Differential Repression by Freud-1/CC2D1A at a Polymorphic Site in the Dopamine-D2 Receptor Gene*†‡

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Freud-1/CC2D1A is a transcriptional repressor of the serotonin-1A receptor gene and was recently genetically linked to non-syndromic mental retardation. To identify new Freud-1 gene targets, data base mining for Freud-1 recognition sequences was done. A highly homologous intronic element (D2-DRE) was identified in the human dopamine-D2 receptor (DRD2) gene, and the role of Freud-1 in regulating the gene at this site was assessed. Recombinant Freud-1 bound specifically to the D2-DRE, and a major protein-D2-DRE complex was identified in nuclear extracts that was supershifted using Freud-1-specific antibodies. Endogenous Freud-1 binding to the D2-DRE in cells was detected using chromatin immunoprecipitation. The D2-DRE conferred strong repressor activity in transcriptional reporter assays that was dependent on the Freud-1 recognition sequence. In three different human cell lines, the level of Freud-1 protein was inversely related to DRD2 expression. Knockdown of endogenous Freud-1 using small interfering RNA resulted in an up-regulation of DRD2 RNA and binding sites, demonstrating a crucial role for Freud-1 in DRD2 regulation. A previously uncharacterized single nucleotide A/G polymorphism (rs2734836) was located adjacent to the D2-DRE and conferred allele-specific Freud-1 binding and repression, with the major G-allele having reduced activity. These studies demonstrate a key role for Freud-1 to regulate DRD2 expression and provide the first mechanistic insights into its transcriptional regulation. Allele-specific regulation of DRD2 expression by Freud-1 may possibly associate with psychiatric disorders or mental retardation.

Dopamine-D2 receptors function as both pre-synaptic auto-receptors and post-synaptic receptors, and play key roles in regulating dopaminergic neurotransmission. Increased levels of dopamine-D2 receptors or dopaminergic hyperactivity have been implicated in schizophrenia (1–3), and most antipsychotic drugs inhibit dopamine-D2 receptors (4–6). Although transcriptional regulation of the rat dopamine D2 receptor (DRD2) gene has been examined, very little is known regarding the regulation of the human DRD2 gene. A polymorphism in the putative DRD2 promoter confers decreased transcriptional activity and has been negatively associated with schizophrenia (7). However, the transcriptional mechanisms for regulation of the human DRD2 gene have yet to be elucidated.

To identify new transcriptional regulators in the nervous system, we previously characterized the serotonin-1A (5-HT1A) receptor promoter region and identified a novel dual repressor element (DRE) that negatively regulates its expression (8, 9). The DRE consists of adjacent and partially overlapping repressor elements: 5′-repressor element (FRE; major regulator in neuronal cells) and 3′-repressor element (9). Analysis of DRE-binding proteins revealed that a novel protein, Freud-1 (Five prime repressor under dual repression-binding protein-1)/CC2D1A (Coiled-coil and C2 Domain containing 1A) binds to and represses the 5-HT1A receptor gene through the FRE (10). In 5-HT1A-expressing raphe cells, inactivation of Freud-1 by calcium-calmodulin kinase or using antisense to Freud-1 leads to up-regulation of 5-HT1A receptor expression. Thus, Freud-1 helps to establish the basal level of 5-HT1A receptor expression in raphe neurons.

Freud-1 is evolutionarily conserved and contains a variable number of Drosophila melanogaster 14 repeats, a helix-loop-helix, and a C2 (protein kinase C-conserved region 2) calcium-phospholipid binding domain (10, 11). Recently, linkage analysis in patients with autosomal recessive non-syndromic mental retardation has

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5 The abbreviations used are: DRD2, dopamine-D2 receptor; 5-HT1A, sero- tonin 1A receptor; FRE, 5′-repressor element; DRD2, dual repressor element; 5-HT, serotonin; Freud-1, five prime repressor under dual repression-binding protein-1; mFreud-1, mouse Freud-1; anti-hFreud-1, anti-human Freud-1 long antibody; CC2D1A, coiled-coil and C2 domain containing 1A; CHIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility gel shift assay; siRNA, small interfering RNA; QPCR, quantitative PCR; C2, protein kinase C conserved region 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline.

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revealed a deletion mutation that eliminates exons 14–16 of the CC2D1A/Freud-1 gene and encodes a truncated protein lacking the fourth Drosophila melanogaster 14, helix-loop-helix, and C2 domains (12). The C2 domain is essential for Freud-1 DNA binding and repressor functions, implying that the mutated Freud-1 protein is non-functional (10). Linkage of the CC2D1A/Freud-1 gene with non-syndromic mental retardation and its widespread localization in brain, including dopaminergic neurons (10, 12), suggested that Freud-1 may regulate other genes in addition to 5-HT1A receptors (13).

In this study, data base mining identified a highly conserved DRE sequence in the second intron of the DRD2 gene (D2-DRE) and two proximal and previously uncharacterized polymorphisms. We investigated the allele-dependent binding and repression of the D2-DRE by Freud-1 and the role of Freud-1 in regulation of dopamine-D2 receptor expression. We find that Freud-1 functions as a transcriptional regulator of the human DRD2 gene.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs—Cloning of mouse Freud-1 (mFreud-1; NCBI accession ABC56419) into pET30A and pcDNA3 (Invitrogen) has been previously described (10). Luciferase reporter constructs containing D2-DRE incorporated primers containing the A-allele (D2-DRE(A)), G-allele D2-DRE(G) for rs2734836 (5’-cgcttgagataaagccctccttcggtaaagtttgaagcag/gatac-3’), or the mutant D2-DRE(m3) (5’-cgcttgagataaagccctccttcggtaaagtttgaagcag/gatac-3’) were generated (bold letters indicate polymorphic mutations). The complementary D2-DRE oligonucleotides used for reporter constructs were purified by electrophoresis on 19% polyacrylamide gel, or mutated nucleotides). The complementary D2-DRE primers were preincubated with or without double-stranded competitor DNA: D2-DRE(A), D2-DRE(G), D2-DRE(m3), E2F (5’-tattgctcattactttctg-3’), 5-HT DRE (5’-ggcctgtaaagccctccttcggtaaagtttgaagcag/gatac-3’). Antibodies (1 µl of anti-mFreud-1, rabbit IgG, and anti-CC2D1A with or without 1X blocking peptide (Bethyl Laboratories Inc., Montgomery, TX)) were used in a 25-µl reaction containing DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol, and 2 mM dithiothreitol, pH 7.9) and 2 µg of poly(d-I-C)) or 250 ng of herring sperm DNA (Roche Applied Science) and incubated at room temperature for 30 min. 32P-Labeled probe (50,000 cpm/sample) was then added and incubated for an additional 20 min at room temperature. The DNA/protein complexes were separated on a 5% polyacrylamide gel at 4 °C dried and exposed to film (9).

**Antibodies, Western Blot, and Chromatin Immunoprecipitation (CHIP)—**Previously described rabbit anti-mFreud-1 antibody was used for supershift in the EMSA (10). Polyclonal rabbit anti-human Freud-1Long antibody (anti-hFreud-1; 1:20,000) was raised (Cedarlane, Hornby, ON) against bacterially expressed and purified (nickel nitritotriacetic acid beads; Qiagen, Mississauga, ON) S-/His-tagged hFreud-1Long (pTriEX4 vector; Novagen, Madison, WI) as antigen (NCBI accession number Q6P1N0). Anti-CC2D1A antibody was used in CHIP assays (1:1000 dilution) and in supershift EMSAs in the presence or absence of its specific blocking peptide (Bethyl Laboratories, which was preincubated with the antibody overnight at 4 °C. In addition, anti-β-actin (1:20,000; Sigma), anti-c-Raf (1:3,000; BD Biosciences), and anti-histone H1 (1:1,000; Upstate Biotechnology, Lake Placid, NY) were used as controls. Immunoblots were performed as described previously (17). Membranes were incubated with primary antibodies overnight at 4 °C followed by horseradish peroxidase-linked anti-rabbit (1:2000; New England Biolabs) or anti-mouse secondary antibody (1:2000; Jackson Immunoresearch Laboratories, West
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Grove, PA) and tertiary BM chemiluminescence blotting substrate (Roche Applied Science). Specificity of anti-hFreud-1 antibody was assessed by Western blot and immunoprecipitation analyses, where a ~130-kDa band was identified only with immunized serum (supplemental Fig. S1) and verified by mass spectrometry (Ottawa Genomics Innovation Center Proteomics Facility; data not shown).

CHIP assays were performed as described in Upstate protocol (Upstate Biotechnology) with modifications. Cells were washed 3× with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4), cross-linked for 15 min at room temperature in PBS supplemented with 1 mM MgCl2 and 1% formaldehyde (v/v), rinsed 3× with PBS, and lysed (18). Shearing of genomic DNA (100–400-bp fragments; data not shown) was done by sonication on ice with the addition of 212–300-μm-diameter glass beads (Sigma) (19) 15× at the setting of 7 for 20 s time (60 Sonic Dismembrator, Fisher). De-cross-linking was done overnight at 65 °C followed by 1 h of digestion with proteinase K (Sigma) and phenol/chloroform extraction. The results were analyzed using quantitative PCR (QPCR; Rotor-Gene RG-3000; Corbett Life Science, Sydney, Australia) with two sets of oligonucleotide primers; one designed to amplify a 124-bp (5'-ctacctggggctgcctattt-3' and 5'-tgacgctgacgactg-3') and the other to amplify a 206-bp region (5'-catcggcagcgtttagt-3' and 5'-tataagctactgtgctgctg-3') containing D2-DRE. The PCR reaction was performed using qTaq™ DNA polymerase mix (Clontech), and amplification cycles were 92 °C for 10 min, 92 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, 84 °C for 20 s (25 cycles), and terminated at 72 °C for 10 min. The results were visualized using SYBR Green (Molecular Probes, Eugene, OR) incorporation and verified on agarose gel.

Immunoprecipitation—Cells were rinsed 2× with ice-cold PBS and lysed in modified radioimmune precipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with 1× protease inhibitor mixture (Roche Applied Science) on ice for 30 min followed by sonication (60 Sonic Dismembrator, Fisher) once at the setting of 10 for 10 s. Lysates were centrifuged at 10,000 × g for 30 min at 4 °C, and the supernatant was diluted 10× with PBS/protease inhibitor mixture. The lysates were precleared with 40 μl of protein A-agarose beads (GE HealthCare) with rotation at 4 °C for 30 min and combined with anti-hFreud-1 antibody (1:1000) overnight at 4 °C. The following day 20 μl of protein A-agarose beads were added and incubated (2 h, 4 °C). The supernatant was then collected, and the beads were washed with 1 ml of NETIN buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40) 3× with gentle inversion and spun for 10 s at 1000 × g. The beads were then boiled for 5 min in 2× loading buffer (200 mM Tris, pH 6.8, 0.8% SDS, 1.6% 2-mercaptoethanol, 0.04% bromphenol blue, 4% glycerol) and subjected to Western blot analysis.

Nuclear and Cytosolic Fractionation and siRNA—Nuclear/cytosolic fractionation was performed as previously described (20). Briefly, cells were lysed on ice with extraction buffer (10 mM KCl, 10 mM Na-HEPES, pH 7.6, 1.5 mM MgCl2, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture) for 10 min. The resultant nuclei were washed (50 mM NaCl, 20 mM Na-HEPES, pH 7.6, 25% glycerin, 0.2 mM EDTA, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture), lysed with nuclear extraction buffer (500 mM NaCl, 20 mM Na-HEPES, pH 7.6, 25% glycerin, 0.2 mM EDTA, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture), and analyzed for success of fractionation and the presence of Freud-1 by Western blot analysis.

Stealth siRNA targeting hFreud-1 (5'-gggcuccauacagcagcagauag-3') and a scrambled negative control (5'-gggucuccahuacagcagcagagaa-3') were designed online (Invitrogen). A7 and SK-N-AS cells were transfected using HiPerFect (Qiagen) transfection reagent and Y-79 cells using Lipofectamine™ 2000 (Invitrogen) for Y-79, with a final siRNA concentration of 20 and 33 nM, respectively. Transfection efficiency control was performed with BLOCK-it™ Fluorescent Oligo (Invitrogen) demonstrating ~90% efficiency (data not shown). The cells were analyzed 72 h post-transfection.

Ligand Binding Assay—Dopamine-D2 receptor sites were measured by specific binding of the antagonist [3H]spiperone (119 Ci/mmol; Amersham Biosciences). Membranes were prepared from A7, SK-N-AS, and Y-79 cells as previously described (21). Membranes were diluted with 1/5 TME buffer (15 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, and 0.2 mM EDTA) supplemented with 0.1% ascorbic acid and 9000 cpm of [3H]spiperone in the presence or absence of apomorphine (10−6 M; Sigma). After 30 min incubation at room temperature, the samples were filtered through GF/C glass microfiber filters (Whatman, Clifton, NJ) and washed 3× with 5 ml of ice-cold 50 mM Tris, pH 7.4. The filters were then combined with 3 ml of scintillation fluid (InterSciences Inc., Markham, ON), and radioactivity was detected using the Packard TRI-CARB 2100TR scintillation counter (PerkinElmer Life Sciences). The receptor binding was normalized to protein concentration determined by bicinchoninic acid assay (Pierce).

Preparation of RNA, cDNA, and QPCR Analysis—The RNA was isolated using TRizol® reagent (Invitrogen) followed by DNase treatment with TURBO DNA-free™ kit (Ambion, Austin, TX), and cDNA was generated using the Cells-to-cDNA™ II kit (Ambion). The resulting cDNA was analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; control) and dopamine-D2 expression levels by QPCR analysis and SYBR Green detection method. More specifically, amplification cycles were 95 °C for 3 min, 92 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s (40 cycles), and terminated at 72 °C for 15 min performed with PCR primers (IDT, Coralville, IA) for human dopamine-D2 receptor cDNA (5'-tctgccacctgcctagc-3' and 5'-gcttggagctgtagcgcgtattgta-3') and human GAPDH (5'-cagacgctgaccttcttttttt-3' and 5'-gcgcacaccagcaccacatcc-3'). The resulting PCR products were analyzed as previously described, (22) using the 2−ΔΔCt method, validated by analyzing ΔCt (Ct,hFreud-1 − Ct,GAPDH), which produced a slope of 0.0633 (slope < 0.1 validates the use of this method). In addi-
Identification and Function of the D2-DRE—To identify new DNA elements recognized by Freud-1, a Blast Search was performed using the rat 5-HT1A 3'DRE sequence, a known Freud-1 binding site (9). A highly homologous sequence was recognized in the second intron of the human DRD2 (AF050737, 12432) with rat (r) 5-HT1A 3'DRE (AF087675, 2058). Conserved base pairs are in bold; Freud-1 binding site (the 5'-repressor element) is boxed. A, alignment of Freud-1 recognition DNA sequences. Conserved nucleotides, as compared with human 5-HT1A 5'FRE (h5-HT1A 5' (AC122707, 101949)) are highlighted in gray. Nucleic acid consensus is depicted at the top of the sequences. Similarity to human DRD2 FRE (hDRD2 (AF050737, 12433)), rat 5-HT1A 3'FRE (r5-HT1A 3' (AF087675, 2059)), and h-sHT1A is shown as percent identity. FRE sequences: human h5-HT1A 3'FRE (h5-HT1A 3' (AC122707, 101976)); rat 5-HT1A 5'FRE (r5-HT1A 5' (AF087675, 2059)); chimpanzee (c) DRD2 FRE (cDRD2 (NM 001033928)); dog (d) DRD2 FRE (dDRD2 (NM 001003110, 2860)); rat DRD2 FRE (rDRD2 (NM 012547, 57031)), and mouse (m) DRD2 FRE (mDRD2 (NM 010077, 58381)).

To determine whether nuclear proteins bind to the D2-DRE, nuclear extracts from HEK293 or dopamine-D2 receptor-positive A7 cells and labeled complementary D2-DRE oligonucleotides were co-incubated and examined by EMSA (Fig. 2A). In both cell types a major protein-D2-DRE complex was observed (arrow) that was competed with excess unlabeled D2-DRE double-stranded oligonucleotides but not unrelated double-stranded E2F primers. Mutant D2-DRE(m3) double-stranded oligonucleotides incorporating mutations shown to reduce Freud-1 binding to the 5-HT1A 3'DRE (9) did not compete for this major protein-D2-DRE complex compared with the non-mutated D2-DRE. The DNA sequence specificity of the major protein-D2-DRE complex is consistent with the binding of Freud-1 to the D2-DRE.

The transcriptional regulatory activity of the D2-DRE was examined using luciferase reporter assays. A7 and HEK293 cells transiently transfected with D2-DRE-containing plasmid displayed reduced luciferase activity compared with vector-transfected cells, indicating that the D2-DRE confers repressor activity (Fig. 2B). Similarly, strong repressor activity was observed when D2-DRE was placed downstream of the SV40 promoter (data not shown), supporting its role as a position-independent repressor element. The D2-DRE(m3) mutant construct lacked significant activity in A7 cells and marginally reduced transcriptional activity in HEK293 cells, consistent with the weak protein binding activity of this mutant element (Fig. 2A). These results indicate that the sequence specificity of
the D2-DRE conforms to a Freud-1 binding site. Taken together, the protein binding and repressor activities of the D2-DRE suggest its role as a repressor element of the DRD2 gene.

Analysis of the sequence surrounding the D2-DRE (http://www.ncbi.nlm.nih.gov/SNP) revealed two previously uncharacterized single nucleotide polymorphisms, A/G (rs2734836) and A/C (rs2734835). The more proximal polymorphism (rs2734836) is located 8 bp downstream of the D2-DRE. The effect of the rs2734836 polymorphism on D2-DRE repressor activity was analyzed using reporter constructs incorporating D2-DRE with either A- or G-allele (D2-DRE(A) or -(G)). Although both D2-DRE alleles displayed significant repression in A7 cells, the G-allele exhibited significantly less repression than the A-allele. Similarly, in HEK293 cells the A-allele repressed luciferase activity to less than 20% of control, whereas the G-allele displayed only 50% repression (Fig. 2B). These data indicate that the A-allele of the rs2734836 polymorphism has a stronger repressor activity in both cell lines than the G-allele.

Freud-1 Interacts with the D2-DRE—To address whether Freud-1 is present in the protein-D2-DRE complex from HEK293 nuclear extracts, an antibody specific for Freud-1 (anti-mFreud-1 (10)) was included to supershift the complex (Fig. 3A). In the presence of anti-mFreud-1 antibody but not preimmune serum, a slowly migrating protein-D2-DRE complex was observed (solid arrowhead), consistent with the presence of antibody-bound Freud-1 in the complex. Similarly, incubation of nuclear extracts from A7 cells with anti-CC2D1A antibody (human Freud-1) resulted in a mobility shift of the protein-D2-DRE complex (Fig. 3B, solid arrowhead). The supershifted complex was partially displaced by inclusion of antigenic blocking peptide, indicating the specificity of the antibody. These results indicate that Freud-1 is present in nuclear extracts and binds to the D2-DRE.

We examined whether Freud-1 directly interacts with the D2-DRE using in vitro transcribed and translated mouse Freud-1 protein. Incubation of recombinant Freud-1 with labeled D2-DRE revealed a specific protein-DNA complex that was efficiently competed by excess unlabeled D2-DRE (Fig. 3C, arrow) but not by unrelated E2F primers (data not shown), indicating that Freud-1 binds directly to the D2-DRE. The influence of the rs2734836 polymorphism on the binding of recombinant Freud-1 was determined by EMSA with either the labeled A- or G-allele of the D2-DRE (Fig. 3D). The intensity of the Freud-1-D2-DRE complex was greater for D2-DRE(A) than for D2-DRE(G), suggesting a greater binding affinity of Freud-1 for the A-allele (Fig. 3D, lanes 2 versus 7). Consistent with this, a 100-fold excess of unlabeled A-allele completely displaced DRE binding, whereas the G-allele was only partially effective (Fig. 3D, lanes 3 versus 4). Unlabeled rat 5-HT1A 3’DRE oligonucleotides competed as effectively as the D2-DRE A-allele, consistent with specific binding of Freud-1 to both DRE sites (Fig. 3D). These results demonstrate that the G-allele of the rs2734836 polymorphism displays reduced affinity for Freud-1 binding, correlating with a decrease in Freud-1-mediated repression at the G-allele compared with the A-allele (Fig. 2B).

To address whether endogenous Freud-1 is bound to the DRD2 gene in cells, quantitative CHIP assays were conducted. Anti-CC2D1A antibody was used to immunoprecipitate Freud-1-DNA complexes from cell lysates, and D2-DRE content was measured by QPCR analysis. A statistically significant enrichment of the D2-DRE from HEK293 cells in the elution fractions was found upon immunoprecipitation using anti-CC2D1A antibody compared with no antibody control (Fig. 4A). Similar results were obtained using anti-hFreud-1 antibody (data not shown). Similarly, in DRD2-expressing SK-N-AS cells, anti-CC2D1A antibody immunoprecipitated a D2-DRE-containing complex from chromatin as demonstrated by gel electrophoresis of the elution fractions (Fig. 4B). Preincubation with antigenic Freud-1 peptide reduced the immunoprecipitation of D2-DRE/Freud-1 complex (Fig. 4B, lane 3), confirming the specificity of the CHIP assay. These results demonstrate specific binding of endogenous Freud-1 protein to the D2-DRE of the second intron of the dopamine-D2 receptor gene.

Regulation of Endogenous Dopamine-D2 Receptor Expression by Freud-1—To address whether Freud-1 regulates dopamine-D2 receptor expression, three human cell lines expressing different levels of dopamine-D2 receptors were examined (7,
The role of Freud-1 in regulation of dopamine-D2 receptor expression was addressed using a specific Freud-1 siRNA to down-regulate Freud-1 expression. The effectiveness of this siRNA was assessed in HEK293 cells. A marked depletion of Freud-1 protein was observed in cytosolic and nuclear fractions of Freud-1 siRNA-treated cells compared with non-transfected cells or scrambled siRNA control (Fig. 6A). The purity of fractions and equal loading was demonstrated by immunoreactivity of histone H1 (nuclear) and c-Raf (cytosolic). Depletion of Freud-1 protein with Freud-1 siRNA also reduced the amount of protein-D2-DRE complex compared with scrambled siRNA control, as detected by EMSA (Fig. 6B, arrow), further substantiating the presence of Freud-1 in this complex.

The effect of Freud-1 siRNA on dopamine-D2 receptor mRNA expression was examined in D2-expressing cells since the DRD2 gene is transcribed and can be regulated, unlike in HEK293 cells, where the gene is completely silenced. Treatment with Freud-1-specific siRNA reduced Freud-1 protein levels in all cell lines compared with non-transfected cells or cells transfected with scrambled siRNA (Fig. 7A). Conversely, Freud-1-specific siRNA but not scrambled siRNA induced a significant increase in dopamine-D2 receptor mRNA and binding levels compared with untreated controls (Fig. 7B and C). The most pronounced increase in dopamine-D2 receptor RNA, and binding levels was observed in A7 cells, which express high levels of Freud-1, but lower levels of dopamine-D2 receptors (Fig. 5). By contrast, siRNA weakly increased dopamine-D2 mRNA in Y-79 cells, which display the lowest levels of endogenous Freud-1 protein and the highest dopamine-D2 receptor levels (Fig. 5). Thus, the impact of Freud-1 siRNA on dopamine-D2 receptor levels was dependent on the level of endogenous Freud-1 expression. Therefore, Freud-1 represses the DRD2 gene and regulates the basal level of DRD2 receptor expression in dopamine-D2-positive cells. Taken together, the results from reporter, EMSA, and CHIP assays implicate the
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In this study we have identified Freud-1 as an important transcriptional regulator of DRD2 expression at a conserved dual repressor element located in the second intron of the DRD2 gene (D2-DRE). Freud-1 binds to this repressor element in vitro and in intact cells (Figs. 3 and 4). The D2-DRE-mediated orientation-independent Freud-1 repressor activity and point mutations shown to abrogate Freud-1 binding also impaired Freud-1-induced repression at the D2-DRE (Fig. 2). In addition to band shift, supershift, and reporter assays, several results demonstrate the crucial role of endogenous Freud-1 activity at the D2-DRE to regulate dopamine-D2 receptor expression. In particular, CHIP assays using Freud-1-specific antibody identified an interaction between endogenous Freud-1 and the second intron of the endogenous DRD2 gene (Fig. 4). The interaction of Freud-1 with the D2-DRE provides the first evidence of a transcription factor that binds to the human DRD2 gene. Analysis of three different human cell lines revealed an inverse correlation between Freud-1 protein level and endogenous expression of dopamine-D2 mRNA and protein levels (Fig. 5, see “Results”), consistent with its role in basal repression of the DRD2 gene. In response to depletion of Freud-1 protein by siRNA treatment in these cell lines, expression of both DRD2 mRNA (Fig. 7B) and receptor levels (Fig. 7C) were markedly up-regulated. Furthermore, both basal dopamine-D2 receptor expression and siRNA-induced up-regulation were proportional to the cellular content of Freud-1 protein (Figs. 5 and 7, B and C), indicating that Freud-1 is crucial for regulation of basal levels of human DRD2 gene expression.

Very little is known regarding regulation of the human DRD2 promoter, which has relatively weak activity compared with the rat DRD2 promoter (7). The rat DRD2 gene has a robust TATA-less, CCG-rich promoter that is driven by Sp1 factors and is typical of housekeeping genes (27–33). The nucleotide identity between rat and human DRD2 promoters is relatively low (58% over 500-bp versus 74.6% for 5-HT1A promoter), and the human promoter lacks consensus sequences for several functional DNA elements (e.g. GATA, RARE, Sp1(B), AP2) (27, 34). Consequently, DNA elements located distal to the promoter may regulate the human DRD2 gene as observed for other genes (35, 36). Identification of the D2-DRE and its regulation by Freud-1 provides new insight into the importance of distal repressors in DRD2 regulation. Although the role of positive regulatory elements such as Sp1 in the human DRD2 has not been studied, the finding that dopamine-D2 receptor expression is induced upon specific depletion of Freud-1 protein levels using Freud-1 siRNA indicates that Freud-1 is a key determinant of DRD2 regulation in dopamine-D2 receptor-positive cells.

Dopamine-D2 receptors in the brain are strongly expressed in post-synaptic regions such as cortex, striatum, and nucleus accumbens in addition to pre-synaptic dopaminergic neurons of the substantia nigra and ventral tegmental area (37). Freud-1 is also expressed in pre-synaptic dopaminergic cells of the substantia nigra (10) and in embryonic and post-natal striatum and cortex, which express dopamine-D2 receptors (12). The presence of Freud-1 in these dopamine-D2 receptor-expressing regions suggests that Freud-1 could play a role in regulation of pre- and post-synaptic dopamine-D2 receptor expression in vivo.

A Functional Polymorphism Affects D2-DRE Repression—In addition to identification of Freud-1 action to repress the DRD2 gene, we also identified a novel functional polymorphism proximal to the D2-DRE that reduces Freud-1 binding and repressor activity. The A/G variation (rs2734836) is located 8 bp downstream of the D2-DRE, and the G-allele attenuates Freud-1 binding and repressor activity (Figs. 2B and 3D). Nonetheless, D2-DRE G-allele retained repressor activity and weak binding...
of Freud-1. The frequency of the D2-DRE A-allele rs2734836 varies considerably depending on ethnicity (0.042–0.5; NCBI) and is rare in Caucasians. The human cell lines used in this study had the GG genotype. Nevertheless, Freud-1 interacted with the genomic D2-DRE site (Fig. 4), and down-regulation of Freud-1 derepressed dopamine-D2 receptor gene expression (Fig. 7, B and C) in these cells. Thus, although weaker than the A-allele, the D2-DRE G-allele retains significant Freud-1 binding and repressor activity.

Functional polymorphisms in DNA elements of candidate genes can have important effects on expression in vivo, perhaps accounting for predisposition to mental illnesses. For example, a functional 5-HT1A promoter polymorphism (C(–1019)G, rs6295), located at a different site from the 5-HT1A-DRE (which is located at –1519 (10)), has been associated with depression and suicide. The G(–1019) allele completely blocks the binding and transcriptional regulatory function of deformed epidermal autoregulatory factor 1 at the 5-HT1A promoter (20, 38). Interestingly, recent imaging studies associate the GG genotype of the C(–1019)G polymorphism with increased expression of 5-HT1A binding sites in the raphe region of medication-free depressed patients (39, 40). Increased expression of 5-HT1A autoreceptors would reduce raphe firing, decreasing serotonin release as a possible mechanism for predisposition to depression and suicide. Because the A-allele of the D2-DRE displays enhanced Freud-1-D2-DRE interaction, a reduction in dopamine-D2 receptor expression is predicted. A decrease in pre-synaptic dopamine-D2 receptors would favor hyperactivity of dopamine neurons, a condition that is associated with enhanced reward, addiction, and schizophrenia (1, 24, 41). On the other hand, reduced expression of post-synaptic dopamine-D2 receptors may reduce dopaminergic signaling. Future studies could address whether the D2-DRE polymorphism is associated with alterations in pre- or post-synaptic dopamine-D2 receptor expression in vivo or is associated with mental illness.

**Freud-1 Function in Vivo**—These studies together with previous findings indicate that Freud-1 regulates both 5-HT1A and dopamine-D2 receptor gene expression. Although these receptors bear little sequence homology and their expression patterns are quite different, both are regulated by the same transcription factor, Freud-1. Interestingly, both receptors function as pre-synaptic autoreceptors to regulate serotonin and dopamine neurotransmission, respectively (11, 42). Thus, Freud-1 may coordinately regulate the activity of these two systems implicated in behavioral control.

The recent linkage of a deletion mutation in the CC2D1A/Freud-1 gene with non-syndromic mental retardation (12) and broad distribution of Freud-1 RNA and protein in the brain (10, 12) suggest its involvement in brain development and cognitive function. The deletion mutation of Freud-1 protein lacks domains essential for its repressor function (10), which likely results in a non-functional or dominant negative protein that could mediate up-regulation of DRD2 expression in receptor positive brain regions. Transgenic mice engineered to overexpress dopamine-D2 receptors in striatum display impaired working memory (26). This raises the interesting possibility that a reduction in Freud-1-mediated repression of the DRD2 gene may contribute to the mental retardation phenotype or to other developmental disorders in which dysregulation of the dopamine system is implicated, such as attention deficit hyperactivity disorder, autism, or schizophrenia (25). In some cases mental retardation has been linked to global regulators of gene transcription, such as the ATPase/helicase ATRX (alpha thalassemia/mental retardation syndrome X-linked) (43, 44) or the methyl binding repressor methyl CpG-binding protein 2 (45, 46). Likewise, Freud-1 may regulate other genes in addition to 5-HT1A or dopamine-D2 receptor genes to regulate cognitive development. Further studies of the function of DRE-like elements in other genes may reveal additional gene targets for Freud-1 that could be implicated in cognitive development and mental retardation.

In summary, we have identified the human dopamine-D2 receptor as a new gene target for the repressor Freud-1. Our data implicate Freud-1-D2-DRE interactions in determining the level of dopamine-D2 receptors in D2-expressing cell types. Because Freud-1 is expressed in dopamine and serotonin neurons in vivo as well as throughout development, Freud-1 regulation of 5-HT1A and dopamine-D2 receptor genes may coordinate the maturation and function of serotonergic and dopaminergic systems.

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