Differential Regulation of the Serotonin Transporter Gene by Lithium Is Mediated by Transcription Factors, CCCTC Binding Protein and Y-Box Binding Protein 1, through the Polymorphic Intron 2 Variable Number Tandem Repeat

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The serotonergic pathways are possible targets for the action of lithium, a therapeutic agent for treatment of bipolar affective disorders. This study aimed to investigate the molecular mechanisms regulating human serotonin transporter gene (SLC6A4) expression by lithium and, specifically, the role of the variable number tandem repeat (VNTR) polymorphic region in intron 2, which is potentially a predisposing genetic factor for bipolar affective disorders. We demonstrated that addition of lithium to human JAR cells led to changes in the levels of SLC6A4 mRNA and protein. Additional investigations revealed that the intron 2 VNTR domain was a potential target for mediation of a transcriptional response to lithium. Properties of two transcription factors, CCCTC binding protein (CTCF) and Y-box binding protein 1 (YB-1), previously shown to be involved in the regulation of SLC6A4 VNTR, were found to be modulated by LiCl. Thus, levels of CTCF and YB-1 mRNA and protein were altered *in vivo* in response to LiCl. Furthermore, CTCF and YB-1 showed differential binding to the polymorphic alleles of the VNTR on exposure to LiCl. Our data suggest a model in which differential binding of CTCF and YB-1 to the allelic variants of the intron 2 VNTR can be regulated by lithium and in part result in differential and even aberrant expression of SLC6A4. Our study of the regulation of the SLC6A4 VNTR by lithium may improve the understanding of psychiatric disorders and enable the development of novel therapies for conditions such as bipolar affective disorder to target only the at-risk allele.

Key words: CTCF; YB-1; SLC6A4; VNTR; affective disorders; transcription; lithium

Introduction

Mutation or inappropriate expression of human 5HT transporter (SLC6A4) gene is postulated as an etiological factor in affective and other neurological disorders. The intron 2 variable number tandem repeat (VNTR) has been reported to be associated with mood disorders in some studies, with the most recent meta-analysis showing a significant but small effect (Cho et al., 2005; Lasky-Su et al., 2005). However, the effect is probably dependent

on specific environmental exposures, as shown for other polymorphisms in this gene (Caspi et al., 2003).

The intron 2 VNTR exists as three common allelic variants containing 9, 10, or 12 copies of a repeated 16 or 17 bp element (termed Stin2.9, Stin2.10, and Stin2.12, respectively) (see Fig. 2A). We have addressed the function of this polymorphism and demonstrated that different repeat number within the VNTR supports differential expression *in vitro* (Fiskerstrand et al., 1999; Lovejoy et al., 2003) and both differential and tissue-specific expression in a transgenic model (MacKenzie and Quinn, 1999). In addition to VNTR copy number, the different primary DNA sequence of the VNTR elements that constitute the VNTR can support differential reporter gene expression (Lovejoy et al., 2003). Our most recent report has demonstrated that the SLC6A4 intron 2 VNTR is bound and regulated by the transcription factor Y-box binding protein 1 (YB-1) (Klenova et al., 2004) a member of the Y-box binding proteins (Wolffe et al., 1992; Wolffe, 1994; Swamynathan et al., 2002; Kohno et al., 2003), and this regulation is modulated by the transcription factor CCCTC binding protein (CTCF) (Klenova et al., 2004), a known binding partner of YB-1 (Chernukhin et al., 2000). CTCF, in addition to transcriptional

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activation or silencing in a context-dependent manner, organizes epigenetically controlled chromatin insulators regulating imprinted genes (Klenova et al., 1993; Filippova et al., 1996; Ohlsson et al., 2001; Klenova et al., 2002).

We hypothesized that the intron 2 VNTR is a target both for physiological stimuli and pharmaceutical agents that would alter SLC6A4 levels or patterns of expression correlated with the progression of affective disorders. To test this hypothesis, we addressed whether the intron 2 VNTR was a target for lithium regulation using the human cell line JAr, which endogenously express SLC6A4, as a model system (Heils et al., 1995) and assessed the role of CTCF and YB-1 in that regulation. Lithium has been used as a mood-stabilizing drug for the treatment of manic episodes and depression. The targets of lithium are varied and clearly point to lithium modifying signal transduction pathways (Chen et al., 2000; Shamir et al., 2003; Tsuji et al., 2003), which would alter the complement of active transcription factors in the cell (Ikonomov and Manji, 1999). We demonstrate that the intron 2 VNTR is a target for mediating a transcriptional response to LiCl via (at least in part) the transcription factors CTCF and YB-1. *In vivo*, transcriptional variation was correlated with differential binding of both CTCF and YB-1 to the distinct VNTR variants after exposure to LiCl, and we postulate that this could lead to differential allelic gene expression.

Materials and Methods

Plasmids. The expression constructs used for *in vitro* production of recombinant YB-1 and CTCF were as described previously (Klenova et al., 2004). The human CTCF (hCTCF) expression construct was generated by subcloning hCTCF into the pCI expression vector (Promega, Madison, WI). The YB-1 episomal expression construct was generated by subcloning the YB-1 cDNA into pREP9 (Invitrogen, San Diego, CA) from pSV-YB-1, a kind gift from J. Ting (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC). The β -galactosidase expression vector pCH110 (Amersham Biosciences, Piscataway, NJ) was used as a control and to normalize for protein expression. The VNTR reporter gene constructs were produced by cloning the 9, 10, and 12 copy number VNTRs into the multiple cloning site of the pGL3-Promoter vector (pGL3p) (Promega). A neomycin resistance cassette was also inserted to enable the generation of stable cell lines.

Cell culture, stable and transient transfections, and Luciferase assays. JAr cells (ATCC HTB-144; American Type Culture Collection, Manassas, VA) were maintained as monolayers in RPMI (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 1 mg/ml glucose, and 1 mM sodium pyruvate. Transformed human epithelial kidney cells (293T) (ATCC CRL-1573; American Type Culture Collection) were maintained in DMEM supplemented with 10% fetal calf serum and 50 μ g/ml gentamycin.

For stable transfections, cells were transfected using Transfast transfection reagent (Promega), according to the manufacturer's protocol. Briefly, cells were seeded into six-well plates 24 h before transfection, and then incubated with 1 ml of serum-free media per well containing DNA and the lipid reagent for 2 h. Then, an additional 3 ml of media containing serum was added and cells were incubated overnight. Cells were thereafter fed as necessary. After 48 h growth, G418 was added to the growth media to select for cells that had integrated the expression plasmid. Stably transfected JAr cells were maintained in this medium supplemented with 400 μ g/ml G418.

For transient cotransfection assays, 10^5 cells were seeded in 12-well plates, and then transfected according to the calcium phosphate method (Sambrook and Russell, 2001). Cells were then harvested and assayed using the Luciferase Assay System according to the manufacturer's instructions (Promega). Luminescence was measured using a Labsystems Luminoskan luminometer (Life Sciences, Helsinki, Finland). To normalize for cell number and transfection efficiency, 0.25 μ g of β -galactosidase marker gene plasmid (pCH110) was included per well in the transfection solution. β -Galactosidase assays and normalization of luciferase were

performed as described previously (Lovejoy et al., 2003; Klenova et al., 2004). Mean and SD were calculated from the results of three experiments performed in triplicate.

Transfection with short interfering RNA. CTCF Smartpool and nontargeting Smartpool were purchased from Dharmacon RNA technologies. JAr cells were seeded in 12-well plates using 10^5 cells per well and 100 nM short interfering RNA (siRNA) was transfected with Dharmafect reagent 1 according to the manufacturer's protocol. Cells were harvested at 48 h after transfection and total RNA was isolated using the Gentra RNA isolation kit according to the manufacturer's instructions and was subsequently used for real-time PCR.

Lithium treatment. A sterile stock solution of 4 M LiCl was prepared in water. To treat cells, this was diluted using medium to construct a concentration response curve. JAr cells were grown to 80% confluence in 6- or 24-well plates before being serum starved (0.1% serum) for 24 h. Cells were incubated in media containing varying concentrations of LiCl overnight, and then allowed an overnight recovery phase, during which cells were returned to media containing 10% serum. Cells were harvested as described above for luciferase assays or RNA was extracted, as described below. Each set of conditions was performed in quadruplicate, and the mean and SEM were calculated from three separate experiments.

Chromatin immunoprecipitation. DNA and protein from 10^7 JAr cells were cross-linked with 1% formaldehyde at room temperature with agitation for 10 min. Chromatin immunoprecipitation (ChIP) was performed essentially as described previously (Kuo and Allis, 1999; Ohlsson et al., 2001). The antibodies used were the following: monoclonal anti-CTCF (BD Transduction Laboratories, Lexington, KY), polyclonal anti-YB-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti- α -tubulin (Sigma, St. Louis, MO). Briefly, cross-linking was quenched by addition of NH_4OH to 0.5% and nuclei were prepared and lysed in 250 μ l of nuclei lysis buffer (50 mM Tris-Cl, pH 8.1, 10 mM EDTA, 1.5% SDS, and protease inhibitor mixture). Chromatin was sonicated by 10×1 min pulses using a Vibracell sonicator. Efficiency of sonication was determined by agarose gel electrophoresis and chromatin absorbance at A_{260}/A_{280} . Samples were diluted in ChIP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 15.0 mM Tris-Cl, pH 8.1, 150 mM NaCl, protease inhibitors) to a concentration of 1 μ g/ μ l. Blocked fast-flow protein A-Sepharose (Sigma) was added, and the samples were incubated at 4°C overnight. Supernatants were then analyzed using appropriate antibodies. The amount of antibody required for immunoprecipitation was determined using the following formula: $(1/\text{Western titer} \times 4) \times (A_{260}/A_{280}) \times (1/[\text{DNA}] (\text{micrograms/microliter})) \times \text{sample volume} (\text{microliters}) = \text{micrograms of antibody required}$. ChIP cross-linking was reversed by incubation at 67°C for 6 h. The samples were precipitated at –20°C overnight, and the DNA was purified before subsequent PCR analysis.

PCR and RT-PCR. DNA primers for amplification of the Stin2 VNTR from genomic DNA were as follows: Stin2 VNTR [forward (for)], 5'-gtcagatcatcaggtcgag-3'; Stin2 VNTR [reverse (rev)], 5'-tggtctagttcttcagcagtg-3'. PCR was performed using KOD hot start DNA polymerase (Novagen, Madison, WI) in standard reaction mixture supplemented with 6% DMSO. Cycles used were as follows: 98°C for 3 min, 40 times (98°C for 1 min, 55°C for 30 s, 68°C for 30 s), 68°C for 3 min. RT-PCR was performed using 10^6 JAr cells. Briefly, cells were harvested 72 h after transfection, and cytosolic RNA was extracted using the RNAeasy kit (Qiagen, Hilden, Germany), followed by the one-step RT-PCR kit (Novagen). Primers were designed that would generate a fragment spanning exons 3 and 4 of SLC6A4 mRNA, namely, (for), 5'-ggacagtaccacgaaatggatgc-3', and (rev), 5'-gggtgatgtgtctcggagaag-3'. PCR conditions were as above.

Quantitative RT-PCR. Total RNA was extracted using the RNA Isolation kit (Gentra, Minneapolis, MN), and cDNA was prepared using the Reverse Transcription System (Promega); both were followed according to the manufacturers' protocol; cDNA was then adjusted to provide 200 ng per quantitative RT-PCR (Q-PCR) assay. Quantitative real-time PCR was performed using a Opticon qPCR machine (GRI, Braintree, Essex, UK) and the Dynamo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). For each experiment, a standard curve for each primer set was generated and used to derive the relative amounts in the unknown samples. The content of unknown samples was calculated and normalized to

the amount of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used were as follows: GAPDH, (for) 5'-accacgtccatgccatcac-3' and (rev) 5'-tccaccacctgttgctga-3'; CTCF (for) 5'-agatcatgattccagccca-3' and (rev) 5'-tgtgacagtcatgtgaaga-3'; YB-1 (for) 5'-gacgtaagtcgcccgcgattcatcc-3' and (rev) 5'-ctctt-tgggtttaccctgtcagtcg-3'; SLC6A4 (for) 5'-ggacagtaccacggaatggatgc-3' and (rev) 5'-ggatgattgtctcctcgagaag-3'. Primers were obtained from MWG Biotech (High Point, NC). BLASTN searches confirmed the total gene specificity of the primer sequences chosen. Thermal cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of amplification, consisting of 94°C for 10 s, 60°C for 20 s, 72°C for 20 s followed by data acquisition. Results of the Q-PCR assays were analyzed using software supplied with the Opticon machine. The default settings of the program were used to define the baseline value for analysis of the raw data. The expression of the target genes was normalized with respect to 1000 copies of GAPDH or β -actin and was calculated using Microsoft Excel (Microsoft, Redmond, WA). Each experiment was performed in triplicate, and each set of samples was assayed in duplicate, from which the average and SD were calculated.

EMSA. DNA fragments for EMSA were amplified using the KOD hot start DNA amplification system (Novagen), separated on agarose gels, and purified using the Qiagen gel extraction kit as recommended by the manufacturer. These fragments were end-labeled with [γ - 32 P]ATP in a standard T4 polynucleotide kinase reaction as described previously (Klenova et al., 2004). Recombinant YB-1 and CTCF were isolated from *Escherichia coli* BL21 (DE3) and baculoviral systems, respectively, and the EMSA was performed according to Chernukhin et al. (24).

Western blotting analysis. Transfected cells were lysed in SDS-urea lysis buffer [0.1 M Tris-Cl, pH 6.8, 7 M urea, 10% β -mercaptoethanol, 4% SDS, 0.01% (w/v) phenol red] after transfection. Samples were separated on 10% resolving polyacrylamide gels, blotted, and probed with anti-CTCF (BD Transduction Laboratories; Abcam, Cambridge, MA), anti-YB-1 (a kind gift from H.-D. Royer, Breast Cancer Research, Caesar, Bonn, Germany), anti-SLC6A4 (Chemicon International, Temecula, CA), anti- α -tubulin, and anti- β -actin (both from Sigma). Detection was performed with enhanced chemiluminescence reagent (Amersham Biosciences) according to the manufacturer's instructions. Quantification of the bands was performed by using the Image J software (<http://rsb.info.nih.gov/ij/>), and values were obtained from the ratios CTCF: α -tubulin, YB-1: α -tubulin, and SLC6A4: α -tubulin, or CTCF: β -actin, YB-1: β -actin, and SLC6A4: β -actin.

Statistical analysis. For the dose-response, the data were analyzed using GraphPad Prism software to calculate ANOVA in conjunction with Dunnett's multiple comparison test. Differences were considered significant at a value of $p < 0.05$. Statistical differences between the means of two groups were determined by Student's t test; results with a value of $p \leq 0.05$ were assessed as significant. Values were statistically analyzed using Microsoft Excel XP. Exploratory ANOVA analyses and other tests were performed in SPSS, version 12.

Results

Lithium modulates expression of the endogenous SLC6A4 gene and the transcription factors YB-1 and CTCF

The human placental cell line JAr has been used extensively to address SLC6A4 function because it expresses the endogenous gene (Heils et al., 1995; Bradley and Blakely, 1997). We therefore analyzed whether exposure of the cells to lithium, a characterized modulator of behavior, would modulate endogenous SLC6A4 expression. Concentrations of 1–5 mM LiCl were used, because at higher concentrations we observed nonspecific metabolic changes and changes in cell survival. LiCl had differential effects on endogenous SLC6A4 expression when measured by Q-PCR; there is a dose-dependent regulation of expression ($F = 6.59$; $p = 0.004$) (Fig. 1A). We have previously shown that the transcription factors CTCF and YB-1 can modulate reporter gene expression supported by the SLC6A4 intron 2 VNTR (Klenova et al., 2004); therefore, we addressed whether the endogenous expression of CTCF and YB-1 was altered in JAr cells by LiCl. As shown

Figure 1. **A**, Q-PCR of selected genes (SLC6A4, YB-1, and CTCF). Total RNA was extracted from JAr cells after exposure to varying concentrations of LiCl as shown, cDNA was synthesized, and Q-PCR was performed as described in Materials and Methods. The expression of the target genes was normalized with respect to 1000 copies of GAPDH mRNA. Each point on the curve is the average of three experiments performed in duplicate. Error bars indicate SD. A strong significance of $p < 0.01$ versus untreated for all treatment groups was ascertained using a two-factor ANOVA analysis. As shown, the varying LiCl concentrations have differential effects on the expression of RNA from each of the three genes. **B**, Western analysis of JAr cells after exposure to varying concentrations of LiCl as shown. The same membrane was also probed with the β -actin antibody (loading control). The images were quantified using the Image J software. The ratios of the intensity of the CTCF, SLC6A4, and YB-1 bands over the intensity of the corresponding β -actin bands were determined. Numbers below each lane show these results. This Western is representative of three separate Western blot analyses.

in Figure 1A, the expression of both of these factors was also differentially modulated by LiCl. Interestingly, the changes in the expression levels of YB-1 and CTCF were significant and similar in direction to that of SLC6A4 (YB1, $F = 10.91$, $p < 0.001$; CTCF, $F = 10.38$, $p < 0.001$), with an increase in both cases. Similar data were obtained when the samples were normalized to actin mRNA (supplement 1, available at www.jneurosci.org as supplemental material). No change in GAPDH mRNA was observed, indicating that this was not a general effect of LiCl on gene expression (supplement 2, available at www.jneurosci.org as supplemental material). These data also are consistent with the fact that we saw no toxicity over the range of lithium concentrations that were used. Lithium concentrations used clinically are normally between 0.5 and 1.4 mM (Shaldubina et al., 2001); we included 1 mM and extended that concentration to observe more robust changes. However, we did see metabolic changes at 10 and 20 mM LiCl, and these data were not included in the analysis.

To determine whether the changes in mRNA expression level were reflected in protein concentration of the factors, Western analysis was performed under the same conditions. As can be seen in Figure 1B, the protein expression mirrors exactly the changes observed in gene expression.

Lithium is a modulator of reporter gene expression directed by the Stin2 VNTR

Lithium is a modulator of reporter gene expression directed by the Stin2 VNTR. The modulation of CTCF and YB-1 by LiCl suggests that the intron 2 VNTR could mediate a transcriptional response to LiCl. To investigate this proposition, stable cell populations containing the three most common Stin2 VNTR allelic variants (9, 10, and 12 copies) (Fig. 2A) in the luciferase reporter gene construct pGL3p were generated. Exposure to LiCl caused a significant decrease in luciferase activity in all three VNTR variants, but not in the pGL3p alone control (Fig. 2B). However, only the stable cell line with the 9 copies repeat demonstrated a linear reduction in luciferase activity (Pearson's $r = -0.498$; $p < 0.001$), with the 10 repeat cell line showing a significant reduction of luciferase activity in response to lithium, which did not increase when higher concentrations were applied ($t = 2.95$; $p = 0.006$). Furthermore, the 10 repeat cell line did show a significantly reduced response (a 20% reduction vs ~32% for each of the other VNTR cell lines) to lithium presence/absence versus the 9 and 12 cell lines ($t = 2.21$, $p = 0.03$ for 10 versus 9 and $t = 2.35$, $p = 0.02$ for 10 vs 12). The control cell line did not show a significant difference before or after lithium ($p > 0.05$ on t test for presence or absence of lithium). Furthermore, the reduction in luciferase activity was seen after treatment with 1 mM lithium ($p < 0.05$ vs untreated) and became more pronounced after treatment with 2 and 5 mM lithium (both $p < 0.01$ vs untreated). We did not compare the response of the VNTR variants (9, 10, or 12), because they are not directly comparable with one another, because we have yet to develop controls for parameters such as copy number of integrant or location of insertion in the chromatin. We examined these difference by ANOVA and found that, overall, all three stable VNTR cell lines but not the control line show a stronger response to the presence of absence of lithium rather than its absolute concentration ($F = 2.174$, $p = 0.09$ for lithium concentration; $F = 5.717$, $p = 0.018$ for lithium presence vs absence).

CTCF and YB-1 coregulate the SLC6A4 intron 2 VNTR in reporter gene assays

We previously demonstrated that CTCF and YB-1 can differentially affect Stin2 VNTR function to support reporter gene activi-

Figure 2. *A*, Diagram of the 5'-region of the human SLC6A4 gene. Gray boxes, Tandem repeats/element sequences; black boxes, noncoding exons; numbered open boxes, coding exons; white open boxes, intronic/intergenic sequences. Element IDs refer to the individual sequences that occur within the intron 2 VNTR. Positions of primers for Q-PCR are indicated by arrows. *B*, LiCl modulates transcriptional activities of the three VNTR variants (Stin2.9, Stin2.10, and Stin2.12) in stably transfected JAR cells. The cells containing stably integrated VNTR-driven luciferase constructs were treated with LiCl, harvested, and assayed for luciferase activity as described in Materials and Methods. The luciferase activity of the lithium-treated cells is presented as a percentage, in which the untreated cells are 100%. Each bar represents the mean of three experiments performed in quadruplicate, and the error bars indicate SEM. Significant reduction in luciferase activity was seen after treatment with 1 mM lithium ($*p < 0.05$ vs untreated) and 2 and 5 mM lithium (both $**p < 0.01$ vs untreated), as confirmed by a two-factor ANOVA analysis.

ity in cell lines that do not demonstrate endogenous SLC6A4 expression, namely, 293T and COS7 cells (Klenova et al., 2004). We repeated these experiments using transient transfection of JAR cells. Figure 3A illustrates the different levels of reporter gene expression supported by the distinct VNTRs in the 293T and JAR cell lines. In both cell lines, the 12 copy VNTR supported much lower levels of gene expression.

Expression of exogenous YB-1 increased reporter gene expression in JAR cells in all three variants, as seen previously in 293T cells (Klenova et al., 2004). However, in contrast to our previous data in 293T cells in which CTCF alone had little effect on VNTR function (Klenova et al., 2004), in JAR cells we observed a dramatic increase in reporter gene expression to greater levels than that supported by YB-1 (Fig. 3B). Previously, we observed that coexpression of YB-1 and CTCF results in antagonism of their action and an ablation of the observed increased reporter gene expression by each individual factor (Klenova et al., 2004); similarly, here in JAR cells, coexpression ablated the increased reporter gene expression (Fig. 3B).

CTCF differentially modulates expression of the endogenous SLC6A4

We next addressed whether CTCF and YB-1 would influence expression from the endogenous SLC6A4 gene. To investigate this, expression constructs for CTCF and β -galactosidase were transfected into JAR cells and the levels of SLC6A4 mRNA were

Figure 3. *A*, The Stin2 VNTR differentially represses transcription in reporter gene assays. Jar or 293T cells were transiently transfected with the constructs including VNTR allelic variants (Stin2.9, Stin2.10, and Stin2.12) fused to Luciferase gene, harvested, and assayed for luciferase activity as described in Materials and Methods. Bars represent fold changes of luciferase activity in the cells transfected with the VNTR-Luciferase constructs compared with the control pGL3p (taken as 1; columns at the far left side of the graph). Each bar shows an average of three experiments performed in triplicate. Error bars indicate SD. Repression by all three VNTR variants in comparison with the control vector pGL3 was significant in both 293T cells ($p < 0.001$) and Jar cells ($p < 0.001$) as shown by Student's t test. *B*, The Stin2 VNTRs are differentially regulated by the transcription factors CTCF and YB-1. Bars represent fold changes of luciferase activity in the cells transfected with the VNTR-Luciferase constructs and vectors expressing 1.5 μ g of CTCF and/or YB-1 compared with the control cells transfected with the reporter only (taken as 1; bars at the left side of each series). Each bar shows an average of three experiments performed in triplicate. Error bars indicate SD. As assessed by Student's t test, significant activation by CTCF ($p < 0.001$) and YB-1 ($p < 0.001$) with all three VNTR variants and significant repression by a combination of CTCF and YB-1 ($p < 0.001$) with Stin2.10 and Stin2.12 were achieved, compared with the control (empty pCi vector).

measured by Q-PCR, using primers located as outlined in Figure 2A. Before transfection, low but detectable levels of SLC6A4 gene mRNA were observed; however, this dramatically increased after the addition of the CTCF expression construct (Fig. 4A). Treatment of Jar cells with the CTCF Smartpool siRNA resulted in ablation of expression of both CTCF and SLC6A4 mRNA, whereas the nontargeting siRNA had no effect (Fig. 4A). We also tested whether overexpression of YB-1 and β -galactosidase would alter the level of SLC6A4 mRNA; transfection of the YB-1 and β -galactosidase expression constructs into Jar cells did not lead to any changes in the level of SLC6A4 mRNA (data not shown).

Figure 4. CTCF modulates expression of the endogenous SLC6A4 in Jar cells. *A*, Analysis of SLC6A4 and CTCF mRNA expression by Q-PCR. Experiments with the CTCF siRNA and transfection with the CTCF expressing vector were performed on 10^5 Jar cells; RNA was harvested 48 h after transfection, cDNA was prepared, and Q-PCR was performed. Each bar is the average of three experiments performed in duplicate. Error bars indicate SD. *B*, Western analysis of Jar cells transfected with CTCF as per transient transfections. The images were quantified using the Image J software. The ratios of the intensity of the CTCF, SLC6A4, and YB-1 bands over the intensity of the corresponding α -tubulin bands were determined. Numbers below each lane show these results. This Western is representative of three separate Western blot analyses.

The increase in expression of SLC6A4 mRNA in response to increased CTCF levels was reflected in Western analysis: a dramatic increase in SLC6A4 protein level was observed when CTCF was overexpressed (Fig. 4B). However, increased CTCF expression had no significant effect on the levels of the endogenous YB-1 protein. There was no increase in α -tubulin levels when measured as a control in the same Western analysis, indicating that this was not a general effect on protein levels attributable to overexpression of CTCF (Fig. 4B).

Differential binding of YB-1 and CTCF to distinct VNTR variants

We previously demonstrated that YB-1 can bind directly to the SLC6A4 VNTR and that CTCF can affect binding by interacting with the CSD (cold shock domain) of YB-1 (Klenova et al., 2004). In this study, we further investigated CTCF binding to the VNTR using purified baculovirus CTCF (bvCTCF) in EMSA. We found that bvCTCF can bind specifically to all three variants (Fig. 5A).

The binding to all three variants was efficiently competed out using a DNA fragment containing the fourth CTCF binding site from the H19 ICR (Kanduri et al., 2002), which demonstrated the specificity of the interaction observed with the VNTR. YB-1 interaction with the three VNTR variants was confirmed in this assay (Fig. 5*Ai–iii*, lanes 2 and 3). Based on the competition assay using the CTCF binding site from the H19 ICR against the CTCF complexes formed on the VNTR (indicated by arrows), CTCF binds the 12 copy VNTR with the lowest affinity, because full competition is observed with 1 log less competitor.

To substantiate our finding that both CTCF and YB-1 could interact with the Stin2 VNTR, we analyzed these interactions *in vivo*, in JAr cells, using ChIP assays. PCR analysis demonstrated that the JAr cell line is heterozygous at this locus containing alleles for 10 and 12 VNTR variants as shown in Figure 5*B*. Strikingly, in JAr cells under basal growth conditions, YB-1 and CTCF interact predominantly with the Stin2.10 allele as shown by titration of template and antibody both in the presence and absence of serum (Fig. 5*B*). To validate the results of the ChIP analysis obtained with the SLC6A4 VNTR, multiple characterized CTCF sites from other genes were also tested under the same conditions and CTCF binding was detected for all of these sites (data not shown).

When cells were exposed to LiCl at 1 and 2 mM, we observed a dramatic change in the interaction of both YB-1 and CTCF with the specific allelic variants of the VNTR. Interaction was now predominantly with the Stin2.12 allele rather than the Stin2.10 allele (Fig. 5*C*). At 5 mM LiCl, binding of these factors could not be detected to either allele.

Discussion

Lithium is effective in the treatment of mania and in the long-term prophylaxis of bipolar disorder (Baastup et al., 1970), and serotonergic pathways are strong candidates for its action (Collier et al., 1996; Lerer et al., 2001). Consistent with this, in our model, the expression levels of the endogenous SLC6A4 gene varied in JAr cells in response to LiCl (Fig. 1). Lithium has been previously shown to regulate enzymes playing important roles in signal transduction pathways (Agam and Shalitel, 2003; Shamir et al., 2003; Tsuji et al., 2003) and transcription factors such as AP1 (Tamura et al., 2002). To this list we add the transcription factors CTCF and YB-1. Importantly, CTCF and YB-1 pro-

Figure 5. *A*, CTCF interacts directly with the Stin2 VNTR. EMSA analysis was performed as described in Materials and Methods with Stin2.9 (*i*), Stin2.10 (*ii*), and Stin2.12 (*iii*). Lanes: F, Free probes; 1, YB-1 glutathione *S*-transferase (GST); 2, YB-1 GST plus cold probe (competitor); 3, bvCTCF (0.5 μ g); 4, bvCTCF (1 μ g); 5, bvCTCF (2 μ g); 6, bvCTCF (1 μ g) plus 1 $\times$ competitor (H19 ICR); 7, bvCTCF (1 μ g) plus 10 $\times$ competitor (H19 ICR); 8, bvCTCF (1 μ g) plus 100 $\times$ competitor (H19 ICR). The small dark bands indicated by asterisks are most likely primer dimers; the complex indicated by an open triangle possibly represents a nonspecific DNA-protein complex, because it did not compete with the specific competitor. *B*, ChIP analysis of the interactions of CTCF and YB-1 with the Stin2 VNTR in JAr cells. Antibodies were used in increasing concentration (2, 4, and 8 μ g) based on titration of antibody, which was determined as in Materials and Methods. Both CTCF and YB-1 interact with only the Stin2.10 allele *in vivo* in cells grown in the presence of serum (top) or absence of serum (bottom). *C*, CTCF and YB-1 bind to the Stin2 VNTR after treatment with LiCl. JAr cells were treated with LiCl, as described in Materials and Methods, and were then used for ChIP assays. LiCl modulated a “switch” of binding between the two alleles, as shown by the appearance of the Stin2.12 band with both anti-CTCF and YB-1 antibodies used at 2, 4, and 8 μ g. As a control, the characterized CTCF binding domain H19 was also analyzed, and no change was observed in binding in response to LiCl.

A. Potential CTCF binding sites within the Stin2.12 VNTR

Sequence	Repeat Number	Element identity	CTCF site
1 12 17			
GGCTGTGACCCAGGGTG	RP1	a	Site 1
GGCTGTGACCCGAGTG	RP2	b	
GGCTGTGACCCGGGGTG	RP3	c	
GGCTGTGACCC GGGTG	RP4	d	
GGCTGCGACCTGGGGTG	RP5	e	
GGCTGTGACCTGGGATG	RP6	f	Site 2
GGCTGTGACCC GGGTG	RP7	d	
GGCTGTGACCTGGGGTG	RP8	g	Site 3
GGCTGTGACCC GGGTG	RP9	d	
GGCTGTGACCTGGGGTG	RP10	g	Site 4
GGCTGTGACCC GGGTG	RP11	d	
GGCTGTGACCTGGGATG	RP12	f	

B. CTCF “consensus”:

CCGCNNGGNGGCAG

C. Comparison of CTCF sites in Stin 2.12 with the “consensus”

CC-CGGGTGGGCTG
|| |..|..|||..|
CCGCNNGGNGGCAG

Figure 6. Potential CTCF binding sites within the Stin2.12 VNTR. **A**, The Stin2.12 VNTR sequence showing individual repeats (RP1–RP12) and unique elements based on primary sequence (a, b, c, d, e, f, g) (Klenova et al., 2004). Potential CTCF binding sites were determined by using Emboss Pairwise Alignment Algorithms (EMBL-EBI) to compare the Stin2.12 VNTR with the consensus CTCF sequence (Chao et al., 2002). Potential CTCF binding sites are underlined. **B**, CTCF “consensus” sequence. **C**, Comparison of potential CTCF binding sites with the CTCF consensus.

vide a direct link between drug action and a predisposing genetic polymorphic domain in the same clinical disorder.

We demonstrate that variation in SLC6A4 expression in response to LiCl was correlated with differential YB-1 and CTCF expression. Our hypothesis was that CTCF and YB-1 control the transcriptional properties of the intron 2 VNTR, which can be modulated by LiCl. This hypothesis was substantiated by the demonstration that, in a stably transfected JAr cell line model, a reporter gene supported by the VNTRs was a target for LiCl modulation (Fig. 2B). The intron 2 VNTR is one of many potential LiCl regulatory targets in the SLC6A4 locus; however, the interesting feature of this domain is the clinical correlation with affective disorders. The variation in mRNA expression is likely to be reflected in protein concentration and effect behavioral changes in a similar way to an SSRI (serotonin specific reuptake inhibitor) modulating the effective functional concentration of the transporter.

The transcription factor YB-1, but not CTCF, was previously shown to interact with the SLC6A4 intron 2 VNTR and activate the VNTR in a reporter assay in COS7 and 293T cells, although these do not express an endogenous SLC6A4 gene (Lovejoy et al., 2003; Klenova et al., 2004). In this report, we provide evidence that, in a SLC6A4 positive cell line, JAr, CTCF binds the SLC6A4 VNTR and activates this domain. Furthermore, LiCl modulates binding of both CTCF and YB-1 to the endogenous intron 2 VNTR in JAr cells. Indeed, inspection of the SLC6A4 VNTR region reveals potential binding sites for CTCF (Fig. 6). Therefore, regulation of the SLC6A4 VNTR may depend on a particular cellular context and involve different mechanisms; one of them, blocking of YB-1 binding to the VNTR, has been described previously (Lovejoy et al., 2003; Klenova et al., 2004). It is conceivable

able that direct binding of CTCF to the SLC6A4 VNTR could also be important for regulation of SLC6A4 function.

Exogenous expression of CTCF in JAr cells increased SLC6A4 mRNA (Fig. 4A). Consistent with this, overexpression of CTCF increased SLC6A4 protein concentration (Fig. 4B). It is likely that CTCF acts at least in part via the intron 2 VNTR, because all three VNTRs respond to CTCF in a reporter assay (Fig. 3B). Interestingly, much higher levels of induction can be achieved from the Stin2.12 variant. Exogenous YB-1 did not lead to activation of endogenous SLC6A4 (data not shown), although it could still activate all three VNTRs in a reporter assay (Fig. 3B). Similarly to CTCF, YB-1 had stronger effects on the Stin2.12 variant. This discrepancy may be explained by a more complex regulation of the endogenous gene, containing various regulatory elements within the same gene. In agreement with our previous report (Lovejoy et al., 2003; Klenova et al., 2004), coexpression of CTCF and YB-1 in JAr cells resulted in abrogation of the effects of YB-1 and CTCF in a reporter gene assay with all three VNTR variants. It is possible that mechanisms involving both interaction between CTCF and YB-1 and direct competition for the VNTR occur, leading to the inhibition of the ex-

pression from the reporter gene constructs by YB-1. From these experiments we conclude that (1) CTCF plays an important role in the regulation of SLC6A4 in JAr cells, (2) the effects of CTCF and YB-1 are likely to be relayed in part through the intron 2 VNTR domain, and (3) distinct VNTR elements are differentially regulated by CTCF and YB-1.

Regulation modulated by CTCF and YB-1 may be different on the 12 copy VNTR, suggesting a mechanism by which these transcription factors distinguish the function of these domains. Specifically, (1) the 12 copy VNTR supported lower basal expression in reporter gene assays in the two cell lines tested (Fig. 3A); (2) *in vitro* CTCF bound with lower affinity to the 12 copy VNTR than to the 9 and 10 copy (Fig. 5A); (3) perhaps most importantly, in the context of SLC6A4 expression in JAr cells, CTCF and YB-1 are both predominantly bound to the 10 copy allele, which dramatically switched to the 12 copy allele in the presence of LiCl. Because this domain is a transcriptional regulator, it may be one of several *cis*-acting domains contributing to the differential gene response to stress. There are likely to be multiple regulatory domains in the SLC6A4 gene, which synergize to give tissue-specific and stimulus-inducible gene expression; some of these may also be polymorphic [i.e., the 5' promoter domain, termed long or short (Heils et al., 1996, 1998)]. Thus, when transcription factors bind to specific alleles in response to a drug or stress, then the correlation must account for polymorphic regulatory domains acting in *cis*- on the same allele. This may explain in part the conflicting data on the statistical significance of the SLC6A4 intron 2 VNTR with affective disorders.

The location of the VNTR within the SLC6A4 gene strongly suggests that this is the gene whose expression is regulated by this domain. This is consistent with our previous studies, in particular

differential gene expression supported by the intron 2 VNTR in the CNS in the region that exhibited the initial serotonergic lineage (MacKenzie and Quinn, 1999). Delineation of the mechanism(s) regulating Stin2 VNTR function and endogenous SLC6A4 expression could be complex. CTCF is involved in the enhancer-blocking function of vertebrate insulators (Bell et al., 1999), has both transcriptional and epigenetic functions in the control of imprinting, and is associated with diseases ranging from Alzheimer's to cancer (Vostrov and Quitschke, 1997; Klenova et al., 2002). YB-1 has been implicated as a regulator of multiple processes such as development, multidrug resistance, oncogenesis, RNA splicing, DNA repair, and immune responses (Gaudreault et al., 2004; Gimenez-Bonafe et al., 2004; Ohba et al., 2004; Tsujimura et al., 2004; Bader and Vogt, 2005; En-Nia et al., 2005; Huang et al., 2005; Matsumoto et al., 2005). The cointeraction of these factors by direct interaction to regulate DNA targets has been observed for other genes (Chernukhin et al., 2000). Differential binding of these factors to the SLC6A4 intron 2 VNTR may predispose individuals to affective disorders by altering the level of SLC6A4 transcription in specific tissues. It is possible that this change may be attributable to posttranslational modifications in CTCF and/or YB-1, which are influenced by LiCl. Alternatively, administration of LiCl may cause epigenetic changes in the DNA and/or chromatin structure mediated by lithium-sensitive enzymes modifying DNA and chromatin, which allows for changes in the binding properties of CTCF and/or YB-1, or changes in the composition of a complex binding to the SLC6A4 intron 2 VNTR.

The complex nature of the SLC6A4 VNTR is further illustrated because, although SLC6A4 expression was induced by LiCl (Fig. 1), the VNTRs were shown to be repressor elements in a reporter gene assay in a stable cell line model that, on exposure to LiCl, further inhibited their activity (Fig. 2B). In this context, isolation of these elements from their natural gene environment might alter VNTR properties. It will be important to address how the VNTR functions in the context of a promoter fragment encompassing the 5' promoter and the intronic domain that is analyzed in our current study. Furthermore, there is a 5' promoter variant, termed the 5HTTLPR, also correlated with genetic predisposition to similar neurological conditions as the intron 2 VNTR (Heils et al., 1996, 1998). The 5HTTLPR has been associated with lithium response (Serretti et al., 2004; Rybakowski et al., 2005), although as yet the intron 2 VNTR has not been examined for lithium response. The 5HTTLPR contains DNA motifs consistent with its binding to CTCF among other transcription factors. Initial data from our group indicate that CTCF will regulate that 5' domain (J.P. Quinn, personal observations). Therefore, both domains should be considered in clinical correlations for a predisposition to a disorder. These issues will be fully addressed in our future experiments.

We hypothesize the therapeutic action of lithium in affective disorder treatment is mediated in part by modulation of a signal transduction pathway that regulates SLC6A4 expression through interactions of transcription factors with the Stin2 VNTR. Because this is a signal transduction pathway, both genetic and environmental interactions should be factored into future clinical correlation of the SLC6A4 intron 2 VNTR with disorders. Such studies are now gaining prominence in contemporary psychiatric genetics (Eley et al., 2004; McGuffin, 2004).

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