ORIGINAL ARTICLE

Association of Promoter Variants in the α7 Nicotinic Acetylcholine Receptor Subunit Gene With an Inhibitory Deficit Found in Schizophrenia

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Background: The α7 neuronal nicotinic acetylcholine receptor subunit gene (*CHRNA7*) has been implicated as a candidate gene for schizophrenia, and for an auditory sensory processing deficit found in the disease, by both genetic linkage at 15q14 and biochemical data. The expression of *CHRNA7* is reduced in several brain regions in schizophrenic subjects compared with control subjects. This study presents DNA sequence analysis of the core promoter region for *CHRNA7* in schizophrenic and control subjects.

Methods: Single-strand conformation polymorphism analysis and DNA sequencing were used for mutation screening of the core promoter in the *CHRNA7* gene. The sample included subjects from 166 schizophrenic families and 165 controls. Controls had no evidence of current or past psychosis and had auditory evoked potentials recorded.

Results: Multiple polymorphic patterns were identified in the *CHRNA7* core promoter in both schizophrenic and control subjects. Functional analysis of polymorphisms indicated that transcription was reduced. The prevalence of functional promoter variants was statistically greater in schizophrenic subjects than in the controls. Presence of an α7 promoter polymorphism in controls was associated with failure to inhibit the P50 auditory evoked potential response.

Conclusions: Although linkage disequilibrium with other genetic alterations cannot be excluded, the *CHRNA7* core promoter variants, found in this study, may contribute to a common pathophysiologic feature of schizophrenia.

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SCHIZOPHRENIA IS a complex disorder, in which heterogeneity, reduced penetrance, and environmental factors have made identification of genetic defects difficult. The work of many investigators has resulted in the discovery and replication of 8 principal linkage regions in the human genome. These include linkages at 1q21-q22,[1] chromosome 6p22-p24,[2] chromosome 6q21-q22,[3] chromosome 8p21-p22,[4] chromosome 10p11-p15,[5] chromosome 13q14-q32,[4] chromosome 15q13-q15,[6-13] and chromosome 22q11-q13.[14] Additional linkages on 6 other chromosomes may be contributory in some populations.[15] In general, linkage in any given cohort is found in only a subset of the total number of families examined, suggesting that abnormalities in different gene sets may result in the same illness. Identification of pathogenic mutations in candidate genes that lie in the major linkage regions is necessary for a rigorous understanding of how several genes interact in the development of schizophrenia. Herein we present evidence that putative functional polymorphisms in the promoter region of the α7 neuronal nicotinic acetylcholine receptor subunit gene (*CHRNA7* or α7), a candidate gene in the 15q13-q14 linkage region, are more frequently found in schizophrenia and are associated with a sensory deficit found in this common mental illness.

The *CHRNA7* gene cluster maps to a region of replicated linkage in schizophrenia on chromosome 15q13-q14 (**Figure 1**). *D15S1360*, a polymorphic marker in intron 2 of the *CHRNA7* gene, is genetically linked to a sensory deficit trait in the disease, a failure to inhibit the response to repeated auditory stimuli in the immediate environment (lod = 5.3, $\Theta = 0.0$, $P < .001$).[16] Linkage to schizophrenia was also positive in this study of 9 families, although not as significant (lod = 1.33). We have found additional evidence for linkage of this locus to schizophrenia as the phenotype in pedigrees from the National Institute of Mental Health (NIMH) Schizophrenia Genetics Initiative.[9,17] A sib-pair analysis showed that a significant proportion of *D15S1360* alleles were shared identical-by-descent in the schizophrenics (0.58; $P < .0024$).[17] In a transmission

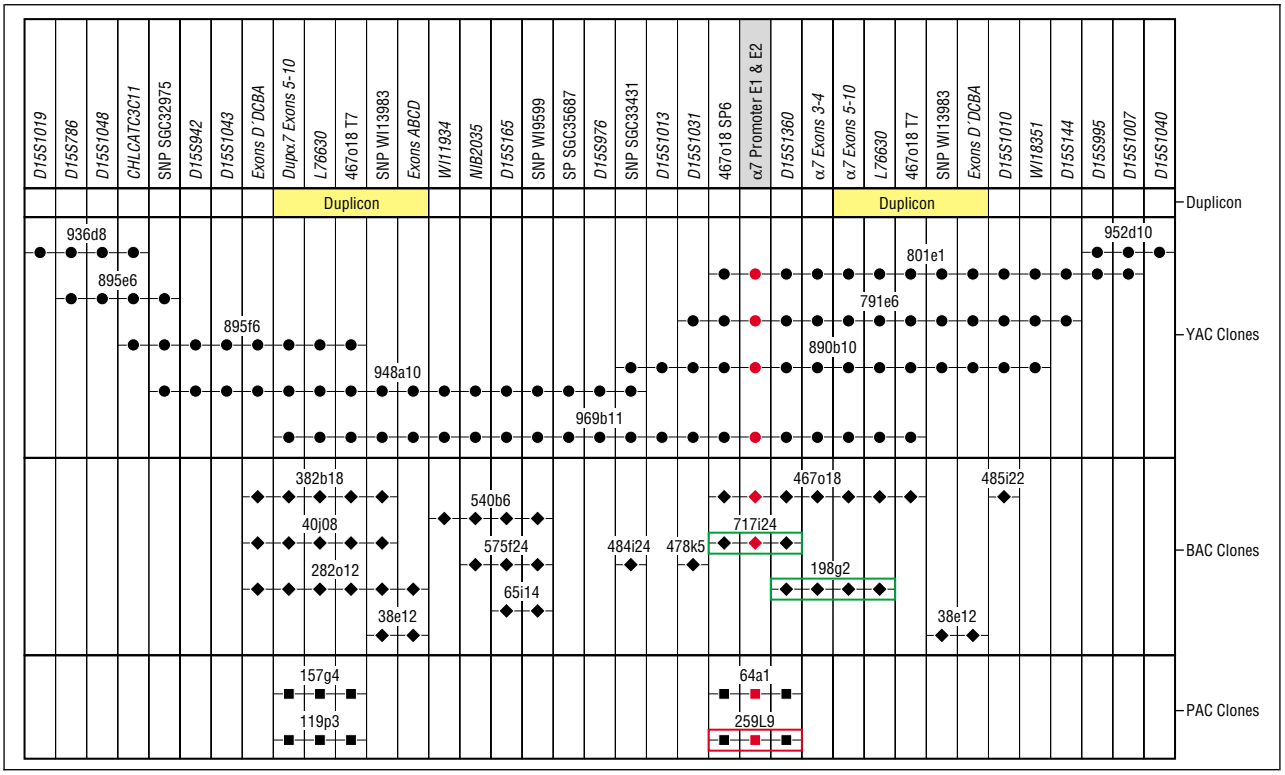

Figure 1. Physical map of the linkage region to schizophrenia on chromosome 15q13-q14. Yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), and P1 artificial chromosome (PAC) marker information was used with marker information generated in our laboratory to construct a physical map of this region. Estimated size of the region is 4 cm. Map location of the α7 nicotinic receptor promoter is shaded; duplicons containing duplicated α7 exons 5 to 10 are shown in yellow; BACs used for mapping of *D15S1360*, green boxes; and PAC subcloned for isolation of α7 promoter sequences, red box.

disequilibrium study of schizophrenia, we found significant genotype-wise disequilibrium ($P<.007$) at *D15S165*, a polymorphic simple sequence marker localized within 1 megabase (Mb) of the α7 nicotinic receptor gene at 15q13-q14.[9] We have recently completed a full genomic linkage analysis of the NIMH Schizophrenia Initiative pedigrees, for which the genotyping was available from Millenium Pharmaceuticals, Cambridge, Mass. We used a parametric genetic analysis and an autosomal codominant model with a diagnosis of schizophrenia and schizoaffective disorder, depressed type, as the affected phenotype. One genetic linkage was found, significant by genome-wide criteria (multipoint lod score, 3.94; $P=.00005$), to the locus on 15q13-q14 within 1 cM of the previous finding for linkage to the locus of the α7 nicotinic receptor gene.[18,19] The linkage finding at 15q13-q15 has been replicated by several independent groups, using nonparametric methods in the NIMH sample,[20] and in other samples.[6-8,10-12] The same region has been linked to juvenile myoclonic epilepsy[21] and more recently to bipolar disorder,[22,23] suggesting that the locus may contain defects in a gene or genes common to several neuronal disorders.

Biological and pharmacologic evidence also supports the *CHRNA7* gene as a candidate gene for schizophrenia.[24,25] Nicotine normalizes a sensory gating abnormality, the P50 inhibitory deficit, found in most patients with schizophrenia and in 50% of their first-degree relatives.[25-28] This trait, which involves inhibition of the response to repeated stimuli presented through the auditory system to the brain, can be measured by means of auditory evoked potentials in a paired pulse paradigm. Electrodes on the scalp record waves with a 50-millisecond latency (P50) following paired auditory stimuli delivered 0.5 second apart.[28,29] In a normal response, the subject decreases the amplitude of the second response (test response), compared with the response to the first stimulus (conditioning response) through the action of an inhibitory neuronal pathway. The results are reported as the P50 test-conditioning (T/C) ratio. More than 85% of schizophrenic patients have abnormally increased P50 ratios, where the test response is greater than expected in the normal population.[30-34] This P50 inhibitory deficit is inherited in families of schizophrenic patients in an apparently autosomal dominant pattern.[16,35,36] Thus, half of family members have aberrant gating of the P50 auditory evoked potential, whether or not they have the disease. The increased incidence in schizophrenic patients and their families suggests that the P50 deficit represents a trait that predisposes to schizophrenia. The deficit is also present, but at much lower levels, in the general population, in subjects with no familial history of schizophrenia.[37] The P50 inhibitory deficit, as previously discussed, is genetically linked to 15q13-q14.[16,38]

The deficit in P50 inhibition appears to reflect decreased activity or expression of the CHRNA7 receptor. Pharmacologic antagonists of the CHRNA7 receptor reproduce the inhibitory deficit in several animal models.[39,40] The DBA/2j mouse strain has 50% lower levels of CHRNA7 than most other inbred strains, it does not show inhibition of its auditory evoked

response to repeated stimuli, and the inhibition is normalized by both nicotine and a specific agonist of the α7 receptor, 2,4-dimethoxybenzylidene anabaseine.[41,42] We have found that the expression of the *CHRNA7* gene is also decreased by approximately 50% in human postmortem hippocampus isolated from schizophrenic subjects, compared with matched control subjects.[43] This finding has now been replicated by 2 other groups in different brain regions, including the reticular thalamic nucleus[44] and frontal cortex.[45]

We isolated a genomic clone for the human *CHRNA7* subunit from a yeast artificial chromosome (YAC) library.[16,46] Mapping of the gene showed that exons 5 to 10 of the *CHRNA7* gene were duplicated as part of a large cassette of DNA. The duplication was inserted approximately 1 Mb proximal to the full-length α7 gene and directly 3′ of 5 novel exons D′-D-C-B-A.[46] The duplicated exons 5 to 10 are expressed with the novel exons D′-A (*dupCHRNA7*) as messenger RNA in both human brain and peripheral tissues. We found that *dupCHRNA7* was homozygotically missing in 5 (4.2%) of 118 schizophrenic patients, but not in 59 control subjects examined. Mutation screening of the amino acid coding region for the full-length *CHRNA7* and *dupCHRNA7* genes and a core promoter region for the full-length gene has been completed. Although multiple polymorphisms were found in the coding region, almost all were synonymous.

A core promoter region for the full-length *CHRNA7* gene was isolated that supports efficient transcription of the reporter gene, luciferase. This 231–base pair (bp) fragment contains consensus binding sites for a number of transcription factors, including stimulating protein Sp1, activator protein AP-4, and a corticosteroid-responsive element, SRE (available at: http://transfac.gbf.de; MatInspector[47]). The regions near the Sp1 binding sites contain several G/C-rich regions, which may also be binding sites for other transcription factors such as Egr1. The location and spacing of these sites with respect to the start of exon 1 are conserved in the bovine α7 gene, where they have been shown to regulate transcription.[48] Mutation screening of this fragment in human DNA samples from control and schizophrenic subjects, reported in the present study, showed a large cluster of polymorphisms, many lying in these putative transcription factor binding sites.

METHODS

SUBJECTS

Subjects were analyzed in a modified case-control study for polymorphisms in the core promoter of the full-length *CHRNA7* gene. A total of 298 schizophrenic subjects from 166 families and 165 control subjects were available for screening (**Table 1**). The sample contained 188 subjects from the NIMH Schizophrenia Genetics Initiative, including DNA samples from 20 families used in a sib-pair analysis positive for schizophrenia.[17] These DNA samples are derived from lymphoblasts in the NIMH collection. Three schizophrenic lymphoblast cultures were obtained from Israel (by P.S.). The remaining DNA samples were isolated from either postmortem brain (33 schizophrenic patients and 13 controls) or lymphoblasts (74 schizophrenic patients and 152 controls), collected in the Denver Schizophrenia Center (Denver, Colo). The locally collected schizophrenic patients includes 25 with childhood onset.

Table 1. Subjects Used for Screening of the *CHRNA7* Gene Promoter*

Source	Subjects	No.	DNA Source	P50 Ratio
NIMH	SZ	188	Lymph	0
Denver	SZ	49	Lymph	34
	SZ	33	Brain	0
	COSZ	25	Lymph	18
Israel	SZ	3	Lymph	0
Total	SZ	**298**	. . .	**52**
Denver	Control	152	Lymph	151
	Control	13	Brain	0
Total	Control	**165**	. . .	**151**

*NIMH indicates National Institute of Mental Health Genetics Initiative for Schizophrenia; SZ, schizophrenia; COSZ, childhood-onset SZ; and lymph, lymphoblasts.

Postmortem brain was donated by the family of the deceased through the Colorado Uniform Anatomical Gift Act (1968) and collected at autopsy. Hospital and autopsy records were reviewed, and family members and physicians were interviewed to determine age, sex, cause of death, and mental illness status (A.O., R.G.R., L.E.A., and R.F.). Brains were weighed, examined for gross pathological features, and divided sagittally. One hemisphere was preserved in formalin for neuropathological analysis. The other hemisphere was sliced coronally into 1-cm slices, from which multiple regions were dissected in blocks, frozen in dry-ice snow, and packaged for storage at –80°C.[49] DNA was isolated from cortex, by means of standard methods.[50] Of the 165 control DNA samples used in the study, 152 were isolated from blood collected in the Denver Schizophrenia Center and had no evidence of current or past psychosis as determined with a Structured Clinical Interview for Axis I *DSM-IV* Disorders—Non-Patient Edition (Version 2.0).[51] In addition, these controls had a Family History–Research Diagnostic Criteria (third edition) interview that showed no evidence of family history of psychosis.[52] All local subjects included in this study provided written informed consent by means of forms approved by the University of Colorado Health Sciences Center Internal Review Board.

Auditory evoked potentials were recorded on 151 of the living controls, by published methods.[28] Briefly, auditory sensory gating is measured by means of the P50 wave of the electroencephalogram response to paired auditory stimuli delivered in the form of clicks. After the second stimulus, delivered 0.5 second after the first, the P50 response is decreased in normal individuals. In most schizophrenic subjects, the response to the second stimulus is not as greatly diminished as in controls; in some subjects the second response is larger than the conditioning response. Control subjects, with no history of mental illness, generally have P50 ratios of the T/C response amplitudes that are less than 0.50. Although some P50 ratios in controls are higher, it has not been known what causes this variation.

Ethnicities of all subjects were recorded from self-report or family interview and represented 3 major groups. White subjects accounted for approximately 65% of the samples from schizophrenic patients and 61% of the controls, and African Americans approximately 31% of the schizophrenic sample and 34% of the control subjects. Hispanics accounted for 4% of samples from schizophrenic patients and 5% of controls. All schizophrenic subjects in each family were screened for polymorphisms to detect the possible presence of different variants in related individuals.

Table 2. Primer Sets Used for PCR, DNA Sequencing, and SSCP*

Primer No.	Primer Sequence	PCR Product Size, bp	Base Location	Purpose
1				
Sense	5′ GGTTGGCAAGACTTCCGAAGCC 3′	618	−553 to −531	PCR and sequencing
Antisense	5′ GTGGCTTTACCGTGCAGGAGCG 3′		+44 to +65	PCR and sequencing
2				
Sense	5′ AGTACCTCCCGCTCACACCTCG 3′	271	−269 to −248	PCR and sequencing
Antisense	5′ ATGTTGAGTCCCGGAGCTGCAG 3′		−20 to +2	PCR and sequencing
3				
Sense	5′ CTGGCCAGAGGCGCGAGGCCG 3′	. . .	−347 to −327	Sequencing
4				
Sense	5′ GGGGCTCGTCACGTGGAGAGGC 3′	180	−170 to −149	SSCP
Antisense	5′ AGCAGCGCATGTTGAGTCCCGGAGC 3′		−14 to +10	SSCP
5				
Sense	5′ GTACCTCCCGCTCACACCTC 3′	176	−268 to −249	SSCP
Antisense	5′ CGGCTCGCGCGCCTTTAAGGA 3′		−112 to −92	SSCP
6				
Sense	5′ AGTACCTCCCGCTCACACCTCG 3′	696	−269 to −248	PCR and sequencing
Antisense	5′ GGAGGCTCAGGGAGAAGTAG 3′		+407 to +427	PCR and sequencing

*PCR indicates polymerase chain reaction; SSCP, single-stranded conformational polymorphism analysis; bp, base pair; and ellipses, not applicable.

MUTATION SCREENING

Genomic DNA was isolated from individuals as previously described,[46] and 231 bases proximal to the α7 ATG translation start site were screened. Single-stranded conformational polymorphism analysis (SSCP) and sequence analysis were used to identify polymorphisms in this core promoter region, as described by Gault et al.[46] Briefly, 2 primer sets for overlapping fragments covered the region from bases −14 to −268 (primer sets 4 and 5; **Table 2**) The 2 primer sets were phosphorylated by means of [γ–^{33}P]-adenosine triphosphate with Promega T4 kinase (Promega Corp, Madison, Wis), then used, separately, to amplify the promoter region using the polymerase chain reaction (PCR). Polymerase chain reaction was done with *Taq* Gold (Perkin-Elmer, Foster City, Calif) and a PCR kit (GeneAmp PCR System 9600; Perkin-Elmer) with the following program: 95°C, 3 minutes; 95°C, 30 seconds; 60°C, 30 seconds; 72°C, 30 seconds, 35 cycles; 72°C, 3 minutes. The products were denatured with loading dye (7.26M urea, 60% formamide, 22mM EDTA, 32mM sodium hydroxide, 0.25% bromophenol blue, 0.25% xylene cyanol) and were separated on detection gels (GeneAmp; Perkin-Elmer) run at 4°C and 25°C by means of a Bio-Rad Power Pac 3000 Power Supply (Bio-Rad Laboratories, Inc, Hercules, Calif) with a temperature probe. The results were similar at both temperatures. Single-stranded conformational polymorphism analysis of DNA samples from both the schizophrenic and control subjects was completed in the same experiment.

Automated DNA sequencing on an Applied Biosystems 377 DNA Seqencer (Applied Biosystems, Foster City, Calif) was used for verification of polymorphisms and determination of the specific base changes, as described in an earlier publication.[46] Generally, a large fragment of 618 bp was generated with the use of primer set 1 (Table 1). A final concentration of 1.25M betaine (Sigma-Aldrich Corp, St Louis, Mo), added to Master Mix 2 in the Expand Long Template PCR System kit (Roche Molecular Biochemicals, Mannheim, Germany), was used to amplify the fragment, with the following PCR program: 93°C, 2 minutes; 38 cycles at 93°C, 30 seconds; 62°C, 30 seconds; 72°C, 1 minute; 72°C, 7 minutes. Briefly, 200 ng of genomic DNA was diluted in a volume of 25 µL to the following final concentrations: 1× Expand Long Template PCR Buffer 3 (Roche Molecular Biochemicals), containing 0.75mM magnesium chloride, 1.67 U of Expand Long Template enzyme mixture (a mix of *Taq* and *Pwo* thermostable DNA polymerases), 0.25mM of each deoxynucleotide triphosphate, 0.4µM of each primer, and 1.25M betaine. An additional primer set 2 was often used for sequencing of a shorter fragment in the proximal promoter region (271 bp). The PCR conditions for the shorter fragment were the same as above.

The G/C (−194) and G/A (−191) variants had indistinguishable SSDP patterns. Samples with these polymorphisms were analyzed with WAVE technology (Transgenomic, Inc, San Jose, Calif). The WAVE detects sequence changes in PCR products based on differential separation through temperature-modulated liquid chromatography and a high-resolution matrix with detection by absorbance at 254 nm. For our analysis, we used PCR products generated by means of primer set 2, with conditions described above. An aliquot of the PCR fragment generated from control or patient was then used for heteroduplex formation in the thermal cycler as follows: 95°C for 5 minutes, ramp slowly from 95°C down to 25°C for 45 minutes, then hold at 4°C. The melting profile of a normal 271-bp promoter sequence was determined with the Wavemaker program (Transgenomics, Inc). A temperature curve was generated for the heterozygous samples containing either G/C (−194) or G/A (−191) at temperatures ranging from 69°C to 73°C. The resulting chromatograms showed the presence of heteroduplex peaks that were resolved optimally at 71°C. A triethylammonium acetate and acetonitrile gradient specified by the manufacturer was used for elution. All subsequent samples were run under identical conditions.

ANALYSIS OF PROMOTER FUNCTION

Promoter function was determined by means of a luciferase reporter gene assay (Promega Corp). To identify a core promoter sequence in the 5′ sequence upstream of the ATG translation start site in the α7 nicotinic receptor gene, we subcloned fragments of this region into the pGL3-Basic Vector from Promega Corp, using PCR and a kit for reporter gene assay (pGEM-T Easy Vector System II kit; Promega Corp). Initially, a 2602-bp fragment was inserted into the pGL3 vector (−2600 to +2; **Figure 2**A). A 1064-bp clone was generated by partial *Pst*I digestion of the original fragment and cloning into the pGL3-Basic Vector. *Pst*I was then used to subclone a fragment of 231

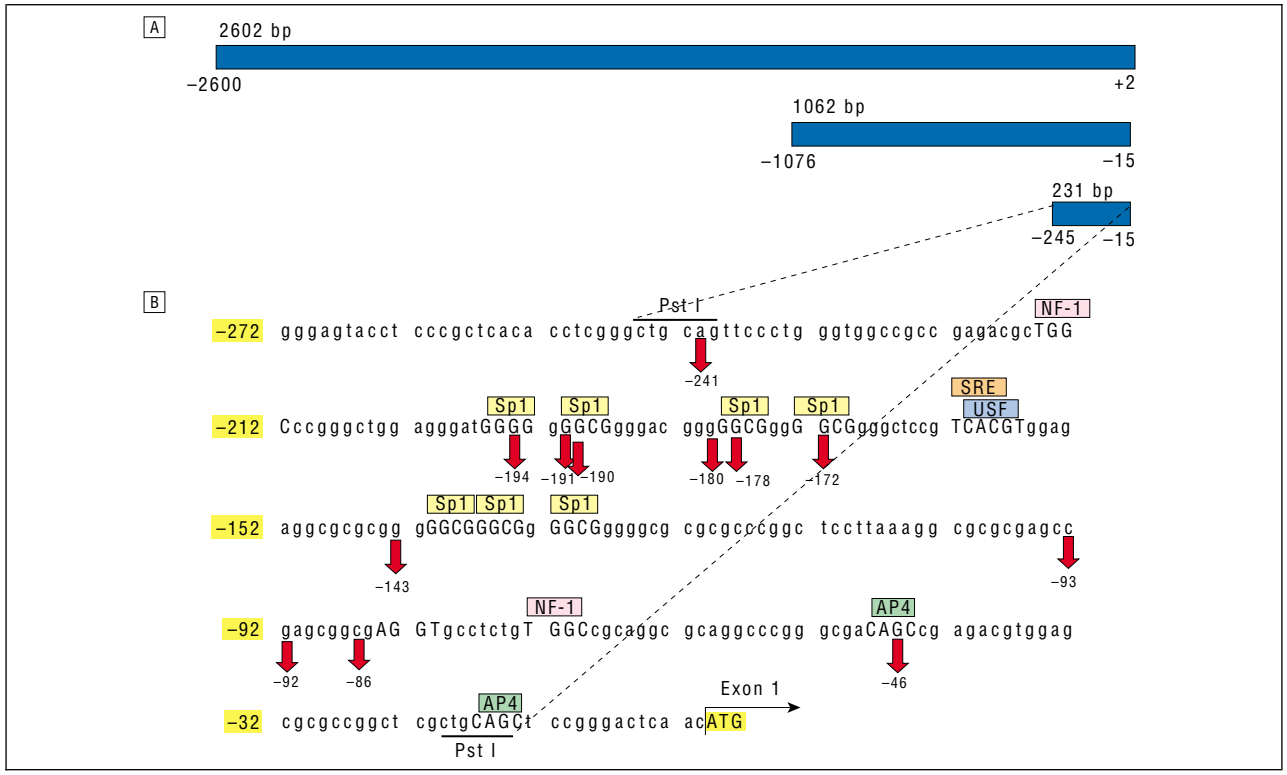

Figure 2. Promoter region of the α7 nicotinic acetylcholine receptor gene (*CHRNA7*). A, Fragments used to identify the core promoter region. B, Core promoter region for the *CHRNA7* gene. Note 2 clusters of stimulating protein Sp1 transcription factor consensus sites. This region of the promoter also contains consensus G/C boxes, which may bind other transcription factors. Polymorphisms isolated in the mutation screen are shown with red arrows. The Sp1 site at −194 is formed by the variant G→C. bp Indicates base pair.

bp, containing the proximal promoter region that is conserved in the bovine α7 gene.[48] Transcription factor consensus sequences in the 5′ upstream region were identified by the TRANSFAC program (available at: http://transfac.gbf.de/TRANSFAC). Variants discovered in the mutation screen were introduced into the normal *Pst*I core promoter clone sequence by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit; Stratagene, La Jolla, Calif). Transient transfections were performed with ProFection Mammalian Transfection Calcium Phosphate System (Promega Corp) with the use of the human neuroblastoma cell line SHSY-5Y[53] grown in 1:1 Ham F12: Dulbecco minimal essential medium, and 10% fetal calf serum, plated to 2×10^5 cells per 35-mm plate. Plasmid DNA (5 µg) prepared with EndoFree Plasmid Kits (QIAGEN Inc, Valencia, Calif) was cotransfected with 1 µg of pRL-TK Vector (Promega Corp). Cells were harvested after 48 hours and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega Corp) and a Turner Designs Luminometer Model TD-20/20 (Turner Designs, Inc, Sunnyvale, Calif).

ANALYSIS OF DOUBLE VARIANTS

Some subjects were found to have more than 1 polymorphism in the *CHRNA7* core promoter. To determine whether these were on the same chromosome, the 2 alleles were examined individually by cloning. The PCR products were generated with the GC-RICH PCR System (Roche Diagnostics, Indianapolis, Ind) with final concentrations of 1× buffer, 2.0mM magnesium chloride, 0.25mM deoxynucleotide triphosphates, and 0.5 µL of enzyme mix in a 25-µL volume. Three primer sets were used (Table 2): primer set 1, core promoter to intron 1; primer set 2, core promoter only; and primer set 6, core promoter to intron 2. The reaction for the smaller product, generated with primer set 2 (0.8µM concentration of each primer), also included 1.0M GC-RICH resolution solution; for the larger products, generated with primer set 1 (0.4µM concentration of each primer) or primer set 6 (0.4µM concentration of each primer), 0.8M GC-RICH resolution solution was included. All PCR products were amplified in a PCR thermocycler (Perkin-Elmer 480; Perkin-Elmer) by means of the following program: 96°C, 3 minutes; 33 cycles at 96°C, 30 seconds; 56°C, 30 seconds; 72°C, 7 minutes. The appropriate PCR bands were gel purified with a rapid gel extraction system (cONCERT Rapid Gel Extraction System; Life Technologies, Inc, Rockville, Md) and cloned into the PCR 4-TOPO vector with the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, Calif). Plasmid DNA was isolated with the S.N.A.P. Miniprep Kit (Invitrogen) and analyzed by DNA sequencing. Approximately 20 clones were sequenced for each double variant cloned.

STATISTICAL ANALYSIS

For the statistical analysis, total counts from schizophrenic individuals included polymorphisms detected in only 1 schizophrenic individual per family unless a second mutation was also present in another affected individual. In this case the second variant was also counted. Subjects homozygous for the common allele were also counted. This strategy was chosen to report the full range of polymorphisms in schizophrenic patients without biasing the results by including multiple individuals who have the same polymorphism based on common ancestry. We used *t* tests to compare means. A Satterthwaite *t* test was used for comparison of means with different variances; χ^2 tests and logistic regression were used to compare prevalence rates. For the double variants in the promoter region, cloning experiments indicated that each polymorphism can be considered as a separate allele.

RESULTS

REFINED PHYSICAL MAP OF THE P50-SCHIZOPHRENIA LINKAGE REGION ON CHROMOSOME 15

Linkage studies have identified a region of chromosome 15q13-q14 that is inherited in subjects with the P50 deficit and with schizophrenia. We previously published a preliminary YAC contig of this region on chromosome 15 that is linked to schizophrenia.[46] Because of the partial duplication of *CHRNA7*, further physical mapping was performed to determine which sequences were and which were not duplicated. The contig now includes multiple bacterial artificial chromosomes and map locations for additional expressed sequence tags and markers (Figure 1). The region is defined by 30 markers and is estimated to be about 4 Mb. The full-length *CHRNA7* gene, implicated in the P50 deficit in schizophrenia, is localized at this site between unique markers *D15S1013* and *D15S1010*. Mapping of α7 exons showed that exons 5 to 10 of *CHRNA7* had been duplicated, along with a large cassette of DNA containing several other genes, and inserted proximal to the full-length gene. The insertion occurred next to novel exons D-C-B-A, with which the duplicated α7 exons are expressed as messenger RNA (*dupCHRNA7*; GenBank Accession No. AF029838).[46] The novel *dupCHRNA7* transcript is found in multiple tissues, including human brain and blood leukocytes, although its function is not yet known.

We have since determined that exons D-C-B-A are also duplicated and expressed with downstream sequences that are not α7 (GenBank Accession No. AA861176). A copy of these novel exons also maps on chromosome 3 by hybrid clone panel analysis (not shown). We hypothesize that exons D-C-B-A, contained in clone AA861176, were duplicated at least once on chromosome 15 with one insertion site near the dinucleotide repeat (*D15S1043*) before the partial duplication of the *CHRNA7* gene. Ultimately, the large cassette containing α7 exons 5 to 10, dinucleotide repeat *L76630*, and expressed sequence tag *WI13983* was duplicated and inserted proximally, interrupting the duplication of AA861176. Additional analysis of exons D-C-B-A indicated that exon D contains 2 exons and an intervening sequence. We have designated these 2 newly defined exons as D′ (proximal) and D (distal). The unique DNA sequence between the full-length *CHRNA7* gene and *dupCHRNA7* is approximately 1 Mb and contains a large number of mapped expressed sequence tags and markers. The site of the marker *D15S1360*, isolated from a YAC containing *CHRNA7*, has been more precisely mapped to intron 2, by means of the sequence available from the National Human Genome Research Institute draft sequence (bacterial artificial chromosomes 717i24 and 198g2; Figure 1). The *D15S1360* repeat has been used extensively for genotyping of both schizophrenic patients and controls in the laboratory; only 2 alleles were ever observed in any one individual. Furthermore, the promoter and exons 1 to 4 of the full-length α7 gene were found only in bacterial artificial chromosomes and P1 artificial chromosomes containing *D15S1360*, all of which map between *D15S1031* and *D15S1040*. We therefore conclude that the region 5′ of exon 4, containing the promoter region of the full-length α7 gene, is not duplicated.

MUTATION SCREENING OF THE α7 GENE CLUSTER

The α7 gene cluster in the 15q13-q14 linkage region was selected as the most likely candidate gene group for mutation studies, based on inclusion of the linkage marker *D15S1360* within intron 2 of the full-length *nAChRα7* gene and the neurobiological evidence described previously that is consistent with diminished α7 expression or function. Because nonsynonymous changes in the coding region were rare and not associated with schizophrenia, attention turned to the promoter region of the gene.

CLONING AND MUTATION SCREENING OF SEQUENCE 5′ OF THE CODING REGION FOR THE FULL-LENGTH α7 GENE

Approximately 2.6 kb (2602 bp) of DNA sequence 5′ of exon 1 in the full-length *CHRNA7* gene was cloned from P1 artificial chromosome 24919 that contains *CHRNA7* exons 1 to 3 (Incyte Genomics, Inc, Palo Alto, Calif). Subclones of this region were constructed for determination of functional domains for gene transcription (Figure 2A). Base pair numbering begins with –1 at the position preceding the translation start in exon 1. The 3 fragments indicated were cloned into the pGL3 Basic Vector from Promega Corp for analysis of promoter sequence effects on the reporter gene luciferase. A fragment of 231 bp, immediately 5′ of exon 1, was identified as the core promoter sequence and is sufficient to drive high levels of transcription in vitro (not shown). Sequences further upstream, included in fragments of 1.0 kb and 2.6 kb, were identified as containing putative repressor elements.

The 231-bp core promoter region is homologous to the bovine α7 core promoter region, including conservation of some transcription factor consensus sequences.[48] The human α7 promoter region is likely to be regulated by Sp1 and AP-4 transcription factors, for which there are 2 clusters of consensus sites (Figure 2B). The regions including the Sp1 sites were also identified as G/C boxes, which may bind other transcription factors. There is a consensus serum responsive element SRE, also found in the bovine gene, but not in chick.[54]

Mutation screening has been completed for the 231-bp core promoter in 195 schizophrenic individuals and 165 controls, demonstrating a complex cluster of variants (**Table 3**). There were 12 different single-nucleotide changes, including 2 insertions and a deletion. Many of the variants lie in putative transcription factor consensus binding sequences (Figure 2B). The variant at –194 G→C introduces a new Sp1 site. In addition, we found that some subjects carried double variants that were combinations of the single variants isolated (8 different combinations). The total numbers of single and double variants found in control and schizophrenic subjects are shown in Table 3 and **Table 4**, respectively, stratified by ethnicity. One polymorphism, an insertion of +CGGG at –140 bp, was found in a single sub-

Table 3. Single Promoter Variants in Control and Schizophrenic Subjects

	Control Subjects				Schizophrenic Subjects			
Variant	**White**	**African American**	**Hispanic**	**Total**	**White**	**African American**	**Hispanic**	**Total**
–46 G→T	0	11	0	**11**	0	9	0	**9**
–86 C→T	9	0	0	**9**	20	1	2	**23***†
–92 G→A	1	0	0	**1**	1	1	0	**2***
–93 C→G	1	0	0	**1**	0	0	0	**0**
–143 G→A	0	0	0	**0**	0	1	0	**1***
–172 +CGGGGG	1	0	0	**1**	0	0	0	**0**
–178 –G	0	3	0	**3**	0	9	0	**9***
–180 G→C	0	0	0	**0**	0	0	1	**1***
–190 +G	0	5	2	**7**	1	2	0	**3**
–191 G→A	0	3	0	**3**	1	6	0	**7***
–194 G→C	9	2	0	**11**	12	1	1	**14***
–241 A→G	0	0	0	**0**	2	0	0	**2***
Total Variants	**21**	**24**	**2**	**47**	**37**	**30**	**4**	**71**
Total subjects	103	54	8	**165**	129	56	10	**195**

*Found more frequently in schizophrenic subjects.
†$P = .04$.

Table 4. Double Variants in Control and Schizophrenic Subjects

	Control Subjects				Schizophrenic Subjects			
Double Variant	**White**	**African American**	**Hispanic**	**Total**	**White**	**African American**	**Hispanic**	**Total**
–46/–178	0	0	0	**0**	0	3	0	**3***
–46/–190	0	0	0	**0**	0	1	0	**1***
–46/–191	0	0	0	**0**	0	1	0	**1***
–86/–194	2	0	0	**2**	1	0	0	**1**
–86/–241	0	0	0	**0**	1	0	0	**1***
–93/–194	1	0	0	**1**	0	0	0	**0**
–178/–191	0	0	0	**0**	0	1	0	**1***
–191/–194	0	1	0	**1**	0	0	0	**0**
Total Variants	**3**	**1**	**0**	**4**	**2**	**6**	**0**	**8**†
Total subjects	103	54	8	**165**	129	56	10	**195**

*Found only in schizophrenic individuals.
†$P = .38$ (not significant).

ject with a diagnosis of psychosis, not otherwise specified (*DSM-IV*, 298.9). As this diagnosis was not included in either our control or schizophrenic samples, this individual was not included in Table 3 or in the statistical analysis, but is mentioned to indicate that additional and perhaps more complex polymorphic patterns may remain to be discovered. Forty-seven of 165 control individuals and 71 of 195 schizophrenic patients had one of the single polymorphisms. Although 1 single variant (–93 bp) and 2 double variants (–93 bp/–194 bp; –191 bp/–194 bp) were found only in control subjects, a larger number of both single and double variants were found in schizophrenic patients than in controls. The difference was not, however, statistically significant. Eight of the 12 variants (–86 bp, –92 bp, –143 bp, –178 bp, –180 bp, –191 bp, –194 bp, and –241 bp), marked with asterisks in Table 3, were more prevalent in schizophrenic subjects. Twenty-seven of 165 control subjects had one of these 8 variants, but 59 were found in the 195 schizophrenic patients. Association of the single variant –86 bp C→T with schizophrenia in the combined ethnic groups reached significance ($P = .04$; Table 3). This polymorphism was examined alone because –86 bp C→T was the most common variant in the region, and it also had the highest prevalence in schizophrenic patients. It is found more frequently in whites than in African Americans. The genotype relative risk for this variant was 2.39 (95% confidence interval, 1.07-5.32). The principal polymorphisms found in African American schizophrenic patients were those at –178 bp del G and –191 bp G→A. Although more variants at these sites were found in schizophrenic subjects than in controls, fewer subjects were carrying each of the polymorphisms and the differences were not significant.

In the individuals examined in this study, there were 8 doubly polymorphic patterns where subjects had more than 1 polymorphism in the core α7 promoter. Five of these double variants were found only in schizophrenic patients (marked with asterisks in Table 4). DNA fragments were cloned and sequenced from individuals with most of the double variant patterns isolated thus far. Three primer sets were used, 1 that amplified the core promoter of 271 bp (Table 2; primer set 2); another set that included the core promoter, exon 1, and part of intron 1

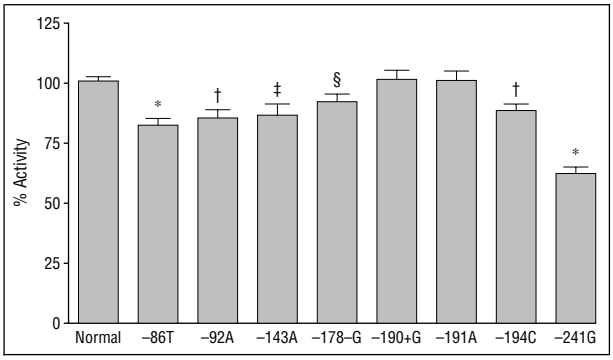

Figure 3. Functional assay of α7 core promoter variants. Mutations were introduced into the normal 231–base pair core promoter by site-directed mutagenesis. These were transfected into SHSY-5Y cells and promoter activity was assayed by measuring luciferase. The activity of the normal promoter sequence was set at 100%. Asterisk indicates $P<.0001$; dagger, $P=.005$; double dagger, $P=.05$; and section mark, $P=.03$.

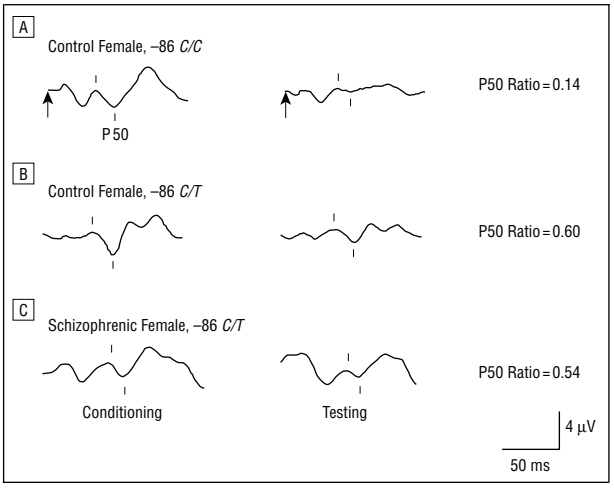

Figure 4. Gating of the P50 auditory evoked potential. Tracings are shown for the conditioning and test responses in control subjects without (A) and with (B) a polymorphism at –86 base pairs in the α7 nicotinic acetylcholine receptor gene (*CHRNA7*) core promoter. C, Tracing from a schizophrenic subject with the same variant. Arrows indicate the paired auditory stimuli.

(primer set 1); and a primer set including the core promoter, exon 1, intron 1, exon 2, and part of intron 2 (primer set 6). Two variants were never found on the same chromosome, and only 2 alleles were present in all cases examined, indicating that the core promoter region is not duplicated in these individuals and, further, that each variant is a separate allele. Thus, polymorphisms in the core promoter of the full-length α7 nicotinic receptor gene are found more frequently in schizophrenic individuals than in subjects with no family history of schizophrenia, and double variants are likely to result from inheritance of 1 mutant allele from each parent.

Thirty-four affected full sib pairs were examined in 30 families that had 1 or more of the promoter polymorphisms. Fourteen of the 34, or 0.41 sib pairs, shared at least 1 of these variants. For the common –86-bp C→T, 6 of 12 sib pairs shared the polymorphism.

FUNCTIONAL SIGNIFICANCE OF VARIANTS IN THE *CHRNA7* PROMOTER REGION

In vitro functional analysis has been performed for several of the polymorphisms found more frequently in schizophrenic subjects. A luciferase reporter gene assay was used to compare the normal core promoter sequence with a fragment containing one of these variants. The results are shown in **Figure 3**. Variants at –86 bp, –92 bp, –143 bp, –178 bp, –194 bp, and –241 bp decreased transcription of the luciferase reporter gene in this in vitro assay, suggesting that presence of one of these polymorphisms in the core promoter region may decrease transcription from the gene. The –86-bp C→T variant resulted in a decrease in luciferase transcription of 20% ($P<.0001$). The functional promoter mutations examined thus far were statistically more prevalent ($\chi^2_1=7.302$, $P=.007$) in schizophrenic patients than in the control subjects.

ASSOCIATION OF PROMOTER VARIANTS WITH P50 INHIBITION

Although promoter variants were found in control subjects, they were fewer in number than in schizophrenic patients. In complex disorders where multiple gene variants may be interacting with environmental factors to produce the disease, it has been suggested that functional polymorphisms are likely to be common in the general population, where each may have a more elementary phenotype, such as a biochemical or electrophysiologic abnormality that is part of the pathophysiology of the illness.[55,56] We, therefore, decided to examine the association of *CHRNA7* promoter polymorphisms in our living control subjects with a functional electrophysiologic assay, inhibition of the P50 response to paired auditory stimuli. We measured P50 auditory sensory gating in 151 of the 152 live controls used in this study. The range of P50 ratios (T/C) for controls was 0.00 to 1.91. Overall mean P50 ratio was 0.22±0.27. There were 38 adult schizophrenic subjects collected locally where P50 recording was done. The mean P50 ratio for these patients was 0.92±1.02, with a range of 0.00 to 4.96. Eighteen patients with childhood-onset schizophrenia, included in the mutation screen, were also recorded. Their mean was 1.05±0.91, similar to that of the adult patients with schizophrenia. The mean ratio for the schizophrenic patients was significantly greater than that of the control subjects ($t_{205}=8.49$, $P<.0001$).

Tracings for subjects with and without the –86-bp C→T polymorphism are shown in **Figure 4**. A control subject with the normal *C/C* genotype (Figure 4A) had a T/C ratio (P50 ratio) of 0.14, indicating that the test response to the second auditory stimulus was being inhibited. However, a control subject carrying a –86-bp C→T heterozygotic genotype (Figure 4B) had a T/C ratio of 0.60, demonstrating a much lower level of inhibition. A schizophrenic patient with the –86-bp C→T genotype also had a higher T/C ratio of 0.54. These results suggest that the presence of a promoter variant might be associated with decreased inhibition in the sensory gating paradigm and, hence, a higher T/C (P50) ratio.

We then examined the relationship between the means for the P50 T/C ratios and the presence of *CHRNA7* promoter variants in the 151 controls (**Figure 5**). The

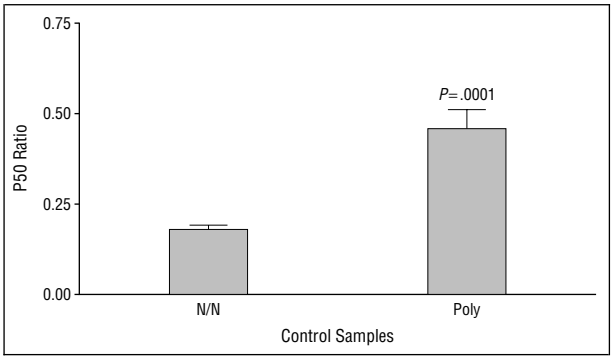

Figure 5. Association of promoter variants in control subjects with P50 gating. Auditory evoked potentials were recorded in control subjects. Mean P50 ratios are shown for control subjects with no polymorphisms in the core promoter (N/N) and with 1 or more polymorphisms (Poly). The difference in the means was highly significant by *t* test ($P=.0001$).

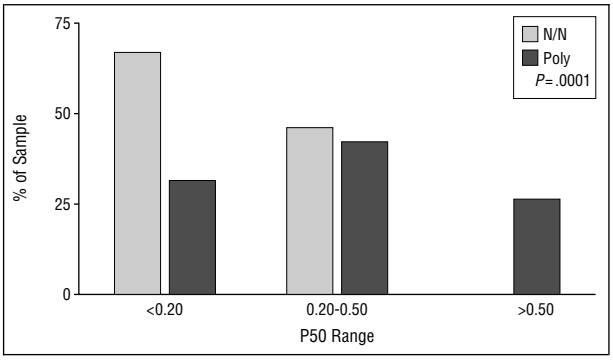

Figure 6. Promoter variants in control subject fit into 3 P50 gating ranges. Logistic regression analysis was used to examine the relationship between the presence of an α7 promoter variant and the P50 test-conditioning ratio (T/C). Statistical analysis suggested that the best fit was into 3 groups ($P<.0001$). Control subjects with no polymorphism (N/N) were found principally in the 2 lower P50 T/C ranges, while subjects with ratios greater than 0.50 all carried a promoter variant (Poly).

mean P50 ratio for controls with no *CHRNA7* promoter variant was 0.179±0.014. However, the mean for control subjects with one of the single or double variants was 0.458±0.055. The results were analyzed, using a Satterthwaite *t* test for samples with different variances. The control subjects with no polymorphisms had a significantly lower mean P50 ratio than control subjects in whom a promoter variant was found ($P<.0001$), suggesting a strong relationship between the presence of a promoter variant and decreased sensory processing.

In the patients with adult-onset disease, where P50 had been recorded, we found that 7 of 8 polymorphisms in the core promoter were found in schizophrenic patients with P50 ratios greater than 0.50. In the 18 patients with childhood-onset disease recorded there were 7 polymorphisms, 5 of which were found in subjects with P50 ratios greater than 0.50. These results are consistent with a similar relationship of the *CHRNA7* promoter polymorphisms to the P50 ratio in both adult- and childhood-onset schizophrenia.

Logistic regression analysis of the control data suggested that the presence of promoter variants is better described by 3 groups than by a regression line on P50 range (**Figure 6**). One group with individual average P50 ratios less than 0.20 appears to have stable auditory gating. A second group with P50 ratios between 0.20 and 0.50 may have a less stable filtering mechanism. A third group with P50 ratios consistently greater than 0.50 exhibits very little auditory gating, similar to what is seen in the schizophrenic population. Control subjects with no polymorphism in the core α7 promoter screened in this study were found to have P50 ratios in the first 2 groups, with most in the less than 0.20 group. However, controls with polymorphisms were more evenly distributed among the 3 groups, and only subjects with a promoter variant were found to have P50 ratios greater than 0.50.

COMMENT

Although schizophrenia has a large genetic component, it is thought to be oligogenic.[19,56] Heterogeneity in the inheritance of prediposing traits further complicates the orderly process of gene identification. At present, there are 14 chromosomes on which genetic linkage has been identified or is suggested.[15] Many of these regions may contain a gene variant contributing to the disease in the linked populations. This suggests that many genes may interact in the disorder, but that not all the gene variants at these loci may be present in a single individual. Furthermore, the actual polymorphism present in any given gene may result in differences in gene expression between subjects that can also be affected by other genes and environmental factors. Some variants may manifest in early development and some during puberty or postpuberty, when schizophrenia is usually first diagnosed. It is also important to recognize that some gene variants may compensate for others, or actually have a beneficial significance.

Three principal issues contribute to a discussion of the contribution of our results. First, the study of a candidate gene for an endophenotype in schizophrenia, rather than the multigenic disease itself, may be more likely to provide isolation of a single gene defect. Endophenotypic traits found in complex disorders have been examined in attempts to simplify the biology and genetics of schizophrenia.[57,58] Examples of such traits are inhibitory gating of the P50 auditory evoked response[58,59] and smooth-pursuit eye tracking,[60,61] both of which are found in the general population at lower levels than in the disease. In control subjects with no history of psychosis, variants in only 1 or a few different genes may be required to produce a specific abnormal phenotype or trait. In a disease such as schizophrenia, interdependence of multiple neurotransmitters in a single brain pathway and the presence of multiple gene defects may worsen performance in a given quantitative trait. However, it is assumed that even in schizophrenia only a subset of the genes involved in the full clinical diagnosis is associated with a specific endophenotype.

Second, the nicotinic acetylcholine receptor subunit gene, *CHRNA7*, was implicated as a candidate gene in the 15q13-q15 linkage region for schizophrenia by genetic and biological data supporting its role in sensory processing deficits in the disease.[62,63] Expression of the *CHRNA7* gene is decreased in postmortem brain isolated from schizophrenic subjects compared with that of controls.[43-45] We presented herein promoter variants in

CHRNA7 that are consistent with decreased expression of this gene. Several of the polymorphisms have been tested in an in vitro reporter gene assay where 6 of 8 variants were found to have decreased transcriptional activity. The most common variant at –86 bp, associated with schizophrenia ($P=.04$), decreased transcription of the luciferase reporter gene by 20% ($P=.0001$). Comparable transcriptional effects have been seen for other gene promoters with single–base pair mutations.[64-66] Many of the promoter variants, both single and double, were found principally in schizophrenic patients. Indeed, the functional variants isolated thus far are statistically more prevalent in schizophrenic subjects ($P=.007$) than in controls. Although this preliminary functional analysis suggests that some single variants may have transcriptional implications, all of the promoter variants, both single and double, require further functional analysis to confirm the hypothesis. The double variants examined thus far, where more than 1 variant was present, were combinations of the known single variants and were found on separate alleles, indicating inheritance of 1 mutation from each parent.

It is possible that some variants in the core promoter region have been missed because of ascertainment bias. Our sample included more schizophrenic subjects than controls, and we had fewer African Americans and Hispanic subjects than whites. Polymorphisms at –92 bp, –143 bp, –180 bp, and –241 bp were found more often in schizophrenic patients but were rare in our sample. It is quite possible that when additional African Americans, Hispanics, and other ethnic cohorts are screened, we will find more subjects with these rare variants and possibly even new variants. Furthermore, we have isolated an additional 2302 bp of sequence upstream of the *CHRNA7* core promoter. Preliminary analysis of 2 subclones indicated the presence of upstream repressor elements. Upstream regulatory elements have been found in several other nicotinic receptor subunit genes that suggest complex developmental and tissue-specific regulation of expression.[53,67] Other functional or more complex variants in schizophrenic subjects may lie in these regulatory regions of the human α7 nicotinic receptor subunit gene, perhaps in disequilibrium with some of the polymorphisms in the core promoter. The frequency of core promoter polymorphisms in multiply affected families was too small to permit determination of their contribution to the transmission of illness. Elucidation of all the genetic variance in this region, including the more extended promoter, in a larger number of families will be necessary for this determination.

Third, because the *CHRNA7* gene was first targeted as a candidate gene by our laboratories as having a biological role in a sensory processing endophenotype seen in most schizophrenic patients and in one half of their first-degree relatives,[25,63] it is significant that a measure of auditory evoked inhibition in humans, the P50 gating phenotype, appears to be correlated with presence or absence of variants in the *CHRNA7* gene core promoter. Inhibition of the P50 response is abnormal in most schizophrenic patients, where the test response is often larger than the conditioning response, resulting in T/C ratios much greater than 0.50. In control subjects with no history of schizophrenia, we found a range of T/C ratios lower than in schizophrenic patients ($t_{205}=8.49$, $P<.0001$). However, the ratios were significantly higher in controls with promoter variants than in controls with no polymorphisms ($P=.0001$). The relationship between the presence of a promoter polymorphism and the P50 T/C ratio appeared to place the control subjects into 3 groups. Whether the grouping indicates a gene dosage effect or additional gene interactions remains to be established. Inhibitory pathways in schizophrenic subjects are likely to be much more complex than in individuals with no history of mental illness. Measurement of the P50 phenotype in control subjects is, thus, expected to be less complicated and more representative of the effect of a few genes or possibly even a single gene defect. The present results suggest that the α7 promoter variants are associated with a measurable phenotype found in the general population, but present more frequently in schizophrenia. Other investigators have noted correlations between higher P50 ratios and schizotypy,[68] particularly in individuals with a family history of schizophrenia.[69]

Last, the design and interpretation of candidate gene association studies, such as the present report, are not obvious. In the human lipoprotein lipase gene, for example, it has been found that the average individual is heterozygous at 17 sites, probably because of a combination of historical population founding, stratification of polymorphic changes, and recombination.[70] Not all of these polymorphisms will be functional, although they may be in disequilibrium with other variants and/or with the disease. This emphasizes the importance of thorough functional analysis of any polymorphisms found to be associated with schizophrenia. Furthermore, the complexity and dependence on the interactions of functional variants contributing to a complex major mental illness is consistent with a hypothesis that many of these functional polymorphisms are likely to be common in the general population.[55,56] In that regard, a variant in the catechol *O*-methyltransferase gene (*COMT*), found in 50% of non–mentally ill subjects, has recently been associated with prefrontal cortical deficits in schizophrenia, but estimated to contribute only a small percentage of the risk for the disease.[71] Our findings of functional variants in the *CHRNA7* gene promoter are similar; promoter polymorphisms were found in 28% of our control subjects with no family history of schizophrenia, but were strongly associated ($P=.0001$) with having a deficit in auditory sensory processing. The genotype relative risk for schizophrenia at one of the polymorphisms, –86 bp, was 2.39 (95% confidence interval, 1.07-5.32), indicating a small contribution to the disorder. This sort of inheritance of gene variants is likely to be the case for many complex disorders. Indeed, a role for calpain 10 in type 2 diabetes has been recently reported, where the aberrant allele was found in 75% of the control population but in 80% of those with diabetes.[72] It may be that the assemblage of a group of functional variants in one individual is required for the development of a complex disease such as schizophrenia.

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