

# Identification of *PIK3C3* Promoter Variant Associated with Bipolar Disorder and Schizophrenia

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**Background:** Genes involved in phosphoinositide (PI) lipid metabolism are excellent candidates to consider in the pathogenesis of bipolar disorder (BD) and schizophrenia (SZ). One is *PIK3C3*, a member of the phosphatidylinositol 3-kinase family that maps closely to markers on 18q linked to both BD and SZ in a few studies.

**Methods:** The promoter region of *PIK3C3* was analyzed for mutations by single-strand conformation polymorphism analysis and sequencing. A case-control association study was conducted to determine the distribution of variant alleles in unrelated patients from three cohorts. Electromobility gel shift assays (EMSA) were performed to assess the functional significance of variants.

**Results:** Two polymorphisms in complete linkage disequilibrium with each other were identified, -432C- > T and a "C" insert at position -86. The -432T allele occurs within an octamer containing an ATTT motif resembling members of the POU family of transcription factors. In each population analyzed, an increase in -432T was found in patients. EMSAs showed that a -432T containing oligonucleotide binds to brain proteins that do not recognize -432C.

**Conclusions:** A promoter mutation in a PI regulator affecting the binding of a POU-type transcription factor may be involved in BD and SZ in a subset of patients.

**Key Words:** Bipolar disorder, Brn, inositol phosphates, lithium, oct-1, phosphatidylinositol, phosphoinositide, POU, schizophrenia

Phosphoinositide (PI) lipids are potential targets for the therapeutic effect of lithium in BD. Lithium inhibits myo-inositol 1-phosphatase, which is involved in the sequential dephosphorylation of inositol phosphates into inositol (Berridge and Irvine 1989; Hallcher and Sherman 1980). Inositol is a precursor of phosphatidylinositol 4, 5-bisphosphate (PIP<sub>2</sub>), which is hydrolyzed into the second messengers inositol 1, 4, 5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (Berridge and Irvine 1989; Nishizuka 1992). Lithium treatment could lead to a reduction in brain inositol levels and subsequently to an inhibition of PI-mediated signal transduction (Berridge and Irvine 1989). Lithium also increases the concentration of soluble inositol phosphate intermediates following ligand activated PIP<sub>2</sub> hydrolysis (Casebolt and Jope 1991; Dixon and Hokin 1997; Jope and Bijur 2002; Manji et al 2001; Lenox and Wang 2003; Sun et al 1992). Lithium has other effects on the PI pathway, including inhibitory and excitatory effects on protein kinase C and inhibition of glycogen synthase kinase, a target of the phosphatidylinositol 3-kinase signal transduction pathway (Jope and Bijur 2002; Klein and Melton 1996; Manji et al 2001).

In addition to its classical role in signal transduction, PIP<sub>2</sub> also plays a role in regulating synaptic vesicle function, affecting both the endocytic and exocytic phases of the synaptic vesicle cycle by binding to synaptic proteins (Cremona and DeCamilli 2001; Eberhard et al 1990; Holz et al 2000; McPherson et al 1996). Transmitter release and vesicle recycling is also affected by other

PIs and high phosphorylated inositol phosphates derived from IP<sub>3</sub> (Hilton et al 2001; Mochida et al 1997).

PIP<sub>2</sub> is synthesized from inositol by phosphorylation at the D<sub>4</sub> and D<sub>5</sub> positions of the inositol ring. Phosphatidylinositol 3-kinases catalyze the phosphorylation of PIP<sub>2</sub> and other PI intermediates at the D<sub>3</sub> position to generate an array of 3-phosphorylated PI signaling molecules (Toker and Cantley 1997).

Considering lithium's multitude of effects on the PI pathway, we have been interested in the genes involved in PI and inositol phosphate metabolism as possible candidates for BD disorder susceptibility. Interestingly, a number of genes that regulate PI lipid levels, including PI kinases and PIP<sub>2</sub> phosphatases, map within a few million nucleotides of markers linked to both BD and SZ (see Discussion).

Based on these observations and theoretical considerations, we have been analyzing genes involved in PI metabolism that map to regions linked to BD or SZ susceptibility (or both). One is *SYNJ1*, a calcium-dependent, synapse-specific PIP<sub>2</sub> phosphatase that plays a key role in synaptic vesicle recycling (Cremona and DeCamilli 2001; McPherson et al 1996). *SYNJ1* maps to 21q22 near markers linked to BD in several studies (Detera-Wadleigh 1997; Straub et al 1994). We identified several rare promoter and splice junction variants found only in a few patients with BD and no control subjects, as well as a common polymorphism near the exon 12-intron 12 splice donor site that showed a modest difference in allele distribution in bipolar patients compared with control subjects (Saito et al 2001).

We also analyzed *PIP5K2A*, a member of the phosphatidylinositol 5-kinase family. *PIP5K2A* maps to 10p in a region of the genome that has been linked to both BD and SZ (Faraone et al 1998, 1999; Foroud et al 2000; Maziade et al 2001; Rice et al 1997; Straub et al 1998). Mutation screening revealed an imperfect CT repeat polymorphism at the exon 9-intron 9 splice donor site that was unequally distributed in patients with BD and SZ, compared with control subjects, small repeats lacking a potential transcription silencing element (CCCTC) found in wild-type alleles were detected more commonly in patients (Stopkova et al 2003). Recently, we also identified a relatively rare promoter variant, -1007C- > T. Homozygosity for -1007C- > T was only detected in two patients with SZ and no control subjects (Stopkova et al, in press). There was also a 30% increase in

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**Table 1.** Primers

<i>PIK3C3</i> Promoter Amplification Primers	
Forward Primer	Reverse Primer
Pro 1 gtcacatgcgcggaagggt	acgccccagcaatcccactc
Pro 2 tggcaaaattccaattttgtgctt	cggaaactccccggaaca
Pro 3 ctagattctaactgggttagg	aacagcttctactggctgcc
Pro 4 tcagcaatgcattagacagc	gcgtgatgtttaactggg
Double Stranded Oligonucleotides Used in EMSA Experiments	
<i>PIK3C3</i> -432C	5'-CCACTGGAATTTAAACCTGGCAAAATCCAA-3' 3'-GGTGACCTTAAATTTGACCGTTTTAAGGTT-5'
<i>PIK3C3</i> -432T	5'-CCACTGGAATTTAAATCTGGCAAAATCCAA-3' 3'-GGTGACCTTAAATTTAGACCGTTTTAAGGTT-5'
<i>PIK3C3</i> -86+/+	5'-GGAACCGGAAGTTCCTGTTGTGGGGCTCA-3' 3'-CCTTGGCCTTCAAGGCACAACACCCCGAGT-5'
<i>PIK3C3</i> -86insC	5'-GGAACCGGAAGTTCCTGTTGTGGGGCTCA-3' 3'-CCTTGGCCTTCAAGGGCACAACACCCCGAGT-5'

Bold signifies polymorphic site.  
EMSA, electromobility gel shift assays.

heterozygotes in the patient group, but the results fell short of statistical significance.

We have now extended our analysis of genes involved in PI metabolism to include *PIK3C3*, a member of the PI 3-kinase family. *PIK3C3* maps to 18q12 approximately 5 centimorgans from markers linked to both BD and SZ, 18q12 is one of three regions on chromosome 18 identified in BD and SZ linkage studies (Badenhop et al 2002; Berrettini et al 1994; Detera-Wadleigh et al 1999; Escamilla et al 2001; Ewald et al 2002; Maziade et al 2001). Screening of the promoter region for mutations resulted in the identification of a variant -432C>T, that was unequally distributed in patients with SZ and BD in three populations of patients, compared with control subjects. Interestingly, -432C>T is strikingly similar to *PIP5K2A*-1007C>T and one of the rare *SYNJ1* promoter variants we previously found in a single patient with BD, -1898T>C (Saito et al 2001). All occur within an octamer sequence containing an ATTT core motif found in promoters that bind to Oct-1 and Brn-2, members of the POU family of transcription factors (Elsholtz et al 1990; Phillips and Luisi 2000; Singh et al 1986; Zhao et al 2002).

## Methods and Materials

### Patients

Patients with BD were diagnosed on the basis of either a Schedule for Affective Disorders and Schizophrenia-Lifetime (SADS-L; Endicott and Spitzer 1978) interview or by unstructured clinical interview modified from SADS-L using Research Diagnostic Criteria (RDC) criteria for diagnosis of either bipolar disorder I or bipolar disorder II (Spitzer et al 1978). The patients were recruited from clinics and private practices affiliated with the Albert Einstein College of Medicine ( $n = 135$ ) and from the Psychiatric Clinic at Charles University in Prague, Czech Republic ( $n = 83$ ). Forty-three subjects were unrelated patients with bipolar disorder I from the Coriell Institute in conjunction with the National Institutes of Mental Health Bipolar Genetics Initiative. U.S. control subjects ( $n = 153$ ) were anonymous blood-bank samples. No formal testing procedure was used to screen U.S. control subjects to rule out individuals who had a personal or family history of mental illness, although the expected frequency of BD and SZ in a population of blood donors would be expected to be 1% or less for each. Control subjects ( $n = 103$ ) from the Czech Republic were blood-bank donors, patients hospitalized for medical reasons (Czech Republic), and students

and hospital workers. Control subjects from the Czech Republic were screened for underlying psychiatric illness using a brief psychiatric clinical interview and were excluded if they had a personal or family history (in a first-degree relative) of psychiatric illness. U.S. patients with SZ ( $n = 129$ ) were recruited from the Bronx Psychiatric Center, Long Island Jewish Hillside Hospital, Rockland State. Diagnosis was established by RDC criteria using the SCID or clinical interview. Subjects were compared with the same U.S. control cohort used in the analysis of the bipolar sample. Israeli patients with SZ ( $n = 124$ ) were recruited from Beer Yaakov Mental Health Center in Tel Aviv. The diagnosis was established by two board-certified psychiatrists using RDC criteria based on clinical interview. Control subjects ( $n = 89$ ) were hospital workers and students who were not formally tested. All patients signed an informed consent approved by the local ethical committees on clinical investigation and institutional review boards.

### Polymorphism Detection and Genotyping

DNA was extracted from whole blood using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota). Overlapping primers (pro 1- > pro 4) were used to amplify the 5'-flanking region (Table 1). To amplify the GC-rich region flanked by the pro 1 primers, the "enhancer" system was used (Invitrogen, Carlsbad, California). Enhancer buffer (1  $\mu$ L) was used in a 20- $\mu$ L reaction. Cycling conditions were 94°, 64°, and 68°C for 30 sec each, following an initial 2-min, 94° denaturing step. For the pro 2 and pro 4 primer sets, routine amplification was performed at 94°, 60°, and 72°C for 30 sec each for promoter 2, and 94°, 56°, and 72°C for 30 sec each for promoter 4, each preceded by 2-min denaturation at 94°C using *taq* polymerase (Invitrogen, Carlsbad, California). To amplify the region encompassed by the pro 3 primer set, we used a "hot start" kit from Qiagen (Alameda, California). A 50- $\mu$ L polymerase chain reaction (PCR) was set up using 10  $\mu$ L of buffer Q according to manufacturer's instructions. The sample was denatured at 95°C for 15 min followed by 30 cycles at 94° and 56°C for 30 sec, and 72°C for 1 min. The primers used in the PCR reactions are shown in Table 1.

The PCR-amplified genomic DNA fragments were screened for polymorphisms using single strand conformation polymorphism (SSCP) analysis originally described by Orita et al (1989); SSCP analysis was carried out on a standard long sequencing apparatus with radio-labeled PCR products, using 5% acrylamide

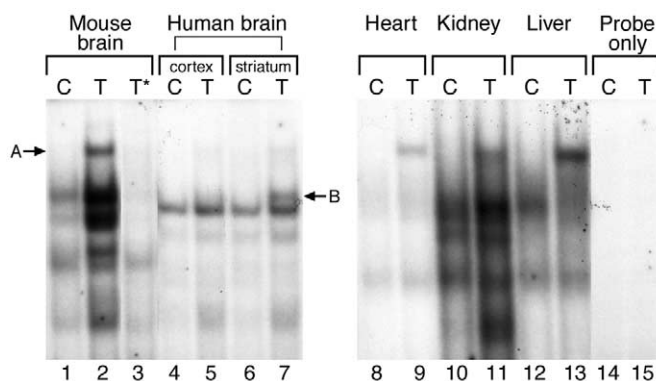
gels containing 5% glycerol. Samples were separated by electrophoresis at 4°C at 5 W for 17 hours, after which the gels were dried and exposed to x-ray film. Thirty DNA samples obtained from unrelated patients with BD and 30 with SZ were used in the initial screening. DNA samples showing a band shift on the gel, indicative of a polymorphism, were then sequenced using fluorescent-labeled dideoxy sequencing and an Applied Biosystems (Foster City, California) model 377 sequencer. Before sequencing, amplimers were purified using a commercially available column (Qiagen) to remove unincorporated primers and other impurities. Both strands were sequenced. The data were compared with available published sequence information using BLAST.

The -432 promoter 3 SNP was analyzed by restriction digestion with the enzyme *Swa1* because the presence of the SNP creates a *Swa1* restriction site. To 17 µL of PCR product, 15 units of *Swa1* were added (1.5 µL of 10 units/µL, along with 1.5 µL of buffer 3 (New England Biolabs, Beverly, Massachusetts) for a total reaction volume of 20 µL. The samples were incubated overnight at 25°C. Samples were then separated by electrophoresis on 1% agarose and the genotype scored by two investigators. Positive control subjects for the SNP were included in every experiment.

The -86 insertion-deletion variant was analyzed by a PSQ HS 96 Pyrosequencer (Pyrosequencing, Uppsala, Sweden). The PCR fragments containing the -86 mutation were amplified under the same conditions described earlier with the exception that pro 1F primer was biotinylated to produce a 5'-biotin labeled template. A sequencing primer, 5'-agctgagccccacaacagg, was annealed to the biotin-labeled strand and used as a template to sequence across the polymorphic site.

**Electromobility Gel Shift Assay**

EMSA was performed according to published procedures (Hope et al 1994). Briefly, double-stranded oligonucleotide probes containing the mutant *PIK3C3* promoter alleles were synthesized (Table 1). Individual strands were constructed to create 3'-recessed ends after annealing. These were filled in with [<sup>32</sup>d]GTP and [<sup>32</sup>P]dCTP using Klenow DNA polymerase to generate a radio-labeled probe, which was used for protein-binding experiments. Nuclear protein extract was isolated from female FVB/NTac FBR inbred mice weighing 40 g using a technique described by Hope et al (1994). Protein was also extracted from human cortex and striatum (tissue kindly provided by Dr. R. Sircar). Protein (40 µg) was mixed with probe (1 ng, approximately 10<sup>6</sup> counts) and incubated for 20 min at room temperature. Probes containing the various alleles were labeled simultaneously using the same amount of DNA and radioactive nucleotides. After purifying the probes, an aliquot was analyzed by scintillation counting to ensure that the labeling efficiency was greater than 10<sup>8</sup> CPM/µg and that the number of counts incorporated into each probe used for allele comparisons were within 20% of each other. Samples were then separated by electrophoresis in a nondenaturing gel system containing 6% acrylamide and 1.6% glycerol. Specificity of the resulting binding activity was demonstrated by competition with nonradioactive probe added in 50-fold excess. All experiments using mouse tissue were repeated on three animals. Experiments on human brains were performed on four samples obtained from two brains. The results shown in Figure 1 were highly reproducible. In supershift experiments, an Oct-1 antibody was used that cross reacts with human and rodent Oct-1 protein (Santa Cruz Biotech-



**Figure 1.** Electromobility gel shift assays experiment with -432 SNP. Protein extracted from whole mouse brain (1-3), human brain (lanes 4-7), and various mouse tissues (lanes 8-13) were used in binding assay. The various alleles are shown above lanes. Lane 3 (T\*) is a binding experiment in which a 50-fold excess of unlabeled primer containing the -432T allele was used to block protein-DNA complex formation. Lanes 14 and 15 were assays set up without protein. The mouse protein-DNA complex detected only with the -432T oligonucleotide is shown by the A arrow; the human protein-DNA complex is shown by the B arrow.

nology, Santa Cruz, California). Oct-6 antibody that binds to human Oct-6 protein was a kind gift from Dr. J. Price.

**Statistical Analysis**

A statistical program, StatXACT-5 (Cytel Software Corporation, Cambridge, Massachusetts), was used to compute the  $\chi^2$  and Fisher statistics and *p* values. Association was tested using the Pearson  $\chi^2$  test (2 × 2 table). All *p* values were two tailed. The level of significance was set at *p* < .05. No corrections for multiple testing were made.

**Results**

The promoter region of *PIK3C3* was screened for mutations in patients with BD and SZ using overlapping PCR amplimers that covered a portion of exon 1 and 860 nucleotides upstream of the *PIK3C3* translation start site. Two polymorphisms were detected, a "C" insert at position -86 nucleotides relative to the ATG translation start site, and a C- > T transition at position -432 (Table 2). The -432C- > T mutation creates an eight-base palindrome with the sequence ATTTAAAT; palindromes are often sites for DNA binding proteins. The polymorphism also creates a 6/8 recognition signal for Oct-1 and Brn-2, members of the POU domain transcription factor family (consensus sequence ATTTGCAT). Samples were genotyped by *Swa1* digestion of PCR-amplified fragments and gel electrophoresis. In the U.S. sample, there was no significant difference in the allele distribu-

**Table 2.** *PIK3C3* Promoter Polymorphisms Detected

-86insC	ggaaccggaagtccggtgtggtgggctc ggaaccggaagtccCgtgtggtgggctc
-432C->T	ggaattt <b>aa</b> aCctggcaaaatt ggaattt <b>aa</b> aTctggcaaaatt

Oct-1/Brn-2/POU consensus (**atttgc**at) in bold type; polymorphism in capital letters. The octamer homology is also an eight-base palindrome with the -432T allele (underlined). Two five-base "hinge" palindromes in wild type -86 allele are underlined.

**Table 3.** Case-Control Comparison of *PIK3C3* -432C-> Single Nucleotide Polymorphism

	CC	CT	TT	C	T
United States <sup>a</sup>					
CONT	.80 (122)	.19 (29)	.013 (2)	.89 (273)	.11 (33)
BD	.79 (107)	.16 (22)	.044 (6)	.87 (236)	.13 (34)
SZ	.74 (96)	.23 (30)	.023 (3)	.86 (222)	.13 (36)
Czech Republic <sup>b</sup>					
CONT	.82 (84)	.17 (17)	.02 (2)	.90 (185)	.10 (21)
BD	.63 (52)	.35 (29)	.02 (2)	.80 (133)	.20 (33)
Israel, Ashkenazi Jews <sup>c</sup>					
CONT	.96 (46)	.04 (2)	(0)	.98 (94)	.02 (2)
SZ	.79 (38)	.21 (10)	(0)	.90 (86)	.10 (10)
Israel, Sephardic Jews <sup>d</sup>					
CONT	.93 (38)	.07 (3)	(0)	.96 (79)	.04 (3)
SZ	.74 (56)	.26 (20)	(0)	.87 (132)	.13 (20)

All results are two-tailed. Number of subjects is given in parentheses.

BD, patients with bipolar disorder; CONT, control subjects; SZ, patients with schizophrenia.

<sup>a</sup>Allele frequency BD vs. CONT,  $O^2 = .46, p = .50$ ; SZ vs. CONT,  $O^2 = 1.31, p = .25$ .

<sup>b</sup>Allele frequency BD vs. CONT,  $O^2 = 6.95, p = .008$ .

<sup>c</sup>Allele frequency SZ vs. CONT in Ashkenazi Jews, Fisher statistic = 5.67,  $p = .02$ .

<sup>d</sup>Allele frequency SZ vs. CONT in Sephardic Jews, Fisher statistic = 5.65,  $p = .02$ . Combined Ashkenazi and Sephardic sample SZ vs. CONT, Fisher statistic = 12.81,  $p = .0003$ .

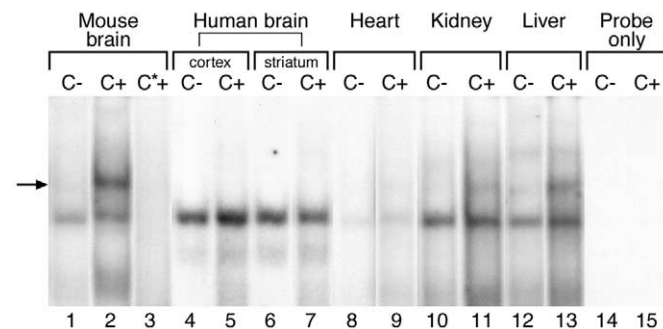
tion between control subjects and patients with SZ and BD; however, there was an increase in the number of bipolar subjects who were homozygotes compared with control subjects (.044 vs. .013, Table 3). We also analyzed a cohort of bipolar patients from the Czech Republic. In this group, we did find a statistically significant difference in allele distribution, although the number of homozygotes was the same (allele frequency,  $\chi^2 = 6.95, p = .008$ ). The control and patient genotypes were in Hardy-Weinberg equilibrium, with the exception of the U.S. bipolar sample, which deviated significantly ( $\chi^2 = 9.1, p = .003$ ).

A statistically significant difference in allele distribution was also detected in a cohort of patients with SZ from Israel. The Israeli sample was separated into Ashkenazi and Sephardic subgroups, the major ethnic subdivisions of Jewish people. Ashkenazi Jews are of eastern European descent, whereas Sephardic Jews trace their ancestry to the Middle East and Africa. In both groups, there was a substantial increase in the frequency of -432T in SZ compared with control subjects, although the sample size was small (Ashkenazi: Fisher statistic = 5.67,  $p = .02$ ; Sephardic: Fisher statistic = 5.65,  $p = .02$ ; Combined sample: Fisher statistic = 12.81,  $p = .0003$ ). There were no homozygotes, consistent with the overall low frequency of -432T in Israeli control subjects.

We also analyzed the -86insC promoter mutation in 320 subjects. The -86insC allele is in complete linkage disequilibrium with -432T, indeed, the two polymorphisms are coupled in the samples analyzed, -86insC was always found with -432T and vice versa ( $r^2 = 1.00$ ). Because of the strong linkage disequilibrium (LD), the -86insC polymorphism was not analyzed in the entire data set; LD between -432T and -86insC was detected in both a heterogeneous Caucasian population from the United States as well as a more ethnically homogeneous group from the Czech Republic (control subjects and bipolar subjects, data not shown).

To determine whether proteins could bind to a DNA fragment containing the -432C->T and -86insC polymorphisms, we used EMSA. Nuclear protein was extracted from human and mouse brains, as well as several other mouse organs, and incubated with radiolabeled double-stranded oligonucleotide probe containing one or the other allele. The samples were then separated by

electrophoresis through 6% acrylamide and 1.6% glycerol under nondenaturing conditions. Unbound oligonucleotide migrates to the bottom of the gel, whereas the migration of protein-bound oligonucleotide is slowed and a band shift is observed. As shown in Figure 1, band shifts indicative of DNA-protein complexes were detected using protein derived from brain and other tissue. A clear, allele-specific pattern was detected. In mouse brain and in other mouse tissue samples, a DNA-protein complex was seen using the -432T-containing oligonucleotide, but not with -432C (labeled "A" in Figure 1). There were several other DNA-protein complexes seen in mouse brain and kidney and liver. Although these were not allele specific like that seen in band A, the signal intensity was greater with the -432T than the -432C oligonucleotide allele, with the exception of liver. In human brain, there was a unique DNA-protein complex seen in the striatum using the -432T oligonucleotide (band B). The common band seen below band B was detected using either allele, although the signal intensity was greater with -432T. We were able to block



**Figure 2.** Electromobility gel shift assays (EMSA) experiment with -86 insert C polymorphism. Protein same as shown in Figure 1. C+ is oligonucleotide with C insert; C- without. Lane 3 (C\*+) is a binding experiment in which a 50-fold excess of unlabeled primer was used to block protein-DNA complex formation. Arrow points to protein-DNA complex only found using the -86C insert oligonucleotide.

**Table 4.** *SYNJ1* and *PIP5K2A* Mutations with Oct-1/Brn-2 homology

*SYNJ1*-1898T->C  
**aagcacaTttgttggta**  
**C**  
*PIP5K2A*-1007C->T  
**cagatttccaCggacgg**  
**T**

Oct-1/Brn-2 consensus (**atttgc**at) in bold type; variants in capital letters. An eight-base palindrome (atttccat) created by -1007T is underlined. An E-box transcription factor consensus (canntg) in -1898 is also underlined.

protein-DNA binding for the bands of interest using an excess of unlabeled oligonucleotide (lane 3), indicating that the interaction between the protein and DNA was specific. Similar results were seen with the human brain tissue (data not shown).

Because of the homology to the binding site for members of the POU family of transcription factors, we attempted to “super-shift” the bands using available antibodies to two members, Oct-1 and Oct-6. Antibodies specific to proteins bound to DNA would further retard the migration of a DNA-protein complex and would appear as a supershifted band on the autoradiogram; however, no supershift was detected (data not shown).

We also used EMSA to analyze the -86insC polymorphism. In this experiment, a substantial, allele-specific difference in the signal intensity of a DNA-protein complex was detected using an oligonucleotide containing the C insert compared with wild type (arrow in Figure 2). The signal was detected in every mouse tissue examined but was most pronounced in the brain. A common band was seen in both mouse and human tissue with a slight increase detected in human cortex using the C insert oligonucleotide.

The -432 SNP is remarkably similar to two other sequence variants we have previously detected in the promoter region of two other PI-regulatory candidate genes, *SYNJ1* and *PIP5K2A*; all cause a change in a sequence resembling an Oct-1-Brn2-POU octamer binding site containing an ATTT motif. *SYNJ1*-1898T>C occurs within the ATTT core consensus and changes a 5/8 base match to the octamer consensus to a 4/8 match. *PIP5K2A*-1007C>T increases the number of bases in the core consensus from 6 to 7 (Table 4). Homozygosity for *PIP5K2A*-1007C>T was previously found in two patients with SZ and no control subjects or bipolar patients, and a single patient with BD has been found with *SYNJ1*-1898T>C (Saito et al 2001; Stopkova et al, submitted). In both cases, similar to the findings shown in Figure 1 for *PIK3C3*-432C>T, substantial allele specific binding to a brain nuclear protein was found for both polymorphisms (Stopkova et al, in press; Lachman and Saito, unpublished data).

The resemblance between the -432 variant reported here and two rare promoter variants found in three patients with BP or SZ (and no control subjects) occurring in PI-regulatory genes that we have analyzed as potential candidates for these disorders on the basis of their chromosomal position and role in PI metabolism suggests that altered binding to members of the POU transcription family affects the expression of PI-regulatory genes to increase susceptibility to BD and SZ in subsets of patients.

## Discussion

The mechanism of lithium's therapeutic effect in BD is not known; however, its multitude of actions on PI and inositol phosphate metabolism suggests that PI-mediated signal transduction or PI mediated synaptic vesicle function are legitimate

targets. If so, these pathways should also be considered in the pathogenesis of BD. Remarkably, a number of genes involved in the synthesis or dephosphorylation of PIP2 and other phosphoinositides map closely to regions of the genome that have been linked to both BD and SZ. These include *SYNJ1* (21q22); *PIK4CA*, a member of the phosphatidylinositol 4-kinase family that maps to 22q11 in a region deleted in the majority of patients with velo-cardio-facial syndrome (a congenital disorder in which both BPD and SZ are observed); *PIB5PA*, a phosphatidylinositol transfer protein (22q12); *PIP5K2A*, a phosphatidylinositol 5-kinase (10p12), and *PIK3C2B* and *PIK3C3*, phosphatidylinositol 3-kinases (1q32 and 18q12 respectively; Berrettini et al 1994; Brzustowicz et al 2000; Detera-Wadleigh et al 1997, 1999; Escamilla et al 2001; Ewald et al 1997; Faraone 1998, 1999; Foroud et al 2000; Lachman et al 1997; Levinson et al 2000; Maziade et al 2001; Rice et al 1997; Schwab et al 1998, Shaw et al 1998; Straub et al 1994, 1998).

In addition, a number of genes involved in the metabolism of IP3 and other inositol phosphate second messengers also map closely to regions linked to BD and SZ in some studies. These include *ITPKB* and *ITPKA*; members of the Ins(1,4,5)P3 3-kinase family (1q41 and 15q14 respectively); KIAA0274, an inositol 3-phosphatase (6q22); synaptic Ins(1,4,5)P3 5-phosphatase (10q26); SAC2, a Sac domain-containing inositol phosphatase (10q26); and myoinositol monophosphatase 2 (IMPA2) (18p11.2; Ekelund et al 2001; Levinson et al 1998; Mowry et al 2000; Numberger and Foroud 1999; Tsuang et al 2001; Yoshikawa et al 2001).

Several chromosomal loci have been linked to both BD and SZ (Berrettini 2000). One explanation for the shared genetic loci is that genetic and biochemical pathways common to both disorders exist, such as abnormalities in PI and IP3 metabolism, that combine with disease-specific genetic factors elsewhere in the genome to cause either BD or SZ. It is conceivable that genetic factors in this pathway could also underlie lithium sensitivity or resistance, and even reduced BD susceptibility. Mutations in PI or inositol phosphate regulatory genes that cause a “lithium-like” effect on PI- or IP3-regulated pathways could conceivably be protective in combination with BD susceptibility genes at other loci. It is interesting to note that an inositol polyphosphate 4-phosphatase, *INPP4B*, maps within 2 megabases of a 4q marker linked to protection against the development of BD in high-risk family members of bipolar pedigrees (Ginns et al 1998).

In this study, we have turned our attention to *PIK3C3*, a member of the PI 3-kinase family. Phosphoinositides phosphorylated at the D3 position of the inositol ring are involved in a host of signaling pathways. 3-phosphorylated PIs increase in cytokine and mitogen stimulated cells and are involved in cell growth and oncogenic transformation (Lin et al 2001; Merlot and Firtel 2003; Wymann and Pirola 1998). Studies also show that 3-phosphorylated PIs play a role in neuronal transmission. For example, phosphoinositide 3-kinases mediate neurotrophin-3-induced synaptic changes at neuromuscular junctions, effect long-term potentiation in the amygdala in response to fear conditioning, and influence long-term depression in the hippocampus (Daw et al 2002; Lin et al 2001; Yang et al 2001). Phosphatidylinositol 3-kinase also plays a role in ligand activated receptor endocytosis, a method used by cells to induce receptor down-regulation (Naga Prasad et al 2001, 2002). Lithium inhibits glycogen synthase kinase, which is activated by a phosphoinositide 3-kinase cascade (Joje and Bijur 2002; Klein and Melton 1996; Manji et al 2001).

*PIK3C3* maps to 18q12, within a few centimorgans of markers linked to both SZ and BD. Escamilla et al (2001) reported a positive linkage to D18S467 in a linkage disequilibrium study conducted on bipolar patients in a genetically isolated population from Costa Rica. The highest lod score in a linkage study conducted in bipolar families by Maziade et al (2001) was at D18S1145, which maps approximately 5 centimorgans from *PIK3C3*. A positive lod was also reported in the region in SZ families. A lod score greater than 1.5 was reported for 18q12 markers in a subset of Australian BD families (Badenhop et al 2002).

The -432C>T polymorphism identified in our preliminary mutation screening showed a heterogeneous and complex distribution pattern in patients with BD and SZ. In the U.S. bipolar cohort, 4.4% of patients, but only 1.3% of control subjects, were homozygous for -432T. No difference in heterozygotes and overall allele distribution was found, however, nor was there a significant difference among control subjects and patients with SZ. In contrast, among the bipolar sample from the Czech Republic, there was a significant increase in the number of heterozygotes compared with control subjects but no increase in the frequency of homozygotes. The frequency of -432T was higher in the Czech bipolar sample than any other group analyzed. In the Israeli SZ sample, there was also a striking increase in the number of heterozygotes with SZ compared with control subjects in both Ashkenazi and Sephardic subgroups; however, the sample size was small when the Israeli sample was broken down into its major ethnic subgroups, increasing the chance of type I error. The significant difference in the distribution of -432T seen in patients with SZ was due to the low frequency of the allele in Israeli control subjects; the frequency of -432T in Israeli SZ patients was only slightly higher than that found in U.S. control subjects. If these data are not due to type I error caused by population stratification and small sample size, the simplest biological interpretation is that other genes are needed to interact with the *PIK3C3* polymorphism to cause psychiatric problems, and the allelic versions of these genes required for psychiatric problems to emerge are unequally distributed in different ethnic groups.

The *PIK3C3* findings by themselves must be viewed with caution considering the high type I error rate associated with case control studies of candidate genes in psychiatric disorders, especially in populations as heterogeneous as the U.S. sample used in this study. Family-based association analyses such as the transmission equilibrium test or a larger case control series in a more homogenous population using genome control subjects for stratification will be necessary for confirmation. The findings are more plausible given the results of our EMSA studies and when viewed in the context of our previous findings, identifying rare promoter mutations in PI candidate genes in patients with BD and SZ that strongly resemble the *PIK3C3*-432 SNP. In each case, the promoter variants occur within or near an ATTT motif found in members of the POU family of transcription factors. In addition, two of the variant alleles, *PIK3C3*-432T and *PIP5K2A*-1007T, both result in palindromes that are often found as core sequences for DNA binding proteins. *SYNJ1*-1898T>C does not affect a palindrome, but the core ATTT motif does overlap with a consensus sequence for the E-box family of transcription factors, as well as resembling a POU binding site. Thus, the promoter variants we have identified may in fact bind several transcription factors in addition to members of POU family. This is supported by our EMSA experiments that show multiple band shifts for most of the promoter variants analyzed. The -86C insert,

which is in linkage disequilibrium with -432T does not affect a POU consensus but does occur within two five-base palindromes flanking a single nucleotide. The important point gleaned from the EMSA experiments that is most relevant to the genetic basis of psychiatric disorders is that promoter polymorphisms found more commonly in patients with BD and SZ have dramatic effects on the binding of brain-expressed nuclear proteins.

Members of the POU family of transcription factors are important regulators of gene expression during development and several, particularly the Brn proteins, play a critical role in brain development (Latchman 1999; Phillips and Luisi 2000). All POU proteins share a common bipartate DNA binding region consisting of a POU domain and a homeobox domain separated by a short amino acid linker or hinge (Herr and Cleary 1995; Malik et al 1997). A number of genes expressed in the brain that are critical for developmental and postdevelopmental neuronal function are influenced by POU proteins including *SNAP-25*, neurofilaments, nicotinic receptor, *KCNN3*, and *Bcl-2* (Latchman 1999). Mouse knockout experiments show that loss of expression of individual POU family members have profound effects on brain development (Latchman 1999).

Although we have focused on the -432 SNP in this discussion because it affects an ATTT motif, the positive association findings, if true, could be due to another allele in linkage disequilibrium with -432T. The -86insC variant that is in complete linkage disequilibrium with -432T would be a candidate because it also affects the binding of a brain protein in an allele specific manner.

The possibility that genes regulated by POU transcription factors could be candidates for BD and SZ susceptibility is consistent with other molecular and genetic findings. SZ is viewed by many as a neurodevelopmental disorder that can be caused by prenatal damage occurring in genetically predisposed individuals. Because members of the POU family play a critical role in brain development, as well as postnatal brain function, genes targeted by POU transcription factors could be a common link in understanding both the developmental and postdevelopmental aspects of psychiatric disorders. Supporting a role for POU factors in SZ is the finding of increased Oct-6 protein in the postmortem brains of patients compared with control subjects (Ilia et al 2002).

Alterations in PI signal transduction fit well with other candidate genes identified in SZ and BD, *Neuregulin 1* and *GRK3* (Barrett et al 2003; Stefansson et al 2002, 2003). *Neuregulin* is a growth factor affecting neurite outgrowth, neuron migration, and apoptosis that functions through activation of receptors in the EGF/ErbB family. This receptor family utilizes PI 3-kinase in its signal transduction cascade (Castellino and Chao 1999; Li et al 2003). Promoter mutations in *GRK3*, a member of the beta-adrenergic receptor kinase family on 22q11, have been implicated in BD (Barrett et al 2003). Beta-adrenergic receptor kinase cooperates with phosphatidylinositid 3-kinase to coordinate clathrin dependent receptor endocytosis (Naga Prasad et al 2001, 2002).

To summarize, our findings suggest that abnormal regulation of PI regulatory genes, possibly by members of the POU family of transcription factors, is a pathway underlying the development of both BD and SZ. This hypothesis fits well with various aspects of SZ and BD pathogenesis, including the therapeutic action of lithium, previous genetic linkage studies, recently identified candidate genes, and neurodevelopmental considerations.

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