Complex haplotypes derived from noncoding polymorphisms of the intronless α_{2A}-adrenergic gene diversify receptor expression

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α_{2A}-adrenergic receptors (α_{2A}AR) regulate multiple central nervous system, cardiovascular, and metabolic processes including neurotransmitter release, platelet aggregation, blood pressure, insulin secretion, and lipolysis. Complex diseases associated with α_{2A}AR dysfunction display familial clustering, phenotypic heterogeneity, and interindividual variability in response to therapy targeted to α_{2A}ARs, suggesting common, functional polymorphisms. In a multiethnic discovery cohort we identified 16 single-nucleotide polymorphisms (SNPs) in the α_{2A}AR gene organized into 17 haplotypes of two major phylogenetic clades. In contrast to other adrenergic genes, variability of the α_{2A}AR was primarily due to SNPs in the promoter, 5′ UTR and 3′ UTR, as opposed to the coding block. Marked ethnic variability in the frequency of SNPs and haplotypes was observed: one haplotype represented 70% of Caucasians, whereas Africans and Asians had a wide distribution of less common haplotypes, with the highest haplotype frequencies being 16% and 35%, respectively. Despite the compact nature of this intronless gene, local linkage disequilibrium between a number of SNPs was low and ethnic-dependent. Whole-gene transfections into BE(2)-C human neuronal cells using vectors containing the entire ≈5.3-kb gene without exogenous promoters were used to ascertain the effects of haplotypes on α_{2A}AR expression. Substantial differences ($P < 0.001$) in transcript and cell-surface protein expression, by as much as ≈5-fold, was observed between haplotypes, including those with common frequencies. Thus, signaling by this virtually ubiquitous receptor is under major genetic influence, which may be the basis for highly divergent phenotypes in complex diseases such as systemic and pulmonary hypertension, heart failure, diabetes, and obesity.

adenylyl cyclase | G protein-coupled receptor | pharmacogenetics | sympathetic nervous system

The α_{2A}-adrenergic receptor (α_{2A}AR) regulates an unusually diverse group of functions compared to most G protein-coupled receptors. These include inhibition of neurotransmitter release from presynaptic nerves, inhibition and stimulation of postsynaptic central nervous system signaling pathways, inhibition of lipolysis in adipocytes, stimulation of platelet aggregation, and inhibition of insulin secretion from pancreatic islet cells (1). Familial clustering of phenotypes and ethnic variability are observed in a number of diseases associated with aberrancies of the above physiologic responses, including heart failure, hypertension, and obesity (2–4). And in familial syndromes such as the familial cardiomyopathies reduced penetrance and generation skipping are not common (2, 5). Furthermore, extensive interindividual variability in risk, progression, and response to therapy is typical in these complex diseases (6). Such observations suggest that underlying polymorphisms within α_{2A}AR-related signal transduction pathways could be responsible for susceptibility or disease modification.

In the central nervous system α_{2A}ARs modulate sympathetic output to peripheral nerves that regulate vascular smooth muscle contraction and thereby peripheral vascular resistance, and centrally acting α_{2A}AR agonists such as clonidine are used for treatment of hypertension. However, the interindividual variability of the antihypertensive response to α_2AR agonists, as well as the central and peripheral adverse effects, has limited the use of these agents (7). In cardiac presynaptic nerve endings, the expressed α_{2A}AR and α_{2C}AR act locally to control norepinephrine release in a negative-feedback manner (8). The α_{2A}AR regulates release due to high neuronal stimulation frequencies, indicating a role when sympathetic activity is high or when it is rapidly changing (8). In heart failure, the characteristic adrenergic response to decreased cardiac output is increased norepinephrine release, acting on cardiac βARs to increase contractility and vascular α_1ARs and (potentially) α_{2A}ARs to increase peripheral vascular resistance. Although acutely this response is advantageous, prolonged elevations of norepinephrine promote cardiomyopathic events and lead to worsening heart failure (9). Thus, genetic variability of α_{2A}AR function would be expected to have significant effects on clinical phenotype or the response to β-blocker treatment. In obesity the balance between lypolytic βAR and antilypolytic α_{2A}ARs is disrupted, with a major effect being attributed to altered α_{2A}AR function, although there is substantial variability in the phenotype (10). Attempts to manipulate fat cell adrenergic receptor signaling for treatment of human obesity have largely been unsuccessful, which may suggest that the interindividual responsiveness may be due to polymorphisms of the target receptors.

The critical roles of α_{2A}ARs in these physiologic processes has prompted association and family studies of receptor genetic variability to identify potential risk factors or disease modifiers and pharmacogenomic studies to identify potential treatment–response loci for multiple central nervous system, cardiovascular, and metabolic diseases (11–23). These studies were performed with single restriction fragment-length polymorphisms (RFLPs) of the α_{2A}AR gene, such as a DraI RFLP, and generally have shown only weak or no associations or a lack of reproducibility. These findings with a few individual single-nucleotide polymorphisms (SNPs) prompted us to consider whether there are other polymorphisms in the gene organized into multiple, potentially common and ethnic-specific haplotypes. Furthermore, we hypothesized that such haplotypes would have functional significance manifested by differential transcript and receptor protein expression. To approach this issue, we resequenced the intronless human α_{2A}AR in a multiethnic DNA repository and found 16 SNPs. These polymorphisms were distributed in 17 different haplotypes grouped into two phylogenic clades. The haplotypic variation was unique compared with other adren-

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Abbreviations: α_{2A}AR, α_{2A}-adrenergic receptor; RFLP, restriction fragment-length polymorphism; LD, linkage disequilibrium; SNP, single-nucleotide polymorphism.

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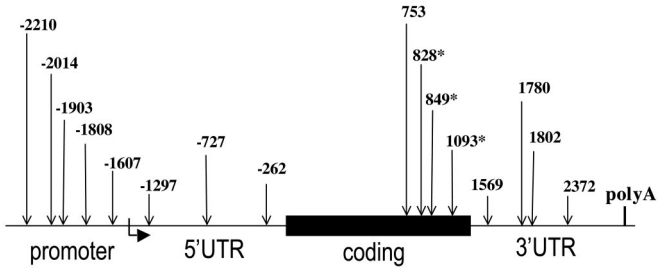

Fig. 1. Organization and localization of polymorphisms of the intronless human α_{2A}AR gene. Indicated are the promoter, 5′ UTR, coding region, and 3′ UTR boundaries and the nucleotide position of the polymorphism. +1 is the adenine of the initiator methionine. *, Synonymous coding.

ergic receptors in that it was almost entirely due to promoter, 5′ UTR, and 3′ UTR SNPs. Despite the compact nature of this intronless gene (≈5.3 kb), local linkage disequilibrium (LD) between a number of SNPs was relatively low, and multiple SNPs or haplotypes showed markedly different frequencies in three ethnic populations. Using whole-gene transfections into a human neuronal cell line we found significant differences in cellular α_{2A}AR mRNA and protein expression between many of the α_{2A}AR haplotypes.

Results and Discussion

The α_{2A}AR Gene Has 16 Single-Nucleotide Polymorphic Sites. The most common sequence of the intronless α_{2A}AR gene was ascertained from resequencing and was assigned as the reference sequence with submitted GenBank accession no. DQ149926. In this report, the first nucleotide of the ORF is nucleotide 1 (which corresponds to nucleotide 2524 of the reference sequence) with the 5′ UTR beginning at nucleotide −1 and continuing in the negative direction. The organization of the α_{2A}AR gene is depicted in Fig. 1, indicating the polymorphisms found from resequencing. All were single-nucleotide substitutions. Altogether there were 16 SNPs: five in the promoter, three in the 5′ UTR, four in the coding region (one nonsynonymous), and four in the 3′ UTR (Table 1). The single nonsynonymous variant at position 753 is the previously described Asn-to-Lys change at amino acid 251 located within the third intracellular loop of the α_{2A}AR, which has been shown to confer

Table 1. Polymorphisms of the human α_{2A} AR gene

		Minor allele frequency, %		
Position	Base*	CA (n = 80)	AA (n = 80)	AS (n = 40)
−2210	T/A	71	18	20
−2014	C/T	0	3	0
−1903	G/C	0	5	0
−1808	G/T	3	14	13
−1607	C/G	0	5	0
−1297	C/G	71	29	20
−727	C/G	11	5	23
−262	G/A	6.3	29	13
753	C/G	0	5	0
828	C/T	0	0	2.5
849	C/G	0	1	0
1093	C/A	1.3	8.8	38
1569	C/A	6.3	29	13
1780	G/A	23	28	68
1802	C/A	3	14	13
2372	A/G	3	10	38

Shown are the positions based on the coding block adenine = +1. n, number of chromosomes genotyped. CA, Caucasian; AA, African American; AS, Asian.

*The first base of the allelic pair is the most common in the total population.

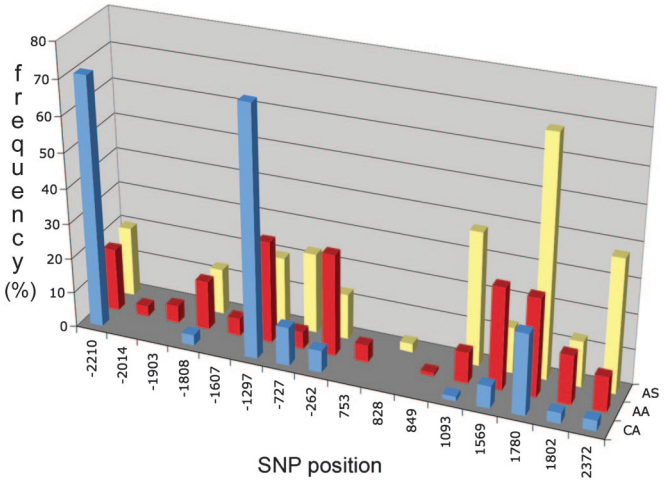

Fig. 2. The frequencies of α_{2A}AR polymorphisms display marked ethnic variation. The polymorphism positions and frequencies are given on the x and y axes, respectively, stratified by ethnic group (z axis). CA, Caucasian; AA, African American; AS, Asian.

enhanced agonist-promoted Gi coupling in transfected cells (24). The polymorphism altering a DraI restriction site has been reported (13) as a G-to-A substitution at position 1780 followed by a G deletion at position 1781 of the 3′ UTR. However, we never found the deletion, but the G-to-A transversion was observed frequently. All SNPs were in Hardy–Weinberg equilibrium ($P > 0.05$) except for −2210 and −1297 in the Asian population, most likely because of the smaller sample size. There are 10 cosmopolitan SNPs, being found in all three ethnic groups, but some significant differences in allele frequencies were notable and are readily visualized by location and ethnic group in Fig. 2. SNPs at positions −2210 and −1297 were >3-fold more common in Caucasians compared with the other two groups. Indeed, in Caucasians the T and C at these two positions, respectively, are by far the common allele, whereas in the other two groups these represent the minor alleles at frequencies ranging from 0.29 to 0.18. Similarly, SNPs −727, 1093, 1780, and 2372 were more common in Asians, with the A at position 1780 being the major allele only in this ethnic group (Fig. 2). The 1093 SNP was ≈20-fold more common in Asians than in Caucasians, and 1780 was ≈2- to 3-fold more common compared with Caucasians and African Americans. Five SNPs (−2014, −1903, −1607, 753, and 849) were found exclusively in African Americans, although the allele frequency of the latter was only 0.01. Compared with the β_1AR (25, 26), β_2AR (27, 28), α_{2B}AR (29), and α_{2C}AR (30), which have high-frequency nonsynonymous polymorphisms, the current data show that the ORF of the α_{2A}AR is virtually invariant at the encoded protein level except for the uncommon substitution at 753, which was found only in those of African descent.

LD was calculated as r^2 and is shown in Fig. 3 grouped by ethnicity. This figure also indicates the numerous monomorphic sites present in Caucasians and Asians (denoted by white squares) as well as the single monomorphic site present at position 828 in African Americans. Among all groups, there were few polymorphic pairs that showed high levels of LD (defined as $r^2 > 0.8$, orange to red blocks). Importantly, multiple SNP pairs that showed moderate ($r^2 \geq 0.5 < 0.8$) or high LD in one ethnic group had low r^2 values in one or both of the other groups. Merging of the data from Fig. 2 identified 11 such instances (Table 2). In Caucasians, moderate to high LD was observed among three SNP pairs, which was low in both of the other groups. In African Americans there were no such instances, but there are two cases where moderate LD was present in this group and Asians but not Caucasians. Asians had three

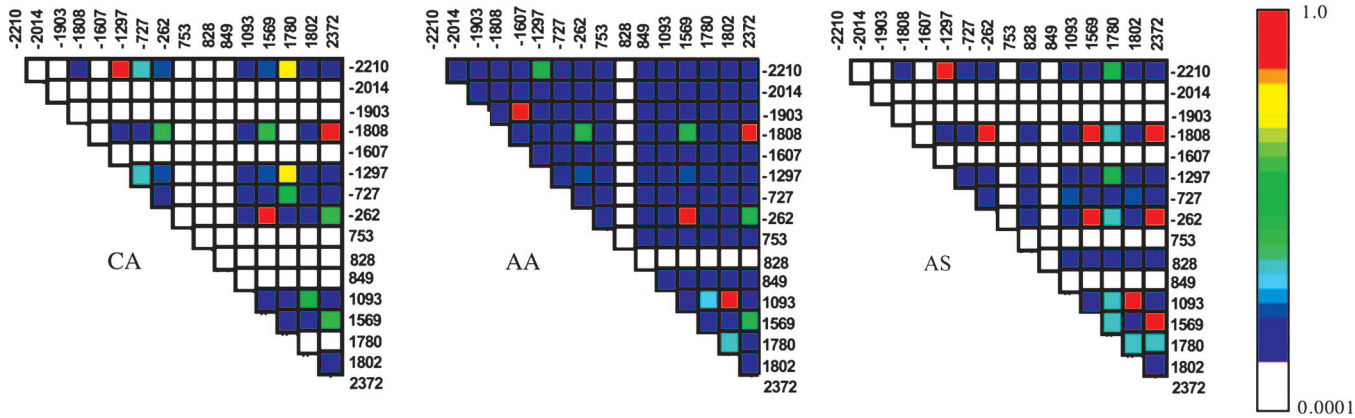

Fig. 3. LD between polymorphisms of the α_{2A}AR gene. LD was calculated as r^2 (see *Materials and Methods*), and data are presented as a matrix such that relative values for any pair of polymorphisms can be determined based on the indicated scale. White squares indicate a monomorphic position in the indicated group.

instances of unique moderate LD and three where at least one of the other two groups had low LD. Thus, despite the compact nature of the α_{2A}AR gene, the disparity by ethnic group in the LD between certain pairs requires an expanded set of sites to be genotyped to obtain accurate genotypic combinations, or haplotypes (see below), in a multiethnic cohort.

α_{2A}AR Polymorphisms Are Organized into Multiple Distinct Haplotypes. The 16 SNPs within the α_{2A}AR gene were found within 17 haplotypes with allele frequencies ≥1% in at least one group. Table 3 shows the frequencies of these haplotypes stratified by ethnic group. There are five cosmopolitan haplotypes (2, 4, 9, 13 and 16), eight haplotypes that are unique to African Americans, and two Asian-specific haplotypes. African Americans displayed the greatest haplotypic variation, with 15 of the 17 possible haplotypes represented. These haplotypes occurred with frequencies ranging from ≈1 to 16%. In contrast, Caucasians and Asians exhibited comparably less haplotypic variation. Seven different haplotypes were present in Caucasians, with haplotype 16 representing the vast majority (≈70%) and the remaining occurring with frequencies between ≈1 and 11%. In Asians, haplotypes 4, 9, and 16 were present at frequencies between 20% and 35%, and the less common haplotypes 2, 6, and 13 were present at frequencies between ≈2% and 8%. Of note, the group formed by haplotypes 1, 3, 5, 7, 8, 10, 11, 14, and 15 represent 48% of African Americans compared with 5% of Caucasians. The nonsynonymous gain-of-function polymorphism (24) at position 753 was present only in haplotype 11. The 3′ UTR polymorphism at position 1780 (DraI RFLP), which has shown equivocal results in association studies (see above), was segregated into six different haplotypes: 2, 4, 5, 6, 9, and 14, ranging in frequencies of ≈1–23% in the various ethnic groups. This partitioning is consistent with this site's having the highest number of disparities in LD between pairs by ethnic group (Table 2). Phylogenetic analysis revealing the relationships between α_{2A}AR haplotypes is shown in Fig. 4. A deep divergence was observed between two major clades of haplotypes with group A composed of haplotypes 1, 3, 7, 8, 10, 11, 12, 13, 15, 16, and 17 and group B consisting of 2, 4, 5, 6, 9, and 14. Such grouping shows the evolutionary relatedness of the haplotypes and is useful for interpreting the expression data (see below). Several probable recombination events were identified. Given that haplotype 12 is Asian-specific and low-frequency, it likely represents an intragenic recombination (in a region between SNPs −1808 and 1802) of haplotypes 13 and 16. Furthermore, haplotype 7 is most readily resolved as a recombinant between haplotypes 8 and 16. We cannot exclude, however, that haplotype 7 is a revertant, representing a second mutation at SNP position 1569 of haplotype 13.

Table 2. Differences in LD between α_{2A}AR SNP pairs by ethnic group

SNP pair	CA	AA	AS
−2210/−727	0.56	<0.1	<0.1
−2210/1780	0.81	<0.1	<0.1
−1808/1780	<0.1	<0.1	0.55
−1297/−727	0.56	<0.1	<0.1
−1297/1780	0.85	<0.1	0.72
−727/1780	0.66	<0.1	<0.1
−262/1780	<0.1	<0.1	0.56
1093/1780	<0.1	0.50	0.54
1569/1780	<0.1	<0.1	0.55
1780/1802	<0.1	0.54	0.54
1780/2372	<0.1	<0.1	0.54

Shown are r^2 values for any SNP pair where at least one discrepant value was found between groups. CA, Caucasian; AA, African American; AS, Asian.

Variation of the α_{2A}AR Gene Alters Transcript and Protein Expression in a Haplotype-Specific Manner. To ascertain potential effects of these haplotypes on receptor expression, whole-gene transfections were carried out. We used the human neuroblastoma cell line BE(2)-C because neuronal cells are a cell type of physiologic importance, they are of human origin, and they express α_{2A}AR mRNA and protein and thus have transcription factors for regulation of the human gene. For these transfections, the vector pBSII KS+ was used, which lacks a eukaryotic promoter; thus, expression of the intronless α_{2A}AR gene is solely under control of its native 5′ and 3′ flanking sequences. The α_{2A}AR haplotypes in these constructs consisted of the entire contiguous ≈5.3-kb sequence of the gene. We considered the mRNA expression to be the primary phenotype because protein expression requires additional processing that may be less than optimal in an overexpression strategy. Transfections were carried out by using constructs corresponding to those haplotypes found to have an allele frequency of ≥5% in any of the three ethnic groups (haplotypes 1, 2, 4, 8–14, and 16; see Table 3). Detection of α_{2A}AR mRNA was by quantitative real-time PCR using various concentrations of BE(2)-C transcripts for the standard curve. Data were normalized to haplotype 16 (the most common in the collective population). Results, which are shown in Fig. 5*A*, indicate significant variability in transcript expression by haplotype. The relationship between haplotype and transcript expression was significant at $P < 0.001$ by ANOVA. Individual analyses were performed for those haplotypes with potential dif-

Table 3. Haplotype of the α_{2A}AR gene

	Promoter region					5′ UTR			Coding region				3′ UTR				Frequency, %		
Haplotype	−2210	−2014	−1903	−1808	−1607	−1297	−727	−262	753	828	849	1093	1569	1780	1802	2372	CA	AA	AS
1	T	C	G	G	C	G	C	G	C	C	C	C	C	G	C	A	0	11.25	0
2	T	C	G	G	C	G	C	G	C	C	C	C	C	A	C	A	10	7.5	7.5
3	T	C	G	G	C	G	C	G	C	C	C	C	A	G	C	A	0	1.25	0
4	T	C	G	G	C	G	C	G	C	C	C	A	C	A	C	G	1.25	8.75	35
5	T	C	G	G	C	G	C	G	C	C	G	C	C	A	C	A	0	1.25	0
6	T	C	G	G	C	G	C	G	C	T	C	A	C	A	C	G	0	0	2.5
7	T	C	G	G	C	G	C	A	C	C	C	C	C	G	C	A	0	1.25	0
8	T	C	G	G	C	G	C	A	C	C	C	C	A	G	C	A	3.75	13.75	0
9	T	C	G	G	C	G	G	G	C	C	C	C	C	A	C	A	11.25	5	22.5
10	T	C	G	G	C	C	C	G	C	C	C	C	C	G	C	A	0	6.25	0
11	T	C	G	G	C	C	C	G	G	C	C	C	C	G	C	A	0	5	0
12	T	C	G	T	C	G	C	A	C	C	C	C	C	G	A	A	0	0	5
13	T	C	G	T	C	G	C	A	C	C	C	C	A	G	A	A	2.5	13.75	7.5
14	T	C	C	G	G	G	C	G	C	C	C	C	C	A	C	A	0	5	0
15	T	T	G	G	C	G	C	G	C	C	C	C	C	G	C	A	0	2.5	0
16	A	C	G	G	C	C	C	G	C	C	C	C	C	G	C	A	70	16.25	20
17	A	C	G	G	C	C	C	G	C	C	C	C	C	G	C	G	1.25	1.25	0

CA, Caucasian; AA, African American; AS, Asian.

ferences (correcting for multiple comparisons), and a grouping of haplotypic phenotypes was made based on similar expression levels. As can be seen, haplotype 10 displayed markedly higher expression ($P < 0.001$), and haplotype 1 displayed lower expression (range of P values was <0.001 to <0.05) compared with all other haplotypes. Expression of haplotypes 8, 9, 12, 13, and 16 did not differ from each other and thus represents expression phenotypes similar to the most common form of the α_{2A}AR gene, haplotype 16 (reference haplotype/phenotype). Haplotypes 2, 4, 11, and 14 did not differ from each other ($P > 0.05$), and each expressed higher mRNA levels compared with all of the reference haplotypes. These haplotypes, then, represent a moderately high expression phenotype compared with the most common haplotype. Taken together, expression of the haplotypes ranged from lower than, equivalent to, and moderately higher than the reference haplotype 16. Except for the drastic high-expression phenotype observed with haplotype 10, the maximal difference in mRNA expression between the other haplotypes was ≈2-fold. Of note, the DraI RFLP has been studied in isolation by using a vector with the reporter gene chloramphenicol acetyltransferase placed 5′ to a portion of the α_{2A}AR 3′ UTR (13). (Transcription was driven by elements within the vector pcDNA3.) Under these conditions the 6.3-kb allele (representing the A at position 1780) resulted in decreased expression of the reporter gene. In our studies, where the full gene was used and expression of α_{2A}AR mRNA was quantitated, the A at 1780 was associated with increased α_{2A}AR mRNA expression (Fig. 4) for three of the four haplotypes (haplotypes 2, 4, and 14) and similar to the reference expression for haplotype 9. This discrepancy may indicate the necessity of examining expression of the gene of interest (rather than a reporter gene) within the context of all of the polymorphisms of distinct haplotypes.

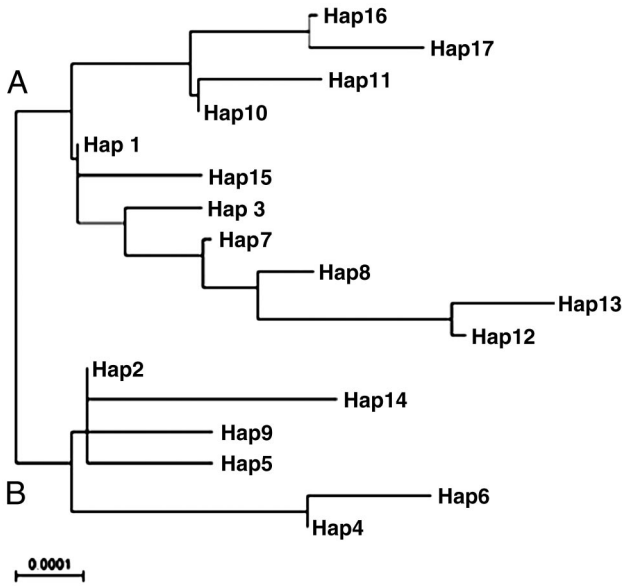

Fig. 4. Phylogenetic analysis of α_{2A}AR haplotypes. The neighbor-joining method was used to reconstruct phylogeny from the full-length nucleotide sequences. Polarity was set by midpoint rooting (see *Materials and Methods*). Two major clades (*A* and *B*) were identified.

α_{2A}AR protein expression was determined by quantitative radioligand binding by using the α_2AR-specific ligand [^{3}H]yohimbine (Fig. 5*B*). There were some clear discrepancies between protein expression and mRNA expression. Most notably, haplotype 10 mRNA expression was greater than all others (Fig. 5*A*), but protein expression did not parallel this profile. Also, haplotype 2 mRNA was higher than haplotype 1, but protein expression, although trending in the same direction, was not statistically significant ($P = 0.1$) and regardless did not approach parity with other haplotypes such as 11 and 14, as was seen with the mRNA results. Haplotype 1 was the lowest-expressing haplotype of all tested, as assessed by both mRNA and protein expression. The haplotypes could be considered in three groups based on similar protein expression profiles: haplotypes 1, 2, 4, and 10 (low expressers), haplotypes 8, 9, 11, 12, and 16 (reference), and haplotypes 13 and 14 (high expressers). The relationship between haplotype and mRNA (or protein) was assessed by post hoc *t* tests, which identified haplotypes whose expressions were or were not different from each other. By so doing, assignment to expression clusters termed low, reference, and high was made. The correlation between haplotype cluster mRNA expression and receptor protein expression was relatively high ($r = 0.8$). The background levels of α_{2A}AR expression in BE(2)-C cells and potentially limited postreceptor signaling elements confound studies of altered signaling (such as inhibition of adenylyl cyclase) by the various haplotypes in these cells. However, like most G protein-coupled receptors, it is well recognized that

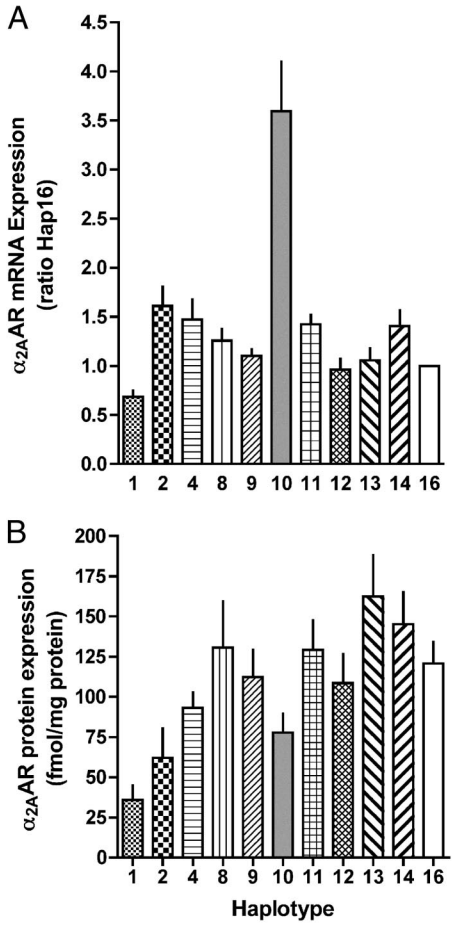

Fig. 5. α_{2A}AR haplotypes display unique profiles of mRNA and protein expression. BE(2)-C cells were transfected with constructs (lacking exogenous promoters) consisting of the ≈5.3-kb contiguous sequence of the α_{2A}AR gene. mRNA was quantitated by RT-PCR by using GAPDH primers as controls for transfection efficiency. α_{2A}AR protein was determined by quantitative radioligand binding with [^{3}H]yohimbine. Both transcript and protein expression were related to haplotype ($P < 0.001$ by ANOVA). Data shown are from five experiments. See *Results and Discussion* for selected individual comparisons.

α_{2A}AR cellular signaling parallels that of receptor expression (31). It is noteworthy that the expression phenotypes (as assessed by mRNA or protein) do not segregate by the two major phylogenetic clades (Fig. 4) or other subgroupings based on the tree. This observation suggests that expression is being affected by SNPs in multiple promoter, 5′ UTR, and 3′ UTR regions, with similar phenotypes resulting from different sets of variants within the gene. Inspection of the commonalities in Table 3 confirms that no SNP (or group of SNPs) defines the expression clusters. Interestingly, although the nucleotide divergence is similar in the four genomic regions (≈0.003 SNPs per base), the number of genotypic combinations by region is greatest in the 3′ UTR, where eight possible combinations are found. Furthermore, haplotypes 1–11 are invariant in the promoter region. There is precedence for 3′ UTRs to have important effects on G protein-coupled receptor expression. For the M_1-muscarinic receptor, an ≈260-bp region in the 3′ UTR, thought to form a stable stem-loop, is critical for mRNA stability (32). For the β_2AR subtype, 3′ UTR sequences have been shown to negatively regulate translation of the receptor (33–35). T cell-restricted intracellular antigen-1 is one RNA binding protein that binds to β_2AR 3′ UTR and acts to repress expression at the translational level (35). The α_{2A}AR 3′ UTR has several A/U-rich regions, but the polymorphisms identified do not disrupt these motifs. Of note, although some limited characterization of the α_{2A}AR promoter has been carried out (36), we are unaware of any studies on control of expression by the 3′ UTR.

The current work now comprehensively defines the genetic variability of the α_{2A}AR gene in terms of individual polymorphisms, their combinations as haplotypes, and the functional consequences of such variability. The results reveal substantial 5′ and 3′ flanking region variability amounting to 16 SNPs organized into 17 haplotypes with frequencies >0.01. This extensive variability, the lack of high LD between some SNPs, and ethnic-specific SNPs/haplotypes may explain the lack of strong associations in studies carried out with individual RFLPs. The most frequently studied is the DraI RFLP in the context of hypertension and related phenotypes. An initial report showed a marginal ($P = 0.03$) association between the DraI RFLP and hypertension in Caucasians but not African Americans (11). In contrast, another study revealed a marginal ($P = 0.02$) association with this RFLP and hypertension in African Americans but not Caucasians (12). However, another study in European Caucasians found no association between this RFLP and hypertension or a family history of hypertension (37). The DraI RFLP has been associated with modest increases in autonomic responses to maneuvers that lower blood pressure, exercise-induced sweat secretion, provocative motion, and platelet aggregation (13, 14). As can be seen from Table 3, the A at SNP position 1780, which represents the minor allele of the DraI RFLP, is present in six different haplotypes with frequencies ranging from 0.0125 to 0.225. This segregation across multiple haplotypes represents significant assignment heterogeneity if subjects are genotyped only at this locus. Several studies with other individual α_{2A}AR RFLPs have failed to find associations with hypertension (24, 38, 39). In addition to these studies, association analysis with single α_{2A}AR RFLPs and a variety of central nervous system and metabolic disorders have been carried out, typically with weakly positive or no associations. These studies include panic and mood disorders (15, 16), attention-deficit/hyperactivity disorder (17), schizophrenia and the response to antipsychotics (18, 19), fat distribution (20, 21), and other obesity-related phenotypes (22, 23). The results from the current work indicate a need for reassessment of potential relationships between these disorders and α_{2A}AR genetic variability now that the full complement of SNPs is known, their haplotypes are delineated, and their effects on expression have been determined. We show that certain haplotypes have similar expression patterns. Such a functional grouping would not be obvious if "related" haplotypes were grouped together, and random grouping would have no biologic basis. Thus, expression clustering as we have elucidated here with the α_{2A}AR haplotypes may be necessary and fully justified if individual haplotypes fail in disease/phenotype association studies.

Materials and Methods

α_{2A}AR Gene Resequencing and Genotype Determination. For polymorphism discovery, resequencing of the α_{2A}AR gene was performed by using DNA samples from 30 ethnically diverse individuals obtained from the Human Variation Panel of the Coriell Institute (http://ccr.coriell.org/nigms/cells/humdiv.html). PCR fragments spanning the α_{2A}AR gene were amplified from genomic DNA and sequenced by using an ABI Prism 3700 (Applied Biosystems). Primer sets and reaction conditions used for α_{2A}AR gene resequencing are shown in Table 4, which is published as supporting information on the PNAS web site. The analyzed sequence included an ≈1,500-bp promoter, an ≈1,000-bp 5′ UTR, a 1,350-bp coding region, and an ≈1,500-bp 3′ UTR that included sequence from the stop codon to the poly(A) termination sequence. Unphased genotypes for each polymorphism were subsequently determined by using an expanded sample set from the above source consisting of 100 unrelated genomic DNA samples representing 40 Caucasians, 40 African Americans, and 20 Asians (see Table 5, which is published as supporting information on the PNAS web site).

LD, Haplotype Assembly, and Phylogenetic Analysis. The LD between all pairwise SNPs was quantitated as r^2 (40) and visualized by the three ethnic groups as a matrix with color scaling. Haplotypes were inferred by the Baysian method of Donnelly and colleagues (41) by using the program PHASE and assuming a stepwise mutation mechanism. All haplotypes that were used for whole-gene transfection had confidence probability values from PHASE of >95%. Data are presented in tabular form with haplotype frequencies given for each of the three populations. The α_{2A}AR haplotypes were used to reconstruct phylogeny by using a distance matrix approach (the neighbor-joining method), which makes no assumptions about the rates of divergence and corrects for transitions and transversions when calculating evolutionary distances (42). Ties were resolved randomly, and polarity was set by midpoint rooting.

Haplotype Expression Constructs and Transfections. To create expression constructs containing the complete α_{2A}AR gene (promoter, 5′ UTR, coding region, and 3′ UTR), a human genomic DNA library (Lambda DASH II; Stratagene) was screened by using a 1.4-kb probe corresponding to the α_{2A}AR coding region. After isolation of an ≈20-kb λ phage clone, restriction enzyme mapping and Southern blot analysis localized the α_{2A}AR gene to an ≈5.7-kb EcoRI/BstZ17 I fragment, which was subcloned into the vector pBSII KS+ (Stratagene). This vector lacks a eukaryotic-responsive promoter, and thus expression is directed by the included α_{2A}AR flanking sequences. Sequence analysis confirmed that the cloned fragment spanned the entire region surveyed during α_{2A}AR resequencing; its sequence was designated here as haplotype 16 (GenBank accession no. DQ149926). This construct was then used as a template for the generation of other α_{2A}AR haplotype constructs. The human neuroblastoma cell line BE(2)-C was used as the host cell for transient transfections, and cells were grown in monolayers. Transient transfections were performed by using 20 μg of each construct as described (43). Two days after transfection, cells were harvested for radioligand binding or real-time RT-PCR.

α_{2A}AR Transcript and Protein Expression. Total RNA was prepared from transfected BE(2)-C cells by using TRIzol reagent (Invitrogen). Reverse transcription of 500 ng of RNA was performed after DNase I digestion by using murine leukemia virus reverse transcriptase and random hexamers. Identical incubations were also performed in the absence of reverse transcriptase. Forward and reverse primers (5′-tggttcggctactgcaacag and 5′-gaaatcgtggttgaagatggtgta) for quantitative real-time PCR were designed by using Applied Biosystems PRIMER EXPRESS V1.0 software and amplified a 63-bp fragment from an invariant portion of the α_{2A}AR coding region. PCRs (50 μl) consisted of a 50-fold dilution of the reverse transcription reaction (≈10 ng), 300 nM each of forward and reverse primers, and 25 μl of SYBR Green Master Mix (Applied Biosystems). Cycle conditions for the ABI 7300 were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was run in triplicate, and after each amplification dissociation curve analysis was performed to confirm the presence of a single amplicon in each reaction. For each amplification, a standard curve was generated by using 0.1–100 ng of cDNA prepared from total RNA isolated from BE(2)-C cells to determine the relative levels of α_{2A}AR present in each sample. Reactions containing primers corresponding to endogenous GAPDH (5′-atggaaatcccatcaccatctt-3′ and 5′-cgccccacttgattttgg-3′) were also performed to control for small variations in the amount of template in each reaction. α_{2A}AR protein expression was determined by saturation radioligand binding with cell membranes as described (30). Briefly, cells were washed twice with PBS, detached with a rubber policeman in 5 mM Tris, pH 7.4/2 mM EDTA buffer, and centrifuged at 30,000 × *g* for 10 min. Membrane pellets were resuspended in 75 mM Tris-HCl, pH 7.4/12.5 mM $MgCl_2$/2 mM EDTA, and radioligand binding using 30 nM [^{3}H]yohimbine (with 10 μM phentolamine to define nonspecific binding) was performed. Reactions were incubated at room temperature for 30 min and terminated by dilution with four volumes of ice-cold 10 mM Tris-HCl (pH 7.4) buffer and vacuum filtration over glass fiber filters.

Statistical Analysis. Agreement between genotypes and those predicted by Hardy–Weinberg equilibrium was assessed by χ^2 tests with 1 df. Comparisons of the results from real-time RT-PCR and radioligand binding were analyzed by repeated-measures ANOVA and post hoc *t* tests by using PRISM software (GraphPad, San Diego). Significance was considered when $P < 0.05$ after correction for multiple comparisons by the Newman–Keuls method. Data are reported as mean ± SE.

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