Allele-Specific Binding of Airway Nuclear Extracts to Polymorphic β_2 -Adrenergic Receptor 5' Sequence

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Like other intronless G protein-coupled receptor genes, the β_2 -adrenergic receptor (β_2 AR) has minimal genetic space for population variability, and has attained such via multiple coding and noncoding polymorphisms. Yet most clinical studies use the two nonsynonymous polymorphisms of the coding region for association analysis despite low levels of linkage disequilibrium with some promoter and 5'UTR polymorphisms. To assess the potential for allele-specific transcription factor binding to β_2 AR 5'-flanking sequence, 3'-biotin-labeled oligonucleotide duplexes were synthesized. Each was centered on variable sites representing major or minor alleles found in the human population with frequencies of 5% or greater (20 polymorphic sites). Electrophoretic mobility shift assays were performed using human airway smooth muscle or airway epithelial cell nuclear extracts. Many of these polymorphisms resulted in an alteration in binding, and both major allele and minor allele dominance were observed. For example, in airway smooth muscle nuclear extracts, 10 polymorphisms decreased and 2 increased binding, whereas 5 showed no differences. Concordance between airway smooth muscle and epithelial cell nuclear extract binding to polymorphic alleles was found in only ~50% of cases. There was no tendency for the rare variants to be more likely to have altered nuclear extract binding compared to the more common variants. Taken together, these results provide potential mechanisms by which β_2 AR 5'-flanking polymorphisms affect obstructive lung phenotypes.

Keywords: asthma; β -agonist; polymorphism

The β_2 -adrenergic receptor (β_2 AR) gene is highly polymorphic in the human population within both the coding and noncoding regions. Within the coding block, two nonsynonymous polymorphisms are common, occurring at nucleotides 46 and 79 relative to the ATG start site (1). These result in Arg or Gly at amino acid 16 and Glu or Gln at position 27, with three of the four possible two-site haplotypes being prevalent in normal cohorts as well as those with asthma and chronic obstructive lung disease. An infrequent, nonsynonymous polymorphism at nucleotide 491 results in a substitution of Ile for Thr at amino acid 164 (1). These polymorphisms have been studied in recombinant systems whereby constructs (without cognate 5'UTR or promoter) were used to stably or transiently transfect model cells, such as fibroblasts (2, 3) and COS cells (4). These studies revealed several phenotypes of the polymorphic receptors, including those related to agonist-promoted receptor trafficking and receptor coupling to G_s (see Refs. 5 and 6 for review). Given the interindividual variability in the response to β -agonists in asthma, of which ~50% has been attributed to genetic variation (7), these polymor-

CLINICAL RELEVANCE

The variability in the response to β -agonists in obstructive lung disease is not fully understood. We show that polymorphisms in the promoter 5'-flanking region of the receptor alter transcription factor binding.

phisms of the β_2 AR have been extensively evaluated in association studies as potential pharmacogenetic predictors of efficacy. In addition, studies have been carried out to assess whether these polymorphisms are risk factors for asthma, or act to impose or modify asthmatic phenotypes. Collectively, results from these studies can be categorized as those that show associations, those that fail to detect associations, and those that show conflicting associations (i.e., opposite phenotypes). These issues have recently been reviewed in detail (8, 9). Although the designs of these clinical studies are often quite different and may be the basis for such incongruities, it is also clear that the full complement of informative genetic variability of the β_2 AR gene is rarely used in these association studies. In part, this may be due to a lack of consistent *in vitro* data (10–13) showing phenotypic effects of the polymorphisms that are within the noncoding regions. The intronless β_2 AR gene has at least 20 polymorphisms in the promoter, 5'UTR, and 5'-leader cistron (collectively termed here as the "5'-flanking region"), which have allele frequencies of 0.05 (5%) in U.S. individuals of European or African descent (11, 14). Clinical studies (11, 15–20) using haplotypes derived from various sets of 5'-flanking and coding single-nucleotide polymorphisms (SNPs) have revealed several, and sometimes discordant, findings (see DISCUSSION). To begin to understand the basis for *in vitro* and clinical phenotypes, in the current study, we have examined each polymorphism within the context of the binding of transcription factors derived from both human airway smooth muscle and airway epithelial cells. We show specific binding to β_2 AR 5'-flanking sequence, which can be dependent on the presence or absence of the polymorphism, as well as the cell-type origin of the nuclear extract. The data provide insight into the potential molecular basis for asthma clinical phenotypes associated with β_2 AR 5'-flanking polymorphisms, and the influence of cell type on phenotypes.

MATERIALS AND METHODS

Polymorphic Sites/Nomenclature

Oligonucleotides were synthesized (see below) based on a reference sequence for the human β_2 AR gene (GenBank accession no. DQ094845) and the polymorphic sites that we have previously identified in the human population (11, 14). In order to maintain a consistent nucleotide numbering system (11), the adenine of the ATG initiation sequence is denoted as +1 (base 4,220 of DQ094845) with the 5'-flanking sequence beginning at -1 and continuing in the negative direction (Figure 1).

Cell Lines and Culture

Human airway smooth muscle cells were obtained from Clonetics (East Rutherford, NJ) and grown in SmGM-2 media (Clonetics) with the

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Figure 1. Localization of polymorphisms of the β_2 AR 5'-flanking region. Shown are the nucleotides listed as major/minor followed by the allele frequency of the minor allele in parenthesis. Where there were differences between allele frequencies between whites and African Americans, the higher frequency is given (information compiled from References 11 and 14). *This polymorphism is within a small open reading frame, the 5'-leader cistron (10), and was not studied.

following recommended supplements: insulin, recombinant human fibroblast and epidermal growth factors, 5% FBS, and 30 μ g/ml gentamycin with 15 ng/ml amphotericin B, as previously described (21). Cells at passages 6–10 were used, which maintained a consistent morphologic appearance of smooth muscle cells. The immortalized human airway epithelial cell line, BEAS-2B, was obtained from American Type Culture Collection (Manassas, VA) and grown, as previously described (22), in HAM's F-12 media supplemented with insulin, transferrin, epidermal growth factor, hydrocortisone, and 10% FBS, with 100 μ g/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained in a 37°, 5% CO₂ atmosphere, and harvested at 90% confluency in batches of 10–20 T-150 flasks for production of nuclear extracts.

Nuclear Extracts

Nuclear extracts were prepared using reagents from the NE-PER extraction system (Pierce, Rockford, IL), as recommended by the manufacturer. Briefly, attached cells were placed on ice, the media aspirated, and washed three times with cold PBS. Cells were removed by gentle scraping and collected by centrifugation at 12,000 $\times$ g for 15 min at 4° and swollen by incubation in CER1 buffer for 10 min. After incubation with the CERII detergent solution for 1 min, preparations were centrifuged at 16,000 $\times$ g for 5 min at 4°. The nuclear pellet was resuspended in nuclear extraction buffer and vortexed for 15 s every 10 min over 40 min, and the preparation centrifuged at 16,000 $\times$ g for 10 min at 4°. The supernatant (nuclear extract) was collected, aliquotted, and frozen at –80° until used. All of the above steps included the protease inhibitors leupeptin and aprotinin at 10 μ g/ml.

Synthesis of Polymorphic Oligonucleotide Duplexes

For each polymorphic site, an oligonucleotide of ~31 bases was synthesized using an Applied Biosystems 3900 synthesizer (Applied Biosystems, Foster City, CA) with the polymorphic site in the middle of the oligonucleotide. The 3' base of the oligonucleotide was labeled with a biotin arm using a 1-dimethoxytrityloxy-3-O-(N-biotinyl-3-aminopropyl)-triethyleneglycolyl-glycerol-2-O-succinyl-polystyrene (BiotinTEG PS; Glen Research, Sterling, VA) column, as described by others (23).

Equal quantities of complementary ([+] and [–] strand) oligonucleotides were mixed, heated to 95° for 5 min, and slowly cooled to 25° over 1 h. A similar set of labeled complementary oligonucleotides, differing only by the polymorphism, were also made in an identical fashion. These sets of annealed oligonucleotides are referred to as oligonucleotide duplexes.

Electrophoretic Mobility Shift Assays

In these experiments, the wild-type and polymorphic duplexes were always studied concomitantly, so as to maintain identical conditions for comparisons. Reactions consisted of 40 fmol labeled oligonucleotide duplex with 0, 3, 5, or 7 μ g nuclear extract in a 10 mM Tris-HCl pH 7.5 buffer containing 50 mM KCl, 1 mM DTT, and 1 μ g poly dI.dC in a final volume of 20 μ l. In some reactions, unlabeled oligonucleotide (1.6 nmol) was included with the 5 μ g nuclear extract condition. Incubations were at 25° for 20 min, and were stopped by addition of loading buffer. Reactions were loaded on native 5% polyacrylamide gels in 0.5 $\times$ Tris borate/EDTA and electrophoresed at 100 V for ~45 min. The products were transferred onto positively charged nylon membrane (Hybond N⁺; Amersham, Piscataway, NJ) in 0.5 $\times$ Tris borate/EDTA at 15 V for 20 min (Trans-Blot; Bio-Rad, Hercules, CA). Transferred DNAs were UV-crosslinked to the membrane for 15 min on a transilluminator equipped with a 312 nm bulb. The biotin-labeled DNA was detected with LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce) according to the manufacturer's instructions. For image capture and quantitation, the Fuji LAS-3000 charge-coupled device camera system (Fujifilm, Stamford, CT) was used, which has a linear range of over 10⁸ counts (in the current studies, typical counts for the lanes with nuclear extracts ranged from ~0.5 to 12.0 $\times$ 10⁶). Counts were also acquired for the unbound, labeled oligonucleotide duplex from reactions that were carried out in the absence of nuclear extract, which were used to correct for any minor differences in labeling efficiency or transfer. Bands representing potential oligonucleotide–nuclear extract complexes were quantitated only if competition with unlabeled oligonucleotide was demonstrated (*see RESULTS*).

Statistical Analysis

For analysis of the digital output from the charge-coupled acquisition device in the EMSA experiments, raw counts were used in paired two-way *t* tests to compare the major versus the minor signals at a given SNP position, with *P* values < 0.05 considered significant. EMSA signals from each experiment were also normalized to the major allele signal in that experiment, and results are given as the mean ratio of the minor allele:major allele signals for the set of experiments representing each of the allelic oligonucleotide duplexes. For discussion purposes, a change was considered relevant when the difference between major and minor signals was $\geq 15\%$ (i.e., minor:major ratios ≤ 0.85 or ≥ 1.15), and *P* < 0.05. Additional analysis was performed on the calculated ratios of the signals from each SNP compared to unity, and the *P* values corrected for multiple comparisons. Data are presented as mean $\pm$ SE.

Miscellaneous

Protein determination was by the copper bicinchoninic acid method (24).

Computer programs were obtained from the following sources: MatInspector (Genomatix, Munich, Germany), MATCH (Biobase, Wolfenbuttel, Germany), TESS (University of Pennsylvania, Philadelphia, PA), PRISM (GraphPad, Palo Alto, CA), and SAS (SAS Institute, Cary, NC).

RESULTS

Shown in Figure 1 are the locations within the β_2 AR 5'-flanking region of polymorphisms with allele frequencies ≥ 0.05 (11, 14) that were used in these studies. The major and minor alleles are indicated, with the allele frequency of the minor allele provided (when the minor allele frequency differs substantially between white and African Americans, the higher frequency is given). Based on the consensus β_2 AR sequence derived from our polymorphism discovery studies, (+) and (–) strand oligonucleotides (~31 bases in length) were designed such that the polymorphic

site was in the middle of the oligonucleotides. These were synthesized with biotin at the 3' ends, and annealed to form the oligonucleotide duplexes for the EMSAs. Two sets (representing the major and minor alleles) of oligonucleotides were synthesized and annealed representing all the indicated polymorphic sites of Figure 1. In the case of the variations at $-3,287$ and $-3,291$, these were too close in proximity to disrupt, and indeed are in 100% linkage disequilibrium (14). Thus, the set of nucleotides synthesized for this double site represent either $-3,287A$ with $-3,291T$, or $-3,287T$ with $-3,291C$. Typical results are shown from studies with nuclear extracts from human airway smooth muscle cells in Figure 2. Shown are instances where the greatest extract binding occurred with the oligonucleotide duplex representing the major allele (Figure 2A), the minor allele (Figure 2B), where the binding was equivalent between the two (Figure 2C), or where binding to either oligonucleotide duplex was not detected (Figure 2D).

Tables 1 and 2 provide the results from all experiments, which involved 38 oligonucleotide duplexes and the nuclear extracts derived from the two different cell types. A difference between extract binding to one oligonucleotide duplex versus its allelic variant was considered relevant if it amounted to + or -15% or greater, and the P value was < 0.05 (the cutoff value of 15% is arbitrary, but is consistent with the accepted practices in DNA microarray analysis [25]). For the human airway smooth muscle cells, 10 major alleles were found to bind nuclear extracts to a greater extent than the corresponding minor alleles (ratio of minor:major signals ranging from 0.77 to 0.19). In two instances (positions $-2,051$ and $-3,251$), the minor allele had greater binding (minor:major signals = 2.155 and 1.385, respectively), and for five oligonucleotide duplexes there was no difference in specific nuclear extract binding between the two allelic variants. In two cases there was no binding to either allelic oligonucleotide duplex. With BEAS-2B cell nuclear extracts, 10 major alleles had binding that was significantly greater than the minor allele (minor:major signal range = 0.827–0.298). There were four instances (positions -468 , $-1,818$, $-2,051$, and $-3,159$) where the minor allele binding was greatest (minor:major signal range = 1.893–3.017). Five sets of oligonucleotide duplexes had equivalent nuclear extract binding for the major and minor alleles. Loci where the greatest difference ($\geq 75\%$) in binding by allele were found to be at positions -406 , -468 , $-1,429$, $-1,818$, $-2,051$, and $-3,459$ (Figure 3A, above and below the dashed lines). Three of these polymorphisms are common and three are uncommon (Figure 1), suggesting no relationship between these binding extremes and the relative frequency of the polymorphism in the population. Indeed, when the frequency of each minor allele is plotted against its binding ratio (Figure 4), no relationship is noted between the nuclear extract binding phenotypes and the allele frequencies of the polymorphisms. A correction for multiple comparisons carried out on the minor:major ratios resulted in a loss of statistical significance for differences in binding between allelic oligonucleotides at positions -20 and $-1,343$ in the airway smooth muscle studies (Tables 1 and 2).

The comparison between results using human airway smooth muscle versus BEAS-2B nuclear extracts by polymorphic site is shown in Figure 3A, and summarized in Figure 3B. There was concordance between human airway smooth muscle and BEAS-2B nuclear extract binding in 10 of the 19 allelic oligonucleotide duplexes. Eight of these showed greater binding to the major allele (minor:major ratio < 1 in Figure 3A; green color in Figure 3B). In only one case (position $-2,051$) was there concordance with greater binding to the minor allele. Among the instances of discordant binding (9 loci) between human airway smooth muscle and BEAS-2B extracts, various combinations were found, as shown in Figure 3B. There were several instances

Figure 2. Electrophoretic mobility shift assays (EMSAs) using β_2AR polymorphic oligonucleotide duplexes and human airway smooth muscle cell nuclear extracts. Shown are representative results where the major allele has greater binding than the minor ([A] position $-3,459$), and where the minor allele has greater binding than the major allele ([B] position $-2,051$). Also shown is a representative experiment showing equivalent binding between the allelic oligonucleotide duplexes ([C] position $-2,387$) and one where there was no detectable binding ([D] position -468). See Tables 1 and 2 for mean data and statistical analysis. NE, nuclear extract; UC, unlabeled competitor oligonucleotide.

where binding was equivalent with extracts from one cell type at both alleles, but different by allele with the other cell type (i.e., loci -20 , -262 , and $-3,251$). Interestingly, there was no instance where the major allele had greater binding from extracts of one cell type and the minor allele had greater binding from extracts of the other cell type.

TABLE 1. RESULTS OF ELECTROPHORETIC MOBILITY SHIFT ASSAY STUDIES USING NUCLEAR EXTRACTS FROM HUMAN AIRWAY SMOOTH MUSCLE CELLS

SNP Position	Alleles		P Value	Minor Versus Major Signals*	Minor:Major Ratio (SE)	n [†]	Corrected P Value Ratio [‡]
	Major	Minor					
-20	T	C	0.024		0.75 (0.098)	8	NS
-262	G	A	0.004		0.49 (0.056)	11	< 0.01
-367	C	T	< 0.0001		0.40 (0.050)	11	< 0.01
-406	C	T	0.001		0.19 (0.070)	10	< 0.01
-468	C	G	NA		NA		
-654	G	A	0.036		0.49 (0.098)	10	< 0.05
-1023	G	A	0.003		0.71 (0.054)	11	< 0.01
-1343	G	A	0.050		0.77 (0.120)	10	NS
-1429	T	A	< 0.001		0.19 (0.030)	9	< 0.001
-1531	C	T	NA		NA		
-1818	A	T	0.715		1.20 (0.133)	10	< 0.01
-2051	4C	5C	0.004		2.16 (0.353)	10	< 0.01
-2274	T	C	< 0.0001		0.38 (0.070)	12	< 0.01
-2387	T	C	0.126		0.89 (0.139)	11	NS
-2633	C	T	0.696		1.15 (0.170)	9	NS
-3159	T	C	0.857		1.06 (0.099)	11	NS
-3251	C	A	0.001		1.39 (0.097)	10	< 0.05
-3287/-3291	A/T	T/C	0.105		0.95 (0.111)	10	NS
-3459	T	C	< 0.0001		0.31 (0.031)	12	< 0.01

Definition of abbreviations: NA, not applicable as no complexes identified; NS, not significant (>0.05) after correction; SNP, single-nucleotide polymorphism.

* Paired *t* test of major versus minor raw acquisition counts.

[†] Number of experiments performed.

[‡] P value for ratio corrected for multiple comparisons.

DISCUSSION

The aim of the current study was to identify potential differences in transcription factor binding from nuclear extracts of human airway epithelial and smooth muscle cells to oligonucleotides representing polymorphisms in the 5'-flanking region of the β_2 AR gene. Most of these 20 SNPs displayed altered binding compared with the major allele, and both major and minor allele dominant binding was observed. There were also substantial qualitative and quantitative differences between binding from

epithelial versus smooth muscle nuclear extracts, indicating cell type-specific interactions. These findings may underlie the basis for clinical phenotypes observed in patients with obstructive lung disease and β_2 AR SNPs, or perhaps the inconsistencies in such studies when the outcome phenotype is "epithelial"- versus "smooth muscle"-dependent.

Reporter-based assays, performed using portions of the 5'-flanking region of the intronless β_2 AR gene, have revealed several intriguing findings. Using luciferase as the reporter and

TABLE 2. RESULTS OF ELECTROPHORETIC MOBILITY SHIFT ASSAY STUDIES USING NUCLEAR EXTRACTS FROM BEAS-2B CELLS

SNP Position	Alleles		P Value	Minor Versus Major Signals*	Minor:Major Ratio (SE)	n [†]	Corrected P Value Ratio [‡]
	Major	Minor					
-20	T	C	0.629		0.99 (0.090)	12	NS
-262	G	A	0.205		0.76 (0.107)	8	NS
-367	C	T	0.02		0.83 (0.075)	12	< 0.05
-406	C	T	0.03		0.35 (0.060)	9	< 0.05
-468	C	G	0.004		1.89 (0.254)	9	< 0.01
-654	G	A	0.0001		0.84 (0.019)	12	< 0.01
-1023	G	A	0.008		0.64 (0.092)	11	< 0.01
-1343	G	A	0.002		0.77 (0.068)	12	< 0.01
-1429	T	A	0.0001		0.29 (0.061)	11	< 0.01
-1531	C	T	0.82		0.99 (0.030)	12	< NS
-1818	A	T	0.0001		1.92 (0.143)	11	< 0.01
-2051	4C	5C	0.0005		3.02 (0.375)	9	< 0.01
-2274	T	C	0.0001		0.57 (0.036)	10	< 0.01
-2387	T	C	0.015		0.86 (0.048)	12	< 0.01
-2633	C	T	0.026		0.82 (0.083)	10	< 0.05
-3159	T	C	0.034		1.20 (0.086)	9	< 0.05
-3251	C	A	0.100		1.35 (0.161)	10	NS
-3287/-3291	A/T	T/C	0.0003		0.39 (0.066)	10	< 0.01
-3459	T	C	0.003		0.70 (0.065)	12	< 0.01

Definition of abbreviations: NS, not significant ($P > 0.05$) after correction; SNP, single-nucleotide polymorphism.

* Paired *t* test of major versus minor raw acquisition counts

[†] Number of experiments performed.

[‡] P value for ratio corrected for multiple comparisons.

Figure 3. Allele-specific binding of epithelial and smooth muscle nuclear extracts to polymorphic β_2 AR oligonucleotides. Shown are the results from EMSAs performed with oligonucleotide duplexes designed around the human polymorphic sites shown (see MATERIALS AND METHODS). Results in (A) are reported as the ratio of the minor allele to the major allele signal. The dashed lines show $\pm 75\%$ changes from unity (solid line). (B) Results are summarized as shown by the color code to indicate differences between EMSA ratios by cell-type nuclear extract (green, minor < major; yellow, minor > major; blue, minor = major; white, no binding). A relevant difference was defined as a $\pm 15\%$ or greater difference ($P < 0.05$). *P* values and the number of experiments are given in Tables 1 and 2.

transfecting COS-7 cells, Scott and colleagues (12) showed the highest activity when 379 bp upstream of the ATG initiator methionine was used in the construct. Additional sequence more 5' resulted in lower activity, suggesting additional transcription binding or repressor events that may control expression as well. Within the 379-bp region lies the -367 SNP, which was studied and found to impose an $\sim 17\%$ decrease in luciferase activity. In another study (26), nuclear extracts from H292 cells were shown to have differential binding to oligonucleotides representing the -367 SNP. However, before nuclear extract harvesting, the cells were exposed to β -agonist, so these results are not readily comparable to others. Nevertheless, in the same study, the -367 SNP was examined in context with other SNPs of the 5'-flanking region in peripheral blood mononuclear cells, and the authors concluded that there was minimal effect of this SNP on transcription (26). In peripheral blood mononuclear cells, Lipworth and colleagues (27) found no statistically significant

differences in 100 μ M isoproterenol-stimulated cAMP accumulation between those with -367C/-47C/+47G/+79G and the comparable -367T haplotype. However, there was a strong trend apparent, with the latter having $\sim 25\%$ lower cAMP response, indicating the potential for this SNP to affect β_2 AR signaling function via altered expression.

The clinical studies with β_2 AR haplotypes, including those using 5'-flanking SNPs, have been critiqued elsewhere in detail (8, 9, 28). In most cases, the SNPs/haplotypes that were used were not the same between studies, nor were the outcomes necessarily measured in the same manner, which may be the basis for the apparent inconsistencies. The limitations of translating results obtained from *in vitro* studies, such as the current study or those using other cells or methods, into clinical context is challenging; indeed, sometimes "opposite" results from *in vitro* results have been found in clinical studies (29–32). One particular focus of clinical studies has been on the bronchodilator response to β -agonist as it relates to β_2 AR variants, including those of the 5'-flanking region, either as individual SNPs or haplotypes. Drysdale and colleagues found associations in patients with asthma with β_2 AR haplotypes and the acute bronchodilator response to albuterol, whereas there was no association with any individual SNP (11). In a family-based association study, Silverman and colleagues (15) found associations with a 5'-flanking SNP (-654) and post-bronchodilator FEV₁, as well as the synonymous SNP, +523 (which is in linkage disequilibrium with several 5'- and 3'-flanking SNPs), with bronchodilator responsiveness. Three multi-SNP haplotypes were prominent in this population. One haplotype was associated with enhanced bronchodilator responsiveness, consistent with the Drysdale results (11). On the other hand, a minor haplotype (Silverman haplotype 3, Drysdale haplotype 6) in this study was associated with decreased bronchodilator response, whereas, in the aforementioned study (11), this haplotype was associated with an enhanced response. Responsiveness to albuterol has also been explored in a cohort of Latino patients with asthma using eight SNPs of the β_2 AR, including three in the 5'-flanking region (16). These haplotypes were associated with bronchodilator response

Figure 4. Absence of a relationship between allele frequency and nuclear extract binding ratio. Shown are results from BEAS-2B experiments, plotted against the minor allele frequency for each polymorphism (higher of the two when different between whites and African Americans).

in Puerto Ricans, but not in Mexicans. However, they were not significantly more predictive than the +46 polymorphism, likely because the haplotypes had SNPs with high degrees of linkage disequilibrium and the major haplotypes could be defined with a more limited set of SNPs. In a study of 45 patients with asthma, β -agonist bronchodilator responses and bronchoprotective effects to methacholine were determined and associations sought with β_2 AR haplotypes encompassing eight SNPs (17). Haplotype associations were noted for the bronchoprotection, but not for the acute bronchodilator effect. Several other studies have shown associations with β_2 AR 5'-flanking SNPs or haplotypes and agonist (or related) phenotypes (18–20). Of note, in these latter studies in particular, haplotypes were often “reduced” to encompass one or two individual SNPs in the coding region based on linkage disequilibrium and the limited number of patients with certain haplotypes. One could just as readily have used SNPs in the 5'-flanking region to completely define the haplotypes, due to this high linkage disequilibrium. The choice to use the coding SNPs to define these limited haplotypes was probably based on *in vitro* studies showing functional effects at these sites (3, 21).

The current study reveals data that support the notion of polymorphisms in the 5'-flanking region of the β_2 AR affecting phenotype via variable transcription factor binding. The results indicate where the greatest of these differences are apparent, as well as a significant cell type dependency of phenotype. Our attempts to precisely define which transcription factors selectively bind to one oligonucleotide versus its allelic variant indicate the complexities of predicting and demonstrating such affects, particularly when multiple transcription factors act together, as appears to be the case in the mammalian genome (33). We used three different programs to predict transcription factor binding sites *in silico*: MatInspector, Match, and TESS. Each provides for flexibility in assigning acceptability of mismatches and methods for calculating probability scores for transcription binding sites within a provided sequence. Despite using various degrees of stringency, there was very little agreement on the transcription factor predicted to bind to a region surrounding each of the β_2 AR 5'-flanking SNPs. A substantial range of core, or matrix, match probabilities between the programs was also observed. Taken together, these issues hampered our ability to identify potential differentially bound individual transcription factors *in silico*. It is also likely, from the amplitude of the signals that we obtained from these extracts, that multiple transcription factors are binding, perhaps in cooperative ways that are not predicted by the current algorithms. Indeed, potential multi-transcription factor interactions, coactivator factors, phosphorylation events, unexplained cell type specificities, and other complexities have resulted in substantial controversy as to the specific mechanisms of regulated gene transcription (33). Even for the first discovered transcription factor (CREB), there appears to be no consensus as to mechanism (for example, see Refs. 25, 34, and 35). The current data, then, with the β_2 AR 5'-flanking SNPs represent an initial first step in dissecting how they may affect β_2 AR expression, and how a clinical phenotype may differ based on its dependence on cell type, given the differences that we find between airway epithelial cells and airway smooth muscle cells. Extending the current findings would necessitate determining the proteome of transcription factors expressed in airway cells (under basal and relevant “pathologic” conditions). Then specific interactions could be considered at various SNP positions. Even then, though, such studies will be confounded by the aforementioned complexities, and the presence of so many polymorphisms along a relatively short region of an intronless gene. Indeed, we have recently shown for the G protein-coupled receptor class of genes, that those that are small and intronless may have evolved highly “compact” mechanisms for attaining

variability (36). Thus, there is little extraneous genomic space (i.e., most sequences may be functional), and so multiple SNP sites may be at play in a given transcription factor function.

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