Genotyping of Essential Hypertension Single-Nucleotide Polymorphisms by a Homogeneous PCR Method with Universal Energy Transfer Primers

CHIKH BENGRA,1 THEODORE E. MIFFLIN,1 YURI KHIRPIN,2 PAOLO MANUNTA,3 SCOTT M. WILLIAMS,4 PEDRO A. JOSE,5 and ROBIN A. FELDER1*

Background: Human hypertension is a complex, multifactorial disease with a heritability of more than 30–50%. A genetic screening test based on analysis of multiple single-nucleotide polymorphisms (SNPs) to assess the likelihood of developing hypertension would be helpful for disease management.

Methods: Tailed allele-specific primers were designed to amplify by PCR six biallelic SNP loci [three in G protein-coupled receptor kinase type 4 (GRK4): R65L, A142V, and A486V; two in angiotensinogen: −6G→A and M235T; and one in aldosterone synthase: −344C→T] associated with essential hypertension. PCRs of SNP loci were coupled (via a common sequence of 21 nucleotide tails) to incorporate Universal Amplifluor™ primers labeled with fluorescein or sulforhodamine in a homogeneous format. Use of Amplifluors in SNP PCRs produced labeled amplicons, the fluorescence of which was quantified by a microplate reader and then analyzed via an Excel macro to provide genotypes for all six SNP loci. Unique restriction endonucleases were identified for five SNP loci that could independently confirm homogeneous PCR results when needed.

Results: We developed six homogeneous PCR assays that were set up, performed, and fluorometrically analyzed in 96-well microplates. Allele frequencies were determined for six SNPs in 60 Italian hypertensive patients and a control group of 60 normotensive persons. A significant correlation (P = 0.034) between one SNP [GRK4 (A486V)] and the hypertensive patients was observed. Genotyping results for five of six SNPs were confirmed by digesting corresponding amplicons with locus-specific restriction endonucleases.

Conclusions: We developed a simple and homogeneous fluorescent protocol that has been used to determine the SNP genotype for six loci in a population of hypertensive and normotensive persons. We also observed a significant association (P = 0.034) between one SNP (A486V) and an Italian population of mildly hypertensive patients.

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Multilocus analysis of single-nucleotide polymorphisms (SNPs) is an adjunct or is definitive for diagnosis of several monogenic diseases such as cystic fibrosis, Gau- cher, and Tay Sachs (1). SNP analysis at multiple loci can also be useful in the study of polygenic diseases by targeting specific families of SNPs or by genome-wide linkage disequilibrium mapping (2, 3). The use of SNPs in genetic disease detection is facilitated by the recent discovery of more than 4 000 000 SNPs in the human genome that have the potential to be a rich source of genetic markers to establish genetic linkage and as indicators of diseases (4). As SNP maps become more accessible with

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1 The University of Virginia, Department of Pathology, PO Box 800214, Charlottesville, VA 22908.
2 Serologicals Corporation, 202 Perry Parkway, Gaithersburg, MD 20877.
3 University “Vita e Salute San Raffaele”, Division of Nephrology, Dialysis, and Hypertension, San Raffaele Hospital, 20132 Milan, Italy.
4 Meharry Medical College, Department of Microbiology, Nashville, TN 37208-3599.
5 Georgetown University Medical Center, Department of Pediatrics and Physiology and Biophysics, 3800 Reservoir Rd., Washington, DC 20007.
*Address correspondence to this author at: University of Virginia, Department of Pathology/MARC, PO Box 800214, Charlottesville, VA 22908. Fax 434-924-5718; e-mail raf7k@virginia.edu.

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6 Nonstandard abbreviations: SNP, single-nucleotide polymorphism; FRET, fluorescence resonance energy transfer; GRK4, G protein-coupled receptor kinase type 4; COM, common; WT, wild type; MUT, mutant; nt, nucleotide; FAM, fluorescein; and SR, sulforhodamine.
finer detail, there is growing recognition that rapid, closed-tube, efficient detection methods are needed to bring SNP analysis into mainstream use in molecular diagnostics laboratories (5, 6). Use of molecular analysis to screen and detect SNPs associated with hypertension is also recognized (7).

We are interested in using SNP analysis as a tool to study essential hypertension, which is a complex disease with a substantial genetic component and a heritability of 30–50%. Numerous genetic studies of hypertension, however, have failed to identify sequence variants that can be uniformly replicated across study populations. It has been hypothesized that the absence of significant linkage disequilibrium may be attributable to an underlying complex genetic interaction among various genes regulating sodium excretion and blood pressure (8). For example, a previous study demonstrated that multilocus interactions are important risk factors in this disease (9). To further study multilocus interactions associated with essential hypertension, we therefore sought a robust genotyping method that would unequivocally distinguish between the allelic variants present in various polymorphisms.

Our review of SNP analysis methods began with several rapid genotyping methods that have been developed recently (6, 10–12). These methods all depend on sequence-specific hybridization of fluorescent oligonucleotide probes. Among SNP genotyping procedures are three popular fluorescence resonance energy transfer (FRET)-based methods that are performed in a single tube (i.e., homogeneous) format: molecular beacons (13), paired probe hybridization, LightCycler (14), and Taq-Man® (5′-exonuclease assay) (15, 16). Although these methods represent a substantial advance in SNP analysis capability, each has its own unique drawbacks as well. The FRET methods that exploit juxtaposition of reporter and quencher moieties on one or two oligonucleotides (e.g., molecular beacons and paired probe hybridizations, respectively) need chemically modified oligonucleotides for each individual SNP. This requirement makes these assays relatively expensive and difficult to optimize for multilocus analyses. Furthermore, hydrolysis-based FRET (TaqMan) assays cannot be performed with all thermostable DNA polymerases because some of these enzymes lack the 5′-exonuclease (proofreading) activity required for the performance of this procedure (17).

From our evaluation of existing SNP genotyping methods, we selected a simple system (Amplifluor™) of universal PCR primers that can be used to genotype virtually any two-allele SNP locus. This homogeneous method of SNP genotyping is based on a combination of a single set of two FRET oligonucleotides (Universal Amplifluor primers) with unique sequence tails that can extend
amplification, beginning with amplicons created from any pair of correspondingly tailed sequence-specific oligonucleotides (Fig. 1) plus a common (COM) opposite primer (18, 19). We therefore sought to adapt this rapid SNP genotyping system to detect six SNPs that have been characterized previously for their association with human hypertension (9, 20–25).

Materials and Methods

Participants and DNA Samples
A cohort of 60 unrelated hypertensive patients and 60 normotensive individuals were recruited at San Raffaele Hospital, Milan, Italy (26, 27). The study protocol was approved by the ethics committee of the San Raffaele Hospital, and informed consent was obtained before the peripheral blood samples were collected. Genomic DNA was extracted and purified from the blood samples and stored at −20 °C before analysis.

Cloned Samples of G Protein-Coupled Receptor Kinase Type 4 (GRK4) SNP Sequences
Plasmid clones of human GRK4 from three SNP loci containing all three genotypes at each locus (clones) were obtained from P.A.J. (Georgetown University). These constructs were provided as cDNA inserts cloned into the pTRE vector (Clontech) that were transfected into Escherichia coli and then amplified, harvested, and purified using a column-based isolation procedure (Plasmid Midi method; Qiagen) at the University of Virginia.

Selection of Hypertension-Related SNPs
We designed primers for PCR amplification of six different SNPs in three genes associated with hypertension (Table 1). Three polymorphisms [448G→T (R65L), 679C→T (A142V), and 1711C→T (A486V)] are located in the binding and membrane targeting domains of the GRK4 gene (28); these polymorphisms, by themselves or via their interactions with genes regulating the renin-angiotensin system, are associated with and may be causal of essential hypertension (9, 20). Three additional polymorphisms selected include a SNP (−344C→T) located in the upstream regulatory element of aldosterone synthase (CYP11B2) and two SNPs [−6G→A and 842T→C (M235T)] located in the angiotensinogen gene (9, 21–25).

PCR Primers and DNA Amplification
The allele-specific PCR primers and the COM (reverse) primers were designed from published gene sequences using OligoTM v6.4 primer analysis software (Molecular Biology Insights). Gene sequences used were GRK4 (GenBank accession no. U33056), angiotensinogen promoter (GenBank accession no. X15323), angiotensinogen second exon (GenBank accession no. 4557286), and aldosterone synthase (GenBank accession no. D13752). PCR primer sequences were synthesized by Midland Certified Reagents.

Sequences of PCR primers [two allele-specific primers, wild type (WT) and mutant (MUT), and a COM opposite primer per SNP] to amplify each of the six SNP loci are shown in Table 1. The allele-specific primers contain 21-nucleotide (nt) regions (identical to the recognition site of one Universal Amplifluor primer; “tailed”) that are different for one of two labeled primers (green or red; Table 1). A different sequence tail is then added to the 5′ end of each allele-specific primer (18). The 21-nt tails on the allele-specific primers are identical with the 21-nt 3′

<table>
<thead>
<tr>
<th>Gene (SNP)</th>
<th>WT (Lwr)*</th>
<th>MUT (Lwr)*</th>
<th>COM (Upr)*</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone synthase (−344C→T)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-[gm tail]CTTATCCTGAGATGAGAGGGGA-3′</td>
</tr>
<tr>
<td>Aldosterone synthase (−344C→T)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-[red tail]CTTATCCTGAGATGAGAGGGGA-3′</td>
</tr>
<tr>
<td>Angiotensinogen (−6G→A)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-GCATGTGCAAGGGCGAGGGGA-3′</td>
</tr>
<tr>
<td>Angiotensinogen (−6G→A)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-GCACTGCTAATCCACCCCGCA-3′</td>
</tr>
<tr>
<td>GRK 4 (448G→T, R65L)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-GCACTGCTAATCCACCCCGCA-3′</td>
</tr>
<tr>
<td>GRK 4 (448G→T, R65L)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-GCACTGCTAATCCACCCCGCA-3′</td>
</tr>
<tr>
<td>GRK 4 (679C→T, A142V)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-AATCTAGATCATTCTCAGAAGGA-3′</td>
</tr>
<tr>
<td>GRK 4 (679C→T, A142V)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-AATCTAGATCATTCTCAGAAGGA-3′</td>
</tr>
<tr>
<td>GRK 4 (679C→T, A142V)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-AATCTAGATCATTCTCAGAAGGA-3′</td>
</tr>
<tr>
<td>GRK 4 (1711C→T, A486V)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-AATCTAGATCATTCTCAGAAGGA-3′</td>
</tr>
<tr>
<td>GRK 4 (1711C→T, A486V)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-AATCTAGATCATTCTCAGAAGGA-3′</td>
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<tr>
<td>GRK 4 (1711C→T, A486V)</td>
<td>X</td>
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<td>X</td>
<td>5′-AATCTAGATCATTCTCAGAAGGA-3′</td>
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<tr>
<td>GRK 4 (1711C→T, A486V)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5′-AATCTAGATCATTCTCAGAAGGA-3′</td>
</tr>
</tbody>
</table>

* Lwr and Upr are terms that refer to particular strands of genomic DNA that contain these sequences. WT contains wild-type (normal) SNP sequence; MUT contains mutant SNP sequence; COM is a common PCR primer; [gm tail] is 5′-gaaggtgaccaagtcatgc; and [red tail] is 5′-gaaggtgaccaagtcatgc.
region of the corresponding Universal Amplifluor (green or red; Fig. 1). Expected sizes of PCR amplicons that include the tagged allele-specific primers and Universal Amplifluor primers (Amplifluors) are listed in Table 2.

Final concentrations of PCR reagents were 200 μM of each deoxynucleoside triphosphate, 1.0 U/reaction of either Taq DNA polymerase (Roche Biochemical) or Platinum® Taq DNA polymerase (Life Technologies), 250 mM of both Universal Amplifluor primers and COM (reverse) primer, and 25 nM of both tagged allele-specific primers in 20 μL. The (1×) reaction buffer was 1.8 mM MgCl₂, 50 mM KCl, and 10 mM Tris, pH 8.30. The Amplifluor reagent system (Seralogicals Corp.) includes two Universal Amplifluor primers [labeled with fluorescein (FAM) or sulforhodamine (SR)], 10× PCR buffer, and deoxynucleoside triphosphates. PCR reactions were set up and performed in optically clear PCR microplates (VWR Scientific Products) and sealed with PCR plate-sealer adhesive tape (Robbins Scientific Corp.).

Amplifications were performed in a PTC-200 gradient thermal cycler (MJ Research) with the following conditions: a pseudo-soft start of 5–10 s at 94°C, denaturation of 4 min at 95°C, then 35 cycles (10 s at 94°C, 20 s at 55°C, and 40 s at 72°C), followed by 3 min of final extension at 72°C. PCR reactions were held at 20°C until fluorescence measurements could be performed.

SNP PCR reactions were optimized by performing PCRs with several 10× PCR buffers [buffer K: 600 mM Tris-HCl (pH 9.5), 150 mM (NH₄)₂SO₄, and 25 mM MgCl₂; buffer N: 600 mM Tris-HCl (pH 10.0), 150 mM (NH₄)₂SO₄, and 20 mM MgCl₂; and buffer I: 100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 18 mM MgCl₂] and then analyzing the PCR products by gel electrophoresis for yield and specificity. One buffer that gave maximum amplicon yield and specificity was subsequently selected for all SNP PCRs. We also performed temperature-gradient PCRs to investigate the potential influence of Amplifluors on amplicon yield and specificity of PCR as a function of annealing temperature (range, 50–70°C). The optimum combination of target amount and cycle number that provided the best yield of PCR amplicon was also determined.

**Table 2. Five hypertension SNP amplicons and their response to restriction endonuclease digestion.**

<table>
<thead>
<tr>
<th>Gene, SNP</th>
<th>Restriction enzyme</th>
<th>Amplicon size, bp</th>
<th>Restriction product sizes, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone synthase, −344C→T</td>
<td>HaeIII</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>WT, SNP (C)</td>
<td>HaeIII</td>
<td>126 + 44</td>
<td></td>
</tr>
<tr>
<td>MUT, SNP (T)</td>
<td>HaeIII</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>Angiotensinogen, 842T→C (M235T)</td>
<td>MwoI</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>WT, SNP (T)</td>
<td>MwoI</td>
<td>65 + 45</td>
<td></td>
</tr>
<tr>
<td>MUT, SNP (C)</td>
<td>MwoI</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>GRK4, 448G→T (R65L)</td>
<td>AatII</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>WT, SNP (T)</td>
<td>AatII</td>
<td>51 + 40</td>
<td></td>
</tr>
<tr>
<td>MUT, SNP (T)</td>
<td>AatII</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>GRK4, 679C→T (A142V)</td>
<td>CviII</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>WT, SNP (C)</td>
<td>CviII</td>
<td>81 + 39</td>
<td></td>
</tr>
<tr>
<td>MUT, SNP (T)</td>
<td>CviII</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>GRK4, 1711C→T (A486V)</td>
<td>AcII</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>WT, SNP (C)</td>
<td>AcII</td>
<td>70 + 40</td>
<td></td>
</tr>
<tr>
<td>MUT, SNP (T)</td>
<td>AcII</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

* Amplicon sizes include Universal Amplifluor primer sequences plus a 21-nt tail. Single letters in parentheses are polymorphic nucleotides.

**Fluorescent measurements and data analysis**

Total fluorescence (as relative fluorescence units) of labeled Universal Amplifluor primer-containing amplicons was quantified through the top of each well of open PCR microplates using a Victor™ 1420 fluorescence microplate reader (Perkin-Elmer Wallac, Inc.). The microplate reader was equipped with the narrow bandpass filters to quantify FAM (excitation, 485 nm; emission, 535 nm) and SR (excitation, 585 nm; emission, 620 nm).

Fluorescence results were transferred to separate Excel worksheets for analysis, and scatterplots for each SNP locus were built as follows. Signals from WT (usually FAM-labeled primer) alleles were plotted along the x axes, whereas signals from MUT (usually SR-labeled primer) alleles were plotted along the y axes. In the typical labeling scheme, fluorescence of samples that have a homozygous WT genotype accumulate along the x axis, whereas signals from samples with the homozygous MUT genotype accumulate along the y axis. Signals from the heterozygous genotypes tend to cluster along a diagonal line between the x and y axes. SIGNALS of no-template (blank) PCRs appear near the x,y origin.

Genotype frequencies were compared with Hardy–Weinberg expectations, and allele frequencies were compared between normotensive and hypertensive groups by the method of Rousset and Raymond (29).

**Sequence confirmation of SNP amplicons**

Confirmation of the WT and MUT amplicon sequences at five of six SNP loci was performed by use of a sequence-appropriate restriction endonuclease to digest the PCR products. After fluorescence quantification, PCR amplicons were typically purified by precipitation using 2× volumes of absolute ethanol and then resuspended with deionized H₂O and restricted with one to two units of an appropriate restriction endonuclease (see Table 2). Restriction endonucleases (AatII, AcII, HaeIII, and MwoI) were from New England Biolabs, and CviII was from Clonex. After incubation, reaction mixtures (volume, 20 μL) were separated by gel electrophoresis on 4% agarose gels in 1× Tris-acetate-EDTA buffer, followed by staining with ethidium bromide. Sizes of digested amplicons were determined by comparison with a 10-bp size ladder (New England Biolabs). The expected digestion patterns and sizes of the SNP-containing amplicons are listed in Table 2 (and illustrated in Fig. 2 for one GRK4 SNP).
PCR amplicons (volume, 20 µL) from microplate wells were purified by precipitation using two volumes of ethanol, resuspended in deionized H₂O, and then digested with AatII for 1 h. Restriction products were separated by electrophoresis in 4.0% agarose gels and then stained with ethidium bromide and visualized with ultraviolet transillumination. The genotype of each sample was assigned using the size and number of restriction products observed in the gel (see Table 3).

**Results**

**PRIMER DESIGN**

By use of Oligo v6.42 software, we designed the forward allele-specific primers and the COM reverse primers to have a broad range of annealing temperatures between 50 and 70 °C. The original allele-specific primers obtained using Oligo software were modified by adding 21-nt sequences at the 5’ ends, which matched the 3’ ends of appropriate Universal Amplifluors (18). Y.K. (Serologicals Corp.) also supplied some guidelines that were used to review the sequence of the tailed allele-specific primers before their synthesis. The modified allele-specific primers were rechecked using Oligo to ensure that no undesired sequence interactions might occur because of the addition of the tailed sequence.

**OPTIMIZATION OF THE PCR ASSAY**

Our goal in optimizing these PCR-based assays was the creation of a single amplicon band for each SNP locus when analyzed in agarose gel electrophoresis. For most of the assay development studies, we performed the PCRs without adding the Universal Amplifluors and analyzed the amplicons by gel electrophoresis. The gels were then stained with ethidium bromide to visualize the PCR products. However, when the Universal Amplifluors were added, it was evident from visual inspection of the gels under ultraviolet light that all PCR products displayed one of three colors: green (FAM) for homozygous WT genotypes, red (SR) for homozygous MUT genotypes, and orange for heterozygotes.

The optimum annealing temperatures to amplify the GRK4 loci ranged from 53 to 60 °C, whereas amplifying the remaining loci was best done at 55 to 65 °C. These annealing temperatures were allele discriminatory and provided efficient amplification; therefore, a consensus annealing temperature of 55 °C was used for genotyping of all six loci.

Each of the six SNP loci PCRs was evaluated for annealing temperature dependence without and with Universal Amplifluors. In the latter arrangement, fluorescence of the homozygous PCR tubes was first quantified in the Victor 2 microplate reader, and then the reaction contents were analyzed via agarose gel electrophoresis. The annealing temperature study of one SNP loci (angiotensigen promoter) is depicted in Fig. 3, which illustrates the variation of amplicon yield with increasing annealing temperature for PCRs assembled without (Fig. 3A) and with (Fig. 3B) Universal Amplifluors. The gel image (Fig. 3A) also shows much nonspecific amplification at annealing temperatures <58 °C. A parallel examination of Fig. 3B revealed virtually no nonspecific amplification in corresponding lanes throughout the range of annealing temperatures studied. A plot of the quantified fluorescence of the PCR amplicon from the Amplifluor-containing label (Fig. 3C) indicates that the maximum signal is obtained with an annealing temperature of 61 °C. Similar results were obtained for two other SNP PCRs.

We varied the template amount (as ng) vs cycle number (results not shown). Fewer PCR cycles (e.g., 27–30) were sufficient when a larger amount of template DNA was used (50 ng or higher). Thirty-five cycles were adequate to amplify all loci using 40 ng of genomic DNA template.

**GENOTYPING OF SNP LOCI**

We optimized this genotyping assay using cDNA clones of known genotypes of the three GRK4 SNPs. The three

![Sample Number](https://academic.oup.com/clinchem/article-abstract/48/12/2131/5642442)

**Fig. 2.** Confirmation of SNP genotype using restriction digests of amplicons.

Confirmation restriction digests of 13 GRK4 SNP (448G>T) amplicons with AatII are shown. PCR amplicons (volume, 20 µL) from microplate wells were purified by precipitation using two volumes of ethanol, resuspended in deionized H₂O, and then digested with AatII for 1 h. Restriction products were separated by electrophoresis in 4.0% agarose gels and then stained with ethidium bromide and visualized with ultraviolet transillumination. The genotype of each sample was assigned using the size and number of restriction products observed in the gel (see Table 3).

**Fig. 3.** PCR annealing temperature study demonstrating influence of Universal Amplifluor reagents on the specificity of the reaction.

(A), PCR annealing temperature study of angiotensigen promoter SNP using only the allele-specific primers and COM downstream primer. Gradient temperature PCRs were performed during the annealing cycle with a temperature range of 50 °C to 70 °C. PCR conditions are listed in the text. Note the amount of nonspecific amplification at temperatures below the annealing temperature of 60 °C. The expected size of the SNP amplicon (Amplicon (+)) is ~90 bp (see dashed line). (B), PCR annealing temperature study of angiotensigen promoter SNP using allele-specific primers and COM downstream primer plus both Universal Amplifluor primers in PCR reaction. The annealing temperature range is the same as described in panel A. Note the absence of nonspecific amplification throughout the annealing temperatures used. The expected amplicon Amplicon (+) is now ~20 bp larger (~110 bp) because of inclusion of the Amplifluors. Panels A and B: lane M, 100-bp DNA ladder. (C), quantification of fluorescence signals (using the Victor microplate reader) from SNP amplicons containing Universal Amplifluors. Note that the maximum fluorescence is near 60 °C, although the signal remains >50% of maximum at both temperature limits.
genotypes for each GRK4 SNP locus yielded clearly distinct data points on the fluorescence scatterplot. The fluorescence of homozygous genotype amplicons, the alleles of which contained FAM, was approximately twice as large as the fluorescence of homozygous genotype amplicons with alleles containing SR (e.g., 1 FAM ≈ 2 SR; Fig. 4). This unequal ratio was observed for all six SNPs but contrasts with the nearly equal intensities reported by Myakishev et al. (18). Heterozygous samples yielded almost equal fluorescent values with both fluorophores (e.g., ~1 FAM = 1.4 SR); Fig. 4).

Typical results of fluorescence scatterplots from three GRK4 SNPs in normotensive and hypertensive populations illustrate that all samples fit within one of three clusters corresponding to the genotype of each sample (Fig. 4). Fluorescence from three blank samples clearly fell away (near the x,y origin) from the clusters of genomic DNA samples in the scatterplots (Fig. 4). Subsequent restriction digests of five of six PCR products confirmed the genotype assessment determined using the Amplifluor method (example shown in Fig. 2).

CLINICAL EVALUATION
A genotyping study of 120 Italian genomic DNA samples was done on blood samples collected from salt-sensitive hypertensive patients and matched normotensive controls (26). Allele frequencies for all six SNPs in both groups were compared with the Hardy–Weinberg principle, and all were compliant except the 842T→C normotensive population that was marginally noncompliant (P = 0.037; Table 2). Allele frequencies were then compared between hypertensive and normotensive individuals for the six SNPs (29). Allele frequencies of normotensive persons differed significantly from those of hypertensive persons at the GRK4 SNP 1711C→T (P = 0.034; Table 3). Allele frequencies that distinguished normotensive persons from hypertensive persons at the angiotensinogen polymorphism 842T→C (M235T) were also close to significance (P = 0.059).

Discussion
The present study describes a SNP genotype analysis system that is simple and easy and also does not require post-PCR processing. Because it is a single-tube method, it facilitates high-throughput SNP genotyping by use of allele-specific primers that differ at their 3' end. In contrast with systems based on other typing processes, such as TaqMan (12) and molecular beacons (13, 30), the Amplifluor detection system requires few materials beyond what is necessary for a standard PCR amplification. Only two labeled oligonucleotides are needed to genotype all possible SNPs, and there are no changes needed in reaction conditions or experimental set-up. Each genotyping assay uses five primers/SNP locus: two (forward) allele-specific tailed primers, two Universal Amplifluor primers, and one COM (reverse) primer. The tails of the allele-specific primers are identical to the 3' regions of either green (FAM) or red (SR) Universal Amplifluor primers (18, 19). The Amplifluor system thus combines the use of allele-specific PCR amplification (31–33) and a novel energy transfer primer system that emits unique fluorescence (FAM or SR) only on incorporation into their respective, allele-specific PCR amplicons (18). Compared with existing genotyping methods, the major advantages of this assay are the elimination of post-PCR processing and the reduction of time required for sample analysis. If the microplate can be scanned while it is still sealed, the risk of amplicon contamination can also be eliminated.

PCR CONDITIONS USING AMPLIFLUORS
To minimize the sequence competition between the Amplifluors and the tailed allele-specific primers for amplimers, the latter are used in one-tenth (25 nmol/L) concentrations of those of the corresponding Amplifluors and COM primers (250 nmol/L) because they are needed only during the first few cycles of amplification. In the early PCR cycles, the extension of the two allele-specific primers yields an amplicon for which one strand is complementary to a corresponding region of the Amplifluors (18, 34). In subsequent cycles of PCR, the amplicons are further amplified by the corresponding Universal Amplifluors and the COM downstream primer (Fig. 1).

Unincorporated Universal Amplifluor primers have minimal fluorescence (19, 35) because of the hairpin stem conformation structure present at their 5' end. When the Universal Amplifluors are free in solution, the fluorophores (FAM and SR; green and red primers, respectively) and the quencher (DABSYL; located on the opposite sides of the 5'-end hairpin structure) are in close proximity, and the fluorescence is efficiently quenched (Fig. 1). When the hairpin unfolds on incorporation into the amplicon, energy transfer diminishes between the fluorophore and the quencher, and subsequently a large increase in fluorescence signal (>20-fold) is generated, as commonly described for other FRET-based methods (36).

Genomic sequences that are closely related to the intended target sequence can also contribute to nonspecific amplification at lower annealing temperatures. We found that even when there is nonspecific amplification or a low yield of amplicon, the fluorescent signal from the target band can be measured easily, probably because of the high selectivity of the tailed primers and/or the sensitivity of the plate reader.

The addition of Universal Amplifluor primers to the allele-specific tailed primers completely eliminates the nonspecific amplification, even at a lower nonoptimum annealing temperature (Fig. 3). This is likely because of the 10:1 excess ratio of Amplifluor-labeled primer to the corresponding allele-specific primer that dominates the amplification reaction after the second round of PCR. Thus, the allele-specific primers define the genomic sequence to be amplified, and the corresponding Universal Amplifluors eliminate nonspecificity (Fig. 3B). Because
Fig. 4. Fluorescence scattergrams for three GRK4 SNPs.

Six scattergrams of fluorescence results for FAM- and SR-labeled Universal Amplifluor amplicons generated from three GRK4 SNPs are shown. For the GRK4 SNPs 448G→T (R65L) and 1711C→T (A486V), the x axes contain the fluorescence data from the FAM-labeled allele (WT), whereas the y axes contain fluorescence data from the SR-labeled allele (MUT). For the GRK4 SNP 679C→T (142V), the x axis contains the fluorescence data from the SR-labeled allele (WT), whereas the y axis contains fluorescence data from the fluorescein-labeled allele (MUT). In each set of panels representing a separate GRK4 SNP, the panel on the left represents data from the normotensive (control) population, whereas each corresponding right-hand panel represents GRK4 SNP data from the hypertensive (patient) population. In each plot, results from blank samples (no-template DNA) are represented by ◦, whereas the samples are represented by ●. RFU, relative fluorescence units.
real-time fluorescence monitoring was not available on the plate reader, we used agarose gel analysis and signal measurements of the Universal Amplifluor-labeled PCR amplicons to assess the contribution of primer oligomerization in no-template samples to the fluorescent signals.

Allele specificity (selectivity) can be greatly influenced by choice of DNA polymerase, salt concentration, and annealing temperature during PCR amplification (37). We recognized that because Taq DNA polymerase has demonstrated poor sequence mismatch discrimination (37), we used it only during PCR optimization. Salt concentration is also important because short PCR products are efficiently synthesized at 50 mM KCl, whereas longer PCR products require minimal KCl concentration for optimal synthesis (37, 38). We therefore used 50 mM KCl in the PCR so that alleles of all six hypertension SNPs were adequately discriminated. During PCR optimization, we examined three buffers, I, K, and N (only I contains KCl). For routine genotyping analysis, we used Platinum Taq DNA polymerase, which exhibits substantially enhanced allele discrimination in the presence of 50 mM KCl found in Buffer I.

During our studies, we found, as others have (37, 39), that a hot-start is essential to minimize formation of primer dimers and other nonspecific amplification reactions that lead to nonspecificity. This result is in accordance with similar approaches taken to prevent primer oligomerization-based backgrounds (34, 37, 40). If the initial copy number of genomic DNA used is sufficient, primer-dimer contribution to the final PCR product should not be significant, and primer dimers do not seem to interfere with the fluorescent signal measurement. We also found that if the number of cycles was >35, primer oligomers are created, and the fluorescent signals from no-template controls are substantially increased, which could lead to false genotyping. In addition to the use of the hot-start process, we recommend that the SNP PCRs be performed with the two-tailed, allele-specific primers at one-tenth the concentration of the Amplifluors and the COM primer (19), as shown by comparing Fig. 3A with Fig. 3B.

**COST ANALYSIS OF SNP TESTING**

Because our method requires only a thermal cycler and fluorescence microplate reader, our instrumentation costs appear lower than other homogeneous methods to detect SNPs associated with hypertension (41). To better identify the costs of performing this assay, we performed a financial analysis of the four major components of this SNP method: (a) instrument costs/depreciation; (b) reagent costs; (c) consumables; and (d) labor. We found that the cost/SNP for each of the four parts was: (a) $0.177/SNP; (b) $2.476/SNP; (c) $0.275/SNP; and (d) $0.469/SNP. Total cost/SNP was $3.40.

Several approaches to reducing this cost include purchasing reagents in large amounts to obtain discount pricing and miniaturizing the assay (42) to perform it in a 384-well microplate format. For the latter option, we estimate that the cost/SNP could be reduced by 60% or more. The cost of the labor component could also be decreased by using a robotic pipetting workstation (see below).

**SAMPLE THROUGHPUT AND USE OF AUTOMATION**

Once allele-specific primers have been designed for a given SNP, a large number of samples can be genotyped relatively quickly. With current instrumentation, this assay is capable of simultaneously genotyping 90 unknown samples for each SNP (each microplate also includes three blanks and three controls). With a 30-min PCR set-up, a 90-min PCR amplification step, and a 20-min genotype scoring and data analysis, one individual can process two to three runs of 96-well plates/day. This genotyping method could also be easily adapted to automated liquid handling (pipetting) workstations (e.g., TECAN Genesis, Beckman Biomek, and others) using 384-well PCR plates. On the basis of our experience using pipetting workstations, all six SNPs could be analyzed using 384-well PCR plates, because the pipetting robots could rapidly deliver the reagents and genomic samples into 384 PCR plates with greater precision in much less time than needed by manual means. Thus, analysis of all six SNPs could be performed on 120 samples in <1 day using two 384-well PCR plates.

**HYPERTENSION GENOTYPE RESULTS**

We have reported that GRK4 SNPs are implicated in genetic hypertension because they participate in the de-
sensitization of G protein-coupled receptors, including D₁ receptors, which leads to sodium retention (20). Additional candidate genes have also been investigated for association with hypertension, including aldosterone synthase (25), angiotensinogen (22, 23), angiotensin receptor (24, 43), and angiotensin-converting enzyme (21). Although demonstrated for isolated populations, each of these SNPs has not been uniformly associated with essential hypertension across many different populations.

To investigate the use of this procedure to perform wide-scale screening, we used the Amplifluor-based SNP method to analyze 120 samples for six SNP loci, all considered linked to essential hypertension. With the PCR-based method described here, genotypes from all 120 DNA samples were successfully determined and then confirmed by locus-specific restriction digests. Of the six SNPs examined in this study, only the GRK4 1711C→T (A486V) polymorphism was significantly different (P = 0.034) for hypertensive persons compared with the normotensive group in this population. Analysis of this SNP may therefore be useful in predicting the onset of hypertensive phenotype in individuals of Italian descent. Salt-sensitive hypertensives and those with low plasma renin have also been associated with polymorphisms of α-adducin (27, 44). It will be of interest to determine whether SNPs of GRK4 and α-adducin interact to create salt-sensitive hypertension.

Variations of SNP allele frequencies are well known in hypertension studies (7). In a study of 163 Caucasians enrolled in the Berlin Salt Sensitivity Trial, Brand et al. (25) studied the aldosterone synthase polymorphism (−344C→T) and found a frequency of 0.55 for the C allele, which is lower than our results of 0.83 for this allele. They found no significant difference in normotensive vs hypertensive groups, which is similar to our findings.

The angiotensinogen gene has been studied extensively, and several allele frequencies have been published for different populations and SNPs (22, 23, 44). In a study of the M235T SNP in 311 normotensive and hypertensive patients, Schmidt et al. (45) found no significant difference between these populations (P = 0.137), which is similar to our results (Table 2; P = 0.059). Their allele frequency for the WT M235 allele in normotensive populations was 0.59, which agrees well with our value of 0.49. When they stratified their populations according to age, they did find, however, a significant association as the age of hypertensive patients decreased to <40 years; the allele frequency also decreased to 0.45, which agrees well with our value of 0.38. It thus appears that age stratification is a useful risk assessment factor when using this SNP for hypertension studies. In another study involving the M235T SNP of angiotensinogen, Jeunemaître et al. (23) found a significant difference in hypertensive persons in a population of 841 French Caucasians (P = 0.004). They found an allele frequency of 0.38 for the MUT T235 allele in the normotensive populations, which is opposite to our value of 0.51 for the T235 allele (Table 2).

Our findings that five of the six SNPs failed to demonstrate an association with hypertension are consistent with other single-locus association studies that fail to retain their significance, especially when tested against different ethnic groups (46). Moreover, not all alleles in one gene may be informative, unless they are in linkage disequilibrium, as discovered for the angiotensinogen SNPs M235T and −6G→A (23). A recent study has demonstrated that analysis of multiple genes may be more predictive than single polymorphisms because nonallelic or epistatic gene interactions may be important in the risk of hypertension (47), particularly when multiple ethnic groups are being examined. Alternative experimental techniques that detect linkage disequilibrium may therefore be useful to complement SNP studies until the number of known, characterized SNPs covers the genome at the 50-kb range (48).

In summary, the implementation of the Universal Amplifluor primer system for genotyping six independent SNP loci with common reaction and cycling conditions leads us to conclude that other SNP loci detected using a previously established PCR assay can be readily genotyped with minimal modifications of their existing experimental protocol. The major task necessary to genotype additional SNP loci using this technique is to create tailed allele-specific primers. We conclude that this approach is an accurate, sensitive, and high-throughput oriented tool for genotyping significant SNPs for association studies or mapping genetic linkages.

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