

$\alpha 7$ Nicotinic Receptor Gene Promoter Polymorphisms in Inbred Mice Affect Expression in a Cell Type-specific Fashion*

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Inbred mouse strains display significant differences in their levels of brain $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) expression, as measured by binding of the $\alpha 7$ -selective antagonist α -bungarotoxin. Variations in α -bungarotoxin binding have been shown to correlate with an animal's sensitivity to nicotine-induced seizures and sensory gating. In two inbred mouse strains, C3H/2Ibg (C3H) and DBA/2Ibg (DBA/2), the interstrain binding differences are linked to a restriction length polymorphism in the $\alpha 7$ nAChR gene, *Chrna7*. Despite this finding, the molecular mechanism(s) through which genetic variability in *Chrna7* may contribute to $\alpha 7$ nAChR expression differences remains unknown. However, studies of the human $\alpha 7$ nAChR gene (*CHRNA7*) previously have demonstrated that *CHRNA7* promoter polymorphisms are associated with differences in promoter activity as well as differences in sensory processing. In the present study, a 947-base pair region of the *Chrna7* promoter was cloned from both the C3H and DBA/2 inbred mouse strains in an attempt to identify polymorphisms that may underlie $\alpha 7$ nAChR differential expression. Sequence analysis of these fragments identified 14 single nucleotide polymorphisms (SNPs). A combination of two of these SNPs affects promoter activity in an *in vitro* luciferase reporter assay. These results suggest a mechanism through which the *Chrna7* promoter genotype may influence interstrain variations in $\alpha 7$ nAChR expression.

The expression of $\alpha 7$ nAChRs,³ as measured by ¹²⁵I-labeled α -bungarotoxin (α -BTX), varies significantly across inbred mouse strains (1, 2), and studies have demonstrated that interstrain differences in $\alpha 7$ nAChR expression are genetically regulated. For example, restriction fragment length polymorphisms in *Chrna7*, the gene that encodes for the mouse $\alpha 7$ nAChR subunit, have been shown to be linked to $\alpha 7$ nAChR expression in genetically segregating populations derived from

the inbred mouse strains C3H and DBA/2 (3). The strain differences in binding appear to be related to differences in $\alpha 7$ nicotinic receptor mRNA levels (2, 4), suggesting that transcriptional or posttranscriptional mechanisms may contribute to the variations in α -BTX expression levels.

Genetically regulated $\alpha 7$ nicotinic receptor expression patterns have been associated with individual variations in neurophysiology and neuroanatomy. Interstrain differences in α -BTX binding are related to variations in sensitivity to nicotine-induced seizures, acoustic startle response (5), and hippocampal sensory gating (2). In addition, genetic variability in *Chrna7* has been implicated in regulating individual differences in cholinergic and GABAergic hippocampal neuroanatomy (6–8). Variations in α -BTX binding have also been associated with differences in neurophysiological function among humans. Schizophrenics, for example, display reduced α -BTX binding levels in post-mortem hippocampus (9). Furthermore, promoter polymorphisms in the gene coding for the human $\alpha 7$ nAChR subunit are associated with an auditory gating deficit that is common among schizophrenics (10). This auditory gating measure is analogous to hippocampal gating of repeated sensory information that is correlated with α -BTX levels in mice. Recently, Leonard *et al.* (11) discovered that *CHRNA7* promoter polymorphisms that led to lower promoter activity are associated with deficits in auditory gating.

In order to establish whether polymorphisms in the *Chrna7* promoter exist between C3H and DBA/2 mice and, if so, whether they affect the function of the *Chrna7* promoter, we have cloned a 947-bp region of the putative *Chrna7* promoter from each strain. Sequence analysis of these regions identified 14 single nucleotide polymorphisms (SNPs). In this report, we describe the effect of the SNPs on the ability of the *Chrna7* promoter to drive the expression of a luciferase reporter gene *in vitro*.

EXPERIMENTAL PROCEDURES

Animals—C3H/2Ibg (C3H) and DBA/2Ibg (DBA/2) mice were maintained in the specific pathogen-free colony at the Institute for Behavioral Genetics at the University of Colorado (Boulder, CO). Animals were weaned at 25 days of age and housed with 1–5 same sex siblings. Mice were maintained on a 12-h light/12-h dark cycle (lights on from 0700 to 1900 h) and had free access to food (Teklad 22/5 rodent diet; Harlan, Madison, WI) and water.

Genomic DNA Isolation and Plasmid Constructions—Mice were sacrificed by cervical dislocation, and their spleens were removed. Genomic DNA was isolated from C3H and DBA/2

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³ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; α -BTX, ¹²⁵I-labeled α -bungarotoxin; SNP, single nucleotide polymorphism; FBS, fetal bovine serum; ANOVA, analysis of variance; HSD, honestly significant difference.

spleens as previously described (3). C3H and DBA/2 *Chrna7* promoter fragments were amplified from the genomic DNA samples using HF2 DNA polymerase (BD Biosciences) and introduced into the vector pCRII TOPO (Invitrogen). Several independent clones from each mouse strain were sequenced in order to distinguish polymorphisms from PCR artifacts. Promoter fragments with the strain consensus sequences were subcloned in pGL3 Basic (Promega, Madison, WI) for reporter gene analysis. The *Chrna7* promoter fragments were introduced into pGL3, such that the native *Chrna7* initiation codon replaced the initiation codon of the luciferase gene. Chimeric clones were generated by standard restriction enzyme digestion (NheI, XbaI, Bsu36I, and NruI; New England Biolabs, Ipswich, MA). XbaI-NruI fragments were subcloned bidirectionally into the pGL3 promoter vector via blunt end ligation.

The 947-, 621-, 422-, and 138-bp *Chrna7* promoter deletion constructs were generated by NheI, XbaI, Bsu36I, and NruI digestion of the 947-bp *Chrna7* promoter fragment, respectively. To produce the 65-bp deletion construct, inverse PCR was used to remove 882 bp from the 5' end of the 947 bp C3H *Chrna7* promoter fragment. This procedure was carried out using 50 ng of the full-length C3H *Chrna7* promoter construct, 125 ng of a sense (5'-phosphate-GAGGCGGCTGGAGCGGC-3') and 125 ng an antisense primer (5'-phosphate-GAAATGT-TCTGGCACCTGCACTTGC-3'), 10× reaction buffer, 1 μl of dNTP mix, 1 μl of *PfuUltra* HF DNA polymerase, and double-distilled H₂O to a final volume of 50 μl. PCR amplification was performed on this reaction using the following parameters: 1 cycle at 95 °C for 30 s; 12 cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 6 min). To select for the DNA template harboring the 882-bp deletion, the parental DNA template was digested with 1 μl of DpnI (10 units/μl) endonuclease for 1 h at 37 °C. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA), and the plasmid was religated using the Quick Ligation Kit (New England Biolabs). The deletion construct was then transformed into XL1-Blue supercompetent cells (Stratagene, La Jolla, CA).

To directly evaluate the effect of the promoter polymorphisms on *Chrna7* promoter activity, specific DBA/2 polymorphisms within -583 to -302 of the α7 promoter region were introduced onto a C3H promoter sequence using the QuikChange II site-directed mutagenesis kit (Stratagene). For the DBA/2 double mutants, the C3H nucleotide variations were introduced onto the DBA/2 promoter sequence. Sense and antisense primers were designed to introduce point mutations between the XbaI and NruI restriction sites (Fig. 1A). Mutagenesis and transformation was carried out as described above. For all constructs described, DNA was purified from bacterial cultures using either the FastPlasmid Mini-Prep Kit (Eppendorf, Westbury, NY) for small scale DNA preparations or the Qiagen Plasmid Maxi Purification kit (Qiagen) for large scale preparations.

Cell Culture and Transfections—GH4C1 were grown in Ham's F-10 medium supplemented with L-glutamine (15%); horse serum (2.5%), fetal bovine serum (FBS), and 1% penicillin/streptomycin. SH-SH5Y, HEK293T, and C2C12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter glucose, 10% FBS, and 1% penicillin/streptomycin.

C2C12 cells were induced to differentiate into myotubes by replacing the FBS in the growth medium with 2% horse serum. PC12 cells were grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 5% FBS, and 1% penicillin/streptomycin. In order to differentiate PC12 cells into a neuronal phenotype, the cells were treated with nerve growth factor (100 ng/ml) in Dulbecco's modified Eagle's medium supplemented with 4% horse serum, 2% FBS, and 1% penicillin/streptomycin for 1 week as previously reported (12). P19 cells were grown in Dulbecco's modified Eagle's medium α supplemented with L-glutamine, ribonucleosides, and deoxyribonucleosides, 7.5% calf serum, 2.5% FBS, and 1% streptomycin. Differentiation of P19 cells into neuronal cells was done by incubation of cell aggregates in media supplemented with 500 nM retinoic acid as described previously (13). All cells types were maintained at 37 °C in 5% CO₂. Cells were fed twice a week with their appropriate culture medium and passaged once per week.

For transfections, 0.5×10^6 cells were seeded per well of a 24-well plate. The next day, the cells were transfected using Lipofectamine Plus reagent (Invitrogen) with 0.2 μg of the test plasmids and 0.2 μg or 10 ng of the transfection control plasmids pCDNA3.1 LacZ or pRL-CMV (*Renilla* luciferase), respectively. All transfections were performed according to the manufacturer's protocol.

Luciferase Assay—Twenty-four hours following transfection, cell extracts were prepared, and assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and either a Beckman Coulter LD400C Luminometer/plate reader (Beckman Coulter, Fullerton, CA) or a PerkinElmer Victor 3^V plate reader (PerkinElmer Life Sciences). All data are reported as the ratio of the luciferase activity of the test plasmid divided by the enzyme activity of the transfected control plasmid. Sample sizes for each experiment are indicated under "Results" and in the figure legends. Each sample was run in duplicate.

Bioinformatic Analysis—Predicted transcription start sites were determined from the ENSEMBL Genome Browser (available on the World Wide Web at www.ensembl.org/) and the data base of transcription start sites (available on the World Wide Web at dbtss.hgc.jp/). Multiple-sequence alignment across species was performed using ClustalW (available on the World Wide Web at www.ebi.ac.uk/clustalw/). Sequence alignment across a pair of species was carried out in VISTA (available on the World Wide Web at genome.lbl.gov/vista/index.shtml).

Promoter sequences were scanned for potential transcription factor binding sites using Patch (available on the World Wide Web at www.gene-regulation.com/cgi-bin/pub/programs/patch/bin), a pattern search program using TRANSFAC 6.0 public sites.

Data Analysis—Statistical analysis was carried out in SPSS version 14.0 (SPSS Inc., Chicago, IL). Data were analyzed by one-way analysis of variance (ANOVA), two-way ANOVA, or Student's *t* test where appropriate. Significant differences were further evaluated using Tukey's HSD *post hoc* test.

Chrna7 Promoter Polymorphisms in Inbred Mouse Strains

A.

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5'
CCAGGGTATTGATGCCACTGCTCCTGCAACCAAGCAGCTGGAGTCACTATCCCCTTCCCACCACTTCC
AGTTTAGTCACATTTTTTTTTTTGATAAGCCTGTCCCTCTTTTTAGTGTGTACTTTCTAACCCATTGGA
GCAACAGTCTGATGACACAGGTGTTATGGAGTCTGTGGGAAGAGAGGA C-755 A-741
CAGAGTAGTCATCAAGGTGTGCTTTCTGTACTTCCGGGGACAAATGGG A-687 A-674 T-667
TGCTCTCATTAGTACC T-583 XbaI TCTAGA ACCAGGGTCTCTGTGGC
TGATCTCCCTCAGT C-321 T-491 A-527
GCTTGCTTTTTCAGAAAGGATTGGAGTAAATGTT C-302 CCAATTTCTGAATCTGTTTAAAGAAAGTGAGAC
TAGGTCAGGCTTTCTGCTGATAAAGTATGTTATG T-371 CTCAGGAAGGAGCTAATCGCTGGAACACCAAGGT
CTTGACAGGAAAACA C-302 TAGGCTTACTCCTGCGTGGTCTACCTTGTCTGGCTGGAGAACTTCTGAGTTT
CGATGCAGATTCTGG NruI TCCGGA TACCAGAGAGTCTGGAGACTGGGTCTGAAAAGTGTACTGGTGTGAGTAC
AGAGCACAGTTGGTAGCAGGAGCAAGTAGCTCGGGGTGAGAGCCATCAACTAGCTGAGCAGGGATGC
AGTTCCCGGGGGCGCCACGGAGCTGCGCGGCTCAGAGAGCGC NruI TCCGGA GGCGGGGGCGCGCTGTCA
CGTGTGGGGGTGCGCGGGCGG GGGCGGGCGCGC AGC GCGTTTAAAGGCGGGAGGCGGCTGGAGC
GGCAGCTCGGCC CCGGCGGACGGCGGGAC G-20 G-12 TCTCCGGCGACATG
3'

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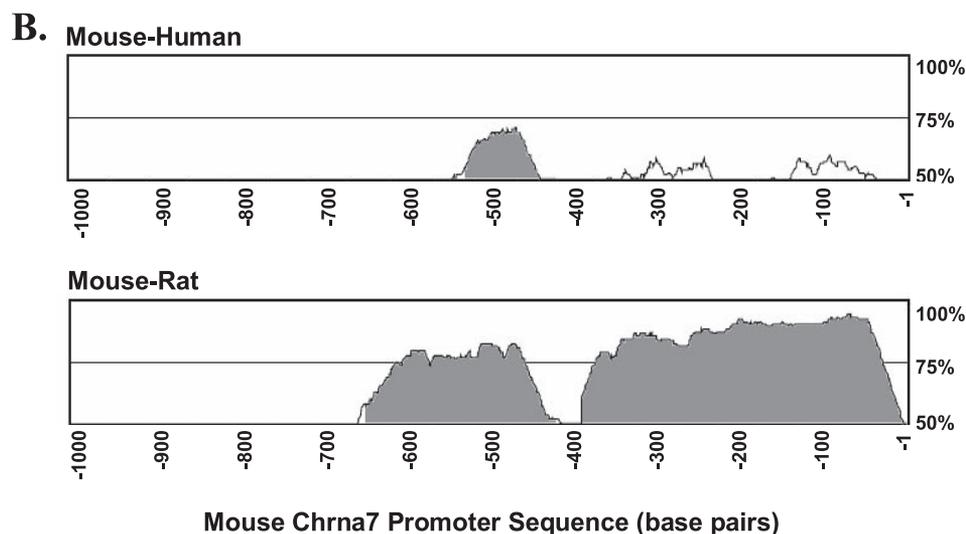


FIGURE 1. Comparison of *Chrna7* promoter sequences across mouse strains and species. *A*, C3H and DBA/1b *Chrna7* promoter sequence comparison. The sequence of the 947 nucleotides immediately upstream of the *Chrna7* initiation codon is shown for the C3H allele of *Chrna7*. SNPs between C3H and DBA/2 in this region of *Chrna7* are indicated in *gray* and in *italic type*. The numbers next to each SNP refer to the nucleotide position from the ATG start site, indicated in *boldface type*. The first three nucleotides of the predicted transcription start sites are *underlined* and in *boldface type*. Reference *XbaI* and *NruI* restriction sites are indicated by *gray boxes*. *B*, the C3H *Chrna7* promoter sequence displayed 61% and 13% identity with the promoters from rat and human, respectively. A comparison of the *Chrna7* promoter sequence across mice, rats, and humans using the VISTA sequence alignment algorithm (available on the World Wide Web at genome.lbl.gov/vista/index.shtml) identified two regions in the promoter that are conserved between mice and rats and one conserved region between mice and humans. The conserved regions between mice and rats include a 235-bp stretch (79.6% sequence identity) between nucleotides -434 and -671 of the mouse promoter (bp -479 and -707 of the rat promoter) and a 441-bp region (83.7% sequence identity) between bp -1 and -428 of the mouse sequence (bp -1 and -440 of the rat sequence). The conserved region between mouse and human (67.8% sequence identity) is 118 bp in length between nucleotides -437 and -546 of the mouse promoter and -723 and -838 of the human sequence.

RESULTS

As a first step in assessing *Chrna7* promoter activity between C3H and DBA/2 mice, we cloned a 947-bp fragment upstream from the initiation codon from both strains (Fig. 1A). This fragment size was selected based upon prior rat and human $\alpha 7$ promoter studies that have reported putative repressor elements within the 1-kb sequence upstream of the initiation

codon (11, 14). Sequence analysis of this region from both mouse strains indicated that it lacked consensus TATA- or CCAAT-boxes. Predicted transcription start sites were located at -37, -80, and -93 bp from the ATG translational start site (Fig. 1A). In addition, the GC content became progressively higher as the sequence neared the ATG translation start site. The region 400 bp upstream was 64.8% GC, the region 300 bp upstream was 70.0% GC, the region 200 bp upstream was 77.5%, and the region 100 bp upstream was 82%.

This sequence displayed 61% and 13% identity with the regions immediately upstream of the rat and human $\alpha 7$ genes, respectively. A comparison of the *Chrna7* promoter sequence across mice, rats, and humans identified two regions in the promoter that are conserved between mice and rats and one conserved region between mice and humans (Fig. 1B). The conserved regions between mice and rats include a 235-bp stretch (79.6% sequence identity) between nucleotides -434 and -671 of the mouse promoter (bp -479 and -707 of the rat promoter) and a 441-bp region (83.7% sequence identity) between bp -1 and -428 of the mouse sequence (bp -1 to -440 of the rat sequence). The conserved region between mice and humans (67.8% sequence identity) is 118 bp in length between nucleotides -437 and -546 of the mouse promoter and -723 and -838 of the human sequence. Comparison of the 947-bp C3H and DBA/2 *Chrna7* upstream regions identified 14 single nucleotide polymorphisms (Fig. 1).

To determine a region upstream of the *Chrna7* ATG translation site that contributed to core promoter activity in GH4C1 cells, a series of 5' cut-downs were derived from the -947 bp C3H *Chrna7* promoter fragment

(Fig. 2). The smallest construct with promoter activity comparable with the full-length promoter was the 138-bp *NruI* fragment (Fig. 2). Removal of an additional 73 bp reduced activity by 85% relative to the full-length promoter (Fig. 2). This 65-bp fragment was the only construct to display significantly reduced promoter activity compared with the full-length C3H promoter (ANOVA: $F_{(1,4)} = 12.2$, $p = 2.0 \times 10^{-6}$; Tukey's HSD: $p = 0.002$).

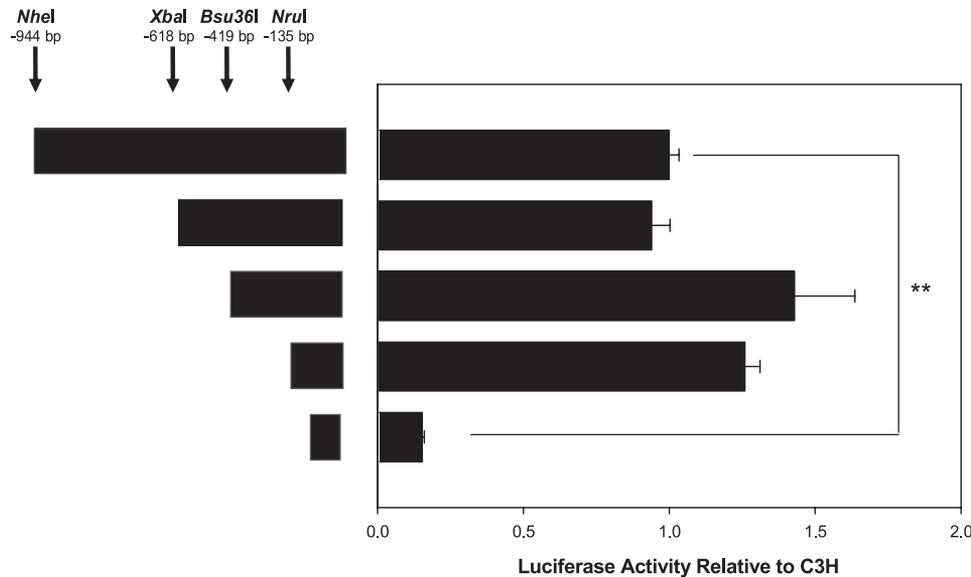


FIGURE 2. *Chrna7* promoter cut-down assays. GH4C1 cells were transfected with a vector containing a firefly luciferase reporter gene driven by the full-length 947-bp C3H *Chrna7* promoter region or a series of 5' cut-downs derived from the full-length fragment. The bp location of each restriction enzyme used in generating the cut-downs is noted above the fragments. Base pair numbering refers to nucleotides proximal to the ATG translational start site. Firefly luciferase activity was normalized relative to *Renilla* luciferase activity of a co-transfected control vector. Data presented display the normalized luciferase activity for each construct relative to the activity of the full-length promoter region. A minimum of six separate transfections were performed per promoter clone. The smallest construct with promoter activity was the 138-bp *NruI* fragment and the removal of an additional 73 bp at the 5' end abolished promoter activity by 85% relative to the full-length region. **, $p < 0.01$.

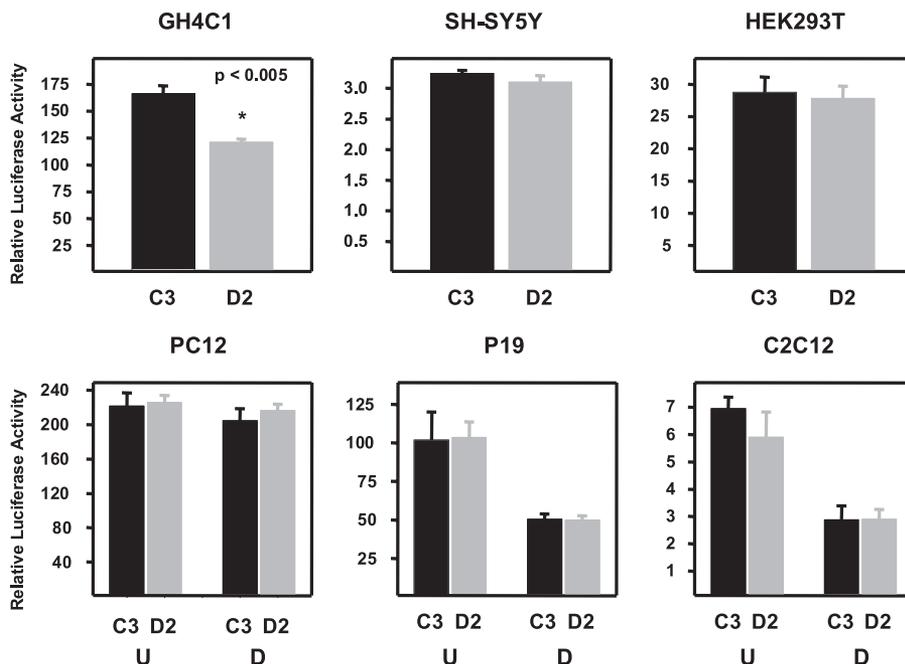


FIGURE 3. Effect of *Chrna7* promoter polymorphisms on reporter gene expression is cell type-dependent. Various cell types were transfected with a vector containing a firefly luciferase reporter gene driven by either the C3H (C3) or DBA/2 (D2) *Chrna7* promoter region. For some cell types, promoter activity was measured in both undifferentiated (U) and differentiated (D) cell phenotypes. Data are reported as the level of luciferase activity relative to the activity of the co-transfected control vector. A significant effect of the *Chrna7* polymorphisms was observed only in the rat pituitary derived cell line, GH4C1. A minimum of six separate transfections were performed per promoter clone per cell type. *, $p < 0.05$.

The 138-bp core promoter region was 88.4% GC and shared 90% sequence identity with rats (data not shown). Approximately 66% and 65% of the sequence was identical to *Chrna7* 5' regions in bovine and human, respectively (data not shown). Moreover, putative binding sites for several transcription fac-

tors, including AP-1, SP1, USF, and Egr-1, were conserved across this region in mice, rats, bovines, and humans (data not shown).

To evaluate whether the variant upstream *Chrna7* sequences from C3H and DBA/2 exhibit differential promoter activity, each strain-derived upstream region was cloned into pGL3 basic, and promoter activity was assessed using luciferase reporter gene assays. The ability of the C3H or DBA/2 *Chrna7* promoter to drive luciferase expression was evaluated across various cell types (Fig. 3), including some cell types that endogenously express the nicotinic receptor subunit (C2C12 (15), SH-SY5Y (16), and PC12 (17)) and several that fail to display detectable *Chrna7* mRNA levels (GH4C1 (17, 18), HEK293 (14), and P19 (19)). For some cell lines tested in this report, promoter activity was measured in both undifferentiated and differentiated cell phenotypes. The *Chrna7* upstream regions from both mouse strains drove the expression of luciferase in all cell types examined. However, a significant effect ($p < 0.05$, Student's *t* test) of the *Chrna7* polymorphisms was observed only in the rat pituitary-derived cell line, GH4C1 (Fig. 3). In this cell line, the DBA/2 *Chrna7* promoter was 22% less active relative to the C3H counterpart. Because an expression difference was only detected in GH4C1 cells, further characterization of the *Chrna7* promoter region was restricted to this cell line.

In order to identify the region of the 947-bp promoter sequence containing the SNP or SNPs that contribute to strain-specific differences in luciferase gene activity, a series of C3H/DBA/2 chimeric *Chrna7* promoters were constructed. Initial analysis indicated that there was a significant effect of promoter construct on luciferase activity (ANOVA: $F_{(1,7)} = 145.2$, $p = 2.85 \times 10^{-13}$). *Post hoc* analysis revealed that sequence differences within the XbaI-NruI fragment of the *Chrna7* promoter appear to be responsible for the observed differences in promoter activities between the C3H and DBA *Chrna7* upstream regions. This is evidenced by the fact that all pro-

Chrna7 Promoter Polymorphisms in Inbred Mouse Strains

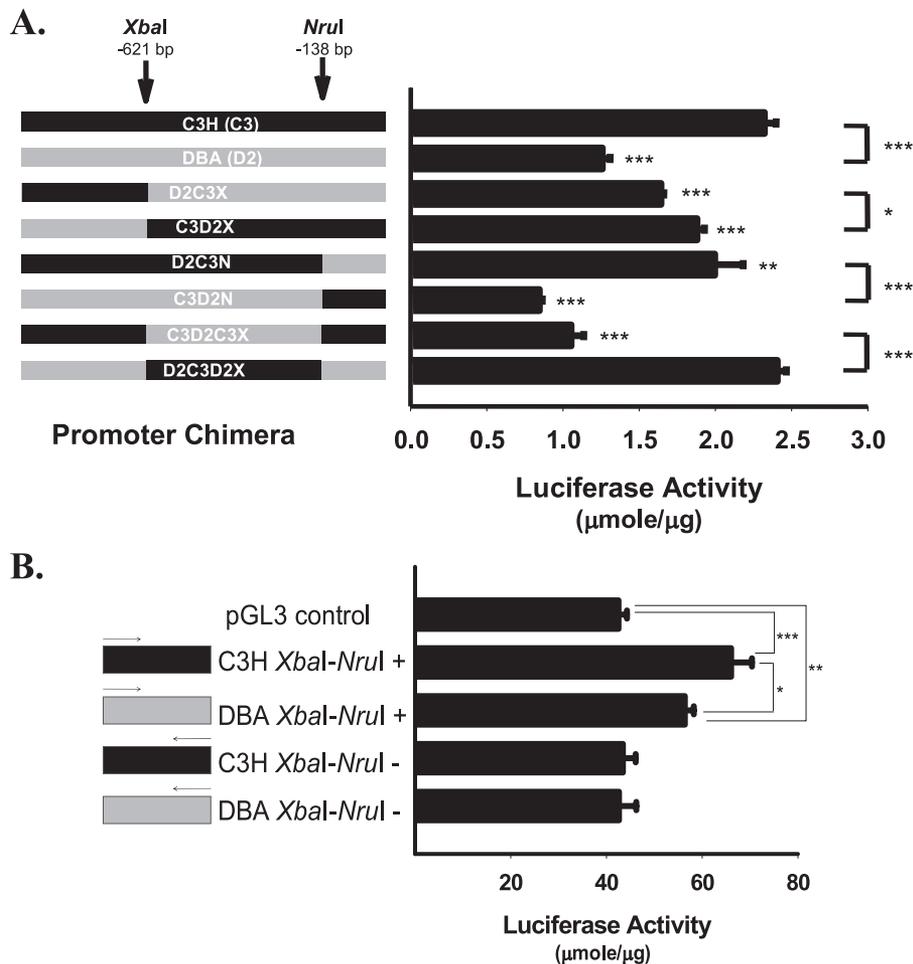


FIGURE 4. A, Luciferase activity driven by chimeric *Chrna7* promoter constructs. GH4C1 cells were transfected with different vectors containing a firefly luciferase reporter gene driven by chimeric *Chrna7* proximal promoter constructs. The strain origin of each region of the chimeric promoter constructs is indicated. Data are reported as the level of firefly luciferase activity relative to *Renilla* luciferase activity of a co-transfected control vector. For all reciprocal clones, the *Chrna7* promoter chimeras that contained the XbaI-NruI fragment from DBA/2 drove lower levels of reporter gene expression than the XbaI-NruI clones derived from the C3H promoter. The bp location of each restriction enzyme used in generating the cut-downs is noted above the fragments. Base pair numbering refers to nucleotides proximal to the ATG translational start site. A minimum of three separate transfections were performed per each promoter clone. Significant differences were determined between reciprocal pairs and compared with the 947-bp C3H promoter. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. B, GH4C1 cells were transfected with different vectors containing a firefly luciferase reporter gene driven by the SV40 minimal promoter or with the XbaI-NruI fragments from either the DBA/2 or C3H strains in either the forward or reverse orientation placed upstream of the SV40 minimal promoter. The differential activity of the XbaI-NruI fragment between strains was dependent upon orientation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

motor constructs containing the DBA/2 XbaI-NruI fragment displayed reduced promoter activity relative to their reciprocal chimeras (Fig. 4A; all comparisons $p < 0.05$, Tukey's HSD).

To further assess whether the XbaI-NruI fragment is responsible for the difference in promoter activity between the C3H and DBA/2 *Chrna7* promoters, the XbaI-NruI fragment was introduced upstream of an SV40 minimal promoter in both forward and reverse orientations relative to the initiation codon. A significant effect of the XbaI-NruI construct, fragment orientation (forward or reverse), and an interaction of construct and orientation on luciferase activity by two-way ANOVA ($F_{(1,4)} = 37.3$, $p = 6.0 \times 10^{-6}$) were detected with these constructs. Both the C3H and DBA/2 XbaI-NruI constructs in the forward orientation significantly enhanced luciferase activity compared with the pGL3 SV40 promoter vector alone (Fig. 4B; $p < 0.01$ for both comparisons, Tukey's

HSD). However, the C3H XbaI-NruI fragment exhibited a significantly greater enhancement of activity than the DBA/2 XbaI-NruI fragment. The influence of both the C3H and DBA/2 XbaI-NruI fragments on SV40 promoter driven luciferase expression was abolished when these constructs were introduced in the reverse orientation (Fig. 4B).

The XbaI-NruI fragment is 479 bp long and spans nucleotides -302 to -583 . This fragment contains 6 of the 14 polymorphisms between these strains. We therefore generated constructs with each of these six DBA/2 SNPs on the C3H *Chrna7* promoter background to evaluate whether a single DBA/2 nucleotide variation within this region is responsible for the reduced reporter gene activity seen in the DBA/2 *Chrna7* promoter. A significant overall effect of SNPs was observed in this region on promoter activity ($F_{(1,6)} = 5.5$, $p = 0.0004$). *Post hoc* analysis revealed that the transfection of GH4C1 cells with a construct harboring the $-302C$ polymorphism resulted in significantly increased relative luciferase activity compared with the C3H *Chrna7* promoter construct (Fig. 5A, $p = 0.002$). None of the DBA/2 SNPs were associated with reduced reporter gene activity (Fig. 5A).

Although no single DBA/2 polymorphism contributed to reduced promoter activity when introduced onto the C3H genetic background, a particular combination of polymorphisms could contribute to the difference in expression between mouse strains.

Therefore, two different SNP combinations from the XbaI-NruI region were selected for further evaluation based upon bioinformatic analyses. One combination, $-491C/T$ and $-527G/A$, were the only polymorphisms located within the region of the mouse *Chrna7* promoter that exhibited significant sequence identity with the human orthologue. Another set of polymorphisms, $-491C/T$ and $-583C/T$, each introduced a putative c-Myb transcription factor binding site only in the DBA/2 promoter. This SNP combination represented the only case in which polymorphisms in one mouse strain introduced multiple binding sites for the same transcription factor. To evaluate whether either combination of DBA/2 polymorphisms leads to reduced promoter activity, reporter gene constructs with DBA/2 double polymorphisms were introduced onto the C3H *Chrna7* promoter background, and

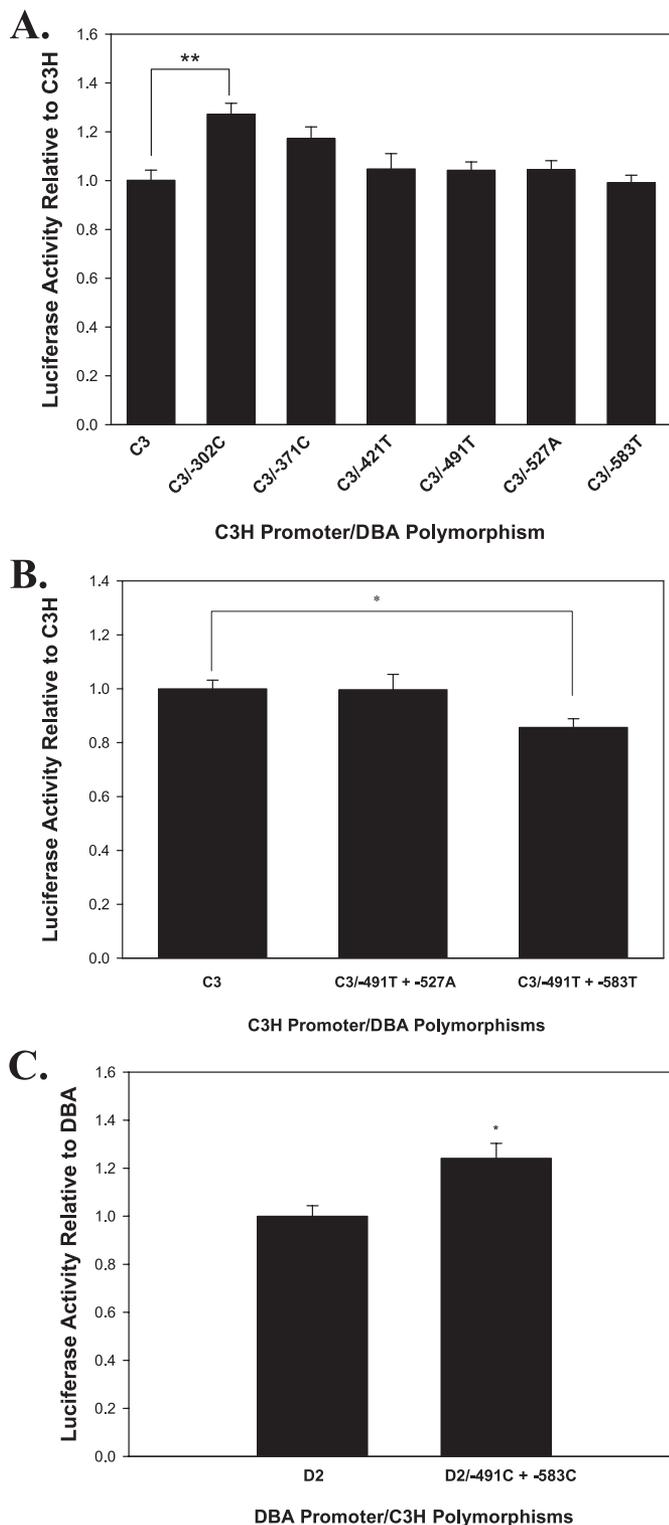


FIGURE 5. The effect of *Chrna7* promoter polymorphisms on luciferase gene reporter activity in GH4C1 cells. A, GH4C1 cells were transfected with the C3H *Chrna7* full-length promoter fragment or one of six different constructs in which a DBA/2 SNP has been introduced onto the C3H *Chrna7* promoter allelic background. The C3H promoter harboring the DBA/2 -302C displayed significantly elevated luciferase activity compared with the wild type C3H promoter. B, GH4C1 cells were transfected with the C3H *Chrna7* full-length promoter fragment or one of two different constructs in which a combination of DBA/2 SNPs has been introduced onto the C3H *Chrna7* promoter allelic background. The DBA/2 -491T and -527A polymorphisms lying within the mouse-human conserved nucleotide sequence contributed to similar reporter gene activity as the C3H *Chrna7* promoter construct,

promoter activity was assessed in GH4C1 cells. *Post hoc* analysis revealed that the DBA/2 -491T and -527A polymorphisms located within the mouse-human conserved nucleotide sequence do not influence promoter activity, whereas the DBA/2 nucleotide variations that introduced the potential c-Myb sites, -491T and -583T, did lead to a significant reduction in promoter function (Fig. 5B, DBA/2 -491T and -583T versus C3H: $p = 0.036$, Tukey's HSD). When the potential c-Myb binding sites were eliminated from the DBA/2 promoter by introduction of the C3H polymorphisms at -491C and -583C DBA/2, a significant increase in promoter activity was observed compared with the DBA/2 promoter (Fig. 5C; $p = 0.01$, Student's *t* test).

DISCUSSION

In this study, luciferase reporter analysis was used to characterize the promoter activity of a 947-bp region immediately upstream of the mouse *Chrna7* initiation codon and to assess whether polymorphisms in this region between the inbred mouse strains C3H and DBA/2 affect promoter function. A 138-bp fragment upstream of the translational start site was found to be sufficient to drive expression of the mouse $\alpha 7$ promoter in GH4C1 cells. However, additional elements upstream of this region appear to contribute to promoter function, as indicated by the observation that polymorphisms outside this region affect promoter activity. Nevertheless, the size of the minimal mouse promoter region is consistent with the *Chrna7* promoter sequence of several different species. For example, a 128- and 178-bp fragment has been shown to be sufficient to drive activity *in vitro* from bovine (20) and rat (21) promoters, respectively. A slightly larger core promoter (231 bp) has been identified for the human *CHRNA7* promoter (11). Based on our bioinformatics analysis in this study, the mouse *Chrna7* promoter shares several other properties with its orthologues, including high GC content, SP1 binding sites, and the absence of TATA- and CAAT-boxes (11, 20, 21).

Within the 947-bp *Chrna7* promoter region, 14 SNPs were identified between the inbred mouse strains C3H and DBA/2. These SNPs affected promoter function in a cell type-specific fashion, since the DBA/2 variant of the *Chrna7* promoter exhibited reduced promoter activity relative to the C3H *Chrna7* promoter in GH4C1 cells but not in other cell lines tested. One possible explanation for the cell type-specific effects of *Chrna7* SNPs on gene expression is that differential expression of transcription factors is contributing to distinct promoter activity in the GH4C1 cells compared with the other

whereas the DBA/2 nucleotide variations introducing the c-Myb sites, -491T and -583T, were associated with a significant reduction in relative luciferase activity. C, GH4C1 cells were transfected with either the DBA/2 full-length promoter, the C3H *Chrna7* full length, or a construct in which C3H SNPs at -491C and -583C have been introduced onto the DBA/2 *Chrna7* promoter allelic background. C3H polymorphisms -491C and -583C on the DBA/2 *Chrna7* promoter sequence significantly increased luciferase activity relative to the DBA/2 wild type promoter. For all *in vitro* reporter assays, luciferase activity was normalized relative to *Renilla* luciferase activity of a co-transfected control vector. Data presented display the normalized luciferase activity for each construct relative to the activity of the wild type promoter region. A minimum of six separate transfections were performed per promoter clone. *, $p < 0.05$; **, $p < 0.01$. C3, C3H; D2, DBA/2.

Chrna7 Promoter Polymorphisms in Inbred Mouse Strains

cell lines evaluated. Variations in transcription factor expression patterns have been hypothesized to contribute to the regional effect of the *Chrna7* alleles on $\alpha 7$ nAChR expression in mouse brain (3). However, the observation that we do not see differential *Chrna7* promoter activity in cell lines that express $\alpha 7$ endogenously could indicate that the polymorphisms that affect $\alpha 7$ expression *in vivo* are not the ones described in this report. Other SNPs in the *Chrna7* promoter or in other regulatory regions could be responsible for the $\alpha 7$ nAChR expression differences between the DBA/2 and C3H brain. The rat $\alpha 4$ nicotinic receptor, for example, is regulated in a cell type-specific manner by an enhancer located in a 3'-untranslated region (24). It is therefore possible that the genotypic variations accounting for region-specific differences in α -BTX levels may be located in the 3'-untranslated region. We have identified a variety of polymorphisms within the *Chrna7* 3'-untranslated region⁴ and are currently investigating whether these SNPs influence gene expression *in vitro*.

GH4C1 is a rat pituitary cell line that does not endogenously express α -BTX binding sites or possess detectable mRNA levels for the $\alpha 7$ nicotinic receptor subunit (17, 18). Because endogenous $\alpha 7$ subunit expression is silenced in the GH4C1 cell line, whereas the 947-bp mouse *Chrna7* fragment possesses promoter activity in these cells, it may be possible that the promoter fragment characterized in this report lacks an important repressor binding site. Alternatively, epigenetic mechanisms of transcriptional regulation, such as DNA methylation, may be responsible for the suppression of endogenous $\alpha 7$ expression in GH4C1 cells. The high GC content of the core $\alpha 7$ promoter region is characteristic of a sequence subject to methylation. Regardless, this study suggests that the GH4C1 cell line harbors the transcriptional machinery necessary for $\alpha 7$ nicotinic receptor subunit expression.

The results from C3H-DBA/2 *Chrna7* promoter chimera constructs indicated that SNPs located within a 479-bp XbaI-NruI fragment of the *Chrna7* promoter are responsible for the differences in activity between the DBA/2 and C3H promoters in GH4C1 cells. This was verified by the fact that the C3H XbaI-NruI fragment enhanced SV40 promoter activity to a greater extent than did the DBA/2 fragment. The effect of both the C3H and DBA/2 XbaI-NruI fragments on SV40 promoter activity was abolished when the fragments were evaluated in the reverse orientation, indicating that this region does not have classical enhancer properties. The SNPs within the XbaI-NruI fragment that were found to affect promoter activity are a combination of SNPs at -491 and -583, which both generate potential c-Myb binding sites in the DBA/2 *Chrna7* promoter. These putative c-Myb binding sites, when introduced in the C3H *Chrna7* promoter, significantly reduced promoter activity. c-Myb is a transcription factor that is known to regulate cellular proliferation and differentiation (22). This molecule is constitutively expressed throughout the adult rat brain (23), and c-Myb mRNA expression was detected in a screen of the rodent cell lines evaluated in this report (*i.e.* C2C12, P19, PC12, and GH4C1) (data not shown). The finding that c-Myb is

expressed in the GH4C1 cells as well as in cell lines that did not display interstrain variations in *Chrna7* promoter activity can be interpreted in several ways: 1) non-GH4C1 cell lines may not express abundant levels of c-Myb protein; 2) differential *Chrna7* promoter activity may be due to a combination of transcription factors that act in concert with c-Myb; or 3) c-Myb is not responsible for the differences in promoter activity observed in this report. Future studies will evaluate the protein expression of this transcription factor across the cell lines examined in this report and will compare protein-DNA interactions in gel shift assays utilizing GH4C1 nuclear extract and DBA/2 and C3H *Chrna7* promoter fragments containing the putative c-Myb binding sites at -491 and -583.

In addition to the SNPs at positions -491 and -583 that generate potential c-Myb sites in the DBA/2 promoter and reduce promoter activity, a DBA/2 polymorphism (*i.e.* -302C) that enhanced promoter activity also was found. This result is consistent with the observation that the relationship between *Chrna7* genotype and level of α -BTX binding in segregating populations of mice derived from C3H \times DBA/2 is brain region-specific. For example, the C3H allele of *Chrna7* is linked to higher levels of α -BTX binding in hippocampus, hypothalamus, and colliculi (3), whereas the DBA/2 allele is linked to higher levels of α -BTX binding in the striatum. Further studies are warranted to investigate whether these region- and strain-specific differences can be attributed to the polymorphisms described in this report and/or regional differences in transcription factors(s) expression. However, the finding that SNPs associated with reduced promoter activity are from a mouse strain with poor sensory gating (DBA/2) is consistent with human studies that have shown that sensory gating deficits among schizophrenic patients are associated with *CHRNA7* promoter SNPs that decrease promoter activity (11).

In summary, this is the first study to characterize the mouse *Chrna7* promoter region. The 947-bp region evaluated shares many properties with many other nicotinic receptor promoters across several different species. Additionally, the results of this study show that a combination of two SNPs in this region, both of which introduce potential c-Myb transcription factor binding sites in the DBA/2 variant, reduce promoter activity relative to the C3H variant. Future examination of the interaction of proteins with this promoter region should provide a clearer assessment of whether these polymorphisms contribute to the interstrain variations in $\alpha 7$ expression levels.

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