

Rapid Molecular Haplotyping of the First Exon of the Human Dopamine D4 Receptor Gene by Heteroduplex Analysis

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The dopamine D4 receptor gene (DRD4) and its products are of great interest in many neuropsychiatric disorders. There are at least five expressed polymorphisms in exons 1 and 3, plus rare expressed variants, all of which may have functional relevance. Several studies have described methods for studying the exon 3 polymorphisms, especially the VNTR; fewer reports have documented the exon 1 polymorphisms and variants of DRD4. We report here a simple, rapid, nonisotopic, nondenaturing heteroduplex method for determining the molecular haplotype composed of the two more polymorphic systems of the first exon of DRD4: the 12 bp duplication and 13 bp deletion. This method will facilitate future research on expressed variation of this gene. *Am. J. Med. Genet.* 74:91–94, 1997.

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INTRODUCTION

The dopamine D4 receptor gene (DRD4) has been cloned and the receptor has also been found to be highly polymorphic [Van Tol et al., 1991, 1992]. The relationships of alleles at DRD4 with schizophrenia [Barr et al., 1993; Cichon et al., 1995; Macciardi et al., 1994; Nanko

et al., 1993a; Petronis et al., 1995; Seeman et al., 1993; Shaikh et al., 1994; Sommer et al., 1993] and other neuropsychiatric disorders, such as alcoholism, bipolar affective disorder, obsessive-compulsive disorder, panic disorders, and parkinsonism, is still of interest [Adamson et al., 1995; Catalano et al., 1993; Cichon et al., 1995; George et al., 1993; Nanko et al., 1993b]. Reports have also appeared claiming an association between alleles at DRD4 and novelty-seeking behavior [Ebstein et al., 1996; Benjamin et al., 1996]. To date, five expressed polymorphisms, any of which may have functional relevance, have been identified. In the third exon, there are a 48-bp VNTR [Van Tol et al., 1992; Lichter et al., 1993] and an amino acid substitution [Seeman et al., 1994]. In the first exon, there are three polymorphisms, a 12 bp duplication [Catalano et al., 1993], a 13 bp deletion [Nothen et al., 1994], and an Arg for Gly substitution [Cichon et al., 1995]. (The 12 bp polymorphism is considered here to be a duplication since the longer allele has two tandem copies of the same sequence and it is unclear which allele is the ancestral form.) Other variants, too rare to be called polymorphisms, have also been described. For example, Cichon et al. [1995] described a single example of a 21 bp deletion in exon 1.

We have investigated the two more common expressed polymorphisms in the first exon region of DRD4 since only the third exon region has been extensively studied [Van Tol et al., 1992; Lichter et al., 1993; Chang et al., 1996]. While the shorter alleles at both exon 1 polymorphisms, *non-dup*(lication) 12 and *del*(etion) 13, are uncommon (about 8% for the 12 bp nonduplication allele [Catalano et al., 1993] and 2% for the 13 bp deletion [Nothen et al., 1994]), the Arg 11 allele is even rarer at roughly 1% in the limited European samples examined so far [Cichon et al., 1995]. According to the sequence data of Van Tol et al. (Genbank accession number L12397) the 12 bp duplication involves nucleotides (nt) 552 to 563 [Catalano et al., 1993], and the 13 bp deletion reported by Nothen et al. [1994] covers nucleotides 711 to 723 (Fig. 1). Because the methods described by Catalano et al. [1993] and Nothen et al. [1994] were found to be too involved for us to use to address these exon 1 markers to our global survey of DRD4 vari-

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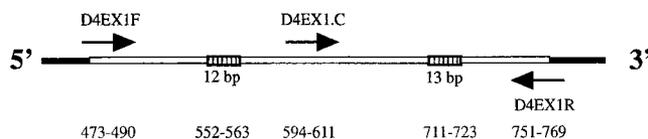


Fig. 1. Schematic of DRD4 exon 1 haplotyping. The figure gives the relative positions and nucleotides (nt) of the two polymorphic sites (cross-hatched areas) and the three primers (arrows) used to examine the first exon of DRD4 according to GenBank sequences L12397 and X58497. The first exon (open area) encompasses nt477 to nt761. The PCR reaction with primers D4EX1F and D4EX1R generates 297 bp or 285/284 bp or 272 bp products. The 272 bp product has not been seen. PCR using primers D4EX1.C and D4EX1R generates 176 or 163 bp products. All PCR reactions used the reaction mix previously described [Chang et al., 1996], a hot start, and the following cycling profile on a PE9600 thermal cycler: denaturing at 94°C for 5 min, then 30 cycles of annealing at 55°C for 20 sec, 72°C for 40 sec, and 94°C for 20 sec, with 72°C for 10 min at the end.

ation (a sample size of nearly two thousand individuals [Chang et al., 1996]), we have developed a simple and rapid nonisotopic method for haplotyping these two exon 1 polymorphisms. Using this method we have confirmed the presence of the 13 bp deletion in two other northern European populations at allele frequencies of roughly 2%, confined to haplotypes with the 12 bp duplication allele.

MATERIALS AND METHODS

Cell Lines and Genomic DNA Preparation

The lymphoblastoid cell lines and genomic DNA from the populations were prepared as previously described [Anderson and Gusella 1984; Chang et al., 1996].

PCR and Nondenaturing Electrophoresis

We first PCR the region using the primers D4EX1F and D4EX1R reported by Catalano et al. [1993], employing a modified protocol involving adding 5% or 10% (v/v) DMSO and 100 μ M 7-deaza-GTP, and using a hot-start procedure. The specific PCR protocol is the same as described for the third exon [Lichter et al., 1993; Chang et al., 1996] and improves yield in the first exon, which is also GC rich. Amplification generates PCR products (from nt473 to nt769 as originally described [Catalano et al., 1993]), encompassing the entire first exon (Fig. 1). The product of the PCR reaction is run in a nondenaturing 4% NuSieve gel (FMC, BioProducts, Philadelphia) with 1 \times TBE using a conventional horizontal electrophoresis apparatus. No special processing is necessary since heteroduplex molecules are produced during the last PCR cycle when product concentrations are high [cf. Kidd and Ruano, 1995].

RESULTS

As shown in Figure 2, most different genotypes of the whole first exon can be distinguished after staining with ethidium bromide. The *non-dup 12* and *del 13* alleles are indistinguishable in this system but the DNA strands with the *non-dup 12* allele and the *del 13* allele, when paired with DNA strands of the more common alleles to form heteroduplexes, migrate differently to give different patterns for the heteroduplex bands. The two heteroduplex bands of the *non-dup 12* allele and the common *dup 12* allele appear to migrate as a single

band that moves more slowly than the apparently paired heteroduplex bands of the *del 13* allele and the normal (*non-del 13*) allele.

To verify that these patterns corresponded to the respective genotypes and to determine the genotype should the rare homozygote be found, we used a second PCR using the antisense primer (D4EX1R) described by Catalano et al. [1993] and the sense primer (D4EX1.C) reported by Nothen et al. [1994]. On either total genomic DNA or product from the first PCR these primers generate 163 bp or 176 bp products covering the second half of exon 1 from nt594 to nt769 (Fig. 1) using the same PCR program and electrophoresis proce-

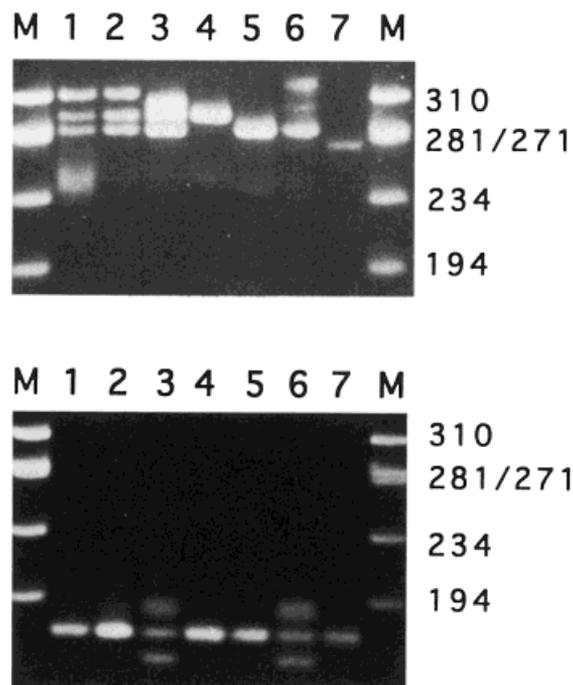


Fig. 2. The heteroduplex patterns of PCR products. Upper panel: the 285, 284, and/or 297 bp products (from nt473 to nt769) following electrophoresis on 4% NuSieve gel (1 \times TBE). (See Table I for correspondences of haplotypes and PCR products.) M: Markers of ϕ X174 *Hae*III digest with band sizes given on the right. Lane 1: Mixture of DNA samples from two homozygotes—the individual in Lane 4 (a 297 bp homozygote), and the individual in Lane 5 (a 285 bp homozygote)—denatured at 95°C for 1 min and allowed to form heteroduplexes by rapidly cooling to 25°C before running. Lane 2: Heterozygote of the 285/297 bp type. Lane 3: Heterozygote of the 284/297 bp type. Lane 4: Homozygote of the 297/297 bp type. Lane 5: Homozygote of the 285/285 bp type. Lane 6: Heterozygote of the 284/285 bp type. Lane 7: A new rare human variant of 273/273 bp, homozygous for still another deletion; note the region around the 13 bp deletion is normal as shown by the second PCR in the lower panel. The patterns of the heteroduplex of the 285 and 297 bp alleles (Lanes 1 and 2) and of the heteroduplex of the 284 and 297 bp alleles (Lane 3) are different: the migration speed of the heteroduplex band in lanes 1 and 2 is slower than the heteroduplex band in lane 3. The pattern of the 284 bp homozygote (not seen in our sample) will be similar to that of the 285 bp homozygote (lane 5), but will be recognizable from the single 163 bp product from the second PCR. Lower panel: the 163 or 176 bp products (from nt594 to nt769) of the second PCR (covering the 13 bp deletion region) following electrophoresis on a 4% NuSieve gel (1 \times TBE). The samples are the same as in the upper panel. As predicted from the results in the upper panel, only Lanes 3 and 6 show heterozygosity for the *del 13* and *non-del 13* alleles; other lanes are all homozygous for the *non-del 13* allele of this segment.

TABLE I. Haplotype Frequencies and Standard Errors for the Two More Common Exon 1 Polymorphisms*

Haplotype		PCR Size (bp)	Finns (2N = 70)	Danes (2N = 96)
<i>dup 12</i>	<i>non-del 13</i>	297	0.914 ± 0.033	0.894 ± 0.030
<i>dup 12</i>	<i>del 13</i>	284	0.026 ± 0.020	0.021 ± 0.015
<i>non-dup 12</i>	<i>non-del 13</i>	285	0.060 ± 0.028	0.085 ± 0.028
<i>non-dup 12</i>	<i>del 13</i>	(272)	0.000 ± 0	0.000 ± 0

* The allelic symbols for both length polymorphisms and the length of the PCR product across the haplotype are given, followed by the frequencies in samples of Finns and Danes. The *non-dup 12, del 13* haplotype was not seen but would be expected to give a PCR product of 272 bp.

dures as before. Those genotypes involving heterozygotes for the 13 bp deletion (Fig. 2, lower panel) are the ones that had shown distinctive patterns in the first typing (Fig. 2, upper panel). A heteroduplex band of the *del 13* and normal DNA strands is also seen on the gel. Representative alleles have been sequenced to confirm the typings.

Using these methods, we have found the *non-dup 12* allele to be present in populations from many parts of the world (unpublished data). However, the 13 bp deletion has only been seen in populations originating from northern Europe. Table I gives the exon 1 haplotypes we have found in these two European samples.

DISCUSSION

By examining the electrophoretic patterns of PCR products that encompass both sites of variation on a nondenaturing NuSieve gel we can uniquely distinguish 8 of the 10 possible genotypes for this two-site haplotype system. The key is the presence of heteroduplex molecules. Of the six possible genotypes predicted by the three common haplotypes (Table I), this method clearly identifies all but two of them unambiguously: the *non-dup 12, non-del 13* homozygote and the *dup 12, del 13* homozygote both give a single band with no heteroduplex bands and the 1 bp difference in size cannot be detected in this system. Similarly, we cannot distinguish the homoduplex bands of these two haplotypes in heterozygotes, but the heteroduplex patterns with the common *dup 12, non-del 13* haplotype are readily distinguishable. If either of these rare homozygous patterns occurs, a second PCR reaction is required to determine the genotype. Should the heteroduplex bands not be visualized, a heterozygote of these uncommon haplotypes would also be confused with the homozygotes for each of them, but be readily identified by a heterozygous pattern of the second PCR. Though we have not seen the *non-dup 12, del 13* haplotype, its homoduplex pattern should be distinctive and hence the genotypes containing it identifiable, if it ever occurs. We have tested these methods on over 200 individuals with either *non-dup 12, non-del 13* or *dup 12, del 13* haplotypes, and the results of the second PCR all fit the prediction of the heteroduplex pattern seen for the product of the first PCR.

In conclusion, we report here a simple, nonisotopic, nondenaturing gel electrophoresis procedure for determining the haplotype by the heteroduplex pattern in a GC-rich region with expressed polymorphisms in the DRD4 gene. We find the analysis of the single PCR

product encompassing both sites can accurately distinguish the *non-dup 12, non-del 13* and *dup 12, del 13* haplotypes, except when homozygous. The second PCR is then required to determine which homozygote is present. Otherwise, the second PCR simply serves to confirm the prediction of the first PCR heteroduplex results, but even then would be required for only the small number of samples that is found to have one of the uncommon haplotypes; most samples will be homozygous for the common haplotype that has the 12 bp duplication and lacks the 13 bp deletion. Thus, large-scale sample screening is quite simple using this method.

Previously, the 13 bp deletion had been reported only in a sample of Germans [Nothen et al., 1994]. Our results on two other northern European populations (Table I) show that it is more widespread in Europe. We did not detect it in any other population, including our two other "European" samples, a sample from near the Caucasus mountains and a sample of a Middle Eastern population, suggesting it is confined to central and northern Europe. Since this is presumably a null allele, a broader survey of European populations is clearly warranted to determine its distribution more precisely.

The method we have described should be useful in further studies to pursue not only the distribution of these alleles, but also some of the hypotheses of behavioral differences associated with the exon 3 VNTR, such as the possible association of novelty-seeking behavior with long VNTR alleles [Ebstein et al., 1996; Benjamin et al., 1996]. None of those studies has controlled for the expressed variation in exon 1.

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