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ORIGINAL RESEARCH ARTICLE

Characterisation, mutation detection, and association analysis of alternative promoters and 5′ UTRs of the human dopamine D3 receptor gene in schizophrenia

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The dopamine D_3 receptor gene (DRD3) is a candidate for a number of psychiatric conditions including schizophrenia, bipolar disorder and alcohol and drug abuse. Previous studies have reported associations between polymorphisms in DRD3 and these disorders, but these findings may have reflected linkage disequilibrium with pathogenic variants that are further upstream. We have isolated and sequenced approximately 9 kb of genomic sequence upstream of the human DRD3 translational start site. Using 5′ RACE, we have identified within this region three additional exons and two putative promoter regions which show promoter activity in three different cell lines. A 5′ UTR identified only in lymphoblasts is spread over three exons and is 353 bp long. A second 5′ UTR, found in adult and fetal brain, lymphocytes, kidney and placenta is spread over two exons and is 516 bp long. A 260-bp sequence within this 9 kb corresponds to a previously reported EST, but corresponding mRNA could not be found in the tissues above. The EST, 5′ UTRs and putative promoter regions have been analysed for polymorphisms, revealing 10 single nucleotide polymorphisms, seven of which were tested for association in a large sample of unrelated patients with schizophrenia and matched controls. No associations were observed with schizophrenia. In addition we failed to replicate previous findings of association with homozygosity of the Ser9Gly variant. The results from this study imply that neither the coding nor the regulatory region of DRD3 plays a major role in predisposition to schizophrenia.
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Introduction

The D_3 dopamine receptor gene (DRD3) has been implicated by association studies as a possible candidate for a number of neuropsychiatric disorders and phenotypes including schizophrenia, bipolar disorder, Tourette syndrome, and substance abuse.[1] The phenotype most intensively investigated in connection with DRD3 is schizophrenia, and since the original report of association[2] there have been more than 24 independent follow-up studies of a non-synonymous A/G polymorphism (Ser9Gly) in exon 1. Although the results of these have been mixed, a meta-analysis concluded in favour of association between homozygosity at this site and schizophrenia.[3] However, the Ser9Gly polymorphism is conservative and does not lie within the regions of the receptor thought to be involved in ligand binding or signal transduction. Moreover, functional differences between the two alleles (or genotypes) have not yet been clearly demonstrated.[4]

The DRD3 gene locus, 3q13.3, is not a region where significant linkage has been reported in schizophrenia. However, these findings raise the possibility that the Ser9Gly variant is in linkage disequilibrium (LD) with one or more susceptibility variants elsewhere in the gene. Previous mutation screens of DRD3 have failed to reveal potentially functional polymorphisms in the coding region that could account for the association data.[5] However these studies were limited by incomplete knowledge of the structure of DRD3, and in particular by the fact that the DRD3 promoters had yet to be identified. Prior to this study, the DRD3 cDNA was believed to consist of six exons (total length 1.2 kb) and five introns (total length $>$ 45 kb). However, neither the extent of the 5′ UTR nor the location of the promoter(s) were known. An additional 1724 bp of 5′ flanking sequence has been recently reported but no gene structure has been attributed to this.[6] In the present study we used 5′ RACE to determine the sequence of the 5′ UTR of DRD3 in several different tissues. By sequencing genomic clones, we have obtained a contiguous genomic sequence containing

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three previously unknown exons which give rise to two alternative mRNAs transcribed from two different putative promoters. We then confirmed the function of the putative promoters by luciferase reporter gene assays. Finally, we searched the putative promoter regions, the exons and flanking intronic regions for novel polymorphisms and carried out association studies between these, the Ser9Gly polymorphism, and schizophrenia in a large case control sample.

Materials and methods

Characterising 5′ UTR

Poly A+ RNA was prepared from human lymphoblasts and lymphocytes using standard procedures. Human kidney, placental, fetal brain and adult brain mRNA samples were purchased commercially (Clontech, Palo Alto, CA, USA). 5′ RACE was performed using three nested antisense primers to the first known exon of DRD3 (SP1: agtaggagagggcatagtag; SP2: gcacctgtgg agttctctg; SP3: tgtagttcaggtggctactc) using the protocols provided with the RACE kit (Boehringer Mannheim). First strand cDNA was synthesised from total RNA using SP1 and purified using the Boehringer Mannheim High Pure PCR purification kit. A homopolymeric A-tail was then added to the 3′ ends of the cDNA using terminal transferase. Tailed cDNA was then used as a template for PCR using primer SP2 and an anchored oligo dT primer (gaccacgcgtatcgatg tcgacttttttttttttttttv). The product of this round of PCR was then re-amplified using primer SP3, and a sense primer comprising the anchor sequence of the anchored oligo dT primer (gaccacgcgtatcgatgtcgac). Good quality RACE products were separated by agarose gel electrophoresis and the resulting bands were then extracted from the gel. Each product was ligated into pGEM T vector and used to transform XL1blue cells. Clones containing the insert were purified and sequenced using primers corresponding to the plasmid, using BigDye chemistry (ABI). Additional 5′ RACE experiments were carried out using primers closer to the 5′ end of the new sequence in order to confirm the 5′ end of the mRNA.

Isolation and sequencing of genomic clones

A gridded human PAC library[7] obtained from the UK HGMP Resource Centre was screened using a 5′ end labelled oligonucleotide (cctggctggcacctgtggag ttctctgccccac) corresponding to bases 44–76 of the coding region of DRD3. Four independent clones were isolated (34e22, 119p1, 138e21, 221m4). Clone 138e21 contained sequence previously designated as exon 1 and an additional 90 kb of 5′ sequence. A total of 9.3 kb of PAC sequence in a 5′ direction from the 5′ end of the known coding region was obtained directly from the purified PAC DNA using BigDye chemistry (ABI) by incrementally designing a series of primers representing the 5′ boundary of known genomic sequence. The same primers were also used to amplify and sequence human genomic DNA to confirm that the PAC clone did not contain rearrangements (data not shown). This sequence has been deposited in the EMBL Nucleotide Sequence Database—accession No. AJ271348.

Promoter analysis

5′ RACE analysis (above) indicated two distinct starts of transcription (see Figure 1). To confirm this, approximately 500 bp of genomic sequence 5′ to both putative starts of transcription were tested for promoter activity in a reporter gene assay. Primers were designed to amplify bases 1300–1945 and bases 2306–2860 (from accession No. AJ271348). Primers for the lymphoblast promoter were forward: gttgcttgggtatgtctgctg and reverse: gtctctgctgggaataattgga. Primers for the promoter seen in the other tissues were forward: gcacatccactgttggcagag and reverse: gttttctcctcagtgctctttt.

Following PCR with Expand High-Fidelity DNA polymerase (Roche), 15 ng of amplimers representing each putative promoter were ligated into a pGL3 basic vector (Promega) modified for T/A cloning (manuscript in preparation). Clones containing the insert were sequenced to confirm their fidelity prior to transfection into three human cell lines originating from tissue known to express the dopamine D3 receptor: HEK293t (human embryo kidney), TE671 (human medulloblastoma) and JEG-3 (human placenta) obtained from the European Collection of Cell Cultures (ECACC). Cell lines were cultured according to ECACC specifications at 37°C with 5% CO_2. Cells were seeded into black, clear bottomed 96-well luminometric plates (Perkin Elmer) at 60% confluencey the day prior to transfection. HEK293t cells were transfected with 40 ng well^{-1} DNA, 1 μl well^{-1} lipofectamine (Gibco), TE671 cells were transfected with 80 ng well^{-1} DNA, 0.25 μl well^{-1} lipofectamine, and 0.75 μl well^{-1} PLUS-reagent (Gibco) and JEG-3 cells were transfected with 80 ng well^{-1} DNA, 0.5 μl well^{-1} lipofectamine, and 0.5 μl well^{-1} PLUS-reagent.

Cell lines were transfected overnight in serum-free medium which was replaced with complete medium and incubated for a further 24 h. Luciferase activity was measured using a Luc Screen assaying kit (Tropix). Plates were read on a TR717 scintillation counting luminometer (Bertoldt).

Mutation analysis

A total of 4750 bp of genomic sequence was screened for mutations (Figure 1), including all novel exonic sequences and flanking intronic and 5′ flanking/promoter regions. The primers and fragment sizes are detailed in Table 1. The sample for mutation screening consisted of 14 unrelated subjects meeting DSM-IV criteria for schizophrenia and 14 subjects meeting DSM-IV criteria for bipolar disorder. All were unrelated British Caucasians born in the UK. Our screening sample for mutation detection contains equal numbers of subjects with schizophrenia and bipolar disorder because our extended research group is interested in both disorders. This decision has little impact on our estimates of power to detect susceptibility alleles for schizophrenia except those with extremely low frequencies.

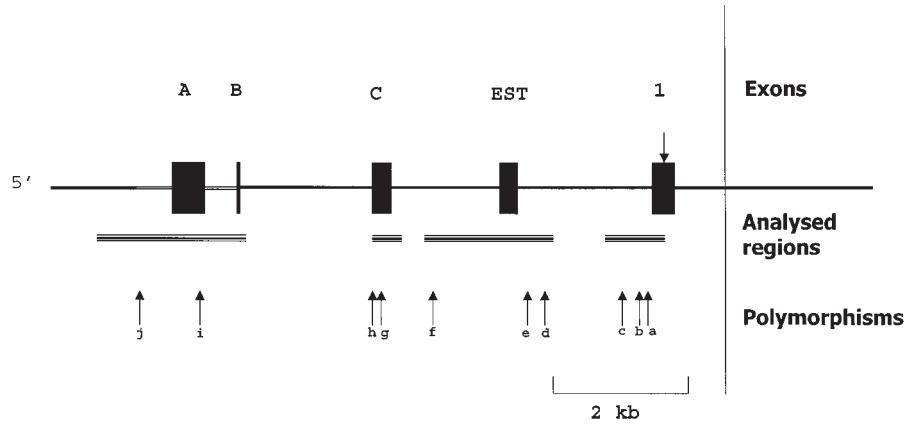

Figure 1 Structure of the human dopamine D_3 receptor gene 5′ to what has previously been called exon 1. Exons identified by this research are shown as blocks (with their designation above) along the genomic sequence obtained from the PAC clone as described above. Double lines represent the putative promoter regions. Triple lines below the gene represent areas where mutation analysis has been carried out. The 5′ ends of exons 1, A, B and C and the EST are 105, 7144, 6195, 4201 and 2377 bp upstream of the start of translation (⇓) respectively. Polymorphism locations are identified by ↑ and are: **a**: A/G –203, **b**: A/G –346, **c**: C/G –710, **d**: A/T –2103, **e**: A/G –2249, **f**: A/G –3461, **g**: C/T –4147, **h**: A/G –4190, **i**: A/G –6733, **j**: C/G –7685. The numbering system starts at the translational start site.

Table 1 Primers used for mutational analysis of the human dopamine D_3 receptor gene. The base pairs shown represent the first and last which were screened for polymorphisms using these primers. The numbering system starts at the translational start site

Sense (forward)	*3′ base*	*Anti-sense (reverse)*	*5′ base*
CCAGGTCAAGACTCAATTGCT	–417	GGCACTGTTGTGAGAGTTGG	–172
GAGTCCAAGCATGGTACTTTCA	–600	AATCAGGAACTCCTCATGGA	–390
TACCCAGACTCAAGGTGTTCTG	–787	TGAGGTAGACAGATTGTGCAGG	–595
TACGTGGCAGGTAGAGGAGG	–2366	GGAAAGGGTGACAAACTTGG	–2040
AGCCCAGCTCAGTTCTGTGTT	–2791	CCACGTAAGAGGGAGGCATA	–2399
GCTCCAGGATATGTGGCAAT	–3177	TGTTTCAACTTCCTCCCTGC	–2750
TGTTTCAACTTCCTCCCTGC	–3494	AGTAGCATACCCCTCCCCAC	–3147
CTTGCCACACAGTAGAAGCCCC	–4246	GGAGTTAAAAGTGGATAGGAGG	–3850
CTGAAGGTTTGGAGAGGCAC	–6243	CAGCAAGTAATGGAAGTCTCACA	–6101
TGTCTGCTGTCAGTAAATGGC	–6720	AGTGCCTCTCCAAACCTTCA	–6262
AAAGCACAGTATCCGAGCAGA	–7057	CTGATTTCTGGAGACCGAGG	–6697
TATTAAGCTTATTTCATCCTTATTGCC	–7397	CTACCTCGCTCTCTTTTCCTCTT	–7047
CATCCAGCAGAGGTCTCCAT	–7807	CATGGCGTATAACTGGCAAT	–7403
AATCAAGGGTCAGCCACTTG	–8042	ACTGCGGTGCAGTTATAGGG	–7784
AAGATGGCATCATGAAGACTGA	–8264	GTCTGAAAGTCACACAGCTAAC	–8043

Under the null hypothesis, alleles in our screening sample will simply be representative of general population allele frequencies. Our sample will therefore have power of 0.8 to detect polymorphisms occurring in the general population with a frequency of 0.03. On the other hand, if the hypothesis is correct, that is alleles of DRD3 are associated with schizophrenia, at least half of our sample will be enriched for susceptibility alleles and therefore this degree of power will be maintained for alleles of even lower frequency. We would also note that because of the huge discordance in their allele frequencies, it is extremely unlikely that alleles with frequencies in this range (<0.03) would permit association to be detected at the *Bal*1 site by virtue of LD.

Denaturing high performance liquid chromatography (DHPLC) was performed at a minimum of two temperatures as described in detail elsewhere[8] using a WAVE DNA fragment analyser (Transgenomic, Santa Clara, CA, USA). Where DHPLC analysis suggested that a subject was heterozygous, the PCR products from that individual were sequenced to determine the nature of the polymorphism using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Cheshire, UK) as described by the manufacturer.

Case control samples

The case control study was undertaken using 368 unrelated Caucasians (266 males and 102 females, age 46 ± 13 years) born in the UK or Ireland/Eire meeting DSM-IV[9] criteria for schizophrenia, and 368 control subjects (266 males and 102 females average age 43 ± 12 years).

Diagnoses were determined following a semi-structured interview and examination of case notes as described.[10] Local Research Ethics Committee approval was obtained, and subjects gave written informed consent to participate. The control individuals were group matched to probands for age, sex, and ethnicity from more than 1400 blood donors recruited from the local branch of the National Blood Transfusion Service (Wales). Controls were not specifically screened for the absence of psychiatric illness, although the policy of the National Blood Transfusion Service is to not receive donations from individuals who are on medication.

Genotyping

During the course of this study, an independent publication by Ishiguro *et al* (2000)[11] reported 800 bp of 5′ DRD3 flanking region, and a significant association between DRD3 haplotypes and schizophrenia. Two of the SNPs studied, in conjunction with Ser9Gly, resulted in a highly significant association. The two SNPs (–712G/C and –205A/G) were independently identified in our study (our nomenclature being –710G/C and –203A/G). These two SNPs along with the Ser9Gly polymorphism were genotyped using the restriction fragment polymorphism (RFLP) method after PCR amplification. The primers, conditions and restriction enzymes are shown in Table 2. The primers and restriction conditions for the –710 G/C and –203 A/G polymorphisms are the same as those used by Ishiguro *et al*.[11] Fragments were separated on either 1.6% agarose or 1% agarose : 2% metaphore gels and visualised by ethidium bromide staining and ultraviolet transillumination.

A further three upstream SNPs (–2249A/G, –4147C/T, –6733G/A) and a recently reported coding SNP (alanine 38 threonine)[11] were genotyped in a subset of the case/control sample (184 patients, 184 controls). The details of the primers, conditions and RFLP assay are also shown in Table 2. These SNPs were selected to permit LD analysis of the region based upon conservative estimates of LD in outbred populations.[12]

Statistics

Genotypic and allelic association was tested by χ^2. Haplotypes were assigned using EH+[13,14] (http:// linkage.rockefeller.edu/ott/eh.htm). The 'Ldmax' option of the 'Gold' package (http://www.well.ox. ac.uk/ssthma/GOLD/) was used to estimate D′.

Results

To allow comparison with previous data, we continue to refer to the previously reported most 5′ exon as exon 1. The novel exons reported here are referred to as exons A, B and C. Exon A is the most 5′ whilst exon C is the most 3′.

Structure and sequence of 5′ region of DRD3

A contiguous sequence of approximately 9 kb (EMBL accession No. AJ271348) was derived from PAC clone 138e21. 5′ RACE of lymphoblast RNA yielded a single product. The sequence is given in Figure 2a.

Comparison with the PAC sequence revealed that the genomic sequence encoding this UTR contains two introns. The UTR is thus encoded by 105 bp of exon 1 and two additional exons of 269 bp (exon C) and 49 bp (exon B) separated by introns of 3826 and 1950 bp respectively (Figure 1).

5′ RACE from several other tissues (adult brain, fetal brain, placenta, kidney and lymphocyte) yielded a product of approximately 500 bp. Subsequent 5′ RACE experiments using new primers 5′ to SP3 extended the 5′ UTR, yielding a total 5′ UTR in these tissues of 587 bases. This is encoded by 105 bp of exon 1 and an

Table 2 PCR primers and conditions used for genotyping

SNP#	*Base change*	*Forward primer*	*Reverse primer*	*Annealing temp*	*Taq*	*Restriction enzyme*
(–6733)	G/A	5′-AAGCTGGAA AAGCAGCACTC	5′-CTCCTGCAGCC ATTTACTG**C**	54°C	HotStar	*Ndel* 1
(–4147)	C/T	5′-CGTCAACTT CCATGCTGCTAT	5′-TAAAAAGGCA GGGGAACAGA	58°C	HotStar	*Hinf*1
(–2249)	G/A	5′-TTGGGCCTC AGCCTGCCTTAAAAGT**C**G	5′-GGAAAGGGTG ACAAACTTGG	54°C	HotStar	*Taq* 1
(–710)	G/C	5′-TTACATGGG AAGAATCTGGAG**TG**CA	5′-GAGGGTGTGA GGTAGACAGATTGTG	57°C	Opt57	*ApaL*1
(–203)	G/A	5′-ATCTCCTCC AGGTCAAGACTCAATT	5′-CCTGTGAGGA GACAGAAAACAATAT	57°C	HotStar	*Apo*1
Ser9Gly*	A/G	5′-GCTCTATCT CCAACTCTCACA	5′-AAGTCTAC TCACCTCCAGGTA	60°C	Qtaq	*Msc*1
Ala38Thr**	G/A	5′-GCCCCAAAG AGTCTGATTTTATAA	5′-TCCTTCAGCAC AGCCATGCACAGCA	57°C	Qtaq	*BstU*1

#Numbering of SNP relates to the position relative to the start of translation. The bases in bold indicate that a mismatched primer was used to introduce a restriction enzyme cut site to identify the polymorphism. *Serine-9-Glycine polymorphism.[29] **Alanine-38-Threonine polymorphism.[11]

a

Lymphoblast 5' UTR – 424 bp

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1    AATTATTCCCAGCAGAGATATGTAGTACTTGAGATGATGATTGTgtatgttc...1950bp...cttacagAAAGAG
51   AACAAATAATTAAAACTCTGTAAGTCTTAATGAGGTGCTAAGGAGGAACCCCACGAATGT
111  TTCAGGAAGACTGTTATTCAGCACTGAGGGATTGAACATCAGCAAAGCAGGACAAATGTC
171  ATAACTGATGGGGACCTGACAACTCTCTGTTCCCCTGCCTTTTTAAATAGGTTATGCACT
231  CACACTAAGAGATGCCAGAACAAACTATAAAGACCAAGATAAATGAATACTTTGTAAGGC
291  ATTTAAAAGCTCATTAAAATGTGTaagtagtgtt...3826bp...caataaataAAAAATGTAACTAAGC
331  AACCAAGCCCCAAAGAGTCTGATTTTATTAATATTGTTTTCTGTCTCCTCACAGGAAGCC
391  CCTTGGCATCACGCACCTCCCTCTGGGCTATG
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b

Fetal brain 5' UTR - 587 bp

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1    AAAGGTAAAAACAGTGGAAAAACTAAGATTAAAAAGAGCACTGAGGAGAAAATATACATG
61   TACTAAAAAGCACAGTATCCGAGCAGATAGAGAGACAAAGAGGAAAAGAGAGCGAGGTAG
121  AAAACGGATACTGCCTATGCCTACTCCATCCCTCTTTCAGCACCAAGGACAGAACCTCTG
181  AGCGGCTGACCCAAGCAACGCTCAGTTTAGGGTCCCTCCCAAATCCTCTAAAGAAAACGG
241  ATACATTCGAAAGCAGCTATGAAACATGCACTAAGGTCTAATAGGGAAGCTGGAAAAGCA
301  GCACTCAAGTAATTTCACCTTAGAGGCAAAAATGGGTGATTTCTTTCTGTTCATTTCATA
361  GTTTCTGAGTCCTGAGAAAGGCAAAGTTTGCTTTGCTTGGGTATGTCTGCTGTCAGTAAA
421  TGGCTGCAGGAGCCGAAGTGGTAAACTCCTCGGTCTCCAGAAATCAGAAGAAAATTTTAG
481  GGtaagtaattt...6527bp...caataaataAAAAATGTAACTAAGCAACCAAGCCCCAAAGAGTCT
521  GATTTTATTAATATTGTTTTCTGTCTCCTCACAGGAAGCCCCTTGGCATCACGCACCTCC
581  CTCTGGGCTATG
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Figure 2 (a) Sequence of the 5′ untranslated region of the human dopamine D_3 receptor obtained from lymphoblasts. (b) Sequence of the 5′ untranslated region of the human dopamine D_3 receptor obtained from brain and other tissues. The translational start site (ATG) is shown by a double underline. Exon sequence is shown in capitals and introns in small letters with the total intron sizes shown.

additional exon of 482 bp (exon A) which is only 467 bp upstream of exon B. The relative positions of the exons are shown in Figure 1.

Comparison of the genomic sequence with various sequence databases identified a previously reported EST (accession number: D81856) obtained from a fetal brain library.[15] This lies between exon C and exon 1. We attempted to PCR between the EST and each of the newly identified exons as well as exons 1 and 2, using both lymphoblast and fetal brain mRNA under a range of PCR conditions. None of these experiments gave a product. PCR analysis of all the cDNA samples described above using primers corresponding to the EST also gave no product. Finally, 3′ RACE was attempted using lymphoblast and fetal brain mRNA with nested primers within this EST, but again no product could be obtained. The failure of these experiments, while not conclusive, suggests that this EST sequence is not represented in dopamine D_3 receptor mRNA and may not be transcribed at all.

Promoter analysis

The relative promoter activities of the two 5′ flanking sequences are shown in Table 3 along with PGL3-SV40 positive control and the promoterless pGL3 basic negative control. Both sequences had much greater activity than pGL3 basic. The lymphoblast promoter drove luciferase transcription significantly higher than the alternative promoter in all three cell lines.

Mutation and association analysis

Ten polymorphisms were found. These are shown in Figure 1. Table 4 presents the data from association analysis of the two 5′ upstream region polymorphisms;

Table 3 Luciferase activity (counts s^{-1}) of the pGL3 constructs ($n = 8$) ± standard deviation

	TE671	*JEG-3*	*HEK293t*
pGL3-SV40	9980 ± 1447	1801 ± 594	5135 ± 1013
pGL3-basic	247 ± 18	33 ± 8	26 ± 10
pGL3-brain promoter	1075 ± 130	261 ± 33	248 ± 71
pGL3-lymphoblast promoter	4356 ± 254	396 ± 35	1033 ± 272

–712G/C, –203G/A, and the exon 1 coding polymorphism (Ser9Gly) in the 368 schizophrenia case/control sample. There was no significant disease association with any of the alleles of the three markers studied. There were more heterozygotes for the Ser9Gly polymorphism in the patient sample ($\chi^2 = 3.998$, $P < 0.05$) and this was reflected by the fact that the patient sample was found to be significantly out of Hardy–Weinberg equilibrium ($\chi^2 = 4.94$, $P < 0.026$). An excess of heterozygotes could occur through partial enzyme digestion causing the miscalling of a Gly/Gly homozygote (fully digested) as a Ser/Gly heterozygote. However the amplimere produced in our assay has a common *Bal*1/*Msc*1 cut site in addition to the cut site introduced by the A/G polymorphism that results in the serine to glycine amino acid change. Therefore any partial digests producing ambiguous heterozygotes would also be seen as partial digests of the common cut site—this was not seen. Half of the sample was regenotyped and this confirmed that deviation from Hardy–Weinberg equilibrium was not a result of genotyping error.

Using the statistical package EH+, we attempted to replicate the haplotypic analysis[11] (Table 5). No significant difference was found in the frequencies of three marker (or two marker; data not shown) haplotypes between the cases and the controls ($P = 0.2150$).

A further four polymorphisms were studied in a subset of the full sample. These were –6733G/A, –4147C/T, –2249G/A polymorphisms and a previously reported A38T missense mutation in exon 1[11] (Table 6). All markers were in Hardy–Weinberg equilibrium. There was no significant genotypic or allelic association with schizophrenia.

Finally, LD max (Gold package) was used to assess the extent of linkage disequilibrium (D′) between the Ser9Gly polymorphism and six other SNPs extending up to almost 7 kb upstream of the Ser9Gly polymorphism. Calculations of D′ between all permutations of the –203, –710 and Ser9Gly variants are shown in Table 7.

Discussion

DRD3 has been implicated by association studies as a possible candidate gene for a number of neuropsychiatric disorders including schizophrenia,[2] alcoholism,[16,17] cocaine abuse,[18] bipolar illness,[19,20] Tourette syndrome,[21] opiate dependence,[22] novelty seeking,[23] anorexia nervosa,[24] Parkinson's disease[25] and others. In most cases the results have been inconclusive, partly due to small subject numbers. However, in schizophrenia, a meta-analysis of the available world literature demonstrated a small excess of homozygosity of the Ser9Gly polymorphism.[3]

Given an absence of a demonstrated functional effect for the *Bal* I polymorphism (Ser9Gly) , we postulated that excess homozygosity in schizophrenia might reflect linkage disequilibrium with a true susceptibility variant or variants polymorphism. In order to test this hypothesis, we sought to clarify the structure of the 5′ flanking region, screen for novel mutations and try to replicate the findings of Ishiguro *et al*[11] who found an association with a three marker haplotype in the 5′ upstream region of DRD3.

Using a combination of 5′ RACE and genomic sequencing, we have identified three novel 5′ exons for DRD3 and have also identified the 5′ end of exon 1. We have also identified two alternative mRNA species designated lymphoblast and brain (Figure 1). Each is encoded by a different terminal 5′ exon which suggest that they are derived from alternative promoters rather than alternative splicing. This interpretation is supported by our observation that both 5′ sequences immediately adjacent to both putative transcriptional start sites drive transcription to a significantly greater extent than pGL3 basic negative control (Table 3).

While we can be confident that both transcripts represent alternative promoter activity experimentally, it is not clear that both transcripts occur *in vivo* because one of the transcripts was only found in lymphoblastoid cell lines. This leaves open the possibility that transformation and culture of lymphoblasts permits transcription from a cryptic promoter that is not used *in vivo*. However, the putative transcription start-site for this transcript has high homology to the 5′-YYCAYYYYY-3′ consensus.[26] Also, an analysis of the sequence immediately upstream of exon B (the lymphoblast expressed first exon) reveals a TATA consensus sequence approximately 30 bp upstream and CAAT motifs further upstream. The sequence immediately upstream of exon A does not contain these elements. However, eukaryotic promoters are known to be highly diverse and difficult to predict from sequence data alone.[27]

The position of the EST between exons C and 1 of the DRD3 initially suggested to us that it may be an exon of another variant DRD3 mRNA species. However, we have not been able to identify any DRD3 cDNA sequence contiguous to the EST which suggests that either it is not part of DRD3, or possibly is not transcribed at all. However, negative PCR reactions cannot exclude the above possibility and it is possible therefore, that this sequence represents either a minor transcript, or one which is transcribed under conditions that are not found in the source tissue of our samples.

Having clarified the 5′ structure of DRD3, we screened this region for polymorphisms using DHPLC

Table 4 Association analysis of the –710G/C, –203G/A and Ser9Gly polymorphisms

SNP	*Population*	*Sample size*	*Genotypes*			*Genotypic* χ^2	*Allele counts*		*Allelic* χ^2	*Homo-zygotes*	*Hetero-zygotes*	*Homozygosity/heterozygosity*	
			G/G	*G/C*	*C/C*		*G*	*C*				χ^{2*}	*Odds ratio (95% CI)*
–710G/C	Controls	363	215	132	16	0.708	562	164	0.674	231	132	0.393	0.907
	Cases	352	198	136	18	$P = 0.701$	532	172	$P = 0.4117$	216	136	$P = 0.53$	(0.6704–1.2287)
			G/G	*A/G*	*A/A*		*G*	*A*					
–203G/A	Controls	365	45	152	168	3.503	242	488	0.0981	213	152	3.202	0.766
	Cases	367	36	177	154	$P = 0.174$	249	485	$P = 0.754$	190	177	$P = 0.073$	(0.572–1.026)
			ser/ser	*ser/gly*	*gly/gly*		*ser*	*gly*					
Ser9Gly	Controls	364	170	153	41	4.5767	493	235	0.0989	211	153	3.998	0.7414
	Cases	360	152	178	30	$P = 0.101$	482	238	$P = 0.753$	182	178	$P < 0.05$	(0.5530–0.994)

*Heterozygotes vs homozygotes.

Table 5 Estimated haplotype frequencies ± SD in the full case/control sample. ± 2 standard deviations are reported when haplotype frequencies are >0.05

3 Marker haplotype (–710G/C, –203A/G, Ser9Gly)	*Controls (n = 361)*	*Schizophrenics (n = 351)*	*Combined sample (n = 712)*
G-A-Ser	0.6660 ± 0.0662	0.6709 ± 0.0615	0.6685 ± 0.0441
C-G-Gly	0.2228 ± 0.0354	0.2450 ± 0.0368	0.2338 ± 0.0267
G-G-Gly	0.0985 ± 0.0237	0.0812 ± 0.0215	0.0900 ± 0.0159
G-G-Ser	0.0097	0.0028	0.0063
C-A-Ser	0.0015	0.0000	0.0008
G-A-Gly	0.0014	0.0000	0.0007

Table 6 Association analysis of –6733G/A, –4147C/T, –2249G/A, Ala38Thre polymorphisms in a subset of cases and controls

SNP	*Population*	*Genotypes*			*Genotypic χ^2**	*Allele numbers*		*Allelic χ^2*
		G/G	*G/A*	*A/A*		*G*	*A*	
(–6733G/A)	Controls	155	28	1	2.587	338	30	2.11
	Cases	164	20	0	$P = 0.274$	348	20	$P = 0.146$
		C/C	*C/T*	*T/T*		*C*	*T*	
(–4147C/T)	Controls	176	8	0	0.297	360	8	0.289
	Cases	178	6	0	$P = 0.862$	362	6	$P = 0.591$
		G/G	*G/A*	*A/A*		*G*	*A*	
(–2249G/A)	Controls	161	21	1	2.148	343	23	1.776
	Cases	168	15	0	$P = 0.342$	351	15	$P = 0.183$
		ala/ala	*ala/thre*	*thre/thre*		*ala*	*thre*	
Ala38Thr	Controls	180	1	0	1.069	361	1	0.958
	Cases	173	3	0	$P = 0.586$	349	3	$P = 0.327$

*Because of small cell sizes, simulated *P* values were estimated using CLUMP.[30] None of the comparisons in Table 6 were significant.

Table 7 Estimation of linkage disequilibrium between –710, –203 and Ser9Gly markers in cases and controls

	Marker 1	*Marker 2*	*P value*	*D′*
Cases	–710	–205	<0.00005	1
(n = 368)	–710	Ser9Gly	<0.00005	1
	–203	Ser9Gly	<0.00005	1
Controls	–710	–205	<0.00005	0.99
(n = 368)	–710	Ser9Gly	<0.00005	0.99
	–203	Ser9Gly	<0.00005	0.99

in 28 unrelated probands with functional psychosis. This sample has greater than 80% power to detect variants with a frequency of 0.03 or more. Ten variants were found of which three had been previously reported. We selected two of the SNPs (–710g/c, –203g/a) in addition to the Ser9Gly polymorphism for genotyping in a sample of 368 cases and controls because haplotypes created with these markers had previously been associated with schizophrenia in a Japanese population. Note that this sample does not overlap with previous publications on DRD3 from our group.[3]

In contrast to our previous findings and that of the meta-analysis[3] we did not find evidence for association between schizophrenia and homozygosity at the Ser9Gly polymorphism. In fact we found the converse, with extremely modest evidence for an excess of heterozygotes in the patient population compared both with controls ($\chi^2 = 4$, 1 df, $P = 0.045$) and with HWE. Given that in the extensive DRD3 literature, there is no support for a finding in this direction, we suggest that this result is attributable to chance rather than true association. We would also note that although the sample is the biggest single sample in which the DRD3 polymorphism has been analysed to date, it is still small compared with that required to have high power for replication.[28] Nevertheless, it is surprising that we did not at least find a non-significant trend towards homozygosity, and the observation that the limit of the 95% confidence interval (0.5530–0.994) in our sample

Table 8 Calculation of D′ in a 7-kb region around the Ser9Gly polymorphism

Marker 1	*Marker 2*	*Cases*			*Controls*		
		Number	*P value*	*D′*	*Number*	*P value*	*D′*
Ser9Gly	Ala38Thr	174	0.11	1	179	0.36957	1
Ser9Gly	−203	177	<0.00005	1	181	<0.00005	0.99
Ser9Gly	−710	174	<0.00005	1	181	<0.00005	0.98
Ser9Gly	−2249	177	0.00004	1	182	<0.00005	1
Ser9Gly	−4147	177	0.0048	1	182	0.00045	1
Ser9Gly	−6733	177	0.25	0.268	182	0.6265	0.077

for an excess of homozygotes, at least casts considerable doubt about the homozygosity hypothesis.

The other SNPs studied by Ishiguro *et al* (−203 and −710) were in highly significant and strong LD with each other and the Ser9Gly polymorphism. However, neither gave evidence for association either on their own or from haplotype analysis. Specifically, Ishiguro *et al* reported the greatest difference in the −710C, −203G, Gly9 haplotype which was more common in their control group.[11] Analysis of our sample in fact revealed a slightly increased frequency of this haplotype in the cases (0.2450 ± 0.0368) compared with the controls (0.2228 ± 0.0354). It seems unlikely therefore that these alleles in the putative promoter region of the DRD3 gene play any major role in the disease state of schizophrenia and that the differences in the haplotype distribution merely reflect the genetic structure of the populations under study.

The main objective of this study was to identify the promoter region of DRD3 and test SNPs in this region for LD with the Ser9Gly polymorphism that might account for other reports of association. Having detected no association with the SNPs or haplotypes of SNPs reported by Ishiguro *et al*,[11] we selected a further three SNPs in the promoter region (−6733G/A, −4147C/T and −2249G/A) to cover approximately 7 kb 5′ to look for LD across this extended region and to look for disease association (Table 8). We also genotyped a missense variant A38T that had been identified in exon 1 in case this had a significant effect in the UK sample. However, none of the SNPs yielded evidence supportive of association with schizophrenia. Clearly, since in this sample no evidence for association was found between schizophrenia and the Ser9Gly polymorphism, we cannot draw firm conclusions as to whether any of these other variants account for association with schizophrenia in other samples by virtue of linkage disequilibrium. However, we would note that with the exception of the −712G/C and −203G/A variants (Table 4), the allele frequencies of the other SNPs and the Ser9Gly polymorphism are so incongruent making it unlikely that findings of association with the latter marker would pick up association as a result of the former.

In summary we have characterised the promoter region of DRD3 and tested the hypothesis that SNPs detected in this region that are in LD with the Ser9Gly polymorphism play a role in susceptibility to developing schizophrenia. Although the promoter region was successfully characterised, we not only failed to show any association with SNPs contained therein and schizophrenia, we failed to replicate previous findings (including our own) that an excess of homozygosity at the Ser9Gly locus was associated with schizophrenia. The results from this study imply that neither the coding nor the regulatory regions of DRD3 plays a major role in predisposition to schizophrenia. However, our data on the genomic structure of DRD3 and the SNPs we have identified constitute important new reagents for further studies on the role of this gene in other forms of psychopathology.

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