Genetics of Autism

GENETICS OF AUTISM:

THE MATERNAL GENOTYPE AT THE DOPAMINE BETA HYDROXYLASE LOCUS MAY BE A FACTOR IN THE ETIOLOGY OF AUTISM AND RELATED PERVASIVE DEVELOPMENTAL DISORDERS

By

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ABSTRACT

Autism is a severe developmental disorder characterized by impairments in reciprocal social interaction and communication, coupled with repetitive stereotypic activities. Evidence from twin and family studies strongly suggest that genetic factors play a significant role in the etiology of autism. The factors involved in the development of autism are also thought to underlie related pervasive developmental disorders (PDD). The affected sib-pair method was used to screen nine autosomal candidate loci in 18 families, each of which have two or more children with autism or a related PDD. Candidate loci were selected on the basis that: (1) the locus is near genetic disorders or chromosomal abnormalities found to co-occur with autism; and/or (2) the gene encodes a protein which has been speculated to play a role in the pathophysiology of autism. Genotypes of the affected children and their parents were determined for the following microsatellite markers which are tightly linked to the candidate genes/regions: 13S118, DRD2, TH, HRAS-1, 22S343, D15S11, GABR*B*3, 16S291, and DBH. No significant concordance between affected siblings was observed for any of the loci tested. During the study, however, many of the families were found to be uninformative at the dopamine beta hydroxylase (DBH) microsatellite locus. A comparsion of DBH allele frequencies observed in the parents to published British values revealed a significant difference between the two groups (L^2=13.16, df=5, $p<0.05$). Given this finding, and the knowledge that serum DBH activity is largely under the control of DNA sequences in or close to the

DBH gene, serum DBH activities were measured in the parents and in an adult control group. Mean serum DBH activity was found to be significantly lower in parents with two autistic/PDD children compared to an adult control group (Student's $t=-1.71$, $df=60$, $p<0.05$). DBH alleles are defined by a polymorphic dinucleotide repeat and the presence or absence of a 19 bp sequence. Upon further analysis the frequency of alleles in which the 19 bp sequence is deleted was found to be significantly increased in the mothers with two autistic/PDD children, compared to both published frequencies ($L^2=11.99$, $df=1$, $p<0.001$) and to a Canadian control group ($L^2=6.96$, $df=1$, $p<0.01$). Subsequent investigation revealed that deletion of the 19 bp sequence is associated with lower mean serum DBH enzyme activity (non-deletion homozygotes 44.5±28.6 iu/L; heterozygotes 30.4±16.0 iu/L; and deletion homozygotes 20.5±15.3 iu/L; $F=5.45$, $df=59$, $p<0.01$). Based on these findings it is proposed that lowered maternal serum DBH activity provides a uterine environment which, in conjunction with genotypic susceptiblity of a fetus, results in autism or a related PDD.

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I also acknowledge the efforts of Lianne Dulsrud who determined DBH allele frequencies in the Canadian control group consisting of blood samples from anonymous newborns. Many thanks also to Chris Schutz who determined sib-pair concordance in the study population for both the FMR-1 and MAO-A loci. I am grateful to Jeanette Holden, not only for providing the samples from Canadian newborns, but also for her invaluable support and feedback throughout this project. Thanks to Brent Murray and Chris Schutz for *many* helpful tips on lab techniques.

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LIST OF ABBREVIATIONS

PDD	Pervasive developmental disorder
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
DBH	Dopamine beta-hydroxylase
TH	Tyrosine hydroxylase
c-HRAS	c-Harvey-Ras oncogene

Note: Refer to Proceedings of the National Academy of Sciences USA (1987) 84: vi-vii for all other abbreviations used in this thesis.

GENERAL INTRODUCTION

In 1943, Leo Kanner described a severe and pervasive childhood psychiatric condition which he termed infantile autism. The cardinal feature of this disorder was extreme withdrawal and an inability to form social attachments to other individuals, including parents. Affected children also demonstrated an obsessive desire for preservation of sameness and a failure to use language to communicate. Kanner stressed that, in most cases, behaviour had been abnormal from early infancy, and it was for this reason he suggested the disorder might be caused by an 'inborn defect' (Kanner 1943).

The etiological factors which result in autism are still unknown, and diagnosis is based entirely on behavioural presentation. The criteria which must be met for a diagnosis of autism have been formalized in two primary reference manuals. In North America, the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) is most commonly used. To be diagnosed as autistic according to DSM-IV guidelines a child must show: (1) qualitative impairment in social interaction (two or more of the specified symptoms must be present); (2) qualitative impairment in communication (one or more symptom); (3) restricted repetitive and stereotyped patterns of behaviour, interests, and activities (one or more symptom); and (4) onset of symptoms must be prior to 3 years of age. As is implied by the list of possible symptoms (Appendix A), there is a great deal of variation in clinical presentation and severity.

Autism is generally viewed as the farthest point on a continuum of conditions referred to as pervasive developmental disorders (PDD) (Szatmari 1992). The etiological factors involved in the development of autism are also thought to underlie Asperger's disorder and atypical autism. A DSM-IV diagnosis of Asperger's disorder is given when all of the criteria for autism are exhibited, except there is no significant delay in language or cognitive development. Atypical autism is diagnosed when the criteria for autistic disorder are not met either because of a late age of onset and/or a subthreshold number of symptoms are present. Frequently profoundly retarded individuals are given a diagnosis of atypical autism because their extremely low level of functioning precludes expression of the specific behaviours required for a diagnosis of autism (APA 1994).

Children with autism or related PDD often exhibit a host of other behavioural problems such as hyperactivity, short-attention spans, and aggressiveness (Jaselskis et al. 1992). Epilepsy is present in about 25% of autistic individuals, with onset typically occurring during adolescence (Lockyer and Rutter 1970, Gillberg and Steffenburg 1987). Approximately 75% of autistic individuals are mentally retarded, with IQ scores usually falling in the moderate range (35-50) (Lockyer and Rutter, 1969). Overall intellectual functioning and the presence of communicative speech are the strongest predictors for prognosis (Bartak and Rutter 1976). However, even among high functioning autistic individuals only a small proportion are able to live independently as adults.

An epidemiological survey estimated the prevalence of autism in the Canadian population to be approximately 10 cases per 10,000 live births

(Bryson et al. 1988). Males are more frequently affected than females, with a sex-ratio of approximately three males to every female (Bryson et al. 1988). Autistic females tend to have lower IQ scores than affected males; however sex differences are not observed on other indices of severity (Lord and Schopler 1985; Volkmar et al. 1993).

Currently, there are two opposing viewpoints regarding the etiology of autism (reviewed by Bailey 1993). Neither side disputes that autism has a biological basis. One position argues that a number of diverse organic etiologies result in the expression of autistic behaviours (Gillberg 1992). The other position proposes that a genetic liability specific for the disorder underlies most cases of autism (Bailey et al. 1995). There is little support for the claim that diverse organic brain dysfunction can account for most cases of autism. Post-mortem studies conducted on the brains of autistic individuals did not find gross destructive lesions, nor abnormalities in gyral configuration (Bauman 1991, Raymond et al. 1989). The most recent twin study reported that only 10% of the pairs had associated medical conditions (Bailey et al. 1995). Furthermore, obstetric hazards did not account for concordance between affected twins, a finding consistent with previous reports (Steffenberg et al. 1989, Folstein and Rutter 1977). Studies with autistic singletons also failed to demonstrate an association with obstetric hazards severe enough to cause the hypothesized brain damage (Bolton et al. 1994, Wing and Gould 1979).

Accumulating evidence strongly supports the concept that genetic factors play a significant role in the etiology of autism. Numerous family studies have found the frequency of autism among siblings of autistic

individuals to be approximately 3 to 4 per cent (Bolton et al. 1994, Baird and August 1985, August et al. 1981, Minton et al. 1982). This represents a 60 to 100 fold increase in risk to siblings, depending on which prevalence estimate is used as the base rate (Smalley et al. 1988). While these studies show that autism is familial, by themselves, they do not necessarily demonstrate the involvement of genetic factors.

The three epidemiological studies of autistic twins, conducted to date, provide more insight about the role of genetics in autism (Bailey et al. 1995, Steffenburg et al. 1989, Folstein and Rutter 1977). In the largest and most recent of these studies, the observed concordance rate for autism for identical (monozygotic, MZ) twins was 69 per cent, while no fraternal (dizygotic, DZ) twins were concordant for autism. The crucial point, is that concordance for autism in MZ twins was much greater than in DZ twins. The two other twin studies also found MZ concordance for autism to be higher than DZ concordance. As MZ twins are genetically identical, while DZ twins on average share 50 per cent of their genes, the much greater concordance for autism in MZ twins strongly suggests the involvement of genetic factors in the etiology of the disorder. Using the data from the 1995 British twin study, Bailey et al. (1995) estimated the heritability of autism and related PDD to be 91 per cent. Twin and family studies also support the concept that autism, Asperger's disorder, atypical autism, and related milder social and cognitive abnormalities have a common etiological basis. Family studies have found approximately 3 per cent of the siblings of autistic individuals met the criteria for a diagnosis of Asperger's or atypical autism (Bolton et al. 1994). Furthermore, in the most recent twin study, 92 per cent of MZ twins were

concordant for a broad spectrum of related cognitive and social abnormalities, as compared to 10 per cent concordance in DZ twins (Bailey et al. 1995). These findings suggest autism is the most extreme manifestation of a genetic liability which can produce a broad range of social and cognitive impairments.

The mode of genetic inheritance for autism and related PDD is unknown. Twin and family data are not consistent with single gene inheritance models. Concordance for autism in MZ twins is less than 100 per cent and recurrence risk to sibling is significantly lower than predicted by either a single dominant or recessive gene (50% and 25% respectively). Although there is a preponderance of autistic males, X-linked recessive inheritance can be excluded based on the population prevalences. Approximately a 1000 fold more females are affected than would be predicted by the frequency of autistic males, assuming Hardy-Weinberg equilibrium (Smalley et al. 1988).

Of the different genetic models which have been proposed for autism, multifactorial inheritance and genetic heterogeneity are the most popular. Multifactorial inheritance proposes the additive effect of a number of genes in combination with environmental factors lead to autism. Low twin concordance rates and low recurrence risk to siblings are consistent with this model (Smalley et al. 1988). However, the association of autism with certain diseases, such as tuberous sclerosis and Fragile X, which have a known genetic basis suggests the existence of genetic heterogeneity in the autistic population (Gillberg et al. 1994; Reiss and Freund 1990).

An argument against the primary role of genetics in the etiology of autism has been the apparent association of obstetric complications (OCs) with the disorder (Tsai 1987). Bolton et al. (1994) cites a number of lines of evidence suggesting the opposite, that OCs may be a consequence of an abnormality in the fetus rather than a cause. First, OCs associated with autism are comparatively mild and are unlikely to result in brain damage (Bailey et al. 1995). Second, obstetric data from individuals with chromosomal abnormalities indicate that fetal maldevelopment caused by genetic factors can lead to OCs (Bolton and Holland, 1994b). Third, twin studies have shown that OCs do not contribute to concordance for autism. Furthermore, the most recent twin study reported that minor congenital anomalies, which indicate early fetal abnormality, are more frequent in autistic twins than in the unaffected twins (Bailey et al. 1995). The type of microscopic abnormalities which have consistently been found in the brains of autistic individuals also suggest impairment of early fetal development (Bauman 1991).

To summarize, available data suggest genetic factors are a significant component in the etiology of autism. Phenotypic expression of this genetic liability is now believed to extend beyond autism per se to include Asperger's disorder and atypical autism, which are milder forms of PDD (Bolton et al. 1994, Bailey et al. 1995). Although the mode of inheritance is unknown, available evidence suggest the presence of both multifactorial inheritance and genetic heterogeneity (Smalley et al. 1988, Bolton et al. 1994). The objective of this research project was to screen candidate genes which could be involved in the etiology of autism in an attempt to provide new information about the

genetic component of autism. To accomplish this goal, the affected sib-pair method was used to examine nine autosomal candidate loci in families which each have two or more children with autism or a related PDD.

CHAPTER 1 :
Affected sib-pair method used to screen nine candidate loci

INTRODUCTION

Family and twin studies have indicated the involvement of genetic factors in the etiology of autism and related pervasive developmental disorders (Bailey et al. 1995, Bolton et al. 1994). Like other psychiatric disorders, autism appears to be genetically complex and heterogeneous (Smalley et al. 1988, Jorde et al. 1991). Genetic investigations of complex disorders are problematic because traditional lod-score linkage methods work best on homogeneous, single gene disorders. A large drawback of lod score methods is they can be extremely unreliable when the mode of inheritance is misspecified.

An alternative strategy is to study families with two or more affected siblings, thereby, decreasing etiological heterogeneity by focusing on cases which are most likely to have a genetic basis. The affected sib-pair method allows linkage to be detected in such families without requiring that a genetic model be specified (Penrose 1935, Blackwelder and Elston 1985). According to this method, linkage of the marker and disease locus is suggested if the number of alleles that are identical by descent and are shared by affected siblings at the marker locus is significantly greater than the number expected

by random segregation. This method was used to investigate nine autosomal candidate loci which could be involved in the etiology of autism and related PDD (Table 1). Two strategies were taken to select the candidate loci: (1) the marker locus is near genetic disorders or chromosomal abnormalities found to co-occur with autism; and/or (2) the gene encodes a protein which has been speculated to play a role in the pathophysiology of autism.

Tuberous sclerosis is an autosomal dominant disorder for which two genes have been identified, either of which is sufficient to cause the disorder (Povey et al. 1994). This condition is characterized by benign tumor-like growths in many organs including the brain, kidney and skin, with phenotypic expression showing a high degree of variability (Smalley et al. 1994). There appears to be a significant association between autism and this disorder. At least 50 per cent of individuals with tuberous sclerosis demonstrate autistic behaviours (Hunt and Dennis 1987), and approximately four per cent of the autistic population have tuberous sclerosis (Gillberg et al. 1994). This frequency is much higher than what would be predicted based on the prevalence of 1/10,000 for tuberous sclerosis and 4/10,000 for autism.

Cytogenetic examination of autistic individuals suggests the association of anomalies in chromosome 15 with the development of the disorder. There have been a number of case reports of individuals with autism having duplications of the 15q11-13 region (Bundey et al. 1994, Baker et al. 1994, Gillberg et al. 1991, Cantu et al. 1990, Schreck et al. 1977). This region is well characterized as it includes the critical region which is commonly deleted in Prader-Willi and Angelman syndromes (Ozcelik et al. 1992, Wagstaff et al. 1993).

Table 1: Summary of nine candidate loci surveyed in families with two autistic/PDD children

Candidate gene/region	Chromosomal location	Microsatellite marker[a]	Repeat sequence	% HET (PIC)	Significance of gene product or chromosomal region
Serotonin receptor, 5HT2	13q14.1-q14.2	13S118	$(CA)_n$	73%	• elevated 5HT serum levels in >30% of autistic individuals may reflect abnormalities in central serotonergic pathways
Dopamine D2 receptor	11q22-q23	DRD2	$(TG)_n$	(0.62)	• abnormalities in central dopaminergic pathways has also been proposed
Tyrosine hydroxylase	11p15.5	TH	$(TCAT)_n$	78%	• enzyme involved in synthesis of catecholamines
c-Harvey-Ras-1	11p15.5	HRAS-1	$(GGGCCT)_n$	52%	• published report of an allelic association with autism
Catechol-O-methyltransferase	22q11.2	22S343	dinucleotide*	95%	• enzyme involved in the breakdown of catecholamines
Prader-Willi syndrome region	15q11-q13	D15S11	$(CA)_n$	74%	• a number of case reports of autistic individuals having a duplication of this region
$GABA_A$ receptor *B*3	15q11-q13	GABRB3	$(CA)_n$	82.5%	• GABA receptor gene within Prader-Willi region
Tuberous sclerosis locus, TSC2	16p13.3	16S291	$(GT)_n$	79%	• tuberous sclerosis and autism frequently co-occur
Dopamine beta-hydroxylase	9q34	DBH	$(AC)_n$	69%	• enzyme involved in synthesis of norepinephrine and epinephrine • linked to tuberous sclerosis locus,TSC1

[a] refer to Chapter 3 for references

* repeat sequence not specified (Buetow 1993)

To date, the only positive finding from genetic studies of autism has been a reported allelic association with a restriction fragment length polymorphism (RFLP) in the c-HRAS oncogene on chromosome 11 (Hérault et al. 1993). This oncogene initially generated interest because of: (1) its close proximity to the gene encoding tyrosine hydroxlyase, the rate-limiting enzyme in the synthesis of catecholamines; and (2) the reported allelic association between c-HRAS and bipolar affective disorder (Egeland et al. 1987). Subsequent studies, have yielded conflicting results regarding the possible association of c-HRAS with bipolar disorder (Kelsoe et al. 1989, Mendlewicz et al. 1991, Meloni et al. 1995).

Speculation about genes which could be involved in the pathophysiology of autism derives mainly from the results of neurochemical studies. The most consistent finding has been that 30-50 per cent of autistic individuals have increased whole blood and platelet levels of serotonin (Schain and Freedman 1961, Hanley et al. 1977, Anderson et al. 1987); and this hyperserotonemia has been shown to be familial (Cook et al. 1988, Piven et al. 1991). The relationship between increased serotonin and the behavioural symptoms of autism, however, is as yet unknown. Neurochemical studies have also reported abnormal levels of two other principal neurotransmitters — dopamine and norepinephrine, albeit far less consistently (reviewed by Martineau et al. 1992). These findings combined with evidence suggesting that neuroleptics sometimes alleviate certain symptoms led to the proposal that central neurotransmitter systems are dysfunctional in autistic individuals (Anderson et al. 1984, Ornitz 1983). Based on these studies, I chose as potential candidate loci genes encoding enzymes involved in the

metabolism of serotonin, dopamine, and norepinephrine, as well as genes encoding receptors for these neurotransmitters.

MATERIAL AND METHODS

Description of families

The study group consisted of 18 families, each of which have two or more children with autism or a related PDD (atypical autism or Asperger's syndrome). Twelve of the families have two affected sons, five families have an affected son and an affected daughter, and one family has two affected daughters and an affected son. An extensive battery of assessments were conducted on the affected children to confirm the diagnoses of autism or PDD using a best-estimate approach with a diagnostic review panel (Szatmari, personal communication).

Additional families recruited during the project were scored only at the DBH microsatellite locus. These include: two more families with two affected sons, and one more family with an affected son and an affected daughter. Six families in which blood samples could be obtained only from the mothers and some of the affected children were also scored at the DBH microsatellite locus. Four of these families have two affected sons, one family has three affected sons, and one has an affected son and an affected daughter. One of the families with an affected son and affected daughter was excluded from DBH allele frequency analysis and subsequent study because one family member has been diagnosed with a mitochondrial disorder (Szatmari, personal communication)

Sample collection

Blood samples from parents and affected children were collected in 7-10 ml venipuncture tubes containing EDTA (lavender top). Blood was transferred to 15 ml plastic tubes and stored at -70°C.

DNA isolation

Blood samples were allowed to thaw for 1 hour at room temperature. An equal volume of 2x lysis buffer (4 M urea, 0.2 M NaCl, 0.1 M Tris-HCl pH 8.0, 0.5% n-laurylsarcosine, 10 mM EDTA) was added, followed by proteinase-K (83 units) digestion at 56°C for 1 hour. Additional proteinase-K (83 units) was added. Samples were placed on a Lab Quaker to provide gentle agitation and incubated at 37°C overnight. DNA was extracted according to the following protocol: (1) a 1:1 ratio of phenol and chloroform (70:30) to aqueous volume was added, samples were gently agitated for 10 minutes, centrifuged for 10 minutes at 3000 rpm, the aqueous (top) layer containing the DNA was removed and placed in a new tube; (2) step 1 was repeated, and then (3) step 1 was repeated again using chloroform instead of phenol/chloroform. DNA was precipitated by adding 0.1 volume of 7.5 M ammonium acetate, and two volumes of 95% ethanol. DNA pellets were washed with 70% ethanol, allowed to air dry, then dissolved in approximately 250 ul of TNE_2 (10 mM Tris-HCl, 10 mM NaCl, 2 mM EDTA, pH 8.0) at 37°C for 3-4 hours with occasional mixing.

Microsatellites

Nine microsatellite loci closely linked to the candidate genes/regions were amplified using the polymerase chain reaction (PCR). The PCR reaction

mixture for 10 samples consisted of 10 ul of 10x PCR buffer (0.1% Triton X100, 10 or 15 mM $MgCl_2$, 0.1 M Tris-HCl pH 8.3, 0.5 mM KCl), 10 ul of 2 mM dNTPs, 2 ul of each primer (6.7 pmol/ul), 0.5 ul Taq polymerase (2.5 units), and sterilized double distilled water was added to a final volume of 75 ul. After vortexing, 7.5 ul of the PCR reaction mixture was added to each sample which consisted of 25 ng of template DNA (10 ng/ul). Annealing temperature and concentration of $MgCl_2$ varied according to the marker locus being assessed (Table 2). Conditions were as follows: (1) 3 minutes at 94°C; (2) 30 cycles of 1 minute at 94°C, 1 minute at the annealing temperature (Table 1), and 1 minute at 72°C; (3) followed by a final extension at 72°C for 10 minutes.

Prior to the PCR reaction the forward primer was end-labeled with ^{33}P according to the protocol described in the Amplitaq cycling sequencing guide by Perkin Elmer. One tube of end-labeled primer (1 ul of primer (6.7 pmol/ul) in a total volume 6.2 ul) was sufficient for 10 samples. Unlabeled forward primer (1 ul of 6.7 pmol/ul) was added to the PCR reaction mixture, so the amount of forward primer equaled the amount of reverse primer used (2 ul of 6.7 pmol/ul). PCR products were separated on 8% denaturing polyacrylamide gels, run at constant power (50 watts). Table 2 summarizes PCR conditions and gel running times used for each locus.

Statistical Methods

The Likelihood ratio (L^2) test was used to determine: (1) if concordance between affected siblings at the test locus was significantly higher than expected under random segregation, and (2) whether microsatellite allele frequencies observed in the parents differed significantly from published

Table 2: **PCR conditions and gel running times for the microsatellite markers**

Microsatellite marker	Allele size range	Annealing temperature	$MgCl_2$ concentration	Gel running time
13S118	187—201 bp	58°C	1.5 mM	4 hrs
DRD2	80—86 bp	58°C	1.0 mM	2 hrs 30 min
TH	244—260 bp	60°C	1.5 mM	4 hr 30 min
HRAS-1	106—118 bp	60°C	1.5 mM	3 hrs
22S343	169—193 bp	56°C	1.5 mM	4 hrs
D15S11	243—263 bp	56°C	1.5 mM	4 hr 30 min
GABRB3	181—201 bp	56°C	1.5 mM	4 hrs
16S291	154—170 bp	65°C	1.5 mM	3 hrs 30 min
DBH	253—280 bp	62°C	1.0 mM	4 hr 30 min

values. This non-parametric test is similar to the chi-square (X^2) test, and has the same probability distribution as the X^2 statistic (Kennedy 1992). There are two primary advantages to using the L^2 test, instead of X^2. First, it can be used to partition the frequency data. Second, it is less sensitive to small expected values than the X^2 statistic (Rolf Morrison, personal communication). The L^2 test of independence and goodness of fit formulas are in Appendix C.

RESULTS

The affected sib-pair approach was used to investigate the possibility of linkage between autosomal markers tightly linked to candidate genes/regions and susceptibility for the broad spectrum of autistic disorders. Genotypes of parents and affected children from 18 families, each having at least two children with autism or PDD, were determined at nine microsatellite loci. For each locus, the number of affected siblings who inherited alleles identical by descent was determined. Standard sib-pair analysis typically classifies affected siblings as sharing 0, 1, or 2 alleles identical by descent (Blackwelder and Elston 1985). A problem in categorizing sib-pairs in this manner is that for a family to be included in the analysis, inheritance of specific alleles from each parent to both affected children must be established. If a parent is homozygous at the marker locus and therefore uninformative, i.e. identity by descent can not be established, the entire family is excluded from the analysis. Due to the small number of families participating in the study, identity by descent between affected siblings (termed 'concordance') was determined for maternal and paternal alleles separately (Appendix B).

Theoretically, at each microsatellite locus, a single band should be amplified for each allele present. Therefore, one band should be observed in homozygous individuals and two bands in heterozygous individuals. Of the nine loci examined, only the tyrosine hydroxylase (TH) microsatellite locus yielded a single product (band) for each allele (Figure 1). This microsatellite is a polymorphic TCAT repeat located in the first intron of the TH gene

(Kobayashi et al. 1988, Polymeropoulos et al. 1991). Five alleles, differing in the number of repeat units present, have been observed at this locus.

The method of scoring is illustrated in Figure 1. In this example (family 25), the mother is heterozygous for alleles A2 and A4, while the father is homozygous for the A1 allele. Genotypes of the affected children are A1 and A4 for child #1 and A1 and A2 for child #2. The children are discordant for the maternal allele, i.e. inherited different alleles, and the paternal genotype is uninformative, since it can not be established if the children inherited the same paternal allele. An example of siblings sharing identical alleles is provided by family 51 (Figure 1). Both parents are heterozygous for different alleles, the mother for A1 and A3 and the father for A2 and A4. Both children inherit the A1 allele from their mother and the A2 allele from their father, so the siblings would be scored as being concordant for both parental alleles.

At the other eight loci, multiple bands were observed for each allele amplified, however the banding pattern was consistent at each locus. Examples of these multiple bands, commonly referred to as 'shadow bands', can be seen in Figures 2 and 3. Alleles of the dopamine beta-hydroxylase (DBH) microsatellite loci (Figure 2) show a commonly observed pattern of one intense band followed by a lighter band 2 bp smaller in size. Shadow bands make determining which alleles are present more difficult. It is essential that the criteria used to establish genotypes at a specific locus remain constant. In this study, the larger and usually more intense band was scored as the allele. For example, in Figure 2, the genotype of individual #3 was recorded as B2 and B7.

Shadow bands can be particularly problematic in determining if an individual is heterozygous for two alleles which differ in size by only 2 bp or is homozygous for the larger allele. When an individual is heterozygous for alleles differing in size by 2 bp the shadow band from the larger allele overlaps with the smaller allele generating a banding pattern which typically shows the presence of three bands with the middle one being the most intense. In comparison, when an individual is homozygous, two bands are normally observed with the larger one being more intense. For example, in figure 2, individual #1 is heterozygous for B7 and B8 while individual #2 is homozygous for B8. The other type of shadow band frequently observed is a faint band 1 bp smaller that the actual allele. Amplification of the GABRB3 microsatellite produced both 1 bp and 2 bp shadow bands, as well as, multiple smaller fainter bands. As at the other loci, the largest band was scored as the actual allele (Figure 3).

Under the null hypothesis of no linkage it is expected that random segregation will result, on average, in 50 per cent of sib-pairs being concordant for a maternal allele and 50 per cent of sib-pairs being concordant for a paternal allele. The Likelihood ratio (L^2) test was used to compare the number of alleles that are identical by descent and are shared by affected siblings and the number expected under random segregation. Observed sib-pair concordance did not deviate significantly from values predicted by random segregation at any of the loci tested (Table 3).

During the genotyping process, a surprisingly high percentage of parents were observed to be uninformative at the DBH locus. A comparison of DBH allele frequencies in the parents to published values revealed a

statistically significant difference between the two groups (L^2=13.16, df=5, $p<0.05$). Due to this result, allele frequencies observed in the parents at the other eight loci tested were compared to published values. No significant differences were found at any loci other than DBH.

Figure 1: Amplification of alleles at the tyrosine hydroxylase (TH) microsatellite locus from genomic DNA of individuals with autism/PDD and their parents. Allele A1 is 260 bp in size, A2 (256 bp), A3 (252 bp), A4 (248 bp), and A5 (244 bp).

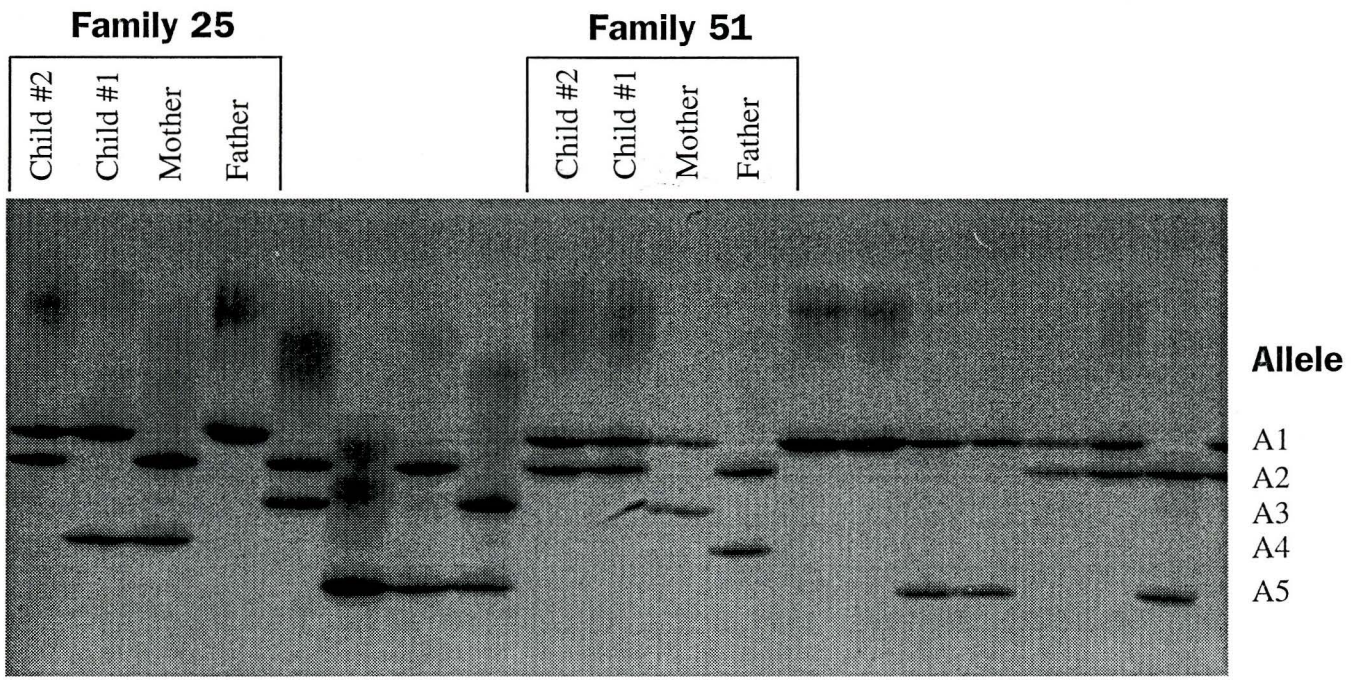
Family 25
Child #2
Child #1
Mother
Father
Family 51
Child #2
Child #1
Mother
Father
Allele
A1
A2
A3
A4
A5

Figure 2: Amplification of alleles at the dopamine beta hydroxylase (DBH) microsatellite locus from genomic DNA of individuals with autism/PDD and their parents. Allele B2 is 276 bp, B7 (259 bp), and B8 (257 bp).

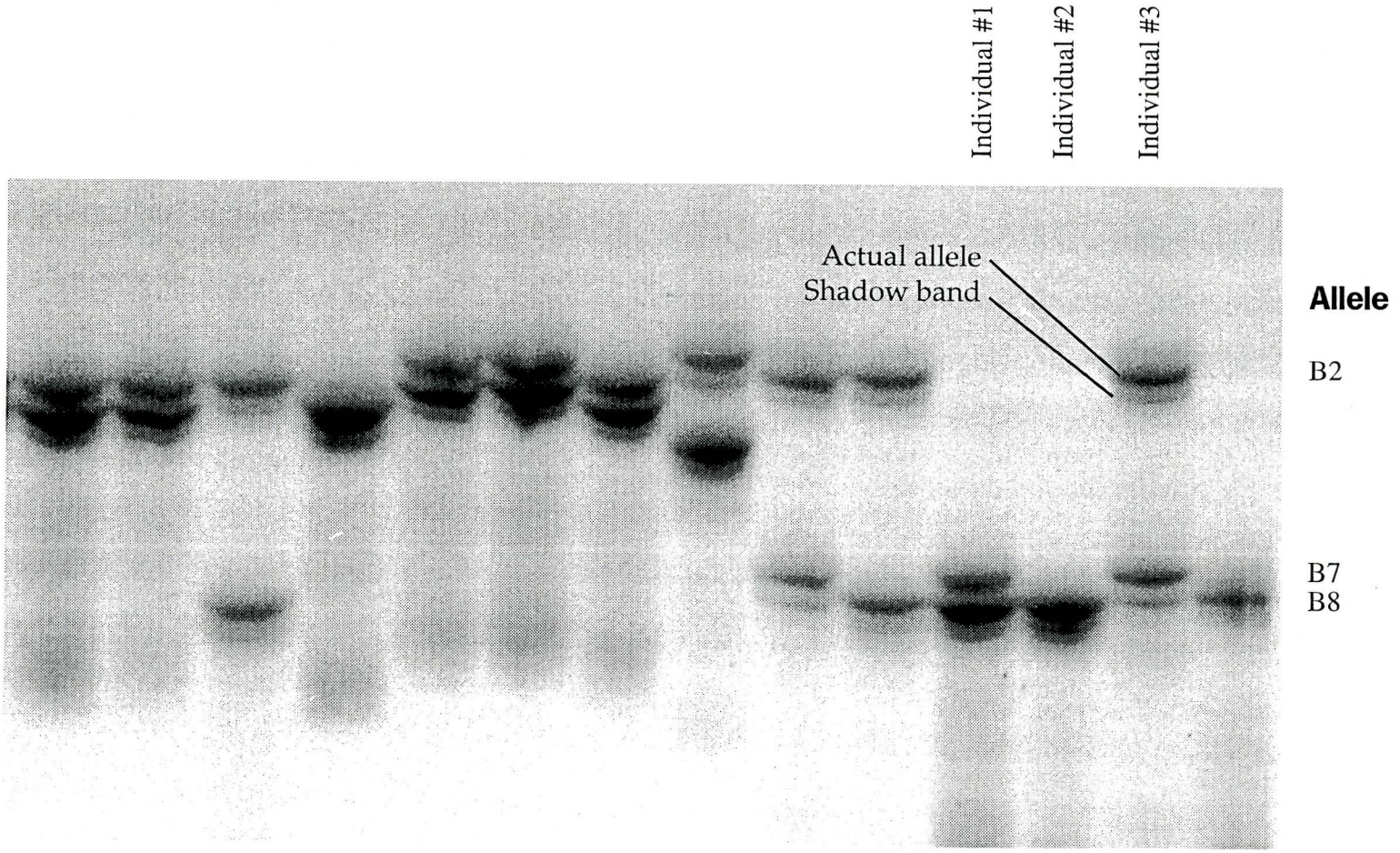
Individual #1
Individual #2
Individual #3
Actual allele
Shadow band
Allele
B2
B7
B8

Figure 3: Amplification of alleles at the $GABA_A$ receptor*B*3 (GABRB3) microsatellite locus from genomic DNA of individuals with autism/PDD and their parents.

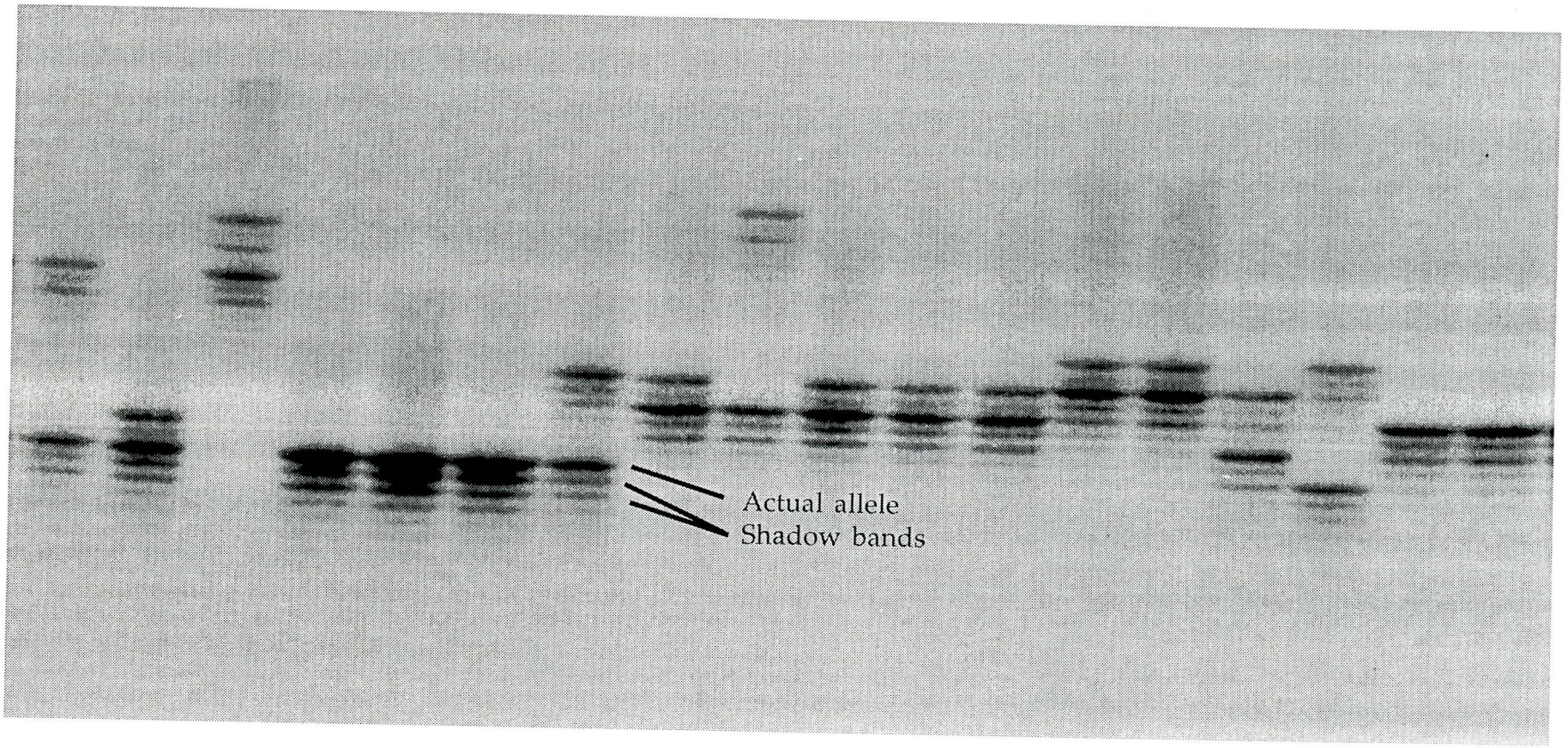
Actual allele
Shadow bands

Table 3: Summary of observed sib-pair concordance

	Maternal allele				Paternal allele			
Microsatellite locus	C	D	NI	L^2 value* (df=1)	C	D	NI	L^2 value* (df=1)
TH	5	10	3	1.70	4	7	7	0.83
HRAS-1	2	7	9	2.94	3	3	12	0
16S291	8	8	2	0	7	9	2	0.25
13S118	5	4	9	0.11	4	8	6	1.36
DRD2	3	6	9	1.02	7	4	7	0.83
GABRB3	10	5	3	1.70	10	4	5	2.66
PWS/AS	7	3	8	1.65	4	5	9	0.11
DBH	**3**	**4**	**11**	**0.14**	**6**	**4**	**8**	**0.40**
22S343	8	10	0	0.22	8	9	1	0.06

* L^2 value must be greater than 3.84 to be statistically significant ($p<0.05$)

DISCUSSION

The affected sib-pair method was used to screen nine candidate loci in 18 families, each of which have two or more children with autism or a related PDD. No significant concordance between affected siblings was observed for any of the loci tested. It is particularly interesting that affected siblings did not inherit the identical alleles more frequently than would be expected by random segregation at the HRAS microsatellite locus. The c-Harvey-Ras gene is thought to be involved in the regulation neural cell differentiation during development (Li et al. 1992). A previous study reported finding an allelic association between a RFLP of the c-HRAS gene and autism (Hérault et al. 1993). As the HRAS microsatellite locus is located within the first intron of the HRAS gene, if this gene were involved in the etiology of autism one would expect to see an increased concordance between affected siblings.

Unfortunately, the heterozygosity of the HRAS microsatellite marker is only 52 per cent. A number of the parents were homozygous, and therefore, uninformative at this locus. Because of the small sample size, caution must be taken in interpreting the lack of significant concordance in affected siblings. While a major locus involved in the etiology of autism could be detected by screening 18 families with two affected children, the number of families studied is not sufficient to exclude the involvement of the HRAS gene, or any of the other loci tested.

Microsatellite markers were used as the test loci to establish inheritance of alleles identical by descent between affected siblings for several reasons:

(1) a large number of highly polymorphic microsatellite markers are currently available; (2) only a small quantity of DNA is required for template, which is extremely helpful since very little blood could obtained from some affected children; and (3) the procedure is relatively quick and inexpensive. A technical problem commonly encountered when amplifying microsatellite repeats is the generation of extra 'shadow' bands. These shadow bands are smaller than the most intense band (allele) and differ in size from the actual allele by multiples of 1 bp or 2 bp. Taq polymerase has been shown to have the ability to add a noncomplementary extra base to the 3'end of blunt-ended molecules (Clark 1988). Furthermore, Weber (1988) demonstrated the one base pair shadow bands, could be eliminated by incubating the amplified products with either Klenow enzyme or T4 DNA polymerase, both of which have 3'—5' exonuclease activity. This treatment decreased the size of the most intense band by one base pair, suggesting the most intense bands in untreated samples are derived from double-stranded DNA with single-base 3' overhangs (Weber 1988). The two base pair shadow bands still remained after treatment with either Klenow enzyme or T4 DNA polymerase. Weber (1988) hypothesized that the two base pair shadow bands were due to the Taq polyermase skipping repeat units. However, to date, little has been published regarding the mechanism responsible for the generation of the two base pair shadow bands.

Chapters 2 and 3 will discuss the results of follow-up studies which explored the possible involvement of the DBH gene in the etiology of autism and related PDD. These studies were prompted by the finding that the parents with two or more affected children had allele frequencies at the DBH

microsatellite locus which were significantly different from published values (L^2=13.16, df=5, $p<0.05$).

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CHAPTER 2

Serum dopamine beta-hydroxylase activity in families with two autistic/PDD children

INTRODUCTION

Dopamine-ß-hydroxylase (DBH) is a tetrameric glycoprotein which catalyzes the conversion of dopamine (3, 4-dihydroxyphenylethylamine) to norepinephrine (Kaufman and Friedman 1965). Unlike other enzymes involved in the formation of catecholamines, DBH is located predominantly within secretory cells of the adrenal medulla and synaptic vesicles of adrenergic and noradrenergic neurons (Axelrod 1972, Hortnagl 1974). Consequently, DBH is concomitantly released with norepinephrine or epinephrine and eventually enters the circulation where it maintains its enzymatic activity (Weinshilboum and Axelrod 1971).

These early findings generated much speculation that serum DBH activity could serve as an index of sympathoadrenal function. Weinshilboum (1979) extensively reviewed studies on experimental animals (mainly rats) and suggested that, although most serum DBH originates from sympathetic nerves, much of the enzyme may enter the circulation by a process which does not involve exocytotic release with catecholamines. Three key findings support Weinshilboum's hypothesis. First, removal of the adrenal glands does not significantly decrease serum DBH activity (Weinshilboum and

Axelrod 1971). Second, serum DBH activity is substantially decreased after treatment with drugs which destroy the sympathetic nerve terminals (Coyle and Axelrod 1972). Lastly, drugs which specifically inhibit the release of norepinephrine in response to nerve stimulation produce little change in serum DBH activity (Reid and Kopin 1974). Coupled exocytotic release with catecholamines is now known to be only one variable which contributes to serum DBH levels.

In humans, genetic factors play a key role determining serum DBH activity levels. The range in serum DBH activities among individuals in the general population is large (Weinshilboum et al. 1975). However, levels within an individual remain remarkably constant over long periods of time (Ogihara et al. 1975). A large study of randomly selected families found the correlation for serum DBH activity between siblings to be 0.50, between father-child pairs to be 0.51, and between mother-child pairs to be 0.48 (Ogihara et al. 1975). Twin studies provided further support for the role of genetic factors in determining serum DBH activity levels. The correlation of serum DBH activity between monozygotic twins was significantly higher than observed between dizygotic twins (Rush and Geffen 1972).

Studies investigating the segregation of serum DBH activity within large multigenerational families have consistently found evidence suggesting the majority of inherited variation can be accounted for by the additive effect at a single gene with two alleles, one for high activity and one for low activity (Wilson et al. 1988, Asamoah et al. 1987, Goldin et al. 1988, Elston et al. 1979). The same studies also reported data indicating linkage between serum DBH activity and the ABO blood group locus located in 9q34. Using *in situ*

hybridization Craig et al. (1988) localized the structural gene for DBH to the same region of chromosome 9.

Together the above findings suggest that an allelic variation at the DBH locus is responsible for more than 50 per cent of the variation in serum DBH activity. This variation could exert its effect in two ways, either by (1) altering protein structure such that catalytic activity is affected, or (2) altering the amount of protein present by either modifying transcriptional regulation or affecting protein stability. Radio-immunoassay studies have demonstrated that differences in serum DBH activity result from quantitative changes in serum DBH protein, rather than alterations in catalytic properties (O'Connor et al. 1983, Dunnette and Weinshilboum 1977). No study published to date, however, has elucidated the molecular polymorphism responsible for this variation in serum DBH activity levels.

Aside from genetic components, factors such as drugs, hormones, and short-term stress have been all been found to affect serum DBH activity levels. Generally, changes in response to these factors are quantitatively small (Weinshilboum 1979). One exception is individuals with thyroid disorders. Hyperthyroidism significantly decreases serum DBH activity, while hypothyroidism significantly increases it (Noth and Spaulding 1974, Nishizawa et al. 1974). Serum DBH activity returns to normal levels when these conditions are treated. Another factor which influences serum DBH activity is age. There are dramatic changes in serum DBH activity over the course of development. Levels of serum DBH activity are extremely low in newborns, but increase substantially during the first two or three years of life (Freedman et al. 1972). Adult levels are reached by the end of the second

decade of life and remain fairly stable, decreasing slightly after the age of 50 (Freedman et al 1972).

As serum DBH activity levels appear to be largely under the control of DNA sequences in or close to the DBH gene, and abnormal allele frequencies at the DBH microsatellite locus were found in parents with two autistic/PDD children, serum DBH activities were measured in both mothers and fathers of these children and in an adult control group. A previous study reported decreased plasma DBH activity in both autistic children and in their first degree relatives (Lake et al. 1977). It should be noted that plasma and serum DBH activity have been shown to be identical, O'Connor et al. 1983). One objective of this study was to attempt to replicate Lake's finding in parents with two autistic/PDD children.

MATERIAL AND METHODS

Sample collection

Blood samples were collected in 5 to 7 ml red-capped tubes which did not contain an anti-coagulant and placed on ice during transport in the lab. If blood samples had not clotted upon arrival, samples were allowed to clot at room temperature for approximately 30 minutes. Blood samples were centrifuged at 3500 rpm for 10 minutes, and serum was drawn off, aliquoted into 1.5 ml eppendorf tubes and frozen at -20°C. Serum can be stored at -20°C for at least three months without any deterioration in DBH activity (Nagatsu and Udenfriend 1972).

Serum samples could not be obtained from the individuals which served as the Canadian control group for DBH allele frequency comparisons (Chapter 1), so a second control group was used for this part of the study. The control group consisted of individuals enrolled in a laboratory assistants course at a local college. Also, as blood had to be retaken from parents to obtain the necessary serum samples, not all parents were available to participate.

DBH assay

Serum DBH activity levels were determined according to the protocol described by Frigon (1987). DBH has a broad substrate specificity allowing it to catalyze the hydroxylation of not only dopamine, but also other beta-phenylethylamine derivatives (Levin and Kaufman 1961). The assay

procedure detailed by Frigon involves the conversion of tyramine to octopamine enzmatically by DBH in the presence of ascorbic acid, which acts as an electron source. Other reagents present include: (1) N-ethylmaleimide which inactivates endogenous DBH inhibitors, (2) catalase which prevents inhibition of DBH by H_2O_2, (3) sodium fumarate for maximal activity, and (4) paragyline which is a monoamine oxidase inhibitor. Octopamine, separated from reactants using ion-exchange columns, is oxidized with periodate to produce p-hydroxybenzaldehyde which is measured spectrophotometrically.

A reaction mixture for the enzymatic step was prepared (Table 1). Ten samples were assayed at the same time, with one being a negative control (no serum) and one being a positive control. Fifty microlitres of serum was added to 800 ul of reaction mixture in a 1.5 ml eppendorf tube, gently vortexed, and incubated for 30 minutes at 37°C. The enzymatic reaction was stopped by adding 200 ul of 20 per cent percloric acid with vortexing. Samples were placed on ice until the remaining samples were processed. The samples were then centrifuged at 3000 g for 20 minutes at 4°C, then 900 ul of the supernatant was applied to 500 ul of AG 50Wx4, 200-400 mesh, H+ resin retained in columns (Biomed). At the beginning of the study the H+ resin was treated with 1N NaOH, washed with ddH_20 (4x), and then washed (20x) with 1 N HCl, followed by approximately 20 washes with ddH_20 until the pH was between 5-7. Columns were washed twice with 1 ml of water, and then the octopamine product was eluted twice with 1 ml of 4 N ammonium hydroxide. The samples were oxidized by adding 200 ul of 2 per cent sodium periodate, incubating at room temperature for 6 minutes and then stopping the reaction by adding 200 ul of 10 per cent sodium metabisulfite. Absorbance

readings were taken at 330 nm in a cuvette of pathlength 1 cm to measure the amount of p-hydroxybenzaldehyde. Serum DBH activity was assayed in duplicate.

Absorbance readings were converted to international units (iu/L), where one unit equals the formation of one micromole of octopamine formed per minute liter serum at 37°C, using the formula:

[ ] p-hydroxybenzaldehyde (umol/min L serum)

$$= \frac{\text{(absorbance at 330 nm)}}{(2.15 \times 10^4\ \text{cm}^{-1}\ \text{mol}^{-1})(1\ \text{cm})} \times \frac{10^6}{30\ \text{min}} \times \frac{2.4 \times 10^{-3}}{50 \times 10^{-6}\ \text{L serum}}$$

The concentration was determined using, an extinction coefficient for p-hydroxybenzaldehyde of 2.15×10^4 and a cuvette with a 1 cm path length.

Table 1: Reaction mixture for DBH assay

Reagent used	Amount per sample	Comment
Sodium acetate, 1 M, pH 5.0	178 ul	
Tyramine hydrochloride, 0.2M	89 ul	prepare weekly
Ascorbic acid, 0.2 M	44 ul	prepare daily
Sodium fumarate, 0.2 M	44 ul	
N-Ethylmaleimide, 0.2 M	133 ul	prepare twice weekly
Catalase (approx. 4 mg/ml)	89 ul	
Pargyline, 20 mM	44 ul	
double-distilled water	178 ul	

RESULTS

Serum DBH activity levels were determined for parents with two autistic/PDD children and for an adult control group. DBH activity was measured according to the spectrophotometric method detailed by Frigon (1987). During the initial DBH assays, it was noticed that the resin appeared to be easily disrupted during addition of the supernatant containing the octopamine product. This observation generated concern that a substantial proportion of the octopamine might be passing through areas of the column from which the resin was displaced, and be discarded with liquid from the first washes. To test this hypothesis, DBH activity was determined twice for the same samples, once with the specified 0.2 ml of resin and once using 0.5 ml resin. The amount of product for each sample was higher when 0.5 ml of resin was used and the values obtained were in the range predicted based on published data. Therefore for each individual, serum DBH activity was determined twice using 0.5 ml of resin. The correlation coefficient between the two sets of measurements was 0.96. The average enzyme activity for each individual was used in subsequent statistical analyses.

In total, serum DBH activity assayed in 19 of the 26 mothers and 14 of the 20 fathers with two autistic/PDD children, and in 29 adult controls (Table 2). In the control group, the mean and median serum DBH activities were 34.4 iu/L and 30.1 iu/L, respectively. In comparison, the mean serum DBH activity in parents with two affected children was 26.0 iu/L, and the median DBH activity was 18.91 iu/L.

Mean serum DBH activity was significantly lower in parents with two autistic/PDD children than in controls (Student's t=-1.71, df=60, $p<0.05$, 1-tailed). Mean serum DBH activity of the mothers did not differ significantly from the fathers (Student's t=-0.326, df=31, n.s., 1-tailed). However, in the mothers, median serum DBH activity was only 15.9 iu/L, compared to a median activity of 22.6 iu/L in the fathers with two affected children and 30.1 iu/L in the control group. Part of the variation in median and mean DBH activities among the three groups is probably because of the genotypic differences at the DBH microsatellite locus among mothers, fathers and controls (discussed in Chapter 3).

Table 2: Serum DBH activity in parents with two autistic/PDD children and an adult control group

	Sample size (n)	Mean serum DBH activity (iu/L)	Standard deviation (iu/L)	Median serum DBH activity (iu/L)
Parents	33	26.0	16.6	18.9
Mothers	19	25.2	18.0	15.9
Fathers	14	27.2	15.2	22.6
Control group	29	34.4	22.0	30.1

DISCUSSION

Mean serum DBH activity was found to be significantly lower in parents with two autistic/PDD children compared to an adult control group (Student's $t=-1.71$, $df=60$, $p<0.05$, 1-tailed). This result is in accord with Lake's (1977) finding of decreased plasma DBH activity in the immediate family members (parents and siblings) of autistic children. Like other studies of serum DBH activity, the range in activity levels among individuals was very large (4.5—63.6 iu/L in parents and 4.4—104.0 in controls) (Weinshilboum et al. 1975). Due to the diversity in enzyme activity levels and the fairly modest number of individuals assayed, mean serum DBH activity might not be the most informative measure.

To address this concern, both mean and median serum DBH activities were determined for the parents and controls. Mean serum DBH activity in the control group was 34.4±22.0 iu/L, which is comparable to mean DBH activity levels reported in previous studies (Weinshilboum 1979). Median serum DBH activity in the control group was 30.1 iu/L. In comparison, mean and median serum DBH activities in parents with two affected children were 26.0 iu/L and 18.9 iu/L, respectively. The mean DBH activity of mothers with two autistic/PDD children was 25.2 iu/L, however, the median activity revealed that 50 per cent of these mothers had serum DBH activity levels lower than 15.9 iu/L. In the fathers, mean activity was 27.2 iu/L and median activity was 22.6 iu/L. The variation in serum DBH activity among the

mothers, fathers, and controls may be partially be due to genotypic differences among these groups at the DBH microsatellite locus.

A physical mapping study placed the DBH microsatellite locus proximal to the 5′ end of the DBH gene, lying somewhere within 25 kb of that gene's transcription start site (Murrell et al. 1995) (Figure 1A). Using *in situ* hydridization, Craig et al. (1988) localized the DBH gene to 9q34. The orientation of the gene is such that the 5′ end is centromeric and 3′ end telomeric (Murrell et al. 1995). Spanning approximately 23 kb, the DBH gene contains 12 exons which encode a 578 amino acid protein (Kobayashi et al. 1989, Lamouroux et al. 1987). Alternate use of two polyadenylation sites within the untranslated region of exon 12 account for the production of the two mRNA types differing in size by approximately 300 bp (Kobayashi et al. 1989). The functional significance of having multiple mRNAs with different 3′ untranslated regions is unknown. Comparative amino acid sequence analysis demonstrated that DBH shares no homology with the other catecholamine synthesizing enzymes (Lamouroux et al. 1987).

Transcription of the DBH gene is restricted to noradrenergic and adrenergic neurons of the central nervous system, sympathetic ganglia, and adrenal medulla chromaffin cells (Axelrod 1972, Hortnagl 1974). The regulatory elements governing DBH expression are complex and have not been fully elucidated (Figure 1B). A combination of positive and negative regulatory elements function to control the tissue-specific pattern DBH expression (Hoyle et al. 1994; Ishiguro et al. 1993; Shaskus et al. 1992). A study using transgenic mice demonstrated the existence of distal regulatory elements up to 6 kb upstream from the DBH transcriptional start site (Hoyle

et al. 1994). The exact sequences of these distal regulatory elements have not been characterized to date. The results from this transgenic mice study suggest that the 1.1 kb region immediately 5′ to the transcriptional start site is sufficient to restrict DBH gene expression to the appropriate neurons (Hoyle et al. 1994). However, Hoyle et al. (1994) speculate that one of the distal elements (element C in Figure 1B) may play a role in increasing the level of expression of the DBH gene.

In the proximal promoter region, the transcription element AP-2 has been found to be involved in maintaining basal levels of DBH expression in the rat (Greco et al. 1995). Mutation of the AP-2 site in the rat resulted in a sevenfold reduction in basal DBH transcriptional activity. The AP-2 site in the rat, however, does not appear to play an essential role in second messenger induction of DBH gene transcription (Greco et al. 1995). A consensus AP-2 binding site is located in approximately the same position (-126 to -118 bp) of the human DBH gene (Greco et al. 1995; Kobayashi et al. 1989).

DBH gene expression is influenced by a variety of environmental stimuli, which act either at the level of transcription or translation. Cyclic AMP, glucocorticoids, and immobilization stress all have been shown to increase DBH mRNA levels (Lamouroux et al. 1993; McMahon and Sabban 1992; Nankova et al. 1993). Sequence analyses of the 5′ flanking region of the DBH gene confirmed the existence of the expected cis-acting response elements including cyclic AMP response elements (CREs) and glucocorticoid response elements (GREs) (Ishiguro et al. 1993; Kobayashi et al. 1989). DBH activity is also increased by stimulation with nerve growth factor and

reserpine, which act by increasing DBH protein synthesis (Acheson et al. 1984; Biguet et al. 1986).

In summary, there are multiple regulatory elements controlling DBH gene expression. The interactions amongst these elements are not fully understood. However, Hoyle et al. 1994 suggested the distal regulatory elements may function to increase the level of DBH gene expression rather than aiding in directing tissue-specific expression. As these distal regulatory elements have not yet been sequenced, it is possible the DBH microsatellite locus may encompass one of these regulatory elements. In this study, parents with two autistic/PDD children were found to differ significantly from control groups in both mean serum DBH activity, and in allele frequencies at the DBH microsatellite locus. Chapter 3 integrates these two results, discussing the relationship between serum DBH activity and DBH microsatellite genotype.

Figure 1A: Basic Structure of DBH Gene

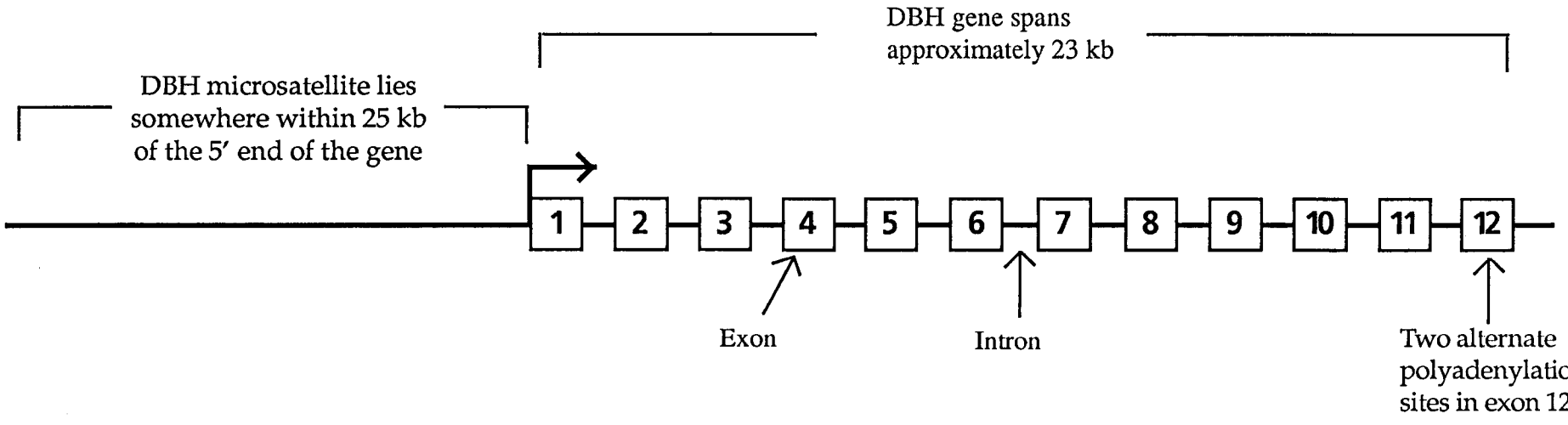
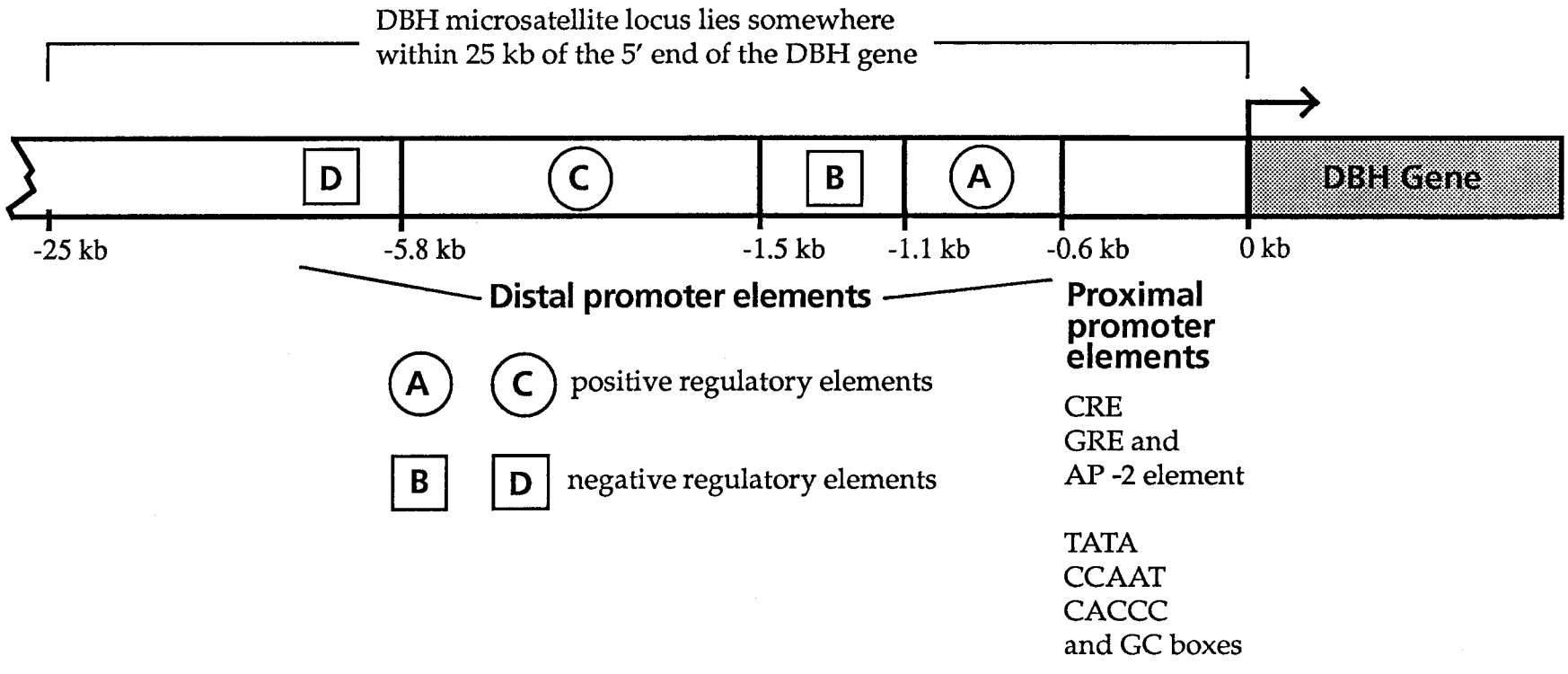


Figure 1B: DBH Regulatory Elements



For further information on distal promoter elements refer to Hoyle et al. 1994 (Chapter 2)

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CHAPTER 3

(to be submitted to *Nature Medicine*)

Low levels of maternal serum dopamine beta-hydroxylase may be a factor in the etiology of autism and related pervasive developmental disorders

ABSTRACT

The affected sib-pair method was used to screen eleven candidate loci in 18 families, each of which have two or more children with autism or a related pervasive developmental disorder (PDD). During the study, it was observed that many mothers were uninformative at the dopamine beta-hydroxylase (DBH) microsatellite locus. DBH alleles are defined by a polymorphic AC repeat and the presence or absence of a 19 bp sequence in the 5′ flanking region of the gene. Mothers had a significantly higher frequency of alleles containing the 19 bp deletion compared to a Canadian control group (0.65 vs. 0.46, L^2=6.96, df=1, p<0.01). The 19 bp deletion was found to be associated with lower mean serum DBH enzyme activity (non-deletion homozygotes 44.5±28.6 iu/L; heterozygotes 30.4±16.0 iu/L; and deletion homozygotes 20.5±15.3 iu/L; F=5.45, df=59, p<0.01). We propose that lowered maternal serum DBH activity provides a uterine environment which, in conjunction with genotypic susceptibility of a fetus, results in autism.

INTRODUCTION

Autism is a developmental disability characterized by impairments in reciprocal social interaction and communication, coupled with repetitive stereotypic activities. Twin and sibling studies have provided strong evidence for the role of genetic factors in the etiology of autism and related PDD[1,2]. However, the fairly low recurrence risk to siblings of autistic/PDD individuals (approximately 4%) and the male-biased sex ratio (4 males : 1 female) are incompatible with simple Mendelian inheritance models[3]. The etiology of autism/PDD appears to be heterogeneous, involving both environmental and genetic factors.

This study used the affected sib-pair method[4] to screen eleven candidate loci which could be involved in the etiology of autism (Table 1). Two strategies were taken to select the candidate loci: (1) the gene encodes a protein which has been speculated to play a role in the pathophysiology of autism; and/or (2) the marker locus is near chromosomal abnormalities or genetic disorders found to co-occur with autism. First, as biochemical studies have reported abnormal levels of serotonin, dopamine and norepinephrine in autistic individuals[5,6], genes for enzymes involved in the metabolism of these neurotransmitters were tested. Second, since genetic conditions such as tuberous sclerosis and fragile X syndrome have been reported to co-occur with autism[7,8], markers close to these disease loci were also examined.

In total, 11 candidate loci were screened using microsatellites closely linked to the candidate genes. No significant concordance between affected

siblings was observed for any of the loci tested (data not presented). However, about half of the families were uninformative at the highly polymorphic DBH microsatellite locus. This observation prompted a comparison of the allele frequencies to published values for all the loci tested, and significant differences were found at the DBH locus.

Several factors suggest the dopamine beta-hydroxylase gene, which has been mapped to 9q34, could be involved in the etiology of autism. The DBH gene encodes a protein which catalyzes the conversion of dopamine to norepinephrine[9]. Decreased serum DBH enzyme activity levels have been reported in autistic individuals and their parents[10]. Further, the DBH gene is closely linked to one of the loci for tuberous sclerosis, a disorder frequently associated with autism[7,11]. This report presents data which suggest that lowered maternal serum DBH activity contributes to the etiology of autism in some individuals.

RESULTS

Comparison of DBH microsatellite allele frequencies

As the DBH microsatellite locus is located in the 5′ flanking region[20], the 19 bp sequence could affect transcriptional regulation of the gene. DBH alleles were grouped by the presence or absence of the 19 bp sequence (Table 2). The frequency of alleles in which the 19 bp sequence was absent (hereafter referred to as 19 bp deletion alleles) was significantly increased in mothers compared to a Canadian control group (L^2=6.96, df=1, $p<0.01$). The frequency of 19 bp deletion alleles was not significantly elevated in fathers (L^2=1.21, df=1, n.s.). Further, the frequency of 19 bp deletion genotypes observed in the affected children was not significantly different from the genotypic frequencies predicted by random segregation of parental alleles (L^2=2.98, df=2, n.s.). The frequency of 19 bp deletion alleles in the Canadian control group was not significantly different from published values[20] (L^2=2.18, df=1, n.s.).

Although the frequency of 19 bp deletion alleles in the fathers was not significantly elevated compared to the control group, seventy-five per cent of the fathers were heterozygous for the 19 bp deletion (Table 3). Further analysis revealed that the genotypic frequencies observed in the fathers differed significantly from those predicted assuming Hardy-Weinberg equilibruim (L^2=6.36, df=2, $p<0.05$). The frequency of 19 bp deletion genotypes in the mothers did not differ significantly from those predicted from observed maternal allele frequencies assuming Hardy-Weinberg equilibruim (L^2=0.97, df=2, n.s.).

Correlation of genotype with DBH enzyme activity

Lake (1977) reported finding lower serum DBH activity levels in the first degree relatives of autistic children[10]. We were interested in determining: (1) if Lake's finding could be replicated in parents with two autistic/PDD children; and (2) whether the 19 bp deletion genotype is associated with lower serum DBH activity. Serum DBH activity levels were measured in the parents and in a control group. Serum samples were not attainable from the individuals which served as the Canadian control group for DBH allele frequency comparisons, so a second control group was used for this part of the study. Since blood had to be retaken from parents to get serum samples, unfortunately not all parents were available to participate.

Mean serum DBH activity was significantly lower in the parents than in controls (Student's $t=-1.71$, $df=60$, $p<0.05$, 1-tailed). The mean and median serum DBH activities in the control group were 34.4 iu/L and 30.1 iu/L, respectively. In the mothers, mean serum DBH activity was 25.2 iu/L and the median activity was 15.9 iu/L. The mean and median serum DBH activities observed in fathers were 27.2 iu/L and 22.6 iu/L, respectively.

Mean serum DBH activity was then compared between parents and controls in each of the genotypic groups. The mean serum DBH activity for 19 bp deletion heterozygotes did not differ significantly between parents and controls (30.2 iu/L vs. 30.6 iu/L, Student's $t=-0.09$, $df=33$, n.s.); nor did the mean DBH activity of 19 bp deletion homozygotes (20.9 iu/L vs. 19.3 iu/L, Student's $t=0.175$, $df=15$, n.s.). A comparison between non-deletion homozygote mean DBH activities could not be made as only one parent had this genotype.

In order to further assess the association between genotype and serum DBH activity levels, the data from parents and controls were pooled. A one-way ANOVA with the 19 bp deletion genotype as the independent variable and serum DBH activity as the dependent variable revealed significant differences in serum DBH enzyme activity among the three genotypes ($F=5.45$, $df=59$, $p<0.01$). Individuals homozygous for non-deletion (+/+) alleles showed the highest mean DBH serum enzyme activity (44.5±28.6 iu/L), heterozygotes' (+/del) mean enzyme level was intermediate (30.5±16.0 iu/L), and homozygotes for 19 bp deletion alleles (del/del) had the lowest mean enzyme activity (20.5±15.3 iu/L) (Table 4).

DISCUSSION

The genotype of mothers at the dopamine beta-hydroxylase gene may be a significant component in the etiology of autism/PDD in some affected individuals. This study found that mothers with two autistic/PDD children had a marked elevation in the frequency of 19 bp deletion alleles at the DBH microsatellite locus (L^2=6.96, df=1, $p<0.01$), located in the 5′ flanking region of the DBH gene. Furthermore, mean serum DBH enzyme activity was found to be significantly associated with the 19 bp deletion genotype (F=5.45, df=59, $p<0.01$). Individuals who were homozygous for the 19 bp deletion alleles had the lowest mean enzyme activity.

DBH catalyzes the conversion of dopamine to norepinephrine[9]. Studies using fetal rats have demonstrated that dopamine acts as a neural morphogen during brain development[27]. It is plausible that low levels of maternal DBH could result in increased levels of dopamine, which could lead to abnormal stimulation of fetal dopamine receptors. Recent studies using DBH knockout mice provide evidence that maternal DBH genotype can influence fetal development[28].

Serum DBH activity is extremely low in newborn infants, and adult levels are not reached until the second decade of life[29]. There is a wide range of DBH serum activity levels in the general population[30]. However, the level of serum DBH activity within a given adult remains remarkably constant over time[31]. Using segregation anaylsis, Elston (1979) estimated that greater than 50 per cent of the variation in serum DBH activity could be accounted

for by a single gene with two alleles[32]. Subsequent linkage studies suggested that the DBH gene itself is responsible for inheritance of very low serum DBH activity[33,34], and that individuals with low DBH activity also have decreased levels of DBH protein[35].

The location of the DBH microsatellite locus in the 5' flanking region of the DBH gene[20] suggests that the 19 bp deletion could affect transcriptional activity of the DBH gene, much in the same way that the minisatellite at the diabetes susceptibility locus IDDM2 is thought to regulate transcription of the INS gene[36].

The type of microscopic neuroanatomic abnormalities found in brains of autistic individuals support the hypothesis that impaired development of central neuronal pathways in autism occurs or begins prenatally[37]. We propose that a lowered level of maternal serum DBH activity provides a uterine environment which, in conjunction with genotypic susceptibility of the fetus, leads to autism/PDD. The increased frequency of 19 bp deletion heterozygotes in the fathers with two autistic/PDD children suggests the 19 bp deletion genotype of the child may be one of the contributing susceptibility factors. However, the frequency of 19 bp deletion genotypes observed in the affected children did not differ significantly from the genotypic frequencies predicted by random segregation of parental alleles (L^2=2.98, df=2, n.s.). These apparently contradictory findings might be the result of selecting for families with two or more affected children. Multiple susceptibility factors are likely to be present in such families, not all of which may need to be transmited for a child to be affected. Further studies are needed to determine if the 19 bp deletion genotype of the child is a susceptibility factor. While the proposed

model cannot account for etiology of autism/PDD in all families, since not all of the mothers had low serum DBH activity, it may help to define more homogenous subgroups.

METHODS

The study group consisted of 26 families, each of which have two or more children with autism or a related PDD (includes atypical autism, Asperger's syndrome, and PDD variant). Nineteen of the families have two affected sons, and one family has three affected sons. Five families have an affected son and an affected daughter, and one family has two affected daughters and an affected son. An extensive battery of assessments were conducted on the affected children to confirm the diagnoses of autism or PDD using a best-estimate approach with a diagnostic review panel[38]. Affected individuals were screened for fragile X, tuberous sclerosis, and for chromosomal abnormalities. If present, the family was excluded from this study.

Blood samples were taken from parents and their affected children. In eighteen of these families, blood samples were available for both parents and affected children. All of the candidate loci were scored in these families. In the remaining eight families DNA samples were not available from one or more family member, and therefore, could not be included in the affected sib-pair analysis. Polymorphic microsatellite markers closely linked to candidate genes were amplified using the polymerase chain reaction (PCR). The PCR products were separated on 8% denaturing polyacrylamide gels.

Canadian control group allele frequency data were generated by screening Guthrie spots, obtained from the Ontario Ministry of Health, from 134 anonymous newborns. The control group for the DBH serum activity

study consisted of students enrolled in a laboratory assistants course at a local college.

DBH activity was measured using 50 ul of serum according to the spectrophotometric method of Frigon (1987)[38], with the exception that 0.5 ml of AG 50W X4, 200-400 mesh, H+ resin was used in the ion-exchange columns. Each sample was assayed twice and average readings were used in the statistical analyses. DBH activity is expressed as international units/liter of serum (iu/L), where one iu is equal to the conversion of one micromole of tyramine to octopamine per minute at 37°C.

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Table 1: **Summary of eleven candidate loci surveyed in families with two autistic/PDD children**

Candidate gene/region	Microsatellite marker	Chromosomal location	Significance of gene product or chromosomal region
Serotonin receptor, 5HT2	13S118 [12]	13q14.1-q14.2	• elevated 5HT serum levels in >30% of autistic individuals may reflect abnormalities in central serotonergic pathways
Dopamine D2 receptor	DRD2 [13]	11q22-q23	• abnormalities in central dopaminergic pathways has also been proposed
Tyrosine hydroxylase	TH [14]	11p15.5	• enzyme involved in synthesis of catecholamines
c-Harvey-Ras-1	HRAS-1 [15]	11p15.5	• published report of allelic association with autism[23]
Catechol-O-methyltransferase	22S343 [16]	22q11.2	• enzyme involved in the breakdown of catecholamines
Prader-Willi syndrome region	D15S11 [17]	15q11-q13	• a number of case reports of autistic individuals having a duplication of this region[24]
$GABA_A$ receptor *B3*	GABRB3 [18]	15q11-q13	• GABA receptor gene within Prader-Willi region
Tuberous sclerosis locus, TSC2	16S291 [19]	16p13.3	• tuberous sclerosis and autism frequently co-occur
Dopamine beta-hydroxylase	DBH [20]	9q34	• enzyme involved in synthesis of norepinephrine and epinephrine • linked to tuberous sclerosis locus,TSC1
Fragile X mental retardation	CGG repeat [21] FMR1	Xq27.3	• some individuals with fragile X demonstrate autistic features
Monoamine oxidase-A	MAO-A [22]	Xp11.23-11.4	• enzyme involved in metabolizing biogenic amines

Table 2: Comparison of observed DBH microsatellite allele frequencies to published values

Allele size	Published frequencies[a]	Canadian sample (n=268)*	Fathers (n=40)	Mothers (n=52)[b]	Autistic/PDD children (n=100)
280 bp	not seen	not seen	not seen	not seen	not seen
278 bp	not seen	0.004	not seen	not seen	not seen
276 bp	0.159	0.108	0.250	0.096	0.150
274 bp	0.451	0.425	0.200	0.250	0.280
272 bp	not seen	0.008	not seen	not seen	not seen
Total without 19 bp deletion	**0.610**	**0.545**	**0.450**	**0.346**	**0.430**
261 bp	0.018	0.037	0.050	not seen	0.030
259 bp	0.111	0.078	0.025	0.096	0.060
257 bp	0.159	0.224	0.325	0.385	0.320
255 bp	0.097	0.116	0.150	0.173	0.160
253 bp	0.004	not seen	not seen	not seen	not seen
Total with 19 bp deletion	**0.390**	**0.455**	**0.550**	**0.654**	**0.570**

* In this table, (n) refers to the number of chromosomes

[a] published allele frequencies estimated from 226 chromosomes from 113 unrelated individuals[20]

[b] $p<0.001$ when compared with expected frequencies using the Likelihood ratio ($L^{2)}$ test [25, 26]

Table 3: DBH microsatellite genotypes grouped by the presence of the 19 bp deletion

	19 bp deletion genotype		
Group	+/+	+/del	del/del
Canadian sample	30% (41/134)	48% (64/134)	22% (29/134)
Autistic/PDD children	18% (9/50)	52% (26/50)	30% (15/50)
Fathers	5% (1/20)	75% (15/20)	20% (4/20)
Mothers	8% (2/26)	54% (14/26)	38% (10/26)

Table 4: DBH serum activity by 19 bp deletion genotype

19 bp deletion genotype	Sample size (n)	Mean DBH serum activity (iu/L)	Standard Deviation
+/+	10	44.5	28.6
+/del	35	30.5	16.0
del/del	17	20.5	15.3

* One-way ANOVA, F=5.45, df=59, $p<0.01$

SUMMARY AND CONCLUSIONS

There is compelling support from family and twin studies that genetic factors play a significant role in the etiology of autism and related PDD (Bolton et al. 1994, Bailey et al. 1995). The data from these studies are not consistent with single gene inheritance models. Both multifactorial inheritance and the existence of genetic heterogeneity have been proposed to account for the observed frequency of autism/PDD among relatives of affected individuals and to explain the anomalous sex ratio (3 affected males to 1 affected female) (Smalley et al. 1988, Bolton et al. 1994). Over the last decade a number of studies have attempted to elucidate the genes and environmental stresses which are involved in the etiology of autism/PDD. Unfortunately, these factors have remained elusive and diagnosis of these disorders is still based entirely on behavioural presentation.

This research investigated the possibility of linkage between nine autosomal markers tightly linked to candidate genes/regions and susceptibility for the broad spectrum of autistic disorders, using the affected sib-pair method. There was no evidence of linkage, as no significant concordance between affected siblings was observed for any of the loci tested. However, an unexpectedly large number of the families were found to be uninformative at the DBH microsatellite locus. Results from subsequent investigation suggest the genotype of mothers at the DBH gene may play a significant role in the etiology of autism/PDD in some affected individuals. Based on the results discussed in Chapter 3, it was proposed that a lowered level of maternal serum activity provides a uterine environment which, in

conjunction with genotypic susceptibility of the fetus, results in autism or related PDD.

The results of this research and the proposed maternal effect model raise many questions which cannot be answered from the data generated in this study. Whether the 19 bp deletion at the DBH microsatellite locus, which was found to be associated with decreased serum DBH activity, is itself responsible for the observed decrease or instead is in linkage disequilibrium with a causal sequence remains to be determined. Another area which needs further exploration is whether or not the genotype of an affected child at the DBH locus contributes to susceptibility for the broad spectrum of autistic disorders. The observation that the frequency of 19 bp deletion genotypes in the affected children did not differ significantly from the genotypic frequencies predicted by random segregation of parental alleles suggests that this is not the case. However, speculation about the possible role of the child's genotype at the DBH locus arose from the fact that three-quarters of the fathers were heterozygous for the 19 bp deletion, and the frequency of 19 bp deletion genotypes among the fathers were significantly different from genotypic frequencies predicted assuming Hardy-Weinberg equilibrium. If the child's genotype at the DBH locus does contribute to the etiology of autism, then the lack of evidence for preferential transmission of 19 bp deletion alleles to affected children observed in this study might be explained by the type of families studied (i.e. families with two affected children.) The concept of familial loading, briefly discussed in Chapter 3, suggests that multiple susceptibility factors are likely to be present in families where multiple children are affected. The crucial point is that not all of the

susceptibility factors necessarily needed to be transmitted for the child to be affected. Studying the frequency of 19 bp deletion genotypes in families which have only one affected child could help to clarify this issue.

If the proposed maternal effect model is correct, it suggests the existence of genetic heterogeneity within the autistic population, as not all of the mothers had low serum DBH activity. In terms of the maternal effect model, since males are more susceptible to environmental insults during development than females, it is tempting to speculate that this model might be able to explain the male-biased sex ratio observed in the autistic population. A number of testable predictions can be generated about the expected relative importance of the maternal DBH genotype in different types of families which have only one autistic/PDD child. For example, in families where there is an affected male child and an unaffected female child, the maternal DBH genotype would be predicted to contribute to the etiology of a substantial portion of these cases. Conversely, if the affected child is female and there is an unaffected male sibling, a mechanism other than the proposed maternal effect model would be predicted to account for the majority of these cases. In a similar manner, expectations can be derived for the other families types which have only one affected child.

As is suggested by the preceding discussion, the results of this research have generated many new questions. Of these, the foremost question is whether the results can be replicated in other study populations.

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APPENDIX A: DSM-IV diagnostic criteria for autism, Asperger's disorder, and atypical autism

Autism:

A. A total of six (or more) items from (1), (2), and (3), with at least two from (1), and one each from (2) and (3):

(1) qualitative impairment in social interaction, as manifested by at least two of the following:

a) marked impairment in use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction

b) failure to develop peer relationships appropriate to developmental level

c) a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing or pointing out objects of interest)

d) lack of social or emotional reciprocity

(2) qualitative impairments in communication as manifested by at least one of the following:

a) delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)

b) in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others

c) stereotyped and repetitive use of language or idiosyncratic language

d) lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

(3) restricted repetitive and stereotyped patterns of behavior, interests, and activities, as manifested by at least one of the following:

a) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus

b) apparently inflexible adherence to specific, nonfunctional routines or rituals

c) stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)

d) persistent preoccupation with parts of objects

B. Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years: (1) social interaction, (2) language as used in social communication, (3) symbolic or imaginative play.

C. The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder.

Asperger's Disorder:

A. Qualitative impairment in social interaction, as manifested by at least two of the following:
   1) marked impairment in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction
   2) failure to develop peer relationships appropriate to developmental level
   3) a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by lack of showing, bringing, or pointing out objects of interest to other people)
   4) lack of social or emotional reciprocity

B. Restricted repetitive and stereotyped patterns of behavior, interests, and activities, as manifested by at least one of the following:
   1) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus
   2) apparently inflexible adherence to specific, nonfunctional routines or rituals
   3) stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)
   4) persistent preoccupation with parts of objects

C. The disturbance causes clinically significant impairment in social, occupational, or other important areas of functioning.

D. There is no clinically significant general delay in language (e.g., single words used by age 2 years, communicative phrases used by age 3 years).

E. There is no clinically significant delay in cognitive development or in the development of age-appropriate self-help skills, adaptive behavior (other that in social interaction), and curiosity about the environment in childhood.

F. Criteria are not met for another specific Pervasive Developmental Disorder or Schizophrenia.

Pervasive Developmental Disorder Not Otherwise Specified (Including Atypical Autism):

This category should be used when there is a severe and pervasive impairment in the development of reciprocal social interaction or verbal and nonverbal communication skills, or when stereotyped behavior, interests, and activities are present, but the criteria are not met for a specific Pervasive Developmental Disorder, Schizophrenia, Schizotypal Personality Disorder, or Avoidant Personality Disorder. For example, this category includes "atypical autism" — presentations that do not meet the criteria for Autistic Disorder because of late age at onset, atypical symptomatology, or subthreshold symptomatology, or all of these.

APPENDIX B: Concordance between affected siblings at the nine microsatellite loci examined

Table B1. microsatellite locus 22s343

Male-male affected sib pairs	Concordance for maternal allele	Concordance for paternal allele
AUT-20	discordant	concordant
AUT-22	concordant	concordant
AUT-23	discordant	concordant
AUT-25	concordant	concordant
AUT-28	discordant	discordant
AUT-40	discordant	discordant
AUT-43	discordant	concordant
AUT-44	concordant	uninformative
AUT-45	discordant	discordant
AUT-53	concordant	discordant
AUT-57	concordant	discordant
AUT-60	concordant	discordant
Male-female affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-13	discordant	concordant
AUT-16	concordant	concordant
AUT-19	concordant	discordant
AUT-31	concordant	concordant
AUT-50	discordant	discordant
AUT-56 *	one discordant	one discordant

* Family with three affected siblings (1 male and 2 females)

Legend:

concordant — siblings inherit the same allele identical by descent

discordant — siblings inherit different alleles

uninformative — identity by descent could not be established

Table B2: microsatellite locus 13S118

Male-male affected sib pairs	Concordance for maternal allele	Concordance for paternal allele
AUT-20	concordant	uninformative
AUT-22	uninformative	discordant
AUT-23	uninformative	discordant
AUT-25	discordant	uninformative
AUT-28	concordant	concordant
AUT-40	uninformative	concordant
AUT-43	uninformative	discordant
AUT-44	concordant	discordant
AUT-45	uninformative	discordant
AUT-53	concordant	uninformative
AUT-57	uninformative	discordant
AUT-60	discordant	discordant
Male-female affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-13	discordant	discordant
AUT-16	discordant	uninformative
AUT-19	uninformative	concordant
AUT-31	concordant	uninformative
AUT-50	uninformative	concordant
AUT-56 *	uninformative	uninfomative

* Family with three affected siblings (1 male and 2 females)

Legend:

concordant — siblings inherit the same allele identical by descent

discordant — siblings inherit different alleles

uninformative — identity by descent could not be established

Table B3: microsatellite locus DRD2

Male-male affected sib pairs	Concordance for maternal allele	Concordance for paternal allele
AUT-20	uninformative	uninformative
AUT-22	concordant	concordant
AUT-23	uninformative	concordant
AUT-25	discordant	uninformative
AUT-28	uninformative	concordant
AUT-40	uninformative	discordant
AUT-43	discordant	uninformative
AUT-44	uninformative	uninformative
AUT-45	discordant	concordant
AUT-53	uniformative	concordant
AUT-57	concordant	concordant
AUT-60	discordant	concordant
Male-female affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-13	discordant	discordant
AUT-16	uniformative	uninformative
AUT-19	concordant	discordant
AUT-31	uninformative	uninformative
AUT-50	discordant	discordant
AUT-56 *	uninformative	uninformative

* Family with three affected siblings (1 male and 2 females)

Legend:

concordant — siblings inherit the same allele identical by descent

discordant — siblings inherit different alleles

uninformative — identity by descent could not be established

Table B4: **microsatellite locus TH**

Male-male affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-20	discordant	concordant
AUT-22	concordant	concordant
AUT-23	discordant	uninformative
AUT-25	discordant	uninformative
AUT-28	discordant	discordant
AUT-40	concordant	uninfomative
AUT-43	concordant	uninformative
AUT-44	concordant	concordant
AUT-45	discordant	discordant
AUT-53	discordant	discordant
AUT-57	uninformative	uninformative
AUT-60	discordant	uninformative
Male-female affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-13	discordant	discordant
AUT-16	discordant	discordant
AUT-19	uninformative	discordant
AUT-31	uninformative	uninformative
AUT-50	discordant	concordant
AUT-56 *	all concordant	one discordant

* Family with three affected siblings (1 male and 2 females)

Legend:

concordant — siblings inherit the same allele identical by descent

discordant — siblings inherit different alleles

uninformative — identity by descent could not be established

Table B5: microsatellite locus HRAS-1

Male-male affected sib pairs	Concordance for maternal allele	Concordance for paternal allele
AUT-20	discordant	concordant
AUT-22	uninformative	uninformative
AUT-23	uninformative	uninformative
AUT-25	uninformative	discordant
AUT-28	discordant	discordant
AUT-40	uninformative	concordant
AUT-43	concordant	uniformative
AUT-44	concordant	uniformative
AUT-45	uninfomative	discordant
AUT-53	discordant	uninformative
AUT-57	uninformative	uninformative
AUT-60	discordant	uninformative
Male-female affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-13	uninformative	uninformative
AUT-16	discordant	uninformative
AUT-19	discordant	uninformative
AUT-31	uninformative	concordant
AUT-50	discordant	uninformative
AUT-56 *	uniformative	uninformative

* Family with three affected siblings (1 male and 2 females)

Legend:

concordant — siblings inherit the same allele identical by descent

discordant — siblings inherit different alleles

uninformative — identity by descent could not be established

Table B6: **microsatellite locus AS/PWS**

Male-male affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-20	uninformative	discordant
AUT-22	concordant	discordant
AUT-23	uninformative	uninformative
AUT-25	uninformative	discordant
AUT-28	concordant	concordant
AUT-40	concordant	uninformative
AUT-43	uninformative	uninformative
AUT-44	uninformative	discordant
AUT-45	concordant	concordant
AUT-53	uninformative	concordant
AUT-57	concordant	uninformative
AUT-60	uninformative	uninformative
Male-female affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-13	concordant	uninformative
AUT-16	discordant	concordant
AUT-19	discordant	uninformative
AUT-31	discordant	uninformative
AUT-50	concordant	uninformative
AUT-56 *	uninformative	one discordant

* Family with three affected siblings (1 male and 2 females)

Legend:

concordant — siblings inherit the same allele identical by descent

discordant — siblings inherit different alleles

uninformative — identity by descent could not be established

Table B7: microsatellite locus GABRB3

Male-male affected sib pairs	Concordance for maternal allele	Concordance for paternal allele
AUT-20	concordant	uninformative
AUT-22	concordant	discordant
AUT-23	uninformative	uninformative
AUT-25	concordant	discordant
AUT-28	discordant	concordant
AUT-40	concordant	concordant
AUT-43	uninformative	uninformative
AUT-44	discordant	discordant
AUT-45	concordant	concordant
AUT-53	concordant	concordant
AUT-57	uninformative	concordant
AUT-60	concordant	concordant
Male-female affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-13	concordant	concordant
AUT-16	concordant	concordant
AUT-19	discordant	concordant
AUT-31	discordant	uninformative
AUT-50	concordant	uninformative
AUT-56 *	one discordant	all concordant

* Family with three affected siblings (1 male and 2 females)

Legend:

concordant — siblings inherit the same allele identical by descent

discordant — siblings inherit different alleles

uninformative — identity by descent could not be established

Table B8: microsatellite locus 16S291

Male-male affected sib pairs	Concordance for maternal allele	Concordance for paternal allele
AUT-20	discordant	discordant
AUT-22	concordant	concordant
AUT-23	concordant	discordant
AUT-25	concordant	discordant
AUT-28	discordant	discordant
AUT-40	uninformative	uninformative
AUT-43	concordant	uninformative
AUT-44	discordant	concordant
AUT-45	concordant	discordant
AUT-53	discordant	concordant
AUT-57	concordant	concordant
AUT-60	discordant	discordant
Male-female affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-13	discordant	concordant
AUT-16	discordant	concordant
AUT-19	concordant	discordant
AUT-31	uninformative	discordant
AUT-50	concordant	concordant
AUT-56 *	one discordant	one discordant

* Family with three affected siblings (1 male and 2 females)

Legend:

concordant — siblings inherit the same allele identical by descent

discordant — siblings inherit different alleles

uninformative — identity by descent could not be established

Table B9: microsatellite locus DBH

Male-male affected sib pairs	Concordance for maternal allele	Concordance for paternal allele
AUT-20	uninformative	discordant
AUT-22	uninformative	concordant
AUT-23	uninformative	uninformative
AUT-25	discordant	concordant
AUT-28	uninformative	concordant
AUT-40	uninformative	uninformative
AUT-43	uninformative	uninformative
AUT-44	discordant	discordant
AUT-45	uninformative	uninformative
AUT-53	discordant	discordant
AUT-57	uninformative	concordant
AUT-60	uninformative	uninformative
Male-female affected sib pairs	**Concordance for maternal allele**	**Concordance for paternal allele**
AUT-13	concordant	discordant
AUT-16	concordant	uninformative
AUT-19	uninformative	uninformative
AUT-31	concordant	uninformative
AUT-50	uninfomative	concordant
AUT-56 *	discordant	concordant

* Family with three affected siblings (1 male and 2 females)

Legend:

concordant — siblings inherit the same allele identical by descent

discordant — siblings inherit different alleles

uninformative — identity by descent could not be established

Appendix C: Likelihood (L^2) ratio test formulas (also termed G-statistic)*

(1) Test for independence:

$$L^2 = 2\left(\sum_{ij} F_i \ln F_i - \sum R \ln R - \sum C \ln C + N \ln N\right)$$

where F_i = frequency of each table cell
R = row totals
C = column totals
N = total sample number

The Likelihood ratio test of independence was used to compare observed microsatellite allele frequency data to published values.

(2) Goodness of fit test:

$$L^2 = 2\sum^{a} f_i \ln\left(\frac{f_i}{\hat{f}_i}\right)$$

where
f = observed cell frequency
$\hat{f}_i$ = expected cell frequency basis on null hypothesis

The Likelihood-ratio goodness of fit test was used to determine if observed sib-pair concordance deviated significantly from values expected if parental alleles were segregating randomly.

*Further explanation of these non-parametric test statistics can be found in: Sokal, R. and Rohlf, F.J. (1981) Biometry (Second edition). Chapter 17. W.H. Freedman and Company. New York.