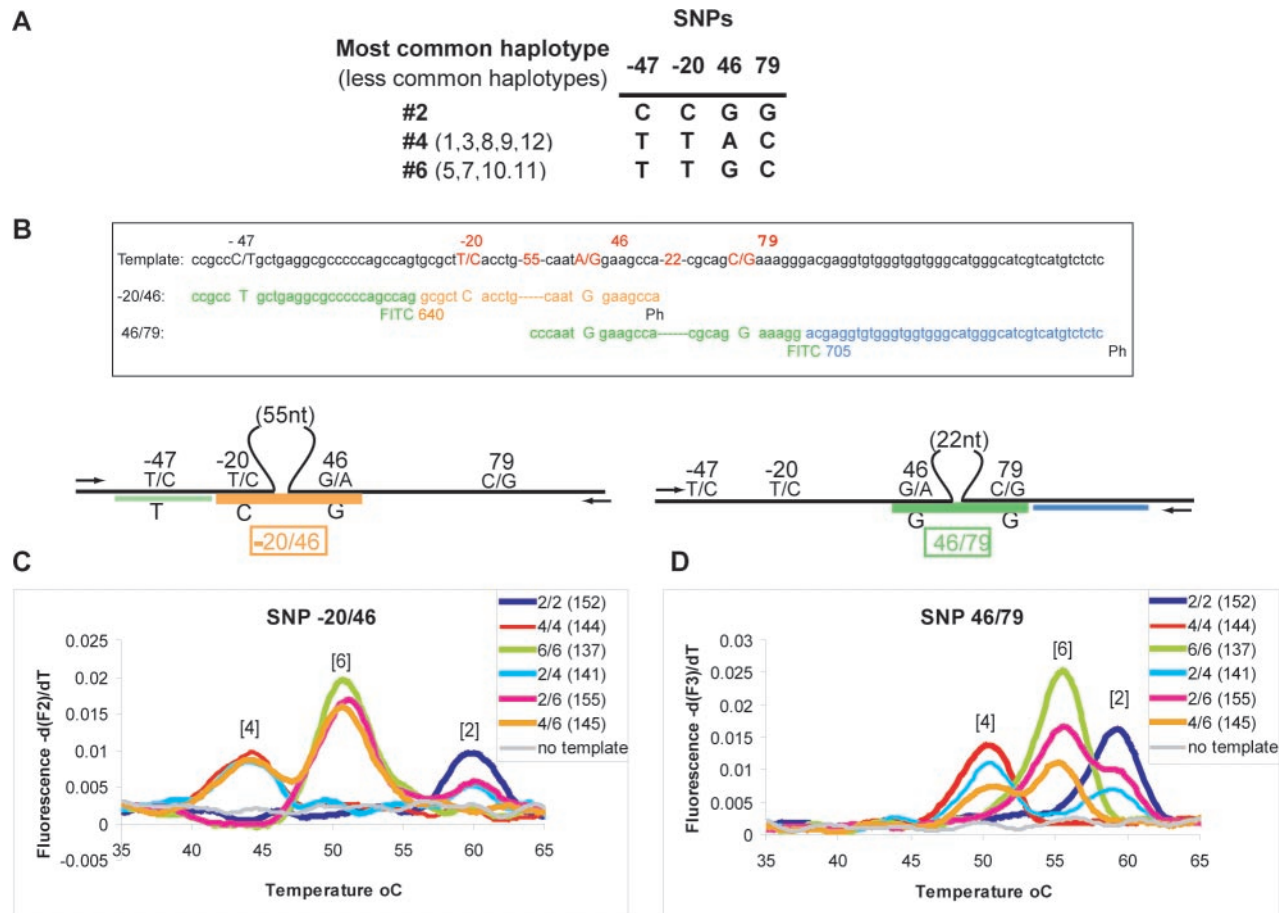
Figure 1. Analysis of β2AR haplotypes 2, 4 and 6 by melting curves of two SNP pairs. (**A**) SNPs characteristic to the three main haplotypes are presented. Less common haplotypes (in parenthesis) are not distinguished using these three SNPs (1). (**B**) All sequences are in the conventional 5′–3′ orientation. The sequence of the template is in black with polymorphisms in capital letters and the three SNPs detected by the probes in red. The polymorphism at position −47 is underneath anchor probes and not analyzed by the assay. The numbers inserted in the template indicate the number of nucleotides omitted in probes. Sequences of all probes (colored) are given with the bases corresponding to the SNPs in capital letters. Fluorescent labels (FITC, LCred640 and LCred705 are indicated under the 5′–3′ extremities of the probes. Ph indicates the presence of a 3′ phosphate group. The dashed lines indicate the sequences of the template missing in the probes. In the schematic representation of the probes and template complexes shown, probes are color coded as above. (**C**) Derivative melting curve analysis of the SNP −20/+46 probe (left) and the SNP +46/+79 probe (right) hybridized to samples homozygous or heterozygous for each haplotype.

haplotypes 4 and 6 in Figure 1C), indicates that the probe dissociates as a unit rather than as two domains.

Determination of the phase of three SNPs using a complex 'looping out' probe

To test further the observation that haplotyping probes dissociate from their template as a unit, we designed a probe set to interrogate the three SNPs (positions −20, +46 and +79) simultaneously (Figure 2). Both deletions are present in the −20/+46/+79 probe that has sequences hybridizing with the three SNPs. Haplotype 2 perfectly matches the −20/+46/+79 probe at all SNPs. Haplotype 6 has two mismatches with the probe and haplotype 4 mismatches the probe at each of the three SNP positions.

The probe that spans the three SNPs is labeled at both extremities with FITC and analyzed in the presence of two acceptor probes (Figure 2A). One of the acceptor probes is labeled at the 5′ end with LCred 640 and the other at the 3′ end with LCred 705. This labeling system allows us to analyze the stability of the −20/+46/+79 complex probe independently on the SNP −20 side in the F2 channel and the SNP +79 side in

the F3 channel. The 30 initial samples and 96 additional ones were tested with this probe set. Identical T_ms are observed in each channel, indicating that this probe also dissociates from the template as a unit (Figure 2B).

Confirmation of haplotypes using other technologies

Several approaches were used to confirm the haplotypes in samples with two derivative curves. Haplotypes 4 and 6 differ only at SNP +46; therefore, the haplotypes can be inferred by genotyping at this position. The PCR products from five hap4/hap6 samples were sequenced and were all heterozygous at SNP +46 (data not shown). In addition to SNPs −20, +46 and +79, haplotype 2 differs from haplotypes 4 and 6 at position −47 (Figure 1A). An haplotyping allele-specific amplification approach was used to confirm the haplotypes in samples determined as hap2/hap4 and hap2/hap6 by melting curve analysis (Figure 3). This haplotyping approach has been used successfully previously (20,23,25,31,33–35). Thirteen hap2/hap4 and two hap2/hap6 samples were amplified using, in different reactions, the non-allele-specific primers or the haplotype 2-specific or the haplotypes 4- and
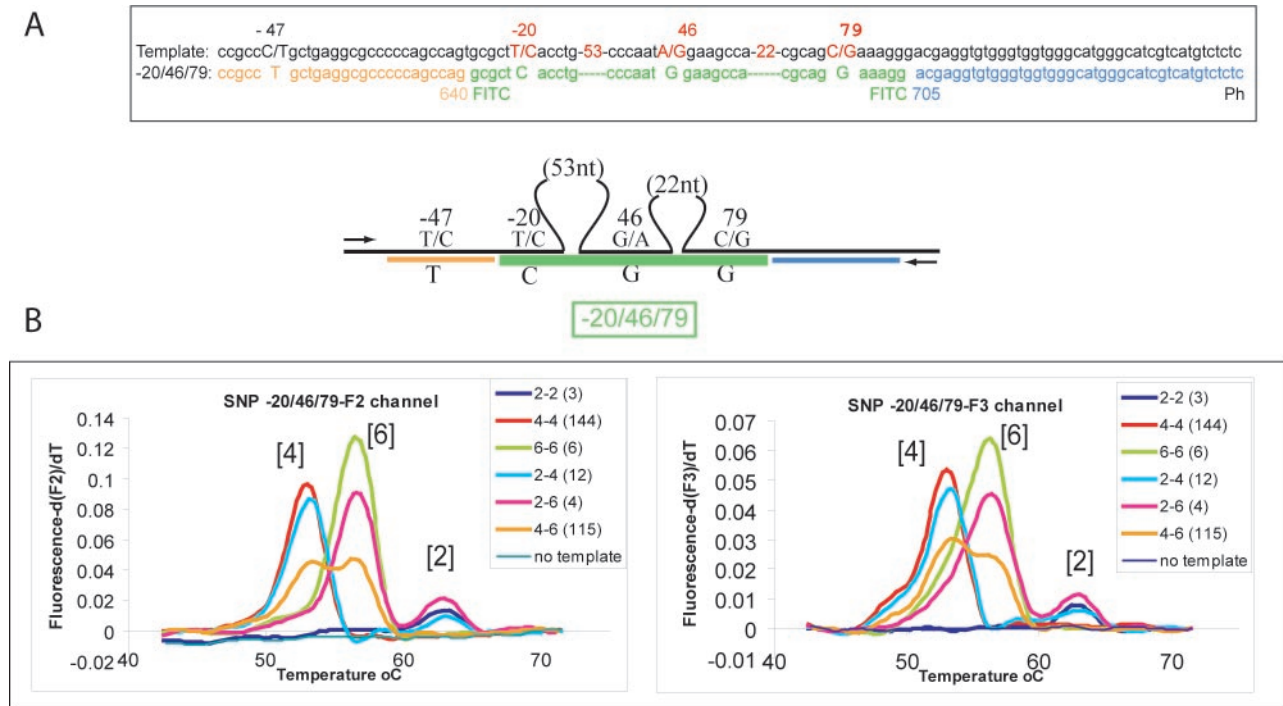
Figure 2. Analysis of β2AR haplotypes 2, 4 and 6 by melting curves of three SNPs simultaneously. (**A**) Representation of sequences and probes is similar to Figure 1. Note that the complex probe −20/+46/+79 is labeled with FITC at both extremities. (**B**) Derivative melting curves obtained by analysis of the SNP −20/+46/+79 probe. The left graph corresponds to the melting analysis in the F2 channel, the right graph to the melting analysis in the F3 channel.
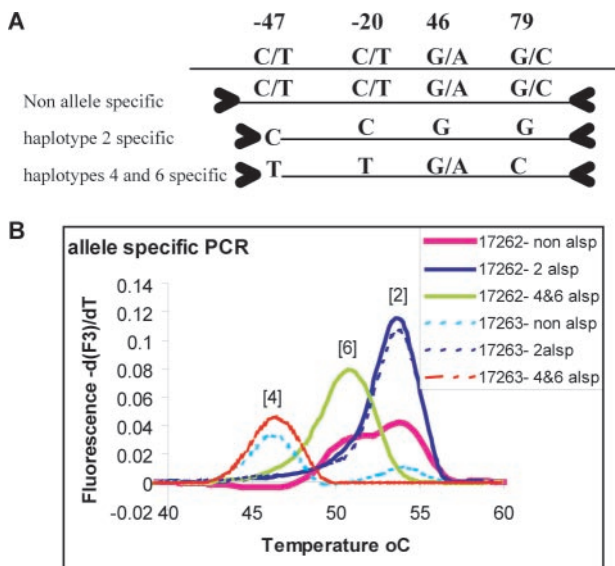

Figure 3. Haplotyping with an allele-specific strategy. (**A**) The design of the experiment is presented. The three PCRs are represented under the map of the relevant section of the β2AR gene. Primers are indicated with thick arrows. The non-allele-specific PCRs amplify both alleles. The haplotype 2-specific forward primer ends in C in 3′ and amplifies only haplotype 2 templates. The haplotype 4- and 6-specific primer amplify both haplotypes. (**B**) An example of allele-specific analysis by melting curve is shown. The samples are from Coriell's Caucasian DNA panel.

6-specific forward primers (Figure 3A). Analysis of the allele-specific amplification reactions by sequencing or melting curve analysis using the complex probe revealed only one sequence, or one melting curve that corresponded to one of the

haplotypes. One example of each haplotype combination is presented on Figure 3B.

The frequencies of haplotypes 2, 4 and 6 vary in different ethnic groups (1). Using the complex probe set, we established the haplotypes of 10 individuals of the following groups; African American, Chinese and Caucasian. Results are shown in Table 1. As observed previously (1), haplotype 2 is more frequent in the Caucasian population and haplotype 6 more represented in Asian and African individuals. Haplotypes of samples from the Caucasian panel were confirmed using the allele-specific PCR approach that discriminates haplotype 2 from haplotypes 4 and 6.

To further confirm the ability of probes to establish haplotypes, a small pedigree with both parents and four children was analyzed with the complex probe set (data not shown). Both parents carried an haplotype 2 and an haplotype 4. Two children had the same haplotype combination and two children had only haplotype 4. These results are consistent with the segregation of haplotypes 2 and 4 through a generation.

Reproducibility of melting curve analysis and discovery of two new haplotypes

Using as controls, samples with haplotypes confirmed by sequencing or allele-specific amplification, we analyzed 102 random samples using the complex probe set. Eight samples did not amplify and derivative melting curves were analyzed from 96 samples. The average T_ms and SD of controls were determined in both the F2 and the F3 channel (Figure 4A) using data from three independent experiments. The haplotypes of 94 samples were easily categorized and are as follows: haplotype 4 (T_m − F2 = 53.38 ± 0.24, F3 = 53.36 ± 0.3) was present on 44.8% of all chromosomes, haplotype 2

Table 1. Haplotypes in different ethnic groups

Ethnic groups	Haplotypes						Haplotypes frequencies %		
	2/2	**4/4**	**6/6**	**2/4**	**2/6**	**4/6**	**2**	1, 3, **4**, 8, 9, 12	5, **6**, 7,10, 11
African American	0	3	3	1	2	1	15 (6.3)	40 (61)	45 (32.9)
Asian	0	2	1	2	1	4	15 (10)	50 (57.5)	35 (32.5)
Caucasian	1	2	0	4	2	1	40 (48.3)	45 (34.6)	15 (16.6)

Ten individuals from each ethnic group were analyzed with the complex haplotyping probe. The number of individuals' per haplotype composition is reported on the left part of the table. On the right, haplotype frequencies were calculated. Rare haplotypes (not in bold) are not distinguished from haplotype 4 and 6 by our assay. Number in parenthesis correspond to published frequencies (1).
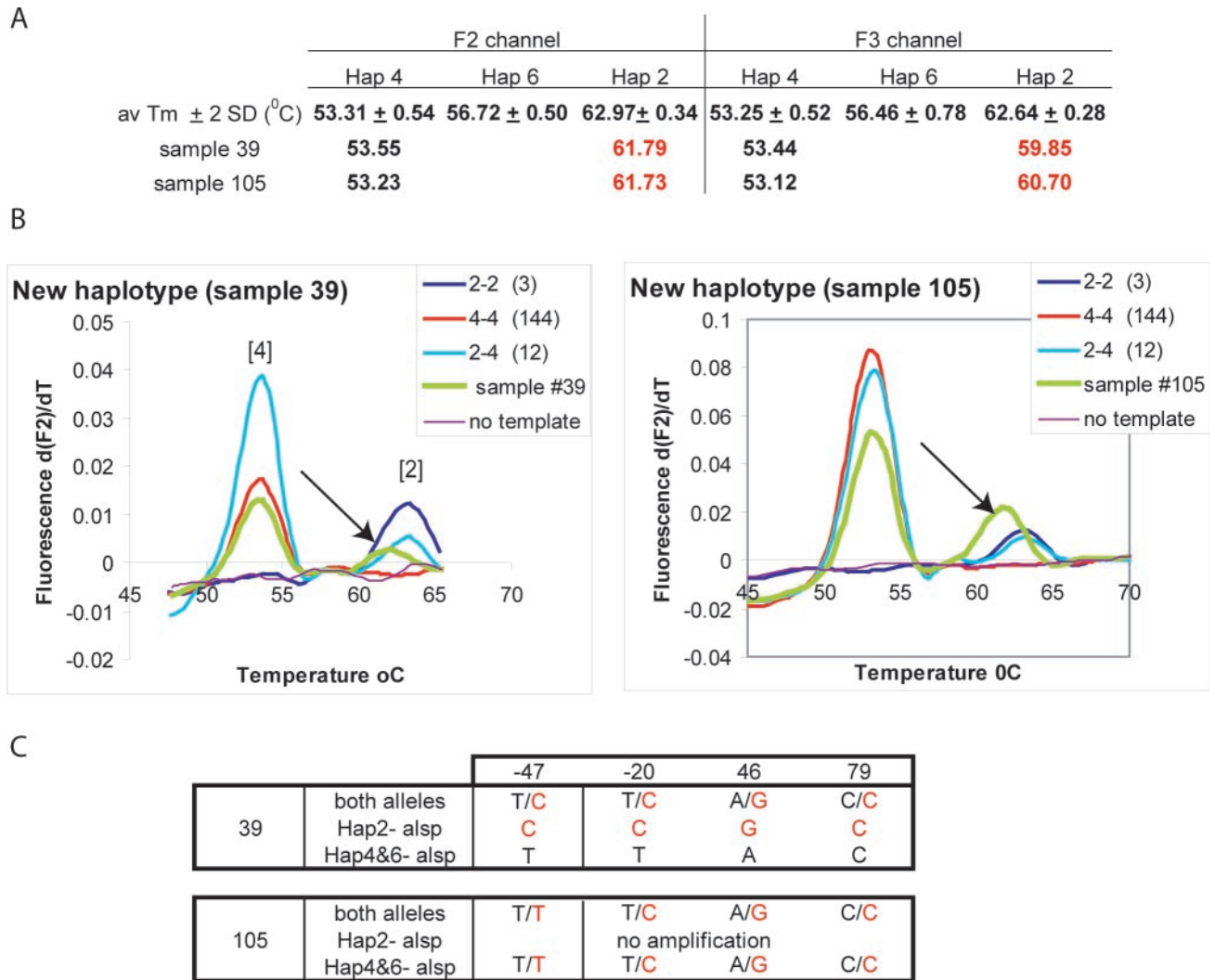

Figure 4. Haplotype 2 variants. (**A**) T_ms of controls, samples 39 and 105 are presented. (**B**) Melting curve analysis of samples 39 and 105. An arrow indicates the derivative melting curve with a lower T_m than expected for haplotype 2. (**C**) Sequence obtained from non-allele-specific and allele-specific PCR at the specified positions.

($T_m - F2 = 63.10 \pm 0.26$, F3 = 62.82 ± 0.26) was present on 32.8% and haplotype 6 ($T_m - F2 = 56.89 \pm 0.25$, F3 = 56.83 ± 0.32) was present on 22.4%. As the ethnicity composition in our random population is not known, these results are difficult to compare with the previously published data. Two samples (39 and 105) were difficult to interpret (Figure 4). The T_m of one derivative curve indicated the presence of an haplotype 4 on one chromosome, while the other derivative curve had a T_m lower than expected for haplotype 2

(Figure 4A and B). Sequences of both samples were compatible with the presence of haplotype 4 (T–T–A–C) but indicated the presence of variant haplotypes 2 (Figure 4C). Sequences of allele-specific reactions confirmed the presence of haplotype 4 on one chromosome and provided identity of these variants. In the variant present in sample 39, position +79 is a C instead of the G, which is typical of haplotype 2. In sample 105, haplotype 2 allele-specific amplification was not successful and as the haplotypes 4 and 6 allele-specific reaction amplified
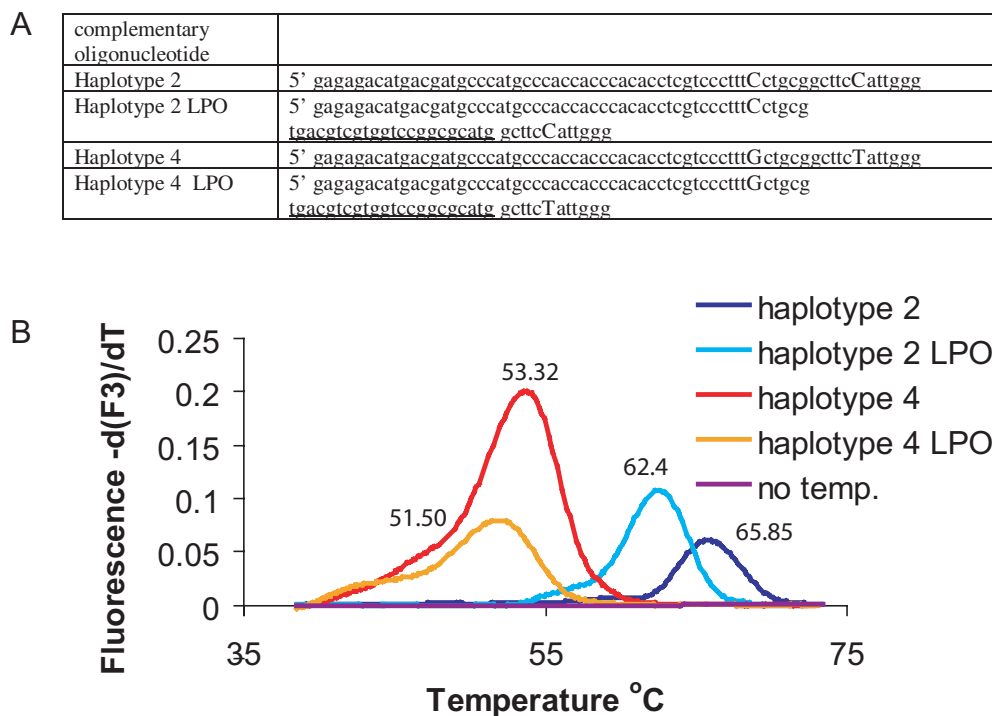
A

complementary oligonucleotide	
Haplotype 2	5' gagagacatgacgatgcccatgcccaccacccacacctcgtcccttt**C**ctgcggcttc**C**attggg
Haplotype 2 LPO	5' gagagacatgacgatgcccatgcccaccacccacacctcgtcccttt**C**ctgcg <u>tgacgtcgtggtccggcgcatg</u> gcttc**C**attggg
Haplotype 4	5' gagagacatgacgatgcccatgcccaccacccacacctcgtccctttG**c**tgcggcttcTattggg
Haplotype 4 LPO	5' gagagacatgacgatgcccatgcccaccacccacacctcgtccctttG**c**tgcg <u>tgacgtcgtggtccggcgcatg</u> gcttcTattggg

B

Figure 5. Melting analysis of oligonucleotides. (**A**) Sequence of the oligonucleotides used as template with the SNP 46/+79 probe. SNPs specific for the haplotypes are indicated with capital letters. The sequence absent in the probe is underlined. LPO stands for 'loop out'. (**B**) Derivative melting curves of the oligonucleotide series. T_ms are indicated above each curve.

both alleles, we concluded that, in addition to C+79, a T is found at position −47 in the variant haplotype. Only SNP +79 is interrogated by the probe and it is expected that the probe demonstrates a similar stability with both variants. These two variants have not been reported previously.

Effect of the 'loop' on probe stability

To evaluate the destabilizing effect of the 'loop' on the probe stability, we used a series of oligonucleotides complementary to the +46/+79 probe set (Figure 5A). These oligonucleotides mimic haplotype 2 (the most stable) and haplotype 4 (the least stable). For each haplotype, two complementary oligonucleotides were tested with the probe; one hybridizes continuously with the probe, whereas the other contains the 22 nt that loops out upon hybridization. Data are presented in Figure 5B. Probes that force out a 22 nt loop from the template dissociate 2–3°C lower than probes that continuously bind the templates.

DISCUSSION

Here, we have presented a method that establishes directly and in a single step the haplotypes of two or three polymorphisms in close proximity. The method relies on the effect of mismatches on the thermodynamic stability of an oligonucleotide with its template (36–38). Many genotyping applications using single mismatches have been reported, but few data address stability of multiple mismatches (36,37). Here, we report the ability of hybridization probes to establish the phases (haplotype) of two or three loci separated by up to 100 nt.

Although this study does not address the mechanism of the hybridization and dissociation of the probe, we hypothesize

that the 22 and 55 nt present in the templates and not in the probes bulge or loop out during binding and that the probe dissociates as a unit. It may be possible to incorporate larger loops and thus to analyze sites even farther apart.

Previously, small insertion/deletion mutations (1–6 nt) have been detected by derivative melting curves using FRET hybridization probes (39–42). In these cases, the stability of the probe with a template containing the deletion is reduced, because the few deleted nucleotides are unavailable for binding. Comparison of the stability of the haplotyping probes with the oligonucleotide series (Figure 5) shows that the presence of extra nucleotides in the template is sufficient to significantly reduce the stability of the probe. These data suggest that relatively large insertions can also be detected with hybridization probes.

Surprisingly, two new haplotypes were discovered during this study that included a total of 252 chromosomes from a random population. It is not clear as to why these haplotypes have not been reported previously. Drysdale *et al.* (1) reported a strong linkage desequilibrium between SNP +79 and SNP −47 and subsequent studies inferred haplotypes from direct analysis of SNP −47 (25) or SNP +79 (31). Furthermore, association of SNP +79C with +46G (samples 39 and 105) could be interpreted as haplotype 6 in the absence of direct analysis of SNP −20 or −47. Our study was not aimed to haplotype the β2AR for clinical purpose but the discovery of these two new haplotypes might have clinical implication. Changes at both SNPs +79 and SNP −47 alter amino acids. The change from SNP G+79 (haplotype 2) to C+79 changes amino acid 27 from a gln to a glu. This position has been implicated in response to beta blockers. The change of SNP −47 from a C (haplotype 2) to a T (other haplotypes) changes

the last amino acid of the β2AR upstream peptide (BUP) encoded in the β2AR promoter. Interestingly, this change of a cysteine to an arginine affects the β2AR protein expression (43).

Our data show that hybridization probes that span several polymorphisms and force template sequences to loop out act as a unit, allowing definite determination of different haplotypes. Other probe systems, such as fluorescein-labeled probes (44,45), MGB-Eclipse-probes (46,47) and molecular beacons (48,49) allow genotyping by melting analysis and could be designed for haplotyping. The thermodynamic property of the 'looping out' probes demonstrated here can also be used to identify closely related genes (or organisms) that differs by at least two polymorphisms in a 100 nt range or to avoid non-significant polymorphisms by omitting them from the probe. For haplotyping purposes, introduction of melting analysis of probes in allele-specific assays that typically require multiple reactions (31,33) or of the intramolecular ligation products described by McDonald *et al.* (26) would significantly reduce the number of reactions needed to establish haplotypes. Furthermore, our system has the advantage to directly determine haplotypes of multiple polymorphisms in a closed-tube environment without additional DNA manipulation after PCR, making it appealing for the development of clinical genetic tests from individual samples. Analysis of haplotypes with hybridization probes on high-throughput instruments would allow analysis of large number of samples needed in association studies and pharmacogenomics clinical trials.

ACKNOWLEDGEMENTS

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