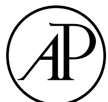

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Promoter and intronic variants affect the transcriptional regulation of the human dopamine transporter gene

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Abstract

We have attempted to identify regions involved in the transcriptional regulation of the *DAT1* (HUGO approved symbol *SLC6A3*) gene that may harbor functional variants predisposing to several neuropsychiatric disorders by examining haplotypes of various 5′ and intronic regions for their effect on expression in a dopaminergic cell line. A 1.5-fold difference in regulatory activity was observed between haplotypes of the proximal promoter/intron 1 region, representing the two previously identified 5′ clades. Although we found no effect on transcription with inclusion of the 9- and 10-repeat alleles of the 3′ VNTR, introns 9, 12, and 14 appear to contain enhancer elements capable of increasing expression approximately 2-fold with respect to the promoter constructs. Differences in expression were also observed between two alleles of intron 14. These results thus suggest that it may be the particular combination of polymorphisms in a haplotype across the gene that ultimately affects *DAT1* gene expression.

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Keywords: Dopamine transporter; Dopamine; Bipolar disorder; Genetic variation; Single-nucleotide polymorphism; Gene expression; Transcriptional regulation

The dopamine transporter (DAT) mediates the active reuptake of dopamine from the synapse and thereby plays a key role in the regulation of dopaminergic neurotransmission. Dopamine is an important mediator of motor behaviors through projections to the striatum and of emotional and attentional behaviors largely through projections to a variety of limbic targets. Cocaine, amphetamine, and other stimulants block the action of DAT, thus increasing synaptic dopamine concentrations. The neuropsychiatric effects of these drugs, as well as a wide array of other data, argue for the importance of dopamine in many neuropsychiatric illnesses. A better understanding of the elements controlling the expression of this gene may provide insight into the pathophysiology of these disorders.

The DAT gene is expressed exclusively in the central nervous system, primarily in midbrain dopaminergic neurons of the substantia nigra and ventral tegmental regions [1]. This highly restricted pattern of DAT gene expression is presumably regulated by a unique combination of positive and negative regulatory factors, which have thus far remained elusive. Characterization of the 5′ flanking sequence of the human DAT gene (*DAT1;* HUGO approved symbol *SLC6A3*) has provided some insight as far as which sequences or regions may be involved in transcriptional regulation. In vitro experiments have demonstrated that proximal *DAT1* sequences constitute a strong core promoter capable of initiating expression without obvious neurospecificity [2,3]. The presence of a strong nonspecific promoter suggests the existence of silencing elements that define the specific expression of *DAT1,* an idea supported by the observed silencing of basal proximal promoter activity in reporter gene constructs with the inclusion of 5′ flanking sequences [2,3]. Enhancer elements also appear to play a role in regulating the expression of the *DAT1* gene. The transcriptional activity of *DAT1* gene constructs has been shown to be enhanced in the presence of the nuclear receptor nurr1, an interaction that appears to be mediated by an as yet unidentified nurr1-responsive element located within 1 kb upstream [3,4]. Although regulatory elements have pre-

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dominantly been localized in the 5′ flanking regions of genes, there are a growing number of examples of intronic and 3′ sequences that play a significant role in defining the tissue-specificity of gene expression [5–9]. In vitro analyses of intron 1 of the *DAT1* gene have revealed a putative neural-specific silencer in this region [2]. However, the existence of more 3′ regulatory elements has not been investigated.

The *DAT1* gene has been implicated in a variety of neuropsychiatric disorders. Cook et al. [10] first reported the association of a 40-bp repeat in the 3′ untranslated region of the gene with attention deficit hyperactivity disorder (ADHD). This association has since been supported by several independent studies [11–13]. Although less robustly, *DAT1* has also been implicated in cocaine-induced paranoia [14], alcoholism [15], the severity of alcohol withdrawal [16,17], schizophrenia [18], and Parkinson's disease [19,20].

We have previously reported evidence for linkage of bipolar disorder to the *DAT1* locus [21]. Our subsequent attempt to identify possible functional variants and anonymous markers for linkage disequilibrium (LD) analyses led to the initial identification of 43 sequence variants across the gene in individuals from the two families with suggestive evidence of linkage to bipolar disorder [22]. Analysis of the LD structure across the gene revealed the presence of two haplotype blocks defining the 5′ (promoter through intron 6) and 3′ (exon 9 through exon 15) regions of the gene [23]. We used this segmental LD structure to construct a limited number of haplotypes for use in association studies of bipolar disorder. Transmission/disequilibrium test analysis of a sample of 50 parent-proband triads revealed a strong association (empirical $p = 0.0004$) for a particular set of haplotypes comprising five SNPs deriving from the 3′ region of *DAT1,* exon 9 through exon 15 [22]. The absence of nonsynonymous coding sequence variants in any of the individuals examined, in combination with the LD data, suggested the presence of one or more regulatory variants. These data are consistent with other reports of comprehensive mutational analyses of the gene that suggest the presence of regulatory variants [24]. However, the segmental nature of the observed LD impeded our attempts to refine the region of interest further within the 3′ block of the *DAT1* gene using genetic methods.

For these reasons, we chose to examine the implicated regions of the gene for the presence of functionally active transcriptional regulatory elements that might be candidate regions for disease susceptibility variants. We have constructed a series of reporter plasmids containing different intronic and upstream segments and examined their effect on transcription in a dopaminergic cell line. We now present functional data that support the role of multiple variants in the transcriptional regulation of the *DAT1* gene. We have analyzed two common haplotypes comprising promoter and intron 1 SNPs, as well as alleles of several 3′ intronic regions and the widely studied VNTR in exon 15 of *DAT1.* These results demonstrate the potential impact of haplotypes on *DAT1* gene transcription.

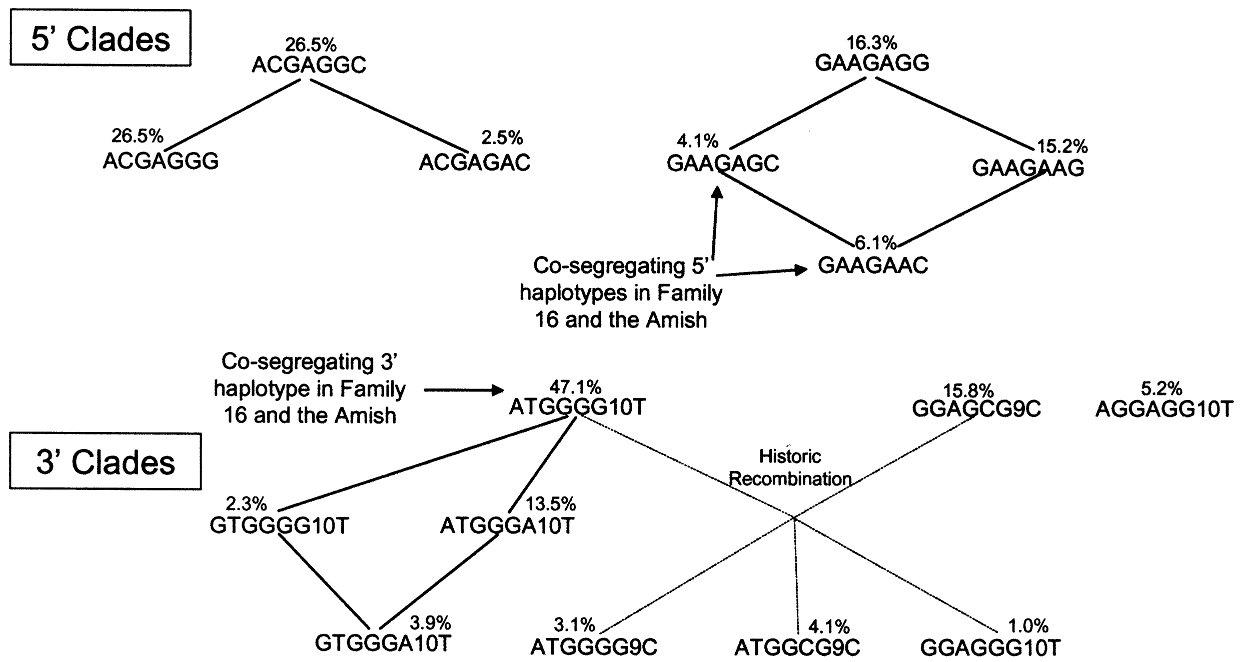
Results

We have previously examined the haplotype structure of the gene and found a segmental distribution of linkage disequilibrium consistent with the presence of separate 5′ and 3′ haplotype blocks [23]. Fig. 1A illustrates the cladistic structure of the haplotypes identified in this previous report. The existence of two distinct 5′ clades exhibiting very little recombination may be an indication that haplotypes of the 5′ regulatory regions of *DAT1* have differential effects on gene expression, which has served to maintain them in the population. At least two, and possibly three, 3′ clades also exist within the *DAT1* gene, again raising the possibility of a functional difference between haplotypes of these clades. We therefore chose to assay a representative haplotype from each 5′ clade, as well as several polymorphic 3′ intronic regions and the widely studied VNTR in exon 15, to determine whether functional differences exist between different haplotypes in these gene regions.

In a study finding suggestive evidence for linkage to bipolar disorder, two extended pedigrees were identified as segregating a similar haplotype at the *DAT1* locus [21]. These two families, Old Order Amish Pedigree 110 and Family 16 of the UCSD bipolar collection, both derive from southeastern Pennsylvania and share a common ancestry. Subsequent genotyping of the previously reported 14 SNPs and VNTR revealed a single haplotype, GAAGAGC-ATGGGG10T, to segregate exclusively with illness in Family 16 [22]. A similar haplotype differing only at one SNP in intron 2, GAAGAACATGGGG10T, was found to segregate with bipolar disorder in at least a portion of the Amish pedigree. As it is possible that these haplotypes share a functional variant that predisposes to bipolar disorder, we chose a representative from each family for a comparison of the "affected" versus "unaffected" haplotypes for these individuals. Partial haplotypes for these individuals are shown in Fig. 1.

To determine whether haplotypes of the two 5′ clades differentially affect *DAT1* gene regulation, we cloned these regions from two subjects, each of whom had been previously determined through extensive sequencing to be homozygous for one of the 5′ haplotypes. The GAAGA haplotype was cloned from an affected subject, 0244, representative of Family 16, and the ACGAG haplotype was cloned from an unaffected control subject, 3230. The putative 5′ regulatory regions for analysis were chosen based on the results of previous analyses of the *DAT1* promoter region and intron 1 [2] and inserted upstream of the luciferase reporter gene to generate three constructs for each haplotype. A detailed diagram of the *DAT1* genomic regions cloned into these promoter plasmids is shown in Fig. 2A.

Previous analysis of a sample of 50 parent-proband triads



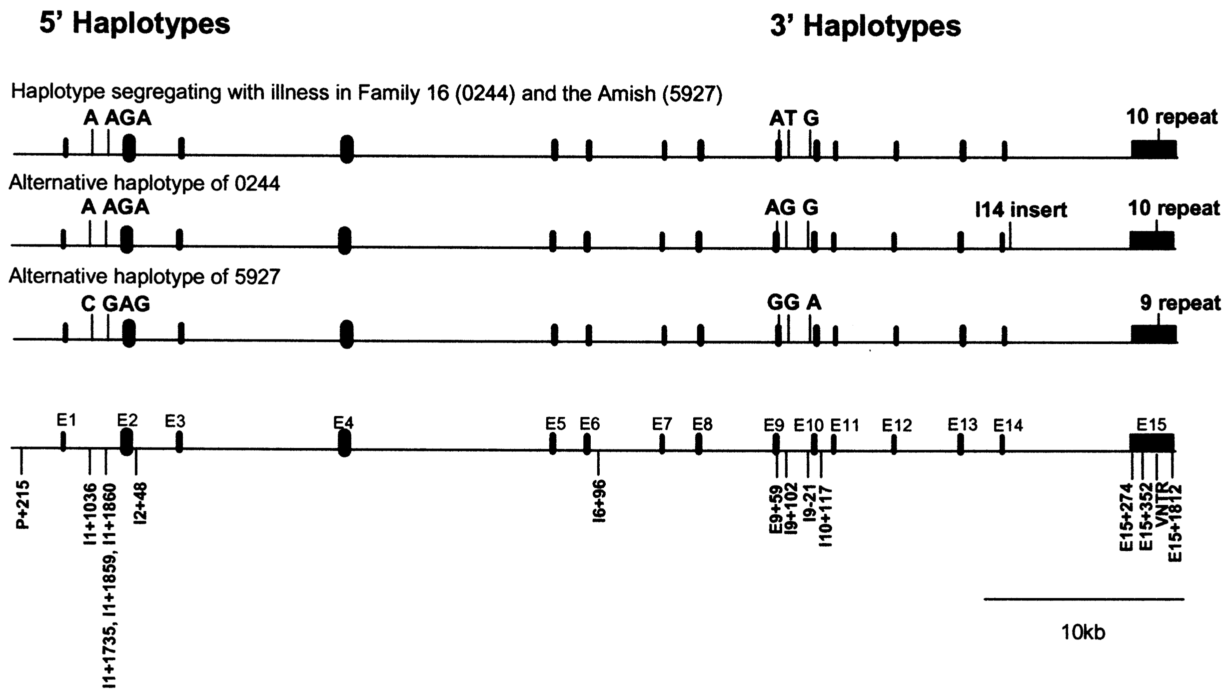
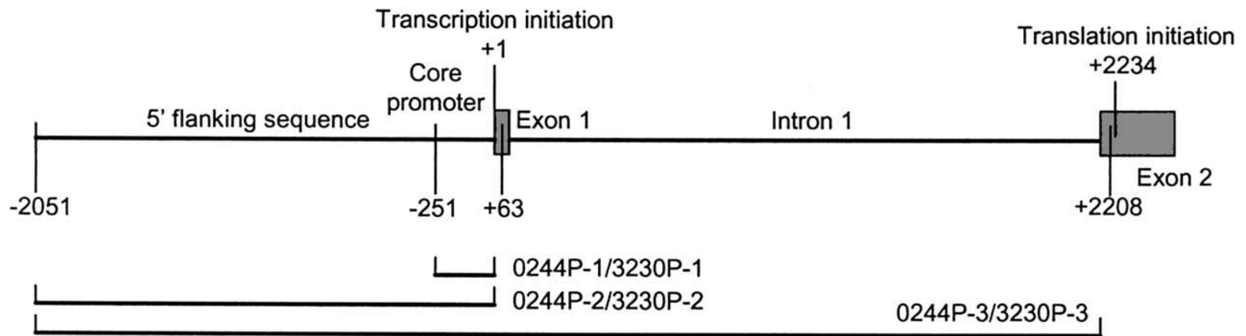


Fig. 1. (A) Illustration of the haplotype structure of the 5′ (promoter through intron 6) and 3′ (exon 9 through exon 15) regions of the *DAT1* gene, indicating the previously identified clades in each region [23] and the 5′ and 3′ haplotypes that segregate with illness in the two families with linkage to *DAT1*, UCSD Family 16 and the Old Order Amish. Only haplotypes present in at least 1% of chromosomes are shown, and SNP positions are as indicated in the lower portion of (B). (B) Diagram of the *DAT1* gene showing the positions of all previously characterized SNPs and the three partial haplotypes present in the representative individuals of the two families with linkage to *DAT1*. Only the alleles present in the promoter/intron 1, exon 9/intron 9, intron 14, and VNTR constructs are indicated.

A.



B.

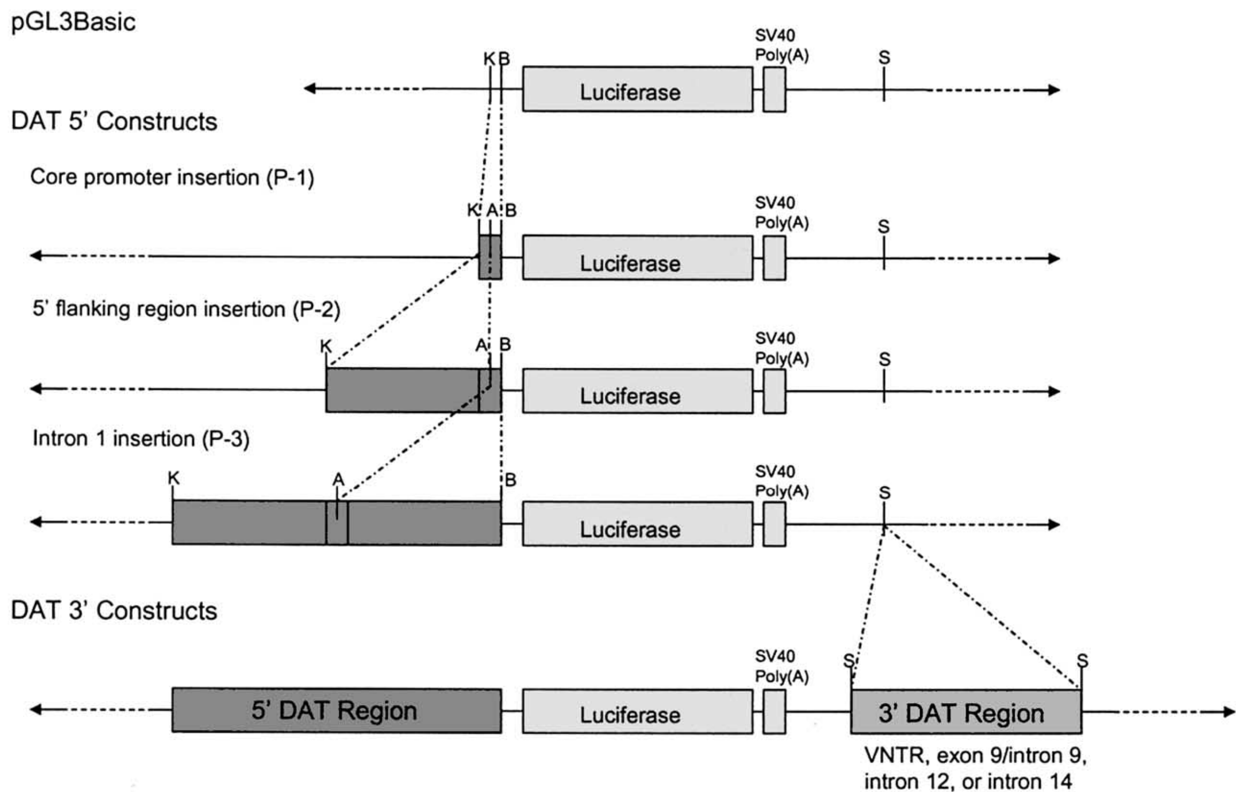


Fig. 2. (A) Details of the design of the three *DAT1* promoter constructs from each subject. P-1 designates promoter constructs extending from −251 to +63, P-2 designates promoter constructs extending from −2051 to +63, and P-3 designates promoter constructs extending from −2051 to +2208. For each type of promoter construct, the alleles of 0244, representing the GAAGA 5′ clade, and 3230, representing the ACGAG 5′ clade, were cloned from genomic DNA. (B) Diagram of the design of the 5′ and 3′ *DAT1* gene constructs showing the sequential insertion of each genomic region. The three *DAT1* promoter regions shown in (A) were cloned into the multiple cloning region upstream of the luciferase reporter gene in the pGL3Basic vector using the *Kpn*I (K) and *Bgl*II (B) sites in pGL3Basic and an *Aat*II (A) site in the core promoter. The four *DAT1* 3′ regions were cloned into the *Sal*I (S) site downstream of the luciferase gene in the respective P-3 promoter constructs.

Table 1
Summary of regions cloned and variants present in all *DAT1* reporter plasmids[a]

Plasmid	Region cloned	Polymorphic variants present in plasmid
0244P-1	Core promoter	P+2459 T
3230P-1	Core promoter	P+2459 A
0244P-2	5′ region through core promoter	P+1686 T, P+2459 T
3230P-2	5′ region through core promoter	P+1686 C, P+2459 A
0244P-3	5′ region through intron 1	P+1686 T, P+2459 T, I1+478 T, **I1+1036 A,** I1+1473 A, **I1+1735 A, I1+1859 G, I1+1860 A,** I1+1965 C
3230P-3	5′ region through intron 1	P+1686 C, P+2459 A, I1+478 G, **I1+1036 C,** I1+1473 G, **I1+1735 G, I1+1859 A, I1+1860 G,** I1+1965 T
0244VNTR-10	Exon 15	**9-repeat VNTR allele**
0244VNTR-9	Exon 15	**10-repeat VNTR allele**
3230VNTR-10	Exon 15	**9-repeat VNTR allele**
3230VNTR-9	Exon 15	**10-repeat VNTR allele**
0244I9-ATCG	Exon 9/Intron 9	**E9+59 A, I9+102 T,** I9+745 C, **I9−21 G**
0244I9-AGCG	Exon 9/Intron 9	**E9+59 A, I9+102 G,** I9+745 C, **I9−21 G**
3230I9-GGAA	Exon 9/Intron 9	**E9+59 G, I9+102 G,** I9+745 A, **I9−21 A**
0244I12-GGAAA	Intron 12	I12+267 G, I12+497 G, I12+580 A, I12+608 A, I12+1381 A
0244I12-TAGGG	Intron 12	I12+267 T, I12+497 A, I12+580 G, I12+608 G, I12+1381 G
0244I14-del	Intron 14	I14+99 deletion
0244I14-ins	Intron 14	I14+99 CAGGGCTGGTGTACA insertion

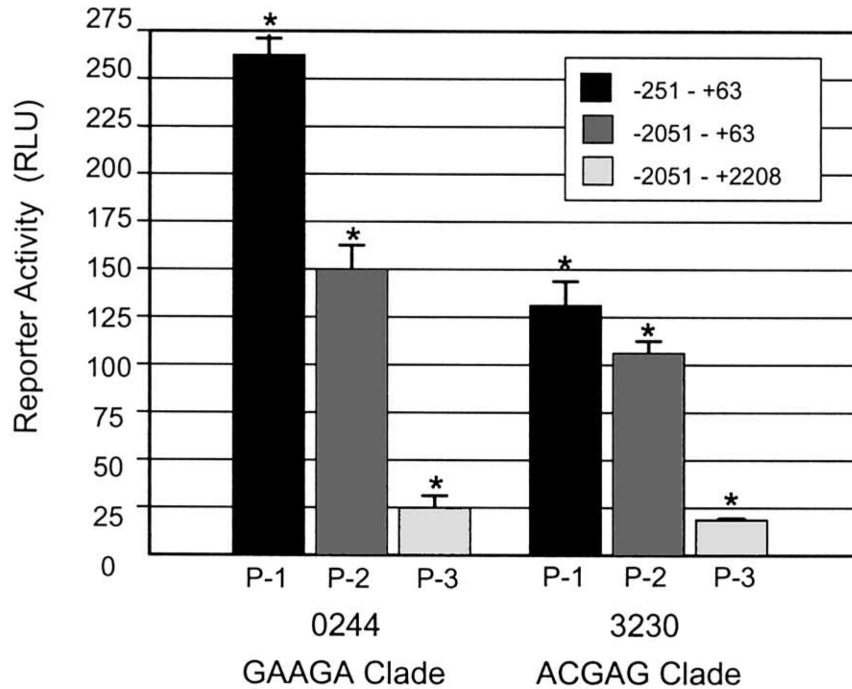
[a] The regions cloned into each plasmid are indicated, along with a complete list of the polymorphic variants represented by the different alleles at each locus in the relevant subjects employed in this study. For each 3′ plasmid, the 5′ variants are as indicated for the P-3 promoter plasmids of the corresponding individual ID in the plasmid name. Variants indicated in bold represent those that were previously reported for the association studies of bipolar disorder [22] and the LD studies of *DAT1* [23].

revealed a significant association with a haplotype comprising SNPs from the 3′ region (exon 9 through exon 15) of the *DAT1* gene [22]. Subsequent analysis of an independent sample of 70 parent-proband triads revealed more modest evidence for association with the region between introns 2 and 9 (data to be presented separately). As these regions exhibit an overlap defined by three SNPs deriving from exon 9 and intron 9, it is conceivable that a functional variant conferring a susceptibility to bipolar disorder exists within this region of the *DAT1* gene. Therefore, the two exon 9/intron 9 alleles of subject 0244, defined by the previously identified ATG and AGG haplotypes, were cloned into the 0244P-3 plasmid to simulate the naturally occurring haplotype structure in 0244 (see Fig. 1B). The ATG-defined allele segregates with illness as part of the affected haplotype in both Family 16 and the Old Order Amish and was shown to be preferentially transmitted with illness in our initial analyses of the 50 triads. We also cloned the GGA-defined allele from a representative Amish subject, 5927, into the 3230P-3 plasmid to simulate the alternative haplotype in this individual and to provide for a comparison between haplotypes representing the two primary 3′ clades.

The 3′ VNTR polymorphism in exon 15 of *DAT1* has been widely employed in association studies of *DAT1,* and although there have been reports of functional activity associated with this repeat, these data are inconsistent and have largely employed nonhomologous cell lines, as well as nonhomologous promoters [25–27]. To determine whether alleles of the VNTR possess regulatory activity, the 10- and 9-repeat alleles were each cloned into the 0244P-3 and 33230P-3 promoter plasmids.

The choice of additional 3′ regions for analysis was based on the availability of a large degree of polymorphic variability in the region as determined by extensive sequencing of these regions in 0244. Introns 12 and 14 were previously found to contain numerous polymorphisms and were thus chosen for assays of regulatory activity. The two alleles of intron 12 were cloned into the 0244P-3 plasmid to simulate the haplotypes in 0244. As intron 14 could not be cloned in its entirety due to its large size, the region surrounding a 15-bp insertion/deletion polymorphism was cloned into the 0244P-3 plasmid. Fig. 2B details the design of all 5′ and 3′ *DAT1* gene constructs, and Table 1 details all of the polymorphisms contained in each construct, including those SNPs that have been used to define the haplotypes.

A comparison of reporter activity driven by each promoter segment in the SN4741 cells is shown in Fig. 3A. Significant differences in expression were observed between the three promoter plasmids derived from 0244 ($F(2,6) = 497.9$, $p < 0.001$), as well as between the three promoter plasmids derived from 3230 ($F(2,6) = 107.2$, $p < 0.001$). The P-1 plasmids containing the proximal 251 bp of the *DAT1* promoter displayed high levels of activity compared to the SV40 promoter used as a control (pGL3Promoter), 9.8-fold for 0244P-1 and 4.8-fold for 3230P-1. Inclusion of an additional 2 kb of flanking sequence in the P-2 plasmids served to reduce expression in these constructs relative to the P-1 plasmids, 1.7-fold ($p <$



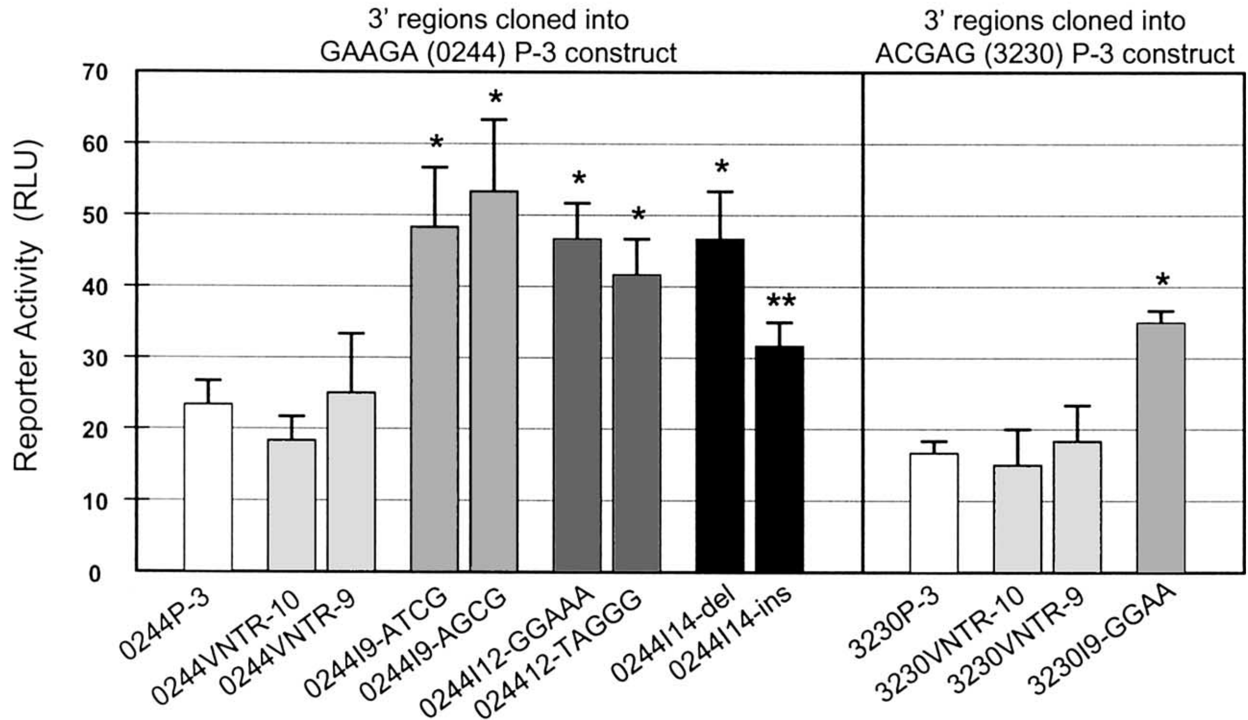


Fig. 3. (A) Comparison of reporter gene activity for the DAT1 gene promoter constructs. The luciferase gene was placed under the control of the indicated segments from the 5′ region of the DAT1 gene. Data are expressed as the mean ± standard deviation from three independent transfection experiments. Analysis by ANOVA and subsequent Tukey post hoc tests revealed a significant difference ($p \leq 0.05$) between all promoter constructs from each subject, as well as between subjects for each promoter region (*). (B) Effects of alleles of the 3′ DAT1 gene regions on reporter gene expression. The alleles of the VNTR and introns 9, 12, and 14 were subcloned downstream of the firefly luciferase gene driven by 5′ sequence −2051 to +2208 from either 0244 or 3230. The panels show expression, indicated as relative light units (RLU), of the 3′ reporter plasmids compared to 0244P-3 (left) or 3230P-3 (right) activity. These data represent the mean ± standard deviation of three to four independent transfection experiments. Each intronic region was analyzed with the appropriate P-3 plasmid by one-way ANOVA and subsequent Tukey post hoc tests. * indicates significance ($p \leq 0.005$) compared to either 0244P-3 or 3230P-3. ** indicates significance compared to 0244I14-del ($p = 0.009$).

0.001) and 1.2-fold ($p = 0.05$) for 0244P-2 and 3230P-2, respectively. The inclusion of intron 1 further decreased expression of the P-3 plasmids with a 6.4-fold decrease seen between 0244P-2 and 0244P-3 ($p < 0.001$) and a 5.4-fold decrease between 3230P-2 and 3230P-3 ($p < 0.001$). This represents an 11-fold reduction of activity between 0244P-1 and 0244P-3 ($p < 0.001$) and a 6.7-fold reduction of activity between 3230P-1 and 3230P-3 ($p < 0.001$). Significant differences in expression between alleles of each promoter region were also observed. A 2-fold difference in expression was observed between the two alleles of the core promoter, 0244P-1 and 3230P-1 ($F(1,4) = 136.0$, $p < 0.001$). This observed difference in regulatory activity was reduced to 1.5-fold with the inclusion of the 5′ flanking sequence in the P-2 constructs ($F(1,4) = 42.09$, $p = 0.003$) and 1.4-fold with the further inclusion of intron 1 in the P-3 constructs ($F(1,4) = 13.104$, $p = 0.022$). However, the trend remained between all constructs with the GAAGA-representative alleles of 0244 having higher expression than the ACGAG-representative alleles of 3230. These results thus indicate the presence of negative regulatory elements both immediately upstream of the *DAT1* gene and within intron 1, as well as apparent allelic differences in regulatory activity between the two 5′ clades.

Fig. 3B shows the activity of reporter constructs containing the indicated 3′ regions and alleles compared to the respective P-3 promoter plasmids into which they were cloned. Inclusion of the 9- and 10-repeat alleles of the VNTR in exon 15 did not result in a significant change in expression compared to the respective P-3 promoter plasmids, nor were there differences in expression between the alleles of the VNTR. By contrast, all alleles of the exon 9/intron 9 region were found to increase expression greater than 2-fold with respect to the P-3 plasmids into which they were cloned ($F(2,8) = 15.8$, $p = 0.002$ for the ATCG and AGCG alleles; $F(1,5) = 159.6$, $p < 0.001$ for the GGAA allele). Although significant differences in activity were observed between the ATCG and the AGCG alleles of exon 9/intron 9 in combination with the 5′ region from 0244 and the GGAA allele of exon 9/intron 9 in combination with the 5′ region of 3230 (1.4-fold, $p = 0.039$ and 1.5-fold, $p = 0.011$, respectively), this most likely reflects the difference in activity of the two promoter alleles rather than a difference between the alleles of intron 9. Analysis of the alleles of intron 12 also revealed significant differences in expression compared to the 0244P-3 promoter plasmid ($F(2,8) = 18.4$, $p = 0.001$); however, no significant difference in activity was observed between these two alleles. Allelic differences in expression were, however, observed for intron 14. The deletion allele of intron 14, which segregates with the affected haplotype in Family 16, was found to increase expression approximately 2-fold ($p = 0.001$) with respect to the 0244P-3 promoter plasmid and displayed a 1.5-fold ($p = 0.009$) higher activity than the allele containing the 15-bp insertion. It thus appears as though a combination of positive and negative regulatory elements in both the 5′ and the 3′ ends of the gene influence *DAT1* transcription and may also play a role in determining its exceptional tissue specificity, although the elements involved remain to be identified.

Discussion

In summary, we have demonstrated the presence of transcriptional regulatory elements in both the 5′ upstream and the 3′ intronic regions of the *DAT1* gene. Two of the regions, the 5′ promoter region and two alleles of intron 14, also displayed differences in activity between different haplotypes present in the Caucasian population. These suggest candidate genomic regions that may include sequence variants that contribute to functional variation in gene expression and possible susceptibility to human disease. However, more studies are needed to define further potential regulatory elements within these regions, identify the transcription factors that bind to those elements, and determine whether differential binding of those factors impacts *DAT1* gene regulation.

Some attention in recent years has been focused on defining the 5′ regulatory elements involved in controlling the expression of the *DAT1* gene [2,3]. However, these studies have employed nonhomologous cell types for their analyses of gene expression. Therefore, further characterization of the previously implicated 5′ regulatory regions in a more homologous system was warranted. The analyses of the *DAT1* 5′ region presented here indicate a strong core promoter and suggest the presence of repressor elements both in the 5′ flanking sequence and within intron 1, somewhat consistent with the results of others [2,3]. The existence of two common 5′ clades exhibiting very little recombination poses the question of whether differences in regulatory activity have served to maintain these clades in the population. These studies provide evidence to suggest that functional differences do exist between haplotypes of the two 5′ clades, with haplotypes of the GAAGA clade, which segregate in Family 16 and the Amish, displaying a significant increase in expression over haplotypes of the ACGAG clade. One SNP, P+2459, located in the core promoter 71 bp 5′ of exon 1 has been recently identified and is the only polymorphism common to all 5′ plasmids in this study (data to be presented separately). This SNP is also present in the 180 bp immediately 5′ of the transcription start site, the only stretch of highly conserved regulatory sequence between the human and the murine DAT genes [28]. It is thus possible that alleles of this SNP result in the differential binding of a transcription factor, thereby influencing *DAT1* gene expression, although this remains to be determined.

The VNTR in the 3′ untranslated region of the *DAT1* gene has been the focus of much attention as a potential regulatory element. Although neither allele of the VNTR seemed to have an effect on *DAT1* gene expression in these

analyses, a recent study by Miller and Madras [27] found an approximate 1.5-fold greater activity for the 10-repeat allele of the VNTR in comparison with the 9-repeat allele. This is in contrast to the findings of Fuke and colleagues [25], who reported the 9-repeat allele to have a greater degree of expression than the 10-repeat allele. However, these observations were made using heterologous promoters and cell lines that lack endogenous DAT expression. A study by Michelhaugh and colleagues [26] found the 9-repeat allele to be capable of enhancing expression in the same cell line employed in these studies, SN4741, yet a heterologous promoter was used and the 10-repeat allele was not tested. The situation is further complicated by the in vivo findings of Heinz and colleagues [29], who found an association between the 9-repeat allele of the VNTR and reduced DAT protein availability, and those of Jacobsen and colleagues [30], who reported the 10-repeat allele to be associated with a reduction in DAT protein. Additionally, a recent study by Mill and colleagues [31] observed an association between the number of 10-repeat alleles and increased *DAT1* expression in vivo. Taken together, these studies suggest that the VNTR may play a role in mRNA stability, rather than transcriptional regulation, a possibility not explored in these analyses. Thus, although the data presented here represent the first in vitro functional analyses of the *DAT1* VNTR using the homologous *DAT1* promoter in a cell line that expresses the DAT gene, and are therefore likely to be more accurate than previous studies of this polymorphism, further investigation in both in vitro and in vivo systems will be required before any conclusions about the functionality of this polymorphism can be drawn.

It is also possible that the 3′ VNTR is not functional itself, but is in linkage disequilibrium with a nearby functional polymorphism. Our data suggest the intron 14 insertion polymorphism, which is in complete linkage disequilibrium with the 10-repeat allele of the VNTR, as a possibility. However, the insertion variant is relatively rare (5% allele frequency in this sample), suggesting that it does not likely explain the reported disease associations.

There are several limitations of the methods employed in these studies. First of all, this is an in vitro model, which may not actually reflect transciptional regulation in vivo. Therefore, the use of a transgenic mouse may be necessary to characterize fully the mechanisms of *DAT1* gene regulation. The cells used for these studies are also derived from mouse substantia nigra, and although there is high coding sequence conservation between the human and the mouse DAT genes, there is very little conserved regulatory sequence [27]. Therefore, a mouse cell line, even one that expresses DAT, may not be ideal for studying the regulation of the human DAT gene. Another important issue is that these constructs are an incomplete assembly of *DAT1* gene regions. Since most of the gene is absent, it is likely that many crucial *cis*-regulatory elements may not be present in the constructs and the observed expression may not reflect the actual regulation of the gene. This problem could be circumvented through the use of a bacterial artificial chromosome containing the entire *DAT1* gene fused to a reporter gene. Finally, these studies analyzed *DAT1* gene regulation only in the basal state. It is thus possible that a cellular signal is required for induction of *DAT1* gene expression, and abnormalities in gene regulation may be evident only in the presence of such a signal.

There may also be an issue with the potential introduction of bias resulting from the initial selection of SNPs that could be unambiguously genotyped by AS-PCR in our previous studies [22,23]. Such a selection bias may have affected our cladistic analyses, ultimately affecting our choice of haplotypes for functional analysis. However, since 14 of the initially attempted 17 SNPs were amplified successfully, this is not likely to be of great concern.

Although our work has focused on the role of *DAT1* in the susceptibility to bipolar disorder, as described above, *DAT1* has also been associated with numerous other disorders, suggesting that variation in this gene may have a general impact on a broad range of neuropsychiatric function. As *DAT1* has been more consistently observed to be associated with ADHD than with bipolar disorder, in which it may play only a minor role in susceptibility, it is possible that *DAT1* may be preferentially associated with a subset of bipolar patients who also have ADHD or other attentional deficits. It is thus likely that the same genetic variability that we have observed in bipolar disorder and the functional differences we report here are also relevant to ADHD and other neuropsychiatric disorders.

Few in vitro studies have assessed the relevance of combinations of multiple SNPs or specific haplotypes in gene expression. However, pharmacogenetic analyses of haplotypes of the β_2-adrenergic receptor promoter and coding region provide precedence for differential effects conferred by different haplotypes [32]. This study suggested that it may be the unique interactions of multiple SNPs within a haplotype that ultimately affect biological phenotype, whereas individual SNPs may have poor predictive power as pharmacogenetic loci. The studies presented here of haplotypes at the *DAT1* gene may also reflect the effect of the interaction of multiple SNPs in a haplotype on gene expression. These results suggest the presence of allelic heterogeneity at the *DAT1* locus and that many SNPs may each contribute a small effect on gene regulation. There may be several 3′ regulatory elements that, in combination with 5′ elements, differentially regulate *DAT1* gene expression. These results thus highlight the likely importance of noncoding sequence variants in complex traits.

Materials and methods

Plasmid construction. All plasmids were constructed based on the pGL3Basic luciferase reporter plasmid (Promega, Madison, WI, USA), which lacks eukaryotic promoter and enhancer sequences. Genomic DNA segments for the con-

structs were amplified from one of three subjects: 0244, a subject with schizoaffective disorder, bipolar type from Family 16 of the UCSD family set; 5927, the proband of Old Order Amish Family 110 with a diagnosis of bipolar I disorder; and 3230, a normal control. Though 0244 has a diagnosis of schizoaffective disorder, bipolar type, this family is primarily segregating typical bipolar I disorder. This individual was chosen based on the large amount of resequencing data available. These subjects were diagnosed using structured interviews and consensus best estimate review following IRB-approved informed consent procedures as described previously [33,34]. The designs of the 5′ and 3′ constructs are illustrated in Fig. 2.

The 5′ regions for analysis were chosen based on the results of previous analyses of the *DAT1* promoter region and intron 1 [2]. These putative 5′ regulatory regions were cloned from the genomic DNAs of the two subjects, 0244 and 3230, who had previously been determined to be homozygous for one of the two identified 5′ haplotype clades, GAAGA and ACGAG, respectively [23]. These regions were then inserted sequentially into the *Kpn*I and *Bgl*II sites in the multiple cloning region upstream of the luciferase reporter gene to generate three constructs for each subject: one containing the core promoter extending from −251 to +63 in the genomic sequence (0244P-1, 3230P-1), another extending from −2051 to +63 and encompassing the core promoter with approximately 2 kb of 5′ flanking sequence reported to contain both enhancer and repressor elements (0244P-2, 3230P-2), and a final construct extending from −2051 to +2208, representing the core promoter, 5′ flanking region, and intron 1, which contains a putative neuron-specific silencer element (0244P-3, 3230P-3).

Each of the four selected 3′ regions were cloned into the *Sal*I site located downstream of the luciferase gene in the promoter/intron 1 constructs. Each intronic allele was cloned into the P-3 plasmid containing the appropriate 5′ haplotype to simulate the haplotype structure observed in the individual from whom they were cloned. All variation was known as these introns had been previously sequenced in their entirety in the relevant individuals. Alleles of intron 9 and a portion of exon 9 were amplified from the genomic DNAs of subjects 0244 and 5927 and cloned into the 0244P-3 and 3230P-3 plasmids, respectively. The two identified alleles of intron 12 were amplified from 0244 genomic DNA and cloned into the 0244P-3 plasmid. A portion of intron 14 containing a polymorphic 15-bp insertion was amplified from 0244 genomic DNA to generate 391- and 406-bp fragments, representing the wild-type and inserted alleles, respectively, that were cloned into the 0244P-3 plasmid. The VNTR in exon 15 (2584–3247 in *DAT1* cDNA sequence NM_001044) [35] was amplified from 5927 genomic DNA to generate 664- and 624-bp fragments, representing the 10-repeat and 9-repeat alleles, respectively, each of which was cloned into the 0244P-3 and 3230P-3 plasmids.

Cell culture. Transient transfection studies were performed using SN4741 cells, an immortalized mouse embryonic substantia nigra-derived cell line that has been shown to endogenously express the mRNAs for several dopaminergic neuron-specific markers, including DAT [4,36]. The cells were grown at 33°C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium, high glucose, containing 10% heat-inactivated fetal bovine serum and supplemented with 0.6% d-glucose (Sigma, St. Louis, MO, USA), 1.4 mM l-glutamine, 10 U/ml penicillin (Invitrogen Life Technologies), and 10 μg/ml streptomycin. All reagents, with the exception of that indicated, were obtained from Invitrogen Life Technologies (Grand Island, NY).

Transient transfections and luciferase assays. SN4741 cells were plated on 24-well plates in 0.5ml growth medium 24 h prior to transfection and subsequently transfected at approximately 70–80% confluence with equimolar concentrations of all *DAT1* reporter plasmids. Each well was transfected with 0.17 μg total plasmid DNA complexed with 3 μl of Lipofectamine reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. The transfection medium was replaced after 6 h with growth medium. The plasmid pRLTK containing the *Renilla* luciferase reporter gene driven by a herpes simplex virus thymidine kinase promoter was cotransfected (5 ng) in all experiments as an internal control for normalization of transfection efficiency. The pGEM plasmid was used as a carrier to keep the total DNA constant between wells. In addition to *DAT1* constructs, the pGL3Promoter vector, a firefly luciferase construct driven by an SV40 promoter, and the pGL3Basic vector were employed as controls. All vectors were obtained from Promega. Every condition was performed in triplicate, and each experiment was repeated three to four times.

Cell extracts were assayed for firefly and *Renilla* luciferase activity 24 h following transfection using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. All relative light unit counts were in the linear range of $>10^3$ to $<10^6$. All data are expressed as the means ± standard deviation of three to four independent experiments. All comparisons between constructs within a group were analyzed by one-way analysis of variance, unless otherwise indicated, followed by Tukey post hoc analysis for pairwise comparisons between specific plasmids.

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