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ORIGINAL RESEARCH ARTICLE

Modified structure of the human serotonin transporter promoter

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Recently, several studies have reported an association between anxiety traits, affective disorders and autism and alleles of a functional promoter polymorphism (5HTT-LPR) in the human serotonin transporter (5HTT, SERT).[1–3] The mechanistic basis for allelic differences in transporter transcription are presently unknown. To explore this issue, we cloned the human 5HTT promoter region from a PAC genomic library[4] and now describe an unreported 381-bp insert between the polymorphic region and the transcription start site. We verified the presence of this novel sequence by Southern hybridization of genomic digests and PCR amplifications from multiple unrelated individuals. Sequence analysis of the novel region reveals a number of canonical transcription factor binding sites (eg AP1, Elk1, NFκB) that may be important in controlling the response of the 5HTT gene to regulatory factors. PCR studies of genomic templates reveal a low level of amplification of a deleted template matching the size of the originally reported 5HTT promoter. This deleted template is absent from PAC amplifications, suggesting that the human 5HTT promoter may exhibit *in vivo* instability. *Molecular Psychiatry* (2000) **5,** 110–115.

The serotonin (5HT) transporter (5HTT, SERT) is the major determinant of 5HT inactivation following release at synapses,[5,6] is the site of action for many tricyclic antidepressants and the SSRIs (serotonin-selective reuptake inhibitor)[7,8] and is also targeted by a number of psychostimulants including cocaine, methylphenidate, and MDMA 'ecstasy'.[9–11] Although no coding mutations[12,13] have as yet been identified in the 5HTT gene that could explain reports of transport and binding deficits in affective disorders,[14–16] a functional polymorphism (5HTT-LPR) has been identified in the human 5HTT promoter.[1,17] Two major alleles (termed 'short' (s) and 'long' (l)) correspond to the presence of 14 *vs* 16 20–23 bp repeat units upstream of the transcription start site. Recently,[18–20] additional low frequency alleles have been identified including a 20-repeat unit 'extra long allele' (el). Allelic status has been correlated with transcription efficiency and consequent transporter levels in heterologous models as well as native lymphocytes.[1,17] To clarify the relationship between the 5HTT-LPR and potential transcriptional regulatory elements, we cloned the human 5HTT promoter from a P1-derived artificial chromosome (PAC) library. Our studies reveal the presence of 381 bp of unreported sequence between the polymorphic region and the transcription start site. The human genomic origin of this sequence was verified by Southern blot analysis of digested genomic samples, PCR reactions with flanking primers, and direct DNA sequencing. Here we report the sequence of the full 5HTT promoter including additional canonical transcription factor binding sites that may be critical for cell-specific or hormonally-regulated 5HTT expression. We also find evidence for instability within the 5HTT promoter that may account for the absence of this material in initial isolates.

Previously,[4] we isolated a PAC clone (No. 6324) encompassing the full human 5HTT gene. This clone contains the major transcription start site[4,21] and the long allele of the 5HTT-LPR. Southern hybridization of restriction digests of the PAC clone using as a probe PCR-amplified 5HTT-LPR revealed anomalously large fragments (Figure 1a). Thus, based on the original report of the 5HTT promoter, a *Bam*HI/*Hind*III digest of long allele PAC DNA hybridized with the 5HTT-LPR should identify a ~1.6-kb band whereas we found a band of ~2 kb. We tested the same digestion panel with human genomic DNA and found an identical migration pattern of hybridizing bands (Figure 1a), suggesting that the anomalous migration pattern of PAC bands was not a result of the process of library creation or amplification. We could limit the origin of the unexpected DNA to within 500 bp of the 5HTT-LPR using an *Eco*RI/*Hind*III double digest (Figure 1b). Figure 1 b also demonstrates the presence of additional material in the 5HTT promoter irrespective of 5HTT-LPR genotype as bands from s/s, s/l and l/l individuals migrated at larger than expected sizes.

To determine the physical basis for the larger than expected 5HTT promoter fragments, we subcloned and sequenced the 2-kb *Bam*HI/*Hind*III fragment from the PAC clone. Results of this analysis are given in Figure 2. We found an unreported 381-bp region (–1255 to –875 bp) upstream of the major transcription initiation site and immediately 3′ of the 5HTT-LPR. BLAST searches of the GENBANK database did not identify sequence with sequences of any other known gene, supporting the contention that the new material did not arise from recombination with *E. coli* DNA during library propagation. This sequence contains a number

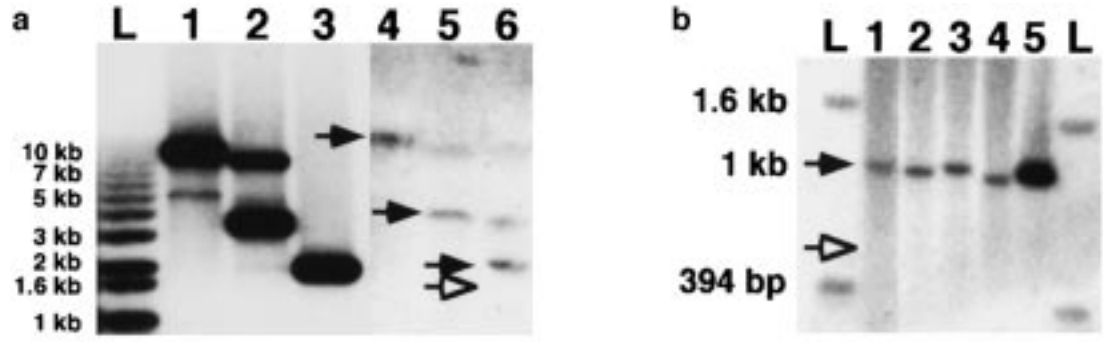

Figure 1 Southern analyses of 5HTT promoter sequences using 5HTT-LPR as probe. (a) Size analysis of restriction fragments from PAC clone 6324 (lanes 1–3) and human genomic DNA (lanes 4–6) digested with *Bam*HI (lanes 1,4), *Hind*III (lanes 2,5) and *Bam*HI/*Hind*III (lanes 3,6). L designates lane containing 1-kb DNA ladder. Solid arrows indicate bands of equivalent size in both PAC and human genomic DNA. Open arrow designates migration position for *Bam*HI/*Hind* III digest based on previously reported sequence (~1.6 kb *vs* 2.0 kb observed in our digests). (b) Southern analysis of *Eco*RI/*Hind* III digested DNA from four unrelated individuals (1–4) compared with the migration of *Eco*RI/*Hind* III digested 5HTT promoter isolated from PAC clone 6324. Solid arrow indicates bands in human genomic DNA similar in size to that liberated from the subcloned 5HTT promoter. Variations in fragment size are accounted for by the allelic status of subjects, genotyped as described in Methods (1 = s/1, 2 = s/s, 3 = l/1, 4 = s/s, 5 = l). Open arrow designates expected migration position for *Eco*RI/*Hind* III digests (~0.6 kb *vs* 1.0 kb observed in our digests), based on previously reported sequence.[17,21]

of canonical transcriptional binding sites (Figure 2b, Table 1), including Elk1, NfkB/Elk1, AP1, AP4 and SOX5 sites, that may play important roles in the endogenous expression of this gene and its sensitivity to allelic status at the 5HTT-LPR.

To extend our analysis of the prevalence of this addition to the 5HTT promoter, we designed a genomic PCR assay using oligonucleotide primers (RB 433 and RB 597) flanking the 5HTT-LPR and the novel sequence (Figure 2a). All 42 individuals genotyped in our assay displayed the novel sequence (data not shown). Amplifications reveal a single 1-kb band by ethidium staining as shown in Figure 3a. Sequence analysis of isolated PCR products revealed the same novel sequences as found in the PAC isolate. Interestingly, when we probed a blot of our PCR reactions with the 5HTT-LPR to verify band origin, for each individual we could detect a faint band migrating at the expected size (~600 bp) for a promoter lacking the novel sequence (Figure 3b). This smaller band does not appear to arise from the PCR reaction itself as PAC DNA amplified in parallel gives only the 1-kb band even though the amplification yields much more of the 1-kb product. Only a single 5HTT gene has been identified in the human genome[9,21] and the smaller size of the faintly-amplified product suggests that it should have been amplified more efficiently were there a duplicate, unexpressed copy of the 5HTT gene in the genome. Lesch and coworkers[21] thus identified a form of the gene lacking what appears to be prevalent DNA sequences in the promoter. Conceivably, rearrangement at the 5HTT locus may have occurred during genomic library production or clone isolation and amplification. In our hands, the 5HTT promoter is difficult to amplify in *E. coli* and we often propagate rearranged templates. However, it is also possible that Lesch and colleagues preferentially subcloned genomic DNA that *originally* lacked these sequences. Since we can identify material after genomic amplifications that matches the expected size of the Lesch promoter, a small, but detectible level of mosaicism at the 5HTT locus may exist, arising from *in vivo* instability of the 5HTT promoter. Mosaicism has, to our knowledge, not been associated with other neurotransmitter related genes, though this is quite common for trinucleotide repeat disorders.[22] It is thus possible that instability could be conferred in a cell or tissue-specific manner linked to the repetitive sequences in the polymorphic region. We cannot at this time rule out that such mosaicism might involve a loss of additional sequences in the 5HTT gene or their duplication elsewhere in the genome, though our Southern analyses verify only a single locus carrying the polymorphic region. That such instability would have functional consequences in the CNS appears likely as Mortensen and coworkers have also identified these novel sequences and found that they contribute to *in vitro* neuronal expression of the 5HTT promoter.[23] Alternatively, DNA extraction itself may precipitate deletions at this locus though the mechanism for such an excision is unclear. We are currently expanding our analyses to look for evidence of increased *in vivo* instability in the 5HTT promoter in disease states associated with altered serotonergic function. Regardless of whether 5HTT promoter instability exists at a sufficient level to compromise 5HTT transcription, transcription factors binding to the novel sequence should be considered as possible mediators of allelic influences on 5HTT transcription given their proximity to both the polymorphic sequences and the major transcription initiation site. Given the possibility that allelic status at the 5HTT-LPR may generate increased risk for affective disorders, substance abuse and autism, the full structure of the 5HTT promoter is essential for advanced studies in this area.

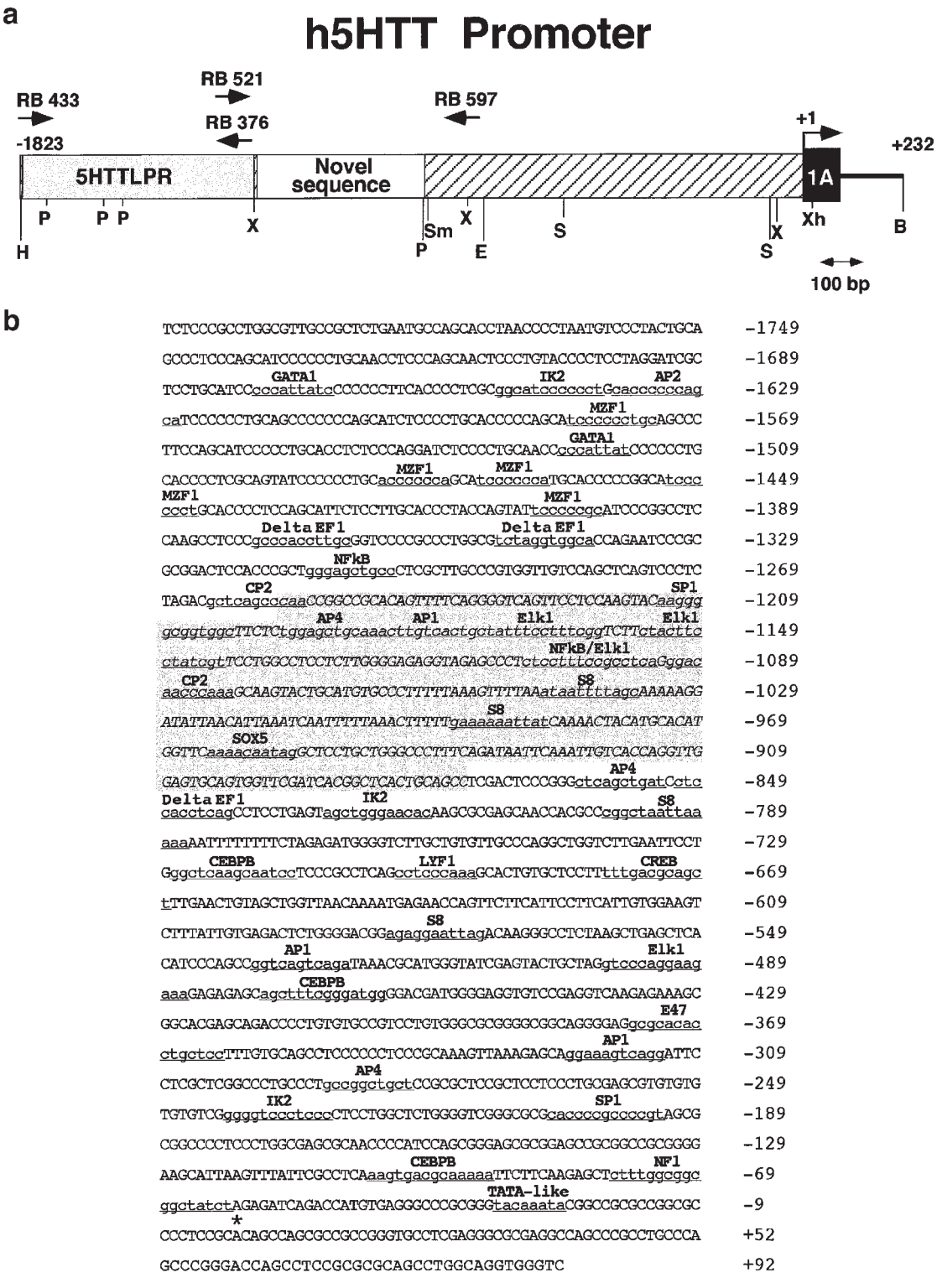

Figure 2 Revised structure and sequence of the 5HTT promoter. (a) Schematic representation of the 5HTT promoter derived from sequence analysis of subcloned *Hind*III (H)/*Bam*HI (B) promoter fragment of PAC isolate 6324. Other enzyme sites listed as landmarks for reference with previous reports include *Pst* I (P), *Xba* I (X), *Sma* I (Sm), *Eco*RI (E), *Sac* I (S), *Xho* I (Xh). 1A designates the first noncoding exon for 5HTT transcripts, with transcription start site at +1. Solid arrows indicate the positions of oligonucleotide primers used for PCR analysis in PAC and genomic templates. (b) Revised sequence of the 5HTT promoter. Sequence is numbered with respect to the transcription start site (*). Shaded sequence represents the novel material identified in this report. A subset of putative transcription factor binding sites is represented (see also Table 1) and designated by underlining and lowercase type.

Table 1 Canonical transcription factor binding sites in novel 5HTT promoter sequence

Locus[a]	*Sequence (CORE)*[b]	*Transcription factor*	*Locus*	*Sequence (CORE)*	*Transcription factor*
−1255	ggccgGTTG	CMYB	−1044	aattttaGCAAaaa	CEBPB
−1236	acTGACccctg	AP1	−1034	aaaagGATAttaac	GATA1
−1235	cttggaggaacTGACccct	ER	−1028	taaTGTTaatat	HFH2
−1226	TTCCtccaa	STAT	−1018	taAATCaattttta	GFI1
−1213	aaggGGCGgtggct	GC	−996	gaaaaaATTAt	S8
−1213	aaggGGCGgtggc	SP1	−994	tGATAAtttt	GATA
−1195	tgCAGCtcca	AP4	−992	gaTAATtt	NKX25
−1185	agTGACaagtt	AP1	−991	gttttgatAATT	OCT1
−1175	tgcTATTtcctt	HNF3B	−964	caaaACAAtagg	SRY
−1173	ccgaaaGGAAatag	ELK1	−963	aaaaCAATag	SOX5
−1155	acgataGGAAgtag	ELK1	−951	ccCAGCagga	AP4
−1152	ggaacGATAggaag	GATA1	−938	ttcaGATAattca	GATA1
−1129	cttGGGGa	MZF1	−933	gaTAATtc	NKX25
−1129	cttgGGGAgcag	IK2	−933	GATAattca	GATA1
−1112	gcggAAAGgagaggg	BARBIE	−932	atttgaATTAt	S8
−1107	tgaggcGGAAagga	ELK1	−926	ggtgACAAttg	SRY
−1097	ctcaGGGAcaac	IK2	−925	ggTGACaattt	AP1FJ
−1093	GGGAcaaccc	NFκB	−925	gtgaCAATtt	SOX5
−1092	ggacaaacCCAA	CP2	−917	accagGTTG	CMYB
−1089	ttggGTTG	CMYB	−917	tccaACCTggt	DELTAEF
−1053	gtttTAAAtaattt	XFD1	−902	agTGGT	AML1
−1046	gctaaaATTAt	S8	−898	gttcGATCac	CDPCR3HD

[a]Sites were selected for presentation due to 100% core sequence identity and total sequence identity for the motif of >85%.
[b]Core sequence in uppercase.

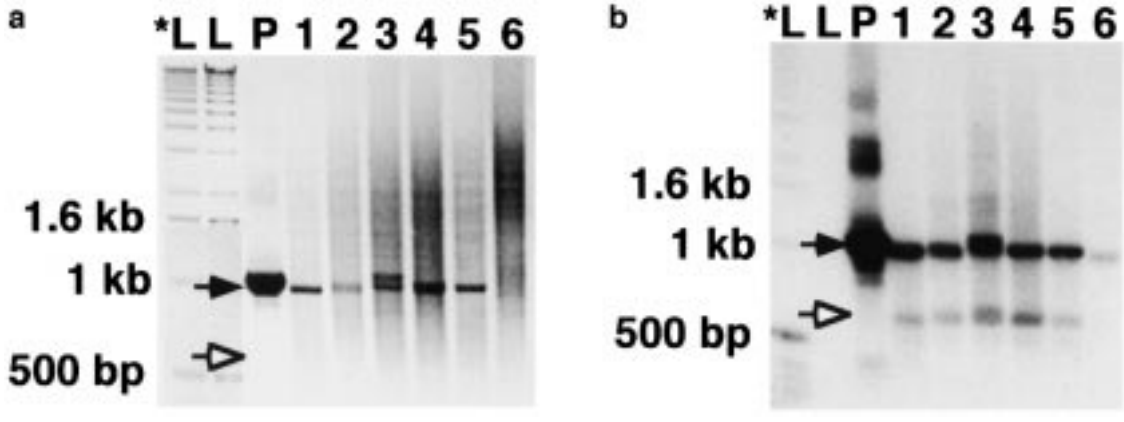

Figure 3 PCR assay for presence or absence of 5HTT novel sequences in human genomic DNA. DNAs were isolated and amplified with PCR primers RB 433 and RB 597 as described in Methods. (a) Ethidium bromide stain of PCR reactions. L* indicates lane with [^{32}P]-labeled DNA ladder. L designates lane with unlabeled ladder. P indicates lane with amplified PAC 6324 DNA. Samples 1–6 represent six unrelated individuals with the following genotypes: 1, s/s; 2, s/l; 3, l/el; 4, l/l; 5, l/l; 6, s/l. (b) Southern blot analysis of gel from panel (a) using [^{32}P]-labeled 5HTT-LPR. Solid arrow indicates observed amplification products. Open arrow indicates the expected size for amplified products based on originally reported 5HTT promoter sequence. Notice the low level of product migrating with a size expected of templates lacking the novel sequence that is only seen with Southern analysis. Note also the differential migration pattern of the smaller bands, which vary with genotype as seen in the larger products, and the lack of smaller amplified products from PAC DNA, supporting the origin of the smaller products from endogenous 5HTT templates lacking the novel sequences.

Methods

Analysis of the 5HTT promoter from PAC and genomic DNA

To identify elements linked to the 5HTT-LPR, the promoter of PAC clone 6324 (Genome Systems, St Louis, MO, USA) containing the human 5HTT gene[4] was isolated by *Bam*HI/*Hind*III digestion and the 2-kb fragment hybridizing with 5HTT-LPR sequences was gel purified and ligated into pBS SKII– (Stratagene, La Jolla, CA, USA). Inserts of positive transformants were sequenced with fluorescent dideoxy chain terminators

and Amplitaq-FS (Perkin Elmer, Foster City, CA, USA) using 25 cycles of 96°C/30 s, 50°C/15 s, 60°C/4 min and analyzed on an ABI 310 automated DNA sequencer. Putative transcription factor binding sites in novel 5HTT promoter sequences were identified using MatInspector Version 2.2 and matrices of the TRANSFAC database version 3.5,[24] available on the World Wide Web at http:transfac.gbf-braunschweig.de/TRANSFAC/index.html.

To validate the structure of the 5HTT promoter, genomic DNA was prepared from whole blood or fresh frozen brain tissue using the Puregene DNA Isolation Kit (Gentra Systems, Inc, Minneapolis, MN, USA). Restriction digests of DNAs, performed as described by the vendor (Promega, Madison, WI, USA; New England BioLabs, Beverly, MA, USA), were ethanol precipitated and electrophoresed in parallel with 1-kb DNA ladder (Gibco BRL, Gaithersburg, MD, USA) on a 0.5% 1× TAE agarose gel containing 0.4 μg ml^{-1} ethidium bromide. In some analyses, DNA ladder was radiolabeled with [^{32}P]α-dCTP using the Klenow fragment of T4 DNA polymerase following standard protocols.[25] After electrophoresis, DNA in the gel was depurinated and denatured by sequential 20-min incubations in 0.2 N HCl and 0.5 M NaOH/1.5 M NaCl. DNA was transferred by vacuum to Hybond-N nylon membrane (Amersham, Arlington Heights, IL, USA), using 20× SSPE (3 M NaCl, 20 mM EDTA, and 200 mM NaH_2PO_4, pH 7.4). Blots were UV-cross-linked (150 mJ; Bio-Rad, Hercules, CA, USA), rinsed with ddH_2O, and prehybridized overnight at 42°C in 50% formamide, 5× SSPE, 0.5% sodium dodecyl sulfate (SDS), 5% dextran sulfate, 5× Denhardt's reagent (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, and 0.2% Ficoll), and 10 mg ml^{-1} herring sperm DNA. To synthesize probe for blots, we amplified in PCR reactions the 528-bp long allele of the 5HTT-LPR from PAC clone 6324 using as oligonucleotide primers, sense 5′-GGCGTTGCCGCTC TGAATGC-3′ (RB 433); antisense 5′GAGGGACTGA GCTGGACAACCCAC-3′ (RB 376). The PCR product was subcloned (pGEM-T-Easy, Promega), sequenced and then reexcised for [^{32}P]-labeling (Megaprime kit, Amersham) and then added directly to prehybridization buffer for an additional 12–24 h at 42°C. Blots were washed twice at room temperature with 2× SSPE, 0.1% SDS buffer for 20 min, once at 42°C with 0.1× SSPE, 0.1% SDS for 20 min, once at 65°C with 0.1× SSPE, 0.1% SDS for 30 min, and once at room temperature with 2× SSPE for 10 min. Blots were then exposed to either a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA, USA) or X-ray film (Amersham).

PCR analysis of genomic DNA for novel 5HTT sequences

The novel sequence identified in the 5HTT promoter from PAC clone 6324 was amplified in human genomic DNA with flanking oligonucleotide primers RB433 and RB597 (5′-CAAGACCAGCCTGGGCAACACAGCAA-GAC-3′) in 50-μl reactions using the Expand Long Template PCR System protocol (Boehringer Mannheim, Indianapolis, IN, USA) and system buffer 3. Amplifications were performed on a Perkin Elmer thermocycler (2400 or 9700) with one cycle at 95°C/2 min followed by 30 cycles of 95°C/30 s, 61°C/30 s, 68°C/2 min, and a final 7-min extension at 68°C. PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide staining, sequenced after gel purification and subjected to Southern blot analysis as described for genomic Southerns, using the long 5HTT-LPR region as probe. The 5HTT-LPR genotypes were determined via agarose (2%) gel analysis of PCR amplifications using oligonucleotides RB433 and RB376 as primers, Taq polymerase (Promega) and cycle conditions as follows: 35 cycles of 94°C/5 min; 95°C/30 s; 61°C/30 s; 72°C/1 min.

The revised 5HTT promoter including the novel sequence has been deposited in GENBANK, accession number AF117826.

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